NEXT-GENERATION PROBIOTICS: FROM COMMENSAL BACTERIA TO NOVEL DRUGS AND FOOD SUPPLEMENTS

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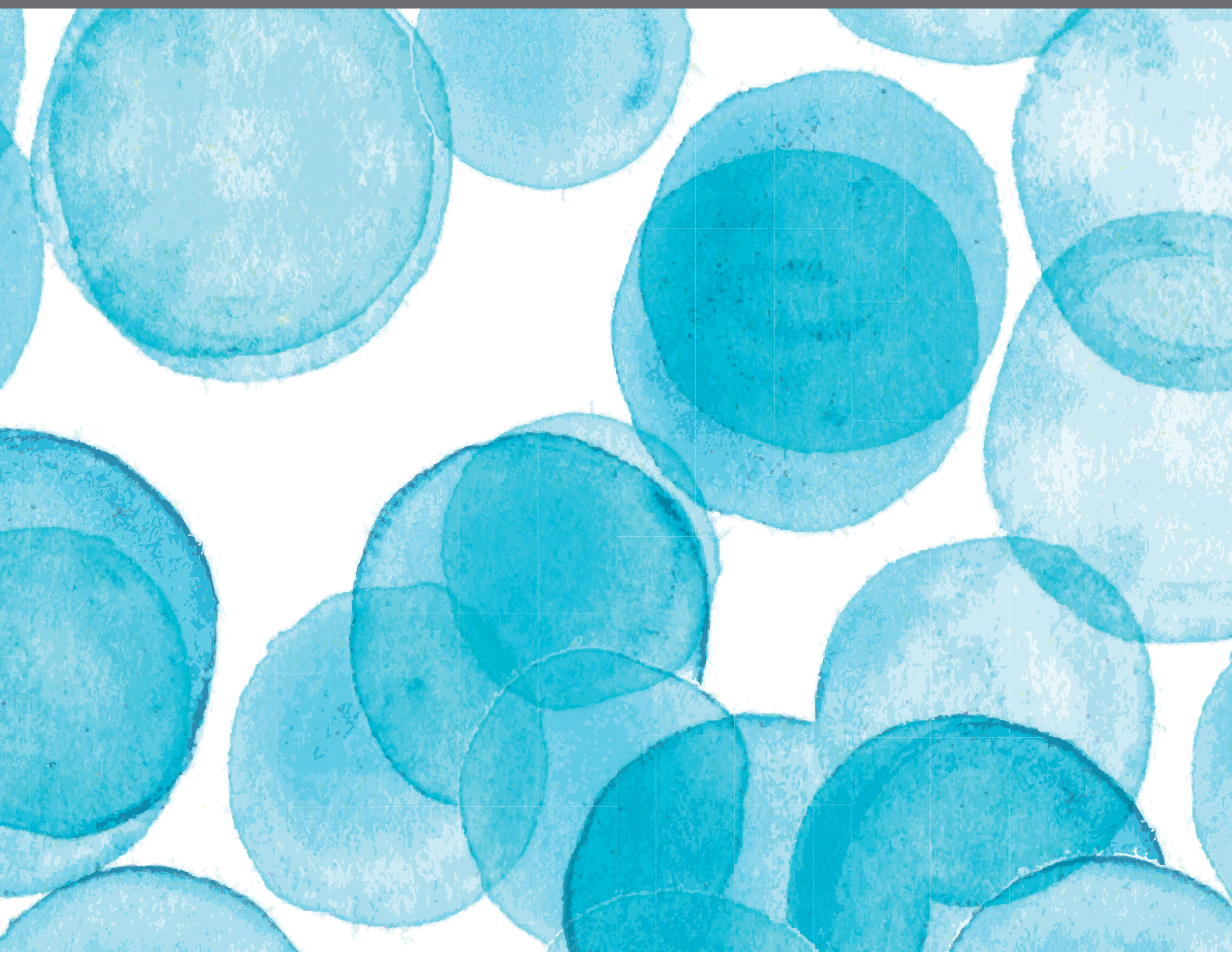

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NEXT-GENERATION PROBIOTICS: FROM COMMENSAL BACTERIA TO NOVEL DRUGS AND FOOD SUPPLEMENTS

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EDITORIAL
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Editorial: Next-Generation Probiotics: From Commensal Bacteria to Novel Drugs and Food Supplements

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Editorial on the Research Topic

Next-Generation Probiotics: From Commensal Bacteria to Novel Drugs and Food Supplements

The concept of traditional probiotics is originally based on the observation that the regular consumption of fermented dairy products with lactic acid bacteria was associated with enhanced health and longevity in elderly Bulgarian people. Since then, the term probiotic has been linked to beneficial bacteria for the host health (Hill et al., 2014). The probiotics field has exploded in the last years due to the increased knowledge of the human gut microbiota and the awareness about health implication of dysbiosis. This new trend highlights that the use of commensal bacteria as probiotics is the natural way to restore a healthy homeostasis situation within the gastrointestinal tract (GIT) opening the door to a new kind of probiotics commonly termed Next-Generation Probiotics (NGP) (Martin and Langella, 2019).

The current Research Topic covers a collection of reviews, mini-reviews, perspectives, opinion, methods, and original research articles focused on the NGPs field. In this sense, two reviews have been focused on the no-scientific concerns. In the first one, El Hage et al. cover current perspectives on the development and assessment of NGPs and the approaches that industry and stakeholders must consider for a successful outcome. In the second one, Brodmann et al. analyze the safety of novel microbes for human consumption with a special focus on the regulatory framework.

Other three non-research articles have been focused on three important NGP candidates. Cani and de Vos wrote a complete state of the art of the well-known beneficial bacterium *Akkermansia municiphila*. Martín et al. focused on the potential bacterial effectors of the multiskilled commensal *Faecalibacterium prausnitzii*. Finally, Rios-Colvian et al. argue about the potential to re-shape the metabolism of *Bacteroides* with specific combinations of dietary carbohydrates-proteins.

Most of the probiotic candidates characterized up today are focused on the GIT. Several research papers published in this topic are focused on the characterization of new strains to be used as probiotic to target GIT related diseases. Nishida et al. describe that *Lactobacillus paraplantarum* 11-1 is able to activate innate immunity and improved survival after infection in silkworm while Bajpai et al. characterize the antibacterial potential of *Pedioccus pentosaceus* 411. Other potential candidates defined on this topic are: *Clostridium butyricum* AQQF01000149 able to block *Salmonella* (Zhao et al.), several strains of *F. praustnizii* characterized from a functional point of view (Martín et al.), isolates of *Enterococcus munditii* that protect insects against *Bacillus thuringiensis* (Grau et al.), and *Bacteroides fragilis* ZY-312 (Wang et al.).

Regarding other ecosystems, Rather et al. have described that *L. sakei* proBio-65 ameliorates the severity of psoriasis-like skin inflammation and López-López et al. reported *Streptococcus dentisani* 7746 and 7747 as an oral probiotic against tooth decay. This strain is able to inhibit the growth of major oral pathogens through the production of bacteriocins, and also buffers acidic pH through

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an arginolytic pathway. Moving to the respiratory tract, Kanmani et al. described the capacity of the commensal bacterium *Corynebacterium pseudodiphtheriticum* 090104 to improve resistance of infant mice to Respiratory Syncytial Virus and *Streptococcus pneumoniae* superinfection. And finally, hepatorenal damage in broilers has been found to be counterbalanced by *L. plantarum* MYS6 by Deepthi et al.

Several research papers have been focused on the delivery method and/or the food matrix employed to administer the probiotic. Baruzzi et al. describe the development of a symbiotic beverage enriched with bifidobacterial and whey proteins. Navarro et al. present the enhancement of the potential probiotic properties of *L. reuteri* ATCC 23272 when it is delivered as a biofilm on dextranomer microspheres with cargo. Likewise, Dinić et al. introduce the use of post-biotics indicating that *L. fermentum* BGHV110 post-biotic-induced autophagy could be a potential approach for treating acetaminophen hepatoxicity.

Nine original research articles have been focused on the mechanisms of action of several probiotic candidates. Three of them describe the major role of bile salt hydrolases (BSH). Allain et al. in two different manuscripts, describe the anti-giardia activity of *L. johnsonii* La1 *in vitro* and *in vivo* and present BSH as novel target to screen anti-giardia lactobacilli. On the other hand, Rani et al. find by docking analysis that the BSH from *L. gasseri* FR4 could be an inhibitory mechanism to be used as a potential alternative to growth promoters for poultry animals.

Several surface molecules have been described as potential effectors in this topic. Castro-Bravo et al. describe by gene replacement and fluorescent labeling that the different exopolysaccharide of *Bifidobacterium animalis* subsp. *lactis* DSM10140 confer variable functional characteristics to the bifidobacterial surface, which may be relevant for its performance. Besides, the surface protein SlpB form *Propionibacterium freudenreichii* CIRM-BIA 129 has been involved in adhesion to intestinal cells by do Carmo et al. while Veljović et al. found that the aggregation promoting factor AggE of *Enterococcus faecium* BGG09-28 enhances adhesion ability to collagen, mucin, and fibronectin and contribute to the increase of biofilm formation.

Other desirable probiotic characteristic is the ability to positively modulate the immune system. Hidalgo-Cantabrana et al. have combined the use of bioinformatics and *in vitro* tools to screen bioactive peptides encrypted in the human gut metaproteome. Thanks to this strategy, they have identified several peptides able to promote Th17 response in commensal bacteria. Besides, Breyner et al. have found that the microbial anti-inflammatory molecule (MAM) from *F. prausnitzii* is able to inhibit NF-κB pathway protecting mice against several types of colitis.

Selma et al. reported the isolation of human gut bacterial strains that belong to *Eggerthellaceae* family capable of producing isourolithin-A, a urolithin with potential beneficial effects.

Finally, this topic also includes methodological articles, such as the method article of Barone et al. in which a new model of chemically-induced chronic colitis with an outbred murine strain is described to test probiotic candidates. Besides, Arnold et al. have used *L. rhamnosus* AMC143 and AMC010 to demonstrate that the use of both classical microbiology and functional genomics methods are key for the characterization of novel probiotics, as variability between strains can dramatically alter bacterial functionality.

In summary, together the articles of this Research Topic make a substantial contribution to the NGP arena as a step toward a better comprehension of this field.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

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REVIEW
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Safety of Novel Microbes for Human Consumption: Practical Examples of Assessment in the European Union

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Novel microbes are either newly isolated genera and species from natural sources or bacterial strains derived from existing bacteria. Novel microbes are gaining increasing attention for the general aims to preserve and modify foods and to modulate gut microbiota. The use of novel microbes to improve health outcomes is of particular interest because growing evidence points to the importance of gut microbiota in human health. As well, some recently isolated microorganisms have promise for use as probiotics, although in-depth assessment of their safety is necessary. Recent examples of microorganisms calling for more detailed evaluation include *Bacteroides xylanisolvens, Akkermansia muciniphila*, fructophilic lactic acid bacteria (FLAB), and *Faecalibacterium prausnitzii*. This paper discusses each candidate's safety evaluation for novel food or novel food ingredient approval according to European Union (EU) regulations. The factors evaluated include their beneficial properties, antibiotic resistance profiling, history of safe use (if available), publication of the genomic sequence, toxicological studies in agreement with novel food regulations, and the qualified presumptions of safety. Sufficient evidences have made possible to support and authorize the use of heat-inactivated *B. xylanisolvens* in the European Union. In the case of *A. muciniphila*, the discussion focuses on earlier safety studies and the strain's suitability. FLAB are also subjected to standard safety assessments, which, along with their proximity to lactic acid bacteria generally considered to be safe, may lead to novel food authorization in the future. Further research with *F. prausnitzii* will increase knowledge about its safety and probiotic properties and may lead to its future use as novel food. Upcoming changes in EUU Regulation 2015/2283 on novel food will facilitate the authorization of future novel products and might increase the presence of novel microbes in the food market.

Keywords: novel microbes, safety, *Bacteroides xylanisolvens*, *Akkermansia muciniphila*, fructophilic lactic acid bacteria, *Faecalibacterium prausnitzii*

INTRODUCTION

Since ancient times, microorganisms have constituted an essential part of human nutrition and been consumed through naturally microbially fermented products containing viable bacteria, such as fermented honey, fruits, berries, and their juices and fermented products of animal origin. Without even knowing of the existence of bacteria, humans historically used them to initiate many food production processes (Selhub et al., 2014). Today, an enormous variety of fermented foods and beverages exists and provides approximately one-third of the human diet globally (Borresen et al., 2012). Certain fermented foods containing significant amounts of viable microbes, especially dairy products and fermented vegetables, are part of daily consumption by millions of people around the globe.

Studies involving new analytical technologies have generated new insights into the human and animal guts and yielded new isolates and candidates that perform special functions in this complex intestinal environment (Marchesi et al., 2016). The composition of human gut microbiota plays an important role in homeostasis and gut functionality, which are also strongly influenced by age, diet, environmental factors, disorders, diseases, and therapies. In recent decades, probiotic microorganisms have been identified as efficient modulators of intestinal microbial balance (Gómez-Gallego and Salminen, 2016). Probiotics are defined as live microorganisms that confer a health benefit to the host when administered in adequate amounts (Cammarota et al., 2014; Hill et al., 2014). These health benefits are provided through relatively common mechanisms found in the vast majority of the investigated probiotics, such as regulation of the intestinal transit, competitive exclusion of pathogens, and production of specific short chain fatty acids (SCFA). In addition, frequently observed mechanisms mostly found on a species level include vitamin synthesis, enzymatic activity, gut barrier reinforcement, and neutralization of carcinogens. The existence of a group of basic benefits shared by strains of the same species in a non-strain specific manner supports the concept of core benefits for specific species. Moreover, mechanisms described exclusively on the individual strain level provide immunological, neurological, and endocrinological effects. Such benefits in general support a healthy digestive tract and immune system (Aureli et al., 2011; Gómez-Gallego and Salminen, 2016).

Traditionally fermented foods, especially fermented dairy products, usually contain viable bacteria. The consumption of such foods is widely connected with the reduction of certain disease types (e.g., childhood obesity, type-2 diabetes, and cardiovascular diseases), improved body composition, and weight loss in adults during energy restriction (Thorning et al., 2016). The International Scientific Association for Probiotics and Prebiotics (ISAPP) panel acknowledged convincing results but also emphasized the difficulty of verifying whether the beneficial results originate from the food matrices, the (viable) microorganisms, or a combination of both. Additionally, traditional fermented foods do not qualify for the term *probiotic* because they may contain varying amounts and changing microbial species over time, and their microbiological composition is not completely known or controlled. A relevant example is traditional Kefir. The ISAPP, therefore, has proposed the term "containing live and active bacterial cultures" for traditional fermented foods (Hill et al., 2014).

Probiotics relevant to the human gut microbiota mainly belong to the genera *Lactobacillus* and *Bifidobacterium*, but several other microbial candidates, such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, could play roles in future probiotic products. Although, commensal gut microbes can provide beneficial health effects, the ISAPP panel suggested conducting strain-by-strain assessment until sufficient research data are available to grant probiotic status on the species level (Hill et al., 2014).

EUROPEAN UNION REGULATIONS OF MICROORGANISMS INTENTIONALLY ADDED TO FOOD

The European Union (EU) is a single-market system consisting of 28 member states and the four members of the European Free Trade Association (Iceland, Liechtenstein, Norway, and Switzerland), which are required to follow European legislation as part of the EU single market. The European Food Safety Authority (EFSA) has the mandate to assess and communicate all risks associated with the food chain in the EU. The EFSA Scientific Committees and Panels are responsible for providing scientific opinions in response to requests from the European Commission, European Parliament, and EU member states. Before the EFSA was established, risk assessment was performed by Scientific Committees assisting the Directorate General for Health and Consumers (DG SANCO). DG SANCO, re-named to DG SANTE, continues to perform a crucial regulatory role regarding microorganisms in food and feed. Standing Committees representing member states, and their interests have strong influence on the work of DG SANTE (von Wright, 2012).

Amid globalization and ongoing technical progress, the number of new food products in the EU has increased significantly, and food safety has become a growing concern. A uniform regulation for novel food was needed to ensure the protection of both European consumers and the market. The introduction of novel foods is governed by Regulation 285/97/EC (European Commission, 1997b) and Regulation 2015/2283 (European Commission, 2015), covering all foods not been used in the EU before 15 May 1997. Under Regulation 2015/2283, the EFSA, rather than competent national authorities, will perform the scientific risk assessment for novel foods; the system of individual authorization is replaced by a system of generic authorization; and the Commission will manage all applicants' files and prepares proposals for the authorization of novel foods found to be safe (European Commission, 2015). To speed decision-making, the EFSA is required to issue its opinion within 9 months of the date of receipt of a valid application.

The guidance lists general requirements for every application: (1) a description; (2) compositional data; (3) production process; (4) specifications; (5) proposed uses and use levels; and (6) anticipated intake of the novel food. Applicants also have

to provide information about the absorption, distribution, metabolism, excretion, nutrition, toxicology, allergenicity, and history of use of novel foods or their source. In addition, all the novel foods that belong to the group of "foods consisting of, isolated from or produced from micro-organisms, fungi or algae" must provide the following information (EFSA Panel on Dietetic Products, 2016): (1) scientific (Latin) name (family, genus, species, strain) according to the international codes of nomenclature; (2) synonyms used interchangeably with the preferred scientific name; (3) for bacteria and yeasts (unicellular organisms), verification of the species and strain identity according to internationally accepted methods; (4) origin of the organism; and (5) if available, deposition in an officially recognized culture collection with an access number.

Qualified Presumption of Safety

EFSA introduced the concept of qualified presumption of safety (QPS) to establish a generic risk assessment approach for biological agents, with the goal to simplify and harmonize assessment of notified biological agents across the EFSA's various Scientific Panels and Units. The QPS approach also enables more focused application of the available resources to agents with higher risk potential (Leuschner et al., 2010), simplifying the assessment of low-risk microorganisms. The QPS concept is intended to establish a safety assessment for microorganisms used in feed and food production. If a specific strain notified for market authorization can unambiguously be connected to a taxonomic unit on the QPS list, the necessary safety assessment steps, if any, are indicated in the list for that taxonomic unit.

Bacteria with a previous history of safe use are usually included on the QPS list. The EFSA's Panel on Biological Hazards assesses the QPS status of a strain and declares that the assigned microorganism displays either no safety concerns or minor concerns defined and addressed with qualifications as expressed in the QPS list. Microorganisms on the QPS list, therefore, need no exhaustive safety assessment except for those specified in the QPS list.

The assessment for QPS suitability is well structured and relies on the following criteria (EFSA, 2007):

(a) Taxonomic unit: This is the most crucial part of the assessment procedure. The genus and all known species, including type species, are listed and described according to their main characteristics. The evaluated taxonomic unit must be unambiguously defined. If the assessed bacterial agent cannot be connected to any known species, it will not be recommended for the QPS list. QPS status is also denied if the taxonomic identification is insufficient.
(b) Body of knowledge: This should be related to the history of use for the assessed bacterial agent, the field of application, the existence of sufficient scientific literature about the microorganism, and the possibility to avoid potential adverse effects for humans, livestock, and the wider environment.
(c) Possible pathogenicity: The bacterial agent's lack of pathogenic and virulence properties is confirmed, and the findings must be supported by clinical data and scientific literature.
(d) Description of end use: This includes the presence and viability of the microorganism in the final product.

Most bacteria recommended for the QPS list belong to the group of Gram-positive non-sporulating bacteria. This group includes many inhabitants of the digestive tract with long histories in food and feed production. For this group, the EFSA's Scientific Committee applies a generic qualification for all taxonomic units on the list, requiring that all strains not carry any transferable antimicrobial resistance unless the final product contains no viable cells (Leuschner et al., 2010). The number of recommended Gram-negative bacteria is very low, with only one, *Gluconobacter oxydans*, on the QPS list. Many members have a long history of safe use, but safety concerns cannot be excluded. *E. coli* strain Nissle 1917, for example, has long been used as a probiotic but can cause a variety of diseases and exhibits versatile virulence mechanisms. Nevertheless, opportunistic bacteria may be placed on the QPS list with additional qualifications, but pathogenic and toxin-producing bacteria are not included.

Microorganisms whose safety properties are not fully understood are subjected to safety assessments. Such assessments need to include a distinct taxonomic classification on the species or the strain level and a comprehensive strain characterization, including whole-genome sequence analysis, to identify potential virulence and antibiotic resistance genes and their horizontal transfer capabilities. However, in the case of new and recently discovered microorganisms from previously unknown microbial groups, the availability of genome sequences might not be enough to identify potential virulence or antibiotic resistance genes. These often require comparison with available data, so functional studies on these microorganisms are needed. For accurate safety evaluation, the food business operator should include the number and the viability of microorganisms in the final product by.

The QPS approach was inspired and influenced by the American Generally Recognized As Safe (GRAS) concept, but differences exist. QPS is defined as an "assumption based on reasonable evidence" (H. C. P. Directorate-General, 2003), providing a helpful assessment tool for the EFSA but, in contrast to GRAS, offers no legal status. The EFSA is responsible for providing the burden of proof, while GRAS lays the responsibility on the food business operator. GRAS, though, requires safety assessment by independent experts to the degree that another panel of independent experts would reach the same conclusions. The QPS assessment's strong focus on the absence of acquired antibiotic resistances and virulence factors is another difference between the two concepts.

MICROORGANISMS AUTHORIZED AS NOVEL FOOD IN THE EU

Leuconostoc mesenteroides

In January 2001, the European Commission authorized market placement of a dextran preparation produced by *L. mesenteroides* as a novel food ingredient in bakery products. Puracor NV (Groot-Bijgaarden, Belgium) filed the application, and the Belgian competent authorities performed the initial assessment. The Scientific Committee for Food found that the dextran

preparation by *L. mesenteroides* was safe for human consumption up to 5% in bakery products. Dextran was identified as a highly digestible bakery ingredient with similar nutritional properties as starch (European Commission, 2001).

L. mesenteroides is a Gram-positive, non-sporulating, coccoid-shaped bacterium in the order Lactobacillales. The organism is often found on the surfaces of various plant parts and is responsible for the fermentation of white cabbage into sauerkraut. *L. mesenteroides* can be also found in artisanal and industrial cheeses, possibly contributing to their safety for long-term consumption (Cibik et al., 2000; D'Angelo et al., 2017). The species can metabolize a broad range of sugars; in particular, sucrose is used to build dextran, and certain strains can produce bacteriocins with anti-listerial activity (de Paula et al., 2015).

Bacillus subtilis Natto

In April 2009, the European Commission allowed placing Vitamin K2 (menaquinone), produced by *B. subtilis* natto, in the market as a novel food ingredient under Regulation (EC) No 258/97. NattoPharma (Oslo, Norway) requested that the Irish competent authorities place *B. subtilis* natto derived Vitamin K2 in the market as a novel food ingredient to be used in foods which served particular nutritional uses and which had added vitamins and minerals (European Commission, 2009). In an initial report, the Irish competent authorities required an additional assessment, so the EFSA was requested to conduct an extended assessment. The Scientific Panel on Dietetic Products, Nutrition, and Allergies concluded that *B. subtilis* natto is a safe source of vitamin K2.

B. subtilis natto is a Gram-positive, aerobic member of the spore-forming genus *Bacillus*. This species, discovered in Japan in 1906, is responsible for production of natto, a sticky fermented soy beans. Natto, produced by *B. subtilis* natto, has long been used in the Japanese diet, so it gained Foods for Specified Health Use approval based on its health benefits from the Japanese Ministry of Health, Labor, and Welfare. *B. subtilis* has a recognized history of safe use, so the EFSA granted it QPS status (EFSA Panel on Biological Hazards, 2010).

Clostridium butyricum

In December 2014, the European Commission authorized placing in the market *C. butyricum* CBM 588 as a novel food ingredient according to Regulation (EC) No 258/97 (European Commission, 2014). The British competent authorities performed the initial assessment after Miyarisan Pharmaceutical Co. Ltd. (Tokyo, Japan) requested using *C. butyricum* on the market as a novel food ingredient used in food supplements. Some beneficial effects reported for *C. butyricum* are related to the production of SCFA and its role in lipid metabolism (Zhao et al., 2014; Shang et al., 2016). No additional assessment was necessary because further explanations provided by the applicant addressed the objections raised by member states. *C. butyricum* was authorized as novel food ingredient in food supplements at a maximum dose of 1.35×10^8 CFU per day. Intervention studies revealed the health benefits of the strain, including prevention of pouchitis in patients with ulcerative colitis and decreased incidence of side effects in patients receiving therapy for the eradication of *Helicobacter pylori* (Shimbo et al., 2005; Yasueda et al., 2016). An antibiotic resistance profile, a study for genes encoding toxins, and an animal study were conducted to ensure the safety of the strain (Isa et al., 2016). The authorized strain is a Gram-positive, spore-forming, obligate aerobic, non-pathogenic, non-genetically modified organism. The product is characterized as a white or pale gray tablet with a specific odor and sweet taste (European Commission, 2014).

Bacteroides xylanisolvens

The most recent authorization for bacteria according to Regulation (EC) No 258/97 was granted in 2015. The European Commission approved placing pasteurized milk products fermented with *B. xylanisolvens* DSM 23964 in the market as a novel food in a heat-treated, non-viable form. Details concerning *B. xylanisolvens* are discussed in the following section.

NOVEL MICROBES: DETAILS AND BACKGROUND REGARDING SAFETY

Bacteroides xylanisolvens DSM 23964

In 2015, pasteurized milk products fermented with *B. xylanisolvens* DSM 23964 were approved as a novel food under Novel Food Regulation No 258/97. Usage of this specific strain was restricted as the starter culture in the fermentation of pasteurized milk products. Only heat-treated and therefore inactivated cells of *B. xylanisolvens* were allowed in the final product (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2015). In addition to the novel food evaluation, the EFSA automatically assessed *B. xylanisolvens* for admission to the QPS list. Interestingly, the EFSA revealed that the available information was not sufficient to include it in the QPS list.

The genus *Bacteroides* is a main inhabitant of the human colon, on average accounting for ∼30% of intestinal microbiota, although large inter-individual variability exists (Sears, 2005). *Bacteroides* spp. is a Gram-negative, obligately anaerobic, bile resistant, non-spore forming rod originally isolated from human stool. This genus can have commensal attributes within the human body but can also promote the development of different diseases. *Bacteroides xylanisolvens* plays a major part in the fermentation and degradation of xylan and other plant fibers (Chassard et al., 2008). *Bacteroides* start colonization of the gut in the newborn child ∼10 days after birth, (Gregory et al., 2015). Polysaccharides in the human diet are an important nutrition source for gut commensals, including *Bacteroides* spp. They can be metabolized into short chain fatty acids and branched chain fatty acids that are reabsorbed through the large intestine and provide an essential part of the host's daily required energy (Hooper et al., 2002).

The levels of Bacteroidetes and Firmicutes seem to be connected to obesity. Obese individuals show lower levels of Bacteroidetes and higher levels of Firmicutes in the gastro-intestinal tract. Turnbaugh and his colleagues (Turnbaugh et al., 2006) suggested that a higher Bacteroidetes/Firmicutes ratio can extract more energy from a given diet and that increased

Bacteroidetes levels in the gut led to weight loss in people with obesity.

While investigating xylan-degrading microorganisms in the human gastro-intestinal tract, Chassard et al. (2008) isolated 6 xylanolytic, Gram-negative anaerobic rods. Subsequent 16S rRNA analysis revealed that the obtained strains belonged to the genus *Bacteroides*, and after proper analysis, the 6 strains were considered to be novel species, and the researchers proposed the name *B. xylanisolvens* based on their xylan-degrading properties. The main characteristics of the designated type strain are shown in **Table 1**.

The German Federal Institute for Occupational Safety in Health (*Bundesanstalt für Arbeitsschutz und Arbeitsmedizin*, BAuA) assessed the safety of *B. xylanisolvens* as a working material (BAuA, 2011). Due to the lack of pathogenicity and diseases, the agency classified the microorganism in the lowest risk group. The recently approved strain *B. xylanisolvens DSM 23964* was first analyzed by Ulsemer and colleagues in 2011 (Ulsemer et al., 2012a,b). They isolated this strain from human feces and performed several analytical techniques to verify that it was new. Biochemical characteristics (no catalase activity, no indole production, and incapable of degrading starch), phylogenetic analysis, and DNA-DNA hybridization classified the strain as *B. xylanisolvens*. So far, the performed analyses have established no differences between the new strain and the type strain. Finally, the Random Amplification of Polymorphic DNA profile revealed significant differences, confirming the existence of the new strain *B. xylanisolvens* DSM 23964.

Scientific Opinion of the EFSA Panel on *Bacteroides xylanisolvens* DSM 23964

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies carried out additional assessment for pasteurized milk products fermented with *B. xylanisolvens* DSM 23964 as a novel food under Regulation (EC) No 258/97 (European Commission, 1997b). This strain has no history of use in the food industry, and no strain in the genus *Bacteroides* has a proven history of use in food production. The novel food status is related to low-fat and skimmed milk products fermented with *B. xylanisolvens* DSM 23964 as the starter culture. After fermentation, the product is heat treated for 1 h at 75°C, and no viable cells of *B. xylanisolvens* are found in the final product.

The relevant phenotypic and genotypic data were provided by the applicant, and the EFSA Panel considered this information regarding the characterization of *B. xylanisolvens* DSM 23964 to be sufficient. The production process was clearly described in the application, and a study to guarantee the effectiveness of the heat treatment was conducted. Consequently, the EFSA Panel deemed the applied techniques to be standardized across the dairy industry, considered that they are sufficiently described, and identified no safety concerns (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2015).

The applicant also provided the anticipated intake of the product. The novel food would be marketed in liquid and semi-liquid forms in fermented, low-fat milk and skimmed milk products (fermented milk, buttermilk, yogurt, and yogurt drinks) or as a spray-dried powder (the fillings and coatings of cereals, cereal bars, fruits, and nuts). Due to a lack of European data, the applicant used consumption data from the United States. A conservative scenario was used to estimate the intake, proposing that the applicant's products would replace all existing products (e.g., yogurt and buttermilk; EFSA Panel on Dietetic Products, Nutrition and Allergies, 2015).

The applicant provided compositional data for spray-dried skimmed milk cultured with *B. xylanisolvens* DSM 23964, including an overview of relevant macronutrients. The applicant also supplied an overview of the vitamin B2, vitamin B12, free lysine, and furosine (as a marker for Maillard reaction) content to ensure that the heat treatment had no impact on these vitamins. Although, lactose content was missing from the analysis (a comparable amount to traditional products was assumed), the EFSA Panel considered consumption of the novel food to not be nutritionally disadvantageous.

The applicant also provided toxicological information about the novel food, including a study on an intraperitoneal abscess formation model in mice (Ulsemer et al., 2012b). Abscess formation is a relevant pathology because some species in the genus *Bacteroides*, such as *B. fragilis*, can induce abscesses on multiple sites in the body. *B. xylanisolvens* DSM 23964 was

TABLE 1 | General characteristics of *Bacteroides xylanisolvens*.

Phylum	Class	Order	Family	Genus	Species
Bacteroidetes	Bacteroidetes	Bacteroidales	Bacteroidaceae	*Bacteroides*	*Bacteroides xylanisolvens*
Type strain	DSM 188367 (XB1AT)				
Biosafety level	1				
Origin	Faecal sample from a healthy adult volunteer				
CHARACTERISTICS					
Morphology	Pleomorphic, Gram-negative, rod-shaped bacterium with rounded ends,				
	1.8–2.5 μm (length), 0.2–0.3 μm (width), no endospores, single or paired cells, no filaments				
Genome	Size: 6,059 kb, ORF's: 4,922, GC content: 41%				
Physiology	Chemo-organoheterotroph, mesophilic (25–42°C—optimum 38°C), pH optimum 6.8, obligate anaerobe				
	Indole negative, catalase negative, xylan positive, D-mannitol positive				
	No starch utilization				

administered in varying doses, but none of the mice developed abscesses. Human studies were also supplied to the EFSA (Ulsemer et al., 2012a,c).

Although, the applicant did not provide material regarding the allergenicity of the product, the EFSA panel judged that it would be unlikely that the allergenic potential would differ from that of other fermented dairy products. The EFSA itself had previously considered the effect of heat treatment on the allergenicity of milk (EFSA Panel on Dietetic Products EFSA Panel on Dietetic Products, Nutrition and Allergies, 2014).

QPS Evaluation of *B. xylanisolvens*

When the EFSA receives an application for a novel bacterial strain or a food product containing a novel strain, it conducts a mandatory, in-depth evaluation based on the QPS scheme. Consequently, after the EFSA Panel on Dietetic Products, Nutrition, and Allergies was asked to perform an additional assessment on *B. xylanisolvens* DSM 23964, the EFSA Panel on Biological Hazards (BIOHAZ) conducted a QPS assessment for *B. xylanisolvens* (EFSA Panel on Biological Hazards, 2014). This panel found that the published studies about *B. xylanisolvens* were not sufficient to include the organism on the QPS list, although no relevant safety concerns could be established.

The *cepA* gene in the genomic DNA of *B. xylanisolvens* DSM 23964 provides the strain with resistance to β-lactam antibiotics (Ulsemer et al., 2012b). This resistance is very common in the genus *Bacteroides* (Wexler, 2007). No mobile elements, such as conjugative transposons and plasmids, were found. The strain was also screened for 8 potential virulence genes common in other *Bacteroides* species, but none was detected, and *B. xylanisolvens* DSM 23964 showed no adhesion to Caco-2 cells in the cell culture model (Ulsemer et al., 2012b). The panel considered transfer of genes unlikely to take place due to the heat inactivation and lack of plasmids.

The panel emphasized that the body of knowledge was insufficient. *B. xylanisolvens* had no history of use in fermentation processes, and the few existing studies were limited to fermented milk products. Although, safety concerns do not seem likely, the panel found the number of published studies to be too low to definitely exclude safety issues. The pilot studies involved only small, healthy cohorts, and the administered bacterial cells were inactivated. Considering all these arguments, the panel did not recommend putting *B. xylanisolvens* on the QPS list (EFSA Panel on Biological Hazards, 2014).

Bacteroides xylanisolvens and Its Beneficial Properties

Most bacteria found in the human intestine are anaerobic. Among the many requirements for a bacterial strain to be categorized as probiotic, an essential characteristic is survival along the gastro-intestinal tract to finally provide beneficial effects for the host. *B. xylanisolvens* DSM 23964 had a survival rate of 90% after spending 3 h in simulated gastric juice and a survival rate of 96% after spending 4 h in simulated intestinal juices (Ulsemer et al., 2012b).

Immunomodulatory properties are important attributes of probiotics. The genus *Bacteroides* achieved a higher induction of mucosal IgA production than *Lactobacillus* when co-cultured with Peyer's patches lymphocytes (Yanagibashi et al., 2009).The fermentation of dietary polysaccharides with production of SCFA by *B. xylanisolvens* is connected to health-promoting effects through lower cholesterol levels, satiety stimulation, and even an anti-carcinogenic effect (Hosseini et al., 2011).

Published studies on fermented milk products containing *B. xylanisolvens* DSM 23964 are limited to inactivated bacterial cells, but by definition, probiotics need to be alive when administered to the consumer. Moreover, despite this microorganism's high survival and ability to ferment carbohydrates, these properties are irrelevant in the context of the approved product because it contains a heat-killed strain; therefore, no survival or metabolic activity is expected.

B. xylanisolvens offers several potential beneficial properties. However, its inability to bind to epithelial cells *in vitro* (Ulsemer et al., 2012b) is a major disadvantage in the assessment of probiotic properties because the capability to bind to intestinal epithelial cells in the host is a key step in probiotic activity. As well, studies with living bacteria are needed to increase the chances of acceptance as a probiotic.

Admission of *B. xylanisolvens* as a Novel Food: Impact on Other Candidates

B. xylanisolvens is only the fourth bacterium, after *L. mesenteroides*, *B. subtilis* natto, and *C. butyricum*, to be approved under Novel Food Regulation No 258/97 (European Commission, 1997b).

The positive outcome of the novel food assessment is independent from the QPS evaluation results. *B. xylanisolvens* and *C. butyricum* have not yet been included on the QPS list. It should be pointed out that during the QPS assessment, only the species itself is analyzed, and the potential food products containing the bacteria are not of interest.

Thermal inactivated bacterial form falls out of the accepted definitions of probiotics described above, because the viability of bacterial cells is an essential condition. Nevertheless, non-viable and therefore non-culturable, and immunologically active microbial cells have been reported to provide health benefits to consumers (de Almada et al., 2016) The use of killed bacteria, as long as the beneficial effect is totally or partially retained, represent and advantage when the use of live bacteria is not authorized or may be potentially harmful for some individuals such as patients with weak immune system or under inflammatory conditions (Taverniti and Guglielmetti, 2011; Tsilingiri and Rescigno, 2012). To define the use of non-viable microorganisms which, when administered in adequate amounts, confer a benefit on the consumer the term "paraprobiotic" has been proposed (Taverniti and Guglielmetti, 2011; de Almada et al., 2016), but it still is not a wide accepted and employed term by scientific community and institutions.

Several mechanistic studies have demonstrated that specific chemical compounds isolated from bacteria can induce specific immune responses (Taverniti and Guglielmetti, 2011). In these cases, the term "postbiotic" has been proposed to refer any factor resulting from the metabolic activity of a bacteria or

any released bacterial molecule capable of conferring beneficial effects to the consumer in a direct or indirect way (Tsilingiri and Rescigno, 2012; de Almada et al., 2016). But, similarly with "parabiotic" concept is not an officially accepted and used term.

The acceptance of the thermal inactivated form of *B. xylanisolvens* as a novel food may pave the way for other novel microorganisms in non-viable form or novel bacterial molecules to exert positive health impacts.

Akkermansia muciniphila

The relatively newly described species *A. muciniphila* is the first discovered taxonomic member in the genus *Akkermansia*. By June 2017, there has been no evaluation according to the QPS status, which would be performed and published by EFSA. Due to the recent description of this microorganism, it has no history of use in the food industry, so it must be treated according to the Novel Food Regulation (European Commission, 1997b).

A. muciniphila was discovered in 2004 during the search for new mucus-degrading bacteria in human fecal samples. The newly isolated organism was named after Antoon Akkermans, a well-respected Dutch microbiologist who has made significant contributions to the field of microbial ecology (Belzer and De Vos, 2012). This oval-shaped, Gram-negative microorganism belongs to the phylum Verrucomicrobia. *A. muciniphila* further belongs to the class Verrucomicrobiae, the order Verrucomicrobiales, and the family Verrucomicrobiaceae. *A. muciniphila* was originally classified as a strict anaerobe, but in the same way as other anaerobic gut colonizers such as *Bacteroides fragilis* and *Bifidobacterium adolescentis*, *A. muciniphila* can tolerate small amounts of oxygen and even can use it at low oxygen concentrations (Ouwerkerk et al., 2016b). This contrasts with some of the butyrate producing bacteria that are really strict anaerobes.

Metagenomic analysis have indicated that at least 8 additional *A. muciniphila*-related species are present in the human intestine (van Passel et al., 2011). Belzer and de Vos suggested that the genus *Akkermansia* consists of 5 distinct clades (Belzer and De Vos, 2012). Four of these 5 clades are associated with *A. muciniphila*, with sequence similarity range of 80–100%.

Nearly all investigated mammals showed *Akkermansia*-related sequences in their intestinal samples. *Akkermansia*-derived sequences are not limited to mammals but also found in other vertebrates: similar sequences were detected in zebrafish and the Burmese python (Costello et al., 2010; Roeselers et al., 2011). From the latter a new species, *Akkermansia glyciniphila* was isolated and characterized from reticulated python (Ouwerkerk et al., 2016a, 2017). These ubiquitous findings suggest that *Akkermansia* played a crucial role in the early stages of vertebrata evolution and that its presence might be related to essential functions in the intestine.

Genomic analyses revealed that the single chromosome of *A. muciniphila* has 2,176 genes with a GC content of 55.8% (Donohue and Salminen, 1996). The secretome responsible for the degradation of mucin involves 61 different proteins, representing 11% of the total protein content (Belzer and De Vos, 2012).

Akkermansia is a relatively new genus, so it is expected that additional species similar to *A. muciniphila* will be found in coming years as is illustrated by the discovery of *A. glyciniphila*. It obviously has no history of use in the food sector, and its potential areas of application should be explored.

Despite the short time since the discovery of *Akkermansia*, its basic characteristics are well-known. This organism is non-motile and specialized in degrading mucin. *A. muciniphila* is anaerobe and chemo-organotroph and can use mucin as its sole nitrogen, carbon and energy source (Derrien et al., 2004). The habitat of *Akkermansia* spp. is also well-documented due to its ubiquitous occurrence in the intestines of many vertebrates (Belzer and De Vos, 2012). The general properties of *A. muciniphila* are listed in **Table 2**.

Safety Aspects

A. muciniphila is a common inhabitant of the human intestine, accounting for 1–4% of the overall colon microbiota (Derrien et al., 2008). Currently, there are no published clinical human trials in which viable cells were administered to people. Such studies have been conducted only with animal models (Everard et al., 2013). Lagier and colleagues reported two cases in humans where *A. muciniphila* accounted for up to 80% of the total

TABLE 2 | General characteristics of *Akkermansia muciniphila* -adapted from Gomez-Gallego et al. (2016).

Phylum	Class	Order	Family	Genus	Species
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Akkermansia*	*Akkermansia muciniphila*
Type strain	MucT (ATCC BAA-835)				
Biosafety level	1				
Origin	Faecal sample from a healthy adult volunteer				
CHARACTERISTICS					
Morphology	Gram-negative, oval-shaped, non-motile				
Genome	Size: 2,664,102 bp, ORF's: 2,176, GC content: 55.8%				
Physiology	Chemo-organotrophic, anaerobic, mesophilic (20–40°C—optimum 37°C)				
	Capable of using mucin as energy, nitrogen, and carbon source; mucolytic in pure culture				
	Sulfate release in free form from mucin fermentation				

intestinal microbiota without any noticeable decline in health (Lagier et al., 2015), and there is no evidence that *A. muciniphila* is connected to any specific disease (Derrien et al., 2010).

There are general concerns because *Akkermansia* shows pathogen-like behavior. Adhesion to mucus is considered to be a crucial step during infection but is also a behavior found in several probiotics. In contrast to pathogens, *A. muciniphila*, as a mucin degrader, stays in the outer layer of the mucus and never reaches the inner layer, which is necessary for a successful infection (Gomez-Gallego et al., 2016). In addition, mucin degradation itself resembles pathogen-like behavior (Donohue and Salminen, 1996) but is regarded as a regular process in a balanced, self-renewing intestine (Gomez-Gallego et al., 2016).

Colorectal cancer patients showed a four-fold increase of *A. muciniphila* in stool samples compared to healthy subjects (Weir et al., 2013). However, patients with colorectal cancer have reduced food intake, and studies have demonstrated that fasting correlates with increased levels *of A. muciniphila* (Remely et al., 2015). In addition, colorectal cancer is related to increased cell proliferation and mucus production; therefore, it is evident that levels of the main mucus-degrading bacterium could increase in this situation (Gomez-Gallego et al., 2016).

Overall, there seem to be no specific safety concerns regarding the genus *Akkermansia* and its type strain *A. muciniphila* MucT. Potential future concerns could be eliminated through individual, case-by-case review. There are no applications so far for *A. muciniphila* and, therefore, no history of safe use, so the QPS status of this species has not yet been determined.

Akkermansia Muciniphila as Candidate for Novel Food

Currently, there are no pending applications for products containing *A. muciniphila* under Regulation (EC) 258/97 (European Commission, 1997b). *A. muciniphila* was only discovered and isolated in 2004, so comprehensive research is still needed to better understand this microorganism.

A. muciniphila was not used for human consumption to any "significant degree" in the EU before 15 May 1997. A food business operator interested in putting a product containing *A. muciniphila* in the market would have to submit an application according to the Novel Food Regulation. The European Commission published Recommendation (European Commission, 1997a) to give food companies the information needed for a successful application. In the case of *A. muciniphila*, there are still many unknown aspects that are needed for Novel Food application, as well as some limitations: (1) specification of the possible novel products which will contain *A. muciniphila*; (2) production processes to keep *A. muciniphila* alive in the final product to fulfill its potential probiotic properties; (3) anticipated human intake taking into account the lack of known intake patterns; (4) the lack of clinical trials in humans (toxicological and nutritional assessments have been conducted only with animal models); and (5) the lack of knowledge about the allergenic potential.

Based on the knowledge acquired so far, though, this microorganism could be very interesting for food companies and consumers. *A. muciniphila* is part of the gut microbiota in all humans and may display probiotic properties due to its contribution to the functional gastro-intestinal tract. In addition, current data indicate that a decline in *A. muciniphila* in the colon correlates with several diseases, such as obesity, type-2 diabetes, and inflammatory bowel disease (Png et al., 2010; Cani and Everard, 2014; Schneeberger et al., 2015). This reduction could be related with a decrease in mucus thickness. But the complex relation between *A. muciniphila* and the mucus layer remains unclear and the administration of *A. muciniphila* might stimulate the recovery of the mucus layer (Everard et al., 2013).

Recently, *A. muciniphila* has been demonstrated to provide beneficial effects even after heat treatment for 30 min at 70°C. The heat-inactivated bacteria could reduce fat mass development, insulin resistance, and dyslipidemia in obese and diabetic mice. Some membrane proteins showed stability and interaction with Toll-like receptor 2 even during the thermal process. This membrane protein also improved the gut barrier and provided ongoing beneficial effects after bacterial inactivation (Plovier et al., 2017). Like *B. xylanisolvens*, therefore, *A. muciniphila* may enter the food market in this non-viable form.

The link between obesity and decreased levels of *A. muciniphila* extends beyond rodents. A Swedish investigation showed that obese pre-school children have significantly lower levels of *A. muciniphila* than their normal-weight peers (Karlsson et al., 2012). Similarly, pre-school infants that used macrolide antibiotics showed a higher BMI than the non-antibiotic users and this was associated with a depletion of *A.muciniphila* (Korpela et al., 2016).

Despite the growing attention to *A. muciniphila*, much research is needed to gain more specific and age-restricted information. No randomized, double-blind, placebo-controlled human clinical trials have studied the effects of *A. muciniphila* intake. Most research has involved animals, and long-term studies on the human level are the next step to take. Despite the lack of information relevant to the European Commission's recommendation on novel food, the microorganism has quite promising prospects for future novel food applications, especially for its potential probiotic health benefits.

It is important to point out that *A. muciniphila* has been detected in breast tissue (Urbaniak et al., 2014) and then has been consumed by humans via breast milk (Collado et al., 2012). The question, therefore, arises whether it is necessary to prove the safety of an organism that is already part of nutrition in the early stages of life. However, the levels of viable cells in breast milk might be much lower than those added to food products. Safety concern may arise due not to the nature of *A. muciniphila* but to the levels used in food and supplements. However, assuming around 10^{13} bacteria to be present in the human intestine, an average healthy adult may carry over 10^{11} *Akkermansia* in its colon. Further discussions are needed, but for such specific cases, European legislation needs to be modified in the future. Placement of *A. muciniphila* on the QPS list under the given conditions may be complicated because a history of safe use cannot be established.

Fructophilic Lactic Acid Bacteria: New Members of the Family

Characteristics

Long before Orla-Jensen described the group of lactic acid bacteria (LAB) in 1919, humankind benefited from their positive attributes in various food production processes (von Wright, 2012). LAB are involved in various industrial fermentation processes, such as fermented milk products and sausages. Along with fermented food production, the growing sector of probiotics represents the biggest operational area for LAB.

Fructophilic lactic acid bacteria (FLAB) constitute a subgroup in the diverse LAB order. FLAB preferably use fructose as a substrate. They show poor growth on glucose, and external electron acceptors (e.g., pyruvate, oxygen, and fructose) dramatically enhance their growth. FLAB, therefore, inhabit fructose-rich niches: flowers, fruits, fermented foods, such as wine and cocoa beans, and even the gastro-intestinal tracts of insects harbor these microorganisms (Endo, 2012). The FLAB group is divided into the genera *Fructobacillus* (its type species: *F. fructosus*) and *Lactobacillus* (Endo et al., 2009).

Four *Fructobacillus* species formerly were members of the genus *Leuconostoc*, but recent analyses suggested that their unique characteristics and phylogenetic positions supported reclassification of this bacterial group (Endo and Okada, 2008). *Fructobacillus* shows a clear preference for fructose over glucose (Endo and Okada, 2008). Several findings related to metabolism, genome size, and gene losses suggest reductive evolution in *Fructobacillus* genus compared to *Leuconostoc*, providing strong support for the reclassification and renaming of *Fructobacillus* (Endo et al., 2015).

The number of species in FLAB subgroup is growing in both genera, *Fructobacillus* and *Lactobacilllus*. Fructobacillus consists of five species: *F. fructosus, F. pseudoficulneus, F. ficulneus, F. durionis,* and *F. tropaeoli* (Endo et al., 2015). *F. fructosus* and *F. pseudoficulneus* are the most frequently detected species in natural sources. These microorganisms have been isolated from figs, bananas, flowers, the honeybee gut, wine, and even taberna, a traditional beverage in Southern Mexico (Alcantara-Hernandez et al., 2010; Endo, 2012). The FLAB members in the genus *Lactobacillus* are *L. kunkeei, L. apinorum*, and *L. florum* (Neveling et al., 2012). *L. kunkeei* was originally isolated from wine and characterized as obligately FLAB (Endo et al., 2012). Interestingly, this microorganism is the major component in the gut microbiota of honeybees (Endo and Salminen, 2013). *L. apinorum* is a recently described species from honeybees, and its fructophilic characteristic were recently reported (Maeno et al., 2017). These two lactobacilli share 98.9% sequence similarity based on 16S rRNA gene sequences. The genomic characteristics of the two species, especially the gene reduction system, are similar to *Fructobacillus* spp. but not to other members of lactobacilli, suggesting that fructose-richness induced an environment-specific gene reduction in phylogenetically distant microorganisms (Maeno et al., 2016). *L. florum* was formerly the only facultatively FLAB. It is differentiated from the obligately FLAB based on the growth ratio on glucose. *L. florum* grows more slowly on glucose than fructose. Like obligately FLAB, electron acceptors enhance growth on glucose.

Practical Impact and Safety

Although, FLAB were only recently discovered, this group of microorganisms might have great potential for the food industry. LAB are generally considered to be safe and have been involved in human food production and nutrition since ancient times (von Wright, 2012). These properties might be extended to FLAB. Recent investigations detected the presence of FLAB in many different food items. For example, several *Fructobacillus* spp. have been observed during the spontaneous cocoa bean fermentation process (Lefeber et al., 2011; Papalexandratou et al., 2011), and *L. florum* has been found in grapes and wine, possibly contributing important properties from an oenological perspective (Mtshali et al., 2012).

The presence of FLAB in the gastro-intestinal tracts of bees, giant ants, tropical fruit flies, and bumblebees indicate possible probiotic characteristics (Endo, 2012), although FLAB has not been detected in vertebrates' intestines (Endo and Salminen, 2013). Bee commensal *L. kunkeei* is considered to be a candidate for honeybee probiotics and paratransgenesis. There is no clinical evidence regarding the pathogenicity of FLAB, which might be an indicator of potential safety. However, this possibility should be treated cautiously because consumers' level of exposure to this bacterial group through food is not well known. Recent studies have suggested that administration of heat-killed *L. kunkeei* YB38 has potential beneficial properties for human health, including increased bowel movements and enhanced immunoglobulin A production (Asama et al., 2015, 2016). As in the case of *B. xylanisolvens*, therefore, *L. kunkeei* might enter the food market in thermal-treated form.

Faecalibacterium prausnitzii

Fusobacterium prausnitzii is the sole member of the genus *Faecalibacterium* and a commensal bacterium of the human gut microbiota (Miquel et al., 2013). This Gram-positive, obligately anaerobe microorganism belongs to class Clostridia. It accounts for 3–5% of total fecal bacteria and, therefore, is a predominant species in human feces (Breyner et al., 2017). *F. prausnitzii* is a non-motile, non-spore-forming bacterium and extremely sensitive to oxygen (Foditsch et al., 2014). *F. prausnitzii* was initially classified as *F. prausnitzii*, but the sequence of the 16s rRNA gene established that it is only distantly related to *Fusobacterium* and more closely related to members of *Clostridium* cluster IV (Miquel et al., 2013). The general properties of *F. prausnitzii* are listed in **Table 3**.

F. prausnitzii can digest dietary fibers producing mostly butyrate, along with other SCFA (Miquel et al., 2013), and has been shown to respond to prebiotic supplementation using a mixed chain length fructan supplement and pectin (Scott et al., 2015). This microbe may play a crucial role in human health because changes in levels of *F. prausnitzii* have been associated with several gastrointestinal diseases, such as Crohn's disease, and depressive disorders (Miquel et al., 2013; Jiang et al., 2015). The microorganism also displays beneficial anti-inflammatory effects on the host, suggesting that it may be used to counterbalance the dysbiosis linked to certain diseases (Sokol et al., 2008; Jiang et al., 2015; Miquel et al., 2015). Butyrate has several beneficial effects on the host, including

TABLE 3 | General characteristics of *Faecalibacterium prausnitzii*.

Phylum	Class	Order	Family	Genus	Species
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Faecalibacterium*	*Faecalibacterium prausnitzii*
Type strain	ATCC 27768				
Biosafety level	1				
Origin	Faecal sample from a healthy adult volunteer				
CHARACTERISTICS					
Morphology	Gram-positive, obligately anaerobe, oval-shaped, non-motile, non-spore-forming				
Genome	Size: 3.05 Mb, ORF's: 2,745, GC content: 56.4%				
Physiology	Chemo-organotrophic, anaerobic, mesophilic (optimum 37°C), pH for growth ranges between 5.7 and 6.7				
	Butyrate, D-lactate, and formate production during glucose fermentation				
	Can utilize pectin, uronic acid, and host-derived substrates for growth				

provision of an energy source for epithelial cells, induction of colonic regulatory T cells, induction of apoptosis in human colonic carcinoma cells, inhibition of inflammatory responses in intestinal biopsy specimens, and improvement of metabolic syndrome. Moreover, recent investigations have revealed possible contributions to protective mechanisms, such as self-defense against inflammatory reactions. *F. prausnitzii* has been involved in the inhibition of pro-inflammatory cytokines and the secretion of bioactive molecules which lead to blockage of the NF-κB pathway, showing protective effects in chemically induced colitis mouse models (Breyner et al., 2017). Further research with *Faecalibacterium* could increase knowledge about its probiotic effects and may lead to its future use in the food industry.

The successful use of *F. prausnitzii* as probiotic most likely will depend on the administration method. Studies related to bacterial resistance to gastric pH *in vitro* recommend oral administration of *F. prausnitzii* after feeding to avoid low gastric pH (Breyner et al., 2017). Moreover, cultivating *F. prausnitzii* is difficult even in anaerobic conditions (Miquel et al., 2013).

The inclusion of *Faecalibacterium* on the QPS list might be difficult due to its lack of a history of safe use and to the multiple antibiotic resistance genes observed in *Faecalibacterium* sp. isolates which could contribute to resistance dissemination (Breyner et al., 2017). In addition, full toxicology assays and characterization of the strain are still needed for regulatory approval (Thomas et al., 2015).

CONCLUSIONS AND OUTLOOK

Remarks on the Candidates

A brief evaluation of the benefits and drawbacks of the new microbial candidates based on existing knowledge is given in **Table 4**. Novel microbes, including many not yet assessed in humans, are increasingly proposed as potential probiotics and assessed as novel food. The introduction of these new species further challenges the traditional selection criteria because the question of their safety and efficacy arises in an untraditional manner, with no previous experience in exposure to these bacteria or their use in foods or supplements. Most probiotics belong to well-known microbial groups (lactobacilli and bifidobacteria) with long histories of safe use, which facilitates preliminary evaluation of their safety and functionality based on the available body of knowledge about these groups. However, some newly proposed probiotics were first described, cultured, and isolated during the past decade and thus present a challenge to both scientific and regulatory frontiers. Nevertheless, *A. muciniphila*, FLAB, and *F. prausnitzii* are regarded as promising candidates. The many proven and potential probiotic properties provide good opportunities for future food authorizations, but their safety in human trials still must be demonstrated.

In the context to the definition of probiotics, Plovier and colleagues made an especially interesting observation: even the pasteurized and non-replicating *Akkermansia* cells were capable of providing beneficial effects. Decreased fat-mass development, dyslipidaemia, and insulin resistance were demonstrated in obese and diabetic mice (Everard et al., 2013). This new insight might have crucial relevance and allow future authorizations because nonviable cells pose rise fewer concerns than living bacteria. Nonviable bacteria are more easily deemed safe in novel food evaluation, albeit usually in case-by-case decisions. In comparison to *B. xylanisolvens*, some information about *A. muciniphila* is still lacking. Due to the absence of practical applications, specification of the novel food and its production process and nutritional information has not yet been performed. However, there is information available about its taxonomy, culturing methods, pathogenic and toxicological nature, and nutritional assessment data in animal models (Donohue and Salminen, 1996). A major key for the approval of novel food is a sufficient number of clinical trials in humans, but *A. muciniphila* lacks enough appropriate human studies. Randomized, double-blind, placebo-controlled clinical trials, dose-response studies, and toxicological studies are still needed to, for example, establish the appropriate number of bacteria to be administered and the right matrix to provide probiotic properties. If the missing data become available, though, *A. muciniphila* has similar chances of acceptance as a novel food as the recently officially accepted *B. xylanisolvens*. Notably the EFSA positively assessed *B. xylanisolvens* although the applicant supplied only two human studies.

FLAB were also discovered and described recently, so it is challenging to predict how they might be accepted as part of future food products. Further research is needed to

TABLE 4 | Examples of potential future probiotics suggested for human use and/or assessed as novel foods in the European Union.

Bacteria species/strain	Safety assessment in European Union	Expected impact on humans	Effects (*in vitro*)	Effects (*in vivo*)	References
Clostridium butyricum/ *Clostridium butyricum* MIYAIRI 588 *Clostridium butyricum* CGMCC0313.1	Yes	Probiotic effects suggested based on Japanese studies	Butyrate production Antitumor effect Reduction on lipogenesis	SCFAs production Prevention of antibiotic-associated diarrhea (combined with *Bifidobacterium*) Protective effect on gastric ulcers Modulation of lipid profile, insulin resistance and colon homeostasis	Nakanishi et al., 2003; Shinnoh et al., 2013; Zhao et al., 2014; Wang et al., 2015; Shang et al., 2016
Bacteroides xylanisolvens/ *Bacteroides xylanisolvens* DSM 23964 *Bacteroides xylanisolvens* XB1A	Yes, but only in the pasteurized form without any viable microbes in the product	Immune protection effects suggested	Dietary fiber degradation and fermentation	Immune modulation Toxicity and safety assessment Tolerance to a regular oral intake assessment	Mirande et al., 2010; Ulsemer et al., 2012a,b,c, 2016; Li et al., 2016
Akkermansia muciniphila	Potential evaluation models suggested Novel—no EFSA evaluation available yet	Weight control, control of low-grade inflammation and prevention of type 2 diabetes	Adherence to the intestinal mucosa and effect on enterocytes	Improvement of glucose tolerance and attenuation of adipose tissue inflammation Reduction of fat-mass gain Regulation of gut inflammation, gut barrier, and gut peptide secretion Reduction of metabolic endotoxemia Attenuation of atherosclerotic lesions	Donohue and Salminen, 1996; Everard et al., 2013; Reunanen et al., 2015; Schneeberger et al., 2015; Li et al., 2016; Yassour et al., 2016
Fructophilic lactic acid bacteria: *Lactobacillus kunkeei* *Fructobacillus fructosus* *Lactobacillus florum*	Novel—no evaluation available	Selective fermentation of fructose Production of antibacterial compounds	Antibacterial activity Fructophilic properties	Reduction of microbial pathogens	Endo et al., 2009, 2010; Endo, 2012; Endo and Salminen, 2013
Faecalibacterium prausnitzii/ *Faecalibacterium prausnitzii* A2-165	Novel—no evaluation available	Beneficial anti-inflammatory effects suggested Butyric acid production	Production of anti-inflammatory compounds Inhibition of IL-17 release in human monocytes Butyrate production	Increase of plasma anti-Th17 cytokines and suppression of IL-17 levels in both plasma and colonic mucosa Normalization of altered colonic permeability Protective effect against colitis	Miquel et al., 2013; Zhang et al., 2013; Laval et al., 2015; Ulluwishewa et al., 2015; Quevrain et al., 2016

The table includes some selected examples of reported positive health effects based on in vitro and in vivo studies.

understand, for example, what differentiates this bacterium from traditional LAB and its exact relevance to the production of fermented foods. Recent studies revealed that heat-killed *L. kunkeei* possess beneficial properties, such as increased bowel movement and enhanced immunoglobulin A production (Asama et al., 2015, 2016). Their special relation to LAB, a very widely used group in the food industry, may lead to inclusion on the QPS list and novel food authorization in the future.

The growing amount of evidence supporting that the modulation of *F. prausnitzii* levels using prebiotics, probiotics, and symbiotics might have prophylactic or therapeutic applications in human health makes this bacterium interesting from the perspective of novel foods. Factors such as sensitivity to oxygen, gastric pH, and bile salts and industrial production for probiotic use need to be optimized, and toxicological assays and antibiotic resistance tests should be carefully conducted before clinical trials in humans.

The potential candidates for novel foods discussed as examples in the present work represent only a small fraction of the potential bacteria that may be included in novel foods in the future. Other potential candidates are *Eubacterium hallii* and bacteria in the genus *Roseburia*. *E. hallii* is a common inhabitant of the human gut microbiota and is known for its versatile utilization of different carbon sources. *E. hallii* can produce butyrate from lactate, acetate, and glucose and is one of the first butyrate producers in the infant gut (Pham et al., 2016). In addition, a recent study showed the impact of *E. hallii* in treating insulin resistance in a mouse model (Udayappan et al., 2016). Its early appearance in the human intestine and production of essential SCFA may enable future novel food applications in the food industry.

The genus *Roseburia* consists of five species which are Gram-positive, obligately anaerobic bacteria and motile due to the presence of subterminal flagella (Tamanai-Shacoori et al., 2017). *Roseburia* spp. are a dominant intestinal bacterial species,

accounting for 2–15% of the total human gut microbiota that produce SCFA (Dostal et al., 2015). Considering the production of essential SCFA and the high levels of *Roseburia* spp. in the human microbiota, members of this genus may have great potential as future novel probiotics.

Remarks on Regulatory Aspects

Ongoing revisions of the regulations on novel food, along with the continuous updating of the QPS list, will accelerate novel food authorization and biological agent safety assessment. Both processes support the work of the EFSA and access to novel products in the food market and ensure the safety of the European consumer. The lengthy process to revise the novel food regulations has demonstrated the difficulty of changing legislation on the European level. Most decisions need to be unanimous, and it is challenging to please all member states. Although, these developments are remarkable, the EU authorization of novel food still seems more comprehensive than the GRAS concept in the United States. The precautionary principle is a fundamental pillar of environmental, food, and health policies in the EU. Prevention of any possible harm or danger to the consumer is the most important priority, especially for marketing of food containing potentially harmful bacteria. The elimination of any possible health threat, therefore, is essential before a product is allowed to enter the European market. The European consumer is the most relevant player in European legislation although companies understandably complain about such strict conditions.

Regulation (EU) 2015/2283 on novel foods (European Commission, 2015), which will come into force on January 1, 2018, is aimed at facilitating the authorization of novel food. The EFSA is responsible for the scientific risk assessment, ensuring the shift from an individual assessment by the national competent authorities to a generic assessment. The new regulation also directs the EFSA to deliver scientific opinions within 9 months after receiving all valid applications. This mandate represents enormous time savings and a significant decrease in the financial costs for food companies submitting applications. The newly introduced data protection for approved novel food authorization will also help companies successfully compete in the food market.

AUTHOR CONTRIBUTIONS

WK, SS, and CG designed the study; TB performed the bibliographical review; TB and CG drafting the manuscript; AE, GV, MG, Wd, WK, and SS contributed with the latest achievements in their field of expertise. AE, MG, GV, Wd, WK, SS, and CG interpreted the main points related with EU legislation discussed in the review for each novel bacteria. AE, MG, GV, Wd, WK, SS, and CG discussed the final version and revising them critically. All the authors give the final approval of the version to be published.

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REVIEW
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Emerging Trends in "Smart Probiotics": Functional Consideration for the Development of Novel Health and Industrial Applications

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The link between gut microbiota and human health is well-recognized and described. This ultimate impact on the host has contributed to explain the mutual dependence between humans and their gut bacteria. Gut microbiota can be manipulated through passive or active strategies. The former includes diet, lifestyle, and environment, while the latter comprise antibiotics, pre- and probiotics. Historically, conventional probiotic strategies included a phylogenetically limited diversity of bacteria and some yeast strains. However, biotherapeutic strategies evolved in the last years with the advent of fecal microbiota transplant (FMT), successfully applied for treating CDI, IBD, and other diseases. Despite the positive outcomes, long-term effects resulting from the uncharacterized nature of FMT are not sufficiently studied. Thus, developing strategies to simulate the FMT, using characterized gut colonizers with identified phylogenetic diversity, may be a promising alternative. As the definition of probiotics states that the microorganism should have beneficial effects on the host, several bacterial species with proven efficacy have been considered next generation probiotics. Non-conventional candidate strains include *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Bacteroides fragilis*, and members of the Clostridia clusters IV, XIVa, and XVIII. However, viable intestinal delivery is one of the current challenges, due to their stringent survival conditions. In this review, we will cover current perspectives on the development and assessment of next generation probiotics and the approaches that industry and stakeholders must consider for a successful outcome.

Keywords: FMT, next generation probiotics, bacterial consortium, synthetic community, CDI

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INTRODUCTION

The gut microbiota plays a significant role in human health, participating in several functions beneficial to the host (Patel and DuPont, 2015; Kristensen et al., 2016). It has been implicated in preventing pathogen colonization (Hand, 2016), shaping our immune system (Round and Mazmanian, 2009; Patel and DuPont, 2015; Macpherson et al., 2017), stimulating the production of gastrointestinal hormones (Saulnier et al., 2013), and regulating brain behavior (De Palma et al., 2014, 2017) through production of neuroactive substances (Steenbergen et al., 2015; Kristensen et al., 2016).

Additionally, the gut microbiota has been involved in the fermentation of non-digestible carbohydrates reaching the colon. This process leads to the production of short chain fatty acids (SCFAs), which elicit health benefits (den Besten et al., 2013). The human gut microbiota can be manipulated through either passive or active processes. Passive factors include hygiene, lifestyle, and diet. For instance, primary colonizers of the gut involved in the immune development are shifted by sanitary practices (Zhou, 2016). In addition, dietary constituents can promote phylogenetic variations in the microbiota (Graf et al., 2015). In this context, prebiotics are defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). Prebiotics act as growth substrates (Patrascu et al., 2017) to enhance the activity of bacterial genera (Scott et al., 2015) such as bifidobacteria and butyrate-producing clostridia (Rivière et al., 2016). SCFA and vitamins resulting from the fermentation of these components are crucial for human health (Graf et al., 2015). In terms of lifestyle factors, physical activity is known to positively impact the diversity of gut microbiota. In fact, gut microbiota of athletes is more diverse than that of non-athletic subjects (Clarke et al., 2014). Amongst the active processes manipulating microbiota composition are antibiotics and probiotics. Antibiotic use has been linked to dysbiosis (Langdon et al., 2016), even leading to low diversity, evenness, and taxonomic richness (Dethlefsen and Relman, 2010; Francino, 2016). Moreover, presence and expression of microbial genes are altered following antibiotic therapy (Reijnders et al., 2016). These detrimental outcomes may lead to decreased SCFA, glycolysis, vitamin production, homeostasis of the immune system, and impaired protection against pathogens (Guarner and Malagelada, 2003). As a result, antibiotic associated diarrhea (AAD) and recurrent infectious diseases like *Clostridium difficile* infection (CDI) may occur (Francino, 2016).

On the other side of the spectrum are probiotics, which can affect the host either directly or through their products, or even influence the activity of resident bacteria in the host (Scott et al., 2015). Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (WHO/FAO, 2006; Hill et al., 2014). The effect of probiotics in preventing metabolic syndromes such as obesity, type 2 diabetes (Kasińska and Drzewoski, 2015), and dyslipidemia has been reported (Asemi et al., 2013). For instance, administration of *Bifidobacterium* (Yin, 2010; Chen et al., 2011; Plaza-Diaz et al., 2014; Reichold et al., 2014; Savcheniuk et al., 2014; Wang et al., 2014) and *Lactobacillus* species reduced body weight gain and adipose tissue in mice fed high-fat diet through stimulation of adiponectin production (Kim et al., 2013; Kobyliak et al., 2016). In addition, lactobacilli have been proven to have therapeutic effects in different pathologies (Di Cerbo et al., 2016). Moreover, probiotics regulate the mucosal immune response (Klaenhammer et al., 2012), improving the activity of macrophages (Sang, 2010) and changing the expression of the genes associated. Even though these outcomes depend on specific bacteria and strains, probiotics may interact with TLR and downregulate the expression of NF-κB and pro-inflammatory cytokines (Ng et al., 2009; Plaza-Diaz, 2014). For instance, peptides of microbial anti-inflammatory molecules (MAMs) that are found in the *Faecalibacterium prausnitzii* supernatant inhibit the NF-κB pathway *in vitro* and *in vivo* (Breyner et al., 2017), confirming the anti-inflammatory and therapeutic properties of *F. prausnitzii* (Martín et al., 2014). These properties and protective effects of *F. prausnitzii* were identified in different models such as dinitrobenzene sulfate (DNBS)-induced colitis model, dextran sodium sulfate (DSS)-induced colitis (Breyner et al., 2017), and 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced acute colitis in mice (Miquel et al., 2015). Additionally, levels of anti-inflammatory cytokines and immunoglobulins, immune cell proliferation, and production of proinflammatory cytokines produced by the T cells may be modulated following probiotic supplementation (Miettinen et al., 1996; Nazemian et al., 2016). Furthermore, probiotics can be alternative strategies for inflammatory disorders, as they upregulate the production of CD4+Foxp3+ regulatory T cells (T_{regs}) (Kwon et al., 2010; Yan and Polk, 2011).

Different effects on the immune function may be species- and strain-related (Klaenhammer et al., 2012). It has been reported that probiotics have therapeutic effect on the central nervous system by reducing the intestinal inflammation. In this way, the regulation of HPA axis and the activity of the neurotransmitters may be improved (Wallace and Milev, 2017). Probiotics from *Bifidobacterium* and *Lactobacillus* genera are usually delivered through fermented products such as yogurts, milk, and cheeses, or they can be delivered as food supplements (Besseling-van der Vaart et al., 2016).

MONOSTRAIN AND MULTISTRAIN PROBIOTICS

Probiotics have been categorized into monostrain or multistrain/multispecies products (Timmerman et al., 2004). Different studies have confirmed positive effects on health when multistrain probiotics are used, due to the symbiosis among strains (Timmerman et al., 2004). Strains in multispecies probiotics can be from different genera. For instance, the efficacy of the multispecies probiotic consortium VSL#3 (*Streptococcus thermophilus*, *Eubacterium faecium*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei,* and *Lactobacillus delbrueckii* subspecies *bulgaricus*) was proven for the treatment of ulcerative colitis (Venturi et al., 1999; Timmerman et al., 2004). Besides, VSL#3 supplementation in women with gestational diabetes mellitus (GDM) may help regulate inflammatory markers and positively influence glycemic control (Jafarnejad et al., 2016). In addition, Chapman et al. (2011) described that probiotic mixtures were more effective than single-strain probiotics in inhibiting pathogen growth and atopic dermatitis, suggesting further application on other diseases like IBD. Another multispecies probiotic called Ecologic®Tolerance/Syngut™ was developed using four different probiotic strains (*Bifidobacterium lactis* W51,

L. acidophilus W22, *L. plantarum* W21, and *Lactococcus lactis* W19). Strains of this consortium have been proven to strengthen the gut barrier function, have beneficial effects on post-immunological induced stress, inhibit Th2, and stimulate IL-10 levels, thus providing beneficial effects in patients with food intolerance (Besseling-van der Vaart et al., 2016). Moreover, a multispecies probiotic consortium, Ecologic AAD (*B. bifidum* W23, *B. lactis* W18, *B. longum* W51, *E. faecium* W54, *L. acidophilus* W37 and W55, *L. paracasei* W72, *L. plantarum* W62, *L. rhamnosus* W71, and *L. salivarius* W24), reduced diarrhea-like bowel movements when administered in healthy volunteers taking amoxicillin (Koning et al., 2008). Multispecies probiotics also prevented rise in fasting plasma glucose (FPG), to decrease high sensitivity C-reactive protein (hs-CRP), and to increase plasma glutathione (GSH) in diabetic patients (Asemi et al., 2013). van Minnen et al. (2007) provided evidence that manipulation of the intestinal flora with multispecies probiotics reduced bacterial translocation, morbidity and mortality in a rat model of acute pancreatitis. Furthermore, multispecies probiotics rapidly relieved IBS symptoms and shifted the microbiota composition (Yoon et al., 2013). According to these results, combining specific probiotic effects from diverse strains can lead to an additive and more synergetic multispecies probiotic consortium (Timmerman et al., 2007).

However, the phylogenetic origin of probiotics is currently limited to conventional formulations of *Bifidobacterium*, *Lactobacillus* species and other lactic acid bacteria (LAB) (Govender et al., 2013) or yeast strains. This may decrease the probiotic effectiveness in the prevention or therapy of diseases entailing severe dysbiosis. Hence, a functionally and phylogenetically diverse probiotic product may be desirable when alterations in the gut microbiota composition are present (Marotz and Zarrinpar, 2016). For instance, CDI and recurrent CDI are major medical conditions that need urgent treatment when conventional antibiotics fail. As a result, development of complex communities with targeted functions is needed.

THE DILEMMA OF FECAL MICROBIOTA TRANSPLANT (FMT)

Fecal microbiota transplant (FMT) or fecal bacteriotherapy is an alternative strategy successfully used for the treatment of CDI (Kelly, 2013). Severe antibiotic therapy and CDI trigger dysbiosis, reducing diversity and functionality of the gut endogenous microbiota (Brandt, 2012). In this case, *C. difficile* spores can germinate, colonize, and thrive in the gut. Treatment of CDI requires additional antibiotics, increasing the risk of recurrent CDI (rCDI) after cessation of treatment, as a result of the dysbiosis caused by antibiotic therapy (Becattini et al., 2016; Francino, 2016), due to infection with the original strain (Barbut et al., 2000; Marsh et al., 2012) or re-infection caused by a different strain (Johnson et al., 1989; Kelly, 2009; Figueroa et al., 2012).

Poor colonization resistance from the gut microbiota and the patient's poor immune response further contribute to CDI risk (Pérez-Cobas et al., 2015). Recurrent CDI risk is 10–20% after initial CDI (Surawicz et al., 2013), and it increases to 45% after a first relapse, and to 60% for those with two or more recurrences (Bartlett, 1990). However, FMT can resolve both CDI and rCDI (Bakken, 2009), with a success rate of 90% when further antibiotic treatments fail (Youngster et al., 2014; Rao and Safdar, 2015). Given the success of FMT, it is now being considered as potential treatment for disorders such as ulcerative colitis (Shi et al., 2016), irritable bowel syndrome (Distrutti et al., 2016), and metabolic syndrome (Hartstra et al., 2015). For instance, FMT induced remission in patients with active ulcerative colitis (Moayyedi et al., 2015), potentially as a result of the introduction of normal flora and the subsequent correction of the imbalance in the microbiota caused by the disease (Bakken et al., 2011). The complexity of the fecal sample can be the key factor behind the positive shift in the microbiota composition generated by the FMT (Marotz and Zarrinpar, 2016). Thus, diversity of the donor microbiome may be crucial (Leszczyszyn et al., 2016). Indeed, some patients do not respond to FMT, probably because only specific bacterial phylotypes can be therapeutic when effectively transferred (Vermeire et al., 2015). Hence, FMT efficacy for treating gastrointestinal disorders is controversial (Sbahi and Di Palma, 2016). Adverse effects after FMT include nausea, vomit, fever, abdominal pain, and diarrhea (Vermeire et al., 2015; Pigneur and Sokol, 2016). Data for long-term effects of FMT is lacking, but theoretically, any disease phenotype from the donor can be transferred to the patient (Sbahi and Di Palma, 2016). This could be expected, as the uncharacterized nature of FMT may result in undetected or unmonitored risk factors such as viruses, pathogens or even allergens being passed to the FMT recipient, causing disease. To overcome this problem, Petrof et al. (2013) developed a synthetic bacteria cocktail with characterized nature to substitute FMT. Alternatively, a thorough pre-screening should be performed on the donor before the actual procedure of the FMT. Thus, the French Group of Fecal microbiota Transplantation (FGFT) was created to secure and evaluate the practice in this field (Sokol et al., 2016). Despite having experience treating CDI, FMT is not yet the top treatment choice of physicians (Zipursky et al., 2014). However, the majority of gastroenterologists and physicians in metropolitan areas were supportive to the idea of creating a fecal transplantation center, and a high percentage of the physicians would refer their patients to those centers (Jiang et al., 2013).

ALTERNATIVES FOR FECAL MICROBIOTA TRANSPLANT (FMT)

Additional microbiome therapeutics using characterized microbial communities of selected fecal bacteria could be developed to replace FMT, and yield the desired outcome (Sbahi and Di Palma, 2016). For instance, Petrof et al. (2013) described a stool substitute constituted by 33 different purified

TABLE 1 | Strains composing the RePOOPulate consortium.

Composition of Stool Substitute (RePOOPulate)
Acidaminococcus intestinalis
Bacteroides ovatus
Bifidobacterium adolescentis (two different strains)
Bifidobacterium longum (two different strains)
Blautia producta
Clostridium cocleatum
Collinsella aerofaciens
Dorea longicatena (two different strains)
Escherichia coli
Eubacterium desmolans
Eubacterium eligens
Eubacterium limosum
Eubacterium rectale (four different strains)
Eubacterium ventriosum
Faecalibacterium prausnitzii
Lachnospira pectinoshiza
Lactobacillus casei/paracasei
Lactobacillus casei
Parabacteroides distasonis
Raoultella sp.
Roseburia faecalis
Roseburia intestinalis
Ruminococcus torques (two different strains)
Ruminococcus obeum (two different strains)
Streptococcus mitis

intestinal bacteria isolated from a healthy donor (**Table 1**), to treat rCDI. In this study, the synthetic bacterial mixture was infused through the colon of the infected patient causing a change in the stool microbial profile. Major shifts reflecting the isolates of the synthetic mixture were still detectable 6 months after treatment. Thus, the concept of "RePOOPulate" the gut microbiome was coined. Authors of the study suggested that using a synthetic stool substitute may be an effective method to replace the use of FMT for treating rCDI. Although further validation is needed, complete resolution of the infection was achieved. Several advantages of this synthetic stool substitute can be highlighted. The composition of the administered bacterial cocktail is accurately characterized, facilitating registration. Further, assembly of the synthetic bacterial cocktail is highly reproducible enabling standardization and upscaling. In addition, patient safety can be guaranteed, because the bacterial mixture can be rendered pathogen- and virus-free (Petrof et al., 2013). These data suggest that a multi-species community such as that in the RePOOPulate study, can be more effective than single-strain probiotics or mixed cultures of probiotic species. This can be because the RePOOPulate community preserved its structure and thus successfully colonized a new environment (Petrof et al., 2013). Moreover, RePOOPulate consisted of a more phylogenetically diverse community including strains with beneficial health effects that can be candidates for next generation probiotics.

NEXT GENERATION PROBIOTICS

Looking at its internationally recognized definition, probiotics are live microorganisms that, when administered in adequate numbers, confer health benefits on the host. Probiotics are usually isolated from our commensal gut bacteria, but cannot be given the definition of probiotics until their stability, content, and health effect are characterized (Sanders, 2008). Probiotics are thought to improve the balance in the host, prevent disturbances, and decrease the risk of pathogen colonization (Goldenberg et al., 2013). They have been referred to as functional foods or beneficial bacteria, and they have been considered for the prevention and treatment of *C. difficile*-associated diarrhea (CDAD) (Goldenberg et al., 2013). Probiotics can be found as capsules or food supplements in health food stores and supermarkets (Goldenberg et al., 2013). Pattani et al. (2013) reported that *Lactobacillus*-based formulations combined with antibiotics reduced the risk of AAD and CDI. They however, suggested that larger studies are needed to decide on the use of probiotic/antibiotic combination as a therapy over the single species probiotic (Pattani et al., 2013). Furthermore, findings from randomized control trials (RCTs) and meta analyses suggest that there is moderate evidence on the ability of probiotics to prevent primary CDI (people at risk of CDI), but there is no enough evidence suggesting the probiotics can prevent secondary CDI (recurrent CDI) (Evans and Johnson, 2015). There are still some evidence gaps for the use of probiotics in the prevention of CDI such as the interaction between specific classes of antibiotics with the probiotics used on CDI risk, the bacterial taxa that provides the best efficacy in the prevention of CDI, and the use of probiotics in immunocompromised or critically ill patients (Rao and Young, 2017). Hence, future RCT should consider these different concerns (Rao and Young, 2017). Besides, probiotics impact the gut-brain axis.

For example, *Bifidobacterium longum* NC3001 had beneficial effects on psychiatric comorbidities, which in turn could temporarily improve the quality of life in IBS patients, indicating that this probiotic reduces limbic reactivity (Pinto-Sanchez et al., 2017).

Overall, classical probiotics show limited effects on the human gut microbiota seeking the need for a better selection and formulation of bacterial strains (Neef and Sanz, 2013). Results from previous studies show promising outcomes in the treatment or prevention of diverse metabolic and inflammatory diseases by specific bacteria (Neef and Sanz, 2013). Those probiotics encompass species different from *Lactobacillus* and *Bifidobacterium* (Cani and Van Hul, 2015; Patel and DuPont, 2015). Nevertheless, the gut microbiome is a complex community, which makes it difficult to define the host–microbe interaction.

The United Nations Food and Agriculture organization (FAO) definition of probiotics is broad, allowing flexibility in terms of the phylogenetic origin of probiotics. Information generated from previous studies assisted in the selection of next generation probiotics, which include members from *Clostridium*

clusters IV, XIVa and XVIII, *F. prausnitzii, Akkermansia muciniphila, Bacteroides uniformis* (Neef and Sanz, 2013; Patel and DuPont, 2015), *Bacteroides fragilis* (Round et al., 2011), and *Eubacterium hallii* (Udayappan et al., 2016). These next generation probiotics were evaluated in preclinical trials and yielded positive outcomes for inflammatory and metabolic disorders (Neef and Sanz, 2013; Patel and DuPont, 2015). In addition, new techniques are required for the development of new probiotic products containing strains from human origin. This is to say, these strains must come from the major groups of the intestinal microbiota, they have to be defined to have a safe status and proven to have potential beneficial effects (Martín et al., 2017). In the following sections, we will discuss some of the most promising bacterial species that are currently under consideration for being used as next-generation probiotics.

Faecalibacterium prausnitzii

Faecalibacterium prausnitzii is an extreme oxygen sensitive (EOS) bacterium (Martín et al., 2017) belonging to the *Clostridium* cluster IV, and it accounts for 3–5% of the total fecal bacteria, and it is one of the predominant groups in the human feces (Breyner et al., 2017). Quévrain et al. (2016) reported low proportions of this species in the fecal and mucosa-associated microbiome in Crohn's disease (CD). *F. prausnitzii* may possess *in vivo* and *in vitro* anti-inflammatory effects. *F. prausnitzii* may possess *in vivo* and *in vitro* anti-inflammatory effects. Breyner et al. (2017) confirmed the anti-inflammatory properties of MAM, and their ability to reduce Th1 and Th17 pro-inflammatory cytokines in Mesenteric Lymphatic Node (MLN) and colon tissues in both DNBS and DSS colitis model. MAM was also able to improve TGFβ cytokine which affects NF-κB activation in DNBS model thus protecting the host and decreasing intestinal inflammation (Breyner et al., 2017). In addition, *F. prausnitzii* can induce the *Clostridium*-specific IL-10-secreting regulatory T cell subset, present in several human colonic cells. Its capacity for lowering IL-12 and IFNγ production indicates that the interaction between *F. prausnitzii* and the host shape and maintain the gut barrier immune function (Quévrain et al., 2016). In this way, anti-inflammatory molecules from *F. prausnitzii* may be used as targeted anti-inflammatory drugs for CD. Moreover, MAM could function as a CD biomarker, predicting loss of *F. prausnitzii* functionality. However, further research should be conducted to elucidate the MAM production mechanisms, before considering it for CD management. Sokol colleagues reported that low proportions of *F. prausnitzii* on a resected ileal Crohn's mucosa were associated with CD recurrence after 6 months. In addition, the oral administration of live *F. prausnitzii* or its supernatant in mice could reduce the severity of trinitrobenzene sulfonic acid (TNBS) colitis and correct the associated dysbiosis (Sokol et al., 2008). The results from this study suggest that *F. prausnitzii* can be considered as a promising probiotic candidate for the treatment of pathologies characterized by chronic gut inflammation (Sokol et al., 2008). Besides, all *F. prausnitzii* strains have proven anti-inflammatory properties, which allows them to further be tested in murine models to determine their beneficial effects before moving to human trials (Martín et al., 2017).

Akkermansia muciniphila

Recent evidence shows that there is a link between the altered gut microbiota and metabolic diseases like obesity, diabetes mellitus, and cardiovascular disease (Schneeberger et al., 2015; Dao et al., 2016; Li et al., 2016). Higher abundance of *A. muciniphila*, a mucin degrading microbe, was associated with healthier metabolic status. Everard et al. (2014) and Schneeberger et al. (2015) studied the effects of high fat diet on metabolic parameters and the gut microbiota composition over time, and they found that *A. muciniphila* was decreased. The negative impact on *A. muciniphila* was associated with expression of lipid metabolism, inflammatory markers in adipose tissue, and different parameters like increased blood glucose, insulin resistance and plasma triglycerides (Schneeberger et al., 2015). This prompted the research toward investigating the putatively positive role of *A. muciniphila* in adipose tissue homeostasis and metabolism. Dao et al. (2016) assessed clinical parameters and *A. muciniphila* abundance before and after a 6-week calorie restriction period, followed by stabilization diet. The results of this intervention study indicate that the higher abundance of *A. muciniphila* at baseline was associated with improvement in blood glucose homeostasis, lipid profile, and body fat distribution after the intervention. Thus, *A. muciniphila* can be used as a prognostic tool for the success of diet interventions (Dao et al., 2016). Moreover, Li et al. (2016) reported that administration of *A. muciniphila* could reverse the atherosclerotic lesions, improve metabolic endotoxemia-induced inflammation, and ultimately restore the gut barrier.

Bacteroides fragilis and *Bacteroides uniformis*

Bacteroides species are commensal bacteria that represent 25% of our gut bacterial population. They are gram negative, anaerobic, bile resistant, and non-spore forming bacteria. *Bacteroides* can be passed from the mother to the child during vaginal delivery, thus becoming primary colonizers of the gut. When retained in the gut, *Bacteroides* act as commensals and can be beneficial for the host (Wexler, 2007). The most common isolate from the clinical specimens is *B. fragilis*, which is the most virulent *Bacteroides* species (Wexler, 2007).

Bacterial colonization of the gut can greatly affect the immune system, either through the direct host–bacteria interaction, or by molecules produced by our commensal bacteria. *B. fragilis* produces polysaccharide A (PSA), which is an immunomodulatory molecule that activates the T-cell dependent immune responses (Troy and Kasper, 2010). Those responses are involved in the development and homeostasis of the host immune system (Troy and Kasper, 2010). Furthermore, Round et al. (2011) demonstrated that *B. fragilis* activates Toll-like receptor (TLR) pathways. This occurs because

PSA signals through TLR2 on Foxp3+ (forkhead box P3) regulatory T cells to boost immunologic tolerance. As a result, PSA can be considered as a model symbiosis factor, because it preserves the balance between T cell types and maintains the immune system homeostasis (Round et al., 2011).

As for *Bacteroides uniformis* (*B. uniformis*) CECT 7771, it is considered a potential probiotic strain originally isolated from the feces of healthy breastfed infants. Oral administration of this specific strain in high fat diet-fed mice improved lipid profile, reduced glucose insulin and leptin levels, increased TNF-α production by dendritic cells (DCs) in response to LPS stimulation, and increased phagocytosis (Gauffin Cano et al., 2012). Thus, administration of *B. uniformis* CECT 7771 can ameliorate metabolic disorder and immunological dysfunction related to intestinal dysbiosis in obese mice (Gauffin Cano et al., 2012; Yang et al., 2016). Furthermore, acute administration of this strain to mice did not promote adverse effects on health status or food intake, and there was no bacteria translocation to blood, liver, or lymph nodes. This indicates that there are no safety concerns for this strain in mice, but further investigation should be completed in humans (Fernández-Murga and Sanz, 2016).

Eubacterium hallii

Eubacterium hallii is an important anaerobic butyrate-producer resident in our gut, which influences the intestinal metabolic balance (Engels et al., 2016). Butyrate has been proposed to lower mucosal inflammation and oxidative status, strengthen the epithelial barrier function, and modulate intestinal motility in addition to being an energy source for colonocytes (Canani et al., 2011). *E. hallii* can yield propionate from a broad range of substrates. This versatility may enhance the host–gut microbiota homeostasis (Engels et al., 2016). Moreover, administration of *E. hallii* in obese and diabetic db/db mice increased energy metabolism and improved insulin sensitivity. However, increasing dosage of *E. hallii* did not impact body weight or food intake, indicating that this strain can a safe and effective alternative for insulin sensitivity (Udayappan et al., 2016).

COCKTAILS OF *Clostridium* CLUSTER IV AND XIVA MEMBERS

As previously described, T_{regs} can regulate immune homeostasis and serve as a therapeutic target for different gut inflammatory disorders. Induction of the colonic T_{regs} is dependent on special properties of our commensal bacteria. *Clostridium* spp. belonging to clusters IV and XIVa (also known as *Clostridium leptum* and *coccoides* groups, respectively) are exceptional inducers of T_{regs} in the colon and can be considered as therapeutic options for IBD and allergies (Atarashi et al., 2011). Previous work indicated that a cocktail of strains isolated for the human gut microbiota can be more effective than a single strain in preventing or treating disease. Thus, Atarashi et al. (2013) isolated 17 strains belonging to Clostridia clusters XIVa, IV, and XVIII from a human fecal sample, which were effective in T_{reg} cell differentiation and accumulation in mouse colon. Authors proposed that the SCFAs produced by this community influenced the expression of *Foxp3*, a key gene controlling T_{reg} cell development (Atarashi et al., 2013). Incidentally, Clostridia clusters XIVa and IV are decreased in fecal samples from patients with inflammatory bowel disease (IBD), and thus the cocktail of the 17 strains could potentially reverse this dysbiosis (Atarashi et al., 2013).

INDUSTRIAL APPLICATIONS AND INTERESTS

Current Developments

Since the manipulation of the gut microbiota has been proven to be promising to prevent and treat different diseases, pharmaceutical and food industries would be interested in the potential therapeutic approaches described before. For instance, Seres health and Rebiotix companies are working on developing a defined microbial cocktail and a standardized commercially prepared FMT, respectively. These therapeutic approaches are intended to treat CDI, and that can be used as an alternative for FMT. Synthetic microbial communities designed for transplants are expected to meet production, mode of action and safety standards (Orenstein et al., 2015; van der Lelie et al., 2017). For instance, Seres health developed SER-109, a novel biological agent proposed to restore the balance in the gut microbiome, promoting resistance to pathogenic invaders like *C. difficile* (Khanna et al., 2016). Seres health also developed SER-287 for the treatment of IBD and in specific ulcerative colitis (Inflammatory Bowel Disease | Seres Therapeutics, 2017). Rebiotix commercially developed RBX2660, a mix of live human microbes for effective treatment of recurrent CDI (Ramesh et al., 2016). Moreover, other formulations including strains belonging to Clostridia classes IV and XIVa were designed to modulate the immune response (Atarashi et al., 2013). The original community of 17 strains (VE202) was developed by Vendanta Biosciences and Johnson and Johnson, and has provided an effective treatment for autoimmune disorders (Reardon, 2014; Ratner, 2015; van der Lelie et al., 2017).

Technical Challenges

Several challenges concerning the stability of the probiotic during the probiotic production are still unsolved. Microorganisms require strict conditions to grow, such as specific nutritional media and environmental conditions (suitable temperature, pH, water activity, oxygen content, among others). The product manufacturing and storage processes may impact the viability of the bacterial strains, influencing probiotic stability and properties. In addition, it is fundamental to consider the viability of the probiotics after consumption. Bacterial strains should remain viable at sufficient numbers through the gastrointestinal tract (GIT) passage. Therefore, the selection of optimal culture medium and cell protectants is crucial

to enhance the efficacy of the probiotic product. Moreover, as most probiotic strains are strict anaerobes or facultative anaerobes, oxygen permeation into carriers should be reduced, or oxygen scavengers should be introduced to reduce the redox potential (Shah et al., 2010). Probiotic bacteria can also be protected by microencapsulation, which has been proposed to improve the stability of the strains and can adapt to the GIT conditions (Heidebach et al., 2012). Nowadays, yogurts and fermented milk are the best-established vehicles for probiotics in the market. However, some probiotic strains are sensitive to the different conditions in fermented products, like oxygen and pH, which can, in turn, affect the stability of probiotics through post-acidification during their storage in the fridge. To minimize this phenomenon, strains that lack the ability to post-acidify should be selected (Damin et al., 2008). As a result, this can cause an economic burden for manufacturers, limiting the addition of probiotics in different products (Gueimonde and Sánchez, 2012). Furthermore, manufacturing the probiotic product in a reproducible manner is a critical aspect (Paulo Sousa e Silva and Freitas, 2014). Several attempts to fix the number of viable probiotic strains throughout the products (Shah et al., 2010) have been attempted, to no avail.

Regulatory Challenges

Probiotics are classified in different categories across countries. Their names and use as functional foods may vary according to different systems. For instance, probiotics fall in the Qualified Presumption of Safety (QPS) list provided by the European Food Safety Authority (EFSA) and referred to as functional foods since there was no legal definition for probiotics. The market for probiotics as functional foods expanded, as a result of probiotic food products like yogurts and fermented milk (Baldi and Arora, 2015), containing conventional LAB. The QPS list is periodically updated according to the safety assessment of the biological products recommended to be added, and not all can be approved (Scientific Opinion on The Maintenance of The List of QPS Biological Agents Intentionally Added to EFSA Panel on Biological Hazards (BIOHAZ), 2013; Ricci et al., 2017). A similar system applies in the United States as Generally Recognized as Safe (GRAS) products should be approved by the FDA. However, if a probiotic is used as a dietary supplement in the United States, then it is considered as "food" and should be regulated by the Dietary Supplement Health and Education Act (DSHEA). If the probiotic was considered to have therapeutic purpose, the probiotic drug should be proven to be safe and effective to be approved by the FDA. Nevertheless, for both the FDA and EFSA, probiotics cannot be used in health claims. On the other hand, Japan acts as a global market leader, where probiotics are considered as both foods and drugs. According to the Japanese regulations, probiotic products are in different category than foods and Foods for Specific Health Uses (FOSHU). Efficacy claims for probiotic products are prohibited on the labeling until the product gets the permission from the Ministry of Health and Welfare (MHLW) to be considered FOSHU, for which efficacy and safety validation is mandatory. FOSHU categorizes the food claims according to the scientific evidence and the strength of the supporting data provided. The government then divided the FOSHU health claims into subcategories, in which their effect could be in GIT, metabolism, cholesterol moderation, or bone health. Japanese regulations also approve new health claims on a regular basis (Baldi and Arora, 2015).

As the definition and classification of probiotics by regulatory agents throughout the world is different, the status of probiotic products is still uncertain. Thus, reservations about probiotic products claims may arise among regulatory bodies, producers, and consumers. Since the probiotic concept is invading the world, further investigation for probiotic traits is needed. Moreover, most probiotics only include LAB, which possess limited phylogenetic diversity and functionality. Hence, critical update of the screenings required by regulatory agents is urgently needed.

Medical Application

Despite the different studies and outcomes of FMT, FDA approval in North America has not been granted. At the beginning, FMT was considered as investigational new drugs (INDs), and FDA authorization was mandatory. Currently, patients unresponsive to standard antibiotic CDI therapies can opt for FMT after completing an informed consent, where they are notified that FMT is still under investigation. However, SERES 109 and RBX2660 have been granted the Orphan Drug designation by the FDA (Rebiotix Media, 2015; Seres Therapeutics, 2015). As for the EMA in Europe, the use of FMT for the treatment of CDI has not been yet regulated (van Nood et al., 2014; Lowes, 2016). Yet, FMT is regularly applied to curb infections across Europe, and it is considered in clinical trials for many other pathologies. In the search for safe FMT alternatives, research on microbiotic medicinal products (MMP) is in full development and novel applications are continuously being considered. These MMP developments require novel views and strategies from the scientific world, the industry, the medical field, and the regulatory bodies. In this context, platforms like the Pharmabiotic Research Institute have been created, to facilitate discussion between different stakeholders (Pharmabiotic Research Institute, 2017). Overall, additional research needs to be conducted before using FMT alternatives containing characterized microbial communities and next generation probiotics, to guarantee their safety and reproducible efficacy.

CONCLUSION

FMT may be replaced with a characterized multispecies bacterial mixture that can be safer, free of allergens or viruses, and capable of treating CDI. With the current *in vitro* and *in vivo* data, next generation probiotics hold promise to treat diverse medical conditions, and they can be more effective than single or multi strains of the commercial probiotics. Moreover, several different strains with proven health benefits can also be considered candidates for next generation probiotics and other microbiota-based drugs. However, additional research is required for an increased understanding of the interactions

among those strains, aiming at producing a successful therapeutic formulation. Research should be conducted to demonstrate whether these probiotics can be applicable to humans, as safety assessments have only been completed in animals. Effective carriage of bacterial strains in food matrices is critical for survival. Thus, optimisation of the growing conditions, and even encapsulation must be considered to promote delivery and release of the live product in the colon. The development of next generation probiotics and MMPs hold promise for innovation in both the food/feed sector and the pharmaceutical industry. A close interaction between academia, industry and regulatory agencies is essential for developing safe and health-promoting products, as both prophylactic and therapeutic strategies.

AUTHOR CONTRIBUTIONS

Conceived and designed the review: REH, EH-S, and TVW. Funding acquisition: TVW. Wrote the paper: REH. Reviewed the manuscript: REH, EH-S, and TVW.

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Next-Generation Beneficial Microbes: The Case of *Akkermansia muciniphila*

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Metabolic disorders associated with obesity and cardiometabolic disorders are worldwide epidemic. Among the different environmental factors, the gut microbiota is now considered as a key player interfering with energy metabolism and host susceptibility to several non-communicable diseases. Among the next-generation beneficial microbes that have been identified, *Akkermansia muciniphila* is a promising candidate. Indeed, *A. muciniphila* is inversely associated with obesity, diabetes, cardiometabolic diseases and low-grade inflammation. Besides the numerous correlations observed, a large body of evidence has demonstrated the causal beneficial impact of this bacterium in a variety of preclinical models. Translating these exciting observations to human would be the next logic step and it now appears that several obstacles that would prevent the use of *A. muciniphila* administration in humans have been overcome. Moreover, several lines of evidence indicate that pasteurization of *A. muciniphila* not only increases its stability but more importantly increases its efficacy. This strongly positions *A. muciniphila* in the forefront of next-generation candidates for developing novel food or pharma supplements with beneficial effects. Finally, a specific protein present on the outer membrane of *A. muciniphila*, termed Amuc_1100, could be strong candidate for future drug development. In conclusion, as plants and its related knowledge, known as pharmacognosy, have been the source for designing drugs over the last century, we propose that microbes and microbiomegnosy, or knowledge of our gut microbiome, can become a novel source of future therapies.

Keywords: *Akkermansia muciniphila*, obesity, diabetes mellitus, type 2, probiotics and prebiotics, gut barrier function

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INTRODUCTION

Overweight and obesity have reached epidemic proportions with more than 600 million of adults and 100 million children of the world's population suffering from obesity (GBD 2015 Obesity Collaborators et al., 2017). Obesity predisposes to the development of type 2 diabetes and cardiovascular diseases. These two pathologies are part of the metabolic syndrome that is also becoming major problem in public health (Abdelaal et al., 2017; Ajala et al., 2017). Gut microbes play an important role in the regulation of host metabolism and low-grade inflammation (Hartstra et al., 2015; Marchesi et al., 2016; Cani, 2017). The perturbation of the composition and the

activity of the gut microbiota, also known as dysbiosis, is thought to be involved in the emergence of the metabolic syndrome (Wen and Duffy, 2017). Nowadays, numerous studies have demonstrated that our dietary habits strongly influence the composition and function of the gut microbiota and eventually may contribute to the onset or the protection against metabolic disorders (David et al., 2014; Korpela et al., 2014; Salonen et al., 2014; Carmody et al., 2015; Zeevi et al., 2015; Cani and Everard, 2016; Thaiss et al., 2016).

Well documented among the potential ways to affect the gut microbiota, is the consumption of selected microbes that are marketed as probiotics defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014). It is worth noting that the current majority of probiotics sold on the market include mainly microorganisms from the genera *Lactobacillus* and *Bifidobacterium* (Douillard and de Vos, 2014). However, other ways such as the consumption of prebiotics have gained considerable attention over the last 20 years (Roberfroid et al., 2010). The prebiotic concept, discovered in Gibson and Roberfroid (1995), has led to a great number of dietary supplements that is an important growth market. The definition of prebiotic is now widely used and has been recently revised as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). Thus, nutritional components that escape the digestion in the upper alimentary tract may have an impact on the gut microbiota by modulating some members of the gut microbiota its composition and its activity. However, the concept of prebiotic has not yet revealed all its secrets. In spite of numerous discoveries of molecular mechanisms explaining how prebiotics and the gut microbiota interact with the host, it remains difficult to identify the bacterial candidate(s) involved in the beneficial effects observed on the energy, glucose, lipid metabolism and immunity.

FROM PREBIOTIC TO NEXT-GENERATION BENEFICIAL MICROBE: FOCUS ON THE IDENTIFICATION OF *Akkermansia muciniphila*

Akkermansia muciniphila is one of the most abundant single species in the human intestinal microbiota (0.5–5% of the total bacteria) and has been isolated and characterized as a mucin-utilizing specialist in 2004 by Muriel Derrien in her Ph.D. research at Wageningen University (Derrien et al., 2004; Collado et al., 2007). This discovery was initiated by the notion that the human body produces its own "prebiotics" or microbial substrates, namely mucus, an abundant glycoprotein that is specifically produced and degraded in the colon (Ouwehand et al., 2005). While germ-free mouse experiments showed that *A. muciniphila* showed immune and metabolic signaling, specifically in the colon, the exact functions of this unusual microbe remained enigmatic (Derrien et al., 2008, 2011).

Further indications for the function of *A. muciniphila* were subsequently determined in other prebiotic studies using inulin-type fructans that were initially characterized as bifidogenic compounds able to increase the abundance of *Bifidobacterium* spp. (Gibson and Roberfroid, 1995). Thanks to the development of novel culture-independent techniques, we decided to revise in depth the impact of such kind of prebiotics on the overall microbial community in mice. Therefore, in search of potential novel bacterial candidates, we combined different techniques (phylogenetic microarray, high-throughput sequencing, gradient denaturation gel and qPCR), which allowed us to analyze and to compare all the bacteria that were present in the intestinal microbiota. The first surprise was to discover that more than 100 different taxa were affected by prebiotics (Everard et al., 2011; Everard et al., 2014). Among these bacteria, we found that the relative abundance of *A. muciniphila* increased more than 100-fold following the ingestion of prebiotics thereby reaching the abundance of up to 4.5% under high-fat diet (Everard et al., 2014), whereas this effect was lower under normal chow diet (0.09–2.5%) depending on the model (Everard et al., 2011, 2014). It is worth noting that these findings are confirmed in different set of experiments (Everard et al., 2013; Liu et al., 2016; Reid et al., 2016; Catry et al., 2017; Zhu et al., 2017). Interestingly, we and others discovered that *A. muciniphila* was less abundant in the intestinal microbiota of both genetic and diet-induced obese and diabetic mice (Everard et al., 2011, 2013, 2014; Schneeberger et al., 2015; Leal-Diaz et al., 2016; Ojo et al., 2016; Song et al., 2016; Singh et al., 2017), however, few studies reported in mice an increased abundance of *A. muciniphila* upon the ingestion of a high-fat high sucrose diet (Anhe et al., 2015; Carmody et al., 2015). It has also been largely demonstrated that inulin-type fructans feeding improves metabolic disorders associated with obesity, including a decreased fat mass, insulin resistance, lower liver steatosis and a reinforcement of the gut barrier (**Figure 1**) (Cani et al., 2004, 2006, 2009; Maurer et al., 2010; Everard et al., 2011; Pachikian et al., 2012; Greer et al., 2016). Importantly, in humans the abundance of *A. muciniphila* was decreased in several pathological situations such as obesity, type 2 diabetes, inflammatory bowel diseases, hypertension and liver diseases (Png et al., 2010; Belzer and de Vos, 2012; Zhang et al., 2013; Dao et al., 2015; Yassour et al., 2016; Grander et al., 2017; Li et al., 2017). Conversely, antidiabetic treatments, such as metformin administration and bariatric surgery were both found to be associated with a marked increase in the abundance of *A. muciniphila* (**Figure 1**) (Shin et al., 2014; Forslund et al., 2015; de la Cuesta-Zuluaga et al., 2017). Therefore, a large body of evidence suggested that *A. muciniphila* may contribute to protect from specific metabolic disorders and cardiometabolic risk factors associated with a low-grade inflammatory tone.

ADMINISTRATION OF *Akkermansia muciniphila*: MULTIPLE EFFECTS ON THE GUT AND BEYOND

Inspired by the numerous indications that the relative levels of *A. muciniphila* decreased during obesity and metabolic disorders

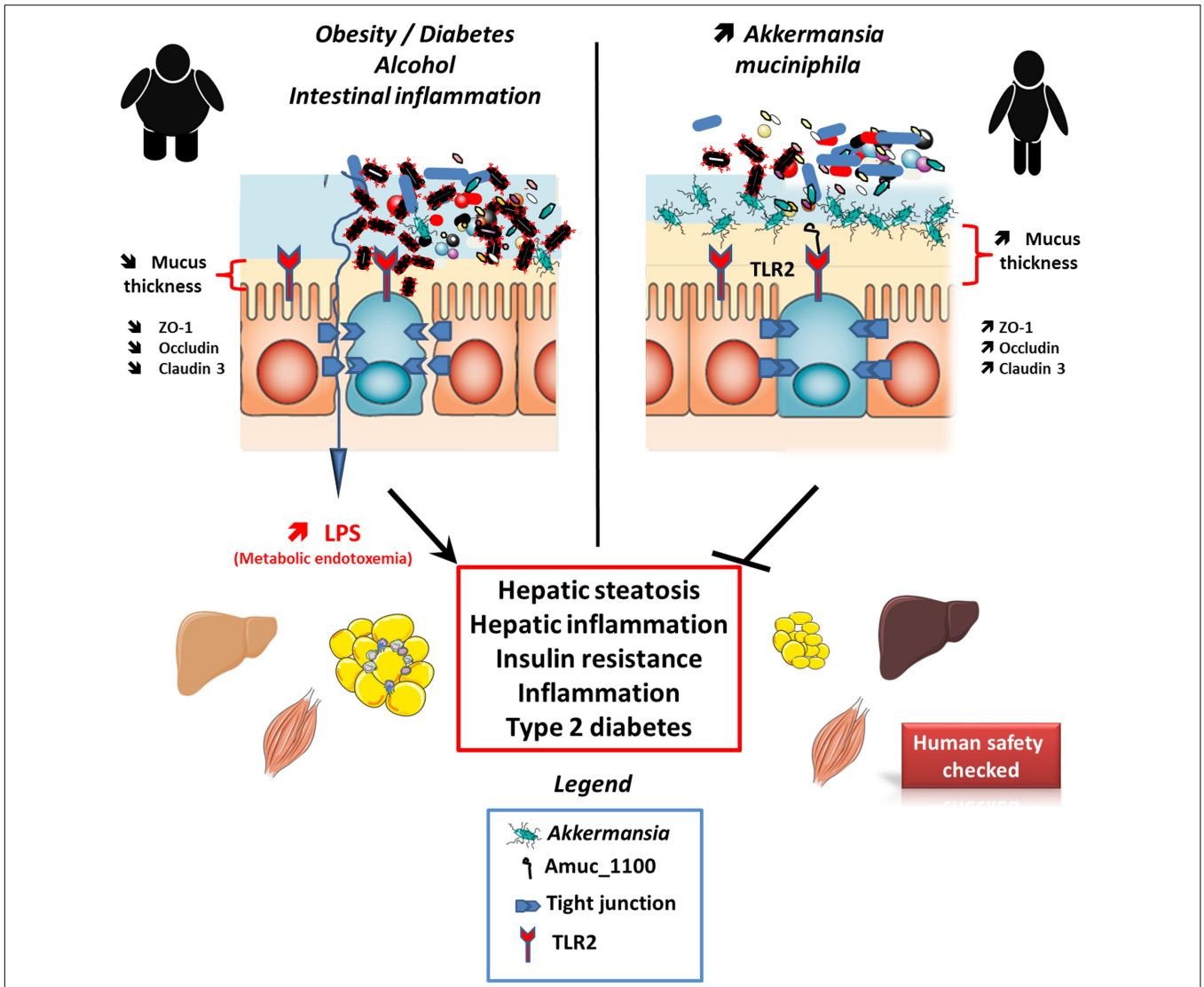

FIGURE 1 | Effects of *A. muciniphila* and derived products on host metabolism. *A. muciniphila* has been found to be lower in several conditions such as during obesity, diabetes, intestinal inflammation, liver diseases, or chronic alcohol consumption. This is associated with an altered gut barrier function leading to an increased plasma LPS levels and eventually triggering low grade inflammation and metabolic disorders. *A. muciniphila* alive or pasteurized as well as Amuc_1100 has been shown to restore gut barrier function likely by acting on TLR2 and restoring appropriate tight junction expression. All these results are associated with an increased mucus later thickness and an improvement of metabolic disorders. It is worth noting that an exploratory human investigation has shown that *A. muciniphila* is apparently safe.

in mouse and man, we decided to study the causal link between *A. muciniphila* and improvements in metabolism. This was done by investigating the impact of a daily oral supplementation with live *A. muciniphila* on the onset of obesity, diabetes and gut barrier dysfunction in mice. We found that the administration of live *A. muciniphila* at the dose of 2.10^8 bacterial cells per day was partly protecting against diet-induced obesity in mice (Everard et al., 2013). Indeed, mice showed a 50% lower body weight gain when treated with live *A. muciniphila* without altering neither their dietary food intake nor the elimination of dietary fats in fecal matter. This protection was mirrored by two times less visceral and subcutaneous fat mass (**Figure 1**), but also by increased markers of fatty acid oxidation in the adipose tissue (Everard et al., 2013). In addition, animals receiving live *A. muciniphila* did no longer exhibited insulin resistance, nor infiltration of inflammatory cells (CD11c) in the adipose tissue, which is a key characteristic of obesity and associated low-grade inflammation (Everard et al., 2013). Interestingly, most of all the metabolic improvements observed following treatment with live *A. muciniphila* were in the range as those observed following oligofructose or inulin treatment (Cani et al., 2009; Dewulf et al., 2011; Everard et al., 2011, 2014), although live *A. muciniphila* was not affecting food intake behavior as do prebiotics like inulin and oligofructose.

Knowing that these metabolic features can be caused by an increased plasma LPS level (i.e., metabolic endotoxemia) or bacterial translocation (Cani et al., 2007; Amar et al., 2011), we next investigated the gut barrier function by measuring several markers. We observed that live *A. muciniphila* prevented the development of metabolic endotoxemia, an effect associated with the restoration of a normal mucus layer thickness (**Figure 1**) (Everard et al., 2013). We also found that administration of live *A. muciniphila* restored the endogenous production of antimicrobial peptides. We then discovered that live *A. muciniphila* increased the endogenous production of specific bioactive lipids that belongs to the endocannabinoid family and are known to have anti-inflammatory activities and regulating the endogenous production of gut peptides involved in glucose regulation and gut barrier, respectively, glucagon-like peptide-1 and 2 (GLP-1 and GLP-2) (Cani et al., 2016). It is worth noting that all these findings have subsequently been confirmed by different groups and extended to other specific disorders such as atherosclerosis, hepatic inflammation and hypercholesterolemia (Shin et al., 2014; Li et al., 2016; Shen et al., 2016; Grander et al., 2017; Plovier et al., 2017).

Collectively all these data reinforce the assumption that live *A. muciniphila* can be considered as a next-generation beneficial microbe with the exceptional particularity that this bacterium can act on numerous facets of the metabolic syndrome and cardiometabolic disorders. Still, these discoveries have raised different fundamental questions that will still have to be studied in humans with the aim to generate new therapeutic tools.

CROSSING THE BARRIER OF SPECIES: FROM MICE TO MAN

Akkermansia muciniphila requires specific culture conditions and complex animal-based medium (i.e., mucin from animal source) and although it may respire under microaerophilic conditions, the cells are relatively sensitive to oxygen (Ouwerkerk et al., 2016). These properties complicate the administration of *A. muciniphila* to human as to evaluate its potential, hence limiting its therapeutic perspectives. In order to solve this problem, a synthetic medium was developed in order to allow the culture of *A. muciniphila* with a high yield and devoid of compounds incompatible with administration in humans (Plovier et al., 2017; Van der Ark et al., unpublished data). Besides the successful development of this synthetic medium, the previous assessment of the efficacy of *A. muciniphila* were performed with cells grown on a mucin-based medium. Therefore, the bacteria cultured on the different media were tested and compared. Interestingly, *A. muciniphila* retains its effectiveness independently of the medium used, and as previously observed, mice treated with the bacterium gained less weight, exhibited an improved glucose tolerance, and insulin resistance under hyperlipidic diet (**Figure 1**) (Plovier et al., 2017).

SERENDIPITY: THE UNEXPECTED ADVANTAGE OF PASTEURIZATION

In 2013, it was showed that the protective effects of *A. muciniphila* disappeared when the bacterium was destroyed by using autoclaving, a heat treatment that destroyes all the constituents of bacteria and spores (Everard et al., 2013). As *A. muciniphila* is a Gram-negative bacterium and hence no spore-former, we were interested what the effects would be of pasteurization, a milder heat inactivation method than autoclaving. Therefore, we tested the impact of administrating pasteurized *A. muciniphila* (30 min at 70°C) cells on diet-induced metabolic disorders in mice. Unexpectedly, this method of inactivation did not abolish the effect of *A. muciniphila* but even exacerbated its beneficial impact. Specifically, mice receiving the pasteurized bacterium and the high-fat diet had a similar body weight gain and fat mass than those observed in mice fed a control diet. Again, these effects were independent of the food intake but pasteurized *A. muciniphila* increased the loss of energy in the feces of the treated mice, indicating a decrease in energy absorption that could contribute to explain the lower weight gain. Pasteurized *A. muciniphila* also strongly improved glucose tolerance, hepatic insulin sensitivity, and completely blocked the diet-induced metabolic endotoxemia. Although, the mechanisms of action of the bacteria are not yet fully elucidated, it is known that *A. muciniphila* express numerous highly abundant protein on its outer membrane (Ottman et al., 2017). Among these proteins, Amuc_1100, implicated in the formation of pili by *A. muciniphila*, was one of the most abundant (Plovier et al., 2017).

Akkermansia muciniphila: A GATE KEEPER THAT DIALOGS WITH THE INNATE IMMUNE SYSTEM

We previously found that *A. muciniphila* was able to restore the expression of specific antimicrobial peptides (Everard et al., 2013). However, Nucleotide oligomerization domain (NOD)-like receptors (NLRs) and Toll-Like Receptors (TLRs) are a specialized group of membrane and intracellular proteins that play a critical role in the regulation of immunity and are directly involved in the recognition of bacterial constituents by the immune system. Therefore, we evaluated the potential of *A. muciniphila* to activate different NOD and TLRs. Strikingly, we found that the bacteria specifically interact with TLR2. TLR2 has been shown to modulate intestinal homeostasis and host metabolism (Caricilli et al., 2011; Brun et al., 2013), thereby participating in the interactions between microbes and host. In addition, to better characterize the interaction between *A. muciniphila* and this receptor, we took advantage of genomic and proteomic analyzes of the external membrane of the bacterium, which may be exposed to host receptors (Ottman et al., 2016). Among these proteins, Amuc_1100 was one of the most abundant. This protein is implicated in the formation of pili by *A. muciniphila* and thus could participate in the interaction between the bacterium and TLR2. This hypothesis was further

confirmed by showing that a version of the genetically engineered protein (called Amuc_1100*) was effectively activating TLR2 and with the same magnitude as *A. muciniphila*. In addition, Amuc_1100* remained stable at the temperature used during pasteurization, and could therefore contribute to the effects of the pasteurized bacterium. Amuc_1100* was also able to replicate almost all the effects of *A. muciniphila* alive or pasteurized in high-fat diet fed mice. *A. muciniphila*, whether live or pasteurized, and Amuc_1100* also decreased high cholesterol levels induced by the high-fat diet. Conversely, the pasteurized bacterium specifically also reduced the triglyceridemia of the treated mice, reinforcing the idea that the pasteurization of *A. muciniphila* reinforces its protective effects. A potential mechanism explaining this could be the exposure of active molecules by the heat treatments, including Amuc_1100, or the inactivation of inhibitory compounds, or combinations thereof.

FIRST ASSESSMENT OF *Akkermansia muciniphila* IN HUMANS WITH METABOLIC SYNDROME

As discussed earlier, *A. muciniphila* has various advantages as compared to other beneficial microbes and specific probiotics, at least in the context of the metabolic syndrome. *A. muciniphila* is present in the human milk, is highly abundant in lean and non-diabetic subjects, and is even highly increased upon metformin treatment of gastric bypass surgery, and this without obvious deleterious impact. This unique character does not preclude the fact the human investigations and safety assessment must be done. Hence, to become a putative future food supplement, the safety must be tested. We evaluated the toxicity and the emergence of possible side effects related to the administration of *A. muciniphila* in humans (20 subjects) as part of an ongoing clinical trial of individuals with metabolic syndrome (Plovier et al., 2017). To this end, we analyzed relevant clinical parameters related to liver, muscles and renal functions as well as markers of immunity and inflammation in individuals who received *A. muciniphila* daily for 2 weeks and then extended to 3 months. Whatever the formulation of *A. muciniphila* (live at 10^9 and 10^{10} bacteria per day or pasteurized at 10^{10} bacteria per day), no changes were observed for the markers tested after 2 weeks or 3 months of daily administration. In addition, the frequency of side effects reported by patients were similar in the different groups. These first data indicate that *A. muciniphila* (active or pasteurized) is tolerated in individuals with metabolic syndrome and is likely not toxic.

While *A. muciniphila* is one of the handful of core microbes identified in the intestinal microbiota of over 1000 human adults (Shetty et al., 2017), the administration of its cells, either in live or pasteurized form, in a dietary supplement may be subject to regulatory frameworks that aim to safeguard the consumer. The regulatory requirements relating to the use of live *A. muciniphila* have recently been addressed (Gomez-Gallego et al., 2016). This review summarized the recent comprehensive studies related to *A. muciniphila* and its safety properties and provided criteria be addressed when *A. muciniphila* cells are to be considered as a novel food by the European Food Safety Authority in Europe. One aspect that is relevant here and applies to other core intestinal microbes as well, is the fact that most if not all healthy subjects carry these anaerobes. So these have to be consumed at some stage and in this context it is important to note that *A. muciniphila* is present in early life microbiota and has been detected in mothers' milk (Collado et al., 2007, 2012; Derrien et al., 2008; Jeurink et al., 2013; Ward et al., 2013). Another aspect relates to the antibiotic resistance of *A. muciniphila* that has been studied to some extent in healthy human subjects that carried high levels of *A. muciniphila*-like bacteria and apparently were sensitive to penicillin and tetracycline derivatives but resistant to vancomycin (Dubourg et al., 2013). This was confirmed in *in vitro* studies on the antibiotic resistance profile with the type strain Amuc^T (Ouwerkerk Ph.D. Thesis Wageningen University 2016). Moreover, inspection of the genome sequence did not reveal antibiotic resistance genes that are linked to known genetically transferrable elements (Gomez-Gallego et al., 2016).

CONCLUSION

Since its discovery in 2004, numerous studies have mostly linked the abundance of *A. muciniphila* with beneficial effects, and this although very few exceptions exist in specific non-physiological models (i.e., gnotobiotic models, specific immune double knock-out models) (Seregin et al., 2017).

Nowadays, *A. muciniphila* is widely considered as a novel potential candidate to improve metabolic disorders associated with obesity, diabetes, liver diseases and cardiometabolic disorders. Indeed, its administration has been shown to profoundly reduce the development of such diseases.

Other important steps toward the development of *A. muciniphila* as a next-generation beneficial microbe have been successfully reached. First, the discovery that *A. muciniphila* remained effective by being grown on a synthetic medium compatible with administration in humans. Second, the discovery that inactivation of the bacteria by pasteurization improved its effects, and thus its stability and potential shelf life. Third, the identification of a key mechanisms of interaction between *A. muciniphila* and its host via the identification of Amuc_1100, and last but not least; fourth, the demonstration that *A. muciniphila* may be safely administered in the human targeted population.

Finally, the pasteurized bacteria and the identification and the isolation of bacterial constituents such as the relatively small 30-kDa Amuc_1100 open the door to putative development of drugs based on *A. muciniphila*-related product that could also target pathologies such as type 1 diabetes, inflammatory bowel diseases or diseases where the intestinal barrier function is compromised.

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PC and WdV: Conceptualized the review content.

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Conflict of Interest Statement: PC and WdV are inventors on patent applications dealing with the use of *A. muciniphila* and its components in the treatment of obesity and related disorders.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OPINION
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Shaping the Metabolism of Intestinal *Bacteroides* Population through Diet to Improve Human Health

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INTESTINAL MICROBIOTA AND THE CONTROL OF GLUCOSE HOMEOSTASIS AND LIPID METABOLISM IN THE HOST

The human intestinal microbiota is dominated by five phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia. In adults, more than 80% of the species belong to just two phyla, Firmicutes and Bacteroidetes. Short chain fatty acids (SCFA) are catabolic end-products from intestinal microbial fermentation. Acetate, propionate and butyrate are the most abundant (Ríos-Covián et al., 2016a) whilst branched chain fatty acids (BCFA; isobutyrate, valerate, and isovalerate), the organic acids lactate, succinate, formate, and gases, can be also formed.

In humans, the main fermentable sources of SCFA are undigested dietary polysaccharides; amino acids and proteins may constitute additional substrates for colonic fermentation, whereas host-derived glycoproteins contribute to a limited extent. BCFA can be formed at considerably lower proportions than SCFA from branched-chain amino acids (BCAA; valine, leucine, and isoleucine). Threonine renders propionate and butyrate, whereas glutamate, histidine, lysine, arginine, and alanine give rise to acetate and butyrate formation; additionally, the intestinal microbiota contributes to the production of amino acids available to the host through *de novo* biosynthesis (Neis et al., 2015). Moreover, metabolic cross-feeding, that is the utilization of end products from the carbohydrate catabolism of a given microorganism by another one, strongly influences the final balance of intestinal SCFA. It occurs mainly for the formation of butyrate from acetate or lactate, is considerably lower for butyrate conversion to propionate, and very scarce between propionate and acetate (Den Besten et al., 2013).

Intestinal SCFA can incorporate into the enterohepatic circulation, being metabolized in the liver and reaching other extra-intestinal locations (Den Besten et al., 2014). Increasing evidence supports a regulatory role for SCFA in glucose homeostasis and lipid metabolism, in which intestinal SCFA ligands FFAR2 and FFAR3 and the glucagon-like peptide are involved. In the liver propionate is gluconeogenic whereas acetate and butyrate are lipogenic. Recent studies evidence that propionate and butyrate activate the intestinal gluconeogenesis (De Vadder et al., 2014), the glucose synthesized serving as a homeostatic signal in the portal system, to control hepatic gluconeogenesis (causal factor of insulin resistance and type 2 diabetes) and improving whole-body glucose homeostasis. Moreover, propionate flux through the liver reduces visceral and liver fat by decreasing intrahepatic triglycerides (Chambers et al., 2015). Propionate inhibits hepatic lipogenesis and cholesterogenesis promoted by acetate (Demigne et al., 1995) whereas propionate and butyrate inhibit lipolysis and lipogenesis and increase the incorporation of glucose mediated by insulin into the adipose tissue (Heimann et al., 2015). These observations prompt to the acetate/propionate ratio as an indicator for the potential contribution of intestinal SCFA to body lipid metabolism. Additionally, the improvement of glucose homeostasis promoted by dietary fiber

seems to be associated with elevated fluxes of SCFA from the intestinal lumen to other organs rather than with the fecal SCFA concentrations (Den Besten et al., 2014).

Several metabolic disorders as obesity, insulin resistance, and metabolic syndrome are associated with impairment of the metabolism of carbohydrates and lipids by the host, and are accompanied by changes in the gut microbiota (Turnbaugh et al., 2009; Bervoets et al., 2013). Higher levels and altered patterns of SCFA (Fernandes et al., 2014; Salazar et al., 2015) and changes in the Firmicutes to Bacteroidetes ratio, have been repeatedly reported in obese individuals (Ley et al., 2006; Turnbaugh et al., 2006). Nonetheless, contradictory results published so far on the relative abundance of both phyla exclude its use as a broadly applicable marker.

Increases in plasma circulating BCAA and aromatic amino acids (phenylalanine and tyrosine) were related with higher risk of type 2 diabetes and insulin resistance (Utzschneider et al., 2016), having been suggested that the altered functionality of the intestinal microbiota (also affecting *de novo* biosynthesis of amino acids) determines these differential profiles of circulating amino acids (Neis et al., 2015).

Increasing protein intake (Pillot et al., 2009) and gastric surgery (Liou et al., 2013) have demonstrated efficacy for weight control and improvement of glucose homeostasis, partly related to the increase of propionate (De Vadder et al., 2014), and enrichment of intestinal Bacteroidetes/*Bacteroides* (Furet et al., 2010; Jumpertz et al., 2011; Liou et al., 2013). In contrast, a significant reduction in butyrate and certain butyrate-producing Firmicutes have been associated with diets containing low amounts of fiber and carbohydrates (Duncan et al., 2007, 2008; Walker et al., 2011). These suggest a microbiota unbalance in obese subjects, or under inadequate diets, which is partly restored following gastric surgery or by introducing weight-loss diets. However, some microbiome alterations seem to persist after dietary interventions, facilitating post-dieting weight regain (Thaiss et al., 2016). This stresses the importance of achieving a full restoration of the intestinal microbiota after dietary treatments, including proper balanced microbial metabolic products, to ensure durable effects.

A FOCUS ON THE GENUS *BACTEROIDES* AND THE PRODUCTION OF PROPIONATE IN THE INTESTINAL MICROBIAL ECOSYSTEM

The order Bacteroidales is the most abundant Gram-negative bacteria, colonizing the human gut at densities up to 5–8 $\times$ 10^{10} CFU per gram of feces (Zitomersky et al., 2011). Among the predominant genera are *Bacteroides* and *Prevotella*. These microorganisms can use a wide range of dietary soluble polysaccharides that are firstly released from vegetable fiber in the intestine by microbial primary degraders (Martens et al., 2011). The genus *Bacteroides* displays a high flexibility to adapt to the nutritional conditions of the intestinal environment (Comstock and Coyne, 2003), being able to use dietary or host-derived glycans according to the nutrient availability (Sonnenburg et al., 2005). *Bacteroides* can also incorporate amino acids from outside (Smith and MacFarlane, 1998) which could be used to maintain cell structures and as an energy source.

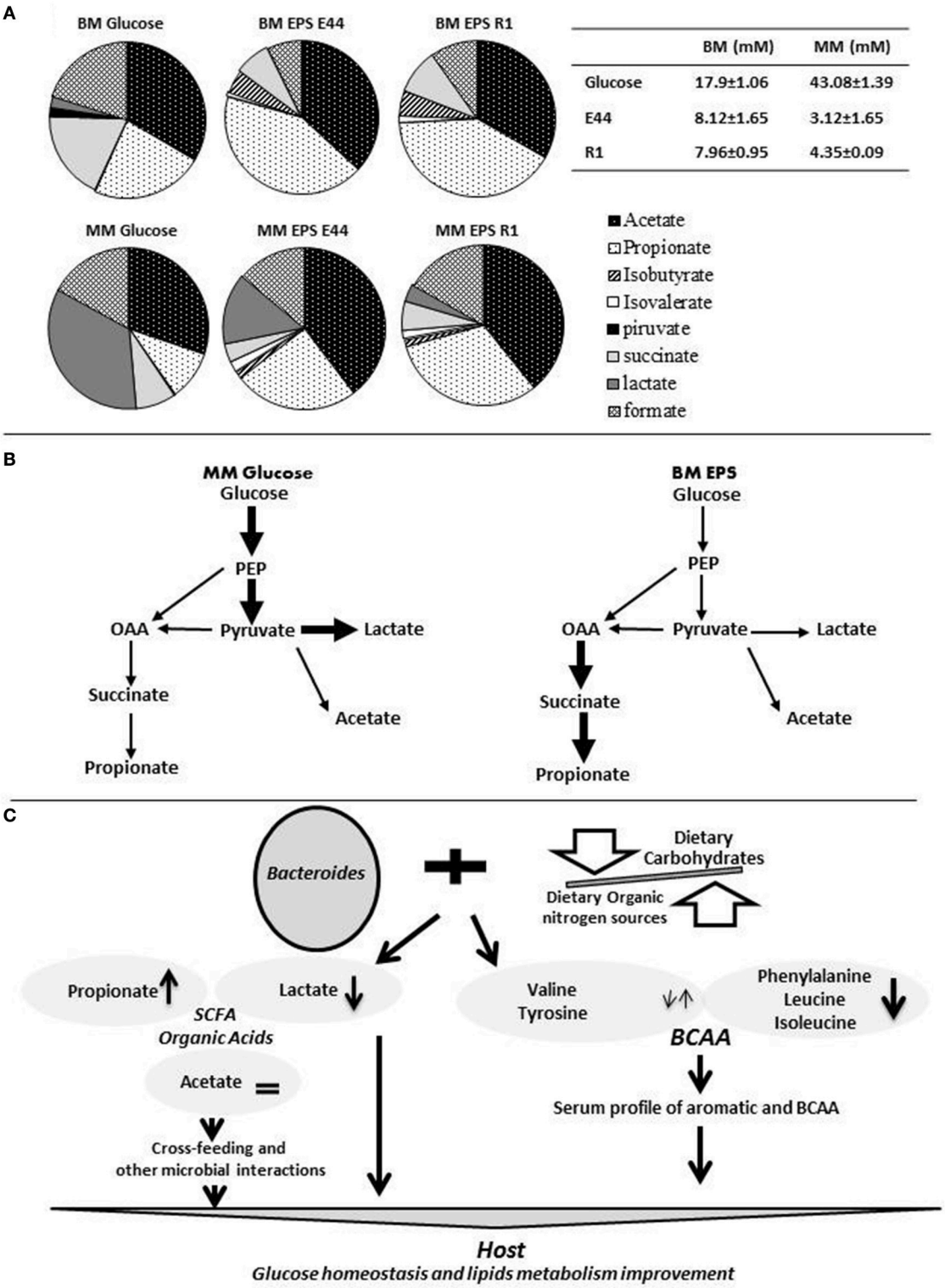
Three different biochemical pathways have been identified in colonic bacteria for propionate formation (Reichardt et al., 2014). The succinate pathway is the only one for propionate production from hexoses by Bacteroidetes, although some Negativicutes (family *Veillonellaceae,* phylum Firmicutes) can form propionate by utilizing succinate. The acrylate pathway is used for the conversion of lactate into propionate by very few bacterial genera within the phylum Firmicutes, whereas deoxy-sugars (fucose and rhamnose) are converted through the propanediol pathway by some Proteobacteria and members of the *Lachnospiraceae* family (phylum Firmicutes). *Akkermansia muciniphila* (phylum Verrucomicrobia) has been identified as a key propionate producing mucin-degrading species (Derrien et al., 2004).

Notably, several studies point to Bacteroidetes as the largest propionate producers in the human gut (Salonen et al., 2014; Aguirre et al., 2016). Interestingly, by modifying microbiota composition with antibiotic treatment in mice, Zhao et al. (2013) found a strong correlation between fecal levels of SCFA and the abundance of *Bacteroides* and other members of the phylum Bacteroidetes.

THE TYPE OF CARBOHYDRATES AND AVAILABILITY OF ORGANIC NITROGEN SOURCES MODIFY *IN VITRO* THE METABOLISM OF *BACTEROIDES*

SCFA and organic acids formed in cultures of *Bacteroides* (acetate, succinate, lactate, and propionate) depend on the type of fermentable substrates, generation time and incubation period (Kotarski and Salyers, 1981; Rios-Covian et al., 2013, 2015, 2016b). Propionate is generally favored at long generation times, with complex carbohydrates, and under carbon source limitation.

We have studied the metabolism of *Bacteroides fragilis* growing in media containing different carbohydrates and nitrogen sources. Catabolic end-products formed in the presence of carbohydrates in non-defined peptone and yeast extract containing medium (BM; Rios-Covian et al., 2015) with respect to a minimal medium without no organic nitrogen source (MM; Rios-Covian et al., 2016b) evidenced higher SCFA and organic acids production in the former medium, when it was supplemented with bacterial exopolysaccharides (EPS), which are complex structures synthesized by some bacteria (**Figure 1A**). Acetate accounted for 30–54% of the total products formed in any condition, constituting a fundamental way for obtaining energy by this bacterium. An inverse correlation was found between the production of propionate plus succinate and that of lactate (Rios-Covian et al., 2015, 2016b; **Figure 1A**), this last being favored in the absence of organic nitrogen sources and with rapid fermentable carbohydrates, as occurs in MM added with glucose. Conversely, a shift toward propionate formation appears to occur in the presence of organic nitrogen when EPS are present. This probably reflects a preferential use of the glucolytic



	BM (mM)	MM (mM)
Glucose	17.9±1.06	43.08±1.39
E44	8.12±1.65	3.12±1.65
R1	7.96±0.95	4.35±0.09

FIGURE 1 | The metabolic versatility of *Bacteroides* and the modulation of its metabolism through diet may impact human health. (A) The relative proportions of the different organic acids and SCFA produced by cultures of *Bacteroides fragilis* at 24 h of incubation in non-defined peptone and yeast extract containing medium (BM; Rios-Covian et al., 2015) and in minimal medium without no organic nitrogen source (MM; Rios-Covian et al., 2016b) and supplemented with glucose, or with exopolysaccharides produced by Bifidobacterium strains (EPS E44 and EPS R1), are represented in shaded circles. The table at the top right side indicates total concentration (mM) of SCFA plus organic acids produced by B. fragilis in the different culture conditions. **(B)** Schematic representation of

(Continued)

FIGURE 1 | Continued
catabolic routes for the formation of SCFA and organic acids by *B. fragilis*. Thick bold arrows indicate pathways probably favored in MM supplemented with glucose (left side) or in BM supplemented with bacterial EPS (right side). **(C)** Schematic representation of the general hypothesis on how re-shaping the intestinal Bacteroides metabolism through the adequate balance of dietary proteins and carbohydrates could influence human health. On the one hand, changes occurring in the profile of SCFA and organic acids produced by this bacterium could act on the host carbohydrates and lipids metabolism directly or through cross-feeding or other microbial interaction mechanisms. On the other hand, the metabolism of intestinal Bacteroides may modify blood circulating amino acids in the host, which have been related with some metabolic disorders. OAA, oxaloacetate; SCFA, short chain fatty acidis; BCAA, branched chain amino acids; PEP, phosphoenolpyruvate.

pathway and acetate formation for obtaining energy and keeping redox balance by *B. fragilis* in the presence of rapidly fermentable carbohydrates; in contrast, when complex/slowly fermentable carbohydrates are available and amino acids are present, carbon skeletons from amino acids could be incorporated at the level of pyruvate; in such conditions the propionate-succinate pathway seems to be potentiated as a way for energy obtaining whilst serving to restore cell redox balance (Rios-Covian et al., 2015; **Figure 1B**). Proteomics and gene expression analyses reinforced the hypothesis of the activation of amino acids catabolism and the succinate pathway in *B. fragilis* grown in BM with EPS (Rios-Covian et al., 2015). Therefore, the preferential metabolic route for energy production and redox maintaining, and the final metabolic products formed by *B. fragilis*, may be largely dependent on carbohydrates and nitrogen sources available. These results suggest the possibility of regulating the metabolism of *Bacteroides* by controlling dietary carbohydrate/protein balance.

Moreover, when analyzing the amino acids in cultures of *B. fragilis* added with different carbohydrates, we found a decrease in the concentration of leucine, isoleucine and phenylalanine after incubation in any condition, whereas valine and tyrosine showed much less increases or slight decreases in EPS as compared to glucose (Supplementary Material Table 2 in Rios-Covian et al., 2015). This points to a potential capacity of *B. fragilis* (as may probably occur with other Bacteroidetes) for regulating BCAA and aromatic amino acids levels in its growth environment.

MODULATION OF THE INTESTINAL *BACTEROIDES* BY DIETARY CARBON/NITROGEN SOURCES: A TOOL FOR RESTORING THE INTESTINAL MICROBIOTA METABOLIC BALANCE

Under sufficient organic nitrogen, the mildly acidic pH (5.5) stimulates butyrate producing species in the human colon curtailing the growth of *Bacteroides* that was however maximized at pH 6.5 (Walker et al., 2005). The pH in the caecum is about 5.7 but gradually increases to 6.7 in the rectum. Dietary fiber fermentation promotes a slight decrease of the luminal pH whereas high protein/amino acids fermentation, favored by low carbohydrate availability, causes slightly pH increases (Smith and MacFarlane, 1998). Interestingly, a recent study with mice demonstrated that diet-microbiome interactions are driven by the pattern of protein and carbohydrate intake (Holmes et al., 2017). Moreover, some experiments with gnotobiotic mice support shifts in *Bacteroides* metabolic functions as a response to dietary changes (McNulty et al., 2013; Wu et al., 2015).

The studies just commented support the interdependence between diet, gut microbiome and host metabolism, and allow to hypothesize that the combination of dietary organic nitrogen sources with appropriate carbohydrates may be used to modify the metabolic activity of colonic *Bacteroides* populations by modifying the profile of organic acids formed and enhancing propionate formation in some parts of the large intestine while promoting shifts toward healthier profiles of serum amino acids (**Figure 1C**).

Within this "scenario," the potential role that the functional control and metabolic reprogramming of *Bacteroides* through diet may play in the regulation of the host metabolism deserves more attention. It is essential to decipher to what extent organic nitrogen sources and carbohydrates could affect the different species of prominent intestinal bacteria, such as the genus *Bacteroides*. An important question raised is whether changes in SCFA and organic acids profile induced by remodeling the metabolic activity of *Bacteroides* through adequate dietary interventions would influence other less nutritionally versatile gut beneficial microbes through the enhancement of cross-feeding or other microbial interaction mechanisms. Omics, including metabolomics/metabonomics, applied to the analysis of microbial cultures, animal models, and human samples are necessary for understanding host and microbiota metabolic remodeling as a response to dietary combinations of organic nitrogen/carbohydrates.

The potential to re-shape the metabolism of *Bacteroides* with specific combinations of dietary carbohydrates-proteins based on their composition, structure and availability in the gut, merits further study. The final aim should be designing diets based on nutrient components targeted at modulating the metabolism of *Bacteroides,* which may interact with other intestinal beneficial microbes, in order to restore the metabolic balance of the microbiota to promote durable host's health effects.

AUTHOR CONTRIBUTIONS

DR, NS, MG, and Cd conceived the idea and designed the structure of the manuscript. All authors contributed significantly to the experimental data compared in the Figure 1A. Cd and DR drafted the manuscript and Figure. All authors have critically red, corrected, and approved the final version of the manuscript and agree with the opinions expressed here.

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PERSPECTIVE
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Searching for the Bacterial Effector: The Example of the Multi-Skilled Commensal Bacterium *Faecalibacterium prausnitzii*

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Faecalibacterium prausnitzii represents approximately 5% of the total fecal microbiota in healthy adults being one of the most abundant bacterium in the human intestinal microbiota of healthy adults. Furthermore, this bacterium has been proposed to be a sensor and a major actor of the human intestinal health because of its importance in the gut ecosystem. In this context, *F. prausnitzii* population levels have been found to be reduced in patients suffering from several syndromes and diseases such as inflammatory bowel diseases. These diseases are characterized by a breakage of the intestinal homeostasis called dysbiosis and the use of *F. prausnitzii* as a next generation probiotic (also called live biotherapeutics) has been proposed as a natural tool to restore such dysbiosis within the gut. Nevertheless, despite the potential importance of this bacterium in human health, little is known about its main effectors underlying its beneficial effects. In this perspective note, we aim to present the actual state in the research about *F. prausnitzii* effectors and the future milestones in this field.

Keywords: probiotic, commensal, *Faecalibacterium*, bacterial effectors, live-biotherapeutics

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INTRODUCTION

Nowadays, humans can be considered as "meta-organisms" composed of 10-fold more microorganisms than human cells (Neish, 2009), which means 150-fold more genes than the human genome itself (Qin et al., 2010; Bruls and Weissenbach, 2011). These microorganisms, named microbiota (and by extension all their genes as a whole, named microbiome) are different depending on the tissue considered. The human gastrointestinal tract (GIT) is one of the most complex ecosystems. The advances of molecular techniques have shown that the collective adult human GIT microbiota is composed of up to 1000–1150 bacterial species (Frank et al., 2007; Qin et al., 2010). The predominant species (46–58%) are those with low GC-content being the clostridium group the most abundant (Zoetendal et al., 2002; Qin et al., 2010). As a consequence of the mutualism established between the host and its microbiota, the GIT micro-ecosystem is key to maintain the homeostasis of a healthy individual (Leser and Molbak, 2009). Indeed, gut microbiota supplies essential nutrients, metabolize indigestible compounds and protects the host against colonization by opportunistic pathogens (Leser and Molbak, 2009; Martín et al., 2013). It also contributes to the development of the intestinal architecture as well as several immuno-modulatory functions (Mazmanian et al., 2005). In some abnormal conditions, an imbalance in the microbial ecosystem may happen leading to a microbial imbalance known as dysbiosis. This dysbiosis is characterized by the growth of different non-predominant bacteria and/or the

depletion of commensal ones that can lead to a situation of illness. As a result, this imbalance can also lead to the lack of some beneficial effects of these commensal bacteria, and thus unchain pathogenic conditions not only due to pathogen overgrowth (Martín et al., 2013).

THE HUMAN GUT MICROBIOTA AS A SOURCE OF NEXT GENERATION PROBIOTICS (NGPs)

As gut microbiota is now considered as a major actor underlying health, the idea of using some well-known microbiota components as next generation probiotics (NGPs) is very promising. Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Recently, this definition has been clarified by an expert panel of the International Scientific Association for Probiotics and Prebiotics (ISAPP) and re-defined as: "live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Most of the traditional probiotics belong to both lactic acid bacteria (LAB) and *Bifidobacteria* groups. These novel probiotics are thus considered as NGPs in contrast to traditional ones, which were not selected on the basis of human microbiota analysis. To consider a strain as probiotic, it should be: (i) well-characterized, (ii) achieve safety requirements, and (iii) confer beneficial effects on the host. However, a careful selection should be made in each case as probiotic properties are usually strain specific and cannot be extrapolated to another strain even belonging to the same species (Pineiro and Stanton, 2007; Gareau et al., 2010).

Similarly, as probiotic therapy is mainly based on restoring the normal balance of the intestinal ecosystem, we consider that the use of commensal bacteria as NGPs is a natural way to restore the dysbiotic situation within the GIT (Miquel et al., 2015a). Nevertheless, in contrast to most of probiotic lactobacilli or bifidobacteria strains, these NGPs are not listed neither on QPS (for Qualified Presumption of Safety), nor on GRAS (for Generally Recognized As Safe organisms) lists. Furthermore, as they have not a long record of safe consumption (precisely no documented safe use in Europe prior to 1997), these NGPs can only be used either as novel foods or drugs and the requirements to allow their market in Europe are more severe than for conventional probiotic strains (Miquel et al., 2015a). Nowadays, the inclusion of *Faecalibacterium* on the QPS list might be difficult due to its lack of a history of safe use. In addition, full toxicology assays and characterization of the strain are still needed for regulatory approval (Brodmann et al., 2017). We have reviewed this problematic in detail elsewhere (Miquel et al., 2015a).

A FOCUS ON *Faecalibacterium praustnizii*

As *Faecalibacterium prausnitzii* is an extremely oxygen sensitive (EOS) bacterium and difficult to grow (Duncan et al., 2002), most of the data about its physiology are based on metagenomic studies (Miquel et al., 2013), with some exceptions (Duncan et al., 2002; Ramirez-Farias et al., 2009; Lopez-Siles et al., 2012; Foditsch et al., 2014; Martín et al., 2017). *F. prausnitzii* is a member of the Clostridium group (phylum Firmicutes, class Clostridia, family Ruminococcaceae), specifically of the *C. leptum* group (Benevides et al., 2017), and represents around 5% of the total fecal microbiota in healthy adults being one of the most abundant bacterium in the human intestinal microbiota in healthy conditions (Hold et al., 2003). The first isolates were classified as *Fusobacterium praustnizzi,* but latter on its close relation with members of the *C. leptum* group was established thorough analysis of 16S rRNA gene (Miquel et al., 2014). The establishment of *F. prausnitzii* along the GIT may result from a combination of environmental factors such as other commensal species, redox mediators, oxygen concentration, mucus layer as well as bile salt concentrations and pH (Duncan et al., 2009; Lopez-Siles et al., 2012). In early infancy, *F. prausnitzii* abundance is very low and increases after the establishment of primo-colonizing bacteria (Hopkins et al., 2005). In the last years, this bacterium has been suggested as a biosensor and a major actor of the human intestinal health (Miquel et al., 2013). Indeed, *F. prausnitzii* levels have been found to be reduced in patients suffering from several syndromes and diseases such as inflammatory bowel diseases (IBDs), irritable bowel syndrome (IBS), colorectal cancer (CRC), obesity, and celiac disease (Balamurugan et al., 2008; Sokol et al., 2008; Neish, 2009; De Palma et al., 2010; Furet et al., 2010; Rajilic-Stojanovic et al., 2011) as well as in frail elderly (van Tongeren et al., 2005). We have more deeply review *F. prausnitzii* physiology and beneficial effect elsewhere (Miquel et al., 2013, 2014). Nevertheless, its EOS condition, make viable intestinal delivery one of the current challenges, due to their stringent survival conditions (El Hage et al., 2017).

Because of its important role in GIT homeostasis, *F. prausnitzii* is considered today as a potential NGPs. Its potential utilization has been already proposed for livestock animals, for instance the isolation and characterization of *F. prausnitzii* strains from stool of calves and piglets have been already performed (Foditsch et al., 2014). Also a specific formulation keeping this EOS bacterium alive at ambient air conditions has been also proposed for patients with intestinal dysbiosis-associated diseases (Khan et al., 2014). In order to evaluate its potential beneficial effects as a NGP, we have successfully used this bacterium in several murine models of IBD and IBS (Sokol et al., 2008; Martín et al., 2014a; Laval et al., 2015; Miquel et al., 2016) and other groups have also clearly demonstrated its beneficial effects *in vivo* (Carlsson et al., 2013; Rossi et al., 2015, 2016). Briefly, we have found that mice treated with either *F. prausnitzii* A2-165 or its supernatant (SN) present lower symptoms of inflammation in both acute and chronic chemically-induced colitis models as well as improved gut permeability and function in a model of gut impairment induced by dinitrobenzene sulfonic acid (DNBS) (Sokol et al., 2008; Martín et al., 2014a, 2015; Laval et al., 2015). We have also observed that A2-165 strain was able to reduce pain sensibility in

partial restraint stress (PRS) and neo-maternal separation (NMS) murine models (Miquel et al., 2016). Furthermore, Carlsson et al. (2013) and Rossi et al. (2015, 2016) have found similar anti-inflammatory results as well as restoration of increased intestinal permeability in dextran sulfate sodium (DSS) induced colitis.

Staring the host pathways involved in the beneficial effects displayed by *F. prausnitzii*, *in vitro* tests have shown that: (i) although *F. prausnitzii* itself had no effect on IL-1β-induced NF-κB activity, its SN abolished it in Caco-2 cells transfected with a reporter gene for NF-κB activity and (ii) peripheral blood mononuclear cell (PBMC) stimulation by *F. prausnitzii* led to significantly lower IL-12 and IFN-γ production levels and higher secretion of IL-10 (Sokol et al., 2008). In this sense, human dendritic cells (DCs) stimulation with A2-165 and HTF-F strains also induced the production of IL-10 (Rossi et al., 2015). IL-10 has been established to be an important immune-regulatory cytokine that successfully suppresses the exacerbated mucosal immune response associated with colonic inflammation (Schreiber et al., 2000). This increasing in IL-10 induced by *F. praustnizii* has also been observed *in vivo* (Sokol et al., 2008). Furthermore, Rossi et al. (2016) found that the strain of *F. prausnitzii* A2-165 has a strong capacity to induce IL-10 in human and murine DCs and influence the T-cell differentiation *in vitro* and *in vivo*. In this sense, *F. prausnitzii* is also able to increase lymphocyte T regulatory (Treg) population *in vivo* after a colonic chemical challenge (Martín et al., 2014a) and has been identified as the major inducer of a specific IL-10 secreting Treg subset named CD4CD8α lymphocytes present in the human colonic lamina propria and blood which is deficient in IBD patients (Sarrabayrouse et al., 2014).

Faecalibacterium praustnizii EFFECTORS: WHERE DO WE STAND?

Since the first study about *F. prausnitzii* performed in our laboratory almost 10 years ago (Sokol et al., 2008), we have sought to identify the bacterial effectors underlying its beneficial effects. We have been focused on its SN which showed anti-inflammatory properties in both *in vivo* and *in vitro* experiments (Sokol et al., 2008; Martín et al., 2013). Our main hypothesis was that *F. prausnitzii* can produce an anti-inflammatory soluble molecule. First, we tried to identify the chemical nature of the molecule by submitting the SN to several enzymatic and physical methods and as shown in **Figures 1A–C**, none of them were able to suppress the anti-inflammatory "properties" from the SN pointing out the possible presence of more than one effector on *F. praustnizii* SN. Of course, this result can also support an important role of butyrate in *F. prausnitzii* effects, although *in vivo* and *in vitro* experiments point out for a more complex situation (see below). Nevertheless, we need to consider that the beneficial effects of *F. prausnitzii* might not be present only in its SN. For instance, we have found that some beneficial effects, such as the anti-nociceptive one, are observed only when the animals where treated with the bacterium but not with its SN (Miquel et al., 2016).

Some of the putative bacterial effectors identified until now are presented in the **Figure 2** and are described in the next paragraphs.

Butyrate

Typically, as *F. prausnitzii* is one of the most abundant butyrate-producing species, its beneficial effects have been first attributed to butyrate. Butyrate is a short chain fatty acid (SCFA) well-known for its pleiotropic and beneficial effects in the GIT (Duncan et al., 2009; Macfarlane and Macfarlane, 2011) as well as its immune-modulatory properties *in vitro* (Bocker et al., 2003). Furthermore, it is involved in the cross-feeding between butyrate producer bacteria and *Bifidobacterium* sp. which favors the co-existence of bifidobacterial strains with other bifidobacteria and with butyrate-producing colon bacteria in the human colon with the consequent benefit in the human health (Riviere et al., 2016). Microbial butyrate is considered key for colonic health, as it is an energy source for epithelial cells and is able to modulate oxidative stress and inflammation (Hamer et al., 2008). However, its role remains controversial as its effects seem to be dose- and time-dependent (Martín et al., 2013). In this sense, it also has different effects depending of the cell line tested (Bocker et al., 2003). For instance, regarding cells from intestinal origin, butyrate was found to decrease IL-8 secretion in Caco-2 and human intestinal primary epithelial cells (HIPECs) and to enhance IL-8 production in both HT-29/p and HT-29 MTX cells (Bocker et al., 2003). Furthermore, not all butyrate producing-bacteria have the same anti-inflammatory profile *in vitro*. For instance, in 6 h TNF-α stimulated HT-29 cells, the SN of *Roseburia intestinalis*, a butyrate producer, does not have anti-inflammatory properties while *F. prausnitzii* SN does (**Figure 1D**).

Although the effect of butyrate is clearly present, the anti-inflammatory capacities of *F. prausnitzii* do not seem to be limited to butyrate only. In previous studies, we have shown that *F. prausnitzii*-mediated butyrate production is not the only beneficial bacterial effector linked to this species in colitis models (Sokol et al., 2008; Martín et al., 2013; Miquel et al., 2015b). For instance, we found that the increase of the presence of 4-hydroxybutyric acid in the feces, colon and caecum of dixenic mice colonized with *F. prausnitzii* A2-165 and a strain of *Escherichia coli* compared to monoxenic mice colonized only by the strain of *E. coli* was not directly linked to health parameters in a rat model of acute trinitrobenzenic acid (TNBS)-induced colitis although the increase of production of butyrate has been also verified by gas liquid chromatography (Miquel et al., 2015b). These results support previous findings, where we found that butyrate did not protect mice from TNBS-induced colitis, in contrast to *F. prausnitzii* A2-165 SN (Sokol et al., 2008).

Other Active Metabolites

The use of a gnotobiotic model including *F. prausnitzii* A2-165 strain and *E. coli* allowed us, using a metabolomic approach, to identify several metabolites that, in contrast to 4-hydroxybutyric acid, are associated to the beneficial effect of *F. prausnitzii* in a TNBS-acute model in rats (Miquel et al., 2015b). These metabolites were the salicylic acid, shikimic acid and raffinose,

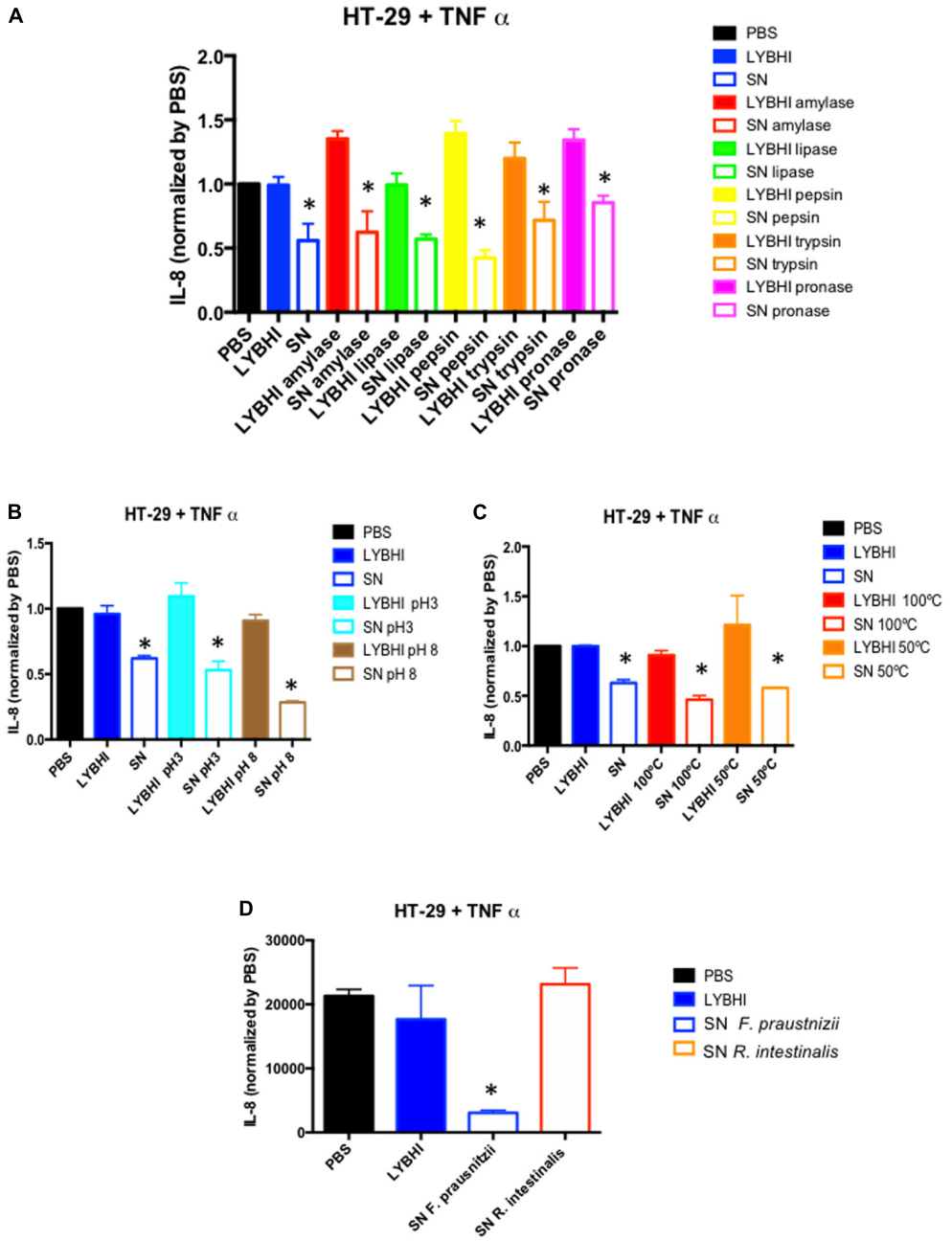

FIGURE 1 | *In vitro* immune-modulatory test on HT-29 cells stimulated with TNF-α. Anti-inflammatory activity of *Faecalibacterium prausnitzii* A2-165 supernatant (SN) and culture medium (LYBHI) submitted to different treatments: **(A)** enzymatic treatments: amylase (red), lipase (green) pepsin (yellow), trypsin (orange) and pronase (pink), **(B)** pH modifications: pH 3 (cyan) and pH 8 (brown), and **(C)** different temperatures: 100°C (red) and 50°C (orange). **(D)** Comparison of *Roseburia intestinalis* DSM14610T SN and *F. prausnitzii* A2-165 SN effect. Bacterial SNs were recovered, filtered and submitted to different treatments (if indicated) before being tested in co-incubation assays with HT-29 cells challenged with TNF-α. IL-8 concentration was determined by ELISA and used as a read-out of the inflammatory status of the cells. The results were normalized by the negative control (PBS) (for material and methods details see Supplementary Data 1). $*p < 0.05$ *versus* LYBHI (bacterial culture medium) ($n = 3*3$).

among others (Miquel et al., 2015b). Salicylic acid is used in the pharmaceutical industry to produce the amine derivate 5-aminosalicylic acid (5-ASA or mesalamine) that it is nowadays used to treat patients suffering from IBD (Messori et al., 1994). Furthermore, shikimic acid is a precursor for the synthesis of several aromatic compounds among which we can also find the salicylic acid through the achorismate synthase pathway (Bochkov et al., 2012). These two compounds point out a key role of *F. praustnizii* in the biosynthesis of salicylic acid, precursor of 5-ASA, both anti-inflammatory molecules that should be linked

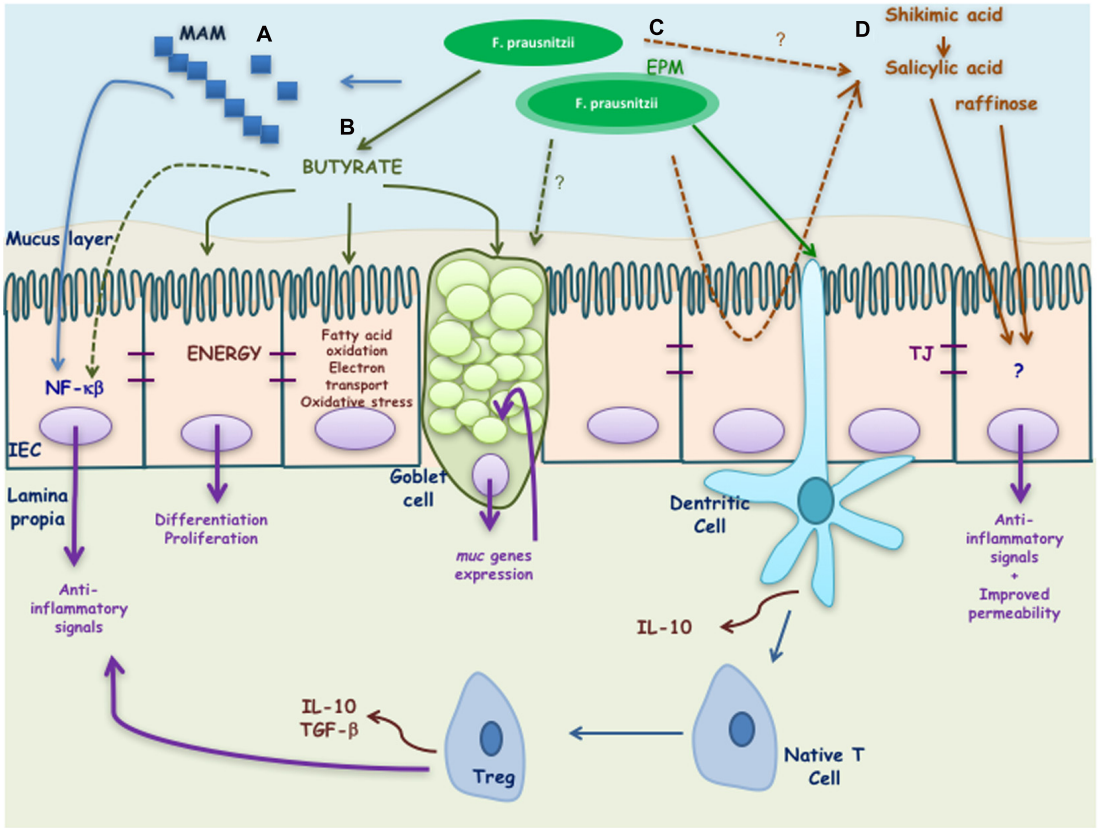

FIGURE 2 | Putative effectors of *Faecalibacterium prausnitzii* and its effects on the host. *F. prausnitzii* exerts its benefic effects by means of different effectors: **(A)** MAM peptides secreted by *F. prausnitzii* block NF-κB activation induced by a pro-inflammatory stimulus. **(B)** Butyrate produced by *F. prausnitzii* inhibits NF-κB activation and interacts with the intestinal epithelial cells (IEC) driving to the activation of different genes involved on the differentiation, proliferation, and restitution of enterocytes. It is also involved on the regulation of fatty acid oxidation, electrons transport chain, oxidative stress, and apoptosis. In goblet cells it has been described to stimulate *muc* genes allowing a high production of mucus. **(C)** EPM produced by *F. prausnitzii* modulates IL-10 cytokine production in antigen presenting cells. Finally, **(D)** salicylic and shikimic acids are anti-inflammatory molecules able to block inflammation induced by a pro-inflammatory stimulus while raffinose is key in maintaining gut permeability.

to the *in vivo* anti-inflammatory effect observed in mice treated with *F. praustnizii*. In contrast, raffinose is an oligosaccharide only fermented by the gut microbiota that is not related to anti-inflammatory effects, but with mucosal permeability, as raffinose permeation is key in maintaining gut permeability (Dawson et al., 1988). The raffinose could thus play a role in the improvement on gut permeability promoted by *F. praustnizii* (Carlsson et al., 2013; Laval et al., 2014). In this sense, we have found that *F. praustnizii* and its SN are able to counterbalance the increase in intestinal barrier permeability in a murine model of gut dysfunction induced by DNBS (Martín et al., 2015). Of note, in this simplified microbiota, we have identified metabolites that can be produced either by the host or by the bacteria, and the direct production of these compounds by *F. prausnitzii* has not yet been proved.

Microbial Anti-inflammatory Molecule (MAM)

Thanks to a peptidomic analysis using mass spectrometry of *F. prausnitzii* A2-165 strain SN, we have successfully identified seven peptides, all derived from the same anti-inflammatory molecule, a protein of 15 kDa named MAM (ZP05614546.1) (Quevrain et al., 2016). Due to the difficulties to test directly the peptides or the protein, mainly due to solubility problems, indirect strategies were performed to determine their biological effect. Transfection of MAM cDNA in epithelial cells led to a significant decrease in the activation of the nuclear factor (NF)-κB pathway with a dose-dependent effect (Quevrain et al., 2016). This inactivation of NF-κB pathway was also observed *in vivo* using a transgenic model of mice producing luciferase under the control of NF-κB promoter (Breyner et al., 2017). Finally, the administration of a food-grade bacterium, *Lactococcus lactis,* delivering a plasmid encoding MAM was able to alleviate DNBS and DSS induced colitis in mice (Quevrain et al., 2016; Breyner et al., 2017). Although these promising results point out for the strong role of MAM on *F. prausnitzii* SN effect, the persistence of the anti-inflammatory effect of the SN after proteolytic treatment and the ability of *F. prausnitzii* SN to block other inflammatory pathways different from NF-κB (Martin et al., 2014b) point that MAM is not the

only bacterial effector mediating *F. praustnizii* SN beneficial effects.

Extracellular Polymeric Matrix (EPM)

Rossi et al. (2015) found that the biofilm-forming strain *F. praustnizii* HTF-F was able to attenuate the clinical symptoms of DSS-induced colitis in a stronger manner than A2-165 strain. Furthermore, the intra-rectal administration of purified extracellular polymeric matrix (EPM) decrease the disease index of the mice indicating that it contributes strongly to the protective effects of HTF-F strain. However, although the authors concluded that the anti-inflammatory effects of *F. prausnitzii* HTF-F strain may be in part be due to the immune-regulating properties of the EPM, they were not able to rule out other possible strain differences such as colonization ability, stress resistance or *in vivo* fitness, among others. In fact, these parameters might also contribute to the efficacy of the strain as improved survival might impact on butyrate or MAM production for instance.

FUTURE PERSPECTIVES

The lack of clearness about *F. prausnitzii* effectors is linked to the lack of knowledge in its biology and phylogeny. Furthermore, as we mentioned above, probiotic characteristics are strain-specific, and therefore individual studies should be performed in order to determine the individual beneficial effects as well as the individual effectors linked to these effects. If we compare to a classic probiotic group, lactobacilli, we can find that although all lactobacilli are able to produce lactic acid (proved to have beneficial effects in some ecosystems such as the vaginal), not all of them are equipped with the same arsenal of bacterial effectors (bacteriocins, biosulfactants, H_2O_2, etc. . .) that are strain specific. In the case of *Faecalibacterium*, the framework should be similar, as even if all the strains are able to produce butyrate, other possible molecules could be responsible of additional strain-specific beneficial effects.

Recently, we have isolated a collection of novel *F. prausnitzii* strains form healthy volunteers that we have characterized (Miquel et al., 2015a) and analyzed for anti-inflammatory properties with the aim of selecting new NGPs candidates (Martín et al., 2017). The deeper phylogenic analysis of the complete genome of this bacterial collection joint to the genomes already present in the public data bases has revealed that there are at least three separate clusters, spanning the classical Phylogroups I and II already found in *F. praustnizii* (Lopez-Siles et al., 2012, 2016; Martín et al., 2017) and that some strains appear to represent a deeper, more divergent branch of the "*Faecalibacterium prausnitzii*" taxon (Benevides et al., 2017). This new truth about *F. prausnitzii* provides evidence that the phylogeny of *F. praustnizii* should be reconsidered. In line with these results, in the future we should take into account the presence of at least three different genospecies inside *Faecalibacterium* group to better characterize their beneficial effects, their crosstalk with the host and the possible effectors underlying these effects. Furthermore, the pangenome analysis have been performed to get a global view of the genome of this genus. A total of 10,366 core-genome codifying DNA sequences have been found (3.33-fold the average total number of genes of the 17 analyzed strains) (Benevides et al., 2017). Nevertheless, up today no extensive genomic description of this taxon has been finished, being an ongoing task with a key role in the future perspectives of the analysis of this genus.

CONCLUDING REMARKS

In this perspective article, we have tended to highlight the importance and problematic of asserting the probiotic characterization of a NGP, a field on the focus of the research of Frontiers in Microbiology audience. Nowadays, the research community recognizes the importance of *F. prausnitzii* in the human health. A decrease of this bacterium in the GIT has been linked to several diseases and syndromes but it is still not clear if this is a cause or a consequence of them. As mentioned above, this special condition, make this species a unique bacterial sensor and actor in the human health, mainly related with intestinal issues, but not only. Taking advantage of this, its use as NGP is being explored for both human and animal use. However, more research in its phylogeny, physiology, safety, and beneficial effects should be performed to fill the lack that currently exists between the knowledge of the biology of the bacterium and the medical interest that it produces. As an example, the analysis of the possible bacterial effectors taking into account the phylogeny and the biological effects related should be performed in more detail to find the best probiotic candidate and refine its use as biomarker in several human disorders.

AUTHOR CONTRIBUTIONS

RM, LB-H, and PL designed the perspective and the experiments. RM wrote the manuscript and performed the experiments. LB-H and PL corrected the manuscript. All the authors approved the last version of the manuscript.

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SUPPLEMENTARY MATERIAL

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The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Health-Associated Niche Inhabitants as Oral Probiotics: The Case of *Streptococcus dentisani*

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Oral diseases, including dental caries and periodontitis, are among the most prevalent diseases worldwide and develop as a consequence of a microbial dysbiosis. Several bacterial strains are being tested as potential oral health-promoting organisms, but usually they are species isolated from niches other than the site where they must exert its probiotic action, typically from fecal samples. We hypothesize that oral inhabitants associated to health conditions will be more effective than traditional, gut-associated probiotic species in key aspects such as colonization of the oral site where disease takes place or the possession of oral health promoting functions, as well as more practical issues like safety and toxicity, and establishing proper doses for administration. As an example of these *active colonizers*, we describe the case of *Streptococcus dentisani*, a new streptococcal species isolated from dental plaque of caries-free individuals. We have detected it in 98% of dental plaque samples from healthy individuals and, as expected, it does not produce any toxic secondary metabolite and does not survive a simulated stomach digestion, preventing potential secondary effects. Besides, this species has a double probiotic action, as it inhibits the growth of major oral pathogens through the production of bacteriocins, and also buffers acidic pH (the primary cause of dental caries) through an arginolytic pathway. We propose the use of *S. dentisani* as a promising probiotic against tooth decay.

Keywords: probiotics, dental caries, pH buffering, arginolytic pathway, bacteriocins, *Streptococcus dentisani*

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INTRODUCTION

Oral diseases such as dental caries (tooth decay) and periodontitis (gum disease) are caused by microorganisms. However, they are currently not considered infectious diseases in classical terms because their etiology is clearly polymicrobial (Fejerskov, 2004; Simón-Soro and Mira, 2015), and because the pathogenic bacteria involved are also found at lower proportions in healthy individuals (Hajishengallis and Lamont, 2012; Camelo-Castillo et al., 2015). Thus, it has been pointed out that antimicrobial strategies may not be effective against oral diseases, and new preventive or therapeutic approaches based on restoring the microbial ecological balance in the oral cavity have been proposed (Marsh, 2015; Marsh et al., 2015). Those new preventive measures could include the use of prebiotic compounds to promote the growth of health-associated bacteria (Santarpia et al., 2014), or the application of probiotic bacteria with beneficial features (Saha et al., 2012). In the case of dental caries, health-associated microbial communities have been identified using omics

approaches (Zaura et al., 2009; Belda-Ferre et al., 2011; Alcaraz et al., 2012), and therefore culturing those microorganisms could provide potential beneficial bacteria to prevent oral diseases.

However, due to the greater development of probiotics in gut pathologies, and the strong safety evidence accumulated for gut bacteria, many microorganisms isolated from human or animal fecal samples with beneficial properties are being developed as potential probiotics to promote oral health. These often include strains of lactobacilli and bifidobacteria, which had previously been shown to have inmunomodulatory, anti-inflammatory and anti-bacterial properties in different *in vitro* studies and also in clinical trials (see **Table 1**; Reid et al., 2011; Cagetti et al., 2013). However, the use of many of these strains in oral diseases like dental caries can be problematic because both *Lactobacillus* and *Bifidobacterium* species have been shown to be acidogenic and to be involved in tooth decay (Badet and Thebaud, 2008; Mantzourani et al., 2009a). Even if weak acidogenic strains are selected, the capacity of gut bacteria to colonize the oral niche and to produce anti-caries effects has still to be demonstrated, and the use of gut probiotics in the oral cavity has been criticized. For instance, when the strain *Lactobacillus salivarius* W24 was tested in an *in vitro* oral biofilm model, it was shown that this strain further lowered the pH and affected the compositional stability of oral communities (Pham et al., 2009). Thus, the identification of novel strains isolated from the oral cavity itself could be instrumental for the development of efficient probiotics applied to oral health.

In the current manuscript, we describe the potential probiotic features of the new species *Streptococcus dentisani* that we recently isolated from the dental plaque of caries-free individuals (Camelo-Castillo et al., 2014). There are currently six isolates for which the genome sequence is available and that robust phylogenomic analysis include within the *dentisani* cluster (Jensen et al., 2016). This cluster forms part of the *S. oralis* clade and differs from the *S. oralis* and *S. tigurinus* clusters mainly in their ability to hydrolyze arginine (Jensen et al., 2016). In this manuscript, we study strains 7746 and 7747^T, which were isolated from two different individuals and that were shown to be different by comparison of their genomes (Camelo-Castillo et al., 2014). We describe their ability to inhibit the growth of caries-producing bacteria, as *Streptococcus mutans* and *Streptococcus sobrinus*, and to buffer extracellular acidic pH, which is the underlying cause of tooth decay. In addition, we show that, being a commensal species in the oral cavity of healthy individuals, their safety features are robust and the appropriate dose for probiotic treatment can be easily determined experimentally.

MATERIALS AND METHODS

Inhibition Assays with *S. dentisani* Supernatants

Inhibition experiments against cariogenic bacteria were carried out with concentrated supernatants of well grown cultures of *S. dentisani* 7746 and 7747 (stationary phase). In order to obtain 5 ml of the concentrated supernatants, single colonies of each *S. dentisani* strain were inoculated into 50 ml of brain hearth infusion broth (BHI, Biolife) and incubated aerobically at 37°C without agitation during 48 h, or until they reached an optical density of around 1.5 at 610 nm ($O.D._{610}$). After the incubation period, the cultures were centrifuged at 4000 rpm 10 min and the pellets discarded. The obtained supernatants were filtered throughout 0.2 μm pore-size filters (Millipore) and ten-fold concentrated by rotary evaporation on a RV 10 digital device (VWR) with the following settings: heating bath at 40°C, 70 rpm of rotation, 100 mbar of pressure, and 30 min of operating time. The resulting 5 ml of concentrated supernatants were filtered throughout 0.2 μm pore-size filters (Millipore) and stored at –20°C until use.

The inhibitory activity of the supernatants was determined by monitoring the growth of *S. mutans* ATCC 25175 and *S. sobrinus* CECT 4034 in the presence/absence of the potential inhibitor by means of optical density measurements. Pre-inocula of *S. mutans* and *S. sobrinus* were obtained by inoculating a single colony of each strain in 10 ml of BHI liquid medium and incubated aerobically overnight at 37°C without agitation. The $O.D._{610}$ of the pre-inocula was measured in a spectrophotometer (BioPhotometer, Eppendorf) and diluted with BHI liquid medium to obtain an optical density of 0.1. To assess the inhibitory effect of *S. dentisani* on the growth of *S. mutans* and *S. sobrinus*, 160 μl of the bacterial suspensions were mixed with 40 μl of the concentrated supernatants and loaded by triplicate into a Nunc Microwell 96-well microplate by triplicate. As controls, 160 μl of the bacterial suspensions were mixed with 40 μl of 10-fold concentrated BHI and loaded by triplicate into the 96-well microplate. The microplate was loaded into a microplate reader (Infinite 200 PRO, Tecan) and incubated at 37°C during 24 h. The $O.D._{610}$ of each inoculated well was measured every 30 min during the incubation time.

To determine the active size fraction, the concentrated supernatant was size-fractionated by sequential filtering throughout 10 KDa and 3 KDa filters (Amicon), following the manufacturer's recommendations. By this way, we obtained three size fractions (>10 KDa, 3–10 KDa, and <3KDa) that were tested by triplicate against *S. mutans* cultures by the same methodology explained above. To confirm the peptidic nature of the inhibitory compounds and discard that the inhibition was produced by hydrogen peroxide we proceed as described in Zhu and Kreth (2012). Briefly, *S. dentisani* was grown on a BHI plate for 24 h and peroxidase (40 μg), peptidase (64 μg), or phosphate-buffered saline were applied beside each colony for 10 min before the other species were inoculated at the same spot. *S. mutans* and *S. sanguinis* were used as controls of proteinaceus inhibitory substance and H_2O_2, respectively.

Besides, we used scanning electron microscopy (SEM) to directly observe the effect of the *S. dentisani* supernatants on the three type strains *S. mutans* ATCC 25175, *Prevotella intermedia* NCTC 13070, and *Fusobacterium nucleatum* DSMZ 20482 cells. Briefly, 160 μl of the bacterial cultures in exponential phase ($O.D._{610} = 0.6$) were mixed with 40 μl of the concentrated supernatant and incubated for 30, 60, and 90 min at 37°C. After the incubation period, the suspensions were centrifuged at 4000 rpm 10 min and the supernatant discarded. The pellets were fixed in 2.5% paraformaldehyde and 0.5% glutaraldehyde,

TABLE 1 | Bacteria tested in clinical trials as oral care probiotics.

Strain	Origin	Brand	Disease	Reference[1]
Lactobacillus rhamnosus GG	Human intestinal tract		Caries	Aminabadi et al., 2011
Lactobacillus rhamnosus LB21	Human intestinal tract		Caries	Stecksén-Blicks et al., 2009
Lactobacillus reuteri 55730 + PTA 5289	Woman breast milk	Prodentis™	Ginvival health, plaque reduction and caries	Krasse et al., 2006; Caglar et al., 2008a
Lactobacillus brevis CD2	Dairy products	Inersan®	Periodontal disease and caries	Riccia et al., 2007; Campus et al., 2014
Lactobacillus paracasei SD1	Human oral cavity		Caries	Ritthagol et al., 2014
Bifidobacterium animalis DN-173 010	Dairy products	ACTIVIA®	Caries	Caglar et al., 2005
Bifidobacterium animalis subsp. lactis BB-12®	Dairy cultures	BB-12®	Caries	Caglar et al., 2008b
Streptococcus salivarius M18	Saliva of a healthy child	BLIS M18®	Plaque reduction	Burton et al., 2013
Lactobacillus salivarius WB21	Mammalian digestive tract		Gingival health, periodontal disease, halitosis and caries	Shimauchi et al., 2008; Mayanagi et al., 2009; Iwamoto et al., 2010; Suzuki et al., 2014
Streptococcus oralis KJ3®, *Streptococcus uberis* KJ2® and *Streptococcus rattus* JH145®	Healthy mouths	ProBiora(3)®	Caries, periodontal disease and halitosis	Zahradnik et al., 2009
Weissella cibaria CMU, CMS-1, CMS-2 and CMS-3	Children's saliva		Halitosis and plaque reduction	Kang et al., 2006a,b
Lactobacillus reuteri DSM 17938 + PTA 5289	Human saliva	BioGaia ProDentis	Halitosis	Keller et al., 2012
Lactobacillus plantarum 7481 and *Lactobacillus brevis* 7480	Children's saliva	AB:Dentis®	Halitosis and gingivitis	WO 2012022773 A1*
Streptococcus salivarius K12	Children's saliva	BLIS K12®	Halitosis	Burton et al., 2006
Lactobacillus acidophilus NK1 + 100ash + K3III24	Human intestinal tract	Acilact (solely Russia)	Periodontal disease	Pozharitskaia et al., 1994
Bifidobacterium bifidum N1 and *Lactobacillus acidophilus* NK1	Human intestinal tract	Bifidumbacterin + Acilact (solely Russia)	Periodontal disease	Grudianov et al., 2002
Lactobacillus casei 37	Human intestinal tract		Periodontal disease	Volozhin et al., 2004
Lactobacillus paracasei GMNL-33 (ADP-1)	Human oral cavity	Dental-Lac™	Periodontal disease	Chuang et al., 2011
Lactobacillus salivarius TI 2711 (LS 1)	Human saliva		Periodontal disease	Matsuoka et al., 2006
Lactobacillus rhamnosus GG (53103) + LC705	Human intestinal tract		Oral candidasis	Hatakka et al., 2007

[1]*Reference = Publication with results of clinical trial; *Patent number.*

washed three times with PBS buffer and exposed to 1% osmium tetroxide in PBS buffer for 1 h. The samples were rinsed with PBS buffer three more times and then moved through a gradual process of dehydration, starting with 30% ethanol and ending with absolute ethanol (multiple rinse steps at each 30, 50, 70, 80, 90, and 100% ethanol). Finally, the samples were mounted on scanning electron micrograph stubs, sputter coated with gold, and viewed on a JEOL JSM 840 scanning electron microscope.

pH Range of Growth and Resistance of *S. dentisani* to the Digestive Process

To know the optimal and pH growth range of *S. dentisani* strains 7746 and 7747 we prepared BHI broth at different pHs (4.7, 5.5, 6, 6.5, 7, and 7.5) by adding NaOH 10 N or HCl 10 N to the commercial medium (Biolife). Pre-inocula were obtained as explained above. Two-hundred microliters of the BHI broths at different pHs were inoculated by triplicate with 20 μl of the inocula into a Nunc Microwell 96-well microplate. The O.D. $_{610}$ of each inoculated well was measured every 2 h and plotted versus the time of incubation to obtain the growth curves.

To evaluate the resistance of *S. dentisani* to the digestive process, the viability of the strains 7746 and 7747 was checked after a treatment with the salivary enzyme alpha amylase, followed by digestion with gastric enzymes under progressive acidic conditions to simulate digestion, following the protocol described in Khalf et al. (2010) and Martínez et al. (2011). The assays were performed in an i*n vitro* model stomach (or dynamic digester) by the external services of the Food Industry Research Association AINIA (Valencia, Spain). Each strain was tested by duplicate whereas the control was assayed once. Colony forming units (CFU) counts were obtained in BHI agar before any treatment (reference cultures), after the chewing step, and during the gastric digestion at three different times (30, 60, and 120 min). The plates were incubated at 37°C during 24–48 h. As the medium used for CFU counts was not selective for *S. dentisani,* the same assay was made without inoculation and used as blank to discount any contamination, obtaining 0 CFUs in this control.

Growth of *S. dentisani* in an Arginine Enriched Medium

Streptococcus dentisani 7746 was grown in 300 ml of BHI medium amended with 5 g/l of L-arginine monohydrochloride 98% (Alfa-Aesar). The pre-inoculum was obtained by inoculating a single colony of the strain in 10 ml of BHI broth and incubating overnight at 37°C without agitation. Inoculation was made with the volume required to obtain an initial O.D. $_{610}$ of 0.02. For comparison, the same volume was used to inoculate 300 ml of BHI without arginine. In both conditions the initial pH was set at 7.3. The cultures were incubated at 37°C during 24 h and aliquots of 1 ml were taken every hour for measuring the pH and the O.D. $_{610}$. Both conditions were assayed by triplicate.

Prevalence of *S. dentisani* and Other Oral Probiotics in the Dental Plaque of Healthy Individuals

To assess the prevalence of *S. dentisani* in the dental plaque of healthy individuals we compared the genomic sequences of the *S. dentisani* strains 7746 and 7747 (1.98 and 1.74 Mb, respectively) against 118 metagenomes of the dental plaque of healthy individuals available at The Human Microbiome Project Consortium (HMP, Turnbaugh et al., 2007). The metagenomic reads were mapped against the sequenced reference genomes using the NUCmer and PROmer v3.06 alignment algorithms (Kurtz et al., 2004) with the default parameters, following the methodology of Belda-Ferre et al. (2011). We considered that *S. dentisani* was present in a sample if at least 20 sequences >100 bp of the metagenome showed a similarity equal to or higher than 99% with the sequenced genomes. For comparison, the same approach was used to analyze the prevalence of *S. salivarius* in dental plaque.

The presence of the genus *Lactobacillus* in different parts of the oral cavity was performed by the analysis of the oral 16S rDNA sequences deposited in the Human Microbiome Project database in year 2014[1]. The niches analyzed were the keratinized gingiva, buccal mucosa, hard palate, palatine tonsils, saliva, supra- and subgingival plaque, and tongue dorsum. The analyses were carried out with a total of 4.3×10^8 sequences. The taxonomic affiliation was performed using the Megablast tool implemented in the NCBI against the SILVA ribosomal database[2], with the following parameters: *e* value <1e^{-10}, percent identity >97%, alignment length >350 bp.

Quantification of *S. dentisani* in the Dental Plaque of Healthy Individuals

Two healthy volunteers, named MG01 and MG02, were selected for sampling. They were males aged 20–30 years, non-smokers, with 28 teeth excluding third molars, with good dental and periodontal health: in both, absence of caries (non-cavitated level), DMF = 0, OHI = 0, GI = 1, and CPI = 1 (following nomenclature by World Health Organization [WHO], 1997). They had not been treated with antibiotics in the 6 months prior to the study nor presented antecedents of routine use of oral antiseptics. The two donors signed a written informed consent and the sampling procedure was approved by the Ethics Committee from the DGSP-CSISP (Valencian Health Authority), with reference 10/11/2009. Supragingival and subgingival dental plaque samples were taken from vestibular (buccal) and lingual (palatine) surfaces of 28 teeth in each volunteer. *Streptococcus dentisani* was quantified in individual free surfaces of each tooth type (incisor, canine, premolar, and molar) from one quadrant and the absolute numbers calculated by multiplying the obtained value by the number of each tooth type in the mouth. The same procedure was followed for the total bacterial content quantification.

Dental plaque samples were resuspended in 100 µl of PBS buffer and DNA was extracted with the MagnaPure LC JE379 instrument and the MagnaPure LC DNA Isolation Kit (Roche). The quantification of the DNA was done with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen), and real-time PCR was performed in a LightCycler 480 System with the LightCycler 480 SYBR Green I Master Mix (Roche). In every step, we followed the manufacturer's recommendations.

The specific primers used for the quantification of *S. dentisani* were designed for this study and targeted the genes for the carbamate kinase (*arcC*): CkSdF 5′-GTAAC CAACCGCCCAGAAGG-3′ and CkSdR 5′-CCGCTTTCGGA CTCGATCA-3′; and the ORF540: Orf540F (5′-ATGTTCA TCGGCTTGACAGGCTT-3′) and Orf540R (5′- TAAGCAA GCATAGAACCGCGCC-3′). Primers specificity was predicted *in silico* by the primerBLAST tool implemented in the NCBI[3] and confirmed by absence of amplification by qPCR with 5 ng/µl DNA from *S. mutans, S. sobrinus*, *S. sanguinis*, *S. salivarius*, *S. mitis, S. pneumoniae, S. infantis, and S. oralis*. The specificity of the primers was also checked by scrutinizing the melting profiles after every assay. Primers for the Ck gene did not amplify any of the tested streptococcal species. Primers for the ORF540 amplified only the DNA of *S. pneumoniae*, which is a rare inhabitant of dental plaque. Amplification was performed in a 20 µl final volume containing 1 µl of template DNA (at concentrations 5–22 ng/µl), 10 µl of the LightCycler 480 SYBR Green I Master Mix, 0.4 µl of each primer, and 7.2 µl of nuclease-free water. The thermocycling protocol used was as follows: an initial step of 95°C for 5 min, and 40 cycles of 10 s at 95°C, 20 s at 65°C, and 25 s at 72°C. All the quantifications were made by duplicate.

The concentration of *S. dentisani* in each sample was calculated by comparison with the Cq values obtained from a standard curve. This was generated using serial 10-fold dilutions of DNA extracted from 2×10^7 CFUs/ml (counted by serial dilutions in agar plates). Finally, the Cq values obtained with the plaque samples were replaced in the standard equation and expressed in absolute numbers per tooth type analyzed.

[1]http://hmpdacc.org

[2]http://www.arb-silva.de

[3]www.ncbi.nlm.nih.gov/tools/primer-blast

RESULTS

Inhibitory Activity of the *S. dentisani* Supernatants

As shown in **Figure 1A**, the addition of the *S. dentisani* 7746 concentrated supernatant produced a marked inhibitory effect on the growth of both *S. mutans* and *S. sobrinus*, as compared to the curves obtained when only concentrated BHI was added. Similarly, the concentrated supernatant completely inhibited the growth of other *S. mutans* strains (strains OMZ175 and ATCC 700610, data not shown). Regarding the curves obtained with the different size fractions, the <3 KDa fraction retained the inhibitory activity when tested against *S. mutans* ATCC 25175, while the 3–10 KDa and >10 KDa fractions did not (**Figure 1B**). The last two even enhanced the growth of *S. mutans*, probably due to a higher availability of nutrients coming from the concentrated culture medium and/or the *S. dentisani* metabolism. The same results were obtained with strain 7747 (data not shown).

Microscopy visualizations revealed that after 30 min of incubation with concentrated supernatants of *S. dentisani*, the cells of *S. mutans*, *F. nucleatum*, and *P. intermedia* were clearly affected, showing pores in the surface of the cells (*S. mutans*), changes in the cell walls' structure (*P. intermedia*) and cell lysis (*F. nucleatum*) (**Figures 2A–C**).

Proteinase treatment of the supernatant provoked the absence of growth inhibition when tested against *S. mutans*, while the peroxidase treatment did not affect the inhibitory activity (**Figure 3**). Taken together, the results indicated that the inhibition is not due to the production of hydrogen peroxide, and supports the idea that *S. dentisani* inhibit oral pathogenic bacteria by the production of inhibitors of peptidic nature, such as bacteriocin-like peptides.

pH Tolerance and Resistance of *S. dentisani* to the Digestive Process

Streptococcus dentisani was tested for its ability to grow at different pHs. The growth curves obtained showed that both strains displayed a very similar behavior, with an optimal growth pH close to 7 (**Supplementary Figures S1A,B**). It is noteworthy that they were able to grow at pH values between 6 and 7.5 but not between 4.7 and 5.5, indicating that *S. dentisani* can endure moderately acidic conditions but is not an acidophilic organism. Surprisingly, the growth of both strains at pH 6 was better than at 6.5, suggesting the activation of a buffering metabolic pathway at pH values around 6 (see Section "pH Buffering Capacity of *S. dentisani*" below).

Regarding the simulated digestive process, and as expected from the pH curves obtained before, *S. dentisani* was not able to maintain the initial viability during digestion simulations. As starting values of viability we obtained 6.5×10^8 and 3.9×10^8 CFUs/ml for the strains 7746 and 7747, respectively. After the chewing simulation with salivary enzymes these values decreased in only two to three orders of magnitude, showing that both strains could remain in the mouth at high levels after chewing, whereas the gastric digestion strongly inhibited their growth, even after the first step of the simulation (30 min, see **Table 2**). As neither strain of *S. dentisani* survived the gastric process, the simulation of an intestinal digestion was not performed.

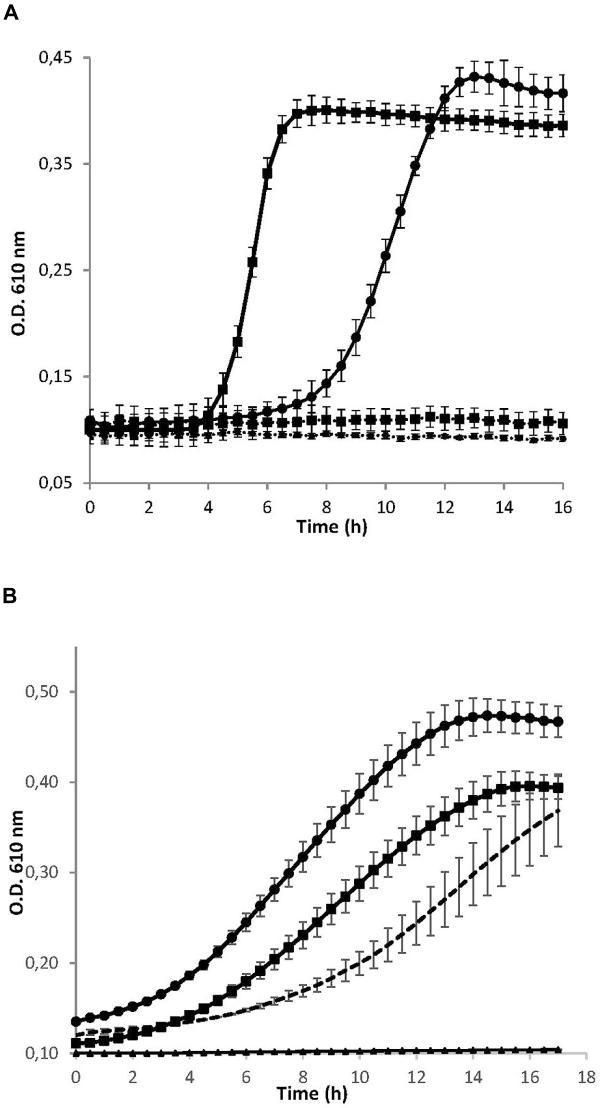

FIGURE 1 | (A) Growth curves of the cariogenic bacteria *Streptococcus mutans* ATCC 25175 (squares) and *S. sobrinus* CECT 4034 (circles) in the presence (dotted lines) and absence (solid lines) of concentrated supernatant of *S. dentisani* strain 7746. **(B)** Growth curves of *S. mutans* ATCC 25175 in the presence of different size fractions of the 10× concentrated supernatant of *S. dentisani* 7746. Circles correspond to the fraction >10 KDa, squares to the 3–10 KDa fraction, and triangles to the fraction <3 KDa. For comparisons, *S. mutans* was grown in the presence of 10x concentrated BHI medium (dotted line). Means ± SD from three independent replicates are plotted.

pH Buffering Capacity of *S. dentisani*

Figure 4 depict the growth curves obtained by triplicate when *S. dentisani* 7746 was grown in presence/absence of arginine. During the initial 6 h of incubation, the growth seemed to be fueled by the sugars present in the BHI medium, as the growth

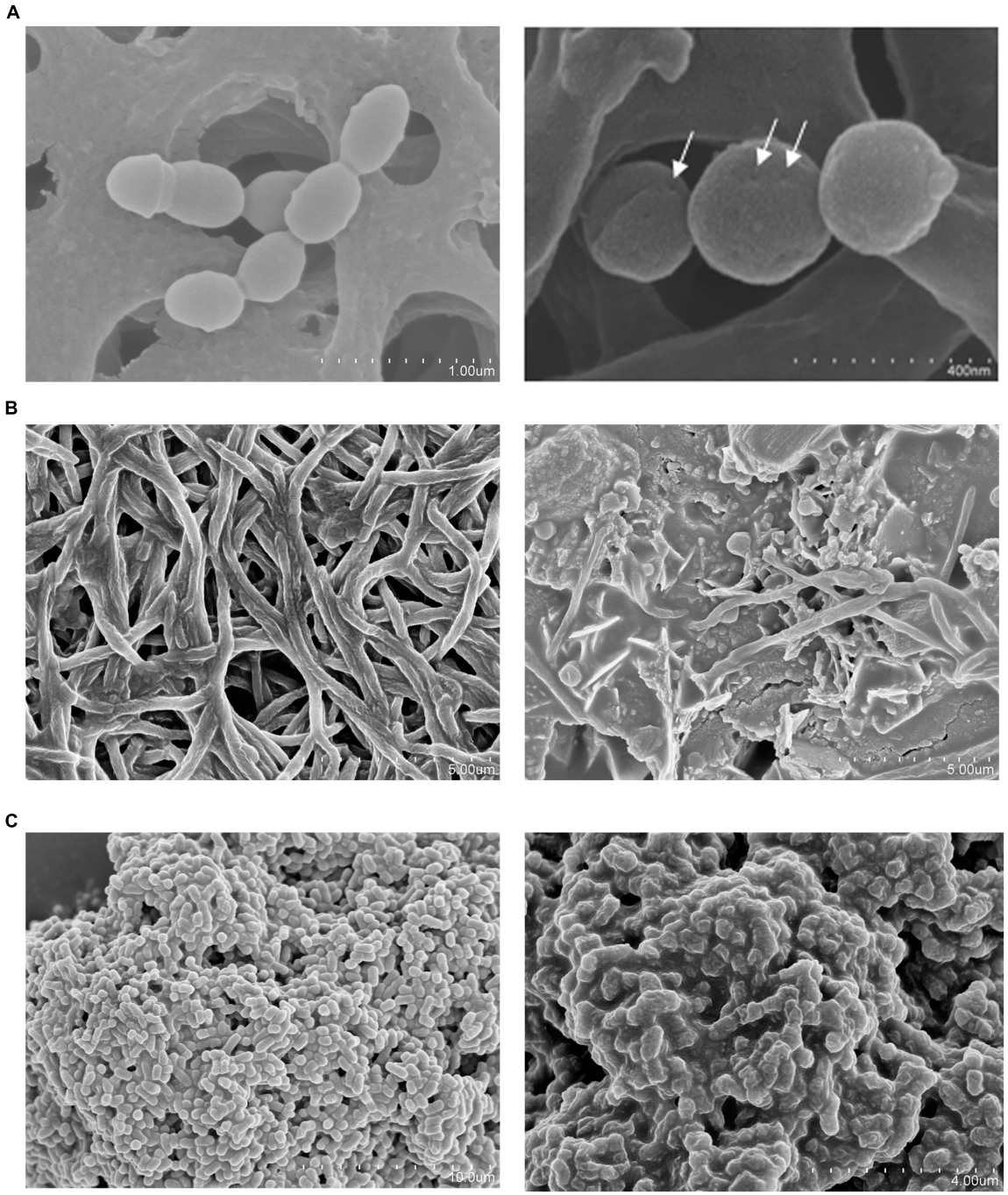

FIGURE 2 | Scanning electron microscopy (SEM) of three oral pathogens before (left) and after (right) a 30-min treatment with 10-fold concentrated supernatant of *S. dentisani* strain 7746. (A) *Streptococcus mutans* (pores are pointed with arrows). **(B)** *Fusobacterium nucleatum*. **(C)** *Prevotella intermedia*.

curves were identical in both conditions (with and without arginine), and the culture pH dropped from 7.3 to about 6.2. Soon after completion of this first phase, in the cultures containing arginine the pH starts to rise, reaching almost the initial pH value 12 h after inoculation. Contrarily, in the medium without arginine the pH continuously decreased to reach a value of about 6.2. In both conditions the O.D. $_{610}$ measurements were similar, with a maximum value of around 1.3 after 12 h of incubation.

The analysis of the genome of *S. dentisani* showed that strains 7746 and 7747^{T} contain the key genes of the arginine deiminase system: *arcA* (arginine deiminase, locus tag HK29_RS02275), *arcC* (carbamate kinase, locus tag HK29_RS02285), and *arcB* (ornithine carbamoyltransferase, locus tag HK29_RS02280),

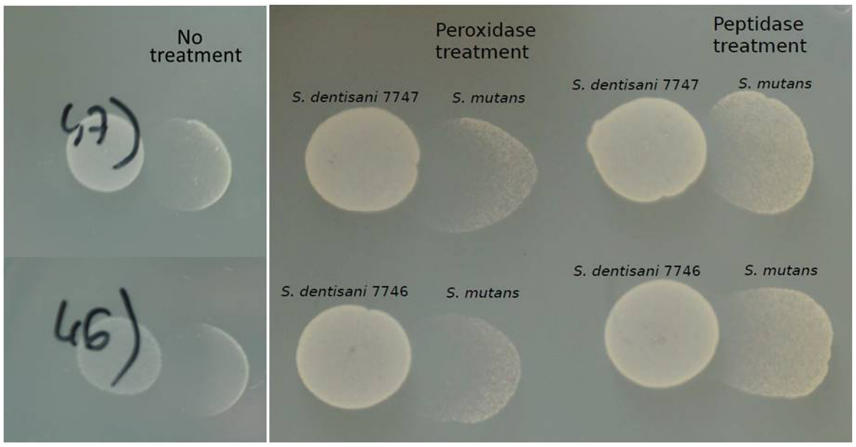

FIGURE 3 | Inhibition of *S. mutans* by *S. dentisani* in the presence of peroxidase and proteinase. The tests were performed with *S. dentisani* strains 7746 and 7747, treating with the enzymes the spots where *S. mutans* would later be grown. The controls on the left panel show the inhibition without treatment.

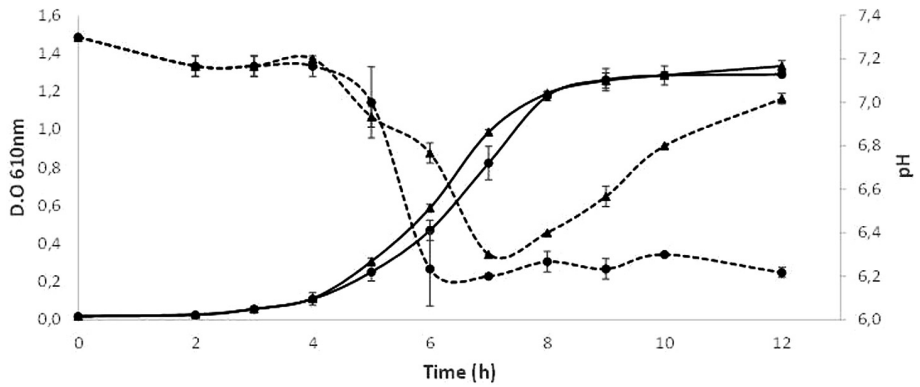

FIGURE 4 | Growth curves of *S. dentisani* strain 7746 in a medium with (triangles) and without (circles) the addition of 5 g/l of arginine. The mean ± SD of O.D. $_{610}$ (solid lines) and pH of the culture (dotted lines) from three replicates are depicted.

involved in the ammonia generation through arginine metabolism, a mechanism that releases ammonnia extracellularly producing the alkalinization of the environment (Liu et al., 2008, 2012).

Prevalence of *S. dentisani* in the Dental Plaque of Healthy Individuals

The recruitment analyses showed that sequences of *S. dentisani* were present in most of the analyzed metagenomes (direct, whole-sequenced DNA coming from dental plaques of healthy individuals at the Human Microbiome Project). At least 20 metagenomic sequences >100 bp were found to have a similarity ≥99% compared with the 7746 genome in 116 out of 118 individuals, and in the strain 7747 this threshold was fulfilled in all 118 individuals. Contrarily, the species *S. salivarius* that has been proposed as a probiotic agent against caries was only detected in three of these metagenomes, in agreement with this species inhabiting mucosal surfaces and not the hard tissues. In addition, we have analyzed the *Lactobacillus* 16S rRNA gene sequences from oral samples contained in the HMP database, as this genus contains species commonly used as oral probiotics (namely *L. acidophilus*, *L. reuteri*, and *L. rhamnosus*). The results showed that *Lactobacillus* spp. is present in very low numbers in the oral cavity, accounting for less than 0.05% of the bacterial genera in the buccal mucosa, hard palate, palatine tonsils, saliva, tongue, and supragingival plaque. The highest numbers of *Lactobacillus* 16S rDNA sequences were obtained in the subgingival plaque, but even here this genus accounted for only 0.94% of the total bacterial population. We analyzed in the same oral niches the prevalence of the genus *Streptococcus*, which was found to be present at very high numbers in the keratinized gingiva, buccal mucosa, hard palatine, palatine tonsil, tongue dorsum, and saliva (59.5, 58.8, 54, 27.1, 26.8, and 19.7 per cent of the total bacterial members, respectively). Furthermore, the percentage of the genus *Streptococcus* in the subgingival and supragingival plaques was high (18.8 and 20.2%, respectively). Overall, the results showed that the genus *Streptococcus* is much more abundant than *Lactobacillus* in every analyzed oral niche, and particularly in those directly affected by the cariogenic processes, being *Streptococcus* between 20 and 600 times more

TABLE 2 | Viability, expressed in CFU/ml, of the two strains of *S. dentisani* after the chewing and gastric digestion processes (G.D.) at three different times.

Strain	Starting culture	Chewing pH 6.9	G.D. 30 min pH 3	G.D. 60 min pH 2.1	G.D. 120 min pH 1.7
S. dentisani 7746	$6.5 \times 10^8 \pm 3.05 \times 10^8$	$5.3 \times 10^6 \pm 2.8 \times 10^8$	<10	<10	<10
S. dentisani 7747	$3.9 \times 10^8 \pm 4 \times 10^7$	$1 \times 10^6 \pm 9.8 \times 10^5$	<10	<10	<10

The values shown are the mean of two independent replicates and the standard deviation is specified.

TABLE 3 | Total cell counts of *S. dentisani* on supragingival dental plaque in the vestibular (V) and lingual (L) parts of different tooth types in two caries-free individuals (MG01 and MG02).

	Incisor	Canine	Premolar	Molar
MG01 (V)	$2.1 \times 10^5/2.8 \times 10^4$	$4.7 \times 10^5/7.9 \times 10^4$	$2.9 \times 10^5/3.46 \times 10^4$	$3.1 \times 10^5/3.1 \times 10^4$
(L)	$2.8 \times 10^5/2.7 \times 10^4$	$1.4 \times 10^5/4.1 \times 10^5$	$4.6 \times 10^5/9.8 \times 10^6$	$4.3 \times 10^5/6.9 \times 10^4$
MG02 (V)	$5.5 \times 10^2/7.5 \times 10^3$	$2.7 \times 10^3/4.4 \times 10^3$	$1.2 \times 10^3/1.3 \times 10^4$	$2.5 \times 10^4/4.5 \times 10^4$
(L)	$3.4 \times 10^4/5.1 \times 10^4$	$2.8 \times 10^4/5.9 \times 10^3$	$7.1 \times 10^4/5.0 \times 10^5$	$4.7 \times 10^4/1.7 \times 10^7$

Data show the estimates of bacterial numbers obtained by qPCR with two different sets of S. dentisani-specific primers (orf540/CK).

abundant than *Lactobacillus* in the subgingival and supragingival plaque, respectively.

Quantification of *S. dentisani* in the Dental Plaque of Healthy Individuals by q-PCR

The absolute numbers of *S. dentisani* cells were obtained for the teeth's free surfaces of two healthy volunteers by the use of two set of *S. dentisani*-specific primers, which provided similar estimates (**Table 3**). The results showed that both individuals contain very similar values of abundance of *S. dentisani* (1.04×10^7 and 3.14×10^7 cells for MG01 and MG02, respectively, as estimated by the ORF540 primers; 4.46×10^7 and 6.94×10^7 cells for MG01 and MG02, as estimated by the carbamate kinase primers). However, although the calculated *S. dentisani* numbers in the mouth of both individuals was highly similar, its distribution was very different. As shown in **Table 3**, MG01 did not show important differences in the amounts of *S. dentisani* between tooth types nor between the lingual and vestibular surfaces. Patient MG02 however, had a more heterogeneous distribution, with higher proportions of *S. dentisani* in premolars and molars, and on the lingual surfaces of every tooth type.

DISCUSSION

Dental caries is considered the most prevalent disease worldwide, with up to 80% of the human population being affected at some point during their lives (Petersen and Lennon, 2004). There are however no efficient means to prevent it, as the polymicrobial nature of the infection and its complex etiology make passive and active immunization strategies (e.g., a caries vaccine) ineffective (Fejerskov, 2004; Simón-Soro and Mira, 2015). Thus, new strategies directed towards the re-establishment of the natural balance in the oral microbiome like the use of pre- and probiotics have been proposed (Marsh et al., 2015). However, as shown in **Table 1**, most of the probiotics proposed to promote oral health are bacteria isolated from environments other than the oral cavity, usually the human gut (Cagetti et al., 2013). In addition, the most commonly used probiotic bacteria to treat dental caries are lactobacilli, due to their well-proven safety characteristics. However, the use of non-oral bacteria may prevent efficient colonization of the oral niche, which is a vital and desirable feature of probiotics. This is supported by the low frequencies of lactobacilli detected in tooth tissues by molecular methods, as reported in the current manuscript. In addition, lactobacilli are important acid-producers and long known to be associated to dental caries lesions (Badet and Thebaud, 2008; Plonka et al., 2012), as well as bifidobacteria (Mantzourani et al., 2009b; Beighton et al., 2010), which are also common oral probiotics. This can be the reason why *in vitro* experiments with these bacteria, even if low-acid producing strains are selected, may result in pH acidification (Pham et al., 2009). These results underline the need to use potential probiotics from the oral cavity, and therefore the isolation of a bacteriocin-producing probiotic *Streptococcus salivarius* which inhibited the cariogenic agent *S. mutans* was promising (Wu et al., 2015). However, the comparison of the *S. salivarius* genome against a high number of metagenomes from supragingival plaque presented in the current manuscript reveals its absence in the tooth, in agreement with its soft-tissues-associated nature. *Streptococcus salivarius* is a typical inhabitant of the buccal epithelium, tongue, and dorsal epithelium (Bowden et al., 1979; Power et al., 2008) and comprises an important part of the total cultivable flora on the soft tissues of the mouth (Wilson, 2005). Probably for this reason, it has been proposed as a probiotic for the pharyngeal mucosa (Guglielmetti et al., 2010) but its inability to colonize the tooth surface may hamper its potential as an anti-caries probiotic. The high heterogeneity of environments in the mouth and the resulting adaptation of microorganisms to those specific microniches (Simon-Soro et al., 2013) underscores the importance of selecting oral bacteria adapted to live in hard tissues as probiotics against dental caries to guarantee a proper colonization. In agreement with this view, our data show that *Streptococcus dentisani*, which was isolated from supragingival dental plaque of caries-free

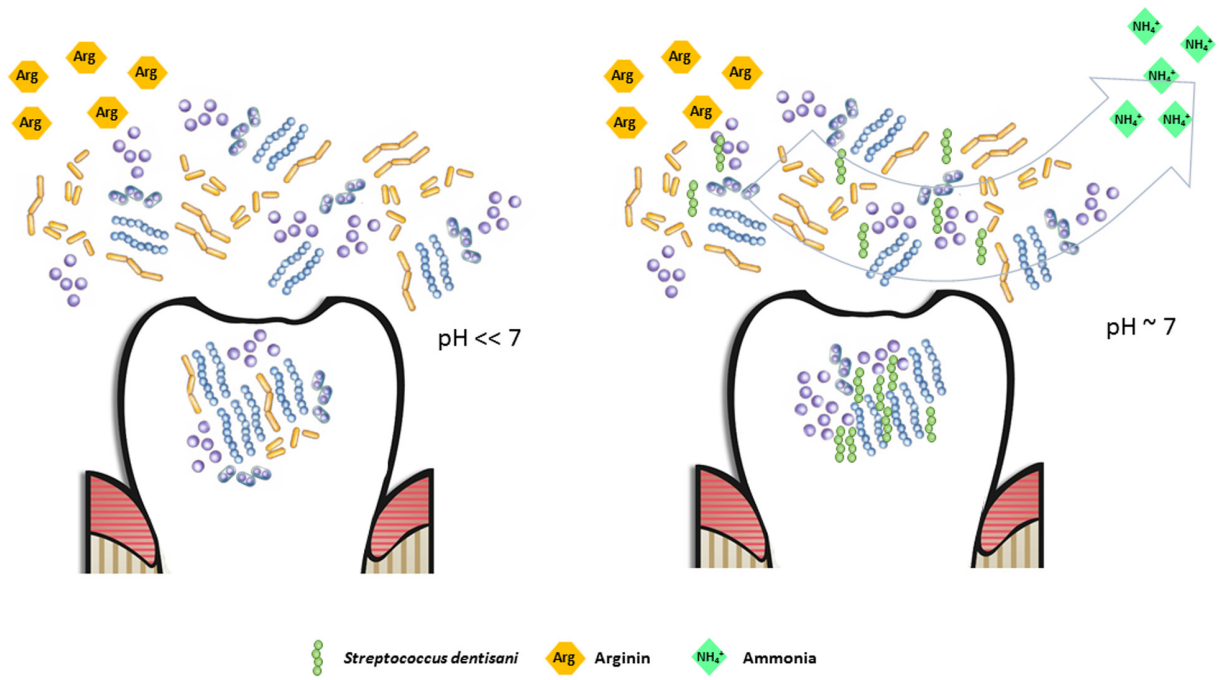

FIGURE 5 | Potential buffering effect in the oral cavity due to the enrichment of the dental plaque bacterial community with *Streptococcus dentisani*. When arginine is available (either as an added prebiotic or as a product of peptide degradation) and *S. dentisani* is established on the tooth surfaces, the pH drop caused by the accumulation of organic acids can be buffered by the production of NH_4^+ through the arginolytic pathway, maintaining the pH close to neutrality (right panel). In the absence of *S. dentisani*, or if present at low proportions, the buffering effect would be diminished, with the consequent acidification and enamel damage. This would be the perfect scenario for the proliferation of acidogenic/cariogenic microorganisms (left panel).

individuals (Belda-Ferre et al., 2011; Camelo-Castillo et al., 2014), was widespread among dental plaque samples from healthy subjects.

Apart from dental colonization, we show that an important probiotic feature of *S. dentisani* is its anti-microbial properties, inhibiting the growth of important oral pathogens like *S. mutans*, *S. sobrinus*, or *Prevotella intermedia*. In addition, the killing of *Fusobacterium nucleatum* is unusual among oral probiotics and may provide an important beneficial effect, as this organism is responsible for a large number of co-aggregation patterns with many oral species and is considered the main "bridge" bacteria between early and late colonizers of dental plaque (Kolenbrander et al., 2002). Thus, its inhibition could contribute to impede the adhesion of pathogenic organisms that co-aggregate with this bacterium and by doing so hamper dental plaque development, whose maturity has been shown to considerably increase the drop in pH after a meal (Firestone et al., 1987). In addition, *F. nucleatum* itself has been associated with halitosis due to the production of volatile sulfur compounds (Krespi et al., 2006), and therefore its growth inhibition should be investigated in the future as a way to diminish the severity of this condition.

The different experiments performed in the current work suggest a peptidic nature of the inhibitory molecules, as the inhibitory effect was significantly reduced by proteinase treatment but was unaffected by peroxidase, indicating that the inhibition was not due to the production of hydrogen peroxide as it is common in other streptococci (Zhu and Kreth, 2012). This, together with the small size of the molecules responsible for the inhibition (<3 KDa) and the appearance of pores in the membrane of sensitive bacteria as identified by SEM, strongly suggest that the inhibition is caused by bacteriocins, and future work should be directed towards identifying and characterizing such antimicrobial peptides.

Dental caries, as well as other oral diseases like periodontitis or halitosis are not considered typical infectious diseases in classical terms, as there is more than one species responsible for their etiology, the microbial consortia causing the disease varies considerably between individuals and even between lesions of the same patient, and pathogenic organisms normally can be isolated also from healthy individuals (Hajishengallis and Lamont, 2012; Simon-Soro et al., 2014; Camelo-Castillo et al., 2015; Marsh et al., 2015; Simón-Soro and Mira, 2015). For these reasons, antimicrobial properties of probiotics may not be sufficient for effectively preventing dental caries (ten Cate and Zaura, 2012) and the ability to restore the microbial ecological balance after pH acidification, for instance by alkali production, is a promising probiotic feature (Huang et al., 2015). Production of ammonia from urea or arginine by several oral bacteria has been shown to efficiently buffer salivary pH and has been associated to a reduction in caries risk (Reyes et al., 2014; Moncada et al., 2015). The analysis of the genome of *Streptococcus dentisani* revealed all genes from the arginolytic pathway, including the arginine deiminase and the carbamate kinase, a feature which appears

to be common to all members of the cluster (Jensen et al., 2016). In addition, the agmatine deiminase gene, involved in the production of ammonia (Liu et al., 2012) was also present in both analyzed strains. In agreement with this, our data show that *S. dentisani* was able to efficiently buffer extracellular pH in the presence of arginine. Arginine is present in saliva in variable concentrations and it has also been added as a prebiotic to toothpaste, with strong clinical evidence for reducing enamel demineralization and buffering acidic pH (Yin et al., 2013; Santarpia et al., 2014). In addition, we have found in the genome of *S. dentisani* genes encoding for different aminopeptidases that can liberate arginine from peptides and proteins (Gonzales and Robert-Baudouy, 1996), which could then enter the arginolytic pathway. This is supported by the higher growth of the bacterium at pH 6 than at pH 6.5 in standard BHI growth medium (**Supplementary Figure S1**). Given that its optimal growth pH is neutral, and that the arginolytic pathway has been shown to be activated by low pH in other species (Liu et al., 2008), the higher growth rates at pH 6 are probably the result of protein degradation liberating arginine, which would allow the production of NH_4, as well as the production of ATP derived from this metabolic route. These results also suggest that the full activation of the arginine degradation genes occurs at pH < 6.5, and the regulation of this pathway should be studied in the future. In **Figure 5**, we have illustrated the buffering effect that could take place in the oral cavity due to the enrichment of the dental plaque bacterial community with *S. dentisani*. When acidification occurs as a consequence of the consumption of dietary carbohydrates, and the pH drops to values around 6, *S. dentisani* would be able to activate the ADS system producing ammonia and, consequently, buffering the pH of its close environment.

The double beneficial action of *S. dentisani* (i.e., anti-microbial and anti-acid) makes it a promising probiotic bacterium. These benefits, together with its dental colonization capacity, derive to a big extent from its oral inhabitance. In addition, the choice of a dental plaque species as probiotic allows the quantification of the species in caries-free individuals, in order to use those levels as the appropriate administration dose, instead of using the dosages normally delivered for gut probiotics. In the current manuscript, we have quantified *S. dentisani* amounts in dental plaque for just two individuals and similar estimates for larger sample sizes could serve to accurately determine the dosage for an appropriate treatment.

For all these reasons, we propose the use of probiotics which are *active colonizers*, that is microorganisms which inhabit the site where the disease takes place, and that are isolated from healthy individuals. We believe that the administration of these organisms will maximize the chance of colonization and the potential beneficial effects for human and animal health. In the case of dental caries, we encourage the search of probiotic bacteria that are normal inhabitants of the human supragingival plaque in healthy individuals and propose the use of *S. dentisani* in clinical trials to test its potential in promoting oral health.

AUTHOR CONTRIBUTIONS

AM conceived the work, was implied in the analyses and interpretation of data and wrote the manuscript together with AL-L. AL-L, AC-C, and MF worked in the designing and performance of the experiments, data acquisition, analyses, and interpretation of the results. AS-S collected the plaque samples and made the bioinformatic analyses.

ACKNOWLEDGMENT

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00379/full#supplementary-material

FIGURE S1 | Growth curves of *Streptococcus dentisani* strains 7746 (A) and 7747 **(B)** in BHI medium at different starting pHs: 4.7 (red), 5.5 (orange), 6 (yellow), 6.5 (light blue), 7 (dark blue), and 7.5 (purple).

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Functional Characterization of Novel *Faecalibacterium prausnitzii* Strains Isolated from Healthy Volunteers: A Step Forward in the Use of *F. prausnitzii* as a Next-Generation Probiotic

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Faecalibacterium prausnitzii is a major member of the Firmicutes phylum and one of the most abundant bacteria in the healthy human microbiota. *F. prausnitzii* depletion has been reported in several intestinal disorders, and more consistently in Crohn's disease (CD) patients. Despite its importance in human health, only few microbiological studies have been performed to isolate novel *F. prausnitzii* strains in order to better understand the biodiversity and physiological diversity of this beneficial commensal species. In this study, we described a protocol to isolate novel *F. prausnitzii* strains from feces of healthy volunteers as well as a deep molecular and metabolic characterization of these isolated strains. These *F. prausnitzii* strains were classified in two phylogroups and three clusters according to 16S rRNA sequences and results support that they would belong to two different genomospecies or genomovars as no genome sequencing has been performed in this work. Differences in enzymes production, antibiotic resistance and immunomodulatory properties were found to be strain-dependent. So far, all *F. prausnitzii* isolates share some characteristic such as (i) the lack of epithelial cells adhesion, plasmids, anti-microbial, and hemolytic activity and (ii) the presence of DNAse activity. Furthermore, Short Chain Fatty Acids (SCFA) production was assessed for the novel isolates as these products influence intestinal homeostasis. Indeed, the butyrate production has been correlated to the capacity to induce IL-10, an anti-inflammatory

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cytokine, in peripheral blood mononuclear cells (PBMC) but not to the ability to block IL-8 secretion in TNF-α-stimulated HT-29 cells, reinforcing the hypothesis of a complex anti-inflammatory pathway driven by *F. prausnitzii*. Altogether, our results suggest that some *F. prausnitzii* strains could represent good candidates as next-generation probiotic.

Keywords: probiotic, commensal, *Faecalibacterium*, molecular and metabolic characterization, immune-modulatory properties

INTRODUCTION

Despite a large number of bacteria, archaea, viruses, and unicellular eukaryotes inhabit the human body, only a few bacterial genera (*Bacteroides, Clostridium, Bifidobacterium,* and *Faecalibacterium*) predominate in the human gut microbiome (Schmidt, 2013). Nowadays it is recognized that *Faecalibacterium prausnitzii* represents around 5% from the total fecal microbiota in healthy adults (Hold et al., 2003). Furthermore, this bacterium has been proposed to be a sensor and an actor of the human intestinal health. Indeed, the levels of *F. prausnitzii* have been found to be decreased in patients suffering from intestinal and metabolic disorders such as inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), colorectal cancer (CRC), obesity, and celiac disease among others (Balamurugan et al., 2008; Sokol et al., 2008; Neish, 2009; De Palma et al., 2010; Furet et al., 2010; Rajilic-Stojanovic et al., 2011) as well as in frail elderly (van Tongeren et al., 2005). Moreover, this species may be a biomarker of choice to assist in Ulcerative colitis (UC) and Crohn's disease (CD) discrimination (Lopez-Siles et al., 2017).

F. prausnitzii has been only described in detail recently probably because it is very difficult to grow as it is an Extremely Oxygen Sensitive (EOS) bacterium (Duncan et al., 2002). Similar to other EOS bacteria, little is known about the biology of *F. prausnitzii* despite its relevance in the human gut ecosystem (Miquel et al., 2014). Most of the data referring *F. prausnitzii* are based on metagenomic studies (Miquel et al., 2013), with only few studies with isolated strains and functional approach (Duncan et al., 2002; Ramirez-Farias et al., 2009; Lopez-Siles et al., 2012; Foditsch et al., 2014). This gap between metagenomic and microbiological data is striking for microbiota-derived EOS bacteria. To reduce this gap, it is now essential to increase the knowledge of several commensal bacterial strains in order to better understand the beneficial effect of this species.

Most of the commercial probiotics do not include dominant commensal human isolates. This is a reason why these probiotic strains do not colonize the human gut and their effects persist only during a short period of time (Schmidt, 2013). Nowadays, there is an increasing interest in the use of commensal bacteria as potential probiotic agents. The reasons are multiple and the most evident is that the role of commensal bacteria in homeostatic crosstalk has started to be unraveled in the last decade (Wrzosek et al., 2013). The domestic probiotic market, with a turnover approaching $7 billion in Europe and $1.7 billion in the US in 2013 (Schmidt, 2013), is expected to grow in the next years. However, these next-generation probiotic-commensal candidates must meet the same criteria than the conventional ones. It means that they should (i) be isolated and well-characterized, (ii) achieve safety requirements, such as the acceptable resistance to antibiotics or the lack of lytic and adhesion capacities, and (iii) show beneficial effects on the host before being considerate as a probiotic. In this sense, the Food and Agriculture Organization of the United Nations (FAO) and the European Food Safe Administration (EFSA) have established several guidelines for the correct definition and evaluation of probiotics on food (FAO/WHO, 2002; Pineiro and Stanton, 2007; Binnendijk and Rijkers, 2013). Regarding *F. prausnitzii*, although little is known about its safety, there is a clear potential of this species as a next-generation probiotic. This was already proposed for livestock animals with the isolation and characterization of *F. prausnitzii* strains from stool of calves and piglets (Foditsch et al., 2014) but also for patients with intestinal dysbiosis-associated illness with the development of specific formulation keeping this EOS bacteria alive at ambient air (Khan et al., 2014). Besides, its beneficial anti-inflammatory effect has been only analyzed *in vitro* and *in vivo* with the reference strain *F. prausnitzii* A2-165 (Sokol et al., 2008) and the biofilm forming strain HTF-F (Rossi et al., 2015). As the probiotic properties are usually strain-specific ones (Pineiro and Stanton, 2007), individual studies are required to assess the anti-inflammatory properties of other *F. prausnitzii* isolated strains.

The aim of this work is to isolate a collection of novel *F. prausnitzii* strains from healthy volunteers in order to characterize them as potential probiotic bacteria in accordance with Novel Food regulatory (Miquel et al., 2015a). We have also validated the collection of viable isolated strains by metabolic and safety tests in order to better understand their biology especially in the gastrointestinal tract. Furthermore, the anti-inflammatory properties of all these strains were validated *in vitro* in order to identify the best potential *F. prausnitzii* strain to be used as a next-generation probiotic.

MATERIALS AND METHODS

Isolation of Novel Extremely Oxygen Sensitive (EOS) Strains

A cohort of healthy volunteers was first established (**Table 1**) to collect freshly emitted fecal samples used as inocula. All volunteers signed informed consent to provide the samples and an agreement of confidentiality. The complete isolation of EOS strain procedure was performed in an anaerobic chamber (N_2 = 90%, CO_2 = 5% and H_2 = 5%). Briefly, fecal samples were homogenized and serial dilutions performed in order to plate dilutions 10^{-8} and 10^{-9} on YBHI [Brain–heart infusion medium supplemented with 0.5% yeast extract (Difco)] agar

TABLE 1 | Studied cohort of healthy humans' volunteers and new *F. prausnitzii* strain identified.

Subject	Sex	Age (years)	Fecal SCFA (mM)			CFU/g	% EOS	Identified *F. prausnitzii* strains	Cultivability of the strain
			Butyrate	Propionate	Acetate				
A	M	81	nd	nd	nd	4.4×10^9	51	CNCM-I4540	Yes
B	F	59	nd	nd	nd	8.7×10^9	30.7	X	
C	M	54	nd	nd	nd	8.0×10^9	67.7	CNCM-I4541	Yes
								CNCM-I4542	Yes
								S3C12	No
								S3G1	No
D	M	54	21.7	14.8	65.4	8.0×10^9	69	X	
E	F	60	nd	nd	nd	3.5×10^{10}	40	X	
F	M	53	3.7	4.4	11.6	3.0×10^9	35.4	X	
G	F	26	3	3.9	14	2.0×10^9	37.5	X	
H	F	56	15.8	10.2	27.6	4.8×10^9	56.2	CNCM-I4574	Yes
								CNCM-I4543	Yes
I	M	59	10.1	9.5	27.3	7.8×10^9	33	S9G3	No
								S9D8	No
J	M	34	9.9	12.3	26.8	7.7×10^9	30.7	CNCM-I4644	Yes
								CNCM-I4544	Yes
								S10H3	No
K	F	60	1.1	2	5.3	2.0×10^9	28.1	X	
L	M	40	1.7	2	6.9	7.4×10^9	29.6	CNCM-I4575	Yes
								CNCM-I4573	Yes
								S13A12	No
								S13E3	No
M	F	51	2.5	3.1	10.8	4.6×10^9	53.1	CNCM-I4546	Yes

All the isolates were obtained from human fecal samples of healthy volunteers consuming omnivorous diets. F, female; M, male; nd, not determined, X, no identified F. prausnitzii strain.

supplemented with rumen fluid 20%. After 4 days of incubation at 37°C, single colonies were obtained on plates and 96 varied colonies were selected and isolated in duplicate on YHBHI supplemented with rumen fluid 20% agar plate. A group of plates was placed brought out of the anaerobic chamber for 1 h to eliminate EOS strains and after a long period of incubation (usually between 48 h and 4 days), we performed a negative screening. The EOS colonies were further re-isolated and a specific *F. prausnitzii* PCR (primers Fprau07/Fprau02) was done to identify strains of this specie (**Table 2**). Finally, a 16S rRNA gene sequencing was performed after complete 16S rRNA amplification using primer FP1 to FP5 (**Table 2**; MWG France). The viable isolates were stocked at −80°C with 16% of glycerol.

Bacterial Strains, Cell Culture, and Growth Conditions

The reference strains A2-165 (DSM 17677; Duncan et al., 2002), L2/6 (Barcenilla et al., 2000) and M21/2 (Louis et al., 2004) and the *F. prausnitzii* isolated strains (**Table 1**) were grown at 37°C in YBHI medium supplemented with cellobiose (1 mg/ml; Sigma), maltose (1 mg/ml; Sigma), and cysteine (0.5 mg/ml; Sigma) in an anaerobic chamber filled with $N_2 = 90\%$, $CO_2 = 5\%$ and $H_2 = 5\%$.

HT-29 (ATCC HTB-38) (LGC-Standars) cell line was grown in Dulbecco's Modified Eagle's minimal essential medium (DMEM) (Sigma-Aldrich) supplemented with 10% (w/v) heat-inactivated fetal bovine serum (FBS) (GibcoBRL, Eragny, France) and with penicillin G/ streptomycin (5,000 IU/mL, 5,000 μg/mL) (Sigma-Aldrich). Cultures were incubated in 25 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) at 37°C in a 5% (v/v) CO_2 atmosphere until confluence.

16S rRNA Gene Analysis

DNA was extracted from isolated colonies of the different *F. prausnitzii* strains by alkaline lysis in 50 μL of NaOH 0.5 M during 30 min and 50 μL of Tris 1M pH7 and 100 μL H_2O were added. 16S rRNA sequences were amplified using FP1 and FP2 primers (**Table 2**) and PCR products purified with the Wizard SV Gel. PCR Clean-Up system (Promega) was used to obtain bidirectional partial 16S rRNA gene sequences by using primers FP1, FP2, FP3, FP4, and FP5 (**Table 2**). All DNA sequences were confirmed by sequencing (Eurofins MWG Operon, Ebersberg, Germany). Sequences for the novel isolates were deposited in the NCBI database under the accession numbers MF185398 to MF186168.

Phylogenetic analysis based on 16S rRNA were performed using the multiple sequence alignment—CLUSTALW (Thompson et al., 1994) integrated in MEGA6 software (Tamura et al., 2013). After that, the most appropriate evolutionary model was defined and the evolutionary history was inferred

TABLE 2 | Oligonucleotides used in this study and PCR product sizes.

Primer	Oligonucleotide sequence (5′–3′)	PCR product size (bp)	Use	References
Fprau07	CCATGAATTGCCTTCAAAACTGTT	141	PCR *F. prausnitzii* specific	Sokol et al., 2008
Fprau02	GAGCCTCAGCGTCAGTTGGT			
FP1	AGAGTTTGATCCTGGCTCAG	1,474	16S rRNA complete sequence amplification and sequencing	This study
FP2	ACGGCTACCTTGTTACGACTT			
FP3	GTTGCGGGACTTAACCCAACATC		16S rRNA sequencing	This study
FP4	GTTTTTCTTGAGTAGTGCAGAGG		16S rRNA sequencing	This study
FP5	GATGTTGGGTTAAGTCCCGCAAC		16S rRNA sequencing	This study

using the Maximum likelihood (ML) criterion, based on the Kimura 2-parameter model (Kimura, 1980), with 1,000 bootstrap replicates. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter = 0.1846)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 64.70% sites). Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and all positions containing gaps and missing data were eliminated. The tree with the highest log likelihood (−3073.67) is shown (**Figure 3**). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 36 nucleotide sequences. There were a total of 1090 positions in the final dataset. In this analysis, sequences used by Lopez-Siles et al. (Duncan et al., 2002; Ramirez-Farias et al., 2009; Lopez-Siles et al., 2012) were included with the objective of compare the new strains to the two phylogroups proposed by that study. *Eubacterium desmolans* was used to root the tree.

Plasmid Presence

The presence of plasmids in the isolated strains were determined following Wizard® Plus SV Minipreps DNA Purification System (Promega) with modifications to adapt it for use with Gram positive bacteria. Briefly, an extra lysis step was performed after centrifugation of liquid overnight (ON) cultures by incubation for 1 h at 37°C with lysozyme (Sigma; 10 mg/ml) in the cell resuspension solution.

Scanning Electron Microscopy

Scanning electron microscopy analyses were performed on the MIMA2 platform (INRA, France) with pure pellet of bacterial culture suspended and fixed in 200 µL of glutaraldehyde and 3% ruthenium red during 2 h in an anaerobic chamber and stored at 4°C. Scanning electron microscopy was performed as previously reported (Joly et al., 2010).

Determination of Antibiotics Resistance

The minimum inhibitory concentrations (MIC) for 13 antibiotics (including tetracycline, kanamycin, chloranphenicol, linezolid, nupri/dalfopri, trimethoprim, gentamicin, erythromycin, cefpirome, clindamycin, streptomycin, vanomycin, and ampicillin) were determined on Wilkins-Chalgren agar (Difco) according to the E-test procedure, in accordance with the conditions recommended by the supplier (Biomerieux, France). The results were recorded after 48 h of incubation.

Anti-bacterial Assays

The anti-bacterial effect of *F. prausnitzii* supernatants were investigated *in vitro* using the bacteriocin activity assay as previously described (Ramirez-Farias et al., 2009). This anti-bacterial effect was tested on six different bacterial species: three aerobic bacteria (*E. coli* Nissle 1917, *E. coli* DH10B, and *Listeria monocytogenes* 11765), one facultative anaerobic bacterium (*Lactococcus* subsp *cremoris* MG1363), and two obligate anaerobic bacteria (*Clostridium perfringens* ATCC13124 and *Bifidobacterium infantis* DSM20088/ATCC15697). YBHI liquid medium alone was used as negative control.

Metabolic Activities

To determine the metabolic activities of the cultivable strains, API-20A galleries and the gelatin degradation test of API-20E galleries were used according to manufacturer's instructions. For detection of DNase and hemolytic activity, the strains were grown ON and then plated into Methyl green-DNA agar plates (Difco) or blood agar plates (Biomérieux) respectively. The results were recorded after 48 h of incubation. The capacity to grow in presence of mucin was assayed using a defined medium (KH_2PO_4: 5.236 g/L, $(NH_4)_2SO_4$: 4 g/L, NaCl: 4 g/L, $CaCl_2$: 30 mg/L, $MgCl_2$: 300 mg/L, $MnCl_2$: 30 mg/L, $FeCl_2$: 8 mg/L, Vitamin B12: 5 mg/L, Vitamin B1: 1 mg/L, Biotin: 1 mg/L, PABA: 1 mg/L, Folic acid: 1 mg/L, Vitamin K: 2 mg/L, cystein 0.5 mg/mL) supplemented with 1.5% mucin (Type II, Sigma-Aldrich).

Short Chain Fatty Acid (SCFA) Analysis

Supernatant concentrations of propionate, acetate, and butyrate were analyzed using gas liquid chromatography (Nelson 1020, Perkin-Elmer, St Quentin en Yvelines, France) as previously described (Lan et al., 2008). Overnight culture (20 h) of *F. prausnitzii* strains were used and culture media as negative control; each measurement for performed at least in triplicate except for fecal samples. SCFA concentrations are expressed in mM.

Dosage of D- and L-Lactate

D- and L-lactate was measured in supernatant of bacterial cultures. This supernatant was precipitated with trichloroacetic acid (10%) and centrifuged at 4,500 g for 20 min at 4°C. Lactate

was then measured in the supernatants with the Biosentec D/L lactic acid enzymatic kits according to the manufacturer instructions (Biosentec, Toulouse, France). Overnight culture (20 h) of *F. prausnitzii* strains were used and culture media as negative control; each measurement was performed at least in triplicate.

Adhesion Assays

Monolayers of HT-29 cells were seeded in 24-well tissue culture plates (Nunc) with 1.83×10^5 HT-29 cells/well and cultivated until confluence, culture medium was changed daily. Monolayers were then infected in 1 ml of the cell culture medium without antibiotics and with heat-inactivated FBS at a multiplicity of infection (MOI) of 100 bacteria per epithelial cell. After, 3 h of incubation at 37°C in anaerobic conditions (as describe above), monolayers were washed three times in phosphate-buffered saline (PBS; pH 7.2). The epithelial cells were then lysed with 1% Triton X-100 (Sigma Chemical Company, St Louis, Mo.) in water. Samples were plated onto YHBHI supplemented agar plates to determine the number of CFU corresponding to the total number of cell-associated bacteria. Adhesion to mucin has been performed as previously described by Radziwill-Bienkowska et al. (2014, 2016) Briefly, after an overnight coating of 96 plates (Nunc) with a solution of 10 mg/ml of mucin [Type III mucin from porcine stomach (lyophilized powder, Sigma-Aldrich)] a bacterial suspension ($OD_{600nm} = 1$) in PBS of each strain was incubated 3-h at 37°C in the anaerobic chamber. Bound cells were stained with crystal violet. Stained bacteria were suspended in 96% ethanol and optical density was determined at 583 nm. All the experiments were performed in triplicate. The adhesion values have been normalized using *Lactobacillus rhamnosus* GG (LGG) a positive control know by their good adhesion properties to mucin (Martin et al., 2015). Results are presented by the mean and the standard deviation.

Immuno-Modulatory Properties Using HT-29 Cells

Anti-inflammatory assays were done following the procedure described by Kechaou et al. (2012). Briefly, 50,000 HT-29 cells per well were seeded in 24-well culture plates (Nunc). Twenty-four h before bacterial co-culture (day 6), the culture medium was changed for a medium with 5% heat-inactivated FBS and 1% glutamine. On the day of co-culture, 10% of bacterial supernatant or bacterial medium (YBHI) were added in DMEM in a total volume of 500 µL. Cells were stimulated simultaneously with human TNF-α (5 ng/ml; Peprotech, NJ) for 6 h at 37°C in 10% CO_2. All samples were analyzed in triplicate. After co-incubation, cell supernatants were collected and stocked at −80°C until further analysis of interleukin-8 (IL-8) concentrations by ELISA (Biolegend, San Diego, CA). Total protein was determined by Bradford Reagent test (Sigma-Aldrich). Experiments have been done at least in triplicate. Results are expressed as IL-8/protein (pg/mg) and have been normalized using as reference value the IL-8 produced after the co-incubation with PBS as a negative control.

Experiments on Peripheral Blood Mononuclear Cells (PBMCs)

The protocol used in this study was adapted from Kechaou et al. (2012). Commercial PBMCs (StemCell Technologies, France) from five healthy donors were used in this assay. Donors presented the following characteristics: men, age under 65, body mass index <30, non-smoking, no drugs with anti-inflammatory known effects taken during the 15 days prior to sampling, and tested negative for HIV, hepatitis A and B viruses. After reception, cells were stored in liquid nitrogen until use. To prepare PBMCs for co-culture experiments with bacteria, the vial were thawed at 37°C in a water bath and then transferred into a medium containing RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 1% L-glutamine and 0.1% Penicillin/Streptavidin (medium components were bought from Lonza, Switzerland). DNase (100 µg/mL, Roche Applied Science, France) was added to this mix to avoid cell clumping. Cells were then centrifuged at 200 g for 15 min, counted using trypan blue and spread on 24-well plates at 1×10^6 cells/well. Supernatants were added in triplicates (three wells per donor) at 10% in a total volume of 1 ml. Plates were incubated for 24 h at 37°C with 10% CO_2. Culture supernatant were collected, mixed with an antiprotease cocktail according to manufacturer's instructions (Complete EDTA-Free protease inhibitor, Roche Applied Bioscience) and stored at −80°C until further analysis of IL-10 concentrations by ELISA (Mabtech, Sweden).

Statistical Analysis

GraphPad software (GraphPad Sofware, La Jolla, CA, USA) was used for statistical analysis. Results are presented as bar graphs ±SEM. Comparisons were realized with the non-parametric Kruskal–Wallis test followed by a Dunn's Multiple Comparison test. Correlation test were performed using spearman test. A $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Construction of EOS and *F. prausnitzii* Libraries

The vast majority of intestinal bacteria are EOS and thus mostly very difficult to culture (Qin et al., 2010). Although metagenomic approaches recently allow identifying some uncultivable organisms, the use of cultivable strains is requested to determine their biological activities. In this study, we report a method for isolation of novel EOS strains from human fecal samples on a complete medium (**Figure 1**). For this, a negative screening was performed through the exposition of bacterial isolates to oxygen and in parallel, these same strains were cultivated in an anaerobic chamber, which maintains a consistent anaerobic environment to ensure proper conditions for optimal EOS growth. We identified between 28.1 and 67.7% of EOS strains in the microbiota of healthy volunteers (**Table 1**). Interestingly, the proportion of EOS strains in the human microbiota was positively and significantly correlated to the amount of fecal acetate ($r = 0.7$; $p = 0.0433$) and tend to be correlated to the amount of fecal butyrate ($r =$

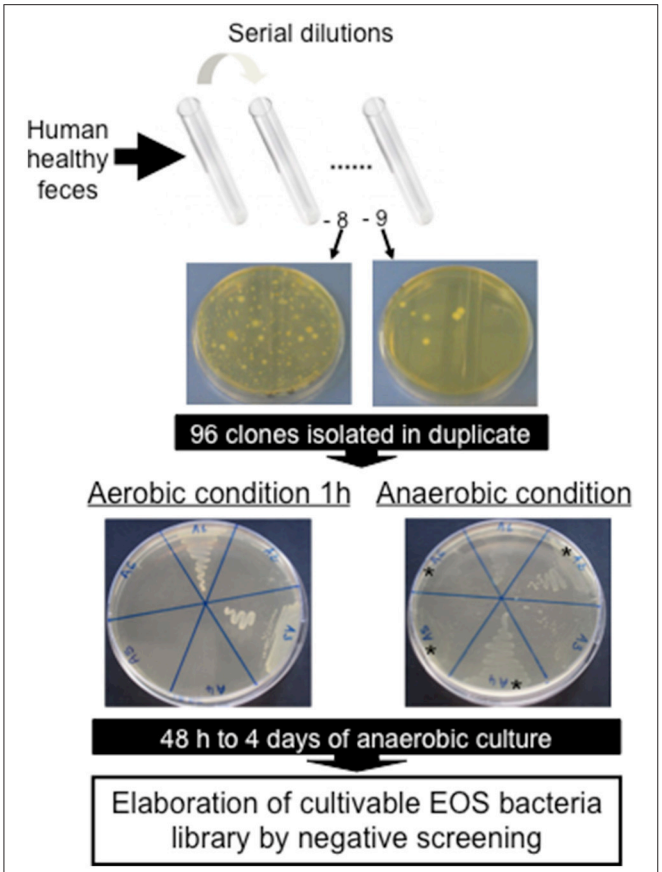

FIGURE 1 | Negative screening for isolation of new Extremely Oxygen Sensitive (EOS) strains from human healthy feces.

0.6833; $p = 0.0503$). These observations suggest that EOS population has an important metabolic impact that could participate to intestinal homeostasis (Wrzosek et al., 2013). The EOS isolates were identified by 16S rRNA gene sequencing and among them *F. prausnitzii* candidate strains were selected for further characterization. These isolation and screening set up can have a small inspecificity rate and no-*F. prausnitzii* strains can be recovered as well as the strain S13E3. After three subcultures, cultivable strains were stored at −80°C in 16% glycerol. Among 17 identified *F. prausnitzii* strains, only 10 were cultivable in the tested conditions (**Table 1**) with an $OD_{600\ nm}$ lower than 2 corresponding to $>1 \times 10^8$ CFU/mL (**Figure 2**). There was no direct correlation between CFU counts and OD_{600nm} due to difference of viability between strains. We substantially increased the number of cultured *F. prausnitzii* isolates from human origin and provided new tools for a better understanding of the diversity and microbial ecology of the colon.

Phylogenetic Diversity of *Faecalibacterium prausnitzii*

Full-length 16S rRNA gene sequences were determined for the 17 isolates of *F. prausnitzii* from healthy individuals (**Table 1**). The sequences from the literature (Barcenilla et al., 2000; Duncan et al., 2002; FEEDAP, 2012; Lopez-Siles et al., 2012) were included in order to classify the new isolates in the two phylogroups proposed by Lopez-Siles et al. (Barcenilla et al., 2000; Duncan et al., 2002; FEEDAP, 2012; Lopez-Siles et al., 2012; **Figure 3**). Each of these 16S rRNA sequences were unique, came from a different colony, and share >97% 16S rRNA sequences similarity. Cultivability of strains was not linked to phylogroups affiliation (**Figure 3**). Of note, all strains have a similar morphotype with cell wall extensions, like "swellings" (**Figure 4**) already described but with yet unknown function (Miquel et al., 2013). The average nucleotide identity between strains of the two phylogroups (S3L/3 and L2/6 = 94%) supports the hypothesis of the existence of two genomospecies without phenotypic properties defined yet (Lopez-Siles et al., 2017). Although, as was previously described for another library, there was a tendency for some sequences to group by isolation and individual with a clustering of strains (subgroup B of the phylogroup II; Lopez-Siles et al., 2012). For example, CNCM I-4574 and CNCM I-4543 strains were isolated from the same volunteer and present 99.8% of homology at 16S rRNA level.

Interestingly, the existence of strains that do not fit in any phylogroup (as CNCM I-4541) suggest that biodiversity of *F. prausnitzii* remains poorly known, maybe since only few strains have been isolated. Moreover, the strain S13E3, could be not an *F. prausnitzii* stain.

Resistance to Antibiotics

The MIC for the different antibiotics tested are represented in the **Table 3**. Concerning the breakpoints for Gram positive bacteria from EFSA (Duncan et al., 2004) which classify bacteria as resistant or not to a specific antibiotic, all *F. prausnitzii* isolates were susceptible to clindamycin, vancomycin, ampicillin, quinupristin+dalfopristin, and chloramphenicol (MICs lower than 0.25, 2, 1, 0.5, and 2 mg/L respectively). Only one isolate, the CNCM I-4541 strain was resistant to erythromycin (MICs > 0.5mg/L). Surprisingly, all tested strains were resistant to streptomycin (MICs ranging from 14 to 50 mg/L) excepted for the CNCM I-4575 isolate. Regarding gentamicin, kanamycin, and tetracycline, different results were obtained for the different isolates: with up to 5 isolates displaying resistance to higher concentrations of the tested antibiotics than the determined breakpoint. Finally, three antibiotics (not included in the EFSA guidance) were also analyzed due to their importance in the clinical treatments: trimetroprim, linezolid, and cefpirome. All strains were resistant to trimethoprim, as expected for an anaerobic bacteria (MICs >32 mg/L; data not shown), while they tended to be susceptible to linezolid (MICs ranging from 0.032 to 3.3 mg/L) and resistant to cefpirome (from 4.66 to >256 mg/L) which, when linked to the general susceptibility to ampicillin, might indicate that the penicillin binding proteins of *Faecalibacterium* are poorly recognized by cephalosporins. Remarkably, CNCM I-4543 and CNCM I-4574 isolates were resistant to cefpirome, a fourth-generation cephalosporin

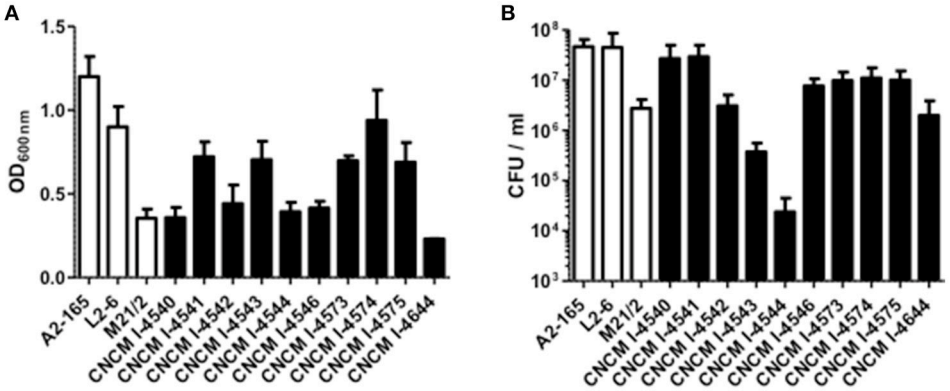

FIGURE 2 | Growth profile of *F. prausnitzii* strains. **(A)** $OD_{600\,nm}$ determination after 20 h growth in YBHI supplemented medium and **(B)** determination of viable bacteria: the CFU/mL numeration in the same cultures. Each measurement have been done at least in triplicate.

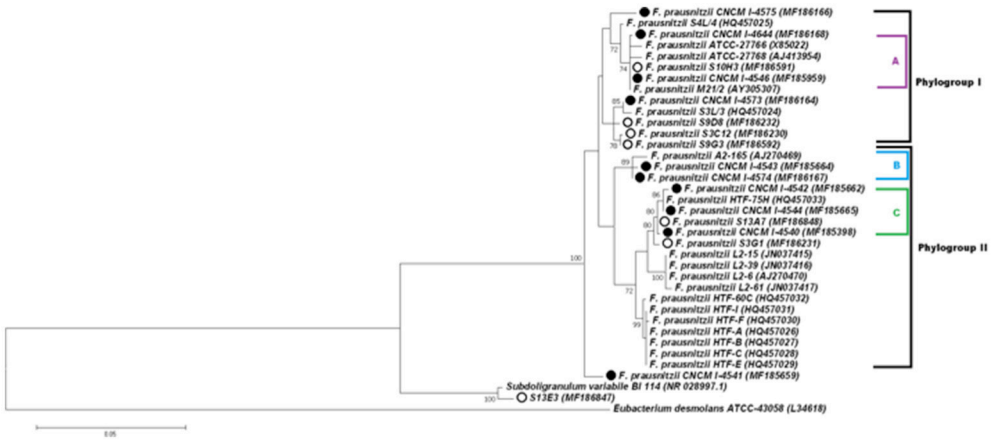

FIGURE 3 | Phylogenetic tree of *F. prausnitzii* strains based on 16S rRNA gene sequences. The tree was constructed with the MEGA6 software package using the Maximum Likelihood method. The bootstrap values above 70% are shown next to the branches. The *F. prausnitzii* isolates incorporated in this study have circles besides. The black circles represent the cultured strains and white circles represent uncultured isolates. Colors (purple, blue, and green) and letters (A, B, and C) indicate the tree groups with high bootstrap values, formed by our cultured strains.

stable against most plasmid- and chromosome-mediated beta-lactamases (Wiseman et al., 1997), with a MIC higher than 256 mg/L.

The analysis of antimicrobial resistance is of major importance due to the fast evolution of antibiotic resistance in response to the extensive use of antimicrobials. However, the microbiological breakpoints marked by the EFSA for most of Gram positive bacteria is probably not the most correct for the analysis of *F. prausnitzii* isolates as no so many information about their natural or acquired resistance patters is reported, to our knowledge, up to day in the literature. Foditsch et al. (2014) have identify that more of the 50% of the *F. prausnitzii* strains that they isolated from fecal samples of healthy calves and piglets were resistant to tetracycline, amikacin, cefepime, and cefoxitin comparing the MIC values with the standard values determined by CLSI for *Bacteroides fragilis* ATCC 25285. This fact highlights the need of more microbiological studies of antibiotic resistance in this species in order to determine a correct standard values for *Faecalibacterium* as well as the search for genes codifying for the most important resistance mechanisms for, at least, some of the antibiotics tested in this study.

Metabolic Activities

Enzymatic activities detected by API-20A gallery system are reported in **Table 4**. Interestingly, only one enzyme was detected and active in all the tested strains: the beta-galactosidase. Otherwise, all the strains were not able to ferment mannose or raffinose, to reduce nitrate and to produce indole (data not shown). Furthermore, all the isolates were negative for the presence of urease, arginine dihydrolase, beta-glucosidase, alpha-arabinosidase, N-acetyl-beta-glucosaminidase, glutamic acid decarboxylase, alkaline phosphatase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosin arylamidase, alanine arylamidase, glutamyl glutamic acid arylamidase, and serin arylamidase (data not shown). These results confirm previous observations where no strain was able

FIGURE 4 | Scanning electron microscopy images of *F. prausnitzii* strains phylogroup II. Strains were grown in YBHI liquid medium 20 h. Scale bars indicate 2 μm. Arrows indicates "swelling."

to metabolize arabinose and raffinose among others as the sole energy source (Duncan et al., 2002; Lopez-Siles et al., 2012).

For all the other enzymes (6 phospho-beta galactosidase, alpha-glucosidase, beta-glucuronidase, arginine arylamiase, leucyl glycerine-arylamidase, glycine-arylamidaseycine, and histidine-arylamidase), differences inter-strains were detected (**Table 4**). Beta-glucuronidase activity has been previously reported in some *F. prausnitzii* isolates (Lopez-Siles et al., 2012). While six strains showed individual profiles, the other seven are included in three different profiles. Two of them corresponds to the group A from phylogroup I (CNCM I-4546 and M21/2). The strains CNCM I-4543 and CNCM I-4574 (group B, phylogroup II), which are the only ones resistant to cefpirome, share also the same metabolic profile and donor. And the third metabolic

TABLE 3 | Minimum inhibitory concentrations (MIC) (mg/L) for the different antibiotics tested.

EFSA Breakpoint (other Gram +)	**GEN**	**STR**	**KM**	**ERY**	**CLI**	**VAN**	**TET**	**QD**	**CM**	**AMP**	**CPO**	**LZD**
	4	**8**	**16**	**0.5**	**0.25**	**2**	**2**	**0.5**	**2**	**1**	**nd**	**nd**
A2-165	1.37 ± 0.12	**96 ± 18.47**	**8.67 ± 1.76**	0.20 ± 0.08	0.016 ± 0	0.27 ± 0.05	0.016 ± 0	0.03 ± 0.01	0.08 ± 0.02	0.11 ± 0.02	9 ± 1	1.25 ± 0.25
L2-6	**4.33 ± 0.88**	**32 ± 7.15**	0.42 ± 0.08	0.10 ± 0.02	0.023 ± 0	0.47 ± 0.12	**4.21 ± 0.62**	0.58 ± 0.46	**20 ± 5.66**	0.06 ± 0.03	24 ± 4.62	0.62 ± 0.12
M21/2	**4.75 ± 1.49**	**21 ± 4.43**	**51 ± 45**	0.05 ± 0.01	0.016 ± 0	0.25 ± 0	1.09 ± 0.63	0.016 ± 0	0.15 ± 0.03	0.04 ± 0.03	16.67 ± 7.86	0.75 ± 0
CNCM I-4540	1.25 ± 0.25	**21.33 ± 2.67**	**122.67 ± 66.83**	0.11 ± 0.02	0.016 ± 0	0.71 ± 0.18	0.016 ± 0	0.011 ± 0.004	0.05 ± 0.04	0.11 ± 0.02	24 ± 4.62	0.04 ± 0.06
CNCM I-4541	1.62 ± 0.47	**24 ± 0**	**14 ± 2**	**20 ± 4**	0.016 ± 0	0.125 ± 0	**8 ± 0**	0.27 ± 0.24	0.17 ± 0.07	0.06 ± 0	40 ± 8	1.25 ± 0.25
CNCM I-4542	2.87 ± 1.12	**23.67 ± 3.26**	**20 ± 4**	0.11 ± 0.07	0.016 ± 0	0.67 ± 0.17	0.016 ± 0	0.016 ± 0	0.29 ± 0.40	0.08 ± 0.03	20.67 ± 7.69	3.17 ± 1.42
CNCM I-4543	2 ± 0.40	**16 ± 0**	**20 ± 4**	0.08 ± 0.02	0.016 ± 0	0.29 ± 0.04	0.2 ± 0.002	0.07 ± 0.03	0.31 ± 0.09	0.12 ± 0	**≥256 ± 0**	1.25 ± 0.38
CNCM I-4544	**6 ± 1.15**	**21.2 ± 7.31**	**100 ± 52.41**	0.12 ± 0	0.016 ± 0	0.92 ± 0.08	0.016 ± 0	0.023 ± 0	0.11 ± 0.02	0.09 ± 0.02	53.33 ± 5.33	0.5 ± 0
CNCM I-4546	**10 ± 66**	**50.67 ± 13.33**	**256 ± 0**	0.22 ± 0.61	0.018 ± 0.002	0.56 ± 0.31	**2.6 ± 0.6**	0.16+0.07	1.25 ± 1.51	0.07 ± 0.02	24.8 ± 6.24	3 ± 1
CNCM I-4573	**7 ± 1**	**32 ± 0**	**234 ± 21.33**	0.01 ± 0.03	0.024 ± 0.007	0.28 ± 0.05	0.028 ± 0.003	0.04 ± 0.005	0.38 ± 0	0.25 ± 0	22 ± 3.83	3.3 ± 0.8
CNCM I-4574	1.75 ± 0.25	**14 ± 0**	6 ± 2	0.07 ± 0.02	0.016 ± 0	0.25 ± 0	0.016 ± 0	0.03 ± 0.01	0.09 ± 0.02	0.22 ± 0.03	**≥256 ± 0**	0.5 ± 0.14
CNCM I-4575	1.25 ± 0.25	5 ± 1	4 ± 1	0.07 ± 0.01	0.016 ± 0	0.5 ± 0	0.032 ± 0	0.03 ± 0.03	0.016 ± 0	0.084 ± 0.02	4.67 ± 1.67	0.03 ± 0.01
CNCM I-4644	0.91 ± 0.08	**9.33 ± 1.33**	**135 ± 69.84**	0.10 ± 0.05	0.026 ± 0.01	0.23 ± 0.02	0.83 ± 0.32	0.04 ± 0.01	0.079 ± 0.04	0.026 ± 0.01	9.333 ± 1.33	0.58 ± 0.08

Gentamicin (GEN), streptomycin (STR), kanamycin (KM), erythromycin (ERY), clindamycin (CLI), vancomycin (VAN), tetracycline (TET), quinupristin/dalfopristin (QD), chloramphenicol (CM), ampicillin (AMP), cefpirome (CPO), and linezolid (LZD). Experiments have been done in triplicate and the results are expressed as the media ± SEM. nd, Not defined. In bold, Resistances.

TABLE 4 | Metabolic capacities of *F. prausnitzii* strains detected by API 32A galleries.

	bGal	bGP	αGLU	bGUR	ArgA	Lga	GlyA	HisA
	beta−galactosidase	beta Galactosidase 6 phosphate	Alpha glucosidase	beta glucuronidase	Arginine Arylamidase	Leucyl Glycine Arylamidase	Glycine Arylamidaseycine	Histidine Arylamidase
A2−165	+	+	+	+	+	+	+	+
L2−6	+	−	+	+	+	+	+	+
M21/2	+	−	−	−	+	−	+	+
CNCM I−4540	+	+	−	−	−	−	−	−
CNCM I−4541	+	+	−	−	+	−	−	−
CNCM I−4542	+	+	−	−	−	−	−	−
CNCM I−4543	+	+	−	+	+	+	+	−
CNCM I−4544	+	−	−	−	−	−	−	−
CNCM I−4546	+	−	−	−	+	−	+	+
CNCM I−4573	+	−	−	−	+	−	+	+
CNCM I−4574	+	+	−	+	+	+	+	−
CNCM I−4575	+	+	−	+	+	−	+	+
CNCM I−4644	+	−	−	−	+	+	+	+

+, Presence; −, absence. Experiments have been done in triplicate.

profile is shared by strains CNCM I-4540 and CNCM I-4542 that belong to the group C of phylogroup II.

It is now well-establish that *F. prausnitzii* is an acetate-consumer and butyrate-producer species (Duncan et al., 2002; Lopez-Siles et al., 2012). Here, we report that in pure cultures, our new isolated strains are also able to produce butyrate and this production is significantly and positively correlated to their growth (OD_{600nm}; $r = 0.8462$; $p = 0.003$; **Figures 5A,B**). It is interesting to highlight that the production level of butyrate was not linked to a particular phylogroup (Phylogroup I 3.91 mM ± 0.43 and Phylogroup II 4.89 mM ± 0.62). Moreover, all strains could metabolize acetate present in the culture medium at around the same level (**Figure 5A**). This consumption was not directly correlated to bacterial growth ($r = -0.3132$, $p = 0.2975$) and tended to be more correlated to butyrate production ($r = -0.544$, $p = 0.0546$). This observation is in agreement with the literature which describes that most of the carbon present in the butyrate produced (around 85%) is derived from external acetate, with only 15% provided directly from glucose (FEEDAP, 2012).

F. prausnitzii can also produce a few amount of D-lactate (FEEDAP, 2012). Indeed, among our strain collection, no L-lactate was detected and only few amounts of D-lactate were detected (1.09 mM ± 0.15 and 1.07 mM ± 0.39 phylogroup I and II respectively; data not shown). This production, not correlated with phylogroup affiliation, was correlated to the OD_{600nm}($r = 0.6209$, $p = 0.0235$). Bacterial D-Lactate production can be viewed as harmful since accumulation of this metabolite into the blood may be neurotoxic and leads to acidosis (Mack, 2004). In particular, humans with short bowel syndrome (in which small intestine has been surgically removed), the D/L fecal lactate ratio seems to be the most relevant index with a higher D-encephalopathy risk (Mayeur et al., 2013). However, in healthy adults, there is no lactate detectable in fecal samples, because lactobacilli (main producer of D-lactate) are minor groups in microbiota and lactate is degraded by other major bacterial groups (36, He, 2008 #41). This observation also suggested that the weak production of D-lactate by *F. prausnitzii* strains, major component of the microbiota, could not have metabolic deleterious impact on the host.

All strains were unable to growth in the presence of mucin as the only carbon source in a defined medium (data not shown). This data agrees with previous results where no evidence of fermentation of porcine gastric mucin by *F. prausnitzii* was detected (Lopez-Siles et al., 2012). Nevertheless, SCFA concentrations and OD_{600nm} measures taken after 2 days of incubation showed the ability of the different strains to survive but metabolically inactive as it could be deduced by the absence of butyrate in the supernatants of the cultures and the almost minimal OD_{600nm} recorded (data no shown). A decrease in butyrate production due to non-optimal growth conditions have been already reported for *F. prausnitzii* A2-165 strain (Lopez-Siles et al., 2012). This characteristic pointed out the intrinsic growth requirements of this species which, in addition to be an EOS, needs strain specific nutritional environment and has the ability to switch between substrates derived from the diet or the host (Lopez-Siles et al., 2017).

Lytic Activities

Gelatin is a heterogeneous mixture of water-soluble protein that is usually used in microbiological procedures to detect the presence of proteolytic activities. None of the strains were able to degrade gelatin in the conditions recommended by the API gallery supplier (data not shown). However, when the strains were inoculated in the gallery in a defined medium instead of API suspension medium, they were able to degrade partially this compound after 3 days of incubation. This fact suggests that the strains are able to hydrolyze gelatin although, maybe due to the growth limitations present in this culture media, the existence of this compound is not enough to allow the metabolic development of this activity in the strains.

The presence of hemolytic activity was tested using blood agar plates. None of the strains showed hemolytic activity under the conditions tested. In contrast, all the strains reveal a DNAse activity in green methyl-DNA medium (data not

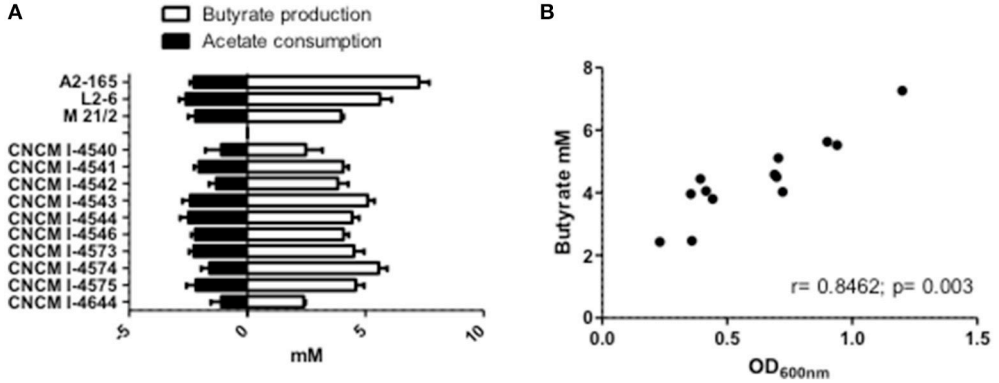

FIGURE 5 | SCFA metabolism of *F. prausnitzii* strains *in vitro*. **(A)** Acetate and butyrate concentrations after 20 h growth of strain in YBHI supplemented medium. The concentration of control media was subtracted for each measurement have been done at least in triplicate. **(B)** Correlation between $OD_{600\,nm}$ and butyrate production.

shown). Furthermore, the presence of a magnesium dependent DNase activity has been previously reported in at least three of five strains already sequenced [A2-165 (gi:257439194), SL3/3 (gi:295105207), and L2/6 (gi:295102777)].

The presence of these extracellular activities is often linked to a virulence status in some bacterial species such as *Enterococcus* spp. (Eaton and Gasson, 2001). However, these factors also contribute to the survival of microorganisms in the mammalian gut being characteristic of several members of the natural microbiota (Sanders et al., 2010). This can be the case of *Faecalibacterium* isolates, which are extremely well-adapted to the gut environment (Lopez-Siles et al., 2012).

Antibacterial Activities

We investigated antibacterial properties of *F. prausnitzii* supernatants, using the bacteriocin activity assay. We did not reveal any antibacterial effect on several anaerobic and aerobic bacterial species under the conditions tested. This fact is a desirable characteristic of a strain to be considered as a probiotic candidate.

Ability to Stimulate the Immune Response

The reference strain *F. prausnitzii* A2-165 is well-known for its immuno-modulatory properties and more specifically for its anti-inflammatory effects both *in vitro* and *in vivo* in different murine models of colitis (Sokol et al., 2008; Martin et al., 2014). To determine whether the newly isolated *F. prausnitzii* strains are able to modulate the immune response, we tested *in vitro* the immuno-modulatory properties of the supernatants from all the isolates in two different cellular models: HT-29 and PBMC. The first one is based on the capacity to block IL-8 production (a pro-inflammatory cytokine) induced by TNF-α stimulation in HT-29 epithelial cells and the second is based on the stimulation of PBMC cells and the measure of the anti-inflammatory cytokine IL-10. As shown in **Figure 6A**, all the strains tend to decrease IL-8 concentrations. However, this decrease was not equivalent in all the strains and does not correlate either with growth ratio ($r = -0.2857$, $p = 0.344$) or butyrate production ($r = -0.3357$, $p = 0.2869$).

For the PBMC assay, although all the strains tend to increase the production of IL-10 cytokine, only four strains (two controls and two new isolates from this study) were able to induce statistically significant increase production of this cytokine (**Figure 6B**) The two most performing strains (A2-165 and 4543) belong to the phylogroup II, group B. Notably, the IL-10 production was correlated with both growth ratio ($r = 0.6813$, $p = 0.0103$) and butyrate production ($r = -0.6923$, $p = 0.0126$). This different phenotype may suggest the presence of different molecule(s) responsible of the anti-inflammatory effects *in vitro*. The anti-inflammatory properties of butyrate have been already reported in the literature (Fusunyan et al., 1999; Kamitani et al., 1999) and its ability to block IL-8 production under the conditions tested in this study were confirmed *in vitro* in similar concentrations to those founds in *F. prausnitzii* supernatants (data not shown). However, its role remains controversial as its effects seems to be dose- and time-dependent as well as depended on the cellular model used (Martin et al., 2013). For instance, regarding cells from intestinal origin, butyrate has been found to decrease the secretion of IL-8 in Caco-2 and HIPEC cells and, in contrast to this study, to enhance IL-8 production in HT-29 and HT-29 MTX cells (Bocker et al., 2003).

However, several authors have found different candidate molecules/structures responsible for *F. prausnitzii* anti-inflammatory effects. MAM protein, found in *F. prausnitzii* supernatant, has been found to block NF-κB activation and the production of the pro-inflammatory cytokine IL-8 (Quevrain et al., 2016). *F. prausnitzii* is also able to produce bioactive anti-inflammatory molecules such as shikimic and salicylic acids (Miquel et al., 2015b). Besides, Rossi and co-workers showed the ability of *F. prausnitzii* strain HTF-F and its extracellular polymeric matrix to develop immunomodulatory effects through the TLR2 dependent modulation of IL-12 and IL-10 cytokine production in human monocyte-derived dendritic cells (Rossi et al., 2015) and *F. prausnitzii* has been found to be a strong inducer of regulatory T cells secreting IL-10 (Sarrabayrouse et al., 2014). All these results point out the complex anti-inflammatory mechanisms underlying this species.

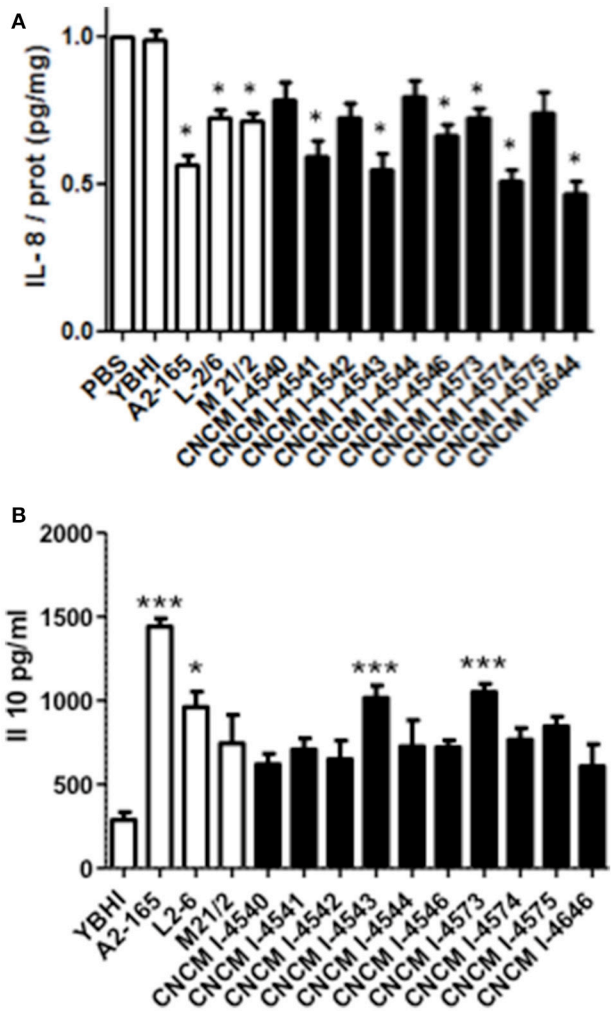

FIGURE 6 | Immuno-modulation capacities of *F. prausnitzii* strains *in vitro*. **(A)** IL-8 production in HT-29 TNF-α stimulated cells. Experiments have been done at least in triplicate. Results are expressed as IL-8/ protein (pg/mg) and have been normalized using as reference value the IL-8 produced after the co-incubation with PBS as a negative control. **(B)** IL-10 production in peripheral blood mononuclear cells. Experiments have been done at least in triplicate. Results are expressed as IL-10 concentration (pg/mL). Significant differences from the control (YBHI) was specified as: $*p < 0.05$ and $***p < 0.001$.

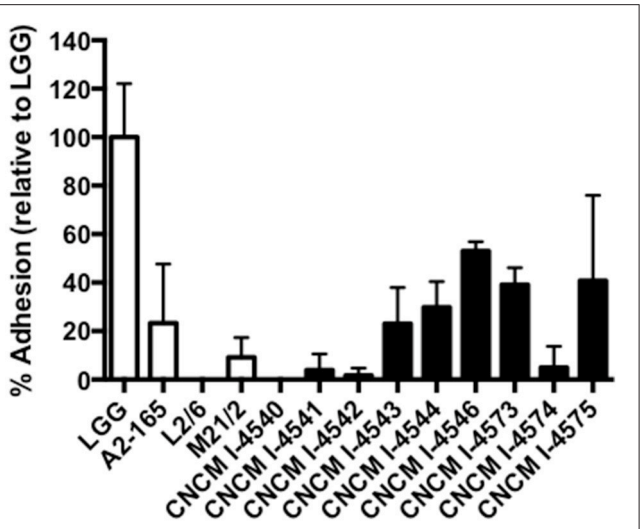

FIGURE 7 | Adhesion to mucin of *F. prausnitzii* strains. Experiments have been done in triplicate. The adhesion values have been normalized using *Lactobacillus rhamnosus* GG (LGG) a positive control know by their good adhesion properties to mucin (50). Results are presented by the mean and the standard deviation.

Adhesion to Epithelial Cells *In vitro*

In parallel, we also sought for the adhesion capacities of the new *F. prausnitzii* isolates to the intestinal epithelial cells HT-29 and mucin. All the tested strains were not able to adhere to HT-29 cells *in vitro* (data not shown) in anaerobic conditions. Regarding mucin, some of the strains were able to adhere to this compound after 3 h of incubation in the anaerobic chamber (**Figure 7**), Even if our conditions were not representative of physiological conditions (death of our eukaryotic cells), this result gives ecological clues about the processes of colonization of the gastro-intestinal tract by *F. prausnitzii*. In fact, this species is a late but major commensal colonizer of the gut which implantation requires a likely copro-cooperation maybe for the establishment of a trophic chain (Wrzosek et al., 2013).

CONCLUDING REMARKS

The development of new probiotic products containing human isolated strains with beneficial properties for the host requires the development of new techniques in order to: (i) isolate strains belonging to the major groups of the intestinal microbiota, (ii) determinate their safe status and (iii) infer in their potential beneficial effects. This study meets these entire three requests. Work with anaerobic and more precisely EOS bacteria are a prerequisite to succeed in the isolation of representative strains that can impact on intestinal homeostasis. For this reason, in this study, we have used a new procedure to isolate EOS strains from feces that has enabled us to build a collection of *F. prausnitzii* strains. The lack of knowledge about this species prompts us to further analyze their genetic diversity by comparing the new isolates with those already available in the databases. This has allowed us to point out the high diversity of our collection ranged on two different phylogroups with different clusters. *F. prausnitzii* strain genomes should be established or/and a metabolic comparison of several strains in the same culture conditions whether the phylogroups belong to genomovars or genomospecies.

Regarding safety concerns, this study is the first step toward a better understanding of *F. prausnitzii* properties. Up to date, little was known about *F. prausnitzii* resistance to antibiotics, lytic activities or adhesion properties. Here, we have shown for the first time the profile of all these characteristics in a collection of human *Faecalibacterium* strains. A positive remark is that all the strains were not antibacterial producers, not hemolytic and weak

producer of D-lactate. Furthermore, although some of the strains were able to adhere to mucin, this trait can be considered as factor favoring durable implantation and a highly effective probiotic (Miquel et al., 2015a). However, further analyses are required to better determine the presence of acquired or natural resistances as well as to distinguish between the pathogenic or adaptative nature of some of the properties detected such as the presence of DNase activity.

Finally, the anti-inflammatory properties of all the strains have been analyzed. There is a well-known correlation between *F. prausnitzii* dysbiosis and a large set of human diseases such as IBD and IBS (Miquel et al., 2013). Recent studies using *F. prausnitzii* strains in *in vivo* models provide arguments concerning its beneficial effect on the host (Sokol et al., 2008; Wrzosek et al., 2013; Martin et al., 2014). The presence of the anti-inflammatory properties of these strains also opens the possibility to test them in murine models in order to further determine their beneficial effects before testing them in human clinical trials.

AUTHOR CONTRIBUTIONS

RM, SM, JC, HS, LGBH, MT, and PL participate in the design of the project. RM, SM, JC, HS, OB, VA, LGBH, MT, and PL designed the experiments. RM, SM, LB, CB, VR, SH, and FC performed the experiments and analysis. RM and SM draft the manuscript. VR, CB, FC, JC, HS, LGBH, MT, and PL revised the manuscript critically. All the authors have read and approved the last version of the manuscript.

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Conflict of Interest Statement: PL and HS are co-founders of the start-up NextBiotiX aiming to use next-generation probiotics to fight and to prevent IBD.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Respiratory Commensal Bacteria *Corynebacterium pseudodiphtheriticum* Improves Resistance of Infant Mice to Respiratory Syncytial Virus and *Streptococcus pneumoniae* Superinfection

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Corynebacterium pseudodiphtheriticum is a Gram-positive bacterium found as a member of the normal microbiota of the upper respiratory tract. It was suggested that *C. pseudodiphtheriticum* may be potentially used as a next-generation probiotic for nasal application, although no deep studies were performed in this regard. We hypothesized that human isolate *C. pseudodiphtheriticum* strain 090104 is able to modulate the respiratory innate immune response and beneficially influence the resistance to viral and bacterial infections. Therefore, in the present study we investigated how the exposure of infant mice to nasal priming with viable or non-viable *C. pseudodiphtheriticum* 090104 influences the respiratory innate immune response triggered by Toll-like receptor (TLR)-3 activation, the susceptibility to primary Respiratory Synsytial Virus (RSV) infection, and the resistance to secondary *Streptococcus pneumoniae* pneumonia. We demonstrated that the nasal priming with viable *C. pseudodiphtheriticum* 090104 differentially modulated TLR3-mediated innate antiviral immune response in the respiratory tract of infant mice, improving their resistance to primary RSV infection, and secondary pneumococcal pneumonia. In association with the protection against RSV-pneumococcal superinfection, we found that viable *C. pseudodiphtheriticum* improved lung $CD3^+CD4^+IFN\text{-}\gamma^+$, and $CD3^+CD4^+IL\text{-}10^+$ T cells as well as $CD11c^+SiglecF^+IFN\text{-}\beta^+$ alveolar macrophages. Of interest, non-viable bacteria did not have the same

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protective effect, suggesting that *C. pseudodiphtheriticum* colonization is needed for achieving its protective effect. In conclusion, we present evidence that nasal application of viable *C. pseudodiphtheriticum* could be thought as an alternative to boost defenses against RSV and secondary pneumococcal pneumonia, which should be further studied and validated in clinical trials. Due to the absence of a long-lasting immunity, re-infection with RSV throughout life is common. Thus, a possible perspective use could be a seasonal application of a nasal probiotic spray to boost respiratory innate immunity in immunocompetent subjects.

Keywords: *Corynebacterium pseudodiphtheriticum,* TLR3, Respiratory Synsytial Virus, *Streptococcus pneumoniae,* respiratory immunity, nasal probiotic

INTRODUCTION

Corynebacterium pseudodiphtheriticum is a non-lipophilic, non-fermentative, urease- and nitrate-positive Gram-positive bacterium with variable shape (rods and cocci), which is found as a member of the normal microbiota of the human skin and upper respiratory tract (Ahmed et al., 1995; Burke et al., 1997; Bittar et al., 2010; Olender and Niemcewicz, 2010). Although there are clinical case reports pointing out at this bacterium as an opportunistic pathogen, it was suggested that *C. pseudodiphtheriticum,* being a natural member of the normal microbiota of nares and throat, may be potentially used as a probiotic for nasal application. In this regard, it has been demonstrated that *C. pseudodiphtheriticum* 090104 "Sokolov" is able to reduce *Staphylococcus aureus* colonization in humans (Uehara et al., 2000). There is also an inverse association between *S. aureus* and corynebacteria suggesting microbial competition during colonization (Liu C. M. et al., 2015). Controversially, some probiotic properties such as adherence to epithelial cells, biofilm formation, certain degree of immune stimulation, competition for nutrients, and adhesion sites are also shared by pathogens. For instance, some of the most known and used species of probiotic bacteria such as *Lactobacillus rhamnosus*, *L. plantarum, Enterococcus faecium, E. faecalis,* and even *E. coli* strain Nissle were also reported, though exceptionally, in clinical case reports. In any case, it has been established that security aspects such as antibiotic resistance or the presence of virulence factors are strain specific. Therefore, in order to propose *C. pseudodiphtheriticum* 090104 as a probiotic strain detailed studies evaluating both its functional properties and security aspects are necessary.

Respiratory syncytial virus (RSV) is a main respiratory pathogen responsible of bronchiolitis and pneumonia causing high morbidity and mortality in children under 3 years old. Currently, there are no available vaccines to prevent RSV infections or specific therapeutic treatments. Both viral and host factors are involved in disease severity. RSV cytopathogenicity is limited, but it elicits a strong immune response, which may result in tissue injury, loss of function and even death (Rutigliano and Graham, 2004; Bem et al., 2011). When exacerbated, immune response turns pathological, and in the case of RSV infection is characterized by high levels of pro-inflammatory chemokines and cytokines such as interleukin (IL)-6, IL-8, macrophage inflammatory protein (MIP)-1, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, and RANTES. At the very early stages of RSV infection, these pro-inflammatory factors participate in virus clearance, but their continuous production leads to increased injury (Rutigliano and Graham, 2004; Bem et al., 2011). In addition, secondary bacterial pneumonia is an important complication responsible for high morbidity and mortality of respiratory infections in infants and children (Liu et al., 2012; Bosch et al., 2013; Liu L. et al., 2015). The prevalence of bacteremia in children with RSV infection reported in the literature is low, ranging between 0.6 and 1.1% when conventional cultures were performed (Levine et al., 2004; Hishiki et al., 2011). However, a recent study showed that one out of every 10 previously healthy children hospitalized due to RSV had bacteremia, and these patients experienced a more severe disease (Cebey-Lopez et al., 2016). The rates of concurrent bacteremia was 10 times higher (10.6%) when molecular methods were applied. These findings are of importance because studies in clinical trials (Weinberger et al., 2013; Cebey-Lopez et al., 2016) and animal models of RSV-*Streptococcus pneumoniae* superinfection (Hament et al., 2005; Smith et al., 2014) showed that enhanced lung injuries and elevated levels of bacteremia are critical factors that determine the severity of infection and the rate of mortality.

Finding alternative strategies for the prevention of primary viral respiratory infections and secondary pneumococcal pneumonia in populations at risk is mandatory. In this regard, we have evaluated the possibility of administering probiotics to enhance the natural respiratory host defenses. In previous work, we showed that oral or nasal application of the probiotic strain *L. rhamnosus* CRL1505 protects adult and infant mice from RSV lethal challenge (Chiba et al., 2013; Tomosada et al., 2013). The main mechanism responsible for protection induced by the CRL1505 strain was enhancement of the mucosal antiviral innate immunity and the reduction of immunopathology. We hypothesized that a commensal bacterium from the respiratory tract such as *C. pseudodiphtheriticum* 090104 would modulate the respiratory antiviral innate immune response and beneficially influence the resistance to secondary bacterial infections. Therefore, in the present study we investigated how the exposure of infant mice to nasal priming with viable or non-viable human isolate *C. pseudodiphtheriticum* 090104

influences the respiratory innate immune response triggered by Toll-like receptor (TLR)-3 activation, the susceptibility to primary RSV infection, and the resistance to secondary *S. pneumoniae* pneumonia.

MATERIALS AND METHODS

Microorganisms

Corynebacterium pseudodiphtheriticum 090104 was cultured in trypticase soy broth and harvested by centrifugation at 3,000 × g for 10 min, washed three times with sterile 0.01 M phosphate buffer saline (PBS, pH 7.2), and resuspended in sterile PBS. Non-viable *C. pseudodiphtheriticum* 090104 was obtained as described previously (Tomosada et al., 2013). Briefly, bacteria were killed by tyndallization in a water bath at 80°C for 30 min and the lack of bacterial growth was confirmed by plating on to soy broth agar plates.

Safety Studies

In order to evaluate the susceptibility to antimicrobials of *C. pseudodiphtheriticum* 090104, the broth microdilution method was followed as recommended by the CLSI for infrequently isolated or fastidious bacteria (M45-A, CLSI-2006). The antimicrobial agents were penicillin (PEN), cefotaxime (CTX), ceftriaxone (CRO), meropenem (MER), vancomycin (VAN), gentamicin (GEN), ciprofloxacin (CIP), erythromicin (ERY), tetracycline (TET). MIC results were interpreted following CLSI guidelines (M45-A, CLSI-2006). Evaluation of bacterial translocation in infant mice was monitored by determining the presence of *C. pseudodiphtheriticum* lung, blood and spleen samples.

Animals and Feeding Procedures

Female 3-week-old BALB/c mice were obtained from the closed colony kept at CERELA (San Miguel de Tucumán, Argentina) or Tohoku University (Sendai, Japan). They were housed in plastic cages at room temperature. Mice were housed individually during the experiments. Viable and non-viable *C. pseudodiphtheriticum* 090104 were nasally administered to mice for 5 consecutive days at a dose of 10^8 cells/mouse/day in 50 µl of PBS. All groups were fed a conventional balanced diet *ad libitum*. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Guidelines for Animal Experimentation of CERELA and all efforts were made to minimize suffering. The Institutional Animal Welfare Committee of CERELA reviewed and approved the protocols used in this study.

Intranasal Administration of Poly(I:C)

Administration of the viral pathogen molecular pattern poly(I:C) was performed on day 6, after a 5 days treatment with viable or non-viable *C. pseudodiphtheriticum* 090104. Mice were lightly anesthetized and 100 µl of PBS, containing 250 µg poly(I:C) (equivalent to 10 mg/kg body weight), was administered dropwise, via the nares (Tomosada et al., 2013). Control animals received 100 µl of PBS. Mice received three doses of poly(I:C) or PBS with 24 h rest period between each administration.

Respiratory Syncytial Virus Primary Infection

Human RSV strain A2 was grown in Vero cells as described by Murawski et al. (2009). Briefly, Vero cells were infected with RSV at a multiplicity of infection (MOI) of 1 in 5 ml of Dulbecco's modified Eagle's medium (DMEM). Cells were infected for 2.5 h at 37°C and 5% CO_2. After infection, 7 ml of DMEM with 10% FBS (Sigma, Tokyo, Japan), 0.1% penicillin–streptomycin (Pen/Strep) (Sigma, Tokyo, Japan), and 0.001% ciprofloxacin (Bayer) were added to the flask and further incubated. When extensive syncytia formed, cells were scraped from the flask and sonicated three times, 5 s per pulse, at 25 W on ice. Cell lysates were centrifuge at 700 × *g* for 10 min at 4°C. Cell-free virus stocks were stored in 30% sucrose at −80°C. Uninfected cells were treated identically to generate a virus- and cell-free supernatant control. Mice were slightly anesthetized and intranasally challenged with 2.4×10^6 PFU RSV strain A2 or an equivalent volume of Vero cell lysate on day 6 after viable or non-viable *C. pseudodiphtheriticum* 090104 treatment.

Intact lung tissue was removed and stored in 30% sucrose for plaque assays. RSV immune-plaque assay was done as previously explained (Chiba et al., 2013). Briefly, lungs were homogenized using a pellet pestle and centrifuged at 2,600 × *g* for 10 min at 4°C. Twenty-four-well tissue culture plates were seeded with 1.5×10^5 Vero cells/well in DMEM (10% FBS, 0.1% Pen/Strep, and 0.001% ciprofloxacin) and virus plaques assays were performed in triplicate. Plates were incubated at 37°C and 5% CO_2 for 2.5 h. After incubation, supernatant was removed, and 1 ml of fresh DMEM medium supplemented with 10% FBS, 0.1% Pen/Strep, and 0.001% ciprofloxacin was overlaid on monolayers. When extensive syncytia developed, the overlay was removed and monolayers were fixed with ice-cold acetone:methanol (60:40). Primary RSV anti-F (clones131-2A; Chemicon) and anti-G (Mouse monoclonal [8C5 (9B6)] to RSV glycoprotein, Abcam) antibodies were added to wells for 2 h, followed by secondary horseradish peroxidase anti-mouse immunoglobulin antibody (Anti-mouse IgG, HRP-linked Antibody #7076, Cell signaling Technology) for 1 h. Plates washed twice with PBS containing 0.5% Tween 20 (Sigma) after each antibody incubation step. Individual plaques were developed using a DAB substrate kit (ab64238, Abcam) following manufacture's instructions. Results for immune-plaque assay were expressed as $\log_{10}$ PFU/g of lung.

Streptococcus pneumoniae Secondary Infection

Streptococcus pneumoniae serotype 6B (ANLIS, Argentina) was obtained from the respiratory tract of a patient from Hospital del Niño Jesús, Tucumán, Argentina. Pneumococci were grown on blood agar for 18 h. Colonies were suspended in Todd Hewitt broth (Oxoid), incubated overnight at 37°C, harvested and washed with sterile PBS. Cell density was adjusted to 4×10^7 CFU/ml. Challenge with pneumococci was performed 5 days

after the last administration of poly(I:C) or RSV challenge. Viable or non-viable *C. pseudodiphtheriticum*-treated as well as control infant mice were challenged intranasally with the pathogen by dripping 25 µl of inoculums containing 10^3 CFU (log phase) in PBS into each nostril.

Treated and control mice were sacrificed 2 days after *S. pneumoniae* infection. Lungs were excised, weighed and homogenized in sterile peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar and incubated for 18 h at 37°C. *Streptococcus pneumoniae* was identified by standard techniques and the results were expressed as log of CFU/g of lung. Bacteremia was monitored in blood samples obtained by cardiac puncture which were plated on blood agar. Results were reported as negative or positive hemocultures.

Cytokine Concentrations in Broncho-Alveolar lavages (BAL)

BAL samples were obtained as described previously (Villena et al., 2005) by performing lavages of lungs with sterile PBS. After centrifugation, cell-free supernatants were kept at −70°C. Tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IFN-β, IFN-α, interleukin (IL)-6, and IL-10 concentrations in serum and BAL were measured by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's recommendations (R&D Systems, MN, USA) (Salva et al., 2011).

Lung Cell Suspensions

Single lung cells were prepared using the previously described method (Villena et al., 2012). Briefly, mice were anesthetized and lungs were removed, finely minced and incubated for 90 min with 300 U of collagenase (Yakult Honsha Co., Tokyo, Japan) in 15 ml of RPMI 1640 medium (Sigma, Tokyo, Japan). After removal of debris, erythrocytes were depleted by hypotonic lysis. The cells were washed with RPMI medium supplemented with 0.1% Pen/Strep and suspended in a medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Cells were counted using Trypan Blue and adjusted to 5×10^6 cells/ml.

Flow Cytometry Studies

Single lung cells from mice were prepared as previously described (Villena et al., 2012; Zelaya et al., 2014, 2015). Lungs were removed, finely minced and incubated for 90 min with 300 U of collagenase (Yakult Honsha Co., Tokyo, Japan) in 15 ml of RPMI 1640 medium (Sigma, Tokyo, Japan). To dissociate the tissue into single cells, collagenase-treated minced lungs were gently tapped into a plastic dish. After removal of debris, erythrocytes were depleted by hypotonic lysis. The cells were washed with RPMI medium supplemented with 100 U/ml of penicillin and 100 mg/ml of streptomycin and then resuspended in a medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Cells were counted using Trypan Blue exclusion and then resuspended at 5×10^6 cells/ml.

Lung cell suspensions were pre-incubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) for 15 min at 4°C. Cells were incubated in the antibody mixes for 30 min at 4°C and washed with FACS buffer. Then, cells were stained with fluorochrome-conjugated antibodies against CD3, CD4, CD8, CD11c, CD11b, CD103, MHC-II, IFN-γ, IL-10, sialic acid-binding immunoglobulin-like lectin F (SiglecF) (BD Bioscience), IFN-β, and CD45 (eBioscience). Cells were then acquired on a BD FACSCalibur™ flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number determined for each tissue (Villena et al., 2012; Zelaya et al., 2014, 2015).

Lung Tissue Injury Studies

To measure increased permeability of the bronchoalveolar–capillarity barrier, we quantified albumin and protein content in cell-free BAL. Furthermore, lactate dehydrogenase (LDH) activity was quantified as an indicator of general cytotoxicity (Villena et al., 2012; Zelaya et al., 2014, 2015). Lung wet:dry weight ratio was determined as described before. Briefly, mice were euthanized and exsanguinated; lungs were removed, weighed (wet weight), dried at 55°C for 7 days, and weighed again (dry weight). The wet:dry weight ratio is a measure of intrapulmonary fluid accumulation. Histopathological examination was also performed in order to evaluate tissue damage during respiratory superinfection. Lungs were aseptically removed, fixed in 4% formalin and embedded in histowax (Leica Microsystems). Histopathological assessment was performed on five-micron tissue sections stained with hematoxylin-eosin.

Statistical Analysis

Experiments were performed in triplicate and results were expressed as mean ± standard deviation (SD). Five to six animals were used in each replicate per experimental group. Normal distributed data were tested by 2-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) or the Fisher's least significant difference (LSD) test (for multi-comparison) were used to evaluate the differences between the groups. Differences were considered significant at $p < 0.05$.

RESULTS

Influence of Viable and Non-viable *C. pseudodiphtheriticum* on Respiratory Immune Response

In order to determine whether viable or non-viable *C. pseudodiphtheriticum* could modulate mucosal immune responses in the respiratory tract, we quantified key cytokines in broncho-alveolar lavages (BAL) and serum samples as well as immune cell populations in lungs. Both treatments elicited an increase in the levels of the proinflammatory cytokines TNF-α and IL-6 in serum and BAL (**Figure 1**). Furthermore, *C. pseudodiphtheriticum* induced a significant increase in the production of IFN-γ and a slight but still significant enhancement of IFN-β, while no effect was observed in IFN-α levels. The immunoregulatory cytokine IL-10 was also detected at higher concentrations in viable or non-viable *C. pseudodiphtheriticum*-treated infant mice than in control mice without bacterial stimulation (**Figure 1**). In general, the effect of

the live bacterium was stronger than the elicited by non-viable *C. pseudodiphtheriticum*. In addition, the changes in cytokine levels were more pronounced in BAL (**Figure 1A**) than in serum samples (**Figure 1B**).

Flow cytometry indicated that there were no significant differences between the experimental groups when the numbers of lung antigen presenting cells including dendritic cells ($CD11c^+CD103$-$CD11b^{high}$ and $CD11c^+CD103^+CD11b^{low}$ cells) and alveolar macrophages ($CD45^+CD11c^+SiglecF^+$ cells) were compared (**Figure 2**). However, activated lung antigen presenting cells differed in mice nasally treated with *C. pseudodiphtheriticum* (**Figure 2**). For instance, $CD11c^+CD103^+MHCII^+$ and $CD11c^+CD11b^{high}MHCII^+$ populations were significantly greater ($p < 0.05$) in viable or non-viable *C. pseudodiphtheriticum*-treated infant mice than in control mice. In both cases, live bacteria had a stronger influence than non-viable *C. pseudodiphtheriticum* in antigen presenting cells activation. No differences were observed between the groups when total counts of lung $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were compared (**Figure 3**). Nevertheless, *C. pseudodiphtheriticum* differentially modulated specific IFN-γ producing populations of $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes. Viable bacteria significantly induced higher numbers of IFN-γ specific $CD3^+CD4^+$ T cells, whereas heat-killed *C. pseudodiphtheriticum* increased IFN-γ specific $CD3^+CD8^+$ cells (**Figure 3**). A slight but not significant increase of lung $CD3^+CD4^+IL\text{-}10^+$ T cells was observed in *C. pseudodiphtheriticum*-treated mice.

Corynebacterium pseudodiphtheriticum Modulates Immune Response Triggered by Poly(I:C) and Reduces Lung Injury

After treatment with viable or non-viable *C. pseudodiphtheriticum*, mice were nasally challenged for three consecutive days with poly(I:C). In order to evaluate the extent of lung injury induced by TLR3-triggered inflammation (**Figure 4**), we determined total protein and albumin concentrations, and LDH activity in BAL 2 days after challenge. In addition, we also determined the magnitude of edema by calculating the lung wet:dry ratio. Administration of poly(I:C) resulted in water retention due to the inflammatory response in the lungs in comparison with unchallenged mice. Viable *C. pseudodiphtheriticum* somehow prevented lungs from retaining fluids as it is shown in **Figure 4** reaching levels close to the unchallenged lung wet:dry ratios. Other lung injury parameters, such as protein content, albumin and LDH activity were also significantly ($p < 0.05$) reduced when mice were previously treated with viable or non-viable *C. pseudodiphtheriticum,* being the treatment with live bacteria more effective than heat-killed bacteria.

As we have described previously (Tomosada et al., 2013), nasal challenge with poly(I:C) increased the levels of proinflammatory cytokines and IL-10 (**Figure 5**), and immune cells (**Figure 6**) in the respiratory tract when compared to basal levels. The nasal pretreatments of mice with viable or non-viable *C. pseudodiphtheriticum* differentially modulated the profiles of cytokines measured in BAL. IFN-γ was the most influenced cytokine, showing a significant increase in mice treated with live bacteria in comparison to untreated control mice challenged with poly(I:C), whereas non-viable bacteria slightly increased the levels of IFN-γ (**Figure 5**). To a lesser extent, TNF-α, IL-6, IFN-β, and IL-10 concentrations in BAL were also higher in viable *C. pseudodiphtheriticum*-treated mice. In contrast, only TNF-α and IL-6 were modulated by non-viable bacteria.

The numbers of lung $CD11c^+CD103^+MHCII^+$ and $CD11c^+CD11b^{high}MHCII^+$ dendritic cells were significantly greater ($p < 0.05$) in viable or non-viable *C. pseudodiphtheriticum*-treated infant mice than in control mice (**Figure 6**). In addition, higher numbers of $CD45^+SiglecF^+IFN\text{-}\beta^+$ alveolar macrophages and $CD3^+CD4^+IL\text{-}10^+$ T cells were observed when mice were previously treated with viable *C. pseudodiphtheriticum* in comparison to control mice (**Figure 6**). In contrast, non-viable bacteria did not produce significant changes in the numbers of these immune cells populations. $CD3^+CD4^+IFN\gamma^+$ lymphocyte counts increased in mice which received viable or non-viable *C. pseudodiphtheriticum* before poly(I:C) challenge, being this effect stronger with the viable bacteria (**Figure 6**).

Viable *C. pseudodiphtheriticum* Increases Resistance to Primary RSV Infection

Nasal administration of viable *C. pseudodiphtheriticum* improved health state of infant mice infected with RSV as reflected by the increase in body weight during the studied period (**Figure 7**). Mice treated with non-viable bacteria did not show the improvement of body weight. Virus load was significantly lower in mice treated with viable *C. pseudodiphtheriticum* when compared to those receiving heat-killed bacteria or controls (**Figure 7**). The markers of lung tissue damage in RSV-infected mice showed that the viral infection induced a significant cellular damage and alveolar-capillary barrier alterations (**Figure 7**). Both, BAL LDH and albumin concentrations were significantly lower in infant mice previously treated with viable *C. pseudodiphtheriticum* than in RSV-challenged controls or mice receiving heat-killed bacteria (**Figure 7**).

Viable *C. pseudodiphtheriticum* Increase Resistance to Secondary Pneumococcal Infection

Finally, we addressed whether the nasal treatments with viable and non-viable *C. pseudodiphtheriticum* where able to increase the resistance of infant mice to secondary pneumococcal pneumonia. For that purpose, mice were nasally primed with viable or non-viable bacteria, infected with RSV, and 5 days after virus infection, they were challenged with *S. pneumoniae*. Pneumococcal colonization and bacteremia were evaluated on day 2 post-pneumococcal challenge. In addition, RSV titers as well as lung tissue damage were studied before (day 0) and after (day 2) infection with *S. pneumoniae*. RSV was detected in lungs of infected infant mice before and after pneumococcal infection (**Figure 8**). In

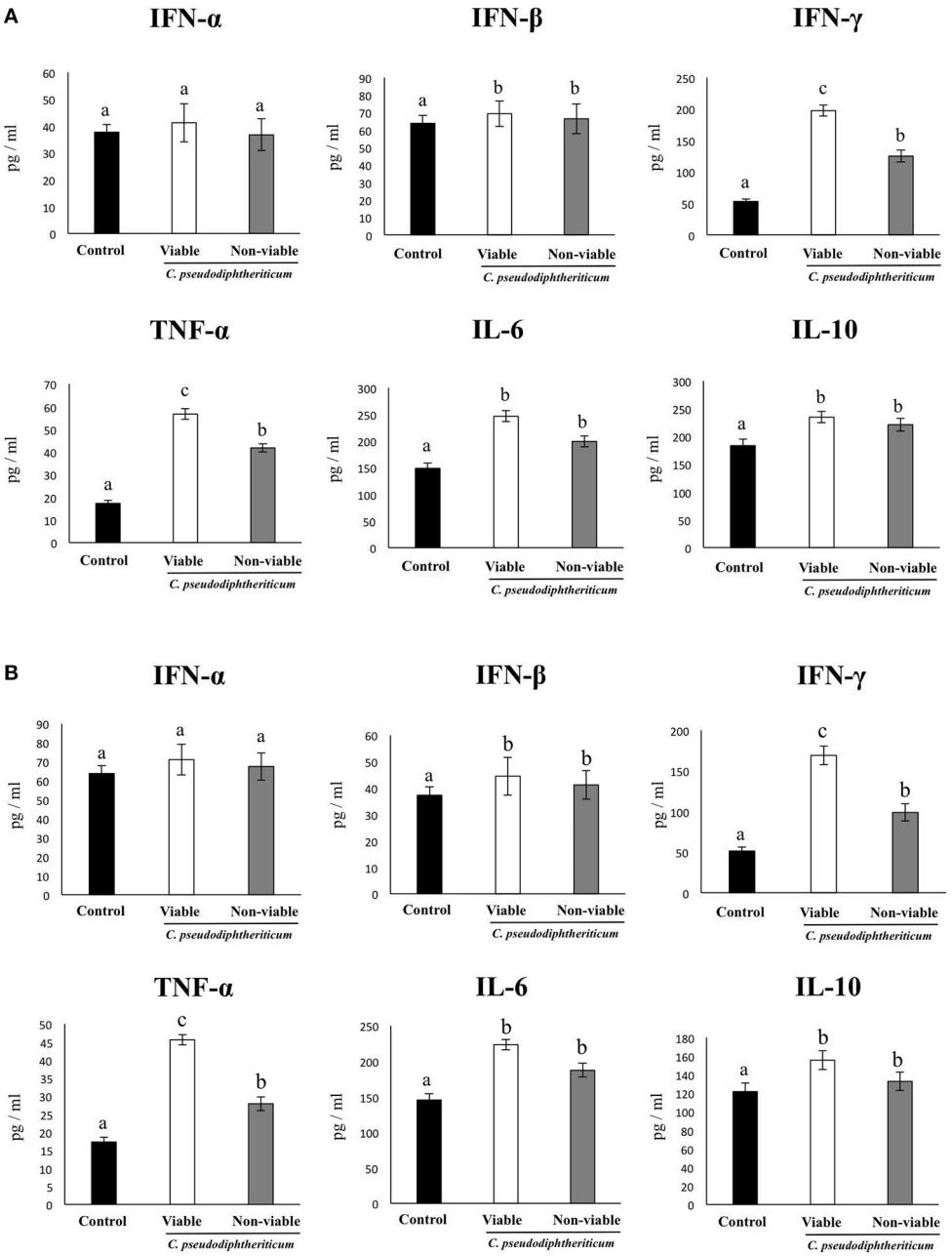

FIGURE 1 | Effect of *Corynebacterium pseudodiphtheriticum* strain 090104 on respiratory and blood cytokines. Viable or non-viable *C. pseudodiphtheriticum* were nasally administered to infant mice during five consecutive days. Non-treated infant mice were used as controls. Levels of tumor necrosis factor (TNF)-α, interferon (IFN)-α, IFN-β, IFN-γ, interleukin (IL)-6, and IL-10 were determined in broncho-alveolar lavages (BAL) **(A)** and serum **(B)**. Experiments were performed with 5–6 mice per group. The results represent data from three independent experiments. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

addition, pneumococci were detected in lungs and blood of control infant mice (**Figure 8**). Viable *C. pseudodiphtheriticum* significantly reduced RSV titers as well as lung bacterial cell counts and prevented the dissemination of *S. pneumoniae* into the blood (**Figure 8**). No protective effect was observed for non-viable *C. pseudodiphtheriticum*. When lung injury was studied it was observed that the secondary pneumococcal pneumonia induced a significant increase of the BAL biochemical parameters that evaluate cellular damage and alveolar-capillary barrier alterations (**Figure 8**). Viable *C. pseudodiphtheriticum* significantly reduced pulmonary damage as demonstrated by the lower LDH and albumin content in BAL when compared

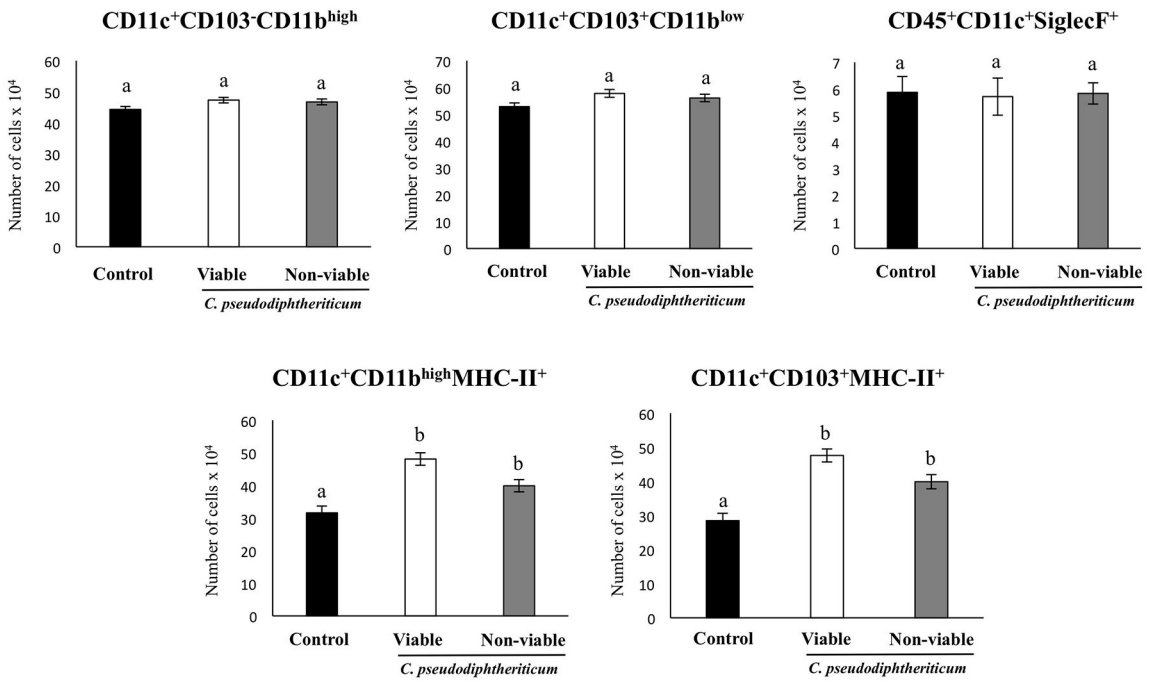

FIGURE 2 | Effect of *Corynebacterium pseudodiphtheriticum* strain 090104 on lung immune cell populations. Viable or non-viable *C. pseudodiphtheriticum* were nasally administered to infant mice during five consecutive days. Non-treated infant mice were used as controls. The numbers of lung antigen presenting cells including MHC-II$^+$CD11c$^+$CD11b^{low}CD103$^+$ and MHC-II$^+$CD11c$^+$CD11b^{high}CD103$^-$ dendritic cells, and CD45$^+$MHC-II$^-$CD11c$^+$SiglecF$^+$ alveolar macrophages were determined by flow cytometry. Experiments were performed with 5–6 mice per group. The results represent data from three independent experiments. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

to controls and non-viable bacteria-treated mice (**Figure 8**). Moreover, histological examination of lung of infected infant mice revealed severe inflammatory cell recruitment around alveoli and blood vessels, focal hemorrhage and a significant reduction of gas exchange spaces (**Figure 8**). Lung histology analysis of viable *C. pseudodiphtheriticum*-tretaed mice showed a significant reduction in the alterations of gas exchange spaces, hemorrhage and inflammatory cells infiltration (**Figure 8**) while mice treated with non-viable bacteria were not different from control animals (data not shown).

DISCUSSION

Respiratory viruses are an important cause of fatal pneumonia in children. They also predispose individuals to suffer bacterial infections by disrupting cells, releasing nutrients and reducing ciliary continuity and kinesia. Further, they alter the innate and adaptive immune systems, which may in turn help promoting bacterial infection (Hament et al., 2005; Smith et al., 2014). Therefore, there is a global need for controlling primary respiratory viral infections and secondary bacterial diseases, and beneficial microbes may offer an interesting alternative (Maragkoudakis et al., 2010; Villena et al., 2012; Chiba et al., 2013; Tomosada et al., 2013; Tada et al., 2016).

There is an increasing amount of evidence indicating that respiratory indigenous microbiota contributes to respiratory health by preventing the overgrowth of pathogens and the inflammation they cause (Pettigrew et al., 2012; Bosch et al., 2013). Commensal bacteria may exert health benefits by opposing to bacterial or viral pathogens directly by blocking adhesion sites and/or indirectly by modulating host immune responses in such a way that pathogen clearance is enhanced but inflammation is simultaneously better controlled (Uehara et al., 2000; Pettigrew et al., 2012; Kiryukhina et al., 2013; Liu C. M. et al., 2015). Therefore, respiratory commensal bacteria, if investigated in depth may be a source for developing next generation probiotic preparations for the improvement of respiratory health. In this study, we demonstrated that *C. pseudodiphtheriticum*, a typical commensal of the nasal human mucosa, is a candidate for enhancing respiratory immune responses and protecting against RSV and *S. pneumoniae* infections.

Although there are some safety concerns about *C. pseudodiphtheriticum* because of a few case reports indicating opportunistic infections by this bacterium, it is well accepted that both probiotic and safety properties are strain specific. Genomic analysis revealed the presence of a hemolysin protein in *C. pseudodiphtheriticum* 090104 genome with similar characteristics to the one found in *Bacillus cereus*, which could become a potential risk for health especially in immunocompromised individuals (Karlyshev and Melnikov, 2013). However, the 090104 strain was safe when used in the mouse model studied here, and in clinical trials in healthy volunteers performed before (Uehara et al., 2000; Kiryukhina

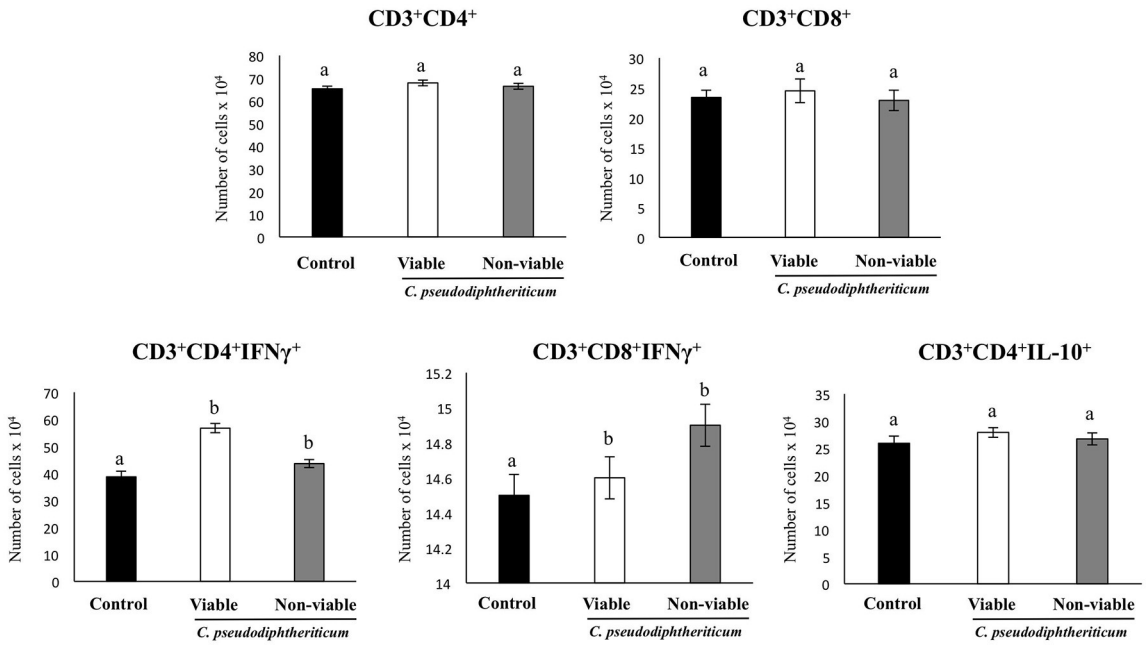

FIGURE 3 | Effect of *Corynebacterium pseudodiphtheriticum* strain 090104 on lung immune cell populations. Viable or non-viable *C. pseudodiphtheriticum* were nasally administered to infant mice during five consecutive days. Non-treated infant mice were used as controls. The numbers of lung T cells including $CD3^+CD4^+IFN\text{-}\gamma^+$, $CD3^+CD4^+IL\text{-}10^+$, and $CD3^+CD8^+IFN\text{-}\gamma^+$ T lymphocytes were determined by flow cytometry. Experiments were performed with 5–6 mice per group. The results represent data from three independent experiments. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

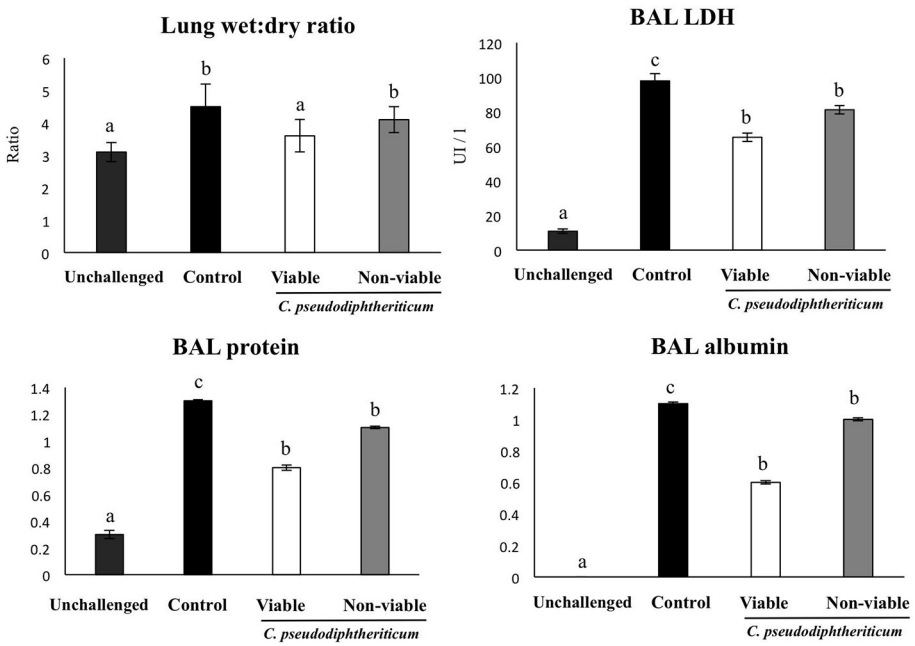

FIGURE 4 | Effect of *Corynebacterium pseudodiphtheriticum* strain 090104 on lung tissue damage induced by the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Infant mice were nasally primed with viable or non-viable *C. pseudodiphtheriticum* during five consecutive days and then challenged with three once-daily doses of poly(I:C). Non-treated infant mice challenged with poly(I:C) were used as controls. Two days after the last poly(I:C) administration lung wet:dry weight ratio, lactate dehydrogenase (LDH) activity and, albumin and protein concentrations in broncho-alveolar lavages (BAL) were determined. Experiments were performed with 5–6 mice per group. The results represent data from three independent experiments. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

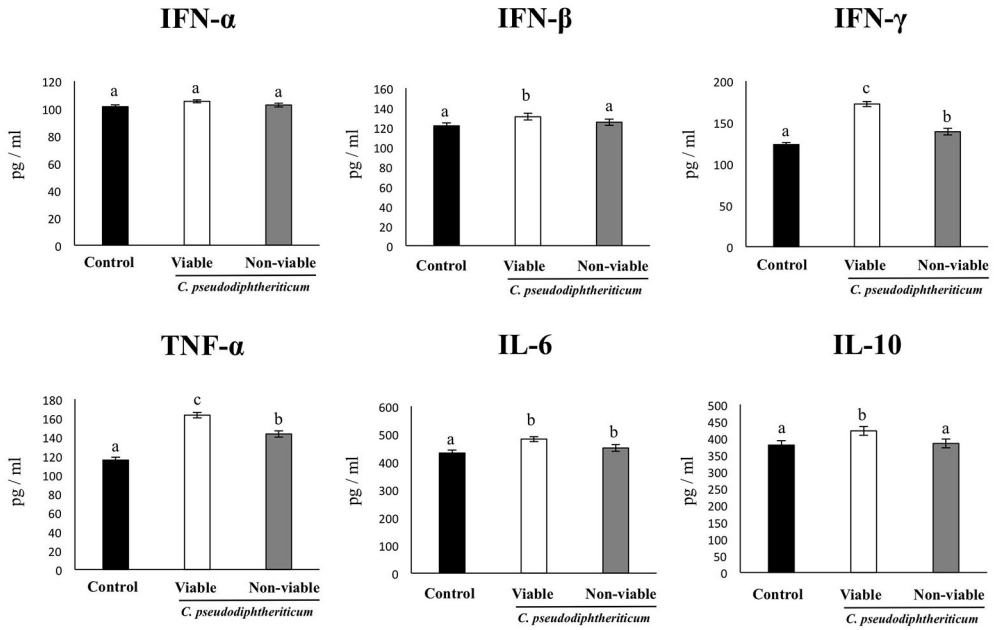

FIGURE 5 | Effect of *Corynebacterium pseudodiphtheriticum* strain 090104 on respiratory cytokines after the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Infant mice were nasally primed with viable or non-viable *C. pseudodiphtheriticum* during five consecutive days and then challenged with three once-daily doses of poly(I:C). Non-treated infant mice challenged with poly(I:C) were used as controls. Two days after the last poly(I:C) administration the levels of tumor necrosis factor (TNF)-α, interferon (IFN)-α, IFN-β, IFN-γ, interleukin (IL)-6, and IL-10 were determined in broncho-alveolar lavages (BAL). Experiments were performed with 5–6 mice per group. The results represent data from three independent experiments. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

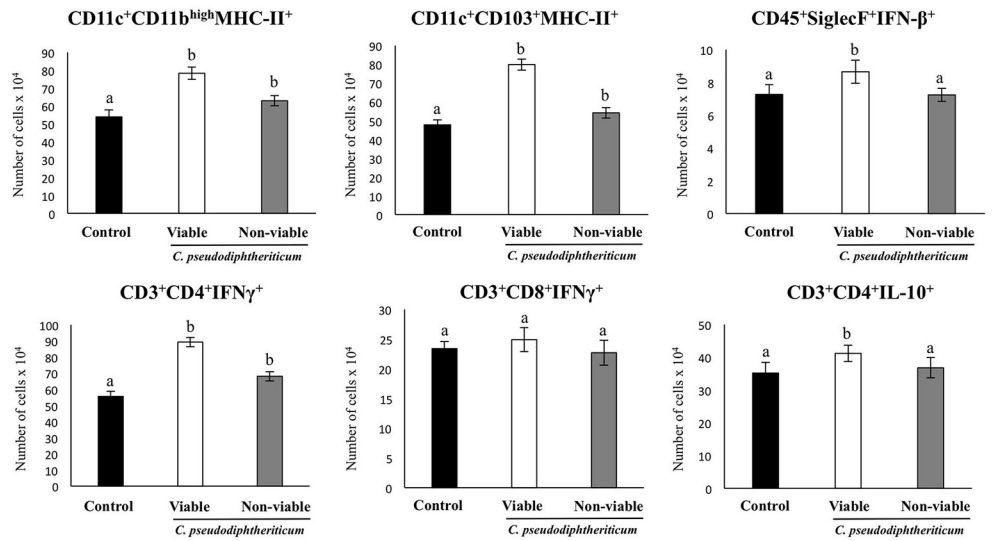

FIGURE 6 | Effect of *Corynebacterium pseudodiphtheriticum* strain 090104 on respiratory immune cell populations after the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Infant mice were nasally primed with viable or non-viable *C. pseudodiphtheriticum* during five consecutive days and then challenged with three once-daily doses of poly(I:C). Non-treated infant mice challenged with poly(I:C) were used as controls. Two days after the last poly(I:C) administration the numbers of lung T cells including $CD3^+CD4^+$IFN-γ^+, $CD3^+CD4^+$IL-10^+, and $CD3^+CD8^+$IFN-γ^+ T lymphocytes, as well as antigen presenting cells including MHC-$II^+CD11c^+CD11b^{low}CD103^+$ and MHC-$II^+CD11c^+CD11b^{high}CD103^-$ dendritic cells, and $CD45^+$MHC-$II^-CD11c^+SiglecF^+$ alveolar macrophages were determined by flow cytometry. Experiments were performed with 5–6 mice per group. The results represent data from three independent experiments. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

et al., 2013; Liu C. M. et al., 2015). Our studies evaluating the potential translocation of *C. pseudodiphtheriticum* 090104 in infant mice after its nasal administration showed no adverse effects (data not shown). In addition, *C. pseudodiphtheriticum* 090104 shows resistance to β-lactam antibiotics and macrolides (data not shown) that are the most prevalent acquired antibiotic resistances described for this species. However, genome analysis did not evidence the presence of antibiotic resistance genes acquired by genetic horizontal transfer. Thus, our studies indicate that the use of this bacterium would be safe.

In previous studies, it has been shown that nasal application of *C. pseudodiphtheriticum* allowed the bacteria to colonize the nasal mucosa reducing *S. aureus* infection (Uehara et al., 2000; Kiryukhina et al., 2013; Liu C. M. et al., 2015). In line with these observations, we showed here for the first time that the nasal priming with *C. pseudodiphtheriticum* 090104 reduced RSV and *S. pneumoniae* colonization in infant mice. Moreover, this is the first study showing immunomodulatory properties for viable *C. pseudodiphtheriticum* as well as its capacity to enhance immunity against primary RSV and secondary pneumococcal pneumonia. According to our results, viable *C. pseudodiphtheriticum* was effective in reducing the burden of RSV infection as reflected by the lower viral load, improved body weight, and reduced pulmonary damage. The ameliorated pulmonary injury was related to the modulation of the inflammatory response that is known to be a main component of damage in RSV infections (Rutigliano and Graham, 2004; Bem et al., 2011; Cervantes-Ortiz et al., 2016). Main secreted proinflammatory cytokines in children coursing a natural RSV infection as well as in experimentally RSV inoculated mice are type I IFNs, TNF-α, IL-6, IL-8, MIP-1, RANTES, and MCP-1. Although these cytokines contribute to virus clearance in the early steps of infection, deregulated cytokine response leads to tissue injury (McNamara and Smyth, 2002). In our experiments, pre-treatment of infant mice with *C. pseudodiphtheriticum* enhanced the secretion of IFN-β, TNF-α, and IL-6 but at the same time, it also enhanced the production of IL-10 in response to RSV infection. It

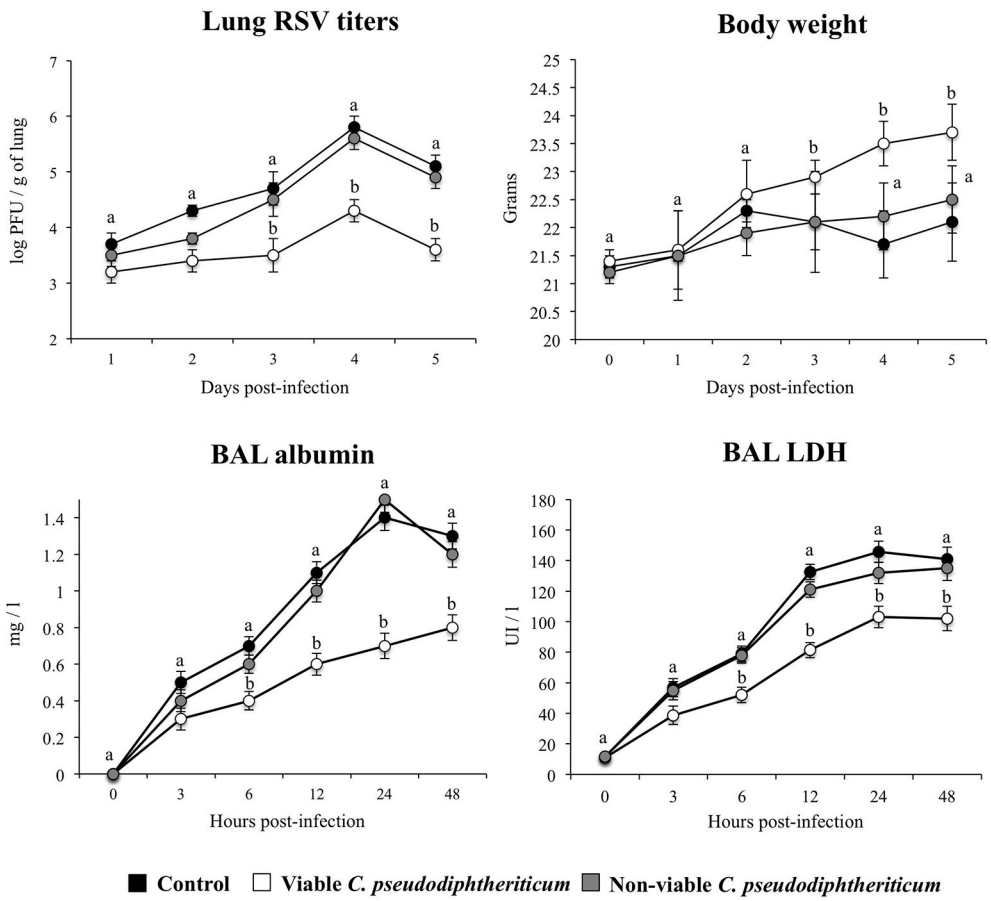

FIGURE 7 | Effect of *Corynebacterium pseudodiphtheriticum* strain 090104 on the resistance to primary Respiratory Syncytial Virus (RSV) infection. Infant mice were nasally primed with viable or non-viable *C. pseudodiphtheriticum* during five consecutive days and then challenged with RSV. Non-treated infant mice challenged with the viral pathogen were used as controls. Lung RSV titers, changes in body weight, and lactate dehydrogenase (LDH) activity and albumin concentrations in broncho-alveolar lavages (BAL) were evaluated on different time points after the viral challenge. Experiments were performed with 5–6 mice per group per each time point. The results represent data from three independent experiments. Values for each time point with different letters were significantly different ($P < 0.05$). Values for each time point with shared letters do not differ significantly.

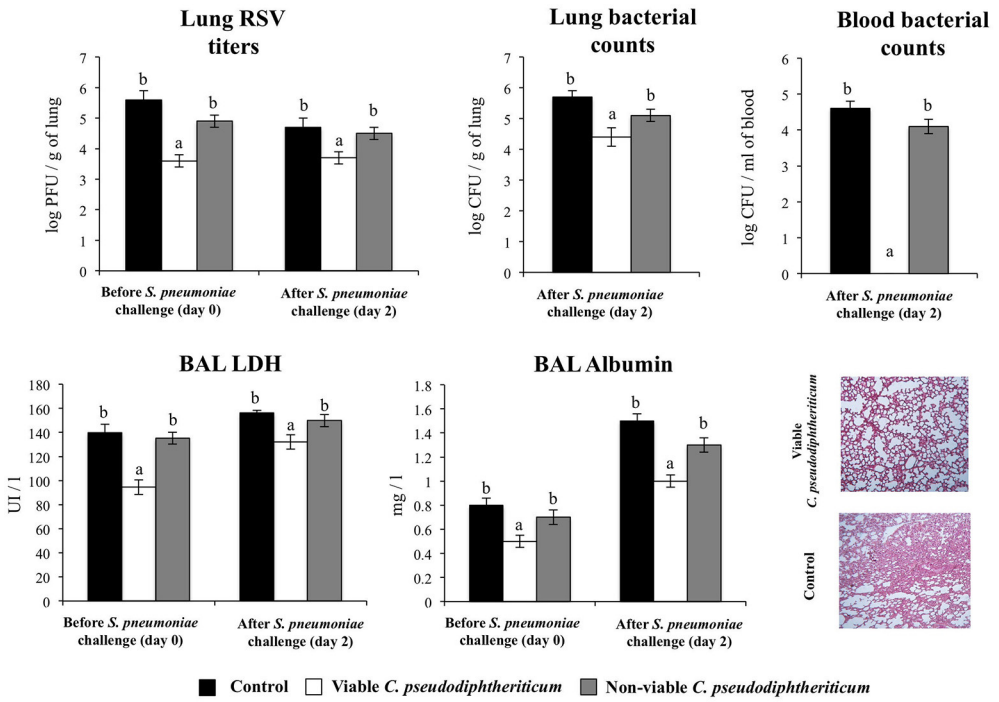

FIGURE 8 | Effect of *Corynebacterium pseudodiphtheriticum* strain 090104 on the resistance to secondary pneumococcal pneumonia after the primary infection with Respiratory Syncytial Virus (RSV). Infant mice were nasally primed with viable or non-viable *C. pseudodiphtheriticum* during five consecutive days, challenged with RSV and, infected with *Streptococcus pneumoniae* 5 days after the viral infection. Non-treated infant mice infected with RSV and challenged with *S. pneumoniae* were used as controls. Lung RSV titers, lung bacterial cells counts, lactate dehydrogenase (LDH) activity and albumin concentrations in broncho-alveolar lavages (BAL), and lung histology were determined on day 2 post-pneumococcal challenge. Lungs histological examination was performed with hematoxylin and eosin stained light micrographs, original magnification ×40. Experiments were performed with 5–6 mice per group. The results represent data from three independent experiments. Values for bars with different letters were significantly different ($P < 0.05$). Values for bars with shared letters do not differ significantly.

was proposed that the most prominent role of IL-10 is its contribution to restrict inflammation during RSV infection, which consequently lowers injury (Stacey et al., 2011; Weiss et al., 2011). The differential regulation of the inflammatory response induced by viable *C. pseudodiphtheriticum* seems to be related to its capacity to modulate TLR3-mediated inflammatory response in the respiratory tract. As others and we demonstrated previously, TLR3 has little effect on RSV clearance but it is necessary to regulate the respiratory immune environment. The absence of an efficient regulation of TLR3 activation significantly contributes to the pulmonary immunopathology associated to RSV infection (reviewed in 42). In fact, respiratory administration of the TLR3 agonist poly(I:C) has been used to mimic the pro-inflammatory and physiopathological consecuences of RSV infections in the lung (Kitazawa and Villena, 2014).

It has also been reported that the exacerbated proinflammatory cytokine/chemokine response is skewed toward a T helper type 2 (Th2) immune response (Cervantes-Ortiz et al., 2016), and that IFN-γ-producing $CD4^+$ and $CD8^+$ T cells contribute to protection during RSV infection (Sun and Lopez, 2016). In our experiments, treatment with viable *C. pseudodiphtheriticum* also increased IFN-γ producing $CD3^+CD4^+$ cells in the lungs of infant mice. The improved numbers of $CD4^+$IFN-γ^+ cells and IFN-γ levels in lung tissue would activate pulmonary macrophages and dendritic cells and induce the enhancement of Th1 cellular response, contributing to the protection induced by *C. pseudodiphtheriticum*.

In line with our previous studies evaluating the effect of beneficial microbes on the susceptibility to viral infections (Villena et al., 2012; Chiba et al., 2013; Tomosada et al., 2013), we found here that the respiratory commensal bacteria *C. pseudodiphtheriticum* improves resistance to RSV infection through the modulation of IFN-β, IFN-γ, and IL-10.

We also demonstrated here that viable *C. pseudodiphtheriticum* significanlty reduced *S. pneumoniae* cell counts in lungs and prevented its dissemination into the blood of infant mice after the primary infection with RSV. The effect of *C. pseudodiphtheriticum* in reducing lung pneumococcal cell counts was modest compared with our own previous studies. We had reported that the nasal administration of viable *Lactococcus lactis* NZ9000 to adult and infant mice reduced in more than two log folds *S. pneumoniae* cell counts in lungs (Medina et al., 2008). Moreover, *C. pseudodiphtheriticum* evaluated in an infant mice model of primary pneumococcal infection also reduced in more than two log folds *S. pneumoniae* cell counts in lungs when compared to untreated controls (data not shown). Despite the modest results obtained here by measuring the burden of

the pathogen in the respiratory tract, *C. pseudodiphtheriticum* treatment was able to significantly reduce lung tissue damage and bacterial dissemination into the blood stream. These findings are of importance because experimental and clinical studies (Hament et al., 2005; Weinberger et al., 2013; Smith et al., 2014; Cebey-Lopez et al., 2016) showed that enhanced lung injuries and elevated levels of bacteremia are critical factors that determine the severity of infection and the rate of mortality.

It could be speculated that the beneficial modulation of the inflammatory response during RSV and the reduced lung injuries induced by *C. pseudodiphtheriticum* administration would be related to the improvement of secondary pneumococcal infection. The different respiratory immune environment (such as the levels of IFN-β, IFN-γ, and IL-10 in the lungs) on *C. pseudodiphtheriticum*-treated mice at the moment of pneumococcal infection would induce an improved immune response and protection. Of note, *C. pseudodiphtheriticum* was able to enhance $CD4^+IFN\text{-}\gamma^+$ cells in the respiratory tract. It has been reported that IFN-γ early during acute *S. pneumoniae* pneumonia induces transcription of target genes in the lungs, which are critical for host defense (Gomez et al., 2015). In addition, *C. pseudodiphtheriticu* stimulated the production of IFN-β in $CD45^+SiglecF^+$ alveolar macrophages after poly(I:C) administration. Some studies have showed that IFN-β is involved in the protection against lung tissue injury as well in the control of pneumococcal dissemination into the blood during secondary pneumococcal pneumonia. No significant differences in *S. pneumoniae* counts in the lungs of $IFNAR1^{-/-}$ and $IFNAR1^{+/+}$ mice were observed after pneumococcal challenge. However, pneumococci were observed earlier and at higher numbers in blood samples of $IFNAR1^{-/-}$ mice compared to wild-type animals (LeMessurier et al., 2013). More detailed studies are necessary to fully understand the immune mechanisms involved in the protection against secondary pneumococcal pneumonia induced by *C. pseudodiphtheriticum* 090104.

Interestingly, non-viable bacteria did not have the same protective effect. Non-viable *C. pseudodiphtheriticum* was unable to induce the increase of the numbers of $CD45^+SiglecF^+IFN\text{-}\beta^+$ and $CD4^+IL\text{-}10^+$ cells neither the levels of IFN-β and IL-10 in the respiratory tract. Only increments in $CD4^+IFN\text{-}\gamma^+$ cells and IFN-γ levels were detected but there were significantly lower when compared with those induced by viable bacteria. These results suggest that *C. pseudodiphtheriticum* 090104 colonization of the nasopharynx is indeed needed for reducing RSV infection and secondary bacterial infection. In line with this finding, it has been reported that the presence of viable *Corynebacterium* spp. in the upper respiratory tract is necessary to protect against pathogens since antibiotic perturbations leading to the reduction of beneficial commensal bacteria such as *Dolosigranulum* spp. or *Corynebacterium* spp. increase the risk of respiratory infections of healthy children (Pettigrew et al., 2012; Teo et al., 2015). Genome sequence analysis of the 090104 strain showed that the bacterium has gene clusters encoding fimbrial subunits and sortase A that are proteins involved in the attachment of fimbria to the cell surface (Karlyshev and Melnikov, 2013). To obtain mutants depleted from these genes and study whether these *C. pseudodiphtheriticum* strains lacking attachment proteins are able to beneficially modulate respiratory immunity is an interesting topic that we intend to evaluate in the immediate future.

In conclusion, we present evidence that nasal application of viable *C. pseudodiphtheriticum* could be thought as an alternative to boost antiviral defenses against RSV and secondary pneumococcal pneumonia, which should be further studied and validated in clinical trials. Due to the absence of a long-lasting immunity, re-infection with RSV throughout life is common. Thus, a possible perspective use could be a seasonal application of a nasal next-generation probiotic spray to boost respiratory innate immunity in immunocompetent subjects.

AUTHOR CONTRIBUTIONS

SA, VM, HK, and JV designed the study. MV, HT, HK, and JV wrote the manuscript. PK, PC, MV, and CR did the laboratory work. PK, PC, and JV performed statistical analysis. HT, MV, and JV contributed to data analysis and interpretation. All authors read and approved the manuscript.

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Protection Mechanism of *Clostridium butyricum* against *Salmonella* Enteritidis Infection in Broilers

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This study was designed to evaluate the protection mechanism of oral administration of *Clostridium butyricum* against *Salmonella* enteritidis (SE) colonization in broilers. In the current study, 180 one-day-old healthy Arbor Acres (AA) broilers were meanly grouped into three, with three replicates of 20 birds each. An negative control group was fed basal diet without SE challenge and a positive control (PC) group was fed the basal diet and challenged with SE [10^6 colony forming unit (CFU)/0.2 mL]. An experimental (EXP) group was fed the basal diet, orally administered with *C. butyricum* (10^6 CFU/mL) and challenged with SE (10^6 CFU/0.2 mL). The results showed that compared to the PC group, the SE loads in livers, spleens, and cecal contents of chickens in EXP group were significantly reduced ($P < 0.05$) except in spleens at the 2-day post-infection; the production of interferon-γ, interleukin (IL)-1β, IL-8, and tumor necrosis factor-α in the livers, spleens, and cecal tissues of chickens in EXP group were decreased to different extents. The results of quantitative real-time polymerase chain reaction further revealed that the inflammation of chickens in EXP group was alleviated by *C. butyricum* via down-regulating TLR4, MyD88, and NF-κB-dependent pathways. Collectively, these findings indicated that oral administration of *C. butyricum* could be a suitable alternative for preventing SE infection in broilers.

Keywords: AA broilers, oral administration, *S.* enteritidis, *C. butyricum*, Q-PCR

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INTRODUCTION

Salmonella, as an important foodborne pathogen, can lead to serious infections in animals and humans worldwide (Mead et al., 1999; Scallan et al., 2011). Poultry have been recognized as an important reservoir for *Salmonella* (Chen and Jiang, 2014). *Salmonella* can cause high morbidity and mortality in poultry breeding industry, especially in young birds within 1 week age (Wigley et al., 2001; Vo et al., 2006). At the early stage of *Salmonella* infection, the production of cytokines, such as interferon (IFN)-γ, interleukin (IL)-1β, IL-8, and tumor necrosis factor (TNF)-α, is of utmost importance for controlling *Salmonella* growth and spread in the host body (Brown et al., 2006; Hu et al., 2015). In addition, Toll-like receptors (TLRs) can play a key role in the protection animals and humans against *Salmonella* infection, and they combat the pathogen through recognition of pathogen-associated molecular patterns (Akira and Takeda, 2004). TLR4, as one important member of the TLRs family, can recognize lipopolysaccharide (LPS) of Gram-negative bacteria and can activate nuclear factor-kappa B (NF-κB) through myeloid differentiation primary response protein 88 (MyD88), and therefore leading to cytokine secretion and inflammatory response (Kawai and Akira, 2007).

As for the combat against *Salmonella* infections, antimicrobials have been widely used in the clinical practice. However, the overuse and even abuse of antibiotics have contributed to the increasing and dissemination of drug-resistant *Salmonella* and have sparked a severe public health concern (Chiu et al., 2002; Tseng et al., 2014). Furthermore, antimicrobials can lead to the loss of commensal gastrointestinal microbiota and potentially to the overgrowth of pathogens (McDonald et al., 2016; Wischmeyer et al., 2016). Therefore, in the recent years, many researchers have been striving to find the substitutes of antimicrobials, and probiotics are being considered as one of the promising substitute for antimicrobials against *Salmonella* infections (Mathipa and Thantsha, 2017).

Probiotics have ability to provide protection effects for the host when administered in adequate amounts (Food and Agricultural Organization/World Health Organization [FAO/WHO], 2002). Numerous studies have showed that the use of probiotics is able to modulate mucosal immune functions, prevent bacterial translocation, and potentially suppress inflammatory cytokine production through modulating LPS-induced inflammation by binding to LPS or directly perturbing the MyD88 signaling pathway (Mainous et al., 1995; Kemgang et al., 2014).

Clostridium butyricum, a strictly anaerobic endospore-forming Gram-positive bacillus, could produce butyric acid. Compared to *Lactobacillus* and *Bifidobacterium*, *C. butyricum* is able to survive at lower pH and relatively higher bile concentrations (Okamoto et al., 2000; Zhang et al., 2016). Previous studies demonstrated that *C. butyricum* can inhibit pathogens propagation and spread in host body and therefore is considered as a potential substitute for antibiotics (Gao et al., 2012; Yang et al., 2012; Zhang et al., 2016). However, the protection mechanism of *C. butyricum* against *Salmonella* enteritidis (SE) colonization in broilers remains to be elucidated. This study was therefore conducted to better understand the protection mechanism by which *C. butyricum* protects chickens against SE infection.

MATERIALS AND METHODS

Ethics Statement

All procedures were approved by the Animal Care and Use committee of Shandong Agricultural University (SDAUA-2016-016).

Bacterial Strains and Growth Conditions

Clostridium butyricum (AQQF01000149) was obtained as a gift from Dalian Sanyi Animal Medicine Company (China) and grown anaerobically at 37°C in liquid fermentation tank for 48 h. The concentration of *C. butyricum* was adjusted to 1×10^6 colony forming unit (CFU)/mL in sterile saline.

A virulent atrichia SE, a isolate from a diseased chicken, was obtained from the Avian Disease Centre of Shandong Agricultural University. While cultivating SE, single colony was picked from xylose lysine deoxycholate agar plate and transferred into a tube contained 5 mL tryptic soy broth and then incubated at 37°C for 12 h. The concentration of SE was adjusted to 1×10^6 CFU/mL in sterile saline.

Experimental Design

The experiment was performed in October, 2016. In total, 180 one-day-old healthy Arbor Acres (AA) chickens (negative for *Salmonella*) were bought from a hatchery in Xintai, China. Chickens were housed in metal cages and provided *ad libitum* with water and commercial starter diet in the animal room of Shandong Agricultural University. The temperature was maintained at 30°C at the first 3 days and gradually reduced to 28°C during the last days of the experiment. The nutrient levels of the basal diets met the nutritional requirement of the broilers (NRC, 1994) (**Table 1**), and the rearing duration lasted 2 weeks. The sanitation of raising environments were regularly cleaned for the health of chicken. Chickens were divided into three treatment groups in random manner: an negative control (NC) group, chickens were orally administrated 0.2 mL sterile saline per chick once every day through day 1 to day 7; a positive control (PC) group, chickens were challenged with 0.2 mL SE enrichment solution (10^6 CFU/0.2 mL) at the 8 day, and were given sterile saline (0.2 mL/chick) during day 1 to day 7; an experimental (EXP) group, chickens were given 0.2 mL *C. butyricum* enrichment solution (10^6 CFU/0.2 mL) once every day from 1 to 7 day, and at the 8 day, chickens were challenged with 0.2 mL SE enrichment solution (10^6 CFU/0.2 mL). For all groups, chickens were euthanized via cervical dislocation, and livers, spleens, as well as cecal tissues and contents were sampled at 2 and 6 days of post-infection. These samples were frozen at −80°C for further analyses.

SE Translocation

SE translocation to livers, spleens, and cecal contents of all SE-challenged groups was determined at 2 and 6 days of post-infection. Livers, spleens, and cecal contents were weighted, homogenized respectively and serially diluted 10-fold with sterile

TABLE 1 | The composition and nutrients of basal diet.

Ingredient	Content (%)	Chemical composition	Content
Corn	55.23	CP, %	20.90
Soybean meal	30.67	ME, Mcal/kg	3.00
Wheat shorts	4.00	Calcium, %	1.00
Fish meal[a]	3.00	Total P, %	0.65
Soybean oil[b]	2.90	Available P, %	0.45
DL-methionine	0.27	Methionine + cysteine, %	0.90
NaCl	0.27	Lysine, %	1.05
Limestone	1.33		
Calcium phosphate	1.33		
Vitamin–mineral premix[c]	1.00		

[a]*Crude protein content is 62.5% and metabolizable energy is 2.79 Mcal/kg.* [b]*Metabolizable energy is 8.8 Mcal/kg.* [c]*Supplied per kilogram of diet: vitamin A (retinyl acetate), 1,500 IU; cholecalciferol, 200 IU; vitamin E (DL-α-tocopheryl acetate), 10 IU; riboflavin, 3.5 mg; pantothenic acid, 10 mg; niacin, 30 mg; cobalamin, 10 μg; choline chloride, 1,000 mg; biotin, 0.15 mg; folic acid, 0.5 mg; thiamine 1.5 mg; pyridoxine 3.0 mg; Fe, 80 mg; Zn, 40 mg; Mn, 60 mg; I, 0.18 mg; Cu, 8 mg; Se, 0.15 mg.*

phosphate-buffered saline (1:10, w/v), and then screened on Brilliant Green Agar plates (Hopebio, Qingdao, China) to count the CFU of SE after incubation at 37°C for 24 h.

Quantitative Real-time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (Q-PCR) was undertaken to relatively quantify the expression levels of cytokine genes including IFN-γ, IL-1β, IL-8, and TNF-α, and the gene expressions of the MyD88-dependent pathway of TLR4, MyD88, and NF-κB in the livers, spleens, and cecal tissues. At 2 and 6 days of post-infection, Trizol reagent (Invitrogen) was used to extract total RNA from livers, spleens, and cecal tissues according to the manufacturer's instruction. Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, MA, United States) was used to determine the concentration and quality of total RNA. SuperScript III First Strand synthesis kit (Life Technologies, Carlsbad, CA, United States) was used to synthesize cDNA with 2 μg of total RNA. The cDNA was stored at −20°C. The Q-PCR was performed with SYBR Green master mix using 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, United States). PCR conditions contained one cycle of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Dissociation analysis of amplification products was performed at the end of each PCR to confirm the specificity of amplicon. The primers for real-time PCR are listed in **Table 2**. mRNA relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The one-way ANOVA and Student's *t*-test of SPSS 15.0 (SPSS Inc., Chicago, IL, United States) were used to perform statistical analyses. The results were shown as mean ± standard deviations (SD). Differences were considered significant at $P < 0.05$.

TABLE 2 | Primers for Q-PCR in this study.

Gene	Sequence (5′–3′)	GenBank No.
TLR4	Forward: AGTCTGAAATTGCTGAGCTCAAAT Reverse: GCGACGTTAAGCCATGGAAG	AY064697
MyD88	Forward: TGATGCCTTCATCTGCTACTG Reverse: TCCCTCCGACACCTTCTTTCTA	EF011109
NF-κB	Forward: CAGCCCATCTATGACAACCG Reverse: TCCCTGCGTCTCCTCTGTGA	NM_205129
IFN-γ	Forward: ATCATACTGAGCCAGATTGTTTC Reverse: ATCATACTGAGCCAGATTGTTTC	NM_205149.1
IL-1β	Forward: GTGAGGCTCAACATTGCGCTGTA Reverse: TGTCCAGGCGGTAGAAGATGAAG	Y15006
IL-8	Forward: ATGAACGGCAAGCTTGGAGCTG Reverse: TCCAAGCACACCTCTCTTCCATCC	AJ009800
TNF-α	Forward: TGCTGTTCTATGACCGCC Reverse: CTTTCAGAGCATCAACGCA	AY765397
β-Actin	Forward: GAGAAATTGTGCGTGACATCAy Reverse: CCTGAACCTCTCATTGCCA	L08165

RESULTS

SE Translocation

The results of SE translocation showed that after 2 and 6 days post-infection, chickens in EXP group significantly reduced the viable count of SE compared to the PC group in the liver, spleen, and cecal content ($P < 0.05$), except in the spleen at 2 day post-infection ($P > 0.05$). In addition, SE was not detected in NC group (**Table 3**).

Gene Expression of Cytokines in the Liver

At 2-day post-infection, gene expression for pro-inflammatory cytokine TNF-α was significantly elevated in PC group compared to NC and EXP groups ($P < 0.05$), but no significant difference was found between NC and EXP groups ($P > 0.05$); with regard to IFN-γ, IL-1β, and IL-8 production, no significant difference was observed among EXP, PC, and NC groups ($P > 0.05$). At 6-day post-infection in the PC group, the gene expressions of IFN-γ, IL-1β, and TNF-α were elevated significantly ($P < 0.05$) compared to NC and EXP groups, but no difference was found between NC and EXP groups ($P > 0.05$); in terms of IL-8, no significant differences were found among EXP, NC, and PC groups ($P > 0.05$) (**Table 4**).

Gene Expression of Cytokines in the Spleen

At 2-day post-infection, gene expression for IFN-γ was significantly increased in PC group compared to NC and EXP groups ($P < 0.05$), but no significant difference was found between NC and EXP groups ($P > 0.05$); in addition, no significant differences were observed in the IL-1β and TNF-α productions among EXP, PC, and NC groups ($P > 0.05$); the expression of IL-8 was significantly mounted in the PC group compared to EXP group ($P < 0.05$), but no significant difference was observed between EXP and NC groups ($P > 0.05$), and the same change was found between PC and NC groups ($P > 0.05$). At 6-day post-infection, gene expressions of IFN-γ, IL-1β, and IL-8 were elevated significantly in the PC group compared to NC and EXP groups ($P < 0.05$), but no significant difference was found between NC and EXP groups ($P > 0.05$); additionally, no significant difference in the TNF-α production was found among EXP, NC, and PC groups ($P > 0.05$) (**Table 5**).

Gene Expression of Cytokines in the Cecal Tissues

At 2-day post-infection, gene expression for IL-1β was significantly elevated in PC group compared to NC and EXP groups ($P < 0.05$), but no significant difference was found between NC and EXP groups ($P > 0.05$); with regard to IFN-γ, IL-8, and TNF-α production, no significant difference was observed among EXP, PC, and NC groups ($P > 0.05$). At 6-day post-infection, gene expressions of IFN-γ, IL-1β, and IL-8 were elevated significantly in PC group compared to NC and EXP

TABLE 3 | Effect of *C. butyricum* on the reduction of SE counts in livers, spleens, and cecal contents of broilers[1].

	Liver		Spleen		Cecal content	
	Log CFU/organ		Log CFU/organ		Log CFU/organ	
Item	2 d post-ch[2]	6 d post-ch	2 d post-ch	6 d post-ch	2 d post-ch	6 d post-ch
PC	1.78 ± 0.29^{a}	1.72 ± 0.44^{a}	0.72 ± 0.47	0.63 ± 0.32^{a}	5.82 ± 0.86^{a}	5.48 ± 0.71^{a}
EXP	ND[b]	ND[b]	0.42 ± 0.20	ND[b]	0.86 ± 0.12^{b}	0.66 ± 0.12^{b}

Mean ± SD in the same line with different superscript letters differ significantly ($P < 0.05$). [1]Each mean represents six birds. PC, birds fed a basal diet and challenged with SE; EXP, birds fed a basal diet with C. butyricum (10^6 CFU/mL) and challenged with SE. Results show the colony counts of SE in different organs, they are expressed as mean (Log_{10} CFU/g of organ) ± SD. [2]The days after challenging.

TABLE 4 | Fold changes of cytokine gene expression in the livers of broilers after challenged with SE[1].

Gene	Age of post-ch[2]	Experimental treats		
		NC	PC	EXP
IFN-γ	2 d	0.001 ± 0.0001	0.001 ± 0.0001	0.0007 ± 0.0001
	6 d	0.60 ± 0.15^{b}	1.36 ± 0.16^{a}	0.49 ± 0.04^{b}
IL-1β	2 d	0.64 ± 0.19	0.71 ± 0.13	0.89 ± 0.18
	6 d	0.55 ± 0.05^{b}	1.09 ± 0.12^{a}	0.31 ± 0.03^{b}
IL-8	2 d	0.34 ± 0.06	0.78 ± 0.11	0.80 ± 0.21
	6 d	0.59 ± 0.19	1.39 ± 0.21	0.76 ± 0.28
TNF-α	2 d	0.56 ± 0.06^{b}	0.92 ± 0.18^{a}	0.30 ± 0.14^{b}
	6 d	0.83 ± 0.06^{b}	2.51 ± 0.32^{a}	1.00 ± 0.08^{b}

Mean ± SD in the same row with different superscript letters differ significantly ($P < 0.05$). [1]Each mean represents six birds. NC, birds fed a basal diet without challenged with SE; PC, birds fed a basal diet and challenged with SE; EXP, birds fed a basal diet with C. butyricum (10^6 CFU/mL) and challenged with SE. [2]The days after challenging.

TABLE 5 | Fold changes of cytokine gene expression in the spleens of broilers after challenged with SE[1].

Gene	Age of post-ch[2]	Experimental treats		
		NC	PC	EXP
IFN-γ	2 d	1.00 ± 0.35^{b}	2.30 ± 0.19^{a}	1.29 ± 0.12^{b}
	6 d	0.39 ± 0.03^{b}	1.31 ± 0.22^{a}	0.38 ± 0.01^{b}
IL-1β	2 d	0.99 ± 0.15	2.19 ± 0.36	1.17 ± 0.34
	6 d	0.63 ± 0.14^{b}	1.64 ± 0.22^{a}	0.32 ± 0.08^{b}
IL-8	2 d	0.58 ± 0.22^{ab}	1.81 ± 0.28^{a}	0.30 ± 0.18^{b}
	6 d	0.57 ± 0.09^{b}	1.95 ± 0.24^{a}	0.79 ± 0.12^{b}
TNF-α	2 d	0.83 ± 0.11	0.80 ± 0.10	1.37 ± 0.49
	6 d	0.008 ± 0.0008	0.009 ± 0.001	0.007 ± 0.001

Mean ± SD in the same row with different superscript letters differ significantly ($P < 0.05$). [1]Each mean represents six birds. NC, birds fed a basal diet without challenged with SE; PC, birds fed a basal diet and challenged with SE; EXP, birds fed a basal diet with C. butyricum (10^6 CFU/mL) and challenged with SE. [2]The days after challenging.

groups ($P < 0.05$), but no significant difference was found between NC and EXP groups ($P > 0.05$); of note, no significant difference in the TNF-α was found among EXP, NC, and PC groups ($P > 0.05$) (**Table 6**).

TABLE 6 | Fold changes of cytokine gene expression in the cecal tissues of broilers after challenged with SE[1].

Gene	Age of post-ch[2]	Experimental treats		
		NC	PC	EXP
IFN-γ	2 d	0.62 ± 0.11	1.33 ± 0.17	0.70 ± 0.22
	6 d	0.49 ± 0.07^{b}	1.37 ± 0.22^{a}	0.56 ± 0.15^{b}
IL-1β	2 d	0.80 ± 0.12^{b}	1.21 ± 0.14^{a}	0.48 ± 0.11^{b}
	6 d	0.66 ± 0.17^{b}	1.29 ± 0.16^{a}	0.54 ± 0.12^{b}
IL-8	2 d	0.67 ± 0.04	0.44 ± 0.10	0.34 ± 0.09
	6 d	0.66 ± 0.15^{b}	1.41 ± 0.16^{a}	0.50 ± 0.06^{b}
TNF-α	2 d	1.00 ± 0.08	0.75 ± 0.17	0.89 ± 0.18
	6 d	0.93 ± 0.08	0.76 ± 0.17	0.89 ± 0.18

Mean ± SD in the same row with different superscript letters differ significantly ($P < 0.05$). [1]Each mean represents six birds. NC, birds fed a basal diet without challenged with SE; PC, birds fed a basal diet and challenged with SE; EXP, birds fed a basal diet with C. butyricum (10^6 CFU/mL) and challenged with SE. [2]The days after challenging.

Expression of Genes of the MyD88-Dependent Pathway in Liver, Spleen, and Cecal Tissues

At 2-day post-infection, no significant difference was observed between EXP and PC groups with regard to the production of TLR4, MyD88, and NF-κB in liver, spleen, and cecal tissues ($P > 0.05$). However, at 6-day post-infection, gene expressions for TLR4, MyD88, and NF-κB in liver, spleen, and cecal tissues were elevated significantly ($P < 0.05$) in the PC group compared to NC and EXP groups ($P < 0.05$), but no significant differences were found between EXP and NC groups ($P > 0.05$) (**Tables 7–9**).

DISCUSSION

In the present study, compared with the PC group, the levels of SE recovered from liver, spleen, and cecal contents were reduced in 1-day-old chickens fed *C. butyricum* for seven consecutive days, which was in agreement with previous reports (Berndt et al., 2007; Tanedjeu et al., 2016). However, the results were different from another study which indicated that the *Salmonella* burden in cecal contents was not affected by probiotic treatments while *Salmonella* infections in liver and spleen were reduced (Yang et al., 2014). The differences may be associated with the types

TABLE 7 | Expression of genes of the MyD88-dependent pathway in livers[1].

Gene	Age of post-ch[2]	Experimental treats		
		NC	PC	EXP
TLR4	2 d	0.78 ± 0.27	1.21 ± 0.25	0.99 ± 0.24
	6 d	0.81 ± 0.23[b]	2.48 ± 0.38[a]	1.35 ± 0.28[b]
MyD88	2 d	0.66 ± 0.09	1.01 ± 0.07	0.63 ± 0.03
	6 d	0.81 ± 0.19[b]	1.65 ± 0.22[a]	0.70 ± 0.10[b]
NF-κB	2 d	0.41 ± 0.04	0.62 ± 0.10	0.57 ± 0.10
	6 d	0.57 ± 0.06[b]	1.18 ± 0.11[a]	0.41 ± 0.01[b]

Mean ± SD in the same row with different superscript letters differ significantly ($P < 0.05$). [1]Each mean represents six birds. NC, birds fed a basal diet without challenged with SE; PC, birds fed a basal diet and challenged with SE; EXP, birds fed a basal diet with C. butyricum (10^6 CFU/mL) and challenged with SE. [2]The days after challenging.

TABLE 8 | Expression of genes of the MyD88-dependent pathway in spleens[1].

Gene	Age of post-ch[2]	Experimental treats		
		NC	PC	EXP
TLR4	2 d	0.75 ± 0.08	0.85 ± 0.05	0.93 ± 0.09
	6 d	0.78 ± 0.18[b]	1.59 ± 0.17[a]	0.66 ± 0.09[b]
MyD88	2 d	1.01 ± 0.07	1.05 ± 0.07	0.91 ± 0.04
	6 d	0.80 ± 0.05[b]	1.46 ± 0.15[a]	0.81 ± 0.07[b]
NF-κB	2 d	1.00 ± 0.67	0.91 ± 0.53	0.84 ± 0.55
	6 d	0.65 ± 0.07[b]	1.54 ± 0.15[a]	0.62 ± 0.13[b]

Mean ± SD in the same row with different superscript letters differ significantly ($P < 0.05$). [1]Each mean represents six birds. NC, birds fed a basal diet without challenged with SE; PC, birds fed a basal diet and challenged with SE; EXP, birds fed a basal diet with C. butyricum (10^6 CFU/mL) and challenged with SE. [2]The days after challenging.

of probiotics used, breed and age of chickens, as well as rearing environments.

IFN-γ is a Th1 cytokine that stimulates macrophages to secret oxidants with antimicrobial activities and is produced by natural killer cells and T-lymphocytes (Alam et al., 2002). In this study, *C. butyricum* significantly decreased SE-induced IFN-γ expression level, which was similar to the report that pretreatment of 1-day-old chickens with probiotics could significantly reduce IFN-γ expression level in *Salmonella* infection period (Chen et al., 2012).

IL-1β is a major mediator of inflammation in birds and mammals, primarily produced by monocytes, tissue macrophages, and enterocytes (Bar-Shira and Friedman, 2006). In this study, *C. butyricum* significantly decreased SE-induced IL-1β expression level, which was consistent with a previous report which showed that treating *Salmonella*-infected chicks with *Lactobacillus* strains could significantly down-modulate the expression level of IL-1β (Chen et al., 2012).

IL-8, as an important member of the chemokines, has chemotactic activity and shares similar structure to cytokines (Baggiolini et al., 1997). The results in this study showed that *C. butyricum* could significantly reduce mRNA level of IL-8, which was also observed in a previous report (Yi et al., 2016).

TABLE 9 | Expression of genes of the MyD88-dependent pathway in cecal tissues[1].

Gene	Age of post-ch[2]	Experimental treats		
		NC	PC	EXP
TLR4	2 d	0.72 ± 0.12	0.86 ± 0.15	0.75 ± 0.14
	6 d	0.93 ± 0.05[b]	2.15 ± 0.23[a]	0.81 ± 0.11[b]
MyD88	2 d	0.52 ± 0.08[b]	0.92 ± 0.06[a]	0.91 ± 0.07[a]
	6 d	0.95 ± 0.05[b]	1.59 ± 0.15[a]	0.46 ± 0.15[b]
NF-κB	2 d	0.92 ± 0.26	1.22 ± 0.82	0.97 ± 0.34
	6 d	0.83 ± 0.07[b]	1.61 ± 0.13[a]	0.74 ± 0.15[b]

Mean ± SD in the same row with different superscript letters differ significantly ($P < 0.05$). [1]Each mean represents six birds. NC, birds fed a basal diet without challenged with SE; PC, birds fed a basal diet and challenged with SE; EXP, birds fed a basal diet with C. butyricum (10^6 CFU/mL) and challenged with SE. [2]The days after challenging.

LPS-induced TNF-α factor, as one kind of vital indicator for evaluating inflammatory response in chickens, can produce the inflammatory response in chickens when infected with pathogens (Feng et al., 2016). In the present study, *C. butyricum* significantly decreased SE-induced the mRNA level of TNF-α in the liver, which was consistent with the report that *Lactobacillus rhamnosus* may decrease *Escherichia coli*-induced TNF-α expression level (Liu et al., 2016).

TLR4 plays an essential role in the innate immune response and hence is likely to be involved in young chickens at risk of *Salmonella* infection (Li et al., 2010). In the study, *C. butyricum* suppressed inflammation by down-regulating TLR4, MyD88, and NF-κB-dependent pathways in chickens with SE infection on day 6 post-infection, which is consistent with the report that indicated that probiotics can decrease pro-inflammatory cytokine levels by inhibiting the expression of TLR4, MyD88, and NF-κB-dependent pathways in LPS-induced macrophages and in mice (Song et al., 2015; Yi et al., 2015).

Although there was a limitation in this study (the 2-week rearing period of SE infection experiment was relatively short), these findings indicated that *C. butyricum* can decrease SE infection by down-regulating cytokine gene expression, and can inhibit inflammation by down-regulating TLR4, MyD88, and NF-κB-dependent pathways. Collectively, *C. butyricum* could be a potential probiotics against SE infection in broiler chickens.

AUTHOR CONTRIBUTIONS

SS, HL, and XZ designed the work. XZ, JY, and LW raised animals. XZ and JY collected samples. XZ analyzed and interpreted data. XZ drafted the article. SS and HL critically reviewed the article.

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Probiotic *Enterococcus mundtii* Isolate Protects the Model Insect *Tribolium castaneum* against *Bacillus thuringiensis*

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Enterococcus mundtii strains isolated from the larval feces of the Mediterranean flour moth *Ephestia kuehniella* show antimicrobial activity against a broad spectrum of Gram-positive and Gram-negative bacteria. The *in vitro* probiotic characterization of one isolate revealed a high auto-aggregation score, a hydrophilic cell surface, tolerance for low pH, no hemolytic activity, and susceptibility to all tested antibiotics. We used the red flour beetle *Tribolium castaneum*, an established model organism, for the *in vivo* characterization of one probiotic *E. mundtii* isolate from *E. kuehniella* larvae. *Tribolium castaneum* larvae were fed orally with the probiotic isolate or the corresponding supernatant and then infected with either the entomopathogen *Bacillus thuringiensis* or *Pseudomonas entomophila*. Larvae exposed to the isolate or the supernatant showed increased survival following infection with *B. thuringiensis* but not *P. entomophila*. Heat treatment or treatment with proteinase K reduced the probiotic effect of the supernatant. However, the increased resistance attracts a fitness penalty manifested as a shorter lifespan and reduced fertility. *T. castaneum* has, pending on further research, the potential as an alternative model for the pre-screening of probiotics.

Keywords: ***Tribolium castaneum*, probiotics, *Enterococcus mundtii*, *in vivo* model, antimicrobial, *Bacillus thuringiensis***

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INTRODUCTION

All animals are associated with a diverse microbial community that promotes their health (McFall-Ngai et al., 2013; Sommer and Bäckhed, 2013). Any disruption to the population of gut microbiota caused by antibiotics or immune system deficiency therefore reduces the fitness of the host (Lozupone et al., 2012; Modi et al., 2014) whereas the administration of probiotic bacteria can increase fitness (Buffie and Pamer, 2013). Probiotics are defined as "live microorganisms, that when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014).

Probiotics have many applications, including the optimization of growth and survival in animal species used for aquaculture and agriculture (Chaucheyras-Durand and Durand, 2009; Pandiyan et al., 2013), and the prevention or treatment of gastrointestinal tract infections in humans (Deshpande et al., 2010; Kotzampassi and Giamarellos-Bourboulis, 2012). Probiotics have several mechanisms of action, including the production of antimicrobial compounds, the inhibition of virulence genes, the enhancing of epithelial barrier functions or the stimulation of the host

immune system (Oelschlaeger, 2010). The best-studied microorganisms with probiotic activity are the bifidobacteria, lactobacilli, enterococci and yeasts (Varankovich et al., 2015). The genus *Enterococcus* (order lactobacillales) is a controversial group that contains both probiotic strains (Hosseini et al., 2009; Strompfová and Lauková, 2009; Al Atya et al., 2015) and pathogenic strains (Higashide et al., 2005; Martin et al., 2005; Gaspar et al., 2009). Enterococci produce organic acids, hydrogen peroxide and up to four different classes of enterocins (Franz et al., 2007), supporting the call for a legislative framework for probiotics (Papadimitriou et al., 2015).

The adult gut microbiota of mammals is essential for the development of the immune system in the offspring (Round and Mazmanian, 2009; Gomez de Aguero et al., 2016). Animals have evolved different ways to transfer beneficial microbes to their offspring, e.g., female mammals can transfer their own beneficial microbes through milk during lactation (Jost et al., 2014). Birds can transfer their microbes via the eggshell or regurgitation (Godoy-Vitorino et al., 2010; Ruiz-De-Castañeda et al., 2011; Kohl, 2012), and insects can transfer beneficial microbes by trophallaxis or coprophagy (Koch and Schmid-Hempel, 2011; Engel and Moran, 2013; Brune and Dietrich, 2015). Insect feces are not only relevant for microbial transmission but they also fulfill a protective function (Rosengaus et al., 2013; Diehl et al., 2015). This is particularly relevant in the case of storage pests, which defecate and live in the same environment.

In vitro assays for the screening of probiotic microorganisms typically test for antimicrobial activity, microbial colonization, and safety. *In vivo* assays are also recommended, because probiotics can negatively affect certain host species, as observed in honeybees (Ptaszyńska et al., 2015) and in humans (Besselink et al., 2008). The regulatory framework governing probiotics varies in different countries, making it difficult to find standardized methods (Baldi and Arora, 2015). *Caenorhabditis elegans*, *Drosophila melanogaster,* and *Galleria mellonella* are currently used for the preclinical screening of probiotics, because they are suitable for large-scale screening therefore reduce costs compared to mammalian models (Papadimitriou et al., 2015; Vilela et al., 2015). Invertebrates are also more suitable for ethical reasons and with respect to the 3Rs strategy (Russell and Burch, 1959). *Tribolium castaneum* is a well-established insect model organism, which is easy and inexpensive to rear in the laboratory. *T. castaneum* is currently used as a model for infection, for transgenerational effects and for the screening of drugs (Zou et al., 2007; Roth et al., 2010; Milutinović et al., 2014; Bingsohn et al., 2016) but not thus far for the screening of probiotics. This model insect is well-suited for screening based on functional genomics because it benefits from a sequenced genome (Tribolium Genome Sequencing Consortium et al., 2008), well-established RNA interference (RNAi) techniques (Bucher et al., 2002; Knorr et al., 2013), a recently established CRISPER/Cas system for gene knockout (Gilles et al., 2015), and the availability of transcriptome datasets generated under various conditions (Park et al., 2008; Altincicek et al., 2013; Dippel et al., 2014). It is also possible to generate axenic strains, which are useful for the analysis of probiotic microbes in isolation (Futo et al., 2016).

Here, we report the *in vitro* probiotic characterization of *Enterococcus mundtii* isolates, sourced from the feces of the Mediterranean flour moth *Ephestia kuehniella*, a common storage pest. We also investigated the *in vivo* protective role of one *E. mundtii* isolate and the corresponding supernatant by oral administration to *T. castaneum* before challenging the beetles with different entomopathogenic bacteria. We conclude that *T. castaneum* is suitable as a high-throughput screening platform for the *in vivo* testing of potential probiotics.

MATERIALS AND METHODS

Insect Rearing

Ephestia kuehniella larvae (provided by the Julius Kühn-Institut, Berlin, Germany) were kept in glass jars at room temperature in darkness, and were fed on wheat grains (Alnatura, Bickenbach, Germany). *T. castaneum* Cro1 beetles collected in 2010 (Milutinović et al., 2013) were maintained on a heat-sterilized standard diet of wheat flour (type 550, Alnatura, Bickenbach, Germany) and 5% brewer's yeast at 32°C and 70% humidity in darkness.

Bacterial Isolation and Identification

Bacteria were isolated from the feces of *E. kuehniella* larvae. Feces were plated with a spatula tip onto casein soya agar and incubated for 48 h at 30°C. Randomly chosen colonies were re-streaked on de Man, Rogosa and Sharpe (MRS) agar to obtain pure isolates. Isolated strains were used to prepare glycerol stocks at −20°C. All isolates were identified as strains of *E. mundtii* based on 16S rDNA analysis. Bacterial DNA was amplified using primers p8FPL 5′-AGT TTG ATC CTG GCT CAG-3′ and p806R 5′-GGA CTA CCA GGG TAT CTA AT-3′ (Relman et al., 1992) yielding a product of ∼800 bp. The following PCR program was used: 94°C/7 min; 35 cycles at 94°C/60 s, 55°C/60 s, 72°C/60 s; and a final extension step at 72°C/10 min. PCR products were separated by 1% agarose gel electrophoresis and stained with SYBR Safe (Thermo Fisher Scientific, Waltham, MA, United States). Prior to sequencing (Macrogen Europe, Amsterdam, Netherlands), the PCR products were purified using a DNA purification kit (Macherey-Nagel, Düren, Germany). Sequences were deposited at GenBank (Supplementary Table S1). A bacterial phylogenetic tree was created using the neighbor-joining method (Saitou and Nei, 1987) by aligning the 16S rDNA sequences against known sequences in the NCBI database using MAFFT v7 (Katoh and Standley, 2013).

Antimicrobial Characterization

The *E. mundtii* isolates were first screened using the agar spot on lawn technique (Schillinger and Lücke, 1989) with the following modifications for antimicrobial activity. Overnight cultures of the isolates grown in MRS medium at 30°C were spotted (7 μl) onto 0.7% MRS agar plates and incubated at 30°C for 24 h under aerobic conditions. The indicator bacteria (**Table 1**) comprised an overnight culture mixed with 1% lysogeny broth (LB) agar at a final concentration of 1×10^5 cells ml^{-1}. Previously spotted isolates were overlaid with 10 ml of the indicator bacteria.

TABLE 1 | Agar spot on lawn test with *E. mundtii* isolates against indicator bacteria.

	Isolated *E. mundtii* strains														
Indicator strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Bacillus thuringiensis (DSM 2046)	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Escherichia coli (strain D 31)	+	+	+	+	+	++	+	+	+	+	+	+	+	+	+
Klebsiella terrigena (DSM 2687)	+	++	++	++	++	+	+	+	+	+	+	+	++	+	++
Listeria grayi (DSM 20601)	+	+	+	−	+	+	−	−	+	+	−	−	−	+	+
Listeria innocua (DSM 20649)	+	+	+	−	−	+	−	−	−	+	−	−	+	+	+
Listeria seeligeri (DSM 20751)	+	−	−	+	+		−	−	−	+	−	−	+	+	+
Micrococcus luteus (DSM 20030)	+	++	++	++	++	+	++	++	+	++	++	++	++	++	++
Pseudomonas aeruginosa (DSM 50071)	+	+	−	+	−	−	+	−	−	++	−	+	−	+	+
Pseudomonas entomophila (DSM 28517)	+	++	++	+	+	+	+	+	+	+	+	+	++	+	++
Salmonella subterranean (DSM 1620)	+	−	+	+	+	+	−	−	−	+	−	−	+	+	+
Staphylococcus aureus (DSM 2569)	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+

Positive inhibition was scored if the radius of the zone of inhibition around the colonies of the isolates was 3–5.99 mm (++) or 1– 2.99 mm (+) and negative inhibition (–) was scored if the zone was 0–0.99 mm. Scores are based on three replicates. The DSM number refers to strains obtained from the DSMZ strain collection, Braunschweig, Germany.

After complete solidification of the upper layer, the plates were incubated for an additional 16 h at 30°C under aerobic conditions. Inhibition was scored positive if the radius of the zone of inhibition around the colonies of the isolates was 1 mm or larger. We carried out three replicates per isolate.

Inhibitory substances from the isolates were further characterized by the agar well-diffusion assay (AWDA) (Tagg and McGiven, 1971) with the following modifications. *E. mundtii* isolates were grown under aerobic conditions for 24 h at 30°C in MRS medium. The cell free supernatant (CFS) was obtained by centrifugation (3200 × *g*, 4°C, 15 min), adjusted to pH 6.5 and passed through a 0.22-μm filter (Carl Roth, Karlsruhe, Germany). Afterward the CFS was concentrated 100-fold, mixed with ethyl acetate (1:1), and shaken vigorously for 1 h. The mixture was stabilized for a further 1 h and then the aqueous phase was removed. The solvent was concentrated under reduced pressure in a rotational evaporator (Büchi Labortechnik AG, Switzerland). To reduce the effect of proteinaceous compounds, 2 ml concentrated CFS was mixed with 80 μl 25 mg ml^{-1} proteinase K (Sigma-Aldrich, Taufkirchen, Germany) for 1 h at 37°C. To reduce the effect of H_2O_2, 2 ml concentrated CFS was treated with 40 μl 2 mg ml^{-1} catalase (Sigma) for 1 h at 37°C. Finally, the heat stability of CFS was tested by heating for 10 min at 98°C. For the AWDA, 40 μl of CFS treated in one of the three ways described above was transferred to 5-mm wells in 1% LB agar plates containing 10^5 indicator bacteria ml^{-1}. The plates were incubated at 30°C for 16 h. The zones of inhibition were measured in mm using a digital caliper, with three replicates per isolate. Sterile concentrated MRS medium was used as a negative control.

Probiotic Characterization

Auto-aggregation correlates based on bacterial adhesion to host cells were determined as described (Del Re et al., 2000; Al Kassaa et al., 2014). *E. mundtii* isolates were grown in MRS medium for 16 h at 30°C. Cells were harvested by centrifugation (3200 *g*, 4°C, 15 min) and washed twice with sterile phosphate buffered saline (PBS; pH 7), re-suspended in PBS and adjusted to 10^8 cells ml^{-1}. The cell suspension (4 ml) was mixed by vortexing and incubated at room temperature for 24 h. After 5 and 24 h, 100 μl of the upper phase was removed and the absorbance at 600 nm was measured. The percentage of auto-aggregation was calculated as follows:

$$\%\,\text{autoaggregation} = [\frac{(OD_1 - OD_0)}{OD_1}] \times 100 \quad (1)$$

where OD_1 is the optical density at 5 or 24 h and OD_0 is the optical density at time point zero. This experiment was performed in triplicate.

To detect bacterial adhesion to solvents, a hydrophobicity assay was carried out as previously described (Rosenberg et al., 1980; Al Kassaa et al., 2014). The bacterial suspension was prepared as described for the auto-aggregation assay, and 3 ml of the suspension was mixed with 1 ml xylene (Carl Roth) by vortexing for 2 min. The aqueous phase was removed after incubating the mixture for 10 min at room temperature, and after incubation for a further 2 h at room temperature the absorbance was measured at 600 nm. The percentage of hydrophobicity was calculated as follows:

$$\%\,\text{hydrophobicity} = [\frac{(OD_1 - OD_0)}{OD_1}] \times 100 \quad (2)$$

where OD_1 is the optical density after 2 h and OD_0 is the optical density before adding the xylene. This experiment was performed in triplicate.

Isolate tolerance to low pH was determined as described (Hosseini et al., 2009) and reveals how well the bacteria can survive under harsh gut conditions. An overnight culture was washed twice with PBS and then resuspended in PBS acidified to pH 2, 3, and 4, respectively. After incubating for 3 h, the bacteria were plated on LB agar in order to count the number of colony-forming units (CFUs).

Safety Characterization

Hemolytic activity was determined by spotting 7-µl overnight cultures of *E. mundtii* onto Columbia sheep blood agar plates as a required safety assay (bioMérieux, Marcy-l'Étoile, France). The plates were incubated for 48 h at 30°C. The presence of clear zones around the colonies indicated hemolytic activity (Abriouel et al., 2008). *Staphylococcus aureus* (DSM 2569) was used as a positive control. The experiment was performed in triplicates.

The sensitivity of the isolates to antibiotics was determined by performing a broth microdilution assay in 96-well plates (Wiegand et al., 2008). The following antibiotic concentration ranges (µg ml^{-1}) was used: ampicillin (0.25–128), rifampicin (0.25–128), erythromycin (0.25–128), kanamycin (2–256), ciprofloxacin (0.25–128); fosfomycin (0.25–128), streptomycin (2–256), tetracycline (0.25–128), and vancomycin (0.25–128). Isolates were grown in LB medium at 30°C overnight and the bacterial suspension was adjusted to 10^6 cells ml^{-1}. A 50-µl aliquot of the suspension was then mixed with 50 µl of the antibiotic solution in a microtiter plate well and incubated for 16 h at 30°C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration that inhibits visible growth.

In Vivo Characterization Following Entomopathogenic Challenge

For the survival assay, 15 glass jars containing approximately 100 adult *T. castaneum* (∼1 month old) were transferred to 100 g flour for oviposition. On the third day after oviposition, the eggs were removed by sieving through a 250-µm mesh (Retsch, Haan, Germany). The eggs collected from all jars were mixed, and ∼400 eggs were transferred to the different probiotic diets (see below) each on 150-mm Petri dishes. After 8 days on these six diets, 96 larvae of similar size were transferred individually to the three challenged diets, one per well of a microtiter plate. A total of 1728 larvae were used in this experiment. The plates were sealed with transparent adhesive foil and punctured with small holes for air supply. Survival was monitored daily for 7 days. The experiment was carried out under standard rearing conditions (32°C and 70% humidity).

Diet Preparation

For the preparation of the different probiotic diets, 0.15 g ml^{-1} of flour (type 405, Alnatura, Bickenbach, Germany) supplemented with 5% brewer's yeast was mixed with: (1) sterile MRS medium as a negative control; (2) 5×10^9 cells ml^{-1} of *E. mundtii* 1 isolate, grown in MRS medium at 30°C overnight, washed with PBS twice, centrifuged (3200 × *g*, 4°C, 15 min) and re-suspended in MRS medium; (3) crude CFS from the same isolate; (4) CFS heated at 98°C for 20 min; (5) CFS treated with 4 ml proteinase K (25 mg ml^{-1}) for 1 h at 37°C; or (6) CFS adjusted to pH 7. The diet was poured into Petri dishes either (150-mm diameter = 100 ml or 90 mm diameter = 20 ml), dried at 37°C and shredded.

The immune challenged diet containing *Bacillus thuringiensis* (DSM 2046) was prepared as previously described (Milutinović et al., 2013). We added 0.15 g flour with 5% brewer's yeast to 1 ml of the bacterial suspension, and adjusted the cell density to 5×10^9 cells ml^{-1}. The diet containing *Pseudomonas entomophila* (DSM 28517) was prepared by growing *P. entomophila* overnight at 30°C in LB, centrifuging the suspension (3200 × *g*, 4°C, 15 min) and washing the pellet twice in PBS before re-suspending in PBS. The cell density was adjusted as described for *B. thuringiensis*. PBS mixed with flour and yeast was used as negative control. We transferred 40 µl of the suspension to each well of a 96-well plate and dried the plates at 37°C overnight.

In Vivo Characterization of Fitness Parameters

For the longevity experiment, 10 glass jars containing ∼100 adult beetles (∼1 month old) were transferred to 100 g flour for oviposition. After 24 h, the eggs collected from all jars were mixed and ∼100 eggs were transferred to the MRS, CFS or *E. mundtii* probiotic diets prepared as described above on 90-mm Petri dishes. Five replicates per diet were incubated under standard conditions. Longevity was measured using a thermotolerance assay at 42°C (Grünwald et al., 2013). Twenty-five age-controlled beetles of mixed sex were transferred after 5 days on the standard diet to the same probiotic diet used to rear the larvae. Survival was recorded daily, and dead beetles were removed from the Petri dishes.

The effect of the probiotic diets (MRS, CFS and *E. mundtii*) on the fitness of *T. castaneum* was determined by measuring the reproductive success of the beetles. Age-controlled virgin beetles were obtained as described for the longevity experiment with the additional sexing of the pupae. Five days after eclosion, virgin beetles (five of each sex, each diet with five replicates) were allowed to mate for 24 h on 10 g of flour in Falcon tubes covered with breathable tissue. After 24 h, the eggs were counted (fertility) and transferred to a standard diet followed by incubation for 8 days. Hatched larvae were counted to determine fecundity (Bingsohn et al., 2016).

Statistics

Statistical analysis was carried out using R (v3.3.2, R Development Core Team, 2008). The survival data were evaluated using Kaplan Meir statistics in the 'survival' package (v 2.40.1 Therneau and Grambsch, 2001) and multiple pairwise comparisons among groups were carried out using log-rank tests. *p*-values were adjusted using the 'holm' correction method. The fertility and fecundity data were analyzed by one-way analysis of variance (ANOVA) and the 'holm' correction method.

RESULTS

Identification of Isolates

BLAST analysis of partial 16S rDNA sequences from 15 isolates showed 100% identity to *E. mundtii*. The phylogenetic relationships shown in **Figure 1** confirm that the isolates are strains of *E. mundtii*.

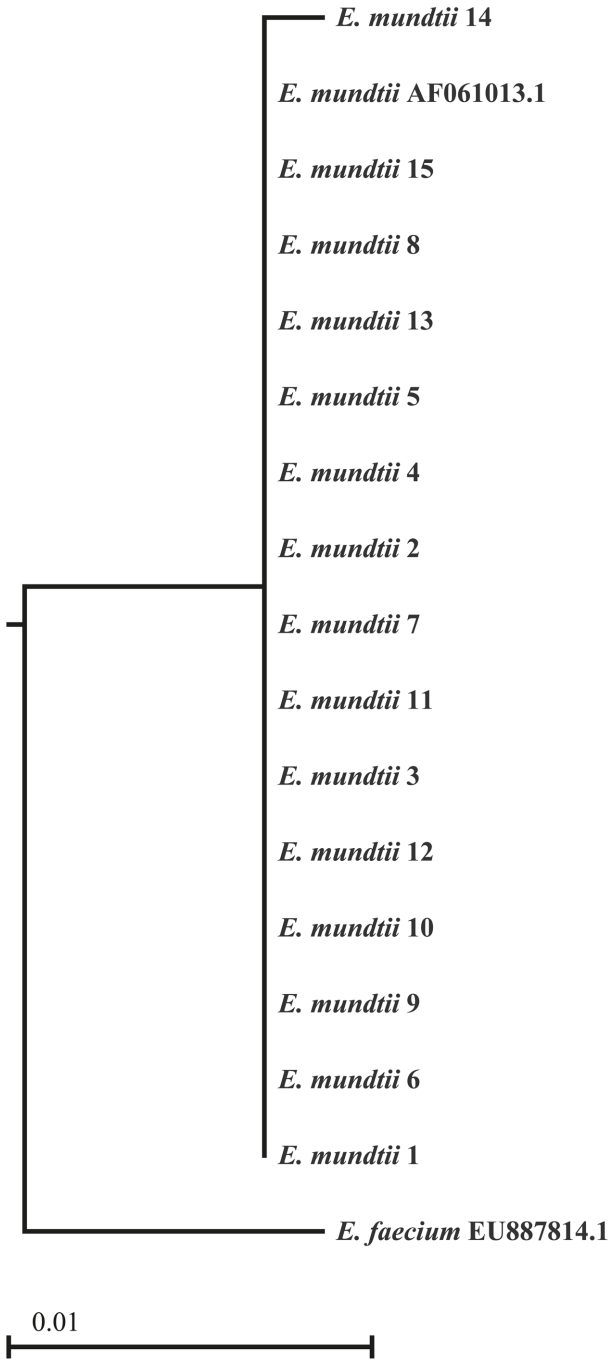

FIGURE 1 | Phylogenetic tree of the 16S rDNA sequences derived from 15 *Enterococcus mundtii* isolates. The sequences from the isolated strains were aligned with two strains identified by their GenBank accession numbers. The scale is based on the bootstrap values from the neighbor-joining method.

Antimicrobial Characterization

An initial screen using the agar spot on lawn method revealed that all 15 isolates showed antimicrobial activity against a broad spectrum of indicator bacteria (**Table 1**). Some isolates also showed activity against entomopathogenic *B. thuringiensis* and *P. entomophila*, as well as human pathogenic bacteria such as *P. aeruginosa* and *S. aureus*. For the further characterization of the antimicrobial compounds, we focused on the CFS of *E. mundtii* isolate 1 using the AWDA (**Table 2**) because this isolate showed the best antimicrobial profile in the agar spot on lawn assay. The crude CFS did not show antimicrobial activity against the indicator bacteria, so we decided to concentrate the CFS by 100-fold. The concentrated CFS was able to inhibit the indicator bacteria to different degrees (**Table 2**). Treatment of the CFS with proteinase K or catalase reduced its antimicrobial activity against some of the indicator bacteria (**Table 2**).

Probiotic Characterization

The cell surface properties of *E. mundtii* 1 isolate are shown in **Table 3**. The isolate showed a high rate of auto-aggregation after 24 h, indicating a significant level of bacterial adhesion to host cells. However, the low level of adhesion to solvents indicated that the surface was hydrophilic. The viability of *E. mundtii* 1 was reduced at pH 2 and slightly reduced at pH 3 (**Table 4**).

Safety Characterization

The microdilution broth assay was used to determine MIC values. *E. mundtii* 1 was susceptible to all the antibiotics listed in **Table 5**. There was no evidence of hemolytic activity on sheep blood agar plated with any of the 15 *E. mundtii* isolates.

In Vivo Characterization

The potential probiotic effect of the *E. mundtii* 1 isolate (and the corresponding supernatant) was tested by measuring the survival of *T. castaneum* when challenged with a pathogen in the presence or absence of the isolate. There were no significant differences in survival when we compared larvae fed on the control diet with or without an earlier exposure to the probiotic diet ($\chi^2 = 5.1$, df $= 5$, $p = 0.399$) (**Figure 2A**) indicating that isolate had no adverse effects on the larvae. In contrast, there was a significant increase in survival rates when we compared larvae fed on the diet containing *B. thuringiensis* with or without an earlier exposure to the probiotic diets ($\chi^2 = 24.8$, df $= 5$, $p < 0.0001$) (**Figure 2B**). The above probiotic effect of the CFS was reduced by treatment with proteinase K or by heating to 98°C. In contrast to the diets spiked with *B. thuringiensis*, there was no significant difference in survival rates when we compared larvae fed on the diet containing *P. entomophila* with or without an earlier exposure to the probiotic diet ($\chi^2 = 4$, df $= 4$, $p = 0.403$) (**Figure 2C**). The precise *p*-values from multiple pairwise comparisons of the Kaplan Meir curves with 'holm' correction are presented in Supplementary Tables S2–S4.

The potential impact of the *E. mundtii* isolate on the longevity of *T. castaneum* was determined using a thermotolerance assay. We observed a significantly shorter lifespan when beetles were reared on diets containing *E. mundtii* ($\chi^2 = 6.4$, df $= 2$, $p = 0.0398$) (**Figure 3**). However, there were no significant differences in longevity among beetles fed on the CFS diet, the MRS control diet or the *E. mundtii* diet (Supplementary Table S5). We also investigated the influence of the probiotic diet on the fitness (fertility and fecundity) of *T. castaneum* and found that

TABLE 2 | Agar well-diffusion assay with CFS from *E. mundtii* isolate 1 with different treatments against indicator bacteria.

Indicator strains	Crude	100 × fold	Proteinase K	Catalase	Heated
Bacillus thuringiensis (DSM 2046)	0	4.75 ± 0.32	4.23 ± 0.09	4.29 ± 0.12	4.58 ± 0.58
Escherichia coli (strain D 31)	0	4.75 ± 0.43	4.81 ± 0.69	4.33 ± 0.27	4.60 ± 0.57
Klebsiella terrigena (DSM 2687)	0	6.3 ± 0.17	6.12 ± 0.08	4.73 ± 1.00	6.14 ± 0.26
Listeria grayi (DSM 20601)	0	2.25 ± 0.53	1.02 ± 0.15	2.43 ± 0.29	2.55 ± 0.06
Listeria innocua (DSM 20649)	0	3.27 ± 0.37	0.56 ± 0.96	2.82 ± 0.13	2.97 ± 0.34
Listeria seeligeri (DSM 20751)	0	2.03 ± 1.19	0.97 ± 0.86	2.71 ± 0.37	2.08 ± 1.05
Micrococcus luteus (DSM 20030)	0	3.92 ± 0.41	3.68 ± 0.69	3.83 ± 0.46	3.69 ± 0.26
Pseudomonas aeruginosa (DSM 50071)	0	4.9 ± 0.22	4.49 ± 0.57	4.07 ± 0.62	4.88 ± 0.39
Pseudomonas entomophila (DSM 28517)	0	3.83 ± 0.42	3.68 ± 0.36	3.62 ± 0.31	3.52 ± 0.38
Salmonella subterranean (DSM 1620)	0	6.72 ± 0.42	6.39 ± 0.31	6.33 ± 0.49	6.48 ± 0.08
Staphylococcus aureus (DSM 2569)	0	0	0	0	0

The radius of the zone of inhibition is measured in mm. Results are mean values of three replicates with standard deviations.

TABLE 3 | Auto-aggregation rate and hydrophobicity of *E. mundtii* 1.

	***E. mundtii* 1**
Auto-aggregation (%) 5 h	12.69 ± 1.28
Auto-aggregation (%) 24 h	89.66 ± 2.83
Hydrophobicity (%)	8.30 ± 9.4

TABLE 4 | Effect of low pH on *E. mundtii* 1.

	***E. mundtii* 1**
CFU start	8.50 ± 0.07
CFU pH 2 after 3 h	4.11 ± 0.04
CFU pH 3 after 3 h	6.49 ± 0.05
CFU pH 4 after 3 h	8.31 ± 0.037

Results are in log CFU ml^{-1} with SD.

TABLE 5 | Antimicrobial susceptibility of *E. mundtii* 1.

Antimicrobial agent	MIC (μg ml^{-1})
Ampicillin	<0.25
Ciprofloxacin	<0.25
Erythromycin	<0.25
Fosfomycin	4
Kanamycin	32
Rifampicin	<0.25
Streptomycin	64
Tetracycline	<0.25
Vancomycin	<0.25

the number of eggs differed significantly in a diet-dependent manner (ANOVA df = 2; $F = 17{,}163$; $p < 0.001$) (Supplementary Figure S1). Multiple pairwise comparisons among the diet groups showed that beetles maintained on the probiotic *E. mundtii* diet laid significantly fewer eggs than beetles on the CFS and control diets ($p < 0.001$). However, there was no significant difference in fecundity among the diet groups (ANOVA df = 2; $F = 0.0327$; $p = 0.968$).

DISCUSSION

We have shown that probiotic bacteria can be isolated from the feces of storage food insects. Based on the analysis of 16S rDNA sequences we isolated several new strains of *E. mundtii*, and characterized their probiotic profiles as well as their broad antimicrobial activity against Gram-positive and Gram-negative bacteria. We also show the potential for *T. castaneum* as a model system for screening of such probiotic bacteria *in vivo*.

The screen for antimicrobial activity revealed a broad spectrum of activity in all 15 isolates we tested. However, we were unable to detect antimicrobial activity in the crude CFS, in contrast to previous studies of *E. mundtii*, which reported a broad spectrum of antimicrobial activity also in the crude CFS (Zendo et al., 2005; Schelegueda et al., 2015). We were able to detect a similar antimicrobial profile in the agar spot on lawn assay and CFS only when the latter was concentrated by 100-fold. *E. mundtii* belongs to the order lactobacillales, whose members are known to produce a variety of heat-stable bacterocins that are sensitive to proteinase K (Franz et al., 2007). However, proteinase K and catalase treatments reduced the antimicrobial activity of only a few of our isolates, and heat treatment had no impact *in vitro*. By adjusting the pH of the extract, we ruled out the possibility that antimicrobial activity was based on organic acids. The major antimicrobial compounds in the isolates are therefore heat stable and resistant to proteinase K, as previously reported (Silva et al., 1987; Ouzari et al., 2008). Further testing is required to identify and characterize the antimicrobial compounds in detail. In many screens for probiotic bacteria, only the crude CFS is tested for antimicrobial activity, which may lead to false negative results given that many genes remain silent under standard laboratory cultivation conditions and the corresponding compounds may not be synthesized (Nett et al., 2009; Seyedsayamdost, 2014; Rutledge and Challis, 2015).

To further characterize the probiotic profile of one of our isolates we investigated the surface properties of *E. mundtii* isolate 1. This isolate showed a high level of auto-aggregation after 24 h and a low level of solvent adhesion. Auto-aggregation is related to the ability of bacterial cells to adhere to epithelial cells and form colonies (Kos et al., 2003), which is a prerequisite

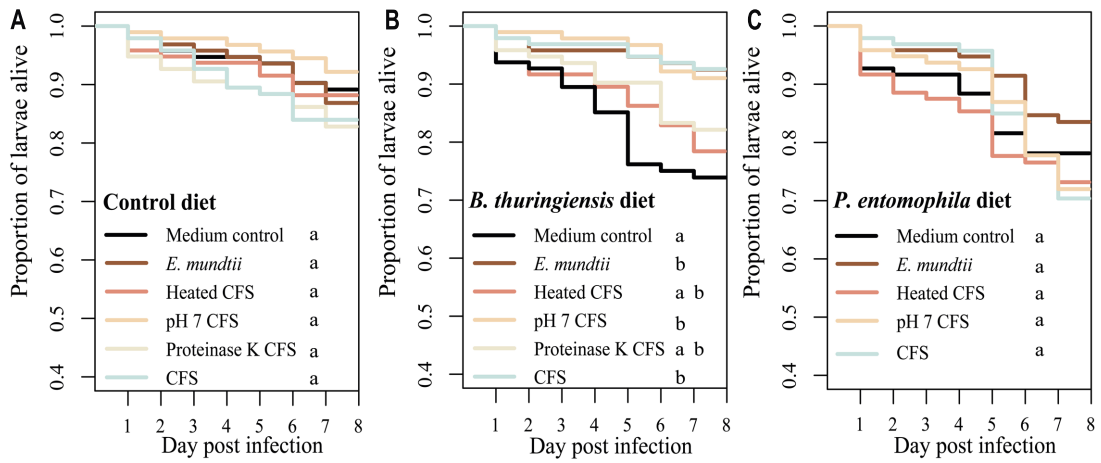

FIGURE 2 | The effect of probiotic diets on *Tribolium castaneum* survival. Survival rates of larvae raised on different probiotic diets based on flour mixed with (i) MRS (control diet), (ii) *E. mundtii*, (iii) CFS pH 7, (iv) CFS treated with proteinase K, (v) CFS heated to 98°C, or (vi) crude CFS. Eight days after exposure to the probiotic diets, larvae (n = 96 per treatment) were challenged with **(A)** the control diet, **(B)** a diet spiked with *Bacillus thuringiensis* diet, or **(C)** a diet spiked with *Pseudomonas entomophila*. Statistically significant differences in the treatments are indicated by differing lowercase letters ($p < 0.05$). Precise p-values for the multiple comparisons are presented in Supplementary Tables S1–S3.

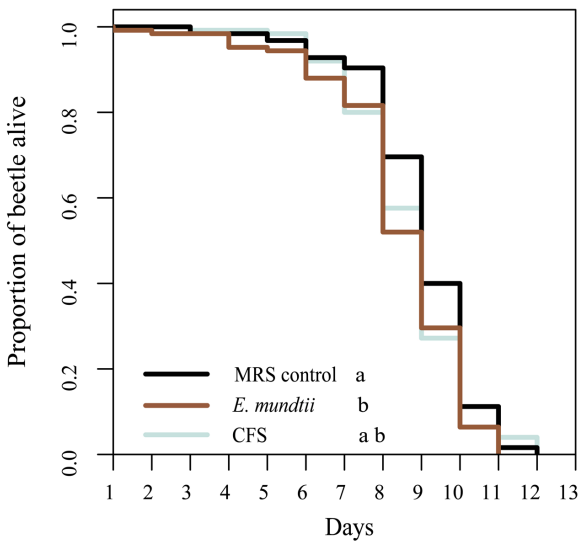

FIGURE 3 | Longevity of *T. castaneum*. Larvae were raised on different probiotic diets, and age controlled adult beetles were maintained on the same diets. Survival was determined by performing a thermotolerance assay (five replicates with 25 beetles of mixed sex). Statistically significant differences between treatments are indicated by differing lowercase letters ($p < 0.05$). Precise p-values for the multiple comparisons are presented in Supplementary Table S4.

for probiotic bacteria because this is how they colonize the gut. The low solvent adhesion indicates the presence of a hydrophilic cell surface based on polysaccharides (Collado et al., 2007, 2008). *E. mundtii* 1 isolate was able to survive in a low-pH environment, making the isolate resistant to the harsh conditions in the digestive system (Conway et al., 1987; Jena et al., 2013). It is also important to evaluate the safety of probiotic bacteria, especially in the genus *Enterococcus*, because the closely related species *E. faecalis* possesses hemolytic activity (De Vuyst et al., 2003). We could not detect hemolytic activity in any of our isolates. There are also concerns that Enterococci may be able to transfer antibiotic resistance genes (Palmer et al., 2010), but *E. mundtii* isolate 1 was susceptible to all the antibiotics we tested.

In vitro assays allow the initial preselection of potentially probiotic strains, but *in vivo* testing is also necessary to check for systemic interactions (Papadimitriou et al., 2015). We introduced the model organism *T. castaneum* as a novel *in vivo* probiotic screening platform. *T. castaneum* is an established model organism that can be used for infection assays and has the capacity for immune priming (Roth et al., 2010; Knorr et al., 2015; Futo et al., 2016). The feeding of *T. castaneum* larvae with either *E. mundtii* 1 or the corresponding CFS protected them against the entomopathogen *B. thuringiensis* but not against *P. entomophila*. A similar result was reported for *C. elegans*, i.e., feeding with Gram-positive probiotics resulted in protection only against Gram-positive pathogens and not against Gram-negative pathogens (Kim and Mylonakis, 2012). The crude CFS showed no *in vitro* antimicrobial activity but nevertheless resulted in a protective function *in vivo*, indicating that the probiotic properties are not based on antimicrobial activity. Both *E. mundtii* and *B. thuringiensis* are Gram-positive bacteria, which should activate the same host immune responses upon contact, in contrast to Gram-negative bacteria as *P. entomophila* (Lemaitre and Hoffmann, 2007; Yokoi et al., 2012). Although the *in vivo* protective effect of the CFS was reduced by heating to 98°C or treatment with proteinase K, the loss of activity was not significant. This may indicate that the protective function of the CFS is based on bacterocins, which are thought to act as signaling peptides for communication with other bacteria or the host immune system (Cotter et al., 2013). The diverse class of bacterocins includes proteinase K-sensitive as well as heat-labile

compounds (Yang et al., 2014). Alternatively, Gram-positive cell wall compounds such as peptidoglycans and lipoteichoic acid are thought to activate the immune system (Leulier et al., 2003; Rao and Yu, 2010). Probiotic bacteria are widely used in aquaculture as food supplements and for the white leg shrimp *Litopenaeus vannamei,* where the supernatant of a probiotic bacterial culture also protects the hosts from pathogens and induces specific immune system genes (Sha et al., 2016). Further research is required to investigate the protective function of the supernatant in more detail, and to determine whether the protective function involves the modulation of the immune system or alters the dynamics of the larval gut microbiota.

Although, our isolate increased host resistance toward an entomopathogen there was a trade-off in terms of beetle fitness. Certain probiotic bacteria have been shown to increase the lifespan of *C. elegans* (Grompone et al., 2012; Park et al., 2015) but our isolate had the opposite effect on *T. castaneum*. Furthermore the fertility of the beetle was also reduced following treatment with the *E. mundtii* isolate. One potential explanation for this phenomenon is the recently observed translocation of bacteria from the *T. castaneum* gut to the eggs, where they elicit an innate immune response that triggers a fitness penalty (Knorr et al., 2015). The potential for such adverse effects highlights the importance of *in vivo* assays for the characterization of probiotic bacteria.

Our results confirm that *T. castaneum* is suitable as an alternative model invertebrate for pre-screening in probiotic research, for human application pending on further confirmation in the mouse model. The short generation time and longevity of *T. castaneum* makes it particularly suitable for long-term studies of probiotics, including potential transgenerational effects and the influence of probiotics on fitness parameters. Here, *T. castaneum* also becomes of interest as test organism with respect to edible insects being closely related to *Tenebrio molitor*, the most promising candidate in mass rearing of insects for feed and food currently (Grau et al., 2017). Our *E. mundtii* isolate showed beneficial probiotic properties *in vitro* and partly also *in vivo*, suggesting that the feces of insect food pests could be a good source for probiotic bacteria in the future.

AUTHOR CONTRIBUTIONS

Planned and conceived the experiments: TG and GJ. Performed the experiments: TG. Analyzed the experiments: TG and GJ. Drafted the manuscript and contributed to the data interpretation: TG, AV, and GJ. All authors read, critically revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Safety Evaluation of a Novel Strain of *Bacteroides fragilis*

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Commensal non-toxigenic *Bacteroides fragilis* confers powerful health benefits to the host, and has recently been identified as a promising probiotic candidate. We previously isolated *B. fragilis* strain ZY-312 and identified it as a novel strain based on 16S rRNA sequencing and morphological analyses. We also determined that ZY-312 displayed desirable probiotic properties, including tolerance to simulated digestive fluid, adherence, and *in vitro* safety. In this study, we aim to investigate whether ZY-312 meets the safety criteria required for probiotic bacteria through comprehensive and systematic evaluation. Consequently, the fatty acid profile, metabolite production, and biochemical activity of strain ZY-312 were found to closely resemble descriptions of *B. fragilis* in Bergey's manual. Taxonomic identification of strain ZY-312 based on whole genome sequencing indicated that ZY-312 and ATCC 25285 showed 99.99% similarity. The 33 putative virulence-associated factors identified in ZY-312 mainly encoded structural proteins and proteins with physiological activity, while the lack of *bft* indicated that ZY-312 was non-toxigenic. *In vivo* safety was proven in both normal and immune-deficient mice. The 11 identified antibiotic resistance genes were located on the chromosome rather than on a plasmid, ruling out the risk of plasmid-mediated transfer of antibiotic resistance. *In vitro*, ZY-312 showed resistance to cefepime, kanamycin, and streptomycin. Finally, and notably, ZY-312 exhibited high genetic stability after 100 passages *in vitro*. This study supplements the foundation work on the safety evaluation of ZY-312, and contributes to the development of the first probiotic representative from the dominant Bacteroidetes phylum.

Keywords: *Bacteroides fragilis*, safety evaluation, probiotic, whole genome sequencing, genetic stability

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INTRODUCTION

Commensal gut microbiota are important for host health. They contribute to maturation of the immune system, building intestinal commensalism, resisting infectious microbes, digesting indigestible carbohydrates, and producing certain nutrients such as vitamins and short-chain fatty acids (Sekirov et al., 2010; Fijan, 2014). An increasing number of reports have confirmed the link between intestinal flora disorder and disease, especially autoimmune and metabolic diseases (Fijan, 2014; Althani et al., 2016). Although it is not clear who takes the leap and how it happens, dominant

members of the intestinal microbiota are now being examined as potential probiotic candidates to assist in the treatment of disease, or become alternatives to antibiotics (McKenney and Pamer, 2015; Miquel et al., 2015).

Approved probiotic products based on intestinal microbiota species mainly include lactic acid bacteria, *Bifidobacterium*, and *Escherichia coli* (Foligne et al., 2013). However, there are currently no probiotic representatives of the phylum Bacteroidetes, the second-largest component of the intestinal flora. Recently, non-toxigenic *Bacteroides fragilis* (NTBF) was shown to have powerful health benefits to the host, and was recommended as a probiotic candidate (Troy and Kasper, 2010; Hsiao et al., 2013; Deng et al., 2016). It is therefore likely that *B. fragilis* will be the first probiotic species from the phylum Bacteroidetes.

We previously isolated a novel *B. fragilis* strain ZY-312, and have carried out basic safety assessments to test its probiotic properties (Deng et al., 2016). Results of 16S rRNA sequence analysis, tolerance to simulated digestive fluid, safety, and adhesion to HT-29 cells suggested *B. fragilis* ZY-312 possessed desirable probiotic properties. However, these results are not sufficient to definitively conclude that ZY-312 is safe for use as a probiotic. According to the framework of evaluations for probiotics from Europe (Miquel et al., 2015) and by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO, 2002), thorough characterization of a strain, including examination of the fatty acid profile, metabolite production, and biochemical activity, evaluation of the potential risk of transferable antibiotic resistance and genetic stability, and verification of safety in animals, especially immunodeficient animals, is an integral part of safety assessment (Miquel et al., 2015). Taking advantage of whole genome sequencing technology, we carried out a thorough characterization and systematic evaluation of novel *B. fragilis* strain ZY-312 to determine whether it meets the safety criteria required for probiotics.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Strain ZY-312 was isolated from the feces of a healthy infant, and was previously identified using 16S rRNA gene sequence analysis (Deng et al., 2016). *B. fragilis* strain ATCC 25285 (also known as NCTC 9343) was purchased from the American Type Culture Collection (ATCC). The culture conditions and materials were as described previously for strains ZY-312 and ATCC 25285 (Deng et al., 2016). For the microbial contamination experiment, *E. coli* CBSLAM00087, *Staphylococcus aureus* (*S. aureus*) CMCC(B) 26058, *Salmonella enterica (S. enterica)* serotype Paratyphi B CBSLAM00994, *Pseudomonas aeruginosa* (*P. aeruginosa*) CBSLAM00818, *Candida albicans (C. albicans)* CMCC(F)98001, and *Clostridium sporogenes* (*C. sporogenes)* CMCC(B) 64941 were obtained from the Academy of Military Medical Science, Beijing, China. All strains were verified by PCR amplification of 16S rRNA gene using the universal primers 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492 R (5′-GGTTACCTTGTTACGACTT-3′) (Lanes D–J). Amplified products were sequenced (Biomed).

Animals

For acute oral toxicity studies, 6- to 8-week-old female specific pathogen-free (SPF) BALB/c mice and nude mice were procured from the Animal Experiment Center of the Academy of Military Medical Sciences, Beijing, China. The animals had free access to tap water and standard rodent diet. All of the animal experiments were performed in accordance with the approval of the Animal Ethics Committee of the Beijing Institute of Microbiology and Epidemiology, Beijing, China.

Genome Sequencing, Assembly, and Analysis

Total DNA was extracted from all strains using a DNA extraction kit (Qiagen, USA) and the complete genome sequence of strain ZY-312 was determined using an Illumina Hiseq 2000 sequencing system (Xu et al., 2015). A genome sequencing library with an average insert size of 400 bp was generated, and raw short-read sequences were filtered using Seqprep[1] and Sickle[2] software. The genome was then assembled *de novo* using SOAP *de novo* software (Luo et al., 2012). Accuracy of the assembled genome sequence was evaluated by mapping all raw reads onto the scaffolds using SOAPaligner (Cui et al., 2013).

Gene prediction was carried out using Glimmer3.0 software (Xu et al., 2015). Putative antibiotic resistance genes and putative virulence factors were identified by BLAST analysis of the antibiotic resistance genes database (ARDB)[3] (Liu and Pop, 2009) and the virulence factors database (VFDB)[4] (Chen et al., 2005), respectively. A BLAST comparison was performed between the genome sequences of strain ZY-312 and *B. fragilis* strain NCTC 9343 (GenBank accession number NC_003228). A Perl script was written and the average nucleotide identity (ANI) was calculated using BLAST. A neighbor-joining phylogenetic tree was built using treebest based on the ZY-312 complete genome sequence, the complete shotgun sequences of other *B. fragilis* strains, and the complete genome sequences of *B. fragilis* strains NCTC 9343 (GenBank accession number NC_003228), YCH46 (GenBank accession number NC_006347), and *B. fragilis* 638R (GenBank accession number NC_016776).

General Characteristics of ZY-312

ZY-312 was cultured on Eosin methylene blue agar for 24 h at 37°C (*E. coli* as positive control), SS agar for 24 h at 37°C (*S. enterica* serovar Paratyphi B as positive control), Columbia agar containing blood and gentamicin for 48 h at 37°C (*C. sporogenes* as positive control), mannitol sodium chloride agar for 24 h at 37°C (*S. aureus* as positive control), NAC agar

[1]https://github.com/jstjohn/SeqPrep
[2]https://github.com/najoshi/sickle
[3]http://ardb.cbcb.umd.edu/
[4]http://www.mgc.ac.cn/VFs/main.htm

TABLE 1 | Major fatty acids (A) and major metabolites (B) of *Bacteroides fragilis* ZY-312.

Peak name	Percentage (%)
(A)	
$C_{15:0}$ anteiso	32.21
$C_{15:0}$ iso	17.29
$C_{16:0}$	11.66
$C_{16:0}$ 3-OH	11.08
$C_{14:0}$	1.82
$C_{17:0}$ ante 3-OH	1.72
$C_{18:0}$	1.68
$C_{15:0}$	1.63
$C_{18:1}$ CIS 9	1.37
Unable to distinguish	19.55

	ZY-312		ATCC 25285	
	Time (s)	Normalized area	Time (s)	Normalized area
(B)				
Acetic acid	1514	67.24×10^5	1513	27.65×10^5
Butanedioic acid	593	498.29×10^5	592	107.27×10^5
propionic acid	1353	2.65×10^5	1352	5.41×10^5
Phenylacetic acid	2404	1.33×10^5	2403	2.06×10^5
lactic acid	424	556.78×10^5	424	557.94×10^5

for 24 h at 37°C (*P. aeruginosa* as positive control), Sabouraud dextrose agar for 72 h at 28°C (*C. albicans* as positive control), rose bengal agar for 96 h at 28°C (*C. albicans* as positive control), and agar agar for 48 h at 37°C (*S. aureus* as positive control) for excluding microbial contamination. The major fatty acids were analyzed using an HP6890 gas chromatograph (ver. A 5.01), as previously described (Tan et al., 2010). Supernatant from late-logarithmic phase ZY-312 and ATCC 25285 cultures was collected by centrifugation for 10 min at 3000 × *g*, and then analyzed by gas chromatography-mass spectrometry. Carbon-source utilization analyses were carried out using a Biolog AN microPlate test panel (Biolog, USA). Bacterial cells were collected from logarithmic phase ZY-312 and ATCC 25285 cultures and resuspended at a concentration of 1.5×10^8 colony forming units (cfu)/mL, and then inoculated into the test plate. After incubating anaerobically for 16–24 h, test results were read using a microplate reader (SpectraMax, M2), using deionized water as a negative control. To test catalase activity, 3–5 drops (about 100 μL) of 3% hydrogen peroxide (freshly prepared) were dropped onto the center of precipitated bacterial cells on microscope slides. To test gelatin liquefaction activity, bacteria were inoculated into gelatin medium, tryptone soy broth (TSB, OXIOD, UK) solidified with 15% gelatin, using a sterilized needle. Plates were incubated at 37°C for 48 h anaerobically, then placed at 4°C for 3–4 h. To determine hemolytic ability, bacteria were anaerobically cultured on tryptone soy agar (TSA, OXIOD, UK) supplemented with 5% (v/v) goat blood for 48 h at 37°C. To determine motility, bacteria were cultured on semi-solid medium (TSB solidified with 0.5% agar) at 37°C for 48 h anaerobically.

Antibiotic Resistance Testing

Antibiotic resistance testing was performed using the minimum inhibitory concentration (MIC) method as described previously (Fernandez et al., 2005). Test antibiotics were chosen based on the antibiotic resistance genes identified in the annotated genome of ZY-312, with the following antibiotics included in the analysis: cefoxitin, ceftriaxone, cefepime, trimethoprim, clarithromycin, chloromycetin, levofloxacin, streptomycin, kanamycin, tetracycline, vancomycin, and polymyxin B (NIFDC, China). Although *bcrA* was identified in the genome, bacitracin was not used because of high toxicity, while fosmidomycin (*rosA*) is still undergoing testing and was therefore also excluded. Quinupristin/dalfopristin, the targets of the *vatB* gene product are not used in China, and there is no corresponding antibiotic for *ykkC*. ZY-312 was cultivated anaerobically at 37°C for 48 h at a concentration of 10^7 cfu/mL with antibiotics at different concentrations. MIC was determined by measuring optical density at 600 nm (OD_{600}) with a microplate reader (SpectraMax, M2).

Acute Toxicity to Normal Mice and Nude Mice

To assess the acute toxicity of ZY-312, SPF BALB/c mice were randomly assigned into five groups ($n = 5$–8). Each group was administered with ZY-312 in a suspension containing 1×10^9, 5×10^{10}, or 5×10^{11} cfu/day, 0.5 mL/day culture supernatant, or 0.5 mL/day saline via oral gavage for 5 days. General condition and body weight were observed daily for 17–18 days. At the end of the experimental period, blood samples were obtained for hematological and serum biochemistry analyses. Stomach, colon, liver, and spleen were collected, weighed, and prepared for histopathological examination. Nude mice were orally administered with ZY-312 suspension at a dosage of 1×10^9 cfu/day for 3 days. General condition and bodyweight were observed for 7 days.

Genetic Stability

To explore whether genetic variation occurs in ZY-312 during *in vitro* passage, we continuously subcultured ZY-312 for 100 generations. Two parallel tubes were inoculated from an original culture of ZY-312 in TSB supplemented with 5% fetal bovine serum, and then incubated at 37°C in an anaerobic glove box (Bugbox, Ruskin) for 24 h. These initial cultures were designated A_0 and B_0. Subsequent passages were performed by inoculating 1% of each culture into fresh medium every 24 h, until A_{100} and B_{100} were obtained. Genetic variation was evaluated by complete genome sequencing of strains A_{10}, A_{25}, A_{50}, and A_{100}, and B_{10}, B_{25}, B_{50}, and B_{100}, and ANI was calculated for each generation. Morphological variations were observed by colony examination, Gram staining, and scanning electron microscopy (SEM) of A_{100} and B_{100}, with wild-type ZY-312 used for comparison. Growth of ZY-312, A_{100}, and B_{100} under anaerobic conditions was also examined over a 24-h period. Acute toxicity of A_{100} and B_{100} was detected in SPF mice at a dosage of 1×10^9 cfu/day for 3 days, with general condition and bodyweight observed for 7 days.

TABLE 2 | Physiological and biochemical properties of *B. fragilis* ZY-312.

	ZY-312	ATCC		ZY-312	ATCC
(A)					
Water	−	−	Dulcitol	−	−
N-Acetyl-D-galactosamine	+	+	D, L-α-Glycerol phosphate	−	−
N-Acetyl-D-glucosamine	/	+	*N*-Acetyl-β-D-mannosamine	−	−
D-Fructose	+	+	L-Fucose	−	−
Adonitol	−	−	D-Galactose	+	+
Amygdalin	+	+	D-Galacturonic acid	−	−
D-Arabitol	−	−	Gentiobiose	+	+
Arbutin	+	+	D-Gluconic acid	−	−
D-Cellobiose	+	+	D-Glucosaminic acid	−	−
α-Cyclodextrin	+	+	α-D-Glucose	+	+
β-Cyclodextrin	+	+	Glucose-1-phosphate	+	+
Dextrin	+	+	Glucose-6-phosphate	+	+
Glycerol	−	−	α-Methyl-D-galactoside	−	−
i-Erythritol	−	−	β-Methyl-D-galactoside	+	+
m-Inositol	−	−	α-Methyl-D-glucoside	−	−
α-D-Lactose	+	+	β-Methyl-D-glucoside	−	−
Lactulose	+	+	Palatinose	+	+
Maltose	+	+	D-Raffinose	+	+
Maltotriose	+	+	L-Rhamnose	−	−
D-Mannitol	−	−	Salicin	+	−
D-Mannose	+	+	D-Sorbitol	−	−
D-Melezitose	−	−	Stachyose	+	+
D-Melibiose	+	+	Sucrose	+	+
3-Methyl-D-glucose	+	+	D-Trehalose	−	−
Pyruvic acid methyl ester	+	+	D-Lactic acid methyl ester	−	−
Acetic acid	−	−	D-Malic acid	−	−
Formic acid	−	−	L-Malic acid	−	−
Fumaric acid	−	−	Propionic acid	−	−
Glyoxylic acid	−	−	Pyruvic acid	+	+
α-Hydroxybutyric acid	/	/	Turanose	+	+
β-Hydroxybutyric acid	−	−	D-Saccharic acid	−	−
Itaconic acid	−	−	Succinamic acid	−	−
α-Ketobutyric acid	+	+	Succinic acid	−	−
α-Ketovaleric acid	+	+	L-Asparagine	−	−
D,L-Lactic Acid	−	−	m-Tartaric acid	−	−
L-Lactic acid	−	−	Urocanic acid	−	−
L-Alaninamide	−	−	L-Methionine	−	−
L-Alanine	/	+	L-Phenylalanine	−	−
L-Alanyl-L-glutamine	+	/	L-Serine	−	−
L-Alanyl-L-histidine	/	/	L-Threonine	−	−
L-Alanyl-L-threonine	+	+	L-Valine	/	+
Succinic acid monomethyl ester	−	−	L-Valine plus L-aspartic acid	−	−
L-Glutamic acid	−	−	2′-Deoxy adenosine	+	+
L-Glutamine	−	−	Inosine	+	+
Glycyl-L-aspartic acid	+	+	Thymidine	+	+
Glycyl-L-glutamine	−	−	Uridine	+	+
Glycyl-L-methionine	−	−	Glycyl-L-proline	−	−
Thymidine-5′-monophosphate	−	−	Uridine-5′-monophosphate	−	/

	ZY-312	ATCC 25285
(B)		
Catalase assay	Positive	Positive
Gelatin liquefaction test	Weakly positive	Weakly positive
Hemolysis test	Negative	Negative
Dynamic test	Negative	Negative

TABLE 3 | Putative virulence-associated genes identified in the genome of *B. fragilis* ZY-312.

Gene ID	VFDB_ID	Name	Function
YDGL001582	VF0091	Alginate algI	Alginate O-acetyltransferase AlgI
YDGL004163	VF0003	Capsule cap8E	Capsular polysaccharide synthesis enzyme Cap8E
YDGL004164	VF0003	Capsule cap8G	Capsular polysaccharide synthesis enzyme Cap8G
YDGL002547	VF0003	Capsule cap8M	Capsular polysaccharide synthesis enzyme Cap8M
YDGL002015	VF0003	Capsule cap8O	Capsular polysaccharide synthesis enzyme Cap8O
YDGL002539	VF0003	Capsule cap8O	Capsular polysaccharide synthesis enzyme Cap8O
YDGL002014	VF0144	Capsule cps4I	UDP-*N*-acetylglucosamine-2-epimerase
YDGL002540	VF0144	Capsule cps4I	UDP-*N*-acetylglucosamine-2-epimerase
YDGL001235	VF0274	Capsule cpsJ	Glycosyl transferase CpsJ(V)
YDGL004140	VF0323	Capsule fcl	Putative fucose syntheses
YDGL002177	VF0323	Capsule glf	UDP-galactopyranose mutase
YDGL001165	VF0072	ClpC clpC	Endopeptidase Clp
YDGL002866	VF0072	ClpC clpC	Endopeptidase Clp
YDGL002842	VF0074	ClpP clpP	ATP-dependent Clp protease
YDGL001133	VF0215	Dispersin aatC	AatC ATB binding protein of ABC transporter
YDGL003303	VF0215	Dispersin aatC	AatC ATB binding protein of ABC transporter
YDGL003305	VF0215	Dispersin aatC	AatC ATB binding protein of ABC transporter
YDGL003564	VF0215	Dispersin aatC	AatC ATB binding protein of ABC transporter
YDGL003567	VF0215	Dispersin aatC	AatC ATB binding protein of ABC transporter
YDGL004218	VF0215	Dispersin aatC	AatC ATB binding protein of ABC transporter
YDGL002299	VF0273	Flagella fleQ	Transcriptional regulator FleQ
YDGL002756	VF0334	HSI-I PA0073	ATP-binding component of ABC transporter
YDGL002122	VF0159	Hsp60 htpB	Hsp60, 60K heat shock protein HtpB
YDGL004141	VF0367	LPS gmd	GDP-mannose 4,6-dehydratase
YDGL001251	VF0106	MgtBC mgtB	Mg^{2+}transport protein
YDGL002018	VF0153	Mip mip	Macrophage infectivity potentiator (Mip)
YDGL003334	VF0298	MprAB mprA	MprB, sensor kinas. MprA, transcriptional factor
YDGL003892	VF0392	*O*-Antigen ddhA	Glucose-1-phosphate cytidylyltransferase
YDGL003891	VF0392	*O*-Antigen ddhB	CDP-glucose 4,6-dehydratase
YDGL003902	VF0392	*O*-Antigen galE	UDP-glucose 4-epimerase
YDGL003150	VF0319	PanC/PanD panD	panD
YDGL003842	VF0169	SodB sodB	Superoxide dismutase
YDGL003057	VF0101	Vi antigen tviB	Vi polysaccharide biosynthesis protein, UDP-glucose/GDP-mannose dehydrogenase

Experimental Replicates and Statistical Methods

All experiments were performed at least in triplicate using independent assays, and values were expressed as the mean ± standard error. An unpaired Student's *t*-test was performed to determine statistically significant differences in the acute toxicity assays. A p-value of <0.05 was considered statistically significant.

RESULTS

Morphological Characteristics

Strain ZY-312 only grew under anaerobic conditions (**Supplementary Figure S1**), with no growth observed under aerobic conditions or in 5% CO_2, implying it was an obligate anaerobe. Microbial contamination was excluded by culturing ZY-312 in different culture media under different conditions (**Supplementary Figure S2**). ZY-312 formed circular, low convex, semi-opaque colonies following anaerobic cultivation on blood agar plates (**Supplementary Figure S1**). Cells were Gram-negative, and shown to be rod shaped with rounded ends by scanning electrochemical microscopy (**Supplementary Figure S1**). This morphology matched the descriptions of *B. fragilis* in Bergey's manual (Krieg et al., 2001).

General Characteristics

The major fatty acids of ZY-312 were $C_{15:0}$ anteiso, $C_{15:0}$ iso, $C_{16:0}$, and $C_{16:0}$ 3-OH (**Supplementary Figure S3** and **Table 1A**). The major products in the culture supernatant of ZY-312 were lactic acid, acetic acid, succinic acid, propionic acid, and phenylacetic acid (**Table 1B**). ZY-312 and ATCC 25285 differed in their ability to metabolize L-valine, salicin, L-alanine, L-alanyl-L-glutamine, and *N*-acetyl-D-glucosamine (**Table 2A**). Both strains were positive for catalase activity, weakly positive for gelatin liquefaction, and negative for hemolytic activity and motility

TABLE 4 | Putative antibiotic resistance genes (A) identified in the genome of *Bacteroides fragilis* ZY-312, and minimum inhibitory concentration values (B) for each of the corresponding antibiotics.

Gene ID	Name	Drug
(A)		
YDGL002306	BcrA	Bacitracin
YDGL000449	BL2e_cepa	Cephalosporin
YDGL000258	dfrA22	Trimethoprim
YDGL003477	MefA	Macrolide
YDGL001150	MexF	Chloramphenicol, Fluoroquinolone
YDGL000604	MexY	Aminoglycoside, Glycylcycline
YDGL003440	RosA	Fosmidomycin
YDGL003171	tet37	Tetracycline
YDGL003704	VanRA	Vancomycin, Teicoplanin
YDGL003646	VatB	Streptogramin_A
YDGL000090	YkkC	na_antimicrobials
Antibiotic	**ZY-312**	**ATCC 25285**
(B)		
Cefoxitin	MIC = 2.0	MIC = 1.5
Ceftriaxone	MIC < 64	MIC < 64
Cefepime	MIC > 32	MIC > 32
Trimethoprim	MIC < 16	MIC < 16
Clarithromycin	MIC < 8	MIC < 8
Chloromycetin	MIC < 32	MIC < 32
Levofloxacin	MIC < 8	MIC < 8
Streptomycin	MIC > 1200	MIC > 1200
Kanamycin	MIC > 50	MIC > 50
Tetracycline	MIC < 16	MIC < 16
Vancomycin	4 < MIC < 8	4 < MIC < 8
Polymyxin B	MIC > 8	MIC > 8

Bacitracin was eliminated because of high toxicity. Fosmidomycin is still being researched and was therefore excluded. Quinupristin/dalfopristin are not available in China and were also excluded. No corresponding antibiotic exists for the ykkC gene product.

(**Table 2B**). The physiological and biochemical characteristics of ZY-312 were in accordance with *B. fragilis* as described in Bergey's manual (Krieg et al., 2001).

Genetic Characteristics

The phylogenetic trees generated from the whole genome sequence of ZY-312 and other *B. fragilis* strains are shown in **Supplementary Figures S5**, **S6**. ZY-312 and ATCC 25285 had an ANI of 99.99%. In total, 33 putative virulence factors (**Table 3**) and 11 antibiotic resistance genes (**Table 4A**) were annotated in the ZY-312 genome based on a minimum of 40% amino acid homology. The complete genome was 4,558,494 bp in length and contained on a single chromosome, with an average GC content of 43.08%, consistent with that of *B. fragilis* (41–44%) (Krieg et al., 2001). All drug-resistance genes were located on the chromosome rather than on plasmids.

Putative Virulence Factors

Through BLAST analysis of the VFDB, a total of 33 virulence factor homologs were identified in the genome of ZY-312 (**Table 3**). Most of these putative virulence genes encoded proteins involved in cellular structure or physiological activities, and none had previously been reported as being related to the pathogenesis of *B. fragilis*. Notably, *bft* was not present in the genome of ZY-312, indicating that it is a non-toxigenic strain.

Antibiotic Resistance

Antibiotic resistance tests were performed based on the antibiotic resistance genes identified in the genome of YZ-312 (**Table 4A**), and results are summarized in **Table 4B**. Based on guidelines for antibiotic resistance breakpoints (Russell and Sambrook, 2002; National Center for Clinical Laboratories, 2006), ZY-312 showed resistance to cefepime, kanamycin, and streptomycin, but was susceptible to ceftriaxone, trimethoprim, clarithromycin, chloramphenicol, tetracycline, and levofloxacin. Without available guidelines for *B. fragilis*, resistance of ZY-312 to vancomycin and polymyxin B could not be confirmed; however, we observed that 4–8 μg/mL vancomycin and >8 μg/mL polymyxin B were sufficient to inhibit ZY-312 growth *in vitro*.

ZY-312 Is Non-pathogenic in Both Normal and Immune-Deficient Mice

To confirm the *in vivo* safety of ZY-312, acute toxicity experiments were performed in both normal SPF BALB/c mice and nude mice. Lacking a thymus and an immune response, nude mice are an ideal animal model for evaluating probiotic safety. No death was observed in the BALB/c mice during the toxicity experiments, no treatment-related toxicity was observed, and no significant difference in body weight was noted between low, medium (**Supplementary Figure S4**), and high (**Figure 1A-1**) dose treatment groups and the control group, respectively. Similarly, no difference was found between the culture supernatant-treated group and the control group (**Figure 1A-2**). In addition, there was no obvious histopathological damage in the stomach, colon, liver, or spleen of BALB/c mice from the high dosage group (**Figure 1B**). There were also no significant differences in blood routine index or hepatorenal function between the high dose and control groups (data not shown). For the immune-deficient mouse toxicity experiments, ZY-312 again had no deleterious effects on body weight (**Figure 1A-3**).

ZY-312 Is Genetically and Phenotypically Stable

To explore whether ZY-312 undergoes major genomic rearrangements during passage, the stability of ZY-312 was examined after 100 generations *in vitro*. A_{100} and B_{100} were morphologically identical to the original ZY-312 strain (**Figure 2A**) based on visual inspection, optical microscopy, and SEM. The growth characteristics of A_{100} and B_{100} (**Figure 2B**) were not significantly different from those of ZY-312. Furthermore, following oral administration of A_{100} or B_{100} at a dose of 1×10^9 cfu/day for 3 days, mice did not show any clinical symptoms or weight loss during the observation period (**Figure 2C**). The calculated ANI values from each generation were at least 99.98% (**Figure 2D**).

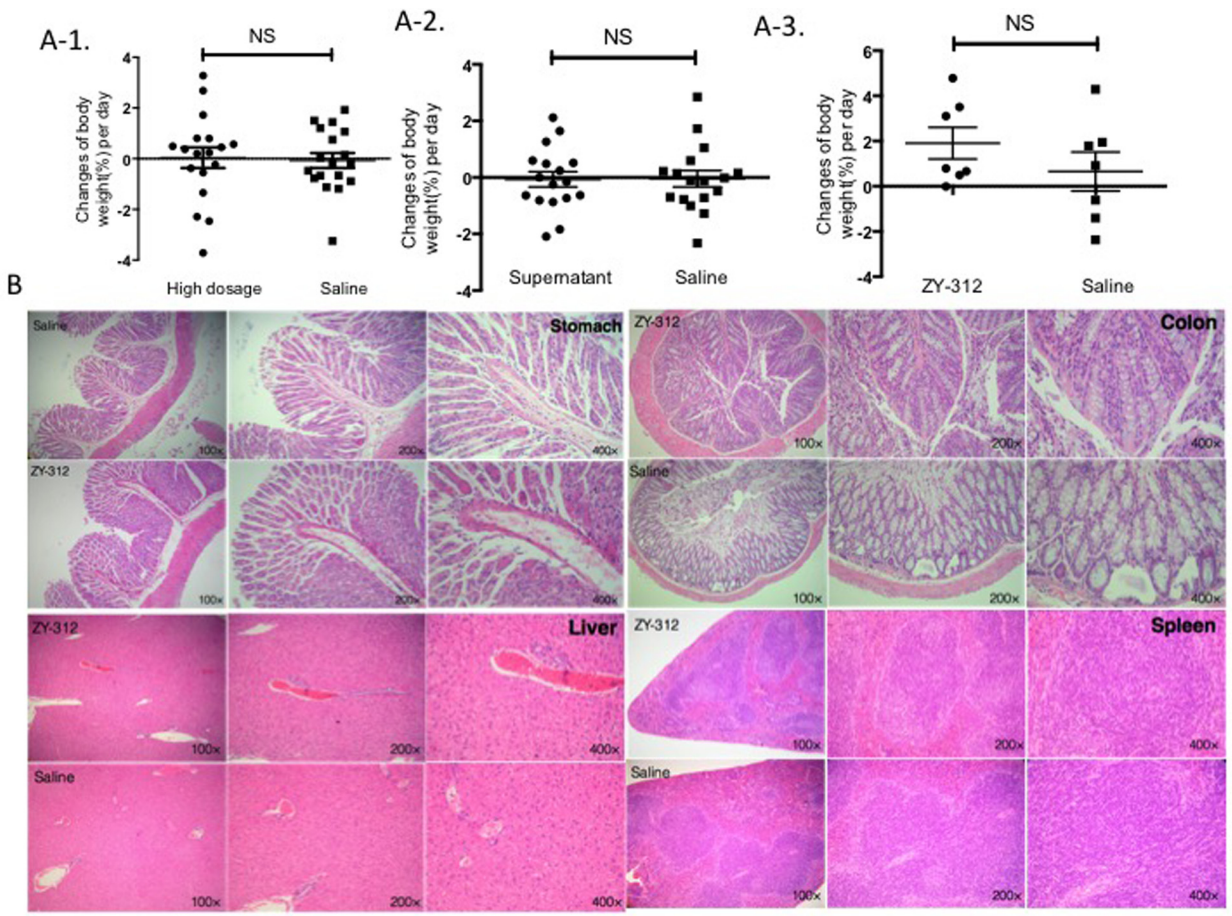
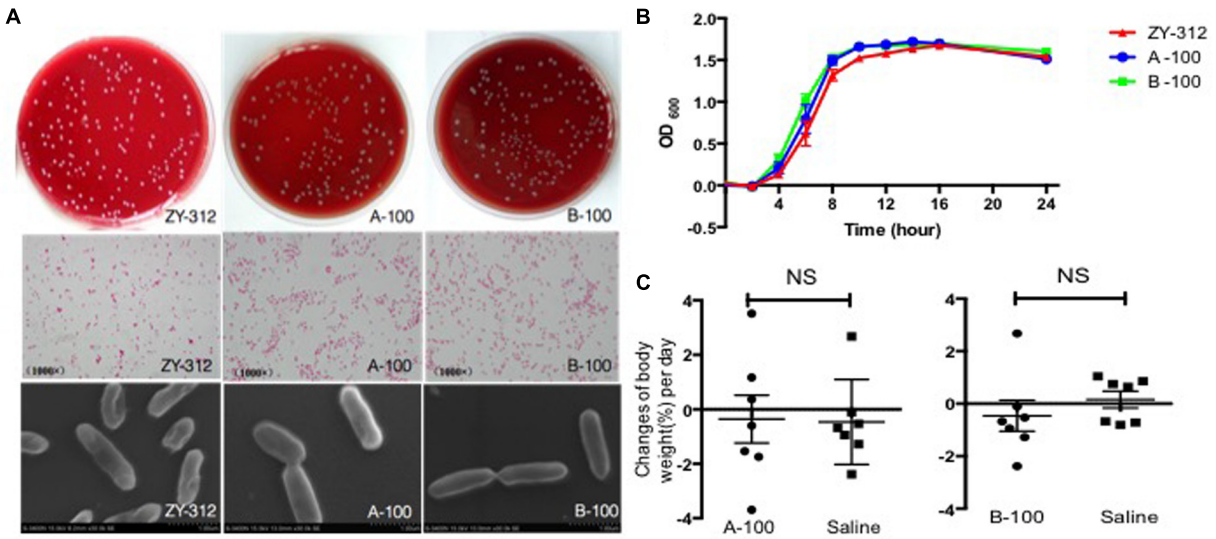


FIGURE 1 | ZY-312 is non-pathogenic in mice. (A) Changes in body weight (%) per day were observed for the experimental and control groups. **(A-1)** Specific pathogen-free (SPF) BALB/c normal mice (n = 8) were treated with 5×10^{11} colony forming units (cfu)/day ZY-312 for 5 days and observed for 18 days. A control group was treated with saline. **(A-2)** SPF normal mice (n = 16) were treated with culture supernatant (0.5 mL/day) for 5 days and observed for 17 days. Tryptic soy broth was used for the control group. **(A-3)** Nude mice (n = 5) were treated with ZY-312 at a concentration of 1×10^9 cfu/day for 3 days and observed for 7 days. The control group were treated with saline. **(B)** Light micrograph images of the stomach, colon, liver, and spleen from mice belonging to the high dosage group (upper) and control group (lower). No significant lesions were observed (mean ± SE; NS, not significant, t-test).

DISCUSSION

According the recent reports, non-toxic *B. fragilis* now is regarded as a commensal constituent with potential as a probiotic candidate (Hsiao et al., 2013), because of powerful immunoregulatory benefits and health-promoting effects it owns. Although *B. fragilis* makes up a very small part of the human intestinal flora, it plays a unique role in the maturation of the host immune system (Mazmanian et al., 2005; Troy and Kasper, 2010). Mono-colonization of mice with *B. fragilis* was sufficient to correct systemic immune defects in germ-free mice by stimulating maturation of splenic $CD4^+$ T cells (Mazmanian et al., 2005) and rebalancing the Th1/Th2 response. Furthermore, *B. fragilis* can direct an anti-inflammatory response, conferring protection in experimental mouse models of colitis (Mazmanian et al., 2008; Round and Mazmanian, 2010) and of autoimmune encephalomyelitis (Ochoa-Reparaz et al., 2010). Those observed effects were absent in mice treated with a *B. fragilis* PSA-deficient mutant strain. PSA is a dominant member of capsular polysaccharide complex (CPC) at the surface of *B. fragilis*, and contains a zwitterionic motif. *B. fragilis* is associated with clinical anaerobic infection and the CPC, especially PSA plays a key role in arising those diseases (Lindberg et al., 1982; Mazmanian and Kasper, 2006). Nevertheless, the role of PSA plays in intra-abdominal abscess probably is beneficial rather than harmful, since the inflammation aroused by PSA helps limit the spread of other gut bacteria and prevent more serious infection (Mazmanian and Kasper, 2006; Deng et al., 2016). Instead of being recognized as virulence factor, recently, PSA has been reconsidered as a symbiosis factor with health-promoting effect (Mazmanian et al., 2008).

Therefore, under overall considerations about the pros and cons, we believe non-toxic *B. fragilis* is a good choice for probiotic candidate and we decided to isolate a new strain of *B. fragilis* named ZY-312 (Deng et al., 2016) and explore its safety whether



D

	ZY-312	A-10	A-25	A-50	A-100	B-10	B-25	B-50	B-100
ZY-312	100	99.994517	99.997925	99.997651	99.988078	99.995188	99.997648	99.982499	99.996197
A-10	99.994517	100	99.998043	99.998044	99.9892	99.99271	99.998292	99.983112	99.99616
A-25	99.997925	99.998043	100	99.996199	99.9868	99.996659	99.993624	99.979244	99.993318
A-50	99.997651	99.998044	99.996199	100	99.989406	99.996487	99.996375	99.9839	99.994727
A-100	99.988078	99.9892	99.9868	99.989406	100	99.996843	99.996995	99.993781	99.995896
B-10	99.995188	99.99271	99.996659	99.996487	99.996843	100	99.996933	99.982974	99.995984
B-25	99.997648	99.998292	99.993624	99.996375	99.996995	99.996933	100	99.982475	99.994383
B-50	99.982499	99.983112	99.979244	99.9839	99.993781	99.982974	99.982475	100	99.9955
B-100	99.996197	99.99616	99.993318	99.994727	99.995896	99.995984	99.994383	99.9955	100

FIGURE 2 | ZY-312 is genetically stable. (A) Gram-stained ZY-312, A_{100}, and B_{100} cells following anaerobic culture on tryptic soy agar (5% sheep blood) for 48 h at 37°C. Observation were made using a light microscope (4000×) and a scanning electron microscope (30000×). **(B)** Growth curves of ZY-312, A_{100}, and B_{100} cultured anaerobically for 24 h. **(C)** SPF BALB/c normal mice (n = 8) were treated with A_{100} or B_{100} at a concentration of 1×10^9 cfu/day for 3 days and observed for 7 days. A control group was treated with saline. **(D)** Average nucleotide identities were calculated between each generation.

satisfy the criteria required for probiotic bacteria. Although the health benefits of *B. fragilis* are generally recognized, much work still needs to be done to obtain certification of this species as a probiotic. Probiotics are defined as non-pathogenic live microorganisms that confer health benefits to the host when administered in adequate amounts (FAO/WHO, 2002). As living microorganisms with the potential for infection or *in situ* toxin production, probiotics should fulfill health and safety claims before entering the market. Safety assessment is the chief task for certifying a probiotic, as any adverse effects should be predicted in advance. Accordingly (FAO/WHO, 2002; Miquel et al., 2015), correct identification, sufficient characterization, and evaluation of potential risk and probiotic properties are integral for evaluation of a new probiotic.

Previously, we demonstrated that novel strain ZY-312, isolated from the feces of a healthy breast-fed infant, possessed similar morphological and growth characteristics to typical *B. fragilis* strains, as well as exhibiting desirable probiotic properties, including tolerance to air, simulated gastric fluid (pH 3.0), simulated intestinal fluid and ox bile (pH 6.8), adhesion, and *in vitro* safety in colon cells (Deng et al., 2016). Based on these results, we carried out a more thorough characterization and systemic evaluation of ZY-312 in the current study. As recommended (FAO/WHO, 2002), phenotypic testing, fatty acid analysis, metabolite production, biochemical activity, and *in vivo* toxicity testing, in combination with genetic analysis, taxonomic identification, and putative virulence and antibiotic resistance gene identification analysis, were performed to determine whether ZY-312 is safe for use as a probiotic. The major fatty acids and metabolic products of ZY-312 closely resemble descriptions of *B. fragilis* in Bergey's manual, as do the results of biochemical activity testing. Our previous work showed that the 16S rRNA sequence of ZY-312 was 99% identical to that of *B. fragilis* strain ATCC 28285. However, 16S rRNA sequence analysis has potential drawbacks for separating closely related species because of low taxonomic resolution. Whole genome sequencing identifies taxa with higher taxonomic resolution, and provides more information about gene function, such as putative virulence

factors and antibiotic resistance genes (Rijkers et al., 2011; Miquel et al., 2015). Taking advantage of whole genome sequencing technology in the current study, we showed that ZY-312 and ATCC 25285 shared 99.99% ANI, and were derived from the same origin (**Supplementary Figure S5**), consistent with previous results. Furthermore, a total of 33 putative virulence factors and 11 antibiotic resistance genes were annotated in the genome of ZY-312 through comparative analysis with the VFDB and the ARDB, respectively. The putative virulence factors consisted of structural proteins and proteins with physiological activity, and none had previously been reported in association with the pathogenesis of *B. fragilis*. Because of the absence of *bft*, ZY-312 was identified as a NTBF strain.

There are many reports claiming probiotic safety based on an assessed lack of infectivity in normal animals (Bernardeau et al., 2002; Shokryazdan et al., 2016). However, confidence would be increased if assessment could be performed in immunodeficient animals (FAO/WHO, 2002). Therefore, we examined the safety of ZY-312 in both normal and immunodeficient mice. The SPF normal mice treated with high doses or supernatant of ZY-312 did not display any significant strain-related toxigenic symptoms, based on the assessment of body weight changes, histopathological examination, blood routine index, and hepatorenal function. Notably, ZY-312 also proved safe in nude mice (**Figure 1A-3**).

The FAO/WHO recommends that probiotic strains should be fully characterized, including determination of antibiotic resistance patterns, and should have no risk of transferring antibiotic resistance (FAO/WHO, 2002). Theoretically, consumption of probiotics with transferrable antibiotic resistance genes might lead to refractory infections if multiple antibiotic resistance genes are transferred to a pathogen (Borchers et al., 2009; Sanders et al., 2010). We demonstrated that there is no risk of ZY-312 spreading antibiotic resistance because all identified drug-resistance genes (**Table 4A**) were located in the chromosome rather than on a plasmid. Moreover, we verified the antibiotic resistance phenotype of ZY-312, and discovered that it is resistant to cefepime, kanamycin, and streptomycin, but susceptible to ceftriaxone, trimethoprim, clarithromycin, chloramphenicol, tetracycline, and levofloxacin. The MICs of vancomycin and polymyxin B for ZY-312 were also identified (**Table 4B**). The inconsistencies between genotype and observed phenotype might stem from the fact that putative antibiotic resistance genes were annotated based on 40% amino acid homology, meaning that some genes were incorrectly identified in the genome of ZY-312, and are likely not present.

As potential genetic variation might lead to unpredictable risk, genetic stability should also be confirmed prior to starting large-scale production of ZY-312-based probiotics (Sanders et al., 2010). We confirmed that following *in vitro* passage of 100 generations from the original strain of ZY-312, there was no significant difference between A_{100}, B_{100}, and the parental strain with respect to morphological characteristics and growth features. No *in vivo* toxicity was observed for A_{100} or B_{100}, and the ANI between each generation was at least 99.98%, demonstrating that ZY-312 has a high degree of genetic stability.

CONCLUSION

We confirmed that ZY-312 is a NTBF strain, without potential virulence factors or risk of spreading antibiotic resistance genes. As well as having desirable probiotic properties (Deng et al., 2016), ZY-312 has a high degree of genetic stability and is non-pathogenic, even to immune-deficient mice. Therefore, ZY-312 is most likely safe for use in future probiotic applications. This study supplements the initial safety assessment work already carried out for ZY-312, and contributes to the development of the first probiotic representative from the dominant Bacteroidetes phylum.

AUTHOR CONTRIBUTIONS

YW did the experiments with DNA and bacteria, analyzed data, and contributed to revising the manuscript; HD did the experiments with bacteria and mice, analyzed data, and wrote the manuscript; ZL did the experiments with mice, analyzed data, and contributed to revising the manuscript; YT, YH, XW, and ZD analyzed data; YL and RY designed the experiments and contributed to revising the manuscript; YjB designed experiments, analyzed data, and provided overall direction, YB and FZ provided overall directions and contributed to revising the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00435/full#supplementary-material

FIGURE S1 | (A) Colonies of ZY-312 on tryptone soy agar (5% sheep blood) following culture in 5% CO_2 **(A-A)**, air **(A-B)**, or anaerobically **(A-C)** for 48 h at 37°C. **(B)** Cells observed under light microscope following Gram staining (4000×). **(C)** Cells observed under scanning electron microscope (5000×).

FIGURE S2 | Confirmation of no microbial contamination. (A) *Escherichia coli* and ZY-312 cultured on Eosin methylene blue agar for 24 h at 37°C. **(B)** *Salmonella enterica* serovar Paratyphi B and ZY-312 cultured on SS agar for 24 h at 37°C. **(C)** *Clostridium sporogenes* and ZY-312 cultured anaerobically on Columbia agar containing blood and gentamicin for 48 h at 37°C. **(D)** *Staphylococcus aureus* and ZY-312 cultured on mannitol sodium chloride agar for 24 h at 37°C. **(E)** *Pseudomonas aeruginosa* and ZY-312 cultured on NAC agar for 24 h at 37°C. **(F)** *Candida albicans* and ZY-312 cultured on Sabouraud dextrose agar for 72 h at 28°C. **(G)** *S. aureus* and ZY-312 cultured on agar agar for 48 h at 37°C. **(H)** *C. albicans* and ZY-312 cultured on rose bengal agar for 96 h at 28°C.

FIGURE S3 | The major fatty acids of ZY-312 as identified by gas chromatography.

FIGURE S4 | Acute toxicity of ZY-312 to mice. Changes in body weight (%) per day were observed for the experimental and control groups. (A) Specific pathogen-free (SPF) BALB/c normal mice ($n = 5$) were treated with ZY-312 at a concentration of 1×10^9 cfu/day for 5 days and observed for 15 days. The control group mice were treated with saline. **(B)** SPF normal mice ($n = 5$) were treated with ZY-312 at a concentration of 5×10^{10} cfu/day for 5 days and observed for 18 days. Tryptic soy broth was used to treat the control group. No significant weight loss was observed in any of the animals (mean ± SE; NS, not significant, *t*-test).

FIGURE S5 | Phylogenetic tree based on complete shotgun sequences showing the relationship between *Bacteroides fragilis* ZY-312 and closely related species. The tree was constructed using the neighbor-joining method.

FIGURE S6 | Phylogenetic tree based on complete genome sequences showing the relationship between *B. fragilis* strain ZY-312, *B. fragilis* NCTC 9343, *B. fragilis* YCH46, and *B. fragilis* 638R. The tree was constructed using the neighbor-joining method.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The property of ZY-312 belongs to Guangzhou ZhiYi biotechnology Co. Ltd. Any use of ZY-312 without permission of Guangzhou ZhiYi biotechnology Co. Ltd. will be illegal.

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Lactobacillus paraplantarum 11-1 Isolated from Rice Bran Pickles Activated Innate Immunity and Improved Survival in a Silkworm Bacterial Infection Model

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Lactic acid bacteria (LAB) have high immune system-stimulating activity and are considered beneficial for human health as probiotics in the gut. The innate immune system is highly conserved between mammals and insects. Microbe-associated molecular patterns (e.g., peptidoglycan and β-glucan) induce cytokine maturation, which, in silkworm larvae, leads to muscle contraction. The purpose of this study is to find a novel probiotic by using silkworm muscle contraction assay. In the present study, we isolated LAB derived from rice bran pickles. We selected highly active LAB to activate the innate immune system of the silkworm, which was assayed based on silkworm muscle contraction. Of various LAB, *L. paraplantarum* 11-1 strongly stimulated innate immunity in the silkworm, leading to stronger silkworm contraction than a dairy-based LAB. Silkworms fed a diet containing *L. paraplantarum* 11-1 exhibited tolerance against the pathogenicity of *Pseudomonas aeruginosa*. These findings suggest that *L. paraplantarum* 11-1 could be a useful probiotic for activating innate immunity.

Keywords: Lactic acid bacteria, *Lactobacillus* sp., silkworm, innate immunity, infection, *Pseudomonas aeruginosa*

INTRODUCTION

Innate immunity is highly conserved between invertebrates and vertebrates. In mammals, dendritic cells and macrophages produce cytokines in response to microbial pathogens (Janeway and Medzhitov, 2002), whereas in insects, hemocytes and fat bodies recognize microbial pathogens and induce an anti-microbial response (Brennan and Anderson, 2004). Especially in silkworms, immune cells produce reactive oxygen species to activate proteases, resulting in cytokine release. Our group discovered an active cytokine, paralytic peptide, that induces muscle contraction in silkworms (Ishii et al., 2008). We used silkworm muscle specimens to screen for innate immunity-activating substances and found that lactic acid bacteria (LAB) strongly induce silkworm muscle contraction (Dhital et al., 2011; Fujiyuki et al., 2012; Nishida et al., 2016). This screening method has several advantages compared with conventional screening using mammalian innate immune cells such as macrophages. First, the silkworm does not respond to lipopolysaccharides, which often produces a false-positive

response in mammalian macrophages. Second, insect whole body assays reflect the absorption, distribution, metabolism, excretion, and toxicity factors that govern the therapeutic effects of medicines. Therefore, substances with less effective pharmacokinetics/pharmacodynamics and/or are toxic in the silkworm muscle contraction assay would be excluded by these tests.

LAB are traditionally used for fermenting foods, dairy products, and probiotics. LAB are Gram-positive, catalase-negative, form no spores, and are immotile. Foods fermented with LAB could be beneficial for human health by activating innate immunity (Ichikawa et al., 2012; Kawashima et al., 2013). In our previous report, we isolated a dairy-based LAB to activate the innate immune system in the silkworm (Nishida et al., 2016). LAB isolated from dairy products have been characterized well, whereas LAB from fermented pickles have not. The purpose of this study is to find a novel probiotic to activate the innate immune system in the silkworm by screening LAB from non-dairy products, such as fermented pickles.

In this work, we isolated LAB from rice bran pickles and Korean pickles (kimchi). We evaluated the innate-immunity stimulating activity of LAB in silkworms. We selected a highly active LAB based on the results of the silkworm muscle contraction assay. Isolated *L. paraplantarum* 11-1 exhibited high activity in the silkworm contraction assay. Silkworms that ingested an artificial diet containing *L. paraplantarum* 11-1 exhibited tolerance against the pathogenicity of *Pseudomonas aeruginosa*. To the best of our knowledge, this is the first report of a probiotic effect of *L. paraplantarum* against *P. aeruginosa* infection. This LAB might be valuable as a probiotic for activating innate immunity. The infection model used in this study has the potential to be used to study a novel probiotic against *P. aeruginosae*.

MATERIALS AND METHODS

Materials

Gifu Anaerobic Medium (GAM) broth and GAM agar were purchased from Nissui (Tokyo, Japan). MRS broth and MRS agar were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). $CaCO_3$-MRS agar was prepared by adding $CaCO_3$ (final concentration: 1%, Wako, Osaka, Japan) to the MRS agar after autoclaving. An AnaeroPak (Mitsubishi Gas Chemicals, Tokyo, Japan) was used for anaerobic culturing on agar plates. Saline was prepared as 0.9% NaCl (Wako, Osaka, Japan). Lysogeny broth (LB) medium was prepared with 1% bacto tryptone (Becton Dickinson, Franklin Lakes, NJ, USA), 0.5% bacto yeast extract (Becton Dickinson, Franklin Lakes, NJ, USA), and 1% NaCl (Wako, Osaka, Japan). An LB agar plate was prepared with LB medium containing 1.5% (w/v) agar (Nacalai Tesque, Kyoto, Japan).

DNA Sequencing

Fragments containing 16S rDNA were amplified with polymerase chain reaction using KOD FX Neo (Toyobo, Tokyo, Japan) with primers 9F and 1541R (Hashimoto et al., 2007). The DNA sequences were determined with direct sequencing, BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (ThermoFisher Applied Biosystems, Foster City, CA, USA). Sequences were analyzed with the NCBI BLASTN 2.2.27+ (Zhang et al., 2000), 16S ribosomal RNA sequences database (Bacteria and Archaea 7545 sequences). DNA sequences are currently in preparation for submission to the GenBank.

Characterization of LAB

Fluid from the pickles was spread on MRS agar. After incubation at 30°C for 2 days, white colonies appeared on each plate. Isolated bacteria were Gram-stained with Gram-color (Merck, Kenilworth, NJ, USA). For scanning electron microscopy (SEM), bacterial cells were pre-fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), post-fixed with 1% osmium tetroxide in the same buffer, and freeze-dried in *t*-butyl alcohol. The sample was examined with a field-emission SEM (JSM- 7500F, JEOL, Japan). The bacterial colony was suspended in 3% H_2O_2 for a catalase test. *Staphylococcus aureus* RN4220 and *Escherichia coli* JM109 were used as controls. Other identification kits, Api Zym and Api 50 CHL, were purchased from bioMérieux (Marcy l'Etoile, France), and the data were analyzed using the Api web v5.1 database (bioMérieux, Marcy l'Etoile, France).

Silkworm Muscle Contraction Assay

Silkworm muscle contraction was measured as previously reported (Ishii et al., 2008). Briefly, autoclaved bacterial suspension (50 μl) was injected into a silkworm muscle specimen. The contraction value was determined as (prelength-postlength)/prelength. The sample amount (mg) that induced a contraction value of 0.15 was defined as 1 unit.

Silkworm Infection Model

The silkworm infection model was described previously (Hamamoto et al., 2004). Pathogens used in the infection model were *P. aeruginosa* PAO1 (Stover et al., 2000) and methicillin-sensitive *S. aureus* 1 (MSSA1) (Akimitsu et al., 1999) from our laboratory stock. Pathogenic bacteria grown in LB medium overnight were diluted with saline (0.9% NaCl) and injected into fifth instar larva ($n = 7$) fed overnight. Survival of silkworm larva was counted for 5 days.

Statistical Analysis

Statistical analysis was performed with Microsoft Excel 2007 for Windows (Redmond, WA, USA) and Excel Statistics 2008 (Social Survey Research Information, Tokyo, Japan). Survival plots were generated by the Kaplan-Meier method and analyzed by the log-rank test. Activity was compared with the Mann-Whitney *U*-test. Differences having a $P < 0.05$ were considered to be statistically significant. Survival curve was plotted using Kaleidagraph 4.1.4 (Synergy Software, Reading, PA, USA) and LD_{50} values were determined from fitting curve of the logistic equation, y = a + (b − a)/(1 + (x/c)^d), y is the fraction of larva killed, x is the number of viable cells injected, c is LD_{50}, a, b and d is the constant of fitting curve. Curve fitting was applied with Levenberg-Marquardt algolism.

RESULTS

Isolation and Characterization of LAB

We selected Gram-positive bacteria on MRS agar, as LAB generally recognized as safe are Gram-positive bacteria. Colonies were re-streaked on $CaCO_3$-MRS medium to confirm lactic-acid fermentation. Lactate-fermenting and Gram-positive bacteria were subjected to the silkworm contraction assay. LAB tested for the contraction assay was sequenced for 16S rDNA (**Table 1**).

In order to investigate morphological and ultrastructural appearance of isolated bacteria, we performed Gram staining and SEM. Gram stains of LAB displayed a Gram-positive bacillus with homogeneous morphology (**Figure 1**). SEM revealed that LAB was characterized as a rod shaped bacterium (**Figure 2**).

The silkworm muscle contraction activity of LAB is shown in **Table 1**. LAB isolated from pickles showed diverse activity. *L. paraplantarum* 11-1 exhibited the highest activity, 165 U/mg, which was higher than that of the LAB in a previous report from our laboratory (Nishida et al., 2016). The activity of the other isolated LABs was as follows: *Lactobacillus sakei* 4, 6.7 U/mg; *Pediococcus ethanolidurans* 11-2, 2.7 U/mg; and *Leuconostoc citreum* A, 43 U/mg.

BLAST analysis of the 16S rDNA sequence of the 11-1 strain revealed that the 11-1 strain had 98% homology with *L. paraplantarum* DSM10667 (NR_025447.1) and *L. plantarum* WCFS1 (NR_075041.1). Strain 11-1 was more similar to *L. paraplantarum* DSM10667 than *L. plantarum* WCFS1. Therefore, we identified the 11-1 strain as *L. paraplantarum*. We then determined the growth characteristics of *L. paraplantarum* 11-1. *L. paraplantarum* 11-1 had strict temperature sensitivity as it grew in MRS medium at 30°C, but not at 16° or 37°C (**Table 2**). Growth of *L. paraplantarum* 11-1 in MRS medium was sensitive to salt higher than 5% NaCl in the medium and resistant to acidic conditions (pH 4.0). In contrast, *L. plantarum* JCM1057 grew at a wide-range of temperatures (16°, 30°, and 37°C) and its growth was resistant to salt (5, 8, and 10% NaCl).

We next examined the ability of *L. paraplantarum* 11-1 to ferment carbohydrates using Api 50 CHL (**Table 3**). *L. paraplantarum* 11-1 exhibited different characteristics from *L. paraplantarum* DSM10667 (CNRZ 1885^T) in 5 of 49 sugars and derivatives utilized. Amygdalin, lactose, melibiose, melezitose, and gluconate were not utilized by *L. paraplantarum* 11-1, whereas α-methyl-D-glucoside was utilized. On the other hand, *L. plantarum* JCM1057 utilized three different sugars than plant-derived *L. plantarum* ATCC14917^T (Bringel et al., 1996; Curk et al., 1996). The carbohydrate fermentation scores of *L. paraplantarum* 11-1 matched 90.5% of those of *Carnobacterium maltaromaticum* in the Api web v5.1 database, whereas those of *L. plantarum* JCM1057 matched 99.2% of those of *L. plantarum* 1. The possibility that strain 11-1 was *C. maltaromaticum* was excluded due to the low similarity of the 16S rDNA sequences. The 16S rDNA sequence of strain 11-1 exhibited 98% similarity with *L. paraplantarum* DSM10667 (NR_025447.1) and 91% similarity with *C. maltaromaticum* DSM 20342 (NR_044710.2).

We also examined enzymatic characteristics of strain 11-1 using the Api Zym test and compared them with those of other related strains (Curk et al., 1996; Oberg et al., 2016) (**Table 4**). The enzymatic characteristics data of *L. paraplantarum* are unavailable, and therefore we compared the data of strain 11-1 and control strain *L. plantarum* JCM 1057, and previously reported data of *L. plantarum* and *L. curvatus*. *L. paraplantarum* 11-1 exhibited different activities (acid phosphatase) of 19 enzymes compared with *L. curvatus* WSU01. In contrast, *L. paraplantarum* 11-1 exhibited 5 different activities (esterase (C4), β-galactosidase, α-glucosidase, β-glucosidase, β-glucosaminidase) of 19 enzymes compared with *L. plantarum* JCM 1057. The enzymatic characteristics of *L. paraplantarum* 11-1 were more similar to those of *L. curvatus* WSU01 than *L. plantarum* JCM 1057.

Probiotic Effect of *L. paraplantarum* 11-1

To evaluate the probiotic effect of *L. paraplantarum* 11-1, we used the silkworm infection model (**Figure 3**). Injection of *P. aeruginosa* into silkworm larvae had time-dependent and dose-dependent silkworm killing effects. Silkworms were fed a diet containing *L. paraplantarum* 11-1 viable cells without apparent problems. Feeding silkworms a diet containing *L. paraplantarum* 11-1 viable cells increased the number of animals that survived after injection of *P. aeruginosa*, resulting in a ∼100-fold higher LD_{50}. We then tested the infection model with the Gram-positive bacteria *S. aureus* (**Figure 4**). Feeding the silkworm a diet containing *L. paraplantarum* 11-1 viable cells increased the number of animals that survived after injection of MSSA, resulting in a ∼2-fold higher LD_{50}.

TABLE 1 | Identification of bacteria and its activity of silkworm muscle contraction assay.

Strain	Origin	Gram stain	Identification (References)	GenBank Accession no.	ID %	Activity (U/mg)
4	Rice bran pickles	Gram-positive Bacilli	*Lactobacillus sakei* 23K (Chaillou et al., 2005)	NR_075042.1	99	6.7
11-1	Rice bran pickles	Gram-positive Bacilli	*Lactobacillus paraplantarum* DSM10667 (Bringel et al., 1996; Curk et al., 1996)	NR_025447.1	98	165 ± 35[a]
11-2	Rice bran pickles	Gram-positive Cocci	*Pediococcus ethanolidurans* Z-9 (Liu et al., 2006)	NR_043291.1	98	2.7
A	Kimchi	Gram-positive Cocci	*Leuconostoc citreum* KM20 (Kim et al., 2008)	NR_074694.1	100	43

[a]*Mean ± SE (n = 2).*

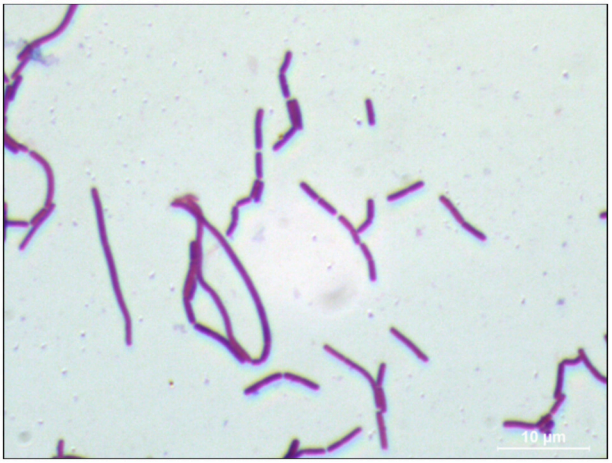

FIGURE 1 | Gram staining of *L. paraplantarum* 11-1. An *L. paraplantarum* 11-1 colony on $CaCO_3$-MRS agar was Gram-stained (Merck). The image was captured with a charge-coupled device camera (Hamamatsu Photonics) using an Olympus phase-contrast microscope at 1,000× magnification.

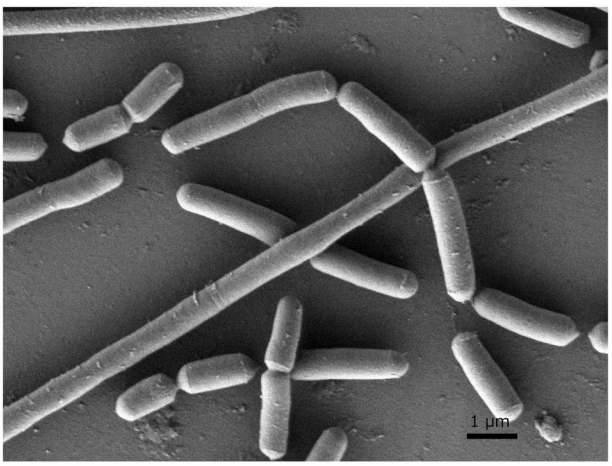

FIGURE 2 | Scanning electron micrograph (SEM) of *L. paraplantarum* 11-1. The sample was examined with a field-emission SEM (JSM- 7500F, JEOL, Japan). The sample was depicted at 10,000× magnification.

TABLE 2 | Growth of LAB in MRS under different conditions.

Growth in MRS	*L. paraplantarum* 11-1	*L. plantarum* JCM 1057
16°C	−	+
30°C	+	+
37°C	−	+
43°C	−	−
pH 4, 30°C	+	+
pH 6, 30°C	+	+
pH 7, 30°C	+	+
5% NaCl	−	+
8% NaCl	−	+
10% NaCl	−	+

DISCUSSION

Isolation of LAB with High Innate Immunity-Stimulating Activity

In general, LAB are thought to be beneficial for human health as probiotics in the gut. LAB were recently reported to have high immunity stimulating activity (Ichikawa et al., 2012; Kawashima et al., 2013). Pickles are a food that is fermented with LAB (Hammes, 2009). Fermented-vegetable foods such as pickles are potential sources of non-dairy LAB. In this study, we isolated LAB from pickles by streaking samples of pickle fluid on MRS agar, a selection plate for LAB (de Man et al., 1960). We confirmed the Gram-positive feature of the isolated bacteria and its lactic acid production on $CaCO_3$-MRS agar, in which lactic acid solubilizes $CaCO_3$ to form a transparent zone around the colony. Gram staining and SEM characterized LAB as a Gram-positive bacillus and a rod shaped bacterium respectively. (**Figures 1**, **2**). Pleomorphism, defined as variation in size or shape of a bacterial cell, is described for different Lactobacillicae in response to the absence of deoxyribosides, vitamin B_{12}, or divalent cation. Culture broth composition is correlated with the morphology of *L. acidophilus* NCFM (Senz et al., 2015). Different sizes in microscopy morphology might depend on nutrient composition in culture medium. Next, we determined the 16S rDNA sequences of each isolate. The identity of strain 11-1 as *L. paraplantarum* was based on 98% similarity between strain 11-1 and *L. paraplantarum* DSM10667 (NR_025447.1).

L. plantarum and *L. paraplantarum* are closely related and heterofermentive. *L. paraplantarum* is isolated from beer and human feces. *L. plantarum* is a nonpathogenic LAB colonizing in fermented foods and in the human mouth and gut. Therefore, *L. plantarum* is normal human gut microbiota (Bernardeau et al., 2008; Hammes, 2009). The *L. plantarum* WCFS1 genome was sequenced and a recombinant DNA technique was established to construct a strain expressing a specific antigen (Grangette et al., 2001; Seegers, 2002; Kleerebezem et al., 2003; Wells and Mercenier, 2008; Siezen et al., 2012).

Innate-Immunity Activation in Silkworms

Multiple studies demonstrate the validity of silkworm contraction assay for innate immune activation (Ishii et al., 2008; Dhital et al., 2011; Fujiyuki et al., 2012). We determined the activity of LAB to stimulate innate immunity in silkworms using a muscle contraction assay (Ishii et al., 2008). When *L. paraplantarum* 11-1 was injected into the silkworm body fluid, the insect cytokine paralytic peptide was activated upon innate immune stimulation, resulting in silkworm muscle contraction. Compared with a conventional method using macrophages, the muscle contraction assay does not require cell culture and is insensitive to lipopolysaccharides, which often cause a false-positive response in test samples. We isolated several LABs that exhibited a variety of muscle contraction activities (Nishida et al., 2016). Among them, *L. paraplantarum* 11-1 exhibited the highest activity (Supplemental Figure 1).

TABLE 3 | Growth characteristics of LAB.

Carbohydrate	11-1 (This study)	*L. plantarum* JCM 1057 (This study)	*L. paraplantarum* DSM 10667^T (CNRZ 1885^T)[a]	*L. plantarum* subsp. *plantarum* ATCC 14917^T[a]
Glycerol	–	–	–	–
Erythritol	–	–	–	–
D-Arabinose	–	–	–	–
L-Arabinose	–	+	–	+
Ribose	±	+	±	+
D-Xylose	–	–	–	–
L-Xylose	–	–	–	–
Adonitol	–	–	–	–
β-Methyl-D-xyloside	–	–	–	–
Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	+	+	+	+
L-Sorbose	–	–	–	–
Rhamnose	–	±	–	–
Dulcitol	–	–	–	–
Inositol	–	–	–	–
Mannitol	+	+	+	+
Sorbitol	–	+	–	+
α-Methyl-D-mannoside	–	+	–	+
α-Methyl-D-glucoside	+	+	–	–
N-Acetyl glucosamine	+	+	+	+
Amygdalin	–	+	+	+
Arbutin	+	+	+	+
Esculin	+	+	+	+
Salicin	+	+	+	+
Cellobiose	+	+	+	+
Maltose	+	+	+	+
Lactose	–	+	+	+
Melibiose	–	+	+	+
Saccharose	+	+	+	+
Trehalose	+	+	+	+
Inulin	–	–	–	–
Melezitose	–	+	+	+
D-Raffinose	–	±	+	+
Starch	–	–	–	–
Glycogen	–	–	–	–
Xylitol	–	–	–	–
β-Gentiobiose	+	+	+	+
D-Turanose	–	+	–	+
D-Lyxose	–	–	–	–
D-Tagatose	–	–	–	–
D-Fucose	–	–	–	–
L-Fucose	–	–	–	–
D-Arabitol	–	±	–	±
L-Arabitol	–	–	–	–
Gluconate	–	+	+	+
2-Keto-gluconate	–	–	–	–
5-Keto-gluconate	–	–	–	–

[a]*Data from Curk et al. (1996).*

TABLE 4 | Enzymatic characteristics of LAB.

Enzyme	11-1 (This study)	*L. plantarum* JCM 1057 (This study)	*L. curvatus* WSU01[a]	*L. planturum* ($n = 7$)[b]	*L. curvatus* ($n = 24$)[b]
Alkaline phosphatase	–	–	–	–	–
Esterase (C4)	–	+	–	–	–
Esterase lipase (C8)	–	–	–	–	–
Lipase (C14)	–	–	–	ND	ND
Leucine aminopeptidase	+	+	+	+	+
Valine aminopeptidase	+	+	+	+	+
Cystine aminopeptidase	–	–	–	–	±
Trypsin	–	–	–	ND	ND
Chymotrypsin	–	–	–	ND	ND
Acid phosphatase	+	+	–	+	±
Phosphohydrolase	+	+	+	±	±
α-Galactosidase	–	–	–	±	–
β-Galactosidase	–	+	–	+	+
β-Glucuronidase	–	–	–	–	–
α-Glucosidase	–	+	–	+	–
β-Glucosidase	–	+	–	+	±
β-Glucosaminidase	–	+	–	+	±
α-Mannosidase	–	–	–	–	–
α-Fucosidase	–	–	–	ND	ND

[a,b]*Data from Oberg et al. (2016) and Papamanoli et al. (2003) respectively.*

Silkworm Acquired Tolerance to Bacterial Infection by Ingesting *L. paraplantarum* 11-1

Use of silkworms as a surrogate animal for animal tests poses the fewer ethical, financial, and logistical problem than mammalian tests. In addition, silkworms are large enough to inject a precise amount of samples compared to other insect (Sekimizu et al., 2012). The infection model described here also has the potential to be used to study novel probiotics for *in vivo* activity against *P. aeruginosae*.

We have used the silkworm as a surrogate animal to test the probiotic effect of LAB (Nishida et al., 2016). Silkworms were fed a diet containing LAB. Silkworms fed LAB exhibited tolerance to the lethality of *P. aeruginosa* and *S. aureus* infections. Our previous results demonstrated that silkworms acquire tolerance to *P. aeruginosa* infection by ingesting a diet containing *Lactococcus lactis* or peptidoglycans of *Lactobacillus plantarum* (Miyashita et al., 2015; Nishida et al., 2016). In this study, we demonstrated that ingesting *L. paraplantarum* 11-1 extended the survival of silkworm after infection with *P. aeruginosa*. These findings suggest that activation of the innate immune system induced tolerance against microbial infection.

LAB in dairy and fermented products are expected to benefit human health. Reports on the probiotic effects of LAB in animal infection models, however, are limited. Oral administration of heat-killed *L. casei* protects against

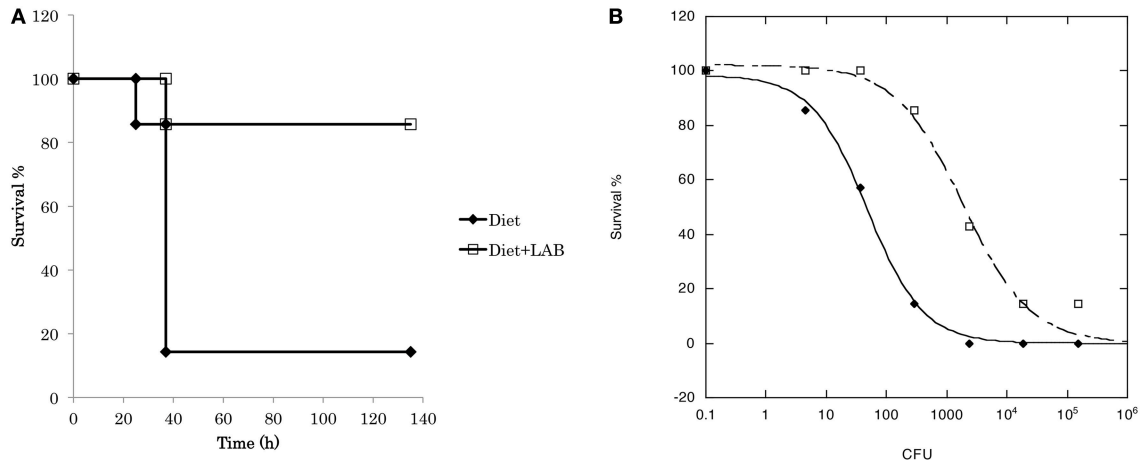

FIGURE 3 | Probiotic effect of *L. paraplantarum* 11-1 on *P. aeruginosa* infection. (A) Time course of survival of silkworms fed a diet with or without *L. paraplantarum* 11-1 viable cells (1×10^7 cfu/larva) after *P. aeruginosa* PAO1 infection. Survival of silkworms fed a diet with *L. paraplantarum* 11-1 was significantly higher than that of silkworms fed a normal diet ($p = 0.038$). **(B)** Dose response of *P. aeruginosa* PAO1 on silkworm survival after 2 days. *P. aeruginosa* PAO1 was injected into 5th instar larva fed a diet with or without *L. paraplantarum* 11-1 viable cells.

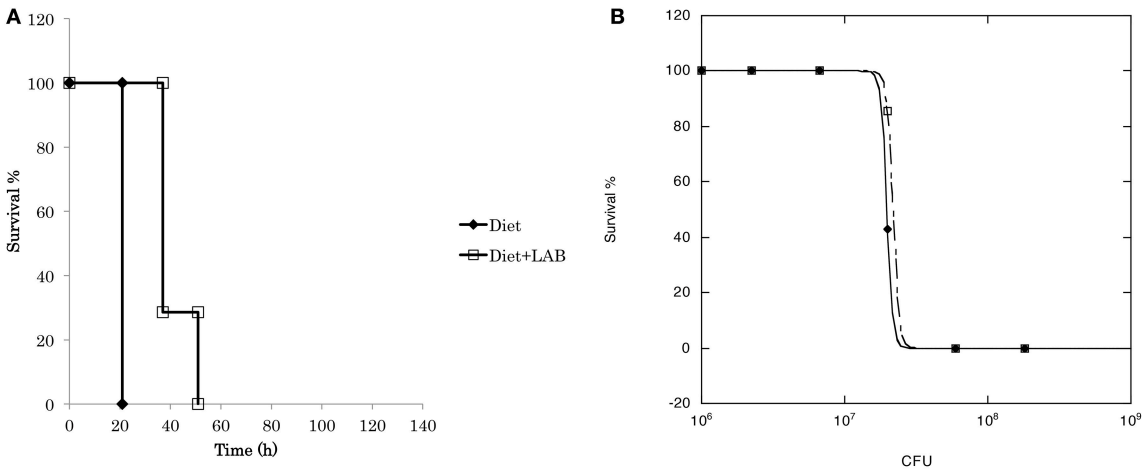

FIGURE 4 | Probiotic effect of *L. paraplantarum* 11-1 on *S. aureus* infection. (A) Time course of survival of silkworms fed a diet with or without *L. paraplantarum* 11-1 viable cells (1×10^7 cfu/larva) after *S. aureus* MSSA1 infection. Survival of silkworms fed a diet with *L. paraplantarum* 11-1 was significantly higher than that of silkworms fed a normal diet ($p = 0.030$). **(B)** Dose response of *S. aureus* MSSA1 on silkworm survival after 2 days. *S. aureus* MSSA1 was injected into 5th instar larva fed a diet with or without *L. paraplantarum* 11-1 viable cells.

P. aeruginosa infection in mice (Miake et al., 1985; Setoyama et al., 1985). *Bifidobacterium longum* prevents *P. aeruginosa* gut-derived sepsis in a mouse model (Matsumoto et al., 2008). *Bifidobacterium* protects germ-free mice from *E. coli* O157 infection (Fukuda et al., 2011). Our data indicate that the silkworm is a useful model animal for evaluating the probiotic effects of LAB. *P. aeruginosa* is the most commonly isolated antibiotic-resistant Gram-negative bacteria in ventilator-assisted pneumonia. Oral administration of a probiotic delays respiratory tract colonization and infection by *P. aeruginosa* in human (Forestier et al., 2008). Searching novel probiotics would be matching potential medical needs. Further study on the prevention of *P. aeruginosa* in silkworm infection model would be required for the translation of probiotics to benefit human health.

ETHICS STATEMENT

This study was exempted by The University of Tokyo Life Science Research Ethics and Safety Committee, and Teikyo University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

SN designed and conducted the experiments, and performed data analysis. MI and YN conducted the experiment of Gram stain and SEM. SN and KS wrote the manuscript. MI, YN, SA, and YO reviewed and edited the manuscript. SN, YO, and KS revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00436/full#supplementary-material

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Lactobacillus plantarum MYS6 Ameliorates Fumonisin B1-Induced Hepatorenal Damage in Broilers

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Fumonisin B1 (FB1), a mycotoxin produced by *Fusarium* species is a predominant Group 2B carcinogen occurring in maize and maize-based poultry feeds. It is shown to be nephrotoxic, hepatotoxic, neurotoxic, and immunosuppressing in animals. In this study, we report the ameliorating effects of a probiotic strain, *Lactobacillus plantarum* MYS6 on FB1-induced toxicity and oxidative damage in broilers. A 6-week dietary experiment consisting of 48 broilers was performed in six treatment groups. Probiotic treatment (10^9 cells/mL) involved pre-colonization of broilers with *L. plantarum* MYS6 while co-administration treatment involved supplementation of probiotic and FB1-contaminated diet (200 mg/Kg feed) simultaneously. At the end of the treatment period, growth performance, hematology, serum biochemistry, and markers of oxidative stress in serum and tissue homogenates were evaluated in all the broilers. The histopathological changes in hepatic and renal tissues were further studied. The results demonstrated that administration of *L. plantarum* MYS6 efficiently improved the feed intake, body weight and feed conversion ratio in broilers. It mitigated the altered levels of hematological indices such as complete blood count, hemoglobin, and hematocrit. Serum parameters such as serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, creatinine, cholesterol, triglycerides, and albumin were significantly restored after administering the probiotic in FB1-intoxicated broilers. Additionally, *L. plantarum* MYS6 alleviated the levels of oxidative stress markers in serum and tissue homogenate of liver. The histopathological data of liver and kidney further substantiated the overall protection offered by *L. plantarum* MYS6 against FB1-induced cellular toxicity and organ damage in broilers. Our results indicated that co-administration of probiotic along with the toxin had better effect in detoxification compared to its pre-colonization in broilers. Collectively, our study signifies the protective role of *L. plantarum* MYS6 in ameliorating the FB1-induced toxicity in the vital organs and subsequent oxidative stress in broilers. The probiotic *L. plantarum* MYS6 can further be formulated into a functional feed owing to its anti-fumonisin attributes and role in mitigating FB1-induced hepatorenal damage.

Keywords: Fumonisin B1, *Lactobacillus plantarum*, poultry, oxidative stress, hepatotoxicity, nephrotoxicity

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INTRODUCTION

Fumonisin B1 (FB1), a potentially hazardous mycotoxin, produced mainly by *Fusarium verticillioides* and *F. proliferatum*, is a common contaminant of maize-based poultry feeds contributing to the unpalatability of feed and reduction in nutrient quality. It affects the alimentary value and organoleptic characteristics of feeds resulting in decreased feed intake and animal performance. FB1 entails the risk of mycotoxicoses and other major diseases in farm animals such as poultry (nephrotoxic and immunosuppressing effects), horses (leukoencephalomalacia), swine (pulmonary edema), cattle etc (Fandohan et al., 2005). High exposure to FB1 is hepatotoxic, and also causes lesions in gastrointestinal tract (Escriva et al., 2015). FB1 has been epidemiologically associated with cancer in humans and classified as Group 2B carcinogen by the International Agency for Research on Cancer (International Agency for Research on Cancer [IARC], 2002). The molecular aspects of FB1-induced toxicity are poorly understood, however, the downstream toxic cellular mechanisms of FB1 have been deduced to be complex involving many molecular sites. Studies have ascribed FB1-induced toxicity to the structural similarity between fumonisins and the sphingoid bases (sphinganine and sphingosine) of sphingolipid layer in cell membrane. FB1 inhibits the synthesis of ceramide by specifically binding to the sphinganine and sphingosine *N*-acetyltransferase enzymes thus deregulating the sphingolipid complex formation (Merrill et al., 2001; Riley et al., 2001; Enongene et al., 2002). This leads to the intracellular accumulation of sphingoid bases which further mediate cytotoxicity, apoptosis, cell proliferation, carcinogenicity, DNA damage (Riley et al., 2001; Voss et al., 2007), and oxidative stress (Poersch et al., 2014; Abdellatef and Khalil, 2016). In addition, Domijan and Abramov (2011) demonstrated that FB1 inhibited the complex 1 of mitochondrial electron transport chain in the cell cultures of rat primary astrocytes and human neuroblastoma (SH-SY5Y). This led to a reduction in the rate of mitochondrial and cellular respiration, depolarization of mitochondrial membrane, over production of reactive oxygen species (ROS) in mitochondria, and deregulation of calcium signaling.

Safe elimination of fumonisins from feeds and/or poultry is of paramount importance as the poultry sector suffers great economic losses due to fumonisins compared to other livestock industries. The most commonly employed detoxification method in poultry industry is the use of mycotoxin binders in feed. Nevertheless, a much promising alternative strategy would be the use of microorganisms such as lactic acid bacteria (LAB) having the potential to detoxify fumonisins. LAB constitutes an important group of probiotic organisms used as dietary supplements for humans and animals. Probiotic strains of LAB should be capable of adhering to the host intestinal epithelial cells and surviving in the gastrointestinal conditions. Beneficial aspects of probiotic LAB include increased feed conversion (Cenesiz et al., 2008), enhancement of host immune system, competitive exclusion of pathogens (Chiu et al., 2007), binding of toxic compounds (Niderkorn et al., 2007), synthesis of pathogen inhibitory metabolites (Todorov et al., 2008; Gerez et al., 2013), and mitigation of stress conditions (Poersch et al., 2014; Abdellatef and Khalil, 2016).

Poultry farming is one of the major agricultural sectors in India contributing greatly to the economy. The majority of studies on mycotoxins from India are aflatoxin-oriented, with less attention being paid to fumonisin B1 contamination in poultry feeds and their toxicity in broilers. A few studies from India demonstrated FB1 toxicity in Japanese quail (Asrani et al., 2006; Sharma et al., 2008). In the present study, we evaluated the *in vivo* potential of a probiotic LAB strain (previously characterized in our laboratory) in ameliorating the FB1-induced toxicity and oxidative stress in broilers.

TABLE 1 | Feed composition of the basal diet during pre-starter, starter, and finisher adopted in the experiment.

Ingredients	Pre-starter (%)	Starter (%)	Finisher (%)
Maize	55.35	59.08	63.44
Soya meal	35	32.5	27.5
Rice polish	4.8	3	3
Rice bran oil	1	2	3
Limestone powder	1.3	1.14	1.1
Digestible crude protein	0.8	0.64	0.4
Salt	0.42	0.37	0.28
Amino acids	0.51	0.48	0.45
Other supplements	0.82	0.79	0.84

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were of the highest purity grade available commercially. Dihydrodichlorofluorescein diacetate (DCFDA) and 4-(2-hydroxyethyl) 1-piperazine ethane sulfonic acid (HEPES) were obtained from Sigma (St. Louis, United States). 2, 4-dinitrophenylhydrazine (DNPH), homovanillic acid (HVA), thiobarbituric acid (TBA), and all other chemicals and solvents were purchased from Sisco Research Laboratories (Mumbai, India). Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), albumin, triglycerides, creatinine, and cholesterol commercial kits were purchased from Swemed Diagnostics (Bengaluru, India).

Diet Preparation and Probiotic Strain

FB1 test diets were prepared using commercially available broiler feed (basal diet; Shresta feeds, Bengaluru). The basal diet composition is given in **Table 1**. Culture jars containing 1 kg of broiler feed ($a_w = 1$, autoclaved at 121°C for 20 min) were inoculated with 20 mL of toxigenic *Fusarium verticillioides* MTCC 1848 (10^6 spores/mL). The jars were shaken thoroughly to ensure complete dispersal of the fungal spores and incubated in dark for 45 days at 28°C ± 2°C. After incubation, jars were dried in hot air oven at 60°C for 8–10 h. The dried feed mixture was ground in a blender to a fine meal. The finely ground feed

TABLE 2 | Treatment groups of *in vivo* study.

Experimental groups	Treatment
Control (C)	Basal diet + PBS control
Positive Control (TOXB)	200 mg FB1/kg feed + 1.0 g/kg feed toxin binder
Group TOX	200 mg FB1/kg feed
Pre-colonization study	
Group LP	10^9 cells/mL *Lactobacillus plantarum* MYS6
Group LP+TOX	200 mg FB1/kg feed + 10^9 cells/mL *L. plantarum* MYS6
Challenge study	
Group TOX_LP	200 mg FB1/kg feed + 10^9 cells/mL *L. plantarum* MYS6

mixture (0.4 g) was taken in a sterile amber vial and suspended in 2.0 mL acetonitrile:water (1:1) and allowed for equilibration overnight in a gel rocker at 28°C ± 2°C. The extracts were syringe filtered using 0.45 μm nylon membrane filters and subjected to liquid chromatography/mass spectrometry (LC/MS) (Waters Acquity/ Synapt G2, United States). Chromatographic separation was achieved on a C18 column maintained at 50°C. Mobile phase A was 0.3% formic acid in water (v/v) and acetonitrile being mobile phase B. The mass spectrometer was operated in the positive electronspray ionization mode (ESI+). The limit of detection (LOD) for FB1 was 10 ng/mL and retention time was found to be 1.77 min. (Deepthi et al., 2016). The cultured broiler feed was mixed with the basal feed to obtain FB1 treatment equivalent to the ingestion of diet containing toxin concentration of 200 mg/kg feed.

The bacterial strain, *Lactobacillus plantarum* MYS6 (LpMYS6) which was previously characterized in our laboratory with respect to its probiotic and antifungal attributes (Deepthi et al., 2016) was used in this study. The bacterium was cultured in de Man Rogosa Sharpe (MRS) broth anaerobically at 37°C for 48 h. Cells were harvested by centrifugation at 8000 rpm for 10 min, washed thrice and re-suspended in phosphate buffered saline (PBS, 100 mM, pH 7.4) to a final concentration of 10^9 cells/mL.

Experimental Design and Treatment

The poultry trial was approved (UOM/IAEC/02/2013) by the Animal Ethical Committee, Department of Zoology, University of Mysore, Mysuru.

One-day old, 48 Cobb variety broiler chicks (male 27, female-21) were used in this study. The 1-day old chicks weighed in a range from 42 to 46 g and were reared in an environmentally controlled room for 42 days. Chicks were randomly distributed into six treatment groups with each treatment having eight chicks. The experimental design involving six treatment groups is described in **Table 2**. The basal diet types included pre-starter feed (0–7 days), starter feed (8–21 days), and finisher feed (22–42 days). Initial 1-week of acclimatization period at high temperature (90°C) was provided to one-day old chicks followed by vaccination on day 7. The experiment was performed in two ways: (a) pre-colonization study which started with the pre-incubation of LpMYS6 from day 8 followed by supplementation of FB1-contaminated feed mixture from day 12 and (b) challenge study involved the co-administration of chicks with LpMYS6 and FB1-contaminated feed simultaneously from day 12. One mL (10^9 cells/mL) of LpMYS6 preparation was administered daily by oral gavage. The positive control consisted of a commercially available multi-spectrum mycotoxin binder (Varishta, Bengaluru, India) that was administered to the chicks from day 12. The toxin binder (TOXB) was a combination of *Picrorhiza kurroa*, activated charcoal, hydrated sodium calcium aluminosilicate (HSCAS), mannan oligosaccharide (MOS), buffered organic acids, and antioxidants. The control (C) group was also a negative control where the chicks were provided with basal diet and PBS. All the chicks were vaccinated for Newcastle disease and Infectious Bursal Disease (IBD) on day 7 and day 14, respectively. Antibiotics and liver stimulants were not supplemented in the basal diet and the basal feed was free from aflatoxins and other major mycotoxins. All the treatment groups were provided with water and basal diet *ad libitum*.

Broiler Performance and Sampling

To evaluate the influence of FB1 and LpMYS6 on broiler performance, body weight of each bird was measured at weekly interval and feed consumption of every pen was monitored throughout the experimental period. The feed conversion ratio (FCR) was calculated for each treatment on the basis of unit feed consumption to unit body weight gain. Treatment groups were observed daily to record the morbidity/mortality. On the conclusion of experiment (day 42), broilers were submitted to pre-slaughter fasting for 18 h. Five birds from each treatment group were randomly selected, weighed, euthanized by cervical dislocation and necropsied for the excision of liver and kidney. Prior to sacrifice, blood samples were collected from the jugular vein in two sets. One set for hematological study was collected in the sterile vials containing the anticoagulant citrate dextrose (ACD) maintained in ice bath. The other set of blood samples was collected in dry sterile tubes, centrifuged at 5000 rpm for 10 min and the serum was separated and stored at −20°C until further use. The excised liver and kidney tissues were blotted free of blood, rinsed with PBS, weighed and stored at −20°C for histopathological studies. The excised liver tissues were homogenized (10% w/v) in ice-cold potassium phosphate buffer (100 mM, pH 7.4) and centrifuged at 5000 rpm for 15 min at 4°C. The resulting supernatant (total liver homogenate) was stored at −20°C for the analysis of different parameters to evaluate oxidative stress.

Determination of Hematological Parameters

Citrated blood samples of five birds from each treatment group were checked for hematological indices using a blood cell counter (ERMA PCE210, Tokyo, Japan). The following parameters were examined: hemoglobin (HGB), red blood corpuscles (RBC), white blood corpuscles (WBC), platelets (PLT), and hematocrit (HCT).

Determination of Serum Biochemical Parameters

Serum samples were screened for routine biochemical parameters such as albumin, triglycerides, creatinine, and

cholesterol. Liver function was assessed by the activities of serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT). All the above parameters were estimated according to the instructions of assay kits (Swemed Diagnostics, Bengaluru, India) using a versatile biochemistry analyzer ARTOS-SBPL/418 (Swemed Diagnostics, Bengaluru, India).

Analysis of Oxidative Stress Markers

Estimation of Reactive Oxygen Species (ROS)

The levels of endogenously generated ROS were measured in liver homogenate and serum as per the protocol described by Driver et al. (2000). In this assay, an aliquot of liver homogenate (20 μL, 0.5 mg protein) was dispensed into a 96 well microtitre plate containing 170 μL of Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$, pH 7.4). To this mixture, 10 μL of DCFDA (10 μM) was added and incubated at room temperature for 30 min. After incubation, fluorescence was measured using a multimode plate reader (Thermo Scientific, United States) with excitation and emission at 480 nm and 530 nm, respectively. To measure the ROS levels in serum, the same assay was followed but the liver homogenate was replaced with 10 μL of serum. Background fluorescence was corrected by the inclusion of parallel blanks. A dichlorofluorescein standard curve was used to quantify ROS levels and the data were expressed as pmol DCF formed per mg protein.

Estimation of Hydrogen Peroxide Level

Another well-known oxidative stress marker, hydrogen peroxide (H_2O_2) was quantified in liver homogenate and serum as per the method followed by Botsoglou et al. (2010) with minor modifications. Here, 20 μL (0.5 mg protein) of liver homogenate was dispensed into 96 well microtitre plate containing HEPES buffered saline (HBS, 145 mM NaCl, 10 mM HEPES, 10 mM glucose, 5 mM KCl, 1 mM $MgSO_4$, pH 7.4) and incubated with 100 μM HVA at room temperature for 45 min. After incubation, reaction mixture was excited at 312 nm and fluorescence was measured at 420 nm. The H_2O_2 levels in serum samples were measured by using 10 μL serum as incubation mixture instead of liver homogenate. The H_2O_2 levels were expressed as nmol H_2O_2 per mg protein.

Assessment of Lipid Peroxidation (LPO)

Lipid peroxidation (LPO) status was assessed in liver homogenate and serum samples by measuring thiobarbituric acid reactive substances (TBARS) and was expressed in terms of malondialdehyde (MDA) content, as described by Ohkawa et al. (1979). In this assay, tubes containing 1.5 mL acetic acid (20% v/v, pH 3.5), 0.2 mL SDS (8% w/v), 1.5 mL TBA (0.8% w/v) were added with 200 μL of test sample (liver homogenate/serum). The reaction mixture was incubated in a boiling water bath for 45 min. After cooling to room temperature, 3 mL of butanol was added to extract adducts formed and were centrifuged at 2000 rpm for 10 min. The absorbance was measured in the supernatant at 532 nm and results were expressed in terms of malondialdehyde equivalents as nmol MDA formed per mg protein.

Measurement of Protein Carbonyl Content (PCC)

Protein carbonyl content (PCC) was estimated in the liver homogenate and serum samples using DNPH following the method described by Levine et al. (1990). Two hundred microliter of sample (liver homogenate/serum; 0.5–1 mg protein) was added into eppendorf tube containing 500 μL of 10 mM DNPH in 2 N HCl and incubated at room temperature for 1 h with intermittent shaking. Corresponding blank was maintained by adding only 2 N HCl to the sample. After incubation, 500 μL 20% TCA was added to the mixture to precipitate proteins followed by centrifugation at 5000 rpm for 10 min. The precipitate was washed twice with acetone and finally suspended in 1 mL of Tris buffer (20 mM, pH 7.4 containing 140 mM NaCl and 2% SDS w/v), vortexed and incubated overnight at 4°C. The absorbance of the mixture was read at 360 nm and expressed as nmol of carbonyl groups per mg protein.

Histopathology

The formalin-fixed tissue samples of liver and kidney were processed routinely, sectioned at 4–5 μm thickness, stained with hematoxylin-eosin dye and observed and photographed using Olympus Bx41 microscope equipped with ProgRes CT3 camera (Tokyo, Japan).

Protein Estimation

The protein concentration of liver homogenate was estimated by the protocol of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard.

Statistical Analysis

The data obtained in this study are the mean of five determinations expressed as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test for multiple comparison, with following probability $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ to be considered statistically significant. The graphs were drawn using GraphPad Prism version 5.03 software (GraphPad Software Inc.).

RESULTS

Growth Performance

On completion of 42 days of feeding experiment, broilers treated with only FB1-contaminated diet (TOX) showed a feed intake 2.725 Kg when compared to the control group (2.948 Kg). Also, FCR was found to be 1.741 ± 0.06 when compared to the control treatment (1.965 ± 0.07). There was no significant difference in the body weight of FB1-intoxicated broilers (TOX) when compared to control. Broilers of pre-colonization treatment (LP and LP+TOX) showed a feed intake of 2.787 and 2.855 Kg, respectively, when compared to the TOXB group (2.933 Kg) and showed a FCR ranging from 1.845 ± 0.27 and 1.860 ± 0.07 when compared to

TABLE 3 | Effect of *L. plantarum* MYS6 and fumonisin B1-supplemented feed on growth performance of broilers after 42 days of feeding experiment.

Groups	Body weight (Kg)	Feed intake (Kg)	FCR	Mortality
C	1.509 ± 0.05	2.948	1.965 ± 0.07	0
LP	1.614 ± 0.17	2.787	1.845 ± 0.27	0
TOX	1.575 ± 0.05	2.726	1.741 ± 0.06	0
LP+TOX	1.545 ± 0.06	2.855	1.860 ± 0.07	0
TOX_LP	1.918 ± 0.01[a]*	2.902	1.507 ± 0.00[b]*	0
TOXB	1.659 ± 0.05	2.933	1.777 ± 0.06	0

*Data are the mean ± SEM of five broiler birds per treatment group. *P < 0.05; [a]Significant when compared to 200 mg toxin control, [b]Significant when compared to TOXB group.*

that of TOXB group (1.777 ± 0.06). Our study showed no significant changes of body weight in the broilers of treatment groups. The body weight, feed consumption and FCR of each treatment are tabulated in **Table 3**. No mortality was recorded in any of the treatment groups during the experiment. Broilers fed with FB1 concentration alone, suffered from dysentery 5 days post toxin treatment (TOX) until the end of experiment. While the broilers of TOXB group and challenge study witnessed recurrent episodes of diarrhea during the experiment. It is important to note that, groups of pre-colonization study administered with LpMYS6 alone and along with toxin did not show any signs of illness throughout the experiment.

Postmortem examination of organs revealed yellowish discoloration of liver in broilers fed with 200 mg FB1-contaminated diet, and also witnessed atrophy of liver (**Figure 3i**). Further, broilers of other experimental groups showed dark brown-enlarged liver when compared to normal sized liver of control group (**Figures 3a,e,m,q**). The effects of TOX on relative organs weight are presented in **Table 4**. Increase in the mean weight of liver of 3.86 ± 0.16 g/100g body weight was observed in broilers fed with 200 mg FB1-contaminated diet (TOX) compared to that of the control (2.84 ± 0.13 g/100g body weight). With respect to mean weight of kidney, substantial differences were not observed in the treatment groups in comparison to the control.

TABLE 4 | Relative organ weights of control and treatment broilers after 42 days of feeding experiment.

	Relative organ weights (g/100g body weight)	
Groups	Liver	Kidney
C	2.84 ± 0.13	0.08 ± 0.01
LP	3.04 ± 0.35[a]*	0.10 ± 0.00
TOX	3.86 ± 0.16[b]**	0.13 ± 0.01
LP+TOX	3.69 ± 0.11	0.11 ± 0.00
TOX_LP	3.20 ± 0.09	0.11 ± 0.00
TOXB	3.61 ± 0.15	0.09 ± 0.03

*Data are the mean ± SEM of 5 broiler birds per treatment group. *P < 0.05; [a]Significant when compared to toxin control. **P < 0.01; [b]Significant when compared to control.*

Hematological Indices

FB1 toxicity in broilers fed with 200 mg toxin damaged the hematological parameters. **Table 5** summarizes the effects of FB1 concentration and LpMYS6 on the hematological indices of broilers. The WBC and PLT count in the control group were 35420 ± 3860 cells/μL and 12000 ± 700 cells/μL, respectively. While 200 mg toxin fed broilers showed a count of WBC up to 40860 ± 850 cells/μL and recorded 9000 ± 410 cells/μL of platelet count. Further, broilers treated with LpMYS6 alone revealed a lesser count in WBC compared to all other groups including control. In case of TOXB group, challenge study and TOX of pre-colonization study, a slight non-significant increase in WBC count with that of control was observed revealing no much differences within these groups. With respect to platelet count, treatment group which received LpMYS6 alone showed no differences when compared to control. Additionally, platelet count was slightly reduced in TOXB group, challenge study and FB1 treatment group (200 mg) of pre-colonization study when compared to the control.

Broilers fed with diet containing 200 mg FB1 concentration alone revealed a reduced (*$P < 0.05$) RBC count, hemoglobin concentration and per cent HCT. Broilers of pre-colonization study fed with only LpMYS6, TOXB group and challenge study showed values close to that of control. However, TOX in broilers pre-colonized with LpMYS6 reestablished the RBC count and hemoglobin concentration while the per cent HCT remained unchanged.

Serum Biochemistry

Forty-two days post-feeding, all the biochemical parameters augmented significantly ($P < 0.001$) in the serum of broilers fed with 200 mg FB1 independently compared to control (**Figure 1**). SGOT and SGPT, were high in the TOX fed with 200 mg FB1 only (**Figures 1A,B**). Treatment with TOXB also caused a significant increase in the levels of SGOT ($P < 0.001$) and SGPT ($P < 0.05$) when compared to control (**Figures 1A,B**). In contrast, the SGOT and SGPT levels were significantly reduced in the TOX (LP+TOX) of pre-colonization study administered with LpMYS6. It is notable that, in the challenge study, LpMYS6 could significantly ($P < 0.001$) alleviate the augmented serum levels of SGOT and SGPT in comparison with TOXB group and pre-colonization study (**Figures 1A,B**).

Damage to kidney was assessed by creatinine level and is depicted in the **Figure 1C**. Here, an increase in the creatinine concentration was observed in the TOXB group and 200 mg FB1-fed groups when compared to the control. In pre-colonization and challenge study, LpMYS6 significantly ($P < 0.001$) lowered the elevated levels of creatinine, however, the reduction level had no much difference within the treatment groups (**Figure 1C**). Broilers fed with FB1 toxin alone showed a significantly ($P < 0.001$) high triglyceride, cholesterol and albumin levels in the serum compared to control (**Figures 1D–F**). TOXBs and LpMYS6 each in combination with toxin lowered slightly the high levels of triglyceride and cholesterol (**Figures 1D,E**). While in case of albumin, a significant decline was noted in

TABLE 5 | Effect of *L. plantarum* MYS6 and fumonisin B1 supplemented feed on hematological indices of broilers after 42 days of feeding experiment.

Groups	WBC10^3/μL	RBC10^6/μL	Hbg/dL	HCT%	PLT10^3/μL
C	35.42 ± 3.86	2.25 ± 0.04	9.94 ± 0.18	29.16 ± 1.96	12.0 ± 0.70
LP	33.3 ± 4.85	2.09 ± 0.39	9.1 ± 1.47	28.52 ± 6.03	12.0 ± 1.94
TOX	40.86 ± 0.85	1.52 ± 0.17[a*]	7.16 ± 0.42[b*]	23.6 ± 0.07	9.0 ± 0.41
LP+TOX	38.04 ± 0.97	1.94 ± 0.09	8.40 ± 0.23	24.04 ± 1.06	10.0 ± 0.31
TOX_LP	38.58 ± 1.22	2.07 ± 0.06	9.2 ± 0.08	26.12 ± 0.66	10.0 ± 0.31
TOXB	38.9 ± 1.17	1.9 ± 0.08	8.64 ± 0.30	28.46 ± 3.15	9.2 ± 0.66

*Data are the mean ± SEM of five broiler birds per treatment group. *P < 0.05;* [a,b]*Significant when compared to control. WBC, white blood cells, RBC, red blood cells, Hb, hemoglobin, HCT, hematocrit, PLT, platelets.*

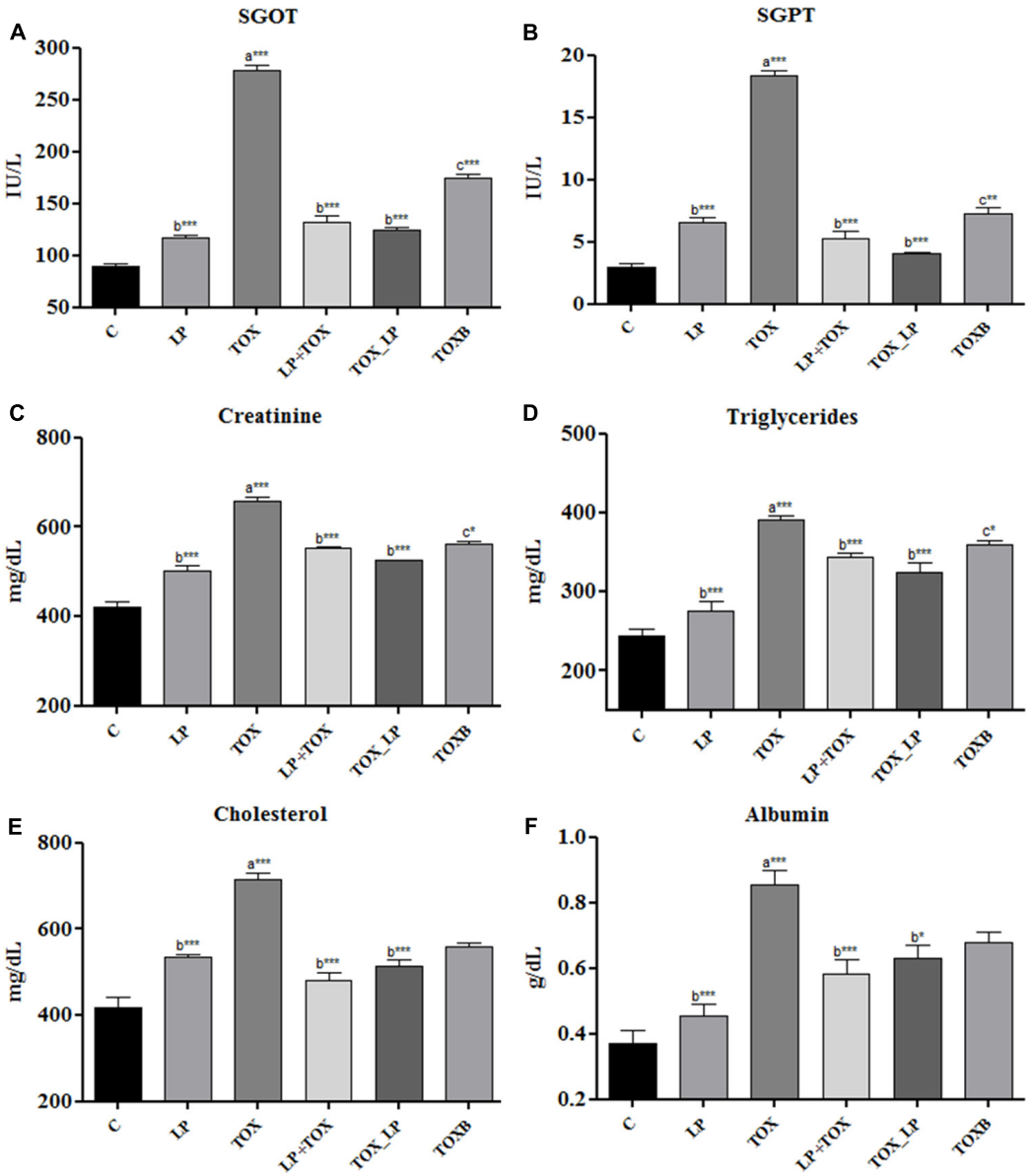

FIGURE 1 | Effect of fumonisin B1, *Lactobacillus plantarum* MYS6 and toxin binder (TOXB) on biochemical parameters of control and treatment broilers. The serum levels of **(A)** SGOT, **(B)** SGPT, **(C)** creatinine, **(D)** triglycerides, **(E)** cholesterol, and **(F)** albumin. Data are the mean ± SEM of five broiler birds per treatment group and were analyzed using one way ANOWA followed by Bonferroni *post hoc* test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; [a]significant when compared to control, [b]significant when compared to 200 mg FB1 toxin control, [c]significant when compared to challenge study).

broilers of pre-colonization and challenge study in comparison to TOXB group as well as broilers treated with FB1 alone (**Figure 1F**).

Markers of Oxidative Stress

Generation of ROS

Our study demonstrated a significantly high generation of ROS in liver homogenate and serum of broilers fed with FB1 alone when compared to the control group. The pre-colonization of broilers with LpMYS6 remarkably reduced the levels of ROS in liver homogenate indicating a better result when compared with the TOXB group (**Figure 2A**). Also, administering broilers with LpMYS6 and 200 mg FB1 simultaneously in challenge study showed a significant ($P < 0.01$) decline of ROS in liver homogenate than other TOX groups (**Figure 2A**). Additionally, pre-colonization of broilers with LpMYS6 in 200 mg FB1 treatment (LP+TOX) resulted in a notable ($P < 0.001$) decline of ROS level in serum. This was less than the ROS of control group and all the other treatment groups (**Figure 2B**).

Generation of Hydrogen Peroxide

A substantial elevation of free radical H_2O_2 was observed in the liver homogenate (**Figure 2C**) and serum (**Figure 2D**) of TOX group (200 mg FB1) in comparison with control. An equal and effective reduction by LpMYS6 was observed in H_2O_2 in the liver homogenate of pre-colonization and challenge study when compared to the TOXB group (**Figure 2C**). However, the levels of reduction in H_2O_2 were nonsignificant between the TOXB group and challenge study. Further, LpMYS6 was more effective in lowering the elevated levels of H_2O_2 in the serum samples of challenge study (TOX_LP) when compared with the LP+TOX group (200 mg FB1) and TOXB group (TOXB group) at $P < 0.001$ (**Figure 2D**).

Lipid Peroxidation

FB1-induced LPO was estimated as an increase in the concentration of MDA in liver homogenate and serum. In the FB1-alone-treated broilers, the concentration of MDA in liver homogenate (**Figure 2E**) and serum (**Figure 2F**) were significantly high compared to the control group. LpMYS6 in pre-colonization study and its co-administration with FB1 in challenge study reduced the levels of MDA both in liver homogenate (**Figure 2E**) and serum (**Figure 2F**) compared to the TOXB-supplemented group. But the reduction level of MDA in serum was observed to be non-significant in all the groups.

Protein Oxidation

Carbonyl content generated as a result of protein oxidation was significantly high in the liver homogenate (**Figure 2G**) ($P < 0.001$) and serum (**Figure 2H**) ($P < 0.01$) of broilers treated with FB1 alone as compared to control group. Compared to the TOXB, LpMYS6 was more effective in reverting ($P < 0.01$) the increased levels of carbonyl content in the liver homogenate to almost normal state as that of control (**Figure 2G**). Besides, LpMYS6 fed broilers of TOX_LP group (challenge study) significantly brought down the elevated levels of PCC in serum (**Figure 2H**). Interestingly, independent administration of LpMYS6 in LP group showed a slight non-significant increase in carbonyl content of serum compared to the TOXs of pre-colonization and challenge studies (**Figure 2H**).

Histopathology

The histopathological examination substantiated the FB1-induced toxicity in hepatic and renal parenchyma. The liver of control group showed a mild irregularity in the hepatocyte arrangement in hepatic cords (**Figures 3b,c**). Also, a mild infiltration of mononuclear cells in the periportal areas and sinusoidal space were noticed due to aging (**Figures 3c,d**). The liver of broilers treated with 200 mg FB1 alone showed severe disorganization in parenchyma compared to the control group revealing progressive stages of FB1 toxicity. Severe necrosis and edema (**Figure 3j**) of liver parenchyma was conspicuous. Biliary hyperplasia accompanied with inflammatory cells infiltration (**Figure 3k**) was evident of toxin insult. Additionally, liver tissue manifested extensive fibrosis surrounded by severe mononuclear cells infiltration suggestive of chronic inflammation (**Figure 3l**). Broilers fed with TOXBs showed a lower severity grade when compared to FB1 alone. Liver tissue of TOXB group displayed inflammatory cell infiltrates (**Figure 3f**), disorganized hepatic cords followed by hepatic necrosis (**Figure 3g**). Besides, moderate bile duct proliferation was observed in the liver of TOXB group (**Figure 3h**). The liver of broilers treated with LpMYS6 alone exhibited a mild hepatic disorganization with mild degeneration and focal to diffused infiltration of mononuclear cells (**Figures 3n–p**). However, LpMYS6 effectively reduced the degree of FB1 toxicity in broilers of pre-colonization (200 mg FB1 treatment) and challenge study. The liver displayed normal hepatocytes architecture though a mild to moderate hepatic disorganization (**Figure 3r**) was noticed. Severity of necrosis and edema was considerably attenuated (**Figure 3t**). A remarkable reduction in the focal inflammation and infiltration of mononuclear cells to the sinusoid was conspicuous (**Figure 3s**). The liver sections showed no fibrosis and biliary hyperplasia compared to the TOXB group and FB1 treatment alone.

Kidney sections of control group showed aging morphology involving normal renal glomeruli and Bowman's capsule space (**Figure 4a**). The increased cellularity of glomeruli and mild desquamation of tubular epithelium was observed (**Figure 4b**). Kidney sections of broilers fed with TOXB showed severe disruption of tubular epithelium and accumulation of granular casts in glomeruli (**Figure 4c**). Focal inflammation, cellular infiltration and mild edema were observed in the interstitium accompanied with distal tubular damage (**Figure 4d**). While broilers fed with 200 mg FB1 alone exhibited severe desquamation of tubular epithelium followed by destruction of renal parenchyma. Sections showed damaged architecture accompanied by acute inflammation with infiltration of inflammatory cells and massive edema (**Figure 4e**). Tubular degeneration proceeded by cytoplasmic vacuolation, fragmented cytoplasm and tubular lumen filled with eosinophilic deposits were frequently encountered as the indicators of renal tubule necrosis (**Figure 4f**). Besides necrosis, cystic glomeruli (**Figure 4g**) and series of renal tubule enlargement involving

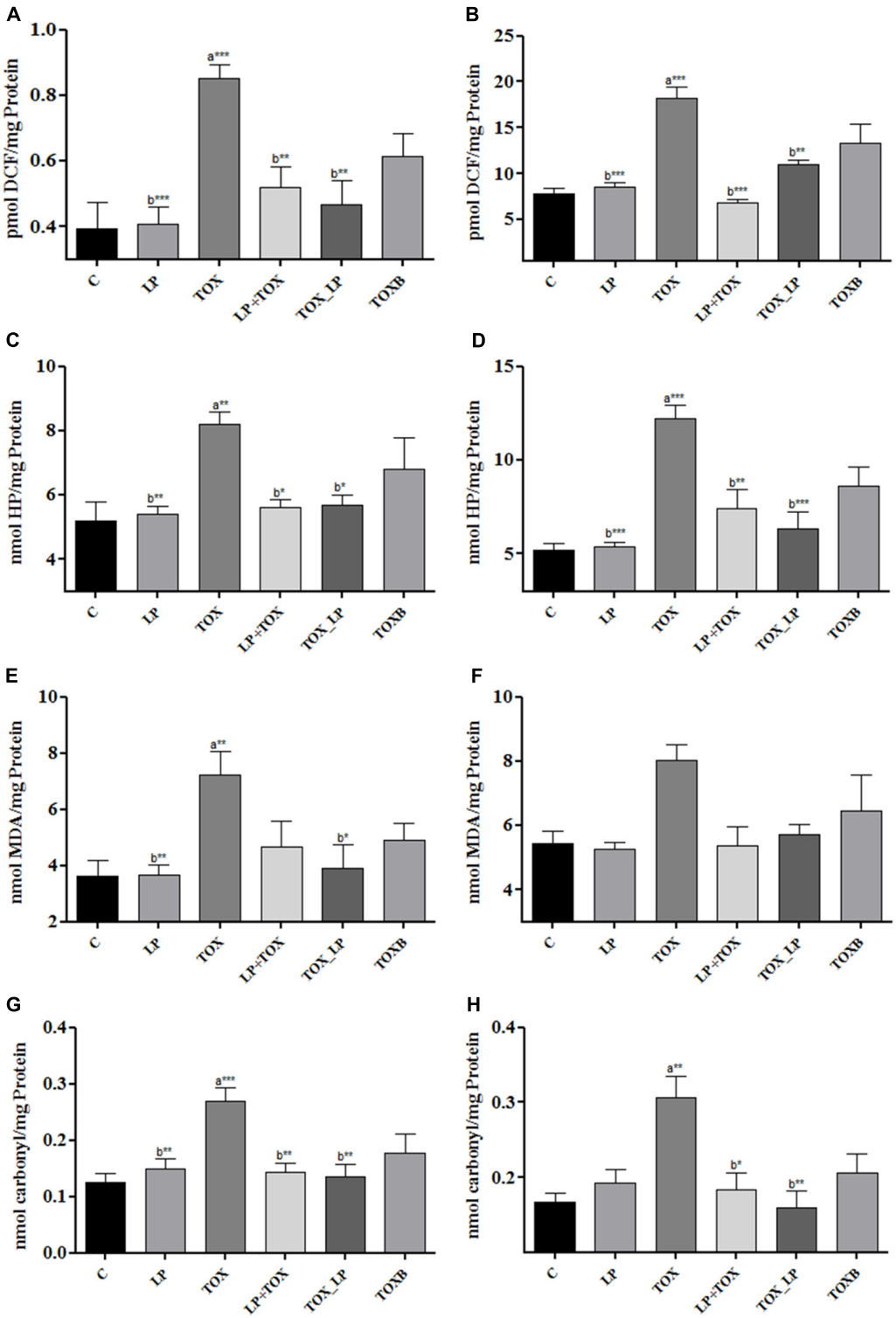

FIGURE 2 | Effect of *L. plantarum* MYS6 on fumonisin B1 induced oxidative stress in the liver homogenate and serum of control and treatment broilers. In the figure, **(A,B)** generation of ROS, **(C,D)** generation of hydrogen peroxide, **(E,F)** peroxidation of lipids, and **(G,H)** oxidation of proteins. Data are the mean ± SEM of five broiler birds per treatment group and were analyzed using one way ANOWA followed by Bonferroni *post hoc* test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; [a]significant when compared to control, [b]significant when compared to 200 mg FB1 toxin control).

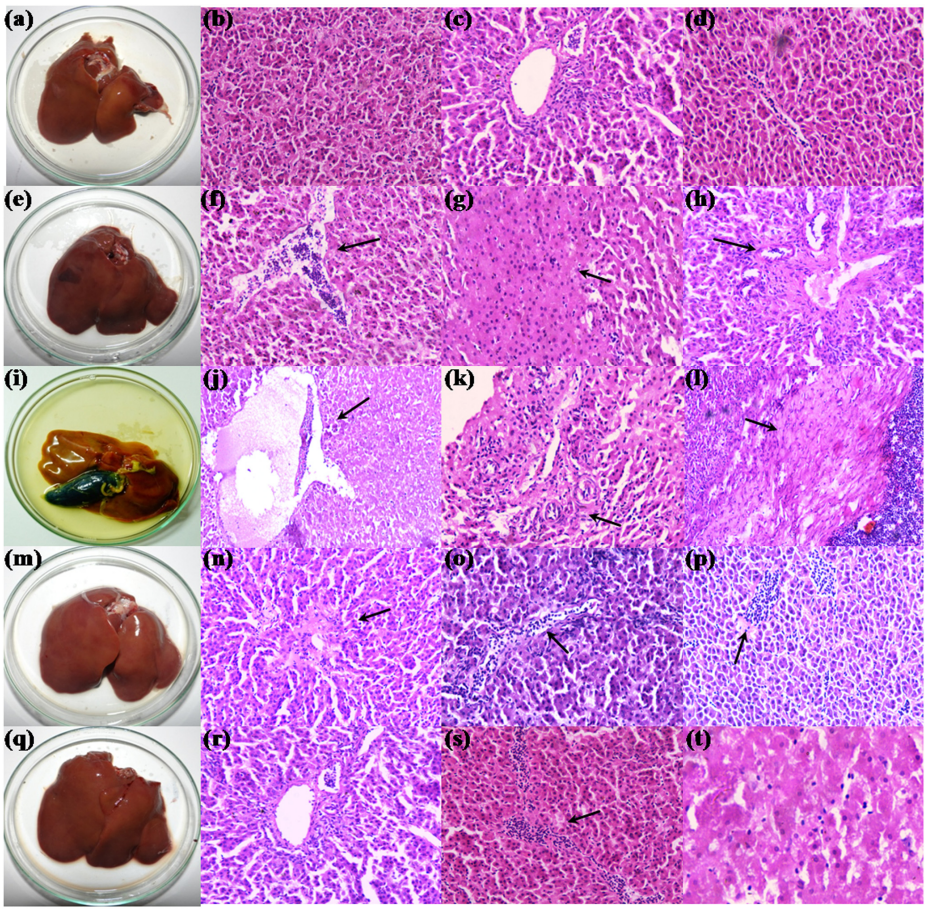

FIGURE 3 | Morphology and histopathology of hematoxylin-eosin-stained photomicrographs of liver of control and treatment broilers. **(a–d)** Control, **(e–h)** TOXB + 200 mg FB1, **(i–l)** 200 mg FB1 alone, **(m–p)** *L. plantarum* MYS6 alone, and **(q–t)** LpMYS6 + 200 mg FB1. Liver sections were observed at 400× magnification.

granular casts and vacoulation of glomeruli (**Figure 4h**) were the other morphologic severity associated with the degeneration of tissue. In contrary, kidneys of broilers treated with LpMYS6 alone depicted a normal morphology of Bowman's capsule in cortex and distal tubules yet age-related mild disruption of renal parenchyma was noticed (**Figures 4i,j**). Furthermore, treatment with LpMYS6 in pre-colonization and challenge study effectively restored the normal histology of kidney tissues. Renal tubule regeneration was clearly observed and included an array of histological changes such as karyomegaly and nuclear crowding along the affected renal tubules (**Figures 4k,l**). In addition, the severity grade of FB1 toxin was extensively reduced and no signs of edema, necrosis, cystic glomeruli, granular casts or any major abnormalities in the renal parenchyma were observed.

DISCUSSION

Fumonisin B1-induced deregulation of sphingolipid complex formation is a well-studied FB1 toxicity in animals and humans. Unlike most mycotoxins which are cyclic compounds, FB1 is a long-hydroxylated hydrocarbon chain having structural similarity to the sphingoid bases. FB1 intoxication also manifests serious consequences of oxidative stress in animals apart from the inhibition of sphinogolipid metabolism (Poersch et al., 2014). FB1 is poorly absorbed in the gastrointestinal tract unlike aflatoxins and is extensively retained as unmetabolized in the tissues such as liver and kidney. The toxin is eliminated in bile through enterohepatic recirculation and exerts its toxicity. It may also end up in the feces and as trace contaminants in urine (Shephard et al., 1994; Enongene et al., 2000). Apparently, poultry ingested with FB1 exhibit lack of appetite, low productive performance, inflamed liver, oral lesions, and immunosuppression leading to adverse health and economic status (Poersch et al., 2014). Hence protection against FB1-induced toxicity and oxidative stress in poultry becomes a prerequisite.

In our study, we observed a non-significant change in feed intake and feed conversion in the broilers treated with FB1 alone compared to control. Previous studies have reported a decrease in feed intake and feed conversion when broilers, ducks, or turkeys were ingested with feed contaminated by FB1 (Tessari et al., 2006; Benlasher et al., 2012). Further, our study showed no significant variations of body weight in broilers of treatment

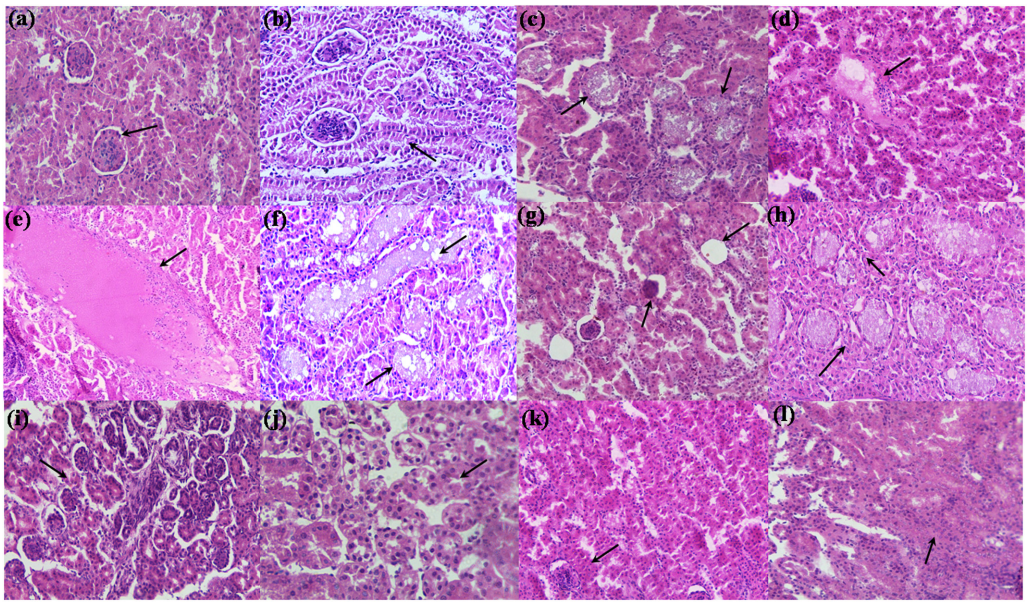

FIGURE 4 | Hematoxylin-eosin stained photomicrographs of kidney of control and treatment broilers. **(a,b)** Control, **(c,d)** TOXB + 200 mg FB1, **(e–h)** 200 mg FB1 alone, **(i,j)** *L. plantarum* MYS6 alone, and **(k,l)** LpMYS6 + 200 mg FB1. Kidney sections were observed at 400× magnification.

groups but health complications such as diarrhea and dysentery was observed in broilers fed with 200 mg FB1 per Kg feed. Earlier reports by Tran et al. (2005) and Tardieu et al. (2007) observed a reduced body weight in ducks and turkeys fed with 0–128 mg of FB1/Kg feed and 0–20 mg of FB1/Kg feed, respectively. Besides, increased relative organ weights of liver and kidney in our study marks FB1 toxicity as these organs are the sites of detoxification of many toxicants including FB1, and also the target organs for toxin effects (Voss et al., 2007). Poersch et al. (2014) also reported a significant increase in the liver weight of broilers treated with FB1 contaminated feed (100 mg/kg feed) showing its toxicity in the target organs.

With respect to hematological indices, broilers fed with 200 mg FB1-contaminated feed showed a diminished RBC count and hemoglobin ($P < 0.05$). This could be due to the toxic effects of FB1 on liver leading to haematopoietic (such as vitamins B12, folic acid, iron) suppression which eventually results in decreased synthesis of hemoglobin and erythropoietin and subsequently affect the RBC production and HCT. Our results are in accordance with the study conducted by Sobrane Filho et al. (2016), where broilers fed with diet containing a mixture of aflatoxin B1 (2 mg/kg feed) and FB1 (100 mg/kg feed) exhibited low values of erythrocyte, hemoglobin, and hematocrit compared to control while no difference was observed in the thrombocyte count. Another study by Broomhead et al. (2002) showed that the above parameters of hematology remain unaffected in broilers when treated with 25 or 50 mg/kg FB1 in diet. FB1 fed broilers in the present study showed a non-significant rise in WBC count probably due to an inflammatory response to the toxin. But Sobrane Filho et al. (2016) reported a decrease in WBC count in the broilers fed with aflatoxin B1 (2 mg/kg feed) and FB1 (100 mg/kg feed) contaminated diet. The inconsistency in the hematology results is tempting us to speculate that hematology would not be a sensitive indicator of FB1 toxicity in broilers.

Further, increased SGOT and SGPT might be the signs of damage of hepatocytes and these could be sensitive indicators of acute hepatic necrosis. In agreement with our results, Khalil et al. (2015) reported a significant increase in serum liver function markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in Sprague–Dawley rats intoxicated with 100 and 200 mg FB1. The increased levels of cholesterol and triglyceride in our study are probable indicators of stress created in broilers due to FB1 feeding affecting lipid metabolism. Also, this could be linked to decreased feed intake and disruption of sphingolipid metabolism because of the interrelationships of these pathways. Meanwhile, a rise in the albumin in FB1-challenged broilers may be due to the damage of hepatocytes and impairment of protein synthesis. A study conducted by Cheng et al. (2006) in broilers fed with 5 and/or 15 mg FB1 showed a significant increase in serum AST, albumin, and cholesterol coupled with poor immunocompetence. Besides, increased levels of creatinine in the present study could be the result of protein catabolism or kidney affliction. A previous study by Khalil et al. (2015) reported a gradual increase in renal products such as urea, uric acid, and creatinine in rats fed with 200 mg FB1 revealing apparent kidney toxicity. Hence, our results clearly signify the damaging effects of FB1 on hepatic and renal tissues.

The FB1 toxicity in broilers was further substantiated by the assessment of oxidative stress markers in serum and liver homogenate. The accumulation of free sphingolipid bases due to FB1 toxicity induces an inhibition of complex 1 of the mitochondrial respiratory chain, CYP450, and NADPH oxidase

system and therefore stimulates the generation of diverse ROS such as superoxide radical, hydrogen peroxide (Domijan and Abramov, 2011; Mary et al., 2012; Rodrigues and Gomes, 2012). The ingestion of 200 mg FB1 induced a significant increase in ROS and H_2O_2 generation, LPO marker (MDA), and carbonyl contents of protein oxidation in serum and liver homogenate in broilers. Recent studies by Theumer et al. (2010) in wistar rats and Kotan et al. (2011) in human lymphocytes indicated that generation of ROS and oxidative stress was a direct consequence of the immunotoxic effects elicited by AFB1 and FB1. Similar results on oxidative stress were observed in rats treated with AFB1 and FB1 (Hassan et al., 2014; Abdellatef and Khalil, 2016) and broiler chicks with FB1 (Poersch et al., 2014). *In vivo* studies of Wistar rats treated with 100 mg FB1, documented a significant increase in MDA level in spleen mononuclear cells, liver and kidney tissues (Theumer et al., 2010; Hassan et al., 2014). Apart from lipids, the other possible major target of oxidative damage is proteins that are further transformed into protein carbonyls. Mary et al. (2012) proposed that 48 h incubation of spleen mononuclear cells with 10 μM FB1 significantly raised the carbonyl content. Also, Domijan et al. (2007) suggested that 200 ng and 50 μg FB1 could cause oxidation of proteins in the kidney of wistar rats.

The histopathological observations of hepatic and renal tissues confirmed the abnormal serum biochemistry, oxidative stress and cellular damage induced by FB1. The intoxicated broilers marked massive destruction of hepatic and renal tissues. Tessari et al. (2006) showed severe damage of liver characterized by disorganization and megalocytosis of hepatocytes, bile duct proliferation, necrosis and inflammation and kidney sections displaying hydropic degeneration in broiler chicks exposed to 50 and 200 mg FB1. Further, Voss et al. (2007) proposed that kidney was the most sensitive organ in Sprague–Dawley and Fischer 344 rats when exposed to FB1, while in BD IX rats, liver was the main target organ.

Our study demonstrates the protective role of a probiotic strain, *L. plantarum* MYS6 (Lp MYS6) against FB1-induced toxicity and tissue damage. The LpMYS6 strain was previously characterized in our laboratory for an array of probiotic attributes, antifungal properties, FB1 detoxification by binding and extraction/purification of low molecular weight antifungal compounds (Deepthi et al., 2016). Probiotic LAB is known to act via diverse mechanisms *in vivo* which include modulation of gastrointestinal physiology by increasing the production of growth factors, competition for nutrients with enteropathogens, bioconversion of available sugars to acids, production of vitamins and organic acids, competitive exclusion for adhesive sites, beneficial immunostimulation of the gut-associated lymphoid tissue, antioxidative and anticancer/antiproliferative activities (Alberto et al., 2007; Oelschlaeger, 2010). Detoxification of mycotoxins by LAB is mainly mediated by the binding of toxin to bacterial cell and is dependent on the cell wall structural integrity of LAB.

In response to probiotic treatment, FB1-challenged broilers showed significant amelioration of toxin ill effects. Broilers of pre-colonization study exhibited a gradual increase in feed intake, body weight and FCR when compared to birds treated with toxin alone. Moreover, the broilers of challenge study showed the highest body weight among all treatment groups. Our results clearly demonstrated that LpMYS6 improved the appetite of FB1-challenged broilers thereby increasing the feed intake and body weight. A recent study by Khalil et al. (2015), reported that *L. delbrueckii* subsp. *lactis* and *Pediococcus acidilactici* significantly helped in improving the body weight and feed intake of rats after three weeks of exposure to 50, 100, and 200 mg concentrations of FB1. Also, our results are in line with the work by Gratz et al. (2006), who demonstrated that *L. rhamnosus* strain GG improved the body weight gain in rats intoxicated with AFB1. LpMYS6 was efficient in reducing the toxicity-induced weight gain in the liver, but there was no significant effect on the relative weight of kidney in all the dietary treatments. With respect to hematological parameters, LpMYS6 efficiently reestablished the RBC count and hemoglobin. The elevated levels of WBC and reduced count of PLT were restored by LpMYS6 administration. Jiang et al. (2014) showed that supplementation of yeast cell wall absorbent had a protective effect on WBC, lymphocytes, PLT, and hemoglobin in broilers fed with mycotoxin-contaminated feed. Further, oral administration of LpMYS6 significantly restored the altered levels of serum parameters such as SGOT, SGPT, creatinine, cholesterol, triglycerides and albumin, both in the pre-colonization and challenge study. The alleviated level of SGPT specifically indicates the rehabilitation of hepatocytes, and also the serum cholesterol. The reduction in elevated levels of cholesterol by LpMYS6 indicate normal lipid metabolism including sphingolipid formation and normalized feed intake. High serum creatinine is an indication of renal trauma. Our strain, LpMYS6 was efficient in reducing creatinine level which may be due to proper catabolism of dietary/tissue proteins and filtration rate in kidney. The above results indicate that LpMYS6 effectively reduced the FB1-induced hepatorenal toxicity. Khalil et al. (2015) reported that co-administration of *L. delbrueckii* subsp. *lactis* and *Pediococcus acidilactici* was efficient in normalizing ALT, AST, albumin, bilirubin levels in rats fed with 200 mg FB1-contaminated diet. Also, Jiang et al. (2014) studied that supplementation of yeast cell wall absorbent to the mycotoxin contaminated diet could significantly improve the serum levels of AST, ALT, ALP, and GGT in broiler chickens.

Furthermore, LpMYS6 effectively scavenged the elevated ROS and H_2O_2 in serum and liver homogenate. Also, the probiotic strain remarkably stabilized the altered levels of LPO and PCC in serum and tissue homogenate of liver. Administration of LpMYS6 was observed to significantly reinstate the normal morphology of liver and kidney. Deabes et al. (2012) reported a significant reduction of oxidative status in liver and kidney of aflatoxin-challenged mice treated with *L. rhamnosus* starin GG by increasing the contents of reduced glutathione (GSH) and superoxide dismutase (SOD). Further, a study by Abdellatef and Khalil (2016), demonstrated the ameliorative effects of *L. delbureckii* subsp. *lactis* DSM 20076 and *Pediococcus acidilactici* NNRL B-5627 on fumonisin B1-induced hepatoxicity and nephrotoxicity in rats.

LpMYS6 induces its protective effects probably by binding FB1 in the chicken crop or gastrointestinal tract and consequently

reducing the bioavailability of FB1. This is supported by our previous observation on the *in vitro* binding ability of *L. plantarum* MYS6 to FB1 toxin wherein the per cent removal of FB1 was 32.9% and 61.7% in 2 and 4 h of incubation time, respectively (Deepthi et al., 2016). Several studies suggest that binding is the main mechanism of detoxification of mycotoxins by LAB (Gratz et al., 2007; Hathout et al., 2011; Abbes et al., 2016; Abdellatef and Khalil, 2016). However, the binding mechanism itself is not thoroughly understood. Zoghi et al. (2014) reported that probiotic LAB binds to the toxin due to the adhesive nature of S-layer proteins in their cell wall. Shetty and Jespersen (2006) suggested that carbohydrate-rich mannoproteins or glucans of LAB are involved in their binding to mycotoxins. Recently, Huang et al. (2017) suggested that *L. plantarum* C88 could effectively detoxify aflatoxin by suppressing the expression of cytochrome P450 1A2 and CYP 3A4 to decrease the production of AFBO (exo-AFB1-8,9-epoxide) and activate GST A3 through Nrf2 signaling pathways. This in turn improves GSH-conjugating activity and reduces toxin mediated oxidative stress. The majority of studies on detoxification of mycotoxins by LAB are aflatoxin-oriented with very limited data existing for LAB-mediated amelioration of FB1-induced toxicity and oxidative stress in broilers. In this context, we made an attempt to evaluate the protective effects of a potent probiotic strain, *L. plantarum* MYS6 against FB1 toxicity and oxidative stress in broilers. Our results showed that protective effects of LpMYS6 were significantly high in challenge study when compared to pre-colonization study. This indicates that the binding of FB1 occurs immediately after administration of LpMYS6 and pre-colonization of LpMYS6 might delay or decrease the binding process. Our outcome is in line with a recent finding by Huang et al. (2017) who reported that *L. plantarum* C88 effectively binds to AFB1 within 2 h post dose in mice and excreted in high levels as fecal AFB1 and lactobacilli. Nevertheless, systemic studies are still needed to understand the precise binding mechanism of FB1 to LAB.

Toxin binders are inert indigestible adsorbents widely employed in the poultry feed industry as detoxicification method. Several studies are available suggesting the application of TOXBs such as HSCAS, clay products, bentonites, zeolites, activated carbon etc (Huwig et al., 2001; Galvano et al., 2001; Avantaggiato et al., 2004; Kabak et al., 2006; Jouany, 2007; Phillips et al., 2008; Devreese et al., 2013; Agboola et al., 2015) as an effective method to minimize mycotoxin-induced toxicity. Interestingly, we found that TOXB was less effective in sequestering the toxin. Moreover, they seemed to induce oxidative stress and organ damage to some extent in our study.

In summary, the present investigation clearly demonstrates the FB1-induced toxicity, oxidative stress and vital organ damage in broilers. Oral administration of *L. plantarum* MYS6 to broilers significantly mitigated the FB1-induced toxicity and organ damage. *L. plantarum* MYS6 also significantly reinstated the imbalanced serum biochemical parameters, oxidative markers and hepatic and renal tissue damage. *L. plantarum* MYS6 was more effective in sequestering the toxin when compared to the commercially available TOXB and had protective health benefits. The protective role of *L. plantarum* MYS6 may be due to its FB1-binding capacity thereby reducing the toxin bioavailability in broilers. To the best of our knowledge, the present study is the first of its kind in employing a probiotic lactic acid bacterium to reduce the effects of FB1-induced oxidative stress and organ damage in broilers. The future prospect of the study is to understand the changes in gut microbiota, mechanism of interaction between LAB and FB1 at molecular level and developing a functional probiotic feed for broilers.

AUTHOR CONTRIBUTIONS

MYS, KSG, and BVD conceived and designed the research; BVD, RS, KPR, and ND performed the experiments and animal work; NKD supervised animal work and necropsy; BVD, RS, NKD, KSG, and MYS analyzed the data; BVD wrote the paper and MYS edited the paper.

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Characterization and Antibacterial Potential of Lactic Acid Bacterium *Pediococcus pentosaceus* 4I1 Isolated from Freshwater Fish *Zacco koreanus*

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This study was undertaken to characterize a lactic acid bacterium 4I1, isolated from the freshwater fish, *Zacco koreanus*. Morphological, biochemical, and molecular characterization of 4I1 revealed it to be *Pediococcus pentosaceus* 4I1. The cell free supernatant (CFS) of *P. pentosaceus* 4I1 exhibited significant ($p < 0.05$) antibacterial effects (inhibition zone diameters: 16.5–20.4 mm) against tested foodborne pathogenic bacteria with MIC and MBC values of 250–500 and 500–1,000 μg/mL, respectively. Further, antibacterial action of CFS of *P. pentosaceus* 4I1 against two selected bacteria *Staphylococcus aureus* KCTC-1621 and *Escherichia coli* O157:H7 was determined in subsequent assays. The CFS of *P. pentosaceus* 4I1 revealed its antibacterial action against *S. aureus* KCTC-1621 and *E. coli* O157:H7 on membrane integrity as confirmed by a reduction in cell viability, increased potassium ion release (900 and 800 mmol/L), reduced absorption at 260-nm (3.99 and 3.77 OD), and increased relative electrical conductivity (9.9 and 9.7%), respectively. Gas chromatography–mass spectrometry (GC–MS) analysis of the CFS of *P. pentosaceus* 4I1 resulted in the identification of seven major compounds, which included amino acids, fatty acids and organic acids. Scanning electron microscopic-based morphological analysis further confirmed the antibacterial effect of CFS of *P. pentosaceus* 4I1 against *S. aureus* KCTC-1621 and *E. coli* O157:H7. In addition, the CFS of *P. Pentosaceus* 4I1 displayed potent inhibitory effects on biofilms formation by *S. aureus* KCTC-1621 and *E. coli* O157:H7. The study indicates the CFS of *P. pentosaceus* 4I1 offers an alternative means of controlling foodborne pathogens.

Keywords: ***Pediococcus pentosaceus* 4I1, *Zacco koreanus*, foodborne pathogens, antimicrobial action, cell free supernatant**

INTRODUCTION

Lactic acid bacteria (LAB) have proven efficacies for food preservation and enhance the nutritive quality of a variety of fermented food products (Gad et al., 2016). Primarily, the LAB exert their antimicrobial effects by producing lactic acid, which increases the acidity of their environment, thereby resulting in the loss of the viabilities of pathogenic bacteria (Ammor et al., 2006;

Gad et al., 2016). Low molecular weight compounds, such as, hydrogen peroxide, carbon dioxide, diacetyl (2,3-butanedione), and bacteriocins also contribute to antimicrobial effects of LAB (Ammor et al., 2006; Sankarankutty and Palav, 2016), as they substantially inhibit the growths of pathogenic bacteria in food system. Nowadays, consumers show considerable interest in the use of LAB as natural additives in food industry due to their generally recognized as safe (GRAS) and antimicrobial effects (Parada et al., 2007; Djadouni and Kihal, 2012).

Recently, severe concerns have been expressed regarding the increasing incidences of diseases associated with foodborne pathogens (Oussalah et al., 2007; da Silveira et al., 2012; Gad et al., 2016). Although adequate biopreservation techniques can prevent food contamination by pathogens, food processors are frequently confronted by situations involving food contamination by pathogenic microorganisms (Runyoro et al., 2010). In addition, the increased resistance shown by pathogenic microbes to commercial antibiotics (Manzoor et al., 2016) has generated much interest in the identification of new classes of natural antibiotics that are capable of combating hazardous pathogens (Millitello et al., 2011).

Recent reports confirm wide consumer acceptance of the use of natural preservatives in foods and the need to reduce the amounts of synthetic additives used (Millitello et al., 2011; da Silveira et al., 2012). Although synthetic additives have been used to retard the growths of foodborne pathogens, they pose serious threats to human health (Carocho et al., 2015). On the other hand, LAB diminish foodborne pathogen proliferation and offer consumers safer and contamination free food products (Callewaert et al., 2000; Mataragas et al., 2003). Furthermore, a number of studies have demonstrated the remarkable efficacies of LAB to control foodborne pathogens *in vitro* and *in vivo* (Callewaert et al., 2000; Mataragas et al., 2003; Ammor et al., 2006; Manzoor et al., 2016).

The present study was undertaken to isolate and characterize a lactic acid bacterium *Pediococcus pentosaceus* 4I1 from *Zacco koreanus*, a freshwater fish, and to examine its antibacterial mechanistic action on selected foodborne pathogens.

MATERIALS AND METHODS

Media, Reagents, and Test Sample Preparation

Bromocresol purple (BCP) agar medium was used for the culture-based initial screening of LAB, whereas nutrient broth (NB) medium was used to cultivate pathogenic bacteria. Both media were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). The de Man, Rogosa, and Sharpe (MRS) agar medium used to isolate and culture LAB was purchased from Difco (USA). Other chemicals and reagents used were of high purity. Spectrophotometric measurements were made using an enzyme-linked immunosorbent assay (ELISA) instrument. A 18∼24 h grown culture of *P. pentosaceus* 4I1 was centrifuged followed by freeze-drying and desired test concentrations of its cell free supernatant (CFS) were prepared in double-distilled sterilized water.

Foodborne Pathogens

The foodborne pathogens tested were *Salmonella enterica* ATCC-4731, *Staphylococcus aureus* KCTC-1621, *Listeria monocytogenes* KCTC-3569, *Bacillus subtilis* KCTC-3569, and *Escherichia coli* O157:H7. Test pathogens were cultured in NB medium and incubated at 37°C. For regular experiments, cultures were maintained on nutrient agar (NA) medium and stored at 4°C.

Sample Collection and Isolation of *P. pentosaceus* 4I1

Zacco koreanus specimens were collected from a local river using catch per unit effort (CPUE) methods following taxonomic identification (Kim and Park, 2002) and isolation procedure of LAB (Cho et al., 2013). Initial screening was performed using the agar spot test on BCP agar medium (Trias et al., 2008). Selected LAB working cultures were then routinely grown in MRS broth and stored as stock cultures in MRS broth containing 15% glycerol (cryoprotective agent) in cryovials at −20°C. Ethical approval regarding "Animal Care and Use" was secured beforehand from the ethical committee of Daejeon National Science Museum Daejeon, South Korea with an approval # NSMD-MSIFP-KOR208. Experiments were performed in accordance with the relevant guidelines and methods.

Morphological, Biochemical, and Molecular Characterization of 4I1

Morphological, biochemical, and molecular characterizations of 4I1 were performed as we previously described (Bajpai et al., 2016a). In brief, 4I1 was identified by gram-staining and based on microscopic observations of colony shapes and cell morphologies (Holt et al., 1994). Further, 4I1 was characterized biochemically using API 50 CHL strips with API 50 CHL medium at the species level according to the manufacturer's instructions (API 50 CHL, BioMerieux, France).

Partial 16S rRNA gene sequencing analysis was used to characterize 4I1 at the molecular level. Briefly, genomic DNA was isolated from 4I1 and then the 16S rRNA gene was amplified by PCR (Bajpai et al., 2016b). PCR reactions were carried out using a Biometra thermal cycler (M Biotech, Inc., Canada) with the following cycle parameters: an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 1 min. The PCR products were sequenced and analyzed, and gene sequences obtained were compared in the National Center for Biotechnology Information (NCBI) for homology using BLAST and multiple-aligned with 16S rRNA gene sequences of different strains for similarity using the ClustalW program coupled with MEGA 5. The neighbor-joining method was used to construct the phylogenic tree using MEGA 5 software.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

A detailed analysis of the chemical composition of the CFS of *P. pentosaceus* 4I1 was performed as described by Sjögren et al. (2003) using a gas chromatography–mass spectrometry (GC/MS) system (Jeol JMS 700 mass spectrometer, Agilent

6890N, Agilent Technologies, Santa Clara, CA, USA) equipped with a fused silica capillary column (30 m length × 0.25 mm ID × 0.25 μm film thickness). The GC–MS conditions used were as previously described (Bajpai et al., 2013). Relative proportions of the extract constituents were expressed as percentages by peak area normalization. Extract components were identified based on GC retention times and computer matching of mass spectra using the Wiley and National Institute of Standards and Technology Libraries for the GC–MS system used.

Determination of the Effect of 4I1 CFS on Antibacterial Activity

The standard agar well-diffusion method was used to determine the antibacterial efficacy of 4I1 CFS (Murray et al., 1995). To obtain the CFS of 4I1, supernatant from a 24 h grown culture of 4I1 was collected by centrifugation (8,000 × *g*; 10 min) and freeze-dried (lyophilized) (Saadatzadeh et al., 2013). Briefly, NA medium (20 mL) was poured into Petri-plates and allowed to solidify. Plates were then dried and 1 mL of standardized bacterial inoculum (10^7 CFU/mL) was poured and uniformly spread onto agar surfaces, and then allowed to stand for 5 min. Wells were made in the agar by using a sterilized borer and 100 μL CFS of *P. pentosaceus* 4I1 was poured into each well against each of the tested pathogen. Negative controls were prepared using the same medium (sterilized distilled water or MRS medium) employed to dissolve the samples. Antibacterial activities were evaluated by measuring the diameters of zones of inhibition (including diameter of well: 6 mm) against the tested bacteria. All assays were performed in triplicate.

Determination of the Effect of 4I1 CFS on Minimum Inhibitory (MIC) and Minimum Bactericidal (MBC) Concentrations

The MICs of CFS of *P. pentosaceus* 4I1 were determined using the twofold serial dilution method (Bajpai et al., 2013). Freeze-dried CFS of *P. pentosaceus* 4I1 (4 mg) was first dissolved in 1 mL distilled water as stock, and incorporated into NB medium to an initial concentration of 2,000 μg/mL, and then was serially diluted to 1,000, 500, 250, 125, 62.5, 31.25, 15.62, and 7.81 μg/mL concentrations of the CFS of 4I1. A 10 μL standardized bacterial suspension of each tested pathogen (10^7 CFU/mL) was transferred to each tube. The treatment and control tubes which contained only bacterial suspensions were incubated at 37°C for 24 h. The lowest concentration of CFS, which did not show any visible growth of tested organisms after macroscopic evaluation, was determined as MIC, and was expressed in μg/mL. Further, the concentrations showing complete inhibition of visual growth of bacterial pathogens were identified, and 50 μL of each culture broth was transferred onto the agar plates and incubated at 37°C for 24 h. The complete absence of growth of bacterial colonies on the agar surface is the lowest concentration of the sample and was defined as MBC. Each assay in this experiment was replicated three times.

Determination of the Effect of 4I1 CFS on Pathogen Viabilities

Freshly grown bacterial colonies of the selected pathogenic bacteria were inoculated in NB medium at 37°C for 24 h, and then bacterial cultures were serially diluted to 10^7 CFU/mL (Shin et al., 2007). To determine the effect of CFS of *P. pentosaceus* 4I1 on cell viabilities, two selected foodborne pathogenic bacteria, *S. aureus* KCTC-1621 and *E. coli* O157:H7 were used. Briefly, each of the tubes containing bacterial suspension (10 μL; approximately 10^7 CFU/mL) of *S. aureus* KCTC-1621 and *E. coli* O157:H7 was inoculated with 100 μL of CFS of 4I1 at its MIC in 890 μL NB broth at 37°C. Samples for viable cell counts were taken out at 0, 40, 80, 120, 160, and 200 min time intervals. Viable plate counts were monitored on NB agar as we previously described (Bajpai et al., 2013). Colonies were counted after incubation for 24 h at 37°C. The controls were inoculated without CFS of 4I1 for each pathogenic bacteria using the same experimental condition. Assay were performed in triplicate.

Determination of the Effect of 4I1 CFS on Potassium Ion Efflux

The effects of CFS of *P. pentosaceus* 4I1 on the efflux of potassium ion from *S. aureus* KCTC-1621 and *E. coli* O157:H7 were determined as we previously described (Bajpai et al., 2013). Concentration of free potassium ion in bacterial suspensions of *S. aureus* KCTC-1621 and *E. coli* O157:H7 was measured after exposing bacterial cells to CFS of *P. pentosaceus* 4I1 at their MICs in sterile peptone water (8.5 g NaCl + 1 g peptone in 1 L sterilized distilled water) for 0, 30, 60, 90, and 120 min. At each pre-established interval, the extracellular potassium concentration was measured by a photometric procedure using the Calcium/Potassium kit (Quantofix, GmbH, Wiesbaden, Germany). Similarly, control was also tested without adding CFS. Results were expressed as the amount of extracellular free potassium (mmol/L) in the growth media in each interval of incubation.

Determination of the Effect of 4I1 CFS on the Release of 260-nm Absorbing Materials

The release of 260-nm-absorbing materials from *S. aureus* KCTC-1621 and *E. coli* O157:H7 cells was monitored in aliquots of 2 mL of the bacterial inocula in sterile peptone water (0.1 g/100 mL). The reaction solution containing MIC of CFS of *P. pentosaceus* 4I1 was incubated at 37°C. At 0, 30, and 60 min time interval of treatment, cells were centrifuged at 3,500 × *g*, and the absorbance of the obtained supernatant was measured at 260 nm using a 96-well plate ELISA reader (Bajpai et al., 2013). Controls were treated in the same manner without CFS of *P. pentosaceus* 4I1. Results were expressed in terms of optical density (OD) of 260-nm absorbing materials in each interval with respect to the ultimate time.

Determination of the Effect of 4I1 CFS on Cell Membrane Permeability

The effects of CFS of *P. pentosaceus* 4I1 on cell membrane permeability of *S. aureus* KCTC-1621 and *E. coli* O157:H7 were determined as described previously (Patra et al., 2015), and expressed in terms of relative electrical conductivity. Upon action of CFS on the cell membrane of tested pathogens, it may cause drastic release of cytosolic materials such as protein, DNA and other essential metabolites, resulting in the cell death. Prior to the assay, cultures of test pathogens were incubated at 37°C for 10 h, followed by centrifugation (5,000 × *g*) for 10 min, and washed with 5% glucose solution (w/v) until their electrical conductivities reached close to 5% glucose solution to induce an isotonic condition. MICs of CFS of *P. pentosaceus* 4I1 acquired for both the tested pathogens were added to 5% glucose (isotonic solution), incubated at 37°C for 8 h, and the electrical conductivities (L_a) of the reaction mixtures were determined. Further, electrical conductivities of the bacterial solutions were measured at 2 h of intervals for a total duration of 8 h (L_b). The electrical conductivity of each test pathogen in isotonic solution killed by boiling water for 5 min served as a control (L_c). The relative electrical conductivity was measured using an electrical conductivity meter. The permeability of bacterial membrane was calculated according to the following formula:

$$\text{Relative conductivity (\%)} = L_a - L_b/L_c \times 100.$$

Scanning Electron Microscopic (SEM) Analysis

Scanning electron microscopic (SEM) study was executed according to Kim et al. (2007) to examine the effects of CFS of *P. pentosaceus* 4I1 on the morphological changes in the cell wall of the selected pathogens, *S. aureus* KCTC-1621 and *E. coli* O157:H7. Control samples were prepared without CFS of *P. pentosaceus* 4I1. Microscopic examination was performed using a S-4300 SEM Analyzer (Hitachi, Japan).

Determination of Growth Phase-Dependent Inhibitory Effects of CFS of 4I1

To determine whether CFS of *P. pentosaceus* 4I1 has growth phase-independent inhibitory effects, CFS of *P. pentosaceus* 4I1 at MIC + pathogenic bacteria (overnight grown single bacterial colony) were inoculated in their respective culture tubes containing 20 mL sterile NB medium followed by incubation at 37°C until 24. After every 4 h, samples were withdrawn and analyzed for the bacterial counts of pathogenic strains. Colonies were counted after growth at 37°C up to 24 h, and the log10 CFU was plotted against incubation period to prepare growth curves of individual strains. As a control, pathogenic bacterial strains without CFS of *P. Pentosaceus* 4I1 were used.

Determination of the Effect of CFS of 4I1 on Biofilm Formation Ability of Pathogenic Strains

Pathogenic bacterial strains were inoculated and grown in NB broth and evaluated for their biofilim formation ability, and effects of CFS of *P. pentosaceus* 4I1 on biofilm formation by pathogenic bacteria was determined following the modified method of Aoudia et al. (2016). A 2 mL of bacterial cultures of *S. aureus* KCTC-1621 and *E. coli* O157:H7 (10^6 CFU/ml) grown in NB broth were added to each glass test-tube. On the other hand, to determine the effect of CFS of *P. pentosaceus* 4I1 on biofilm formation, CFS at MIC mixed with 2 mL of bacterial cultures of *S. aureus* KCTC-1621 and *E. coli* O157:H7 (10^6 CFU/ml) grown in NB broth and was added to each glass test-tube. While, 2 mL of NB broth was added in blank test-tubes without bacterial culture (negative control). The tubes were incubated for 48 h at 30°C. To quantify the biofilm formation, the tubes were gently washed three times with 2 mL of sterile distilled water, and attached bacteria were fixed with 2 mL of methanol for 15 min, and then, tubes were emptied and air dried at room temperature or oven dried at 60°C for 30–45 min. Subsequently, 2 mL of a 2% (v/v) crystal violet solution was added to each well and held at ambient temperature for 15 min. Excess stain was then removed by placing the test tubes under gently running tap water. A 2 mL of ethanol was used for destaining. The OD of released adherent cells was measured at 595 nm. Each assay was performed in three individual times on different days under the same conditions, and the negative control was performed in uninoculated NB broth. The cut-off OD was defined as the mean OD value of the negative control. Based on the OD, strain was confirmed for biofilm production ability in presence of CFS as a no-biofilm producer or biofilm producer (strong or week) (OD < OD of negative control confirmed as no-biofilm producer), (ODC < OD × 2 OD of negative control confirmed as week biofilm producer), moderate (2 × OD of negative control < OD ≤ 4 × OD of negative control confirmed as moderate biofilm producer) (4 × OD of negative control < OD confirmed as strong biofilm producer).

Statistical Analysis

All experiments were performed in triplicate and results were expressed the mean ± SD following one-way ANOVA coupled with Duncan's multiple test.

RESULTS

Morphological, Biochemical, and Molecular Characterization of 4I1

Small yellow colonies of similar sizes that appeared on BCP agar using pour-plating method confirmed the presence of the LAB isolate 4I1 which was confirmed to be coccus-shaped by microscopic evaluation. Biochemical analysis of 4I1 was performed using the API 50 CHL strip kit and a

TABLE 1 | Biochemical characterization of *Pediococcus pentosaceus* (4I1) based on carbohydrate interpretation using API 50 CHL kit.

Active ingredient	Result	Active ingredient	Result
Glycerol	–	Salicin	+
Erythritol	–	D-cellobiose	+
D-arabinose	–	D-maltose	+
L-arabinose	+	D-lactose (bovine origin)	+
D-ribose	+	D-melibiose	+
D-xylose	+	D-saccharose	+
L-xylose	–	D-trehalose	+
D-adonitol	–	Inulin	–
Methyl-β-D-xylopyranoside	–	D-melezitose	–
D-galactose	+	D-raffinose	+
D-glucose	+	Amidon (starch)	–
D-fructose	+	Glycogen	–
D-mannose	–	Xylitol	–
L-sorbose	–	Gentiobiose	+
L-rhamnose	–	D-turanose	+
Dulcitol	–	D-lyxose	–
Inositol	–	D-tagatose	–
D-mannitol	+	D-fucose	–
D-sorbitol	–	L-fucose	–
Methyl-α-D-glucopyranoside	–	D-arabitol	–
N-acetylglucosamine	+	Potassium gluconate	–
Amygdalin	+	Potassium 2-ketogluconate	–
Arbutin	+	Potassium 5-ketogluconate	–
Esculin	–		

(–): The bacterium does not use this carbohydrate; (+): The bacterium uses this carbohydrate.

selected strain was identified as a gram-positive and rod-shaped isolate (**Table 1**). API web software confirmed that strain 4I1 utilized carbohydrates, including L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannitol, D-sorbitol, *N*-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, gentiobiose, and D-turanose (**Table 1**). Color change from violet to yellow in the strip capsule indicated complete fermentation of sugar by 4I1. Molecular analysis using partial 16S rDNA gene sequencing showed the selected strain displayed 99.9% similarity with different *Pediococcus* spp. (**Figure 1**), thus, the strain was finally characterized as *P. pentosaceus* 4I1. The derived sequence was submitted to GenBank with nucleotide accession number KT372700.

GC–MS Analysis

The GC–MS analyses of the CFS of *P. pentosaceus* 4I1 identified seven different components accounted for 99.98% of total CFS. The CFS of *P. pentosaceus* 4I1 yielded compounds largely amino acids, organic acids, and fatty acids, as well as pyrrol derivatives which included (*S*)-2-Hydroxypropanoic acid (24.28%), caprolactam (8.83%), D-valine (28.53%), D-leucine (35.71%), 3-pyrrolidin-2-yl-propionic acid (2.43%), pyrrolo [1,2-a]pyrazine-1,4-dione, hexa (19.34%), and 9-octadecenoic acid (8.29%).

Antibacterial Potential

The antibacterial activity of CFS of *P. pentosaceus* 4I1 against the tested foodborne pathogenic bacteria was confirmed by the presence or absence of inhibition zones on the agar well plates. As presented in **Figure 2**, CFS of *P. pentosaceus* 4I1 exhibited potent inhibitory effects against all the tested foodborne pathogenic bacteria. In this assay, *P. pentosaceus* 4I1 exerted consistent antibacterial effects against both gram-positive and gram-negative bacteria, with zone of inhibition diameters ranging from 16.5 to 20.4 mm (**Figure 2**). Sterilized distilled water and/or MRS medium used as a negative control had no inhibitory effect.

The MIC assay revealed different susceptibilities of tested pathogens to the CFS of *P. pentosaceus* 4I1, and exhibited potent inhibitory effect as MIC and MBC values. In this assay, the MIC and MBC values of 4I1 CFS against the tested foodborne pathogens were ranged from 250 to 1,000 μg/mL (**Figure 3**). Furthermore, the CFS of 4I1 exhibited potential antibacterial effects as reflected by MIC and MBC values against all the tested pathogens (**Figure 3**). Interestingly, one of the pathogens *S. enterica* ATCC-4731 was found to be highly susceptible pathogen to the CFS of *P. pentosaceus* 4I1. Notably, both gram-positive and gram-negative bacteria were inhibited by the CFS of *P. pentosaceus* 4I1.

Effect on Cell Viability

In this assay, the CFS of *P. pentosaceus* 4I1 when inoculated at MIC, exhibited significant inhibitory effects against the growth of tested bacterial pathogens, *S. aureus* KCTC-1621 and *E. coli* O157:H7, as confirmed by reduced bacterial cell viability when inoculated at MIC (**Figure 4**). Exposure to CFS of *P. pentosaceus* 4I1 for 0 to 80 min did not elicit severe inhibition of cell viability, but remarkable declines in the cell viable counts of *S. aureus* KCTC-1621 and *E. coli* O157:H7 was observed after exposure to the CFS of *P. pentosaceus* 4I1 for 160 min. Interestingly, the exposure to CFS of *P. pentosaceus* 4I1 for 200 min completely inhibited the cell viabilities of both tested pathogens (**Figure 4B**).

Effect on Potassium Ion Leakage

This test assay confirmed the antibacterial effect of the CFS of *P. pentosaceus* 4I1 by revealing K^+ release from *S. aureus* KCTC-1621 and *E. coli* O157:H7 versus the control (**Figure 5**). The CFS of *P. pentosaceus* 4I1 added at MIC to cell suspensions of tested pathogenic bacteria resulted in rapid K^+ release from the bacterial cells following protracted steady loss (**Figures 5A,B**). However, no leakage of K^+ was observed from *S. aureus* KCTC-1621 and *E. coli* O157:H7 controls (**Figure 5**).

Effect on Release of 260 nm Materials

Release of 260-nm absorbing materials (DNA and RNA) from the cells treated with specific antimicrobials may be an indication of bacterial cell death. Hence, we evaluated the effects of the CFS of *P. pentosaceus* 4I1 on the release of 260-nm absorbing materials from *S. aureus* KCTC-1621 and *E. coli* O157:H7 treated at MIC. Interestingly, exposure of CFS of *P. pentosaceus* 4I1 to *S. aureus* KCTC-1621 and *E. coli* O157:H7 caused rapid loss of

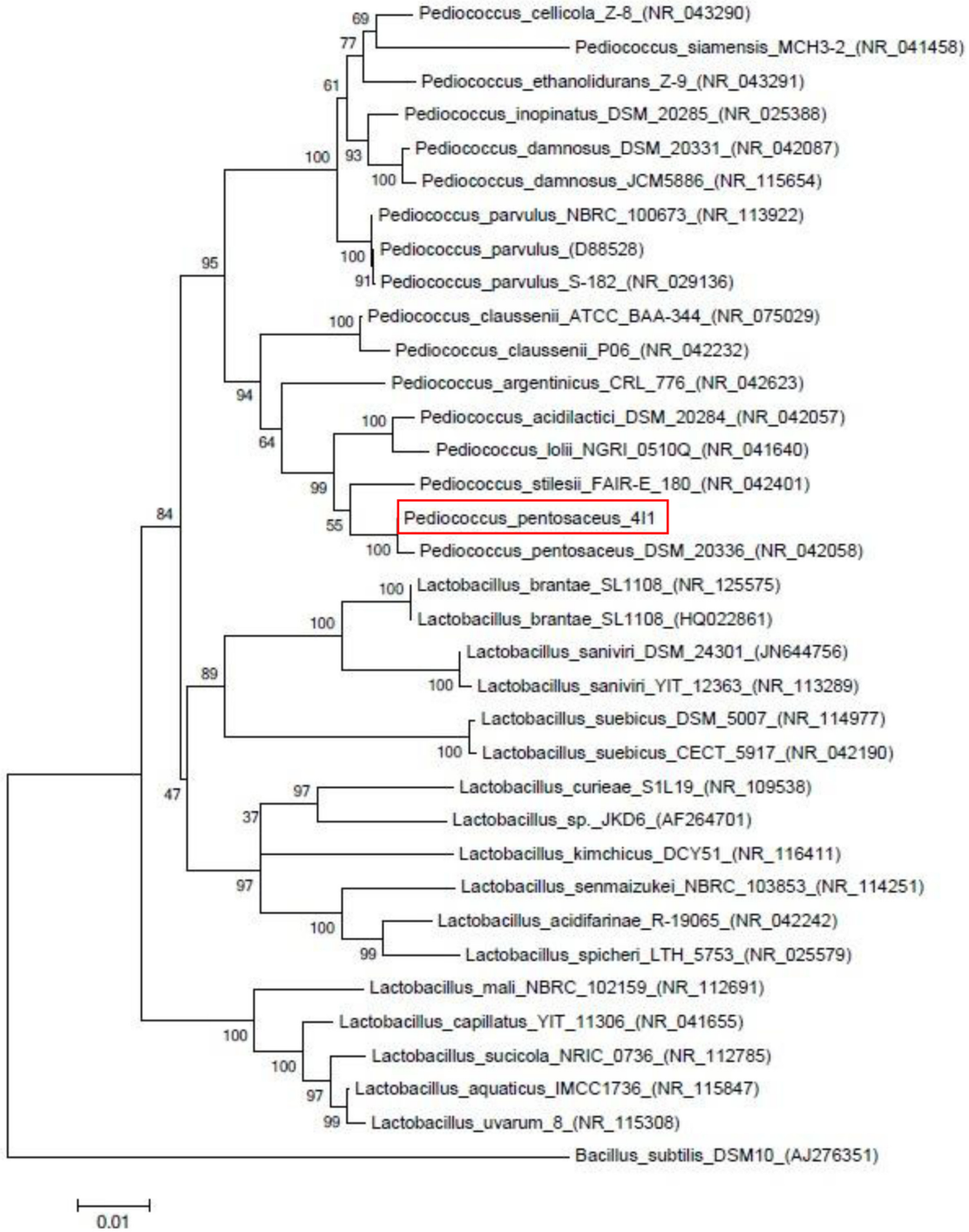

FIGURE 1 | Neighbor-joining phylogenetic tree showing the position of strain *Pediococcus pentosaceus* 4I1 among the different *Pediococcus* strains based on 16s rDNA sequences.

260-nm absorbing materials from the bacterial cells. The ODs of culture filtrates of *S. aureus* KCTC-1621 and *E. coli* O157:H7 cells exposed to the CFS of *P. pentosaceus* 4I1 at 260 nm, revealed a significant time-dependent increase in the release of 260-nm-absorbing materials (**Figure 6**). However, no changes in the OD of control cells of tested pathogens were observed. Notably, exposure for 60 min to the CFS of *P. pentosaceus* 4I1 caused about a twofold increase in the OD of treated bacterial cell culture filtrates as compared with their respective controls (**Figures 6A,B**).

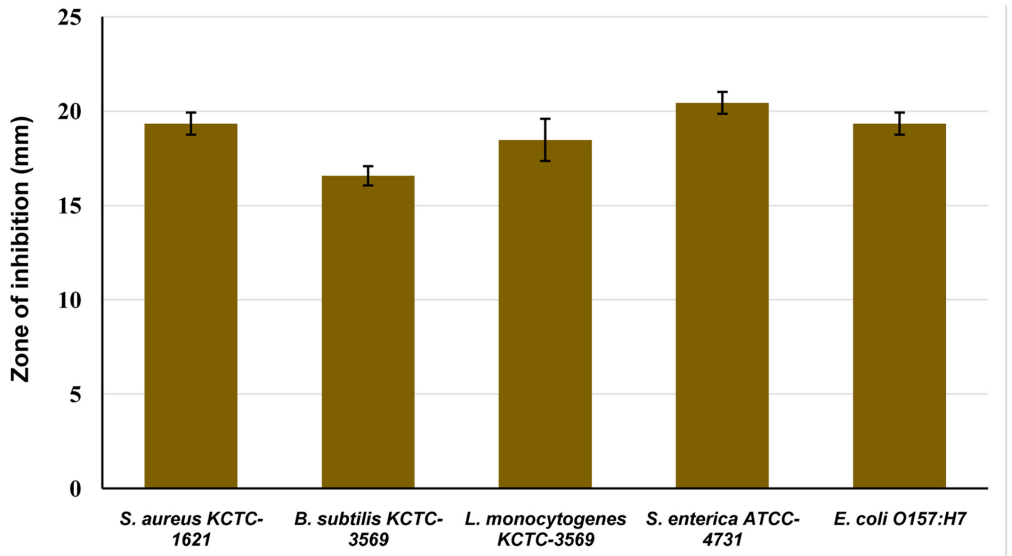

FIGURE 2 | Antibacterial activity of cell free supernatant (CFS) of *P. pentosaceus* 4I1 against foodborne pathogenic bacterial in agar well-diffusion assay. Data are expressed as mean ± SD ($n = 3$).

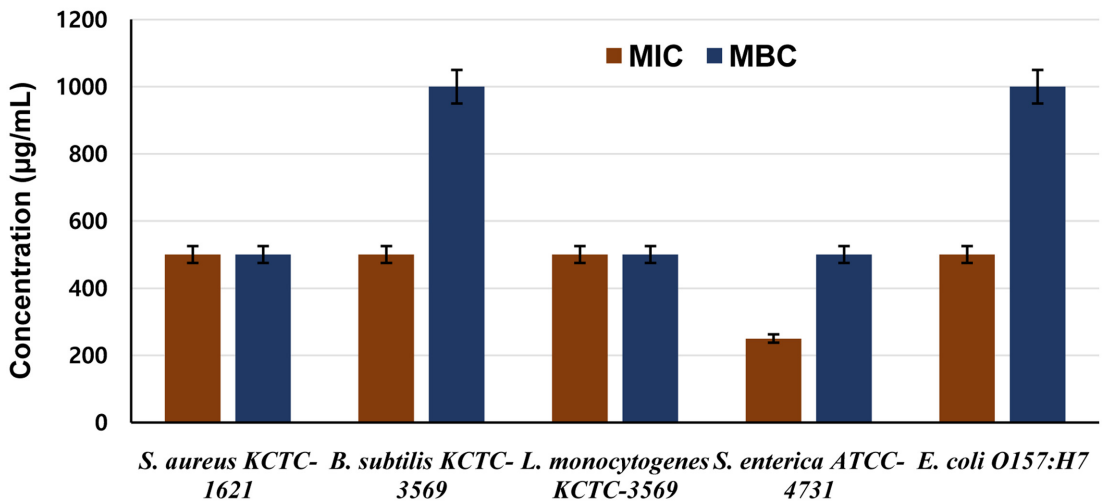

FIGURE 3 | Determination of minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) concentrations of CFS of *P. pentosaceus* 4I1 against foodborne pathogenic bacteria. Data are expressed as mean ± SD ($n = 3$).

Effect on Cell Membrane Permeability

This assay visualized the effect of the CFS of *P. pentosaceus* 4I1 at MIC on the membrane permeabilities of tested pathogens as determined by their relative electrical conductivities. As was expected, the CFS of *P. pentosaceus* 4I1 exhibited time-dependent inhibitory effect on the membrane permeabilities of the tested pathogens, and the relative electrical conductivity of each tested pathogen was increased time-dependently. Furthermore, the CFS of *P. pentosaceus* 4I1 exhibited a greater inhibitory effect on cell membrane of *S. aureus* KCTC-1621 (**Figure 7A**) than *E. coli* O157:H7 (**Figure 7B**) as indicated by their relative electrical conductivity values (**Figure 7**). No relative electrical conductivity was observed in untreated controls. In this assay, the CFS of *P. pentosaceus* 4I1 showed an ability to disrupt the plasma membranes of both tested bacteria as confirmed by the changes observed in the relative electrical conductivity values.

Morphological Observation by SEM

Since exposure to an antimicrobial agent may lead to disruption of bacterial cell wall, we turned to SEM analysis to investigate further the effect of the CFS of *P. pentosaceus* 4I1 on the cell wall physiologies and morphologies of *S. aureus* KCTC-1621 and *E. coli* O157:H7 cells (**Figure 8**). As was expected, control bacterial cells not exposed the result of CFS of 4I1 had regular

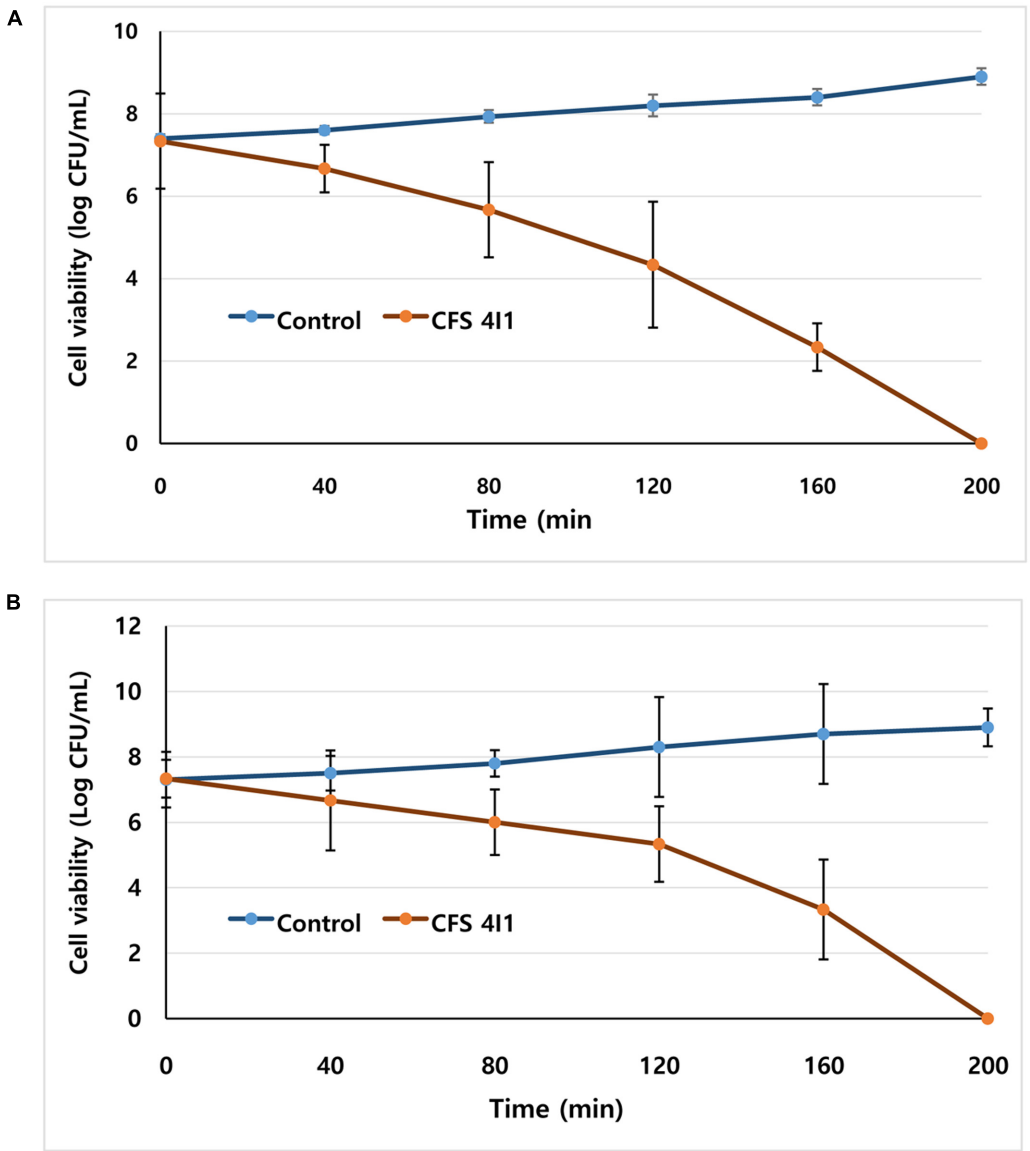

FIGURE 4 | Effect of CFS of *P. pentosaceus* 4I1 on the viability of the tested pathogenic bacteria of *Staphylococcus aureus* KCTC-1621 (A) and *Escherichia coli* O157:H7 **(B)**. Control without treatment. Data are expressed as mean ± SD ($n = 3$).

smooth surfaces (**Figures 8A,B**), whereas those treated with CFS of 4I1 at MIC showed cell wall damage and lysis (**Figures 8C,D**).

Growth Phase-Dependent Inhibitory Effect

The growth phase-dependent behavior of CFS of *P. pentosaceus* 4I1 on the tested pathogens was measured at different time intervals as demonstrated in Supplementary Figure S1. In both, mono- and co-culture tubes, the lag phase for *P. pentosaceus* 4I1 lasted about 3–4 h, whereas pathogenic strains grew rapidly (Supplementary Figure S1). However, decreases in pathogen growths were observed in the presence of CFS of *P. pentosaceus* 4I1 after incubation for 6–12 h, and pathogen growth was reduced by almost four log cycles within 24 h of incubation as compared with the control. The effect of CFS of *P. pentosaceus* 4I1 on the growth of *S. aureus* KCTC-1621 was significantly more marked than on that of *E. coli* O157:H7.

Effect of CFS on Biofilm Formation Ability of Pathogenic Strains

The effects of CFS of *P. pentosaceus* 4I1 on biofilim formation by *S. aureus* KCTC-1621 and *E. coli* O157:H7 were analyzed by using crystal violet assay, which showed a potent inhibitory effect of the CFS of 4I1 on the biofilm formation ability of both the tested pathogenic bacteria (Supplementary Figure S2). As a result, the CFS of *P. pentosaceus* 4I1 induced inhibition of biofilm formation

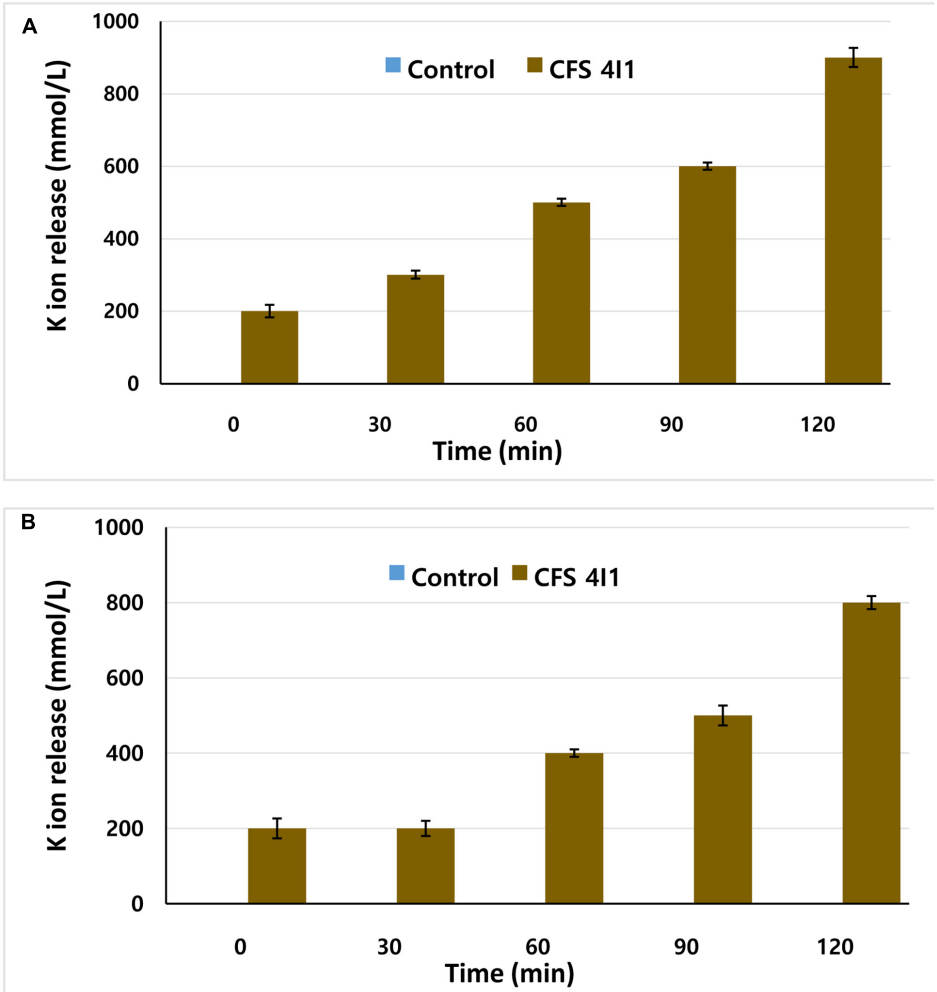

FIGURE 5 | Effect of CFS of *P. pentosaceus* 4I1 on leakage of potassium ions from the tested pathogenic bacteria of *S. aureus* KCTC-1621 (A) and *E. coli* O157:H7 **(B)**. Control without treatment. Data are expressed as mean ± SD ($n = 3$).

by *S. aureus* KCTC-1621 and *E. coli* O157:H7 (Supplementary Figure S2) as confirmed by a significant ($p < 0.05$) reduction in OD values, while in control without CFS of *P. pentosaceus* 4I1, *S. aureus* KCTC-1621 and *E. coli* O157:H7 showed a strong biofilm formation ability (Supplementary Figure S2). Our results indicate that CFS of *P. pentosaceus* 4I1 has potential effect against biofilm formation ability of *S. aureus* KCTC-1621 and *E. coli* O157:H7.

DISCUSSION

In this study, we characterized a lactic acid bacterium 4I1 isolated from freshwater fish *Zacco koreanus* as *P. pentosaceus* 4I1 based on its morphological, biochemical, and molecular characteristics (Bajpai et al., 2016b; Casaburi et al., 2016). Furthermore, LAB isolate 4I1 showed remarkable antibacterial activities against a panel of foodborne pathogens as confirmed by inhibitory zones in the agar well-diffusion assay and different susceptibilities in the MIC/MBC assay. As reported previously, other LAB have shown potential antibacterial effects against a number of foodborne pathogens (Amenu, 2013). Furthermore, a variety of pathogenic and foodborne pathogenic bacteria exhibit susceptibility to LAB (Tadesse et al., 2005; Carina Audisio et al., 2011; Darsanaki et al., 2012; Yah et al., 2014). In subsequent assays, we randomly selected two foodborne pathogenic bacteria, *S. aureus* KCTC-1621 and *E. coli* O157:H7, to evaluate the antibacterial effects of CFS of *P. pentosaceus* 4I1.

This study shows exposure to CFS of 4I1 reduces *S. aureus* KCTC-1621 and *E. coli* O157:H7 viable cell counts. Previous reports have confirmed the inhibitory effects of various LAB strains on the viabilities of foodborne pathogenic bacteria (de Barros et al., 2012). Recently, we also reported that the CFS of lactic acid bacterium isolated from Kimchi (a Korean fermented food) exhibits inhibitory effects (Bajpai et al., 2016b). Other LAB have also been shown to exhibit antibacterial effects with respect to inhibiting the cell viabilities of foodborne pathogenic bacteria (Carina Audisio et al., 2011).

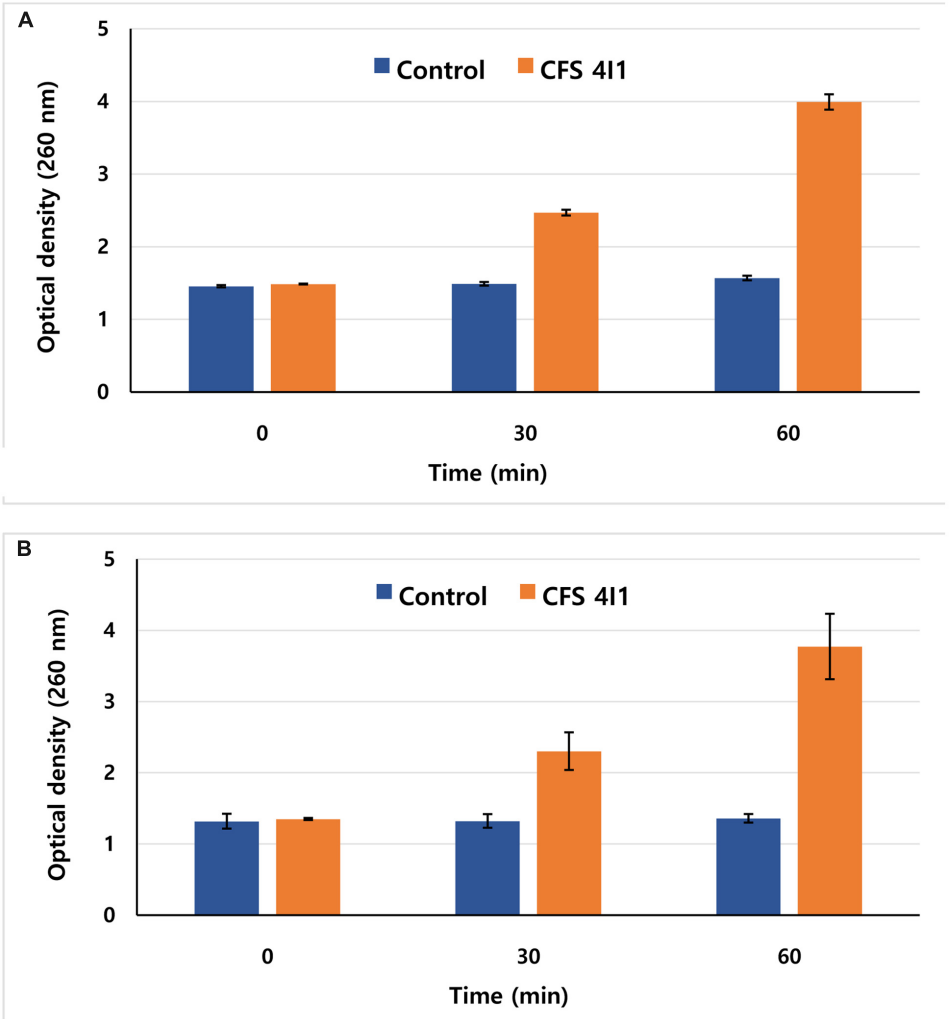

FIGURE 6 | Effect of CFS of *P. pentosaceus* 4I1 on the release rate of 260-nm absorbing material from *S. aureus* KCTC-1621 (A) and *E. coli* O157:H7 **(B)**. Data are expressed as mean ± SD ($n = 3$).

The bacterial plasma membrane plays an important barrier function, for example, it inhibits the transit of various important electrolytes, such as, the potassium ion, which participates in various cell membrane functions and in essential enzymatic activities. The regulation of these important electrolytes is critical, for example, elevated leakage of potassium ion can cause bacterial cell membrane disruption, and thus, it is necessary to maintain the equilibrium of essential ions in order to maintain energy status and survival (Cox et al., 2001). Changes in the structural integrity of the bacterial cell membrane may increase potassium ion release, and abrupt cell metabolism, thus induce cell death (Cox et al., 2001). In a previous study, inhibitory effects of CFS derived from other LAB were confirmed to involve potassium ion efflux (Bajpai et al., 2016b).

In the present study, it was observed the CFS of *P. pentosaceus* 4I1 at MIC had remarkable effects on the release of 260 nm materials (DNA and RNA) from cells of tested pathogenic bacteria, which confirmed its potential role as a potent antibacterial agent. Marked release of 260-nm materials from CFS-treated cells of pathogenic bacteria was supported by observations of loss of cell membrane structural integrity, which would lead to the loss of essential cell electrolytes (Farag et al., 1989). These observations suggest that the release of 260-nm absorbing materials from *S. aureus* KCTC-1621 and *E. coli* O157:H7 might provide sensitive indicators of membrane damage and loss of membrane integrity. Similar results on the inhibitory effect of CFSs or LAB on nucleic acid release from bacterial pathogens have been previously reported (Alakomi et al., 2000; Bajpai et al., 2016b).

In this study, SEM analysis showed marked morphological changes to the cell walls of *S. aureus* KCTC-1621 and *E. coli* O157:H7 resulting in cell wall deformation by the CFS of 4I1. Consistent with our findings, other LAB isolates have also been demonstrated to induce such morphological alterations in several pathogenic microbes (Kim et al., 2009). These morphological alterations may be due to aberrations in membrane lipid

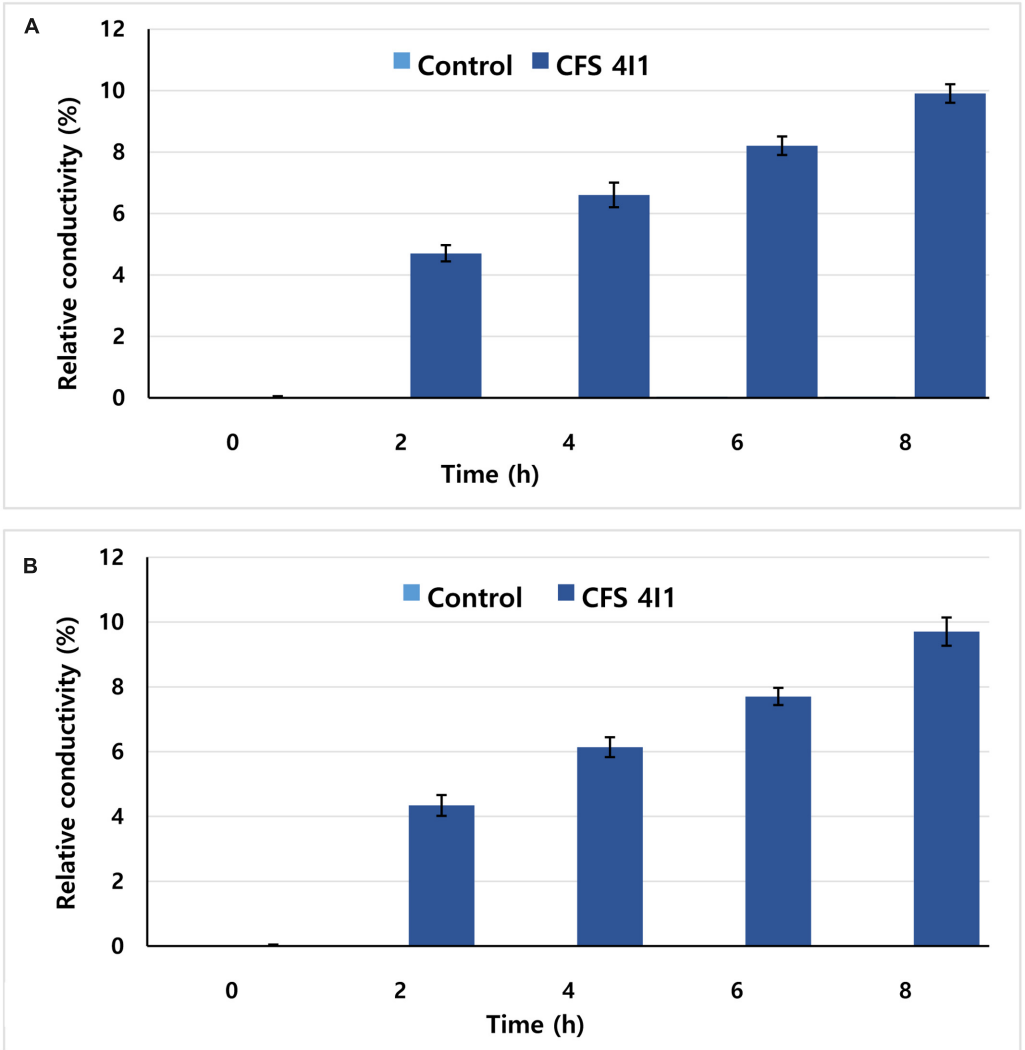

FIGURE 7 | Effect of CFS of *P. pentosaceus* 4I1 on membrane permeability of *S. aureus* KCTC-1621 (A) and *E. coli* O157:H7 **(B)**. Data are expressed as mean ± SD ($n = 3$).

composition, altered membrane fluidity and/or membrane integrity resulting in cell wall lysis and loss of intracellular dense material (Sikkema et al., 1994).

Changes in relative electrical conductivity can adversely affect membrane integrity and eventually result in cell death, and thus, the maintenance of bacterial cell membrane integrity is required to secure normal cell metabolism (Cox et al., 2001). Our results suggest CFS of *P. pentosaceus* 4I1 severely disrupted the plasma membranes of tested foodborne pathogens and that this resulted in increased loss of essential metabolites and necessary ions. These findings agree well with those of Roth and Keenan (1971), who reported that LAB have the ability to cause sub-lethal injury to *E. coli*. In addition, similar results have also been reported for LAB derived organic acids, such as, acetic acid (Przybylski and Witter, 1979). Furthermore, their effects were attributed to disruption of the lipopolysaccharide layer (Przybylski and Witter, 1979). Similarly, LAB-derived CFSs and/or other antimicrobials have also been shown to markedly affect the relative electrical conductivity parameters of foodborne pathogens (Patra et al., 2015; Bajpai et al., 2016b).

This study also revealed growth phase-dependent inhibitory effects of the CFS of *P. pentosaceus* 4I1. The inhibitory effect observed for the tested pathogens in growth-phase dependent inhibition assay began from the early stationary phase, which could be mediated by secondary metabolites, organic acids, or other compounds produced in the CFS of *P. pentosaceus* 4I1. Similar results were observed by Muhsin et al. (2015).

Moreover, the antimicrobial activities of producer strains may be related to the action of various compounds, such as, organic acids, H_2O_2, and bacteriocin-like substances (Anas et al., 2008). A number of reports have confirmed strains of *Lactobacilli* are not able to regenerate hydrogen peroxide under anaerobic conditions, and that antimicrobial activities are unaffected by acidity (Juillard et al., 1987). Furthermore, most *Lactobacilli*

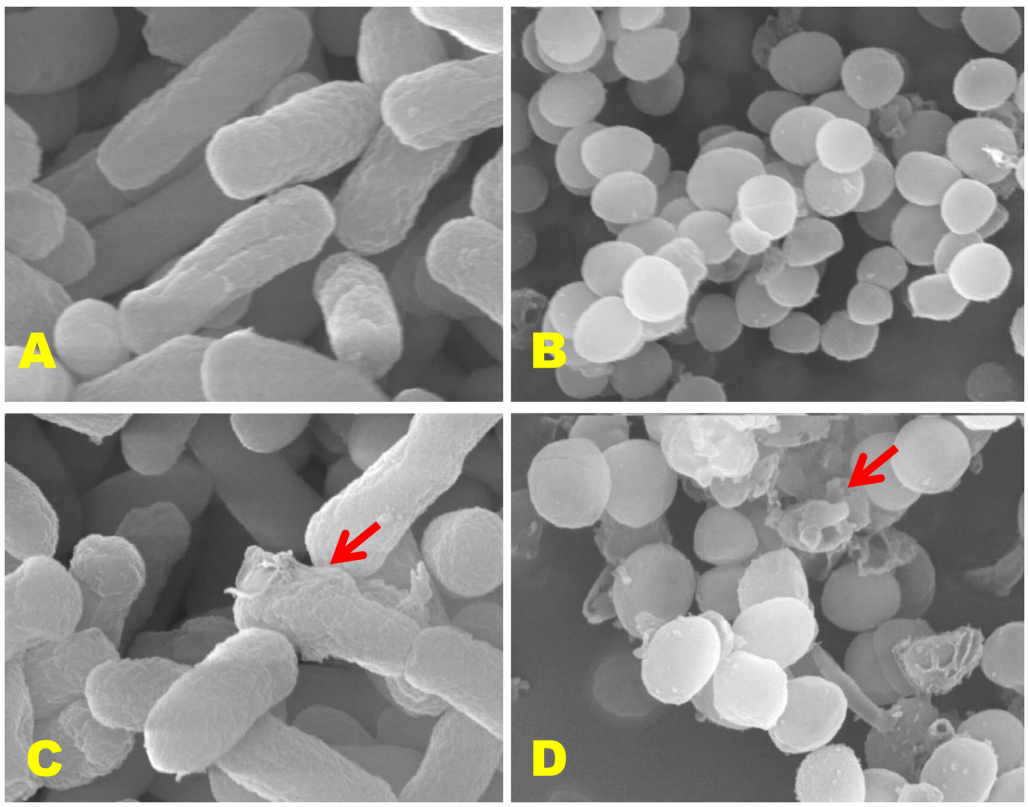

FIGURE 8 | Scanning electron microscopic (SEM) analysis of *E. coli* 0157:H7 and *S. aureus* KCTC-1621 cells treated with CFS of *P. pentosaceus* 4I1. Controls **(A,B)** showing a regular and smooth surface; treated cells **(C,D)** arrows showing disruption and cell lysis, respectively.

strains produce bacteriocins or bacteriocin-like substances with wide antimicrobial spectrums (Klaenhammer, 1988; Piard and Desmazeand, 1991; Nettles and Barefoot, 1993). During our preliminary screening, we treated CFS of 4I1 with trypsin (a proteolytic enzyme) and found lost its activity, indicating the antimicrobial activity of *P. pentosaceus* 4I1 might be due to the production of proteinous substances, such as, bacteriocin-like substances, as was previously reported (Ghrairi et al., 2008).

Another concern regarding H_2O_2-related antimicrobial activity, according to Prado et al. (2000), is that lyophilization promotes the removal of oxygen metabolites and H_2O_2. Rodriguez et al. (1997) emphasized H_2O_2 is rapidly degraded in the MRS broth, which excludes the possibility that the antimicrobial activity demonstrated by lyophilized *Lactobacilli* involves H_2O_2, as in this study, MRS was used as the growth medium for the test producer strain and lyophilization was used to concentrate the supernatant for later resuspension. de Oliveira et al. (2014) also reported similar findings for lyophilized cultures.

The results of the present study indicate the inhibitory effect of CFS of 4I1 might be due to the actions of organic acids produced by LAB strains. The lyophilized concentrated supernatant from strain 4I1 was found to be sensitive to neutralization with 4 M NaOH solution, and 24, 48, and/or 72 h after neutralization, it completely lost its inhibitory ability against tested pathogenic strains (data not shown). Thus, we conclude the antimicrobial activities observed in the present study involved the actions of organic acids and/or the acidification of medium. Similar results were presented by Pereira and Gómez (2007), who evaluated the antimicrobial potential of a commercial *Lactobacillus acidophilus* strain on foodborne pathogens (*E. coli* and *S. aureus*). Organic acids contribute to the control of microorganisms and reduce food pH values, which adversely affects the survival and proliferation of pathogenic microbes, including gram-positive and gram-negative bacteria (Tamanini et al., 2012).

To confirm that the antimicrobial action of CFS of 4I1 was organic acid mediated, we subjected CFS of 4I1 to GC–MS analysis. The obtained confirmed the presence of fatty acids and other organic acids in the CFS of 4I1, importantly responsible for antimicrobial properties of *Lactobacilli* strains. Based on the above, we hypothesized that the antimicrobial activity of 4I1 observed in this study might be mediated by its production of bacteriocin-like substances and/or organic acids. However, the synergistic effects of acids, hydrogen peroxide, and bacteriocin-like substances cannot be ruled out (Anas et al., 2008). Sjögren et al. (2003) also confirmed the antimicrobial properties of 3-(R)-hydroxydecanoic acid, 3-hydroxy-5-*cis*-dodecenoic acid, 3-(R)-hydroxydodecanoic acid, and 3-(R)-hydroxytetradecanoic acid, from *Lactobacillus plantarum* MiLAB 14, and Sharma et al. (2014) confirmed the presence of octadecanoic acid in

Lactobacillus helveticus by GC–MS. Haque et al. (2009) reported that of the organic acids examined, propionic acid (PA) was an effective antimicrobial, and thus, further supported the organic acid-mediated inhibitory effect of *Lactobacilli* strains, and the outcome of the present study.

Under adverse conditions, many pathogenic bacteria have the ability to produce biofilms (Kim et al., 2013), which protect cells by restricting the access of antimicrobials, and thus, biofilm-forming bacteria pose a substantial threat to human health. In the present study, CFS of *P. pentosaceus* 4I1 inhibited biofilm formation by *S. aureus* KCTC-1621 and *E. coli* O157:H7. Woo and Ahn (2013) reported similar results for probiotic strains against *L. monocytogenes* and *Salmonella*. Similarly, Kim et al. (2013) found that *L. acidophilus* A4 exhibited strong anti-biofilm activity against the growth of *E. coli* O157: H7, *Salmonella enteritidis*, *Salmonella typhimurium* KKCCM11806, *Yersinia enterocolitica*, *Pseudomonas aeruginosa* KCCM 11321, *L. monocytogenes,* and *Bacillus cereus*. Also, it has been reported that LAB-derived CFSs produce antimicrobial molecules that have significant potential to inhibit foodborne pathogenic bacteria such as *E. coli* O157:H7, *S. enterica* serovar Enteritidis, *S. aureus* and *L. monocytogenes* (Aoudia et al., 2016).

This study shows the newly identified lactic acid bacterium *P. pentosaceus* 4I1, which was isolated from the intestinal microbiota of the freshwater fish *Zacco koreanus*, has marked inhibitory effects against foodborne pathogenic bacteria in different *in vitro* models. More specifically, the CFS of *P. pentosaceus* 4I1 had a significant inhibitory effect on membrane permeability, as reflected by reduced cell viability and the cellular release of 260-nm absorbing materials and potassium ions, and increased relative conductivity. Morphological alterations (observed by SEM) and protective biofilm formation ability further supported potent antibacterial activity of *P. pentosaceus* 4I1. These findings reinforce suggestions that *P. pentosaceus* 4I1 could be used as an effective antimicrobial agent against foodborne pathogens.

AUTHOR CONTRIBUTIONS

VB, IR, JL, J-IY performed experiments and drafted manuscript; VB, IR, J-HH contributed interpretation, analyzed data and wrote paper, CP, JL, WP, Y-HP contributed for conception, designed experiment, analyzed data, and provided technical support.

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ORIGINAL RESEARCH
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Probiotic *Lactobacillus sakei* proBio-65 Extract Ameliorates the Severity of Imiquimod Induced Psoriasis-Like Skin Inflammation in a Mouse Model

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This study was designed to evaluate the protective effect of ethanol extract (SEL001) isolated from a potent probiotic strain *Lactobacillus sakei* proBio-65 on imiquimod (IMQ)-induced psoriasis-like skin inflammation in a mouse model. Histopathological and histomorphometrical changes in the ear and dorsal skin tissues were observed under hematoxylin and eosin stain for general histopathological architectures or Masson's trichrome stain for collagen fibers. The expression profile of psoriasis-associated specific genes was determined using Real-Time PCR analysis. As a result, topical application of IMQ resulted in a significant increase of mean total and epithelial (epidermis) thicknesses, the number of inflammatory cells infiltrated in the dermis, and the decrease of dermis collagen fiber occupied regions in the ear tissues of IMQ and IMQ plus vaseline treated groups when compared to the intact control group. A significant increase of epithelial thickness and number of inflammatory cells infiltrated in the dermis of dorsal skin tissues were also noticed in IMQ and IMQ plus vaseline treated groups as compared to the intact control group, suggesting classic IMQ-induced hypersensitive psoriasis. IMQ-induced hypersensitive psoriasis related histopathological changes to the ear and dorsal skin tissues were significantly inhibited by the treatment of a standard drug clobetasol and SEL001. Further, mRNA expression analysis indicated a significant increase in gene expression levels of pro-inflammatory cytokines, including IL-19, IL-17A, and IL-23 in IMQ and IMQ plus vaseline treated groups than that of the control. Clobetasol and SEL001 treated groups resulted in a lower gene expression level of IL-19, IL-17A, and IL-23 as compared to IMQ and IMQ plus vaseline treated groups. These results enforce that SEL001 could be a novel treatment for psoriasis and an alternative to other drugs that pose a number of side effects on the skin.

Keywords: psoriasis, skin inflammation, *Lactobacillus sakei*, lactic acid bacteria, imiquimod

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INTRODUCTION

The skin is one of the vital organs of the body that serves as a barrier from the outside environment. Psoriasis is a chronic skin disorder with unknown trigger, which is characterized by inflammation, thickening, and abnormal epidermal proliferation with up to 4% of prevalence in the general population (Griffiths and Barker, 2007). The pathogenesis of psoriasis is multifactorial and, as a perpetual incendiary skin issue, irregularity amongst pro- and anti-inflammatory mediators may play a vital part in the development and progression of this skin disorder (Li et al., 2016). Due to its incomplete etiology, there is still no permanent cure; however, genetic predisposition and environments stimuli might have a disease causing role. While the pathogenesis of psoriasis is not fully elucidated, it is extensively putative that pro-inflammatory cytokines have a key role in both development and maintenance of psoriatic lesions. A number of studies have reported the presence of immune-derived cytokines in psoriatic skin lesions and serum, suggesting their role in the pathogenesis of psoriasis (Kagami et al., 2010; Res et al., 2010; Zhang et al., 2010; Mudigonda et al., 2012; Tokura, 2012; Yoo et al., 2012; El Malki et al., 2013; Wang et al., 2013). Specifically, tissue alterations seen in psoriasis are driven by the exaggerated production of pro-inflammatory cytokines and the lesion development is critically dependent on IL-23 and IL-17 (Lee et al., 2004; Piskin et al., 2006; van der Fits et al., 2009). One of the pro-inflammatory cytokines, IL-19 has a key role in psoriatic pathogenesis and upregulates in psoriatic lesions during disease progression (Li et al., 2005), thus, controlling the level of these pro-inflammatory cytokines is necessary to treat this chronic disease.

The effect of the concurrent presence of IL-17 and IL-19 in psoriasis lesions might have impact on various levels of the pathogenic progression. The two cytokines upregulate the expression of β-defensins and S100A proteins thereby increases the antibacterial competence of keratinocytes. Moreover, their role in impeding infections may uphold the process to infiltrate immune cells to skin and increase the inflammation (Wolf et al., 2008). The process could be further regulated by tight coordination of IL-19 and IL-17 which in turn could attract particular T-helper type 1-cells, dendritic cells and neutrophilic granulocytes thereby directly increasing the secretion of chemokines. In addition, IL-19 and IL-17 promote the maintenance and effector function of T helper type 17-cells synergistically through the production of several mediators. Of note, T helper type 17-cells are critical for pathogenicity, IL-23 drives their proliferation, survival, and cytokine production (Ouyang et al., 2008). In addition, IL-19 and IL-17 are known to induce other interleukins which contributes to the psoriasis typical epidermal alterations. Topical application of IMQ, a TLR7/8 ligand and potent immune activator, can induce and exacerbate psoriasis, a chronic inflammatory skin disorder (van der Fits et al., 2009), and has been used as activator for valuable psoriasis animal model (Qin et al., 2014; Bai et al., 2016). Additionally, psoriasis may be linked with genuine comorbidities, for example, metabolic disorder and cardiovascular sickness, likely mirroring a systemic provocative part of the infection (Perera et al., 2012). At present, there are a number of temporary treatments for psoriasis ranging from tropical medicine, heliotherapy, and systematic to biological treatments (Gustafson et al., 2013). Nevertheless, the outcome of these treatments may result in severe side effects, thus, demands a much safer treatment to render this disease.

Probiotics are known as beneficial microorganisms that, when administered in adequate amounts, confer health benefits to the host (Joint FAO/WHO, 2001). The effect of probiotics on the skin could be mediated by the modulation of both the innate and the adaptive immune responses in the host. Modulation of an immune system is one of the beneficial effects of probiotics in human health. Recently, we reported that oral administration of heat-killed *Lactobacillus sakei* proBio65 inhibited immunoglobulin E-mediated histamine and β-hexosaminidase in NC/Nga mice, suggesting that *L. sakei* has an inhibitory effect on atopic dermatitis-like skin lesion (Park et al., 2008; Kim et al., 2013). Oral administration of *L. casei* has been seen to reduce antigen-specific skin inflammation by controlling the size of the CD8+ effector pool (Chapat et al., 2004). In addition, *L. casei* DN-114001 alleviates T-cell mediated skin inflammation (Hacini-Rachinel et al., 2009). Recently, *L. pentosus* GML-77 has been seen to inhibit skin lesions in IMQ-induced psoriasis in mice (Wu et al., 2017).

Use of probiotics and their cellular components in preventive medicine to maintain a healthy function is well documented (Matsumoto et al., 2005; Tojo et al., 2014). Probiotics have been proposed as therapeutic agents in various pathological conditions, including intestinal chronic inflammation, atopic dermatitis and gut homeostasis (Park et al., 2014; Tojo et al., 2014; Plaza-Díaz et al., 2017). Previously, we confirmed the therapeutic potential of SEL001 like products of plant and microbial origin *in vitro* and *in vivo* using a diabetic animal model (Shukla et al., 2011; Bajpai et al., 2016). Various studies have also shown that probiotics, such as *L. sakei* probio65 exhibit a wide range of pharmacological effects such as anti-atopic dermatitis in animals and humans (Park et al., 2008, 2014; Kim and Pyo, 2012; Kim et al., 2013, 2015c).

Although a number of studies have emphasized the therapeutic role of probiotics in various inflammatory conditions, there is no report available on the anti-inflammatory effects of SEL001 of probiotic origin in a psoriasis model of inflammation. Since the IMQ-induced psoriasis model highly resembles human psoriasis lesions (van der Fits et al., 2009), in this study, we investigated the effect of topical application of SEL001, a probiotic product isolated from *L. sakei* proBio65 in an IMQ-induced psoriasis mouse model.

MATERIALS AND METHODS

Preparation of SEL001

To prepare the ethanolic extract of *Lactobacillus sakei* Probio65 hereby called SEL001, 48 h grown culture of strain *L. sakei* probio65 was mixed with ethanol (1:2 ratio) and the flask containing mixture of bacterial culture and ethanol

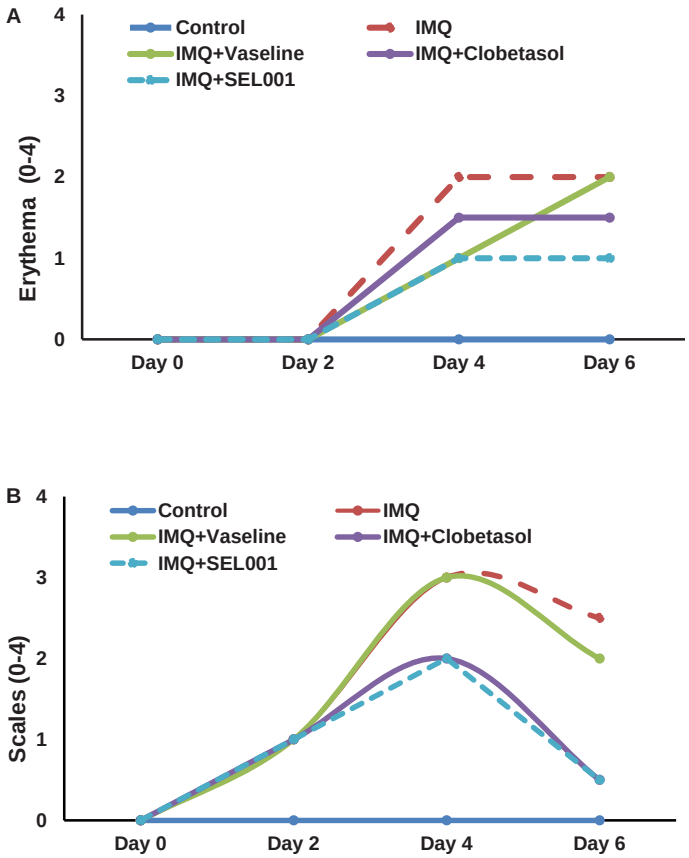

FIGURE 1 | Erythema and scales score of the dorsal skin of the mice. The scoring was performed on 0 day, 2 day, 4 day, and 6 day using the Psoriasis Area Severity Index (PASI). **(A)** Erythema score and **(B)** scales score.

was incubated at 150 rpm for 6 h at room temperature. Following the incubation, the mixture was centrifuged (8,000 *g* for 10 min) and the cell pellet was discarded. Resulting supernatant was filter sterilized and subjected to freeze drying until get a dry-powdered material, which was served as SEL001.

Chemical Composition Analysis of SEL001

Gas chromatography–mass spectrometry (GC–MS) was explored to identify the chemical composition of SEL001. Briefly, dried SEL001 derived from the probiotic strain *L. sakei* probio65 was dissolved in 95% v/v methanol and two microliter of this solution was injected for GC-MS analysis. The protocol was followed as reported by Ravi et al. (2013).

Animals

Thirty ICR mice of 6 weeks age were occupied from Samtaco Bio Co. (Korea), and were kept in cages at constant levels of temperature and humidity on 12 h light/dark cycles. The animals were acclimatized for 1 week and their backs were shaved using pet electric shaver (GSAK CO., LTD., Korea). The animals had free access to water and feed throughout the trial. Animal management and institutional approval for research protocols were supported and approved by the Animal Ethical Committee of the Yeungnam University (YNU-ANETCOMM-2016-00123), Gyeongsan, Korea.

IMQ-Induced Psoriasis Model and Treatment

The mice were divided into five groups; a control group, an IMQ group, an IMQ+vaseline group, an IMQ-clobetasol group, and an IMQ+SEL001. Each group contains 6 mice and were housed as 2 mice per cage.

In this study a total of four topical treatments were used such as, IMQ, vaseline, clobetasol, and SEL001. IMQ was used to induce psoriasis like skin inflammation in mouse mode. Further, the treatment groups received vaseline, clobetasol and SEL001 topically 1 h before topical application of IMQ. Clobetasol propionate, a corticosteroid drug is used to treat various skin diseases, including psoriasis, therefore, it was used a gold standard, and SEL001 was used as a test sample against IMQ-induced psoriasis. Except for the control group, all other groups received a daily dose of 62.5 mg of 5% IMQ cream/cm^2 (Aldara; MEDA AS) applied their backs and 20 mg on the right ear once a day for six consecutive days as previously described (van

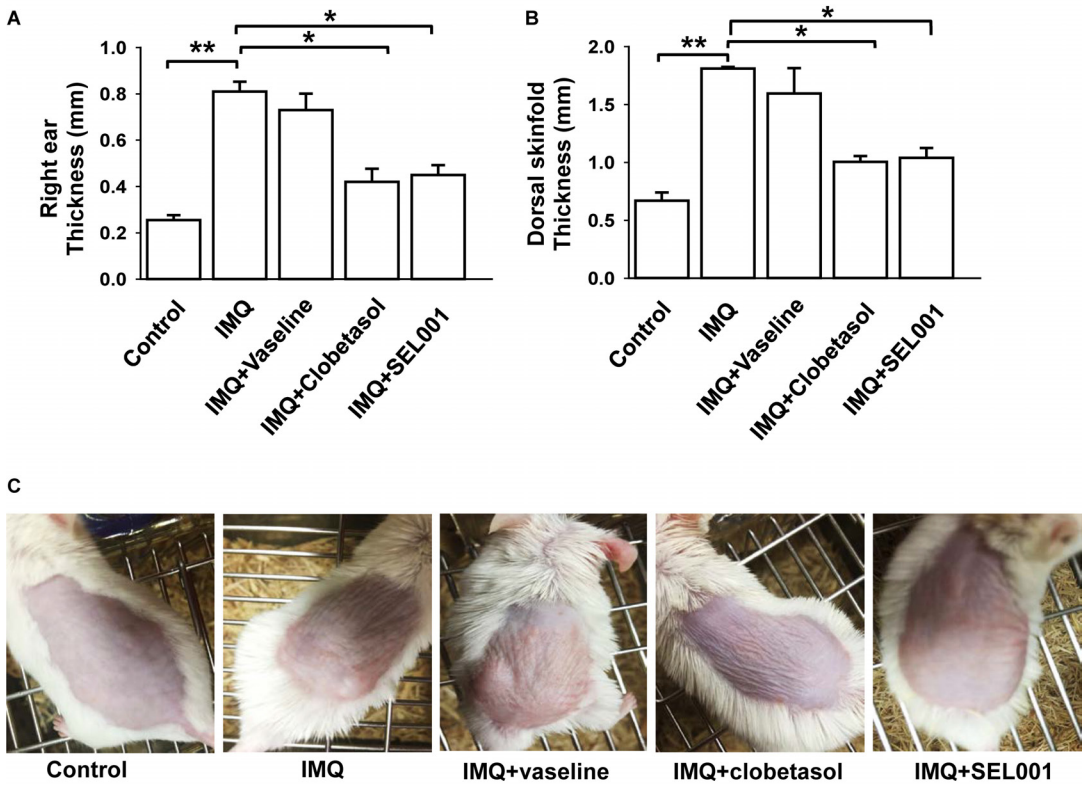

FIGURE 2 | Caliper measurement and visual appearance of skin. **(A)** Skin fold thickness on the backs of the mice, **(B)** right ear thickness, and **(C)** visual appearance on day 6. Columns represent means ± standard deviation of skin/ear measurements day 6. $^{**}p < 0.001$ or $^{*}p < 0.05$ is significant, analyzed by one-way ANOVA test.

der Fits et al., 2009; Nadeem et al., 2015; Rather et al., 2016). Further, IMQ+vaseline group received a daily dose of 62.5 mg of 5% IMQ cream plus vaseline cream (80 mg/cm^2 on back and 20 mg on the right ear); IMQ plus clobetasol group received a daily dose of 62.5 mg of 5% IMQ cream plus clobetasol (80 mg/cm^2 on back and 20 mg on the right ear), and IMQ plus SEL001 group received a daily dose of 62.5 mg of 5% IMQ cream plus SEL001 (50 mg/cm^2 on back and 10 mg on the right ear).

Scoring of Skin Inflammation Severity

On days 0, 2, 4, and 6, all animas were assessed using 2 elements of the Psoriasis Area Severity Index (PASI), to consign a score of 0–4 (0, none; 1, moderate; 3, severe; 4, very severe) for both erythema and scaling parameters. Further, back skin and ear thickness were measured by using electronic caliper (Shenzhen Liweihui Technology Co., Ltd., China). After day 6, animals were euthanized and the shaved skin area of the back and ear was excised. Two lesions of the skin of each mouse were taken for histological and mRNA expression analysis.

Histological Process

Approximated regions of an individual ear and dorsal skin tissues were sampled and they were crossly trimmed. All trimmed ear and dorsal skin tissues were re-fixed in 10% neutral buffered formalin (NBF), again for 24 h. After paraffin embedding, 3–4 μm sections were prepared. Representative sections were stained with hematoxylin and eosin (HE) for general histological architectures or Masson's trichrome (MT) for collagen fibers in the dermis of the ear and dorsal back skin tissues (Kim et al., 2015a; Bai et al., 2016). The histological profiles of individual cross trimmed ear and dorsal skin tissues were observed under a light microscope (Model Eclipse 80*i*, Nikon, Tokyo, Japan). Histological evaluation was performed on the two fields in each part of the ear and dorsal skin tissues; consequently, six histological fields of an ear and dorsal back skin tissues in each group were considered for further histomorphometric analysis. The histopathological analysis was done by the histopathologist who was unaware of group distribution. To observe more detailed changes, mean total and epithelial thicknesses of ear and dorsal skin tissues (μm), mean numbers of inflammatory cells infiltrated in dermis of ear and dorsal skin tissues (cells/mm^2 of dermis) and mean collagen fiber occupied regions in dermis of ear and dorsal skin tissues (%/mm^2 of dermis) were calculated using a computer-based automated image analyzer (*i*Solution FL ver 9.1, IMT *i*-solution Inc., Vancouver, QC, Canada) according to our previously established methods (Qin et al., 2014; Kim et al., 2015b; Bai et al., 2016). At least five

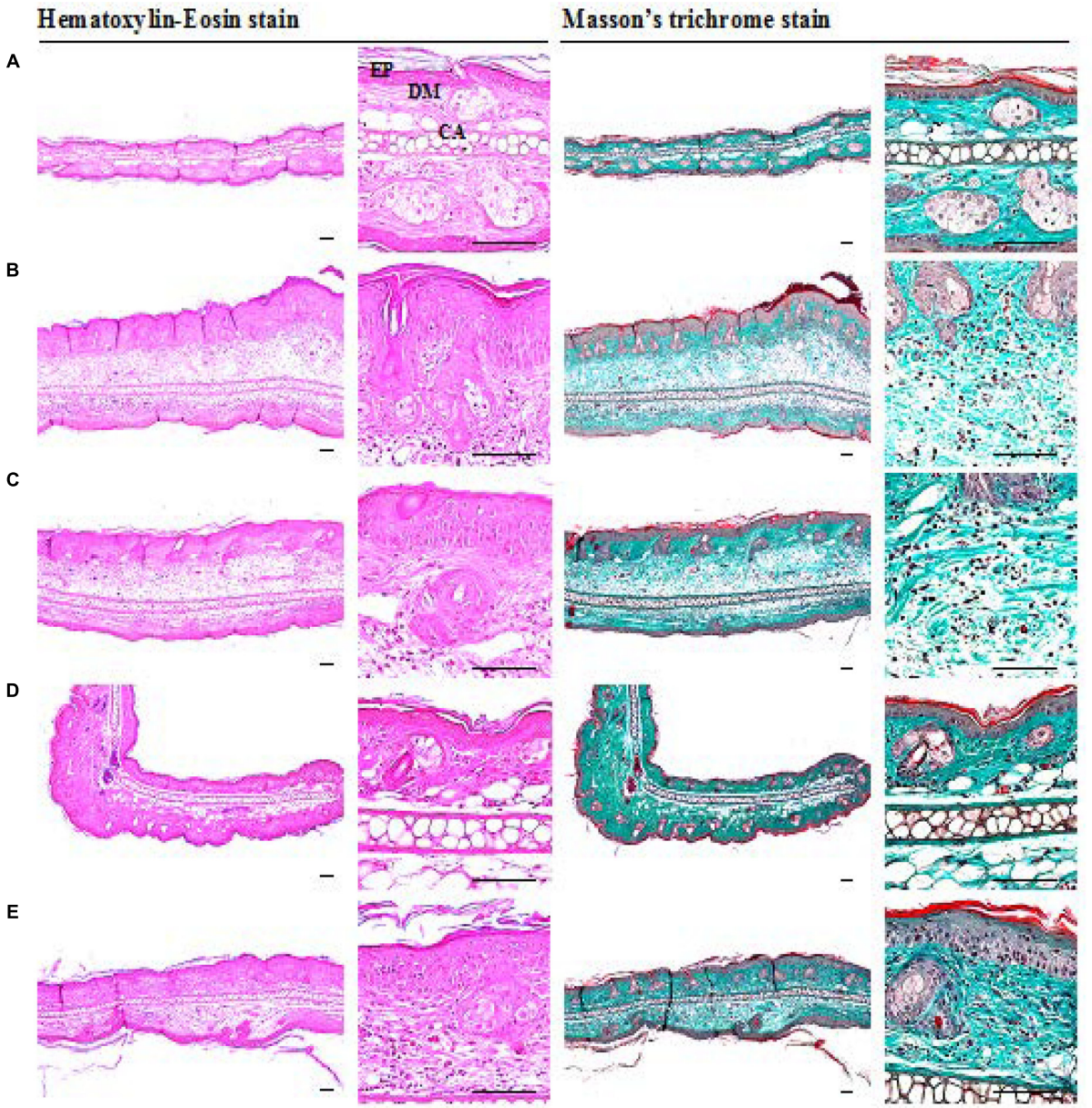

FIGURE 3 | Histopathological images of ear tissues of IMQ-induced psoriasis mice. **(A)** Control, **(B)** IMQ, **(C)** IMQ+vaseline, **(D)** IMQ+clobetasol, and **(E)** IMQ+SEL001. A noticeable increase of total and epithelial (epidermis) thicknesses, inflammatory cell infiltrations in the dermis, and decreases of dermis collagen fibers were observed in ear tissues of IMQ and IMQ+vaseline as compared with intact control, respectively. However, these IMQ-induced hypersensitive psoriasis related histopathological changes on the ear tissues were significantly inhibited by treatment of test material SEL001 and reference drug clobetasol in the current histopathological inspection. No meaningful histopathological changes in the ear tissues were demonstrated in IMQ+vaseline as compared with those of IMQ control [IMQ = Imiquimod; EP = Epithelium/Epidermis; DM = Dermis; CA = Ear cartilage].

repeated measurements were considered to calculate each mean histomorphometric value, whenever possible, in this histopathological evaluation.

Statistical Analysis

The data obtained were analyzed by one-way ANOVA test followed by least significant differences (LSD) multi-comparison test to determine which pairs of group comparison were significantly different. Differences were considered significant at $p < 0.05$.

Quantitative Real-Time (RT) Reverse Transcriptase Polymerase Chain Reaction (PCR)

The extraction of RNA was done using Trizol^{TM} reagent (Invitrogen, United States) following manufacturer's instructions.

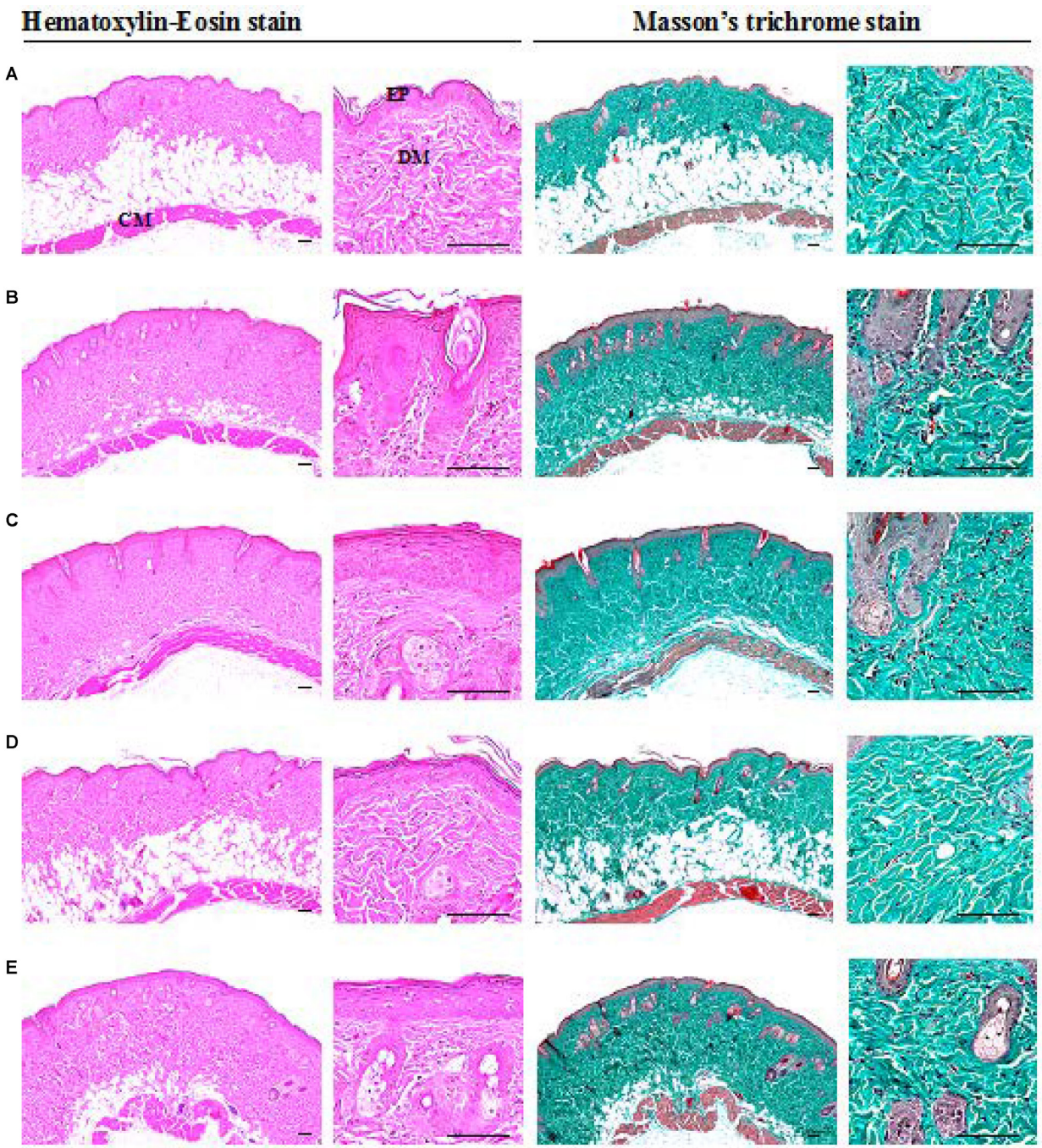

FIGURE 4 | Histopathological images of dorsal skin tissues of IMQ-induced psoriasis mice. **(A)** Control, **(B)** IMQ, **(C)** IMQ+vaseline, **(D)** IMQ+clobetasol, and **(E)** IMQ+SEL001. Increase of dorsal skin epithelial thickness and inflammatory cell infiltrations in dermis of dorsal skin were noticed in IMQ control and IMQ+vaseline as compared with intact control, respectively. But no significant changes on the dorsal back skin total thicknesses and collagen fiber occupied regions in the dermis were demonstrated in IMQ control and IMQ+vaseline as compared with intact control and no significant histopathological changes in the dorsal skin tissues were demonstrated in IMQ+vaseline as compared with those of IMQ control. These IMQ-induced hypersensitive psoriasis related histopathological changes on the dorsal skin tissues were significantly inhibited by treatment of SEL001 and clobetasol.

The extracted RNA was diluted in RNase-free water and quantified using Nano Drop by measuring the absorbance at 260 and 280 nm. Further, 1 μg of RNA was converted to cDNA using Maxime RT premix (iNtRON Biotechnology). Transcription levels of genes were quantitatively determined using RT-PCR (Stratagene 246 mix 3000p QPCR System, Agilent Technologies, Santa Clara, CA, United States) by employing power SYBR green (Roche Diagnostics Gmbh, Mannheim, Germany). The

PCR reactions for each sample were run in duplicate, and for every gene, the transcription levels were normalized with β-actin. The primers used in this study were purchased from Microgen (Korea).

RESULTS

Chemical Composition of SEL001

The GC–MS analysis of ethanolic extract of a probiotic strain *L. sakei* probio65 (SEL001) resulted in the identification of various compounds which included organic acids, amino acids, phenols, imidazole derivatives, and sugar alcohols along with some other organic compounds. The major composition of the SEL001 contained D-lactic acid (33.86%), propanoic acid (16.65%), glycerol (16.6%), L-lactic acid (6.03%), *myo*-inositol (1.86%), 1,3-butandiol (1.35%), 4-(methylsulfanylphenyl) carbamic acid (1.32%), 3,6-dioxa-2,7-disilaoctane (1.31%), pyrrolo[1,2-1] pyrazine-1,4-dione (1.26%), and valine (1.12%).

Erythema and Scaling

The severity of psoriasis during the 6-day trial was scored on 0, 2nd, 4th and 6th day following Psoriasis Area Severity Index (PASI). By applying IMQ on dorsal skin, psoriasis-like skin became apparent on second day onward. No significant changes in skin condition were seen on the second day in groups applied with vaseline, clobetasol or SEL001. However, from day three onward, erythema and scales were more visible. The erythema and scaling on mice back showed a dramatic increase in all groups as seen on day 4. However, a significant increase in erythema and scaling scores was seen in IMQ group when compared to control. In case of IMQ+vaseline group, a steady rate in erythema and slight decrease in scaling was observed from day 4 to day 6. In case of IMQ+clobetasol and IMQ+SEL001 the erythema scores were significantly less when compared to IMQ group ($p < 0.01$), and the scaling scores significantly decreased from day 4 to day 6+. Nevertheless, the erythema score in IMQ+clobetasol and IMQ+SEL001 was not completely normalized when compared to control group. The total mean scores for erythema and scales are shown in **Figures 1A,B**, from 0 day to day 6.

Skin and Ear Thickness

The ear thickness of right ear significantly increased in IMQ and IMQ+vaseline groups. In case of IMQ+clobetasol and IMQ+SEL001, the ear thickness was significantly reduced (**Figure 2A**). In addition, similar results were seen in dorsal skin thickness. The dorsal skinfold thickness of the mice in IMQ group and IMQ+vaseline showed a significant increase by topical application of IMQ treatment as compared with the intact control group ($p < 0.01$). The skinfold thickness in IMQ+clobetasol and IMQ+SEL001 group was significantly reduced compared to the IMQ group ($p < 0.01$) (**Figure 2B**). **Figure 2C** shows the visual appearance of IMQ-induced skin after 6 days of treatment. The skinfold thickness and ear thickening in IMQ+clobetasol and IMQ+SEL001 were not completely normalized when compared to control group. Nevertheless, the effect of clobetasol and SEL001 was promising.

Histological Analysis

A significant increase in epithelial thickness and number of inflammatory cells infiltrated in the dermis of ear tissues were observed in IMQ and IMQ+vaseline group compared

TABLE 1 | Histomorphometrical analysis of ear tissues from control or IMQ-induced psoriasis mice.

Groups	Total thickness (μm)	Epithelial thickness (μm)	Inflammatory cell numbers (cells/mm^2 of dermis)	Collagen occupied regions (%/mm^2 of dermis)
Control	228.70 ± 23.62	20.14 ± 2.67	21.83 ± 3.54	66.92 ± 5.43
IMQ	651.42 ± 133.75^{a}	84.97 ± 13.00^{a}	566.50 ± 125.43^{a}	42.56 ± 4.54^{a}
IMQ+vaseline	653.42 ± 104.38^{a}	83.30 ± 13.78^{a}	550.17 ± 135.19^{a}	41.65 ± 6.66^{a}
IMQ+clobetasol	343.09 ± 86.69^{bcd}	34.43 ± 10.71^{bcd}	156.83 ± 38.59^{bcd}	60.88 ± 1.07^{cd}
IMQ+SEL001	426.78 ± 90.28^{acd}	46.16 ± 13.00^{acd}	286.00 ± 71.55^{acd}	53.56 ± 6.61^{acd}

Values are expressed as mean ± SD of six ear histological fields. IMQ = Imiquimod, SEL001 = Test material; LSD = Least-significant differences multi-comparison. $^{a}p < 0.01$ and $^{b}p < 0.05$ as compared with control by LSD test; $^{c}p < 0.01$ as compared with IMQ by LSD test; $^{d}p < 0.01$ as compared with IMQ+vaseline by LSD test.

TABLE 2 | Histomorphometrical analysis of dorsal skin tissues taken from control or IMQ-induced psoriasis mice.

Groups	Total thickness (μm)	Epithelial thickness (μm)	Inflammatory cell numbers (cells/mm^2 of dermis)	Collagen occupied regions (%/mm^2 of dermis)
Control	807.56 ± 104.99	24.30 ± 6.19	43.33 ± 10.44	68.91 ± 10.00
IMQ	797.82 ± 105.24	65.11 ± 5.47^{a}	319.83 ± 139.20^{d}	67.82 ± 11.00
IMQ+vaseline	814.14 ± 76.68	66.41 ± 10.91^{a}	320.17 ± 116.10^{d}	67.51 ± 11.54
IMQ+clobetasol	792.12 ± 91.95	34.44 ± 10.47^{bc}	37.00 ± 8.44^{ef}	69.07 ± 10.28
IMQ+SEL001	803.39 ± 78.65	44.54 ± 11.40^{abc}	79.67 ± 17.01^{def}	70.84 ± 10.42

Values are expressed as mean ± SD of six ear histological fields. IMQ = Imiquimod, SEL001 = Test material; LSD = Least-significant differences multi-comparison. $^{a}p < 0.01$ as compared with control by LSD test; $^{b}p < 0.01$ as compared with IMQ by LSD test; $^{c}p < 0.01$ as compared with IMQ+vaseline by LSD test; $^{d}p < 0.01$ as compared with control by MW test; $^{e}p < 0.01$ as compared with IMQ by MW test; $^{f}p < 0.01$ as compared with IMQ+vaseline by MW test.

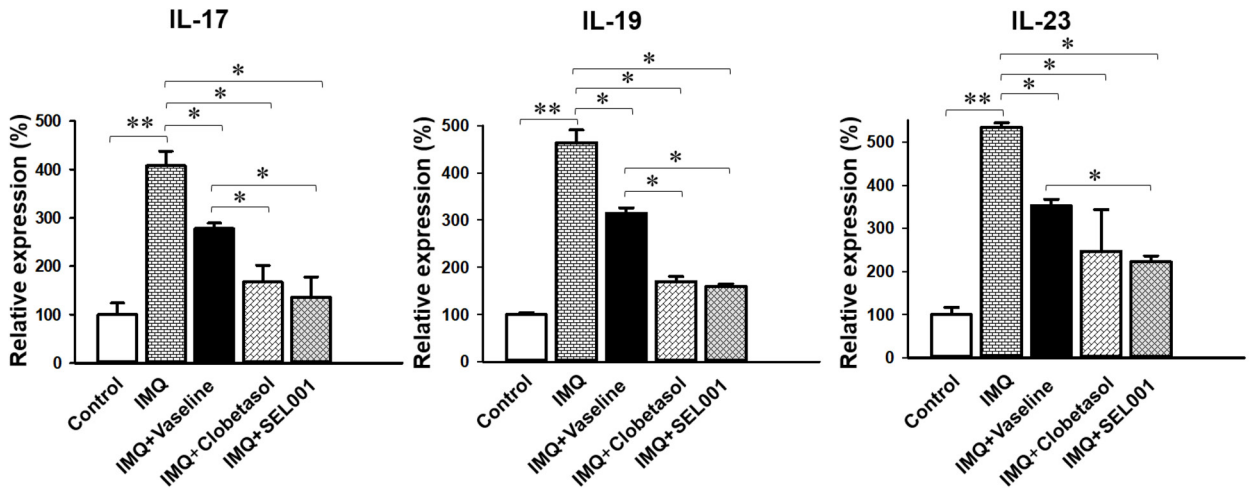

FIGURE 5 | mRNA expression profile. The data is expressed as mean ± standard deviation, $^{**}p < 0.001$ or $^{*}p < 0.05$ is significant, analyzed by one-way ANOVA test.

to control group ($p < 0.01$), as shown in **Figure 3**. In addition, a significant increase in dorsal skin epithelial thickness and the number of inflammatory cells infiltrated in dermis of dorsal skin were also noticed in IMQ group and IMQ+vaseline group as compared with control group ($p < 0.01$), as shown in **Figure 4**. Moreover, no significant changes in total dorsal skin thickness and collagen fiber occupied regions in the dermis were observed in the IMQ group and IMQ+vaseline group as compared with the control group, and no significant histopathological changes in the ear and dorsal skin tissues were observed in the IMQ+vaseline group as compared with those of IMQ group. These IMQ-induced hypertensive psoriasis related histopathological changes in both ear and dorsal skin tissues were significantly inhibited in IMQ+clobetasol and IMQ+SEL001 groups ($p < 0.01$) as shown in **Tables 1**, **2**.

Gene Expression Analysis

Topical treatment of IMQ on the dorsal skin of mice showed an intense change in the gene expression level compared to the intact control, and/or mice treated with IMQ+clobetasol and IMQ+SEL001. The RNA expression analysis indicated an increase in gene expression of IL-19, IL-17A, and IL-23 in the IMQ group when compared with control group a pronounced drop of IL-19 was induced by clobetasol and SEL001 as seen in IMQ+clobetasol and IMQ+SEL001 groups. Similarly, a downregulation of gene expression level of IL-19, IL-17A, and IL-23 was seen in IMQ+clobetasol and IMQ+SEL001 groups compared to IMQ group and IMQ+vaseline group (**Figure 5**).

DISCUSSION

We have previously shown that *L. sakei* pro65 has a potent effect against atopic dermatitis both in the mouse model as well as in human clinical trial (Park et al., 2008; Woo et al., 2010). *Lactobacillus sakei* pro65 significantly reduced the IgE expression level in DNCB induced AD animal model (Kim et al., 2013). Furthermore, in our previous study, we showed *L. sakei* pro65 extract has antioxidant, anti-diabetic and tyrosinase inhibitory effects (Bajpai et al., 2016). Therefore, this study was designed to evaluate the effect of SEL001 on IMQ-induced psoriasis-like skin inflammation in a mouse model. Interestingly, chemical composition analysis of SEL001 confirmed the presence of various bioactive substances in SEL001, including organic acids, amino acids, phenols, imidazole derivatives, and sugar alcohols. As reported previously, these compounds or SEL001 like products of different origins containing phenolics and organic acids have been found to exhibit significant anti-inflammatory effects in various *in vitro* and *in vivo* models (Lee et al., 2006; Beh et al., 2017; Hearps et al., 2017). However, no effective agent has been developed against chronic psoriasis using the probiotic approach, which are known to be Generally Recognized As Safe (GRAS) in nature with no or less adversary effect for human application. Moreover, the SEL001 was found to contain important sugar alcohols, including *myo*-inositol. It has been reported that *myo*-inositol has been implicated with curing inflammatory disorders in animal mouse model (Claxson et al., 1990), suggesting that anti-inflammatory effect observed in this study might be mediated through *myo*-inositol. However, synergistic effects of other active components present in the SEL001 cannot be ruled out. As expected, the application of IMQ resulted in an increase in skin thickness and PASI score for both erythema and scaling. IMQ induced psoriasis model is considered as similar to human psoriasis (Leslie van der et al., 2009; Rather et al., 2016). Nevertheless, it was shown that IMQ induced skin lesions in humans differ to some extent from native psoriasis plaques (Vinter et al., 2015).

In the current histopathological inspection, skin protective effects of SEL001 were observed on the IMQ-induced

hypersensitive psoriasis in a mouse model through well-documented histopathology-histomorphometric methods (Qin et al., 2014; Kim et al., 2015a,b; Bai et al., 2016). The results of representative histological profiles and histomorphometric analysis of the ear and dorsal back skin tissues are shown in **Figures 3** and **4**, respectively.

As reported previously, histopathologically, IMQ-induced hypersensitive psoriasis-like inflammation increased the epidermis hyperplasia and hypertrophy, and dermis inflammatory cell infiltrations in the ear and dorsal skin (van der Fits et al., 2009; Kim et al., 2015a; Bai et al., 2016), and similar results were observed in IMQ and IMQ+vaseline groups in this study. Significant increase of mean total and epidermal thicknesses, numbers of inflammatory cells infiltrated in the dermis, and decrease of dermis collagen fiber occupied regions were observed in the ear tissues of IMQ and IMQ+vaseline groups when compared with the control group. In addition, a significant increase of dorsal skin epithelial thicknesses and number of inflammatory cells infiltrated in the dermis of dorsal back skin were also noticed in IMQ and IMQ+vaseline groups as compared with control group, suggesting classic IMQ-induced hypersensitive psoriasis.

Moreover, no significant changes in the dorsal skin total thicknesses and collagen fiber occupied regions in dermis were demonstrated in IMQ group and IMQ+vaseline group as compared with control group. Also, in the present histopathological measurement, no significant histopathological changes in the tissue of both ear and dorsal skin were observed in IMQ+vaseline group as compared with those of IMQ group, suggesting that vaseline treatment did not show any effect on IMQ-induced hypersensitive psoriasis related histopathology in the ear and dorsal skin tissues. On the other hand, these IMQ-induced hypersensitive psoriasis related histopathological changes in both ear and dorsal skin tissues were significantly inhibited by the treatment of clobetasol and SEL001. Based on histopathological findings, SEL001 has obvious protective effects on the IMQ-induced hypersensitive psoriasis related histopathology in the ear and dorsal skin tissues. The decrease of collagen fibers in the dermis has been indicated edematous changes related to inflammations, as also considered in previous reports (Kim et al., 2015a,b). Decrease in the gene expression of IL-19, IL-17A, and IL-23 levels further confirms the potent effect of SEL001 on IMQ-induced psoriasis.

CONCLUSION

In summary, in this study, the SEL001 ameliorates the severity of IMQ-induced psoriasis like skin inflammation in mice. Topical application of SEL001 significantly reduced the skin thickening, and improved the erythema and scaling scores. These findings were further supported by improvements in histoclinical symptoms. SEL001 significantly inhibited IMQ-induced skin epithelial thicknesses and dermis inflammatory cell infiltrations. Furthermore, treatment with SEL001 decreased the expression level of psoriasis-associated pro-inflammatory cytokines, such as IL-17A, IL-19, and IL-23, proposing that altogether these changes might be mediators of the positive effects of SEL001 observed in the psoriasis-like skin inflammation model used in this study.

AUTHOR CONTRIBUTIONS

IR and VB performed the experiments and analyzed the data. EB and WP helped in the interpretation of the data. JL and Y-HP participated in the design of the study and the interpretation of the data. IR and VB wrote the manuscript. All authors read and approved the manuscript.

FUNDING

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Enhanced Probiotic Potential of *Lactobacillus reuteri* When Delivered as a Biofilm on Dextranomer Microspheres That Contain Beneficial Cargo

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As with all orally consumed probiotics, the Gram-positive bacterium *Lactobacillus reuteri* encounters numerous challenges as it transits through the gastrointestinal tract of the host, including low pH, effectors of the host immune system, as well as competition with commensal and pathogenic bacteria, all of which can greatly reduce the availability of live bacteria for therapeutic purposes. Recently we showed that *L. reuteri*, when adhered in the form of a biofilm to a semi-permeable biocompatible dextranomer microsphere, reduces the incidence of necrotizing enterocolitis by 50% in a well-defined animal model following delivery of a single prophylactic dose. Herein, using the same semi-permeable microspheres, we showed that providing compounds beneficial to *L. reuteri* as diffusible cargo within the microsphere lumen resulted in further advantageous effects including glucosyltransferase-dependent bacterial adherence to the microsphere surface, resistance of bound bacteria against acidic conditions, enhanced adherence of *L. reuteri* to human intestinal epithelial cells *in vitro*, and facilitated production of the antimicrobial compound reuterin and the anti-inflammatory molecule histamine. These data support continued development of this novel probiotic formulation as an adaptable and effective means for targeted delivery of cargo beneficial to the probiotic bacterium.

Keywords: ***Lactobacillus reuteri*, microsphere, reuterin, glucosyltransferase, maltose, dextranomer**

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INTRODUCTION

Probiotic bacteria are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Araya et al., 2006). Commercially, probiotics can be diverse genera of bacteria both alone and in combination are utilized for the treatment of numerous ailments and diseases, such as diarrheal diseases (McFarland, 2006; Johnston et al., 2012), infant colic (Savino et al., 2010; Sung et al., 2014), allergies (Soh et al., 2009; Stefka et al., 2014), and elevated LDL-cholesterol (Agerholm-Larsen et al., 2000; Nguyen et al., 2007).

Orally consumed beneficial bacteria face a gauntlet of challenges in the host, such as low pH in the stomach, effectors of the host immune system and competition with commensal and pathogenic

bacteria (Ding and Shah, 2007). All of these factors negatively impact the ability of ingested probiotic bacteria to be sufficiently sustained within the host and thus reduce the potentially beneficial effects conferred. Commercially, encapsulation of lyophilized probiotics is used as the primary means to protect bacterial viability (Cook et al., 2012; Kailasapathy, 2014), however there is no evidence of improved probiotic persistence within the host.

We have devised a novel delivery method that utilizes hollow semi-permeable, biocompatible and biodegradable microspheres comprised of cross-linked dextran (dextranomer microspheres or DMs). We have previously shown that allowing the probiotic bacterium *Lactobacillus reuteri* to adhere to DMs prior to oral delivery reduces the incidence of experimental necrotizing enterocolitis (NEC) (a disease of high mortality in premature infants born under 1,500 g, Neu and Walker, 2011) by 50% with a single dose in a rat model of the disease (Olson et al., 2016). Importantly, in the same study, a single dose of planktonic *L. reuteri* showed no prophylactic effect. We surmised that it was delivery of the probiotic bacteria in the biofilm state that facilitated the significantly increased therapeutic value of *L. reuteri* in this model system. According to our model (as shown in **Figure 1**), bacteria on the surface of the DMs in the form of a biofilm (i.e., an adhered community of bacteria that produces a self-forming protective matrix to resist adverse environmental conditions, Hall-Stoodley et al., 2004), would also have ready access to any beneficial compounds that diffused from the lumen of the DMs.

The choice to pair the Gram-positive bacterium *L. reuteri* with DMs is many fold. *L. reuteri* is a popular choice for commercialization due to its production of lactic acid and the antimicrobial compound reuterin, a metabolite of glycerol metabolism known chemically as 3-hydroxypropionaldehyde (3-HPA), which typically exists in equilibrium with other downstream products such as 3-HPA hydrate and acrolein (Talarico et al., 1988; Engels et al., 2016). Reuterin induces oxidative stress in a broad range of microorganisms (Schaefer et al., 2010), and is highly effective at inhibiting growth of many gastrointestinal pathogens (el-Ziney and Debevere, 1998; Arques et al., 2004; Spinler et al., 2008; De Weirdt et al., 2012). In addition to the antimicrobial reuterin, *L. reuteri* also produces anti-inflammatory factors including histamine that modulate cytokine production *in vitro* (Jones and Versalovic, 2009) and ameliorate the symptoms of inflammatory bowel disease (Ghouri et al., 2014). Indeed, in animal models, *L. reuteri* can prevent the exacerbating effects of the physiological stress response on colonic inflammation. However, once administration of *L. reuteri* is terminated, colonic inflammation is again exacerbated (Mackos et al., 2016). Thus, strategies to enhance the ability of *L. reuteri* to better persist in the GI tract may have more impactful therapeutic value.

Integral to our probiotic formulation strategy is *L. reuteri*'s extracellular glucosyltransferase (GTF) protein, which in the strain of *L. reuteri* used in this study (DSM 20016, containing GTFW encoded by *gtfW*) (Leemhuis et al., 2013; Bai et al., 2015) catalyzes the formation of exopolysaccharides of glucose (glucans) from its sole known substrate maltose. Importantly,

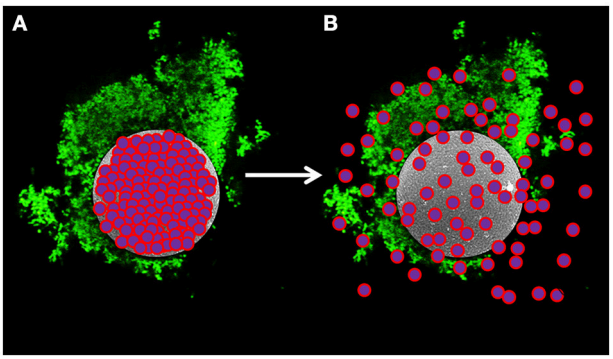

FIGURE 1 | Model of novel probiotic delivery formulation. (A) A biofilm of *L. reuteri* (green) adhered to the surface of a dextranomer microsphere (gray) that contained beneficial compounds as cargo (purple and pink spheres) within the lumen of the microsphere. **(B)** Over time, the cargo will diffuse out of the porous microsphere thereby facilitating ready access by the adhered bacteria.

GTF proteins typically have a glucan binding domain that recognizes its own produced exopolysaccharide (Monchois et al., 1999; Kralj et al., 2004). The GTF protein, its substrate, and resulting glucan product are highly strain-specific in *L. reuteri*; some are characterized as producing dextran (primarily α-1,6 linkages), mutan (primarily α-1,3 linkages), or the aptly named reuteran (primarily α-1,4 linkages) (Kralj et al., 2002, 2004). Cell aggregation, biofilm formation, and gut colonization are directly linked to the activity of GTFA in *L. reuteri* strain TMW1.106; inactivating *gtfA* significantly diminishes the ability of *L. reuteri* to aggregate, form biofilms, and colonize the GI tract *in vivo* (Walter et al., 2008).

Our novel approach was to choose DMs (a macroscopic porous microsphere that is sold commercially for size exclusion chromatography, Porath and Flodin, 1959) as a biocompatible surface so as to take advantage of *L. reuteri's* GTFW native ability to bind to this cross-linked dextran (Tieking et al., 2005; Schwab et al., 2007; Walter et al., 2008). Our strategy is based on concurrent research which shows that the highly similar GTFs of *Streptococccus mutans*, an oral pathogen that contributes to tooth decay, binds to DMs with high affinity (Mooser et al., 1985) and, as a consequence of GTF being cell-associated, results in strong binding of *S. mutans* to DMs (Mashburn-Warren et al., submitted). Here in we show that GTFW-dependent binding of *L. reuteri* to DMs results in: one, selectivity of binding to DMs and as a result better binding of *L. reuteri* to colonic epithelial cells; two, protection against low pH and three, the ability of *L. reuteri* to acquire the luminal contents of the DMs at sufficiently high concentrations to enhance *L. reuteri*'s probiotic effects.

MATERIALS AND METHODS

Strains and Culturing Conditions

Bacterial strains, plasmids and oligonucleotides used are listed in **Table 1**. *L. reuteri* (ATCC 23272) and *Lactobacillus rhamnosus* GG (ATCC 53103) were grown in MRS (de Man, Rogosa, Sharpe) medium (De Man et al., 1960) (BD, Franklin Lakes, NJ) for

TABLE 1 | Bacterial strains, cell lines, plasmids, and oligos used in this study.

Bacterial strains	Description	Source/Reference
Lactobacillus reuteri ATCC 23272	Wild type (GTFW)	American Type Culture Collection
LMW500	*L. reuteri* 23272 Δ*gtfW*; Cm^R	This study
LMW501	*L. reuteri* 23272 + pWAR501 Cm^R	This study
LMW502	*E. coli* ER2566 + pWAR502	This study
LMW503	*L. reuteri* 23272 + pWAR503 Cm^R	This study
Lactobacillus rhamnosus GG ATCC 53102	Wild type (non-dextran forming GTF)	G. Rajashekara J.S. Gunn
Salmonella enterica serovar typhi TY2 ATCC 700931	Wild type (non-dextran forming GTF)	
Citrobacter rodentium ATCC 51459	Wild type (non-dextran forming GTF)	American Type Culture Collection
Clostridium difficile R20291 (BI/NAP1/027)	Wild type (non-dextran forming GTF)	J.K. Spinier
Human cell lines	**Description**	**Source/Reference**
DLD-1 ATCC CCL-221	Human colonic epithelial cells (colorectal adenocarcinoma)	G.E. Besner
FHs 74 Int ATCC CCL-241	Human fetal small intestinal epithelial cells	G.E. Besner
Plasmids	**Description**	**Source/Reference**
pWAR500	pFED760 (Mashburn-Warren et al., 2012) derivative containing *cat* and DNA fragments flanking *gtfW* to create insertion mutant; see Materials and Methods; Cm^R, Erm^R	This study
pWAR501	pJC156 (Mashburn-Warren et al., 2012) derivative containing the promoter region of *gtfW* upstream of the click beetle luciferase; see Materials and Methods; Cm^R	This study
pWAR502	pTXB1 derivative containing *gtfW* (with its stop codon); see Materials and Methods; Amp^R	
pWAR503	pJC156 (Mashburn-Warren et al., 2012) derivative containing the promoter region of elongation factor Tu (EF-Tu) upstream of the click beetle luciferase; see Materials and Methods; Cm^R	This study
Oligos	**DNA sequence***	**Source/Reference**
oSG1082	GCGTG**GCGGCCGC**CATTATTTTCATGTAGTGTATT T	This study
oSG1083	GCGTG**GTCGAC**CTTTTTTATGTCCATAATCTATT	This study
oSG1084	GCGTG**GTCGAC**GAAAATATTTAATATGAAAATGA	This study
oSG1085	GCGTG**CTCGAG**CCAAGCACTATTTCACGAGAAT	This study
LMW34	GCGTG**GTCGAC**GATGAAAATTTGTTTGATTT	Mashburn-Warren et al., 2012
LMW35	GCGTG**GTCGAC**TTATAAAAGCCAGTCATTAG	Mashburn-Warren et al., 2012
oSG1102	GCGTG**CTCGAG**CAACAAGAGTATCAGGGTAAAGC	This study
oSG1103	GCGTG**GTCGAC**TCCTTCCCAATAGATGATTGATT	This study
oSG1067	GCGTG**GTCGAC**ATGGTAAAACGTGAAAAAAATGT	This study
oSG1068	**GCGGCCGC**TCCGCCAGCTTTTTCTAATAACT	This study
oSG1120	GCGTG**GCTAGC**ATGAACCTGCCAACAATTCCTAA	This study
oSG1126	GCGTG**GCTCTTCCGCA**TTAAATATTTTCTTGGTTT	This study
oSG1069	GCGTG**CTCGAG**CGCAACAAATACAGTTTCTAATA	This study
oSG1070	GCGTG**GTCGAC**AAACCTCCTGATAATTTACAAGT	This study

Cm^R, chloramphenicol resistant; Erm^R, Erythromycin resistant; Amp^R, Ampicillin resistant.
**Sequences in bold indicate restriction enzyme sequences.*

16 h at 37°C, 5% CO_2. *Salmonella typhi* (strain JSG698) and *Citrobacter rodentium* (ATCC 51459) were grown in Lysogeny broth (LB) at 37°C, 5% CO_2. *Clostridium difficile* (strain R20291) was grown in degassed brain-heart infusion (BHI) medium (BD, Franklin Lakes, NJ) at 37°C in an anaerobic chamber (Thermo Forma Scientific, 1025 Anaerobic System, Hampton, NJ) established with an atmosphere of 5% H_2, 85% N_2, and 10% CO_2. DLD-1 (ATCC CCL-221) human colonic cells were grown in RPMI medium supplemented with 10% fetal bovine serum at 37°C, 5% CO_2. FHs 74 Int (ATCC CCL-241) human

fetal small intestinal cells were grown in Hybri-Care medium (ATCC 46-X) supplemented with 30 ng/ml epidermal growth factor (EGF) and 10% fetal bovine serum at 37°C, 5% CO_2. The *gtfW* deletion strain (LMW500) was constructed by insertion of a chloramphenicol resistance cassette (cat) into the *gtfW* open reading frame by allelic exchange as described previously (Mashburn-Warren et al., 2012). Briefly, 1kb fragments upstream and downstream of *gtfW* were amplified by PCR using oligos oSG1082-1083 and oSG1084-1085, followed by cloning into pFED760 (Mashburn-Warren et al., 2012) using NotI/SalI and SalI/XhoI restriction sites, respectively. The *cat* cassette was amplified from pEVP3 (Mashburn-Warren et al., 2012) using oligos LMW34-35, followed by cloning into pFED760 that contained the upstream and downstream fragments of *gtfW* using the SalI restriction site. The resulting *gtfW* knock-out construct plasmid (pWAR500) was then introduced into *L. reuteri* ATCC 23272 by electroporation. *L. reuteri* electrocompetent cells were prepared by growing 5 ml of culture in MRS at 37°C with 5% CO_2 until OD_{600nm} of ∼1.0. Cells were then pelleted and resuspended in 10 ml of sterile cold 0.5 M sucrose and 10% glycerol twice, followed by a final resuspension in100 μl sterile cold 0.5 M sucrose and 10% glycerol. To this resuspension 1 μg of pWAR500 was added and the cell/DNA mixture was placed into an ice cold 2 mm electroporation cuvette (BioRad, Hercules, CA). Cells were electroporated at 2500V, 25 μF and 400 Ω using a BioRad Gene Pulser Xcell (BioRad, Hercules, CA). Immediately after electroporation, cells were resuspended in 1 mL of MRS and incubated at 30°C for 2 h, followed by serial dilution and plating onto MRS agar containing 5 μg/ml chloramphenicol and incubated at 30°C. The mutant was selected and confirmed as previously described (Chang et al., 2011).

To estimate transcription from the *gtfW* promoter (P_{gtfW}), the P_{gtfW}-*CBluc* reporter plasmid was constructed by amplifying the promoter region 350 bp upstream of the *gtfW* start codon (including the native ribosome binding site) by PCR using oligos oSG1102-1103. The resulting DNA fragment was inserted into pJC156 using the XhoI/SalI restriction sites. The click beetle luciferase (CBluc) gene was amplified from the *Streptococcus mutans strain* ldhCBGSm (Merritt et al., 2016) using oligos oSG1067-1068 and inserted downstream of the *gtfW* promoter region in pJC156 using SalI/NotI restriction sites. The resulting reporter plasmid pWAR501 was transformed into *L. reuteri* 23272 as described above to create the reporter strain LMW501.

The *E. coli gtfW* overexpression strain (LMW 502) was created by amplifying the *L. reuteri gtfW* open reading frame (including the stop codon) using primers oSG1120-1126. The resulting DNA fragment was inserted into pTXB1 (New England BioLabs, Ipswich, MA) using NheI/SapI restriction sites. The resulting plasmid, pWAR502 was then transformed into the *E. coli* expression strain ER2566 (New England BioLabs, Ipswich, MA) and selected on LB agar containing 100 μg/ml ampicillin and confirmed by DNA sequencing. This strain allows the overexpression of tagless GTFW protein.

To produce a *L. reuteri* strain constitutively expressing click beetle luciferase, a reporter plasmid was constructed by amplifying the promoter region 250 bp upstream of the elongation factor Tu (EF-Tu) start codon (including the native ribosome binding site) by PCR using oligos oSG1069-1070. The resulting DNA fragment was inserted into pJC156 using the XhoI/SalI restriction sites. The click beetle luciferase (CBluc) gene was amplified from the *S. mutans strain* ldhCBGSm (Merritt et al., 2016) using oligos oSG1067-1068 and inserted downstream of the EF-Tu promoter region in pJC156 using SalI/NotI restriction sites. The resulting reporter plasmid pWAR503 was transformed into *L. reuteri* 23272 as described above to create LMW503.

Microsphere Preparation and Application

Anhydrous dextranomer microspheres (DMs; Sephadex® G-25 Superfine) were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Anhydrous cellulose microspheres (CMs; Cellulobeads D50) were obtained from Kobo Products, Inc. (South Plainfield, NJ). Anhydrous microspheres were hydrated in growth medium or water at 50 mg/ml then autoclaved for 20 min. For conditions with microspheres that contained maltose, sucrose, fructose, or glucose only, microspheres previously autoclaved in water were removed from solution on a vacuum filter apparatus and approximately 50 mg were collected via sterile loop into 1ml of filter-sterilized 1M solution of the sugar (see Figure S1). The microsphere mixture was then vortexed vigorously and incubated for 24 h at room temperature to reach equilibrium.

For application with *L. reuteri*, microspheres loaded with water, 1M maltose, 1M sucrose, 1M glucose, or 1M fructose were removed from solution on a vacuum filter apparatus and collected via a 10 μl sterile loop. Approximately 5 mg of hydrated microspheres were then added to 1 ml of 2×10^9 CFU *L. reuteri* from an overnight culture that had previously been pelleted by centrifugation at 3220 × g for 10 min, washed twice with sterile 0.9% saline, and resuspended in 1 ml sterile saline. For experiments involving eukaryotic cell lines, 2×10^9 CFU of bacteria were resuspended in 1 ml RPMI instead of saline. For experiments with no microspheres but equivalent volume of cargo, 10 μl of cargo was added to 1 ml of bacteria either in sterile saline or RPMI. For all experiments, the bacteria and microsphere mixture were incubated together at room temperature for 30 min (unless otherwise stated) to facilitate bacterial adherence and biofilm formation on the microsphere surface prior to use in assays.

Microsphere Adherence Assay

L. reuteri culture was grown and prepared as described above and incubated with microspheres filled with either: water, 1M maltose, 1M sucrose, 1M fructose, or 1M glucose. To examine bacterial adherence to the microspheres, 300 μl of bacteria (from an overnight culture containing ∼2×10^9 CFU) in sterile saline and 5 mg of microspheres were combined and incubated for 5 min in a Micro Bio-Spin column (BioRad, Hercules, CA) (see Figure S2). The columns were then centrifuged (100 × g) for 1 min. The flow-through was serially diluted and plated to calculate the total number of non-adhered bacteria, and this value was subtracted from the total number of starting bacteria to derive the total number of adhered bacteria. For all experiments, a control

preparation that consisted of bacteria with no microspheres was used.

Reporter Assay

The reporter strain LMW501 was grown at 37°C with 5% CO_2in MRS or MRS containing 3% glucose, sucrose, fructose, or maltose and optical densities (OD_{600nm}) of the cultures were measured throughout growth using an Epoch Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT). At the indicated times, 80 μl aliquots of the bacterial cultures were mixed with 20 μl 2 mM D-luciferin in 0.1M citrate buffer, pH 6.0 and placed in a Falcon white flat-bottom 96-well plate (Becton, Dickinson Labware, Franklin Lakes, NJ), followed by luminescence detection using a Veritas Microplate Luminometer (Turner BioSystems Inc., Sunnyvale, CA).

GTF Enzymatic Assay

S. mutans was grown in Todd Hewitt Broth at 37°C with 5% CO_2 until early log phase (OD_{600nm} ∼0.3), *L. reuteri* WT and the Δ*gtfW* mutant were grown in MRS at 37°C with 5% CO_2 until late log phase ($OD_{600\ nm}$ ∼1.0) for optimal *gtf* expression, and the *E. coli gtfW* overexpression strain was grown in LB broth at 37°C shaking (200 rpm) until mid-log phase ($OD_{600\ nm}$ ∼0.4) followed by the addition of 1 mM IPTG to induce *gtfW* expression and was then grown at 37°C shaking for an additional 2 h. Whole cells of *S. mutans*, *L. reuteri* WT, *L. reuteri* Δ*gtfW*, and the *E. coli gtfW* overexpression strain were assayed for GTF activity as previously described (Bai et al., 2015) using Periodic acid-Schiff staining of SDS-PAGE gels.

Cargo Diffusion Assay

The rate of cargo diffusion out of the microspheres was determined by tracking crystal violet, a small molecular weight dye (407.979 g/mol) (Fisher Scientific, Hampton, NJ). The microspheres were loaded with a 0.1% solution of crystal violet by incubating 20 mg of microspheres in 1 ml of 0.1% crystal violet solution either with or without added glycerol (40 or 80% v/v) overnight to reduce the diffusion rate by increasing viscosity. After 16 h, excess crystal violet solution was removed from the microspheres as described above using a vacuum filter apparatus. The crystal violet-loaded microspheres were then placed into 1 ml of water, and aliquots of water were removed and analyzed for diffusion of crystal violet into solution using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT) at OD_{590nm} every hour for 16 h. Percent diffusion was calculated using the equivalent amount of crystal violet within the microspheres (10 μl) in water as a control equivalent to 100% cargo diffusion.

Reuterin Assay

Production of reuterin by *L. reuteri* was measured via a quantitative colorimetric assay (Cadieux et al., 2008). As this assay did not differentiate between similar aldehyde products, measurements included 3-HPA and any potential derivatives, such as acrolein and 3-HPA hydrate. *L. reuteri* was grown overnight in MRS as described above, 1 ml aliquots of 2×10^9 CFU were pelleted at 3,220 × g for 10 min, washed twice with sterile saline, and resuspended in either 1 ml of sterile saline or 1 ml sterile saline containing 2% v/v glycerol. DM containing 0, 2, 10, 20, 30, 40, 50, 60, 70, or 80% glycerol were prepared as described above for other cargo, and added to the resuspended *L. reuteri* in saline (so that the only source of glycerol available for reuterin production was via the microsphere cargo) for 1 h at 37°C. Cells were then pelleted again and the reuterin-containing supernatant was removed, filtered through a 0.45 μm filter, and assayed for reuterin as described in Cadieux et al. (2008) without modification. A standard curve using reuterin at known concentrations was used to extrapolate bacterial-produced reuterin concentrations from DM-glycerol and the 2% v/v glycerol control experimental conditions.

L. reuteri Survival with DM-80% Glycerol

Overnight cultures of WT *L. reuteri* were aliquoted into microcentrifuge tubes, centrifuged, washed twice with sterile saline, and resuspended in either 1 ml saline or 1 ml MRS medium. Five mg of either DM-water or DM-80% glycerol were then added to the tubes and incubated at 37°C. At hourly intervals the tubes were mixed thoroughly and aliquots were taken for subsequent serial dilution and plating for viable CFU of bacteria.

Histamine Assay

Production of histamine from L-histidine by *L. reuteri* was measured via ELISA (Enzo Life Sciences, Inc., Farmingdale, NY). *L. reuteri* was grown overnight in MRS as described above, 1 ml aliquots of 2×10^9 CFU were pelleted at 3220 × g for 10 min, washed twice with sterile saline, and resuspended in one of the following conditions: sterile saline, saline with 3% maltose, saline with 2% v/v glycerol, 4 mg/ml L-histidine (Sigma-Aldrich, St. Louis, MO), 4 mg/ml L-histidine with 3% maltose, or 4 mg/ml L-histidine with 2% v/v glycerol. 5 mg of DM containing either 4 mg/ml or 30 mg/ml L-histidine were added to media lacking L-histidine, so that the only source of L-histidine for *L. reuteri* was as cargo diffusing out of the DMs. Each condition was then incubated at 37°C for 2 h, after which time the contents were pelleted and the supernatant was removed for histamine quantification via a histamine ELISA kit (Enzo Life Sciences, Inc., Farmingdale, NY) following the manufacturer's instructions without modifications. All conditions were done in at least triplicate.

pH Survivability Assay

Bacteria were exposed to a synthetic gastric acid equivalent to determine survival at pH 2. Gastric acid equivalent is a modified version of synthetic gastric fluid (Cotter et al., 2001), composed of 0.1M HCl, 0.1M NaCl, and 0.01M KCl, with pH adjusted to 2 using 0.1M NaOH. For the assay, 1 ml of 2×10^9 CFU of *L. reuteri* from a fresh overnight culture were pelleted at 3,220 × g for 10 min, washed twice with sterile saline, and resuspended in 1 ml 0.9% sterile saline. The cells were incubated for 30 min with approximately 5 mg of loaded or unloaded microspheres as described above, and the bacteria-microsphere mixture was diluted 1:100 directly into gastric acid equivalent. Aliquots of the inoculated acid solution were mixed, serially diluted, and plated

at hourly time points for 4 h to determine the number of viable bacteria. Bacteria without microspheres were used as a control.

Adherence to Intestinal Epithelial Cells

DLD-1 colonic cells and FHs 74 small intestinal cells were cultured as described above. When the adherent epithelial cells reached confluence, the growth medium was removed, cells were washed twice with sterile phosphate buffered saline (PBS), and trypsin-EDTA (0.25%) was added for 10 min at 37°C to dislodge the cells from the culture flask surface. Total epithelial cells were counted using a hemacytometer (Hausser Scientific, Horsham, PA). Cells were then diluted to a concentration of 5×10^5 cells/ml and 1 ml per well was seeded into a 24-well plate and incubated at 37°C, 5% CO_2. After either 48 h (for DLD-1 cells) or 120 h (for FHs 74 cells) of growth, the spent medium was removed and replaced with 1 ml of RPMI or Hybri-Care medium containing 2×10^9 CFU of *L. reuteri* alone, *L. reuteri* with 5 mg water-filled DMs, *L. reuteri* with 5 mg sucrose-filled DMs, or *L. reuteri* with 5 mg maltose-filled DMs. After a 1 h incubation, the spent medium was removed and the well was washed with 1 ml of sterile PBS 3 times to remove non-adhered bacteria. The remaining epithelial cells, with adhered bacteria, were then trypsinized as described above, serially diluted, and plated onto solid MRS medium for enumeration of total adhered bacteria. For confocal microscopy experiments with DLD-1, Nunc Lab-Tek 8-well borosilicate chamber slides (Fisher Scientific, Hampton, NJ) were used in place of 24-well plates. The chamber slides were treated with collagen prior to DLD-1 seeding to improve cellular adherence using the following protocol: a mixture of 100 μl of 7.5% BSA (Sigma-Aldrich, St. Louis, MO), 50 μl of 3.79 mg/ml collagen (Millipore, Temecula, CA), 100 μl of 1 mg/ml rat fibronectin (Biomedical Technologies, Stoughton, MA), and 9.75 ml of PBS was prepared, and 200 μl of this solution was added per chamber slide well. After incubation for 1 h at 37°C, the solution was removed from the well, and epithelial cells were seeded and grown as described above.

Mucin Adherence Assay

Mucin agar plates were created using porcine stomach mucin (Sigma-Aldrich, St. Louis, MO). Mucin agar plates contained 2% mucin and 0.8% agar to simulate the consistency of the mucus layer found *in vivo* (Macfarlane et al., 2005; Van den Abbeele et al., 2009). To assess *L. reuteri*'s ability to bind mucin, 2×10^9 CFU of *L. reuteri* that contained a plasmid that encoded expression of the click beetle luciferase enzyme either planktonically or bound to 5 mg DM-water, DM-sucrose, or DM-maltose were incubated on both mucin agar and agar without mucin stationary at room temperature. After 60 min, the non-adhered *L. reuteri* were removed by washing the plates twice with sterile saline. The luciferase substrate D-luciferin (Sigma-Aldrich, St. Louis, MO) was then added to the plates at a concentration of 0.4 mM to visualize the remaining adhered bacteria. Relative luminosity generated from the bacteria on the plates was measured using a FluorChem E system (ProteinSimple, San Jose, CA) with a 20 min exposure setting. To assess the number of bacteria bound to the mucin within the plate (and not any background binding that may occur to the agar within the plate), the amount of luminescent signal from the agar-only plates was subtracted from the mucin agar plates.

Confocal Microscopy

All confocal laser scanning microscopy (CLSM) was performed using a Zeiss LSM 510 confocal microscope (Ziess AG, Oberkochen, Germany). For fluorescent staining, dextranomer and cellulose microspheres were pre-stained with Congo Red (Fisher Scientific, Hampton, NJ) prior to incubation with the cargo (e.g., sucrose) and experiments with bacteria. *L. reuteri* was stained with SYTO 9 (Life Technologies, Carlsbad, CA). Differential fluorescent visualization was performed using the following settings: Congo Red excitation 554 nm/emission 568 nm, and SYTO 9 excitation 490 nm/emission 525 nm. Samples were fixed using a custom biofilm fixative containing 1.5% paraformaldehyde, 0.025% glutaraldehyde, 4.0% acetic acid, and 0.1M phosphate buffer at pH 7.4 (Devaraj et al., 2015). All microscopy was performed on samples in Nunc Lab-Tek 8-well borosilicate chamber slides (Fisher Scientific, Hampton, NJ). For CLSM experiments with DLD-1 epithelial cells, DLD-1 was stained with 4′,6-Diamidino-2-Phenylindole (DAPI, Life Technologies, Carlsbad, CA), *L. reuteri* was stained with carboxyfluorescein succinimidyl ester (CFSE, Life Technologies, Carlsbad, CA). AxioVision software (Ziess AG, Oberkochen, Germany) and ICY (de Chaumont et al., 2012) were used to analyze images and create figures from CLSM images. COMSTAT (Heydorn et al., 2000) software was used to quantify bacterial biomass in CLSM images.

For *in vitro* biofilm assays, overnight cultures of WT and Δ*gtfW L. reuteri* were diluted into fresh MRS growth medium to 0.01 OD_{600nm}, incubated at 37°C 5% CO_2 for 2.5 h until reaching 0.65 $OD_{600\ nm}$, diluted 1:2,500 into either MRS, MRS + 3% sucrose, or MRS + 3% maltose, seeded into 8-well borosilicate chamber slides and incubated for 1, 3, or 6 h at 37°C 5% CO_2. At the designated time intervals, the bacteria were stained for viability with LIVE/DEAD stain, fixed, visualized via confocal microscopy, and quantified via COMSTAT analysis of the fluorescent signal.

Scanning Electron Microscopy

All scanning electron microscopy (SEM) was performed using a Hitachi S-4800 field emission SEM (Hitachi, Tokyo, Japan). Samples were prepared as described in "Adherence to colonic cells," with the exception that DLD-1 human colonic epithelial cells were grown on 15 mm diameter thermanox coverslips (Electron Microscopy Sciences, Hatfield, PA) placed within the well of a 12-well plate. Samples of DLD-1 cells and adhered bacteria were fixed overnight at 4°C in a solution of 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2). Samples were then washed with double distilled water and stained with a 1% solution of osmium tetroxide (Sigma-Aldrich, St. Louis, MO) in 0.1M phosphate buffer (pH 7.2) for 1 h, washed for 5 min, stained with a 1% solution of thiocarbohydrazide (Sigma-Aldrich, St. Louis, MO), washed for 5 min, and further stained with 1% osmium tetroxide for 30 min. Samples were then dehydrated using a graded series of ethanol: 25% ethanol for 15 min, 50% ethanol for 15 min, 70% ethanol for 30 min, 95% ethanol

for 15 min (twice), 100% ethanol (twice), a 1:1 mixture of 100% ethanol to 100% hexamethyldisilazane (HMDS, Sigma-Aldrich, St. Louis, MO) for 100 min, 100% HMDS for 15 min, and a final immersion in 100% HMDS that was allowed to air dry overnight. Dehydrated sample coverslips were then mounted onto 15 mm diameter metal SEM specimen stubs (Electron Microscopy Sciences, Hatfield, PA) using colloidal silver (Electron Microscopy Sciences, Hatfield, PA). The outer edge, where the stub and coverslip meet, was then coated with a light layer of colloidal silver, and allowed to dry overnight. Samples were sputter coated with gold and palladium for 2 min at 25 mA using an Emitech K550X sputter coater (Quorum Technologies Ltd., Laughton, United Kingdom).

Statistical Analysis

All experiments were conducted a minimum of three times and statistical analysis was performed via a Student's *t*-test using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA), wherein a *P*-value less than 0.05 was accepted as significant.

RESULTS

Maltose or Sucrose within the Lumen of DMs Improved *L. reuteri* Adherence to DMs in a GTF-Dependent Manner

Our strategy was to have probiotic bacteria adhere to a biocompatible surface to induce the formation of a biofilm (**Figure 1**). To investigate this, we differentially stained DMs with Congo Red and *L. reuteri* with SYTO 9, and examined binding via confocal laser scanning microscopy (CLSM). As shown in **Figure 2**, aggregates of bacteria were associated with the surface of numerous DMs which indicated that *L. reuteri* was able to adhere to the DM surface within the time allotted. Since DMs are cross-linked glucan similar to the native reuteran produced by *L. reuteri,* we hypothesized that either an increase in GTFW (for enhanced binding to DMs) or production of glucan to stimulate aggregation and biofilm formation would facilitate the adhered state of *L. reuteri.* To this end, we compared adherence of *L. reuteri* to DMs that contained luminal cargo of either sucrose (an inducer of *gtfW* expression but not a substrate for GTFW; see Figure S3) or maltose (the sole substrate of GTFW). As shown in **Figures 2B,C**, compared to DMs that contained only water within the lumen (**Figure 2A**) there were greater numbers of *L. reuteri* adhered to DMs with either sugar as cargo.

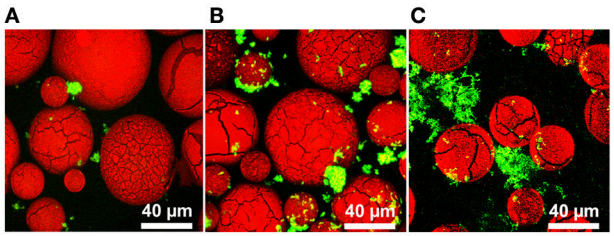

FIGURE 2 | *L. reuteri* binds to dextranomer microspheres. Confocal laser scanning microscopy (CLSM) of *L. reuteri* adhered to DMs. **(A)** Water-filled DMs, **(B)** sucrose-filled DMs, and **(C)** maltose-filled DMs after incubation with *L. reuteri* for 30 min showed that *L. reuteri* adherence to DMs can be enhanced to incorporate biofilm-promoting cargo within the DM lumen (green: bacteria stained with SYTO 9, red: DMs stained with Congo Red).

To further investigate *L. reuteri*'s ability to bind DMs, we tested other DM lumen compounds that we hypothesized should not affect GTFW protein mediated binding and thus unlikely to support increased adherence to DMs. For this assay we chose the monosaccharide subunits of maltose and sucrose (e.g., glucose for maltose, glucose and fructose for sucrose), which the GTF enzyme cannot utilize to catalyze glucan polymers. Interestingly, fructose (and not glucose) was shown to induce *gtfW* expression at a rate similar to sucrose, but did not result in enhanced binding to DMs as was found with sucrose (Figure S3A, **Figure 3A**).

To determine if this GTFW-dependent binding is specific to the glycosyl linkages of DMs, we compared *L. reuteri* binding to cellulose microspheres (CMs), as DMs are composed of polymers of glucose with α-linkages while CMs possess β-linkages between the glucose units (Updegraff, 1969; Kralj et al., 2002). As shown in **Figure 3A**, only ∼10% of *L. reuteri* adhered to CMs regardless of luminal contents. Collectively the data in **Figure 3** indicated that *L. reuteri* does not bind to CMs, binding to DMs was GTFW-dependent and further, that inclusion of maltose or sucrose significantly enhanced the binding of *L reuteri* to DMs. We hypothesized that the predicted glucan binding domain of GTFW is a necessary component of *L. reuteri's* ability to adhere to DMs. To further test if the adherence to DM is GTF-dependent, we created a mutant strain of *L. reuteri* (LMW500) with a chloramphenicol resistance gene inserted in place of the *gtfW* gene. As shown in **Figure 3B**, the Δ*gtfW* strain was not able to bind to DMs as effectively as the wild type (WT) in our spin column assay, regardless of the cargo within the DM lumen. To further demonstrate the difference between the WT and Δ*gtfW*, we examined biofilm formation on glass chamber slides in media supplemented with sucrose or maltose (Figure S4). After a 1 h incubation, the WT had more bacteria present and noticeably more bacterial aggregation when sucrose or maltose was added to the growth medium (Figures S4A,B). After 3 and 6 h with sucrose or maltose supplemented media, the WT displayed a significantly more robust biofilm with greater biomass compared to the *gtfW* mutant under every condition, with significantly more cells present when sucrose or maltose was in the growth medium (Figures S4A,C,D).

We next tested whether bacteria that do not express a similar GTF would lack the adherent phenotype shown in **Figures 3A,B**. To examine this, we performed our DM adherence assay with another probiotic bacterium and three enteric pathogens that *L. reuteri* would likely encounter within the gastrointestinal tract: *Lactobacillus rhamnosus* GG, a Gram-positive bacterium commonly found in the genitourinary system and sold commercially as a probiotic; *Salmonella typhi*, a Gram-negative bacterium responsible for typhoid fever in humans; *Citrobacter rodentium*, a Gram-negative bacterium that causes colitis in rodents; and *Clostridium difficile*, a Gram-positive spore-forming bacterium that can cause severe colitis and recurring infections in humans. As shown in **Figure 3C**, all of

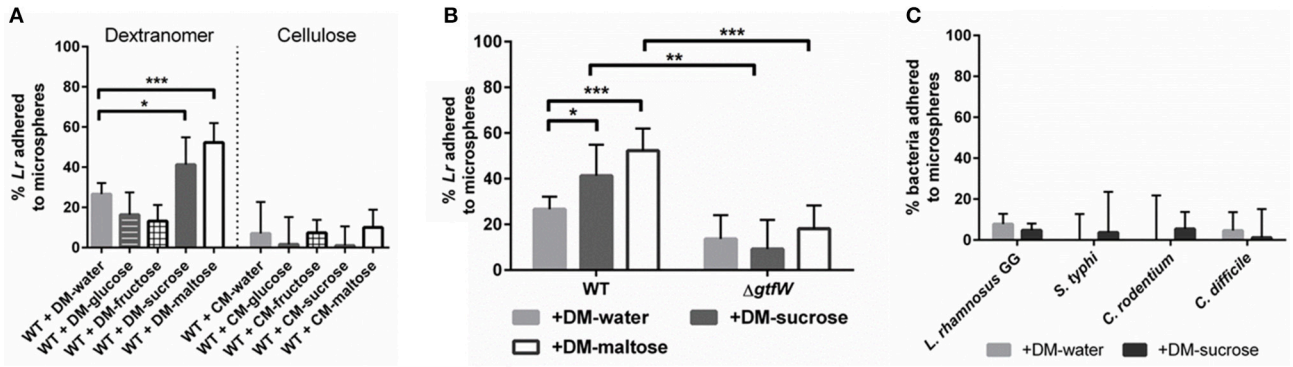

FIGURE 3 | Microsphere composition and lumen cargo affected *L. reuteri* adherence, *L. reuteri* adhered to DMs in GTFW-dependent manner, and bacteria lacking GTF did not bind to DMs. A spin column assay was performed to assess relative bacterial adherence to microspheres. Bacteria were incubated for 5 min with 5 mg of microspheres, centrifuged at 100 × g to separate bound and unbound bacteria, then CFU of non-adhered bacteria were quantified in the flow-through of the spin column. **(A)** Microspheres composed of either cross-linked dextran (DM) or cross-linked cellulose (CM) were filled with water or various sugars at a concentration of 1M to determine which microsphere type supported greatest adherence of *L. reuteri*. **(B)** Relative WT and Δ*gtfW L. reuteri* adherence to DM showed that *L. reuteri* adhered to DMs in a GTF-dependent manner. **(C)** Non-GTF expressing bacteria were similarly tested for microsphere adherence with water-loaded and sucrose-loaded DMs. Error bars represent standard error of the mean. Statistical significance is indicated by the following: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0005$.

the non-GTF expressing bacteria showed minimal adherence to DMs, regardless of cargo present within the DM lumen.

Diffusion of Cargo from DMs

Initial binding of bacteria to DMs is a critical component of our formulation, however equally important is the ability to co-deliver beneficial luminal cargo needed by the adherent bacteria during transit of DMs through the gastrointestinal tract. Targeted delivery of maltose (or any other beneficial compound) via diffusion out of the DMs directly to the probiotic bacterium over time was a desired feature of our system (**Figure 1**). However, since the method of cargo delivery would be diffusion through the porous surface of the microsphere and not its degradation, such as occurs in poly(lactic-co-glycolic) acid (PLGA) microspheres (Danhier et al., 2012), the rate of diffusion is dependent upon the size of the microsphere, the mass of the solute, and the viscosity of the diluent. As proof of concept, we filled the DMs with crystal violet, a small molecular weight stain (407.979 g/mol), and tested the diffusion rate of the dye out of the DMs with and without changing the viscosity of the solution in the DM lumen. As shown in **Figure 4**, the crystal violet diffused out of the DM lumen with a half-life of ∼6 h. When the viscosity was increased by adding 40% glycerol, the half-life of release was increased to ∼8 h. At 80% glycerol, the half-life of crystal violet release was further enhanced to 12 h. By 16 h >95% of all of the crystal violet had been released under all tested conditions.

L. reuteri Produced Reuterin From Glycerol-Loaded Microspheres

An important feature of *L. reuteri*'s function as a probiotic bacterium is its ability to compete with pathogenic bacteria within the host potentially via production of antimicrobials e.g., extracellular reuterin (Cleusix et al., 2007; Spinler et al., 2008). Due to limited glycerol availability, suboptimal endogenous concentrations of glycerol in the GI tract would likely limit adequate reuterin production. In order to obviate the need to provide high levels of glycerol to satisfy *L. reuteri*'s optimal needs, we provided targeted delivery of glycerol directly to the bacteria attached to the surface of DMs. To test this *in vitro*, we utilized a colorimetric assay for reuterin production (Cadieux et al., 2008). As shown in Figure S5, DMs filled with glycerol concentrations ranging from 10 to 80% were able to induce reuterin production. Compared to the 2% glycerol solution control, DMs filled with 80% glycerol produced on average 53% more reuterin in 1 h (average concentration of reuterin produced: 2% glycerol = 40 mM, DM-80% glycerol = 61 mM). To determine if the 80% glycerol or the resulting reuterin/downstream metabolites of glycerol fermentation produced by *L. reuteri* is toxic to *L. reuteri*, we compared hourly colony forming units (CFU) of *L. reuteri* incubated with either DM-water or DM-80% glycerol, in either sterile saline or MRS growth medium. As shown in Figure S6, there was no loss of CFU regardless of DM cargo when *L. reuteri* was incubated in MRS. Incubating *L. reuteri* in saline did result in a steady loss of viable CFU over time, though there was no difference in viability between the DM-water and DM-80% glycerol over this time, suggesting the loss of CFU was not due to any potentially toxic compounds, such as reuterin or acrolein, from glycerol fermentation (Figure S6). As acrolein in particular is known to be toxic to humans and is a byproduct of reuterin production, we next calculated the maximum possible amount of acrolein that could be produced from the dosage of *L. reuteri* and volume of glycerol provided via DMs in our formulation, assuming all available glycerol was converted 1:1 into acrolein. As shown in Figure S7, the amount of acrolein that could possibly be produced via our formulation is a nominal ∼6 μg (for reference, the World Health Organization recommends less than 7.5 μg/kg body weight per day) (Gomes et al., 2002). From these results and the data presented in **Figure 4**, we hypothesized that DMs loaded

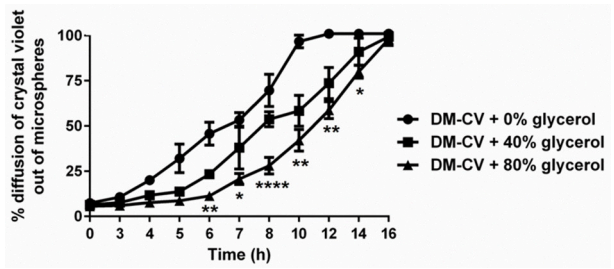

FIGURE 4 | Diffusion of cargo out of microspheres over time. Crystal violet (CV)-loaded DMs with and without glycerol (added to increase viscosity) were assayed to determine the relative rate of CV diffusion from the microspheres. With 0% added glycerol, CV diffused at a higher rate (100% diffusion after 10 h) compared to DMs that contained 40 or 80% glycerol. We observed 100% diffusion from DMs after 16 h regardless of viscosity. Error bars represent standard error of the mean. Statistical significance from DMs with 0% added glycerol is indicated by the following: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

with glycerol would have two beneficial effects *in vivo*, namely slowing the release of beneficial cargo and providing a substrate for reuterin production.

L. reuteri Produced Histamine from L-Histidine-Loaded Microspheres

Histamine produced by *L. reuteri* has previously been shown to inhibit pro-inflammatory cytokines such as TNF via H_2 receptors and reduce colitis in an animal model (Thomas et al., 2012; Gao et al., 2015). Our microsphere-based approach provides a unique method for delivery of the histamine precursor substrate L-histidine to *L. reuteri*. To test this *in vitro*, we filled DMs with 30 mg/ml and 4 mg/ml L-histidine and measured the amount of histamine produced by the bacteria when the only source of L-histidine was via diffusion out of the DMs. As shown in **Figure 5**, DM- L-histidine (4 mg/ml) resulted in histamine levels only slightly lower than those produced when bacteria were incubated in 4 mg/ml L-histidine solution without DMs. When the DMs were loaded with a higher concentration of L-histidine, the amount of histamine produced was 6–7 times greater than the lower 4 mg/ml concentration, consistent with the DM-L-histidine (30 mg/ml) providing ∼7 times more L-histidine than the DM-L-histidine (4 mg/ml) (**Figure 5**). In addition, we tested whether other cargo relevant DM cargo substrates, such as maltose and glycerol, would negatively affect histamine production. Addition of glycerol did not result in reduced histamine production, regardless of whether the L-histidine was in solution or provided via DMs (**Figure 5**). With addition of maltose, histamine production actually increased when L-histidine was provided in solution, but statistically unchanged when L-histidine was provided via DMs (**Figure 5**).

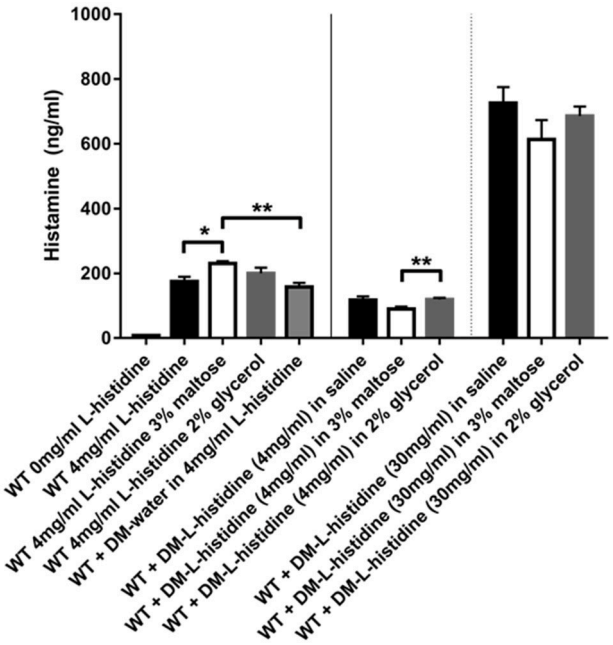

FIGURE 5 | Histamine can be produced by *L. reuteri* from L-histidine delivered via DM. Stationary phase WT *L. reuteri* was incubated for 2 h in either saline with and without 3% maltose or 2% glycerol, or 4 mg/ml L-histidine with and without 3% maltose or 2% glycerol. Histamine production was increased with addition of 3% maltose to 4 mg/ml L-histidine solution (white bar black border) compared to just 4 mg/ml L-histidine (black bar and gray bar black border). When L-histidine at 4 mg/ml was provided via DM the overall levels of histamine produced were significantly lower (middle 3 bars) compared to L-histidine provided in solution (left 4 bars), likely due to less immediate availability of L-histidine to the bacteria. However, when the concentration of L-histidine loaded into DM was increased to 30 mg/ml, significantly more histamine was produced (right 3 bars) despite any caveats related to slower access to L-histidine due to availability only via diffusion out of DM. Error bars represent standard error of the mean. Statistical significance is indicated by the following: $^{*}P < 0.05$, $^{**}P < 0.01$.

Microspheres Filled with Sucrose or Maltose Improved *L. reuteri* Survival at Low pH

Orally consumed probiotics face a significant pH challenge upon reaching the stomach, where pH values are as low as 1.5 when the stomach is empty (Dressman et al., 1990). Enhancing the ability to deliver a maximal number of viable *L. reuteri* to the colon is crucial to its sustainability and effectiveness as a probiotic. We thereby hypothesized that *L. reuteri* bound to the surface of DMs in the form of a biofilm would increase survival upon exposure to acid, and that DMs filled with sucrose or maltose would result in even greater survival in a GTFW-dependent manner. As shown in **Figure 6**, less than 0.1% of WT *L. reuteri* without DMs survived in synthetic gastric acid after 4 h at pH 2, which resulted in a nearly 3 log loss of viable probiotic. Addition of water-filled DMs did not significantly alter the survival rate of WT *L. reuteri* in gastric acid; however, when either DM-sucrose or DM-maltose was delivered with WT, nearly 1 log more survived the acid stress (**Figure 6**). To show that the protective effect is dependent on the microspheres and not the cargo within the DM lumen, we also incubated *L. reuteri* with the equivalent amount of diffusible cargo without the DMs. Acid survival in the presence of cargo only was no different than *L. reuteri* alone (**Figure 6**), which strongly indicated the importance of the bacterial biofilm-on-DM delivery system for the observed protective effect.

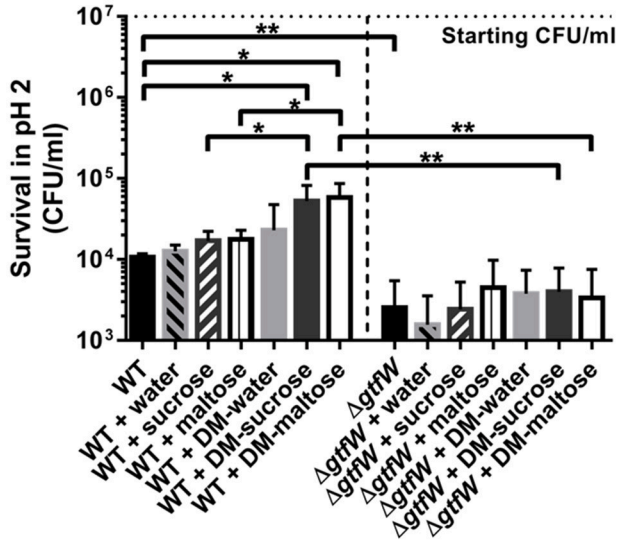

FIGURE 6 | Gastric acid survival. WT and Δ*gtfW L. reuteri* (10^7 CFU/ml) viability after 4 h in pH 2 synthetic gastric acid in the absence or presence of 5 mg of DMs that contained water, sucrose (1M), or maltose (1M) as cargo, or 10 μl of the cargo alone without DMs. Relative survival in acid was enhanced when WT *L. reuteri* was adhered as a biofilm on DMs that contained sucrose or maltose compared to equivalent volumes of the same cargo delivered without DMs, which indicated that the biofilm phenotype contributed to better survival during exposure to low pH. Δ*gtfW* showed decreased resistance to acid compared to the WT, regardless of the presence or absence of either DMs or sugar alone. Error bars represent standard error of the mean. Statistical significance is indicated by the following: $^*P < 0.05$, $^{**}P < 0.01$.

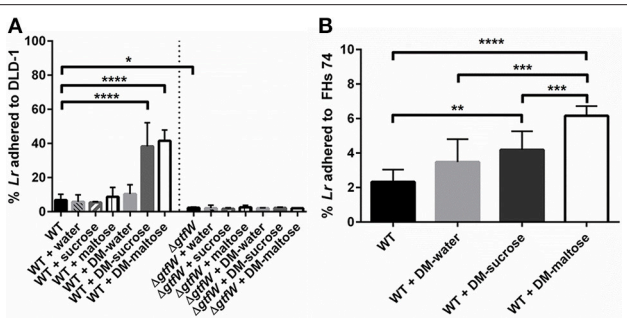

FIGURE 7 | Delivery of *L. reuteri* adhered to DMs as a biofilm supported increased adherence to intestinal epithelial cells. (A) *L. reuteri* WT and Δ*gtfW* adhered as a biofilm on DMs that contained either water, sucrose (1M), or maltose (1M), or the equivalent volume of sugar alone (without DMs), were examined for relative adherence to human colonic DLD-1 cells after incubation for 60 min. Significantly more WT adhered to DLD-1 cells when delivered as a biofilm on the surface of DMs that contained sucrose or maltose, compared to water-filled DMs or the equivalent volume of sugar alone. Significantly fewer Δ*gtfW* mutant cells adhered to DLD-1 cells, regardless of cargo, which indicated that the GTFW protein contributes to *L. reuteri* adherence. **(B)** Adherence of WT to fetal small intestinal FHs 74 cells after 60 min incubation showed that providing *L. reuteri* adherent on the DM surface as a biofilm with either sucrose or maltose as cargo resulted in greater adherence to intestinal cells. Error bars represent standard error of the mean. Statistical significance is indicated by the following: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

To investigate whether this phenotype is GTFW-dependent, we also tested synthetic gastric acid survival using the Δ*gtfW* strain of *L. reuteri* and found that the beneficial effect of DM-sucrose and DM-maltose was lost (**Figure 6**). Interestingly, the mutant also showed deficiency in acid survival without DMs compared to WT, which indicated that GTFW's role in cellular aggregation and biofilm formation (Figure S4) may contribute significantly to survival in synthetic gastric acid.

Microspheres Promote *L. reuteri* Adherence to Human Intestinal Epithelial Cells

Next, we examined what effect the DMs, the DM luminal cargo and the product of the *gtfW* gene have on the relative adherence of *L. reuteri* when delivered as planktonic cells or as biofilms on DMs to the human intestinal cell lines DLD-1 (adult human colonic epithelial cells) and FHs 74 Int (3–4 months gestation, small intestine epithelial cells) *in vitro*. As shown in **Figure 7A**, after a 1 h incubation on DLD-1 cells, significantly more WT *L. reuteri* (without DMs) adhered to the colonic cells compared to Δ*gtfW* either with or without DMs, which indicated that GTFW contributed to host cell adherence. When *L. reuteri* adhered to DMs that contained sucrose or maltose were added to colonic cells, relative adherence of WT *L. reuteri* to the colonic cells was increased by 4.7-fold for DMs that contained sucrose and by 5.2-fold for DMs that contained maltose (**Figure 7A**). Although overall fewer WT *L. reuteri* adhered to the FHs 74 cells than to DLD-1 cells, delivering the bacteria with either DM-sucrose or DM-maltose resulted in 1.8-fold (DM-sucrose) or 2.7-fold (DM-maltose) more adhered bacteria compared to WT bacteria without DM (**Figure 7B**).

To further show that DM luminal cargo of maltose and sucrose improved relative adherence of *L. reuteri* to epithelial cells *in vitro*, we analyzed WT and Δ*gtfW L. reuteri* adherence after 1 h incubation on DLD-1 cells visually, using CSLM (**Figure 8**). As with the CFU data presented in **Figure 7**, delivery of WT *L. reuteri* as a biofilm on maltose or sucrose-loaded DMs supported greater adherence to the DLD-1 cells than those delivered on water-loaded DMs or with no DMs, both by visual inspection (**Figure 8A**) and when analyzed by quantification of bacterial biomass using COMSTAT analysis of CSLM images (**Figure 8B**). The observed adherence was significantly diminished in the Δ*gtfW* mutant compared to the wild type, consistent with measured CFUs (**Figure 7A**).

Finally, we tested the effect of DM adhered WT *L. reuteri*'s ability to bind to mucin. While cellular binding of probiotics likely plays a role in colonization, a healthy GI tract has a mucus layer on the apical surface of epithelial cells, of which the primary constituent is mucin (Turner, 2009). Indeed it is believed that healthy commensals are found primarily within this layer so it is imperative that our formulation maintains it enhanced probiotic effects in the presence of mucin. As mucin adherence is not GTF-dependent, but rather controlled by specific mucin-binding proteins (Miyoshi et al., 2006; Lukic et al., 2012), we hypothesized that being bound to DMs would not have an effect on the ability

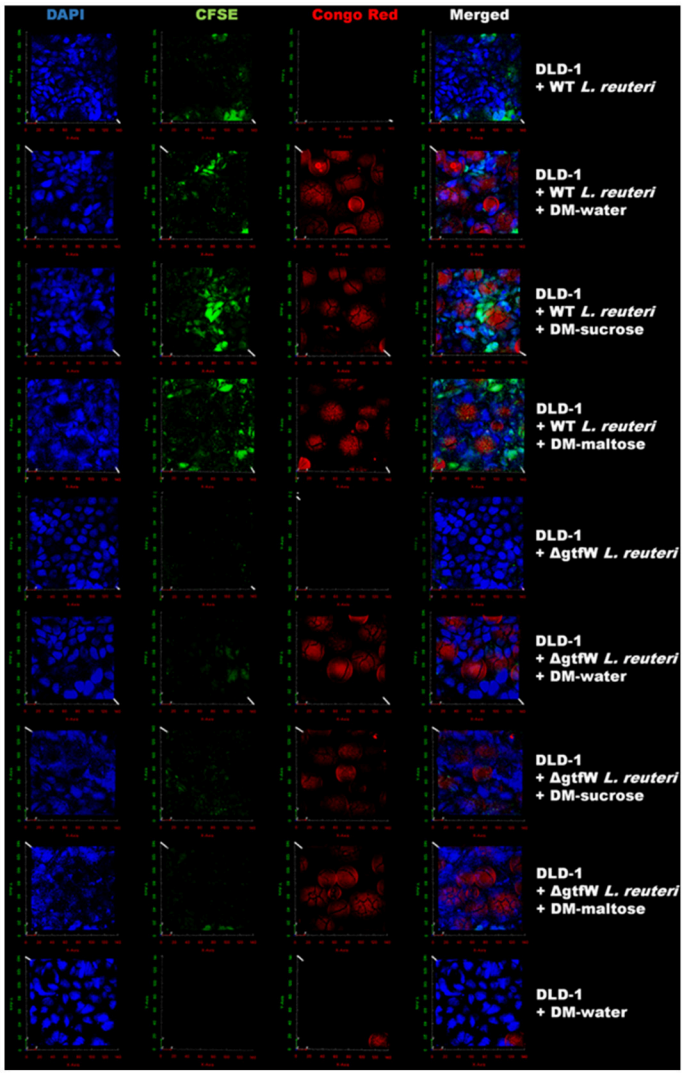

FIGURE 8 | Increased adherence to DLD-1 colonic epithelial cells is observed when *L. reuteri* was delivered as a biofilm attached to DMs. (A) *In vitro* CLSM of DLD-1 epithelial cells (blue, DAPI), *L. reuteri* (green, CFSE), and DMs (red, Congo Red). WT *L. reuteri* (top four rows) compared to Δ*gtfW L. reuteri* (middle four rows) and no *L. reuteri* (bottom row). Bacteria and DMs were pre-stained, incubated for 1 h on pre-stained DLD-1 epithelial cells, washed three times, and fixed for CLSM analysis. **(B)** Comparison of bacterial biomass quantified via COMSTAT analysis of the green channel of CLSM images of WT and Δ*gtfW L. reuteri*. WT without DMs ($n = 10$) resulted in less total bacterial signal compared to either WT + DM-sucrose ($n = 10$) or WT + DM-maltose ($n = 10$). Δ*gtfW* showed no difference in relative number of bacteria adhered to DLD-1 cells, regardless of the presence of DMs. Error bars represent standard error of the mean. Statistical significance is indicated by the following: $^{*}P < 0.05$, $^{**}P < 0.01$.

of *L. reuteri* to adhere to mucin. As shown in Figure S8, there is no significant difference in relative adherence of WT *L. reuteri* to mucin when delivered as either a planktonic bacterial suspension or as a biofilm adhered to DMs after a 60 min incubation on mucin agar plates.

DISCUSSION

We have previously shown that a single dose of *L. reuteri* delivered as a biofilm adhered to DMs reduces the incidence of necrotizing enterocolitis (NEC) by 50% (Olson et al., 2016) in a rat pup model. Here we showed that *L. reuteri* bound to DMs with appropriate luminal cargo promoted significantly increased survival at low pH and supported increased adherence to human epithelial cells *in vitro*. Importantly *L. reuteri* and DMs are considered "generally recognized as safe" (GRAS) by the FDA. In fact, DMs have been used in medical products that are left in the body for long periods of time (years) with no ill effects (Hoy, 2012), such as with Debrisan®, a cicatrizant wound dressing (Jacobsson et al., 1976), Deflux®, a bulking gel used to treat vesicoureteral reflux (VUR) in children (Stenberg and Lackgren, 1995), and Solesta™, a bulking gel injected submucosaly into

the anal canal to treat fecal incontinence (Hoy, 2012). The scope of the research presented here shows a small subset of possible beneficial cargos that can be placed into the DM lumen for utilization by *L. reuteri,* and for many applications it may be as simple as matching the correct lumen cargo precursor to the desired *L. reuteri*-produced effect (e.g., reuterin and histamine). Moreover, this formulation obviates recombinant versions of probiotics, an approach not currently approved by the FDA (Venugopalan et al., 2010).

An exciting feature of our novel formulation is the ability to directly deliver beneficial compounds to the probiotic bacteria that are adhered to the DM surface as a biofilm (**Figure 1**). To combine beneficial compounds (prebiotics) with beneficial bacteria to stimulate growth is a well-established concept in probiotic research and commercial applications (Collins and Gibson, 1999; de Vrese and Schrezenmeir, 2008). There is significant evidence to show that synergism between probiotics and prebiotics effectively increases the overall population of probiotic bacteria (de Vrese and Schrezenmeir, 2008; van Zanten et al., 2014) and promotes effective treatments of diseases such as inflammatory bowel disease (Geier et al., 2007) and necrotizing enterocolitis (Asmerom et al., 2015). However, a major drawback of traditional prebiotics is that they are typically limited to carbohydrates that are non-digestible or absorbable by the host to ensure sufficient availability to the probiotic bacteria in the gut. Our delivery system effectively solves this problem in that the probiotic bacterium *L. reuteri* is now delivered: (1) in association with DMs to which it adheres in greater numbers; (2) in the form of a biofilm which confers resistance to clearance; (3) along with a cargo of nutrients that promotes bacterial growth; (4) with cargos that promote production of the antimicrobial reuterin or histamine; (5) in a format that is resistant to acid-mediated killing thus promoting improved survival during transit through the acidic stomach, and (6) in a manner that appeared to better support adherence to intestinal epithelial cells and thus likely to promote persistence in the gut. With regard to *L. reuteri*-induced release of substance potentially beneficial to the host, reuterin has been suggested to inhibit competition by other gut flora, and histamine has been shown to have anti-inflammatory effects. Although the secondary metabolites produced from glycerol metabolism to generate reuterin (e.g., acrolein) and histamine could result in adverse effects at high levels, the maximum quantities generated with our formulations are $<1\%$ and $<$ 40% less than what is thought to be problematic in humans for acrolein (Figure S7) and histamine, respectively (Maintz and Novak, 2007; Thomas et al., 2012; Engels et al., 2016). Ongoing and future experiments utilizing *L. reuteri* adhered to DMs will test the putative aforementioned beneficial cargos in an *in vivo* animal model (Olson et al., in preparation) to demonstrate both safety and efficacy. Concurrently we are also investigating strategies for long-term storage and downstream application and delivery of our DM-based formulation.

Using maltose as cargo have particular value for several reasons; it is the substrate for this strain of *L. reuteri*'s glucosyltransferase (GTFW) (Leemhuis et al., 2013; Bai et al., 2015), induces *L. reuteri* to aggregate in a GTF-dependent manner (Walter et al., 2008), and causes *L. reuteri* to grow significantly faster and to a higher cell density (CFU/ml). In this study, we show that both maltose and sucrose have a positive effect on *L. reuteri* adherence to microspheres, promote adherence of *L. reuteri* to human intestinal epithelial cells, and improves bacterial survival in gastric acid (**Figures 2**, **3**, **6**, **7**, **8**). We have demonstrated in concurrent work that *S. mutans* binds rapidly and with high affinity to DMs, and the effect is increased in the presence of sucrose in a GTF-dependent manner (Mashburn-Warren et al., submitted). *S. mutans* and *L. reuteri* GTF proteins are very similar in sequence and structure. Sucrose is the sole substrate for *S. mutans* and most *L. reuteri* GTF proteins (Tieking et al., 2005; Walter et al., 2008), and sucrose has previously been shown to cause *L. reuteri* cultures to aggregate rapidly in a GTF-dependent manner (Walter et al., 2008). The positive effect of sucrose to induce GTFW dependent adhesion is likely due to GTFW acting as an adhesin to DMs (via the glucan binding domain) and sucrose's ability to induce *gtfW* expression (Figure S3A). Indeed, failure of sucrose to affect *L. reuteri* adherence to CMs (cross-linked glucan with variant glycosidic linkages) supports this notion. Sucrose-dependent biofilm formation has previously been linked to two-component regulatory systems in the rodent strain 100-23 of *L. reuteri* (Frese et al., 2011; Su and Ganzle, 2014); however, the genes necessary for this phenomenon appear to be absent in the human-derived strain of *L. reuteri* used in this study (23272/DSM 20016). Since sucrose is a preferred carbon source of the *L. reuteri* used in this study via its sucrose phophorylase mediated metabolism (Ganzle and Follador, 2012) it was not surprising that sucrose had a positive impact on biofilm formation and increased adherence to DMs and is likely due to the increased doubling time of *L. reuteri* in the presence of sucrose. The failure of glucose (a carbon source but not a *gtfW* inducer or GTFW substrate) and fructose [an inducer of *gtfW*, but not a carbon source (Figure S3 and data not shown), or substrate for GTFW] to enhance adherence to DMs suggests that understanding bacterial physiology will be critical in selecting beneficial luminal cargos.

Although we describe *L. reuteri* adhered to DMs as a biofilm, we have yet to characterize this physiologic state. We have demonstrated in previous work with the dental pathogen *Aggregatibacter actinomycetemcomitans*, that challenging a host with an already-established pathogenic biofilm results in greater ability of the pathogen to establish disease in an animal model of oral infection (Freire et al., 2011, 2017). While it is clear that being bound to DMs offers multiple advantages in terms of survivability and relative adherence to an epithelial target cell, the actual state of the DM-adhered *L. reuteri* and its phenotype has yet to be determined. There is evidence that microbes such as *L. reuteri* exist naturally as biofilms in the gastrointestinal tract (Macfarlane and Dillon, 2007), but there has thus far been a lack of research as to the composition and dynamics of biofilm communities of the gut (Hall-Stoodley et al., 2004; de Vos, 2015). *L. reuteri* adhered to DMs in our experiments are biofilms by definition and via our observations in **Figure 6**, which showed that bacteria adhered to DMs resisted low pH challenge better than planktonic bacteria. In experiments where *L. reuteri* bound to DMs are incubated with colonic cells we observed both aggregates of bacteria surrounding the DMs as well as those that were adhered to the confluent eukaryotic cell surface (Figure S9). Future work on the biofilm state will include the dlt operon that encodes proteins involved

in D-alanylation of teichoic acid, and shown to be important in biofilm formation, adherence, and host colonization (Walter et al., 2007).

In this study we show that many parameters important to *L. reuteri*'s survivability and sustainability within the host can be improved by delivering *L. reuteri* as a biofilm on the surface of DMs that contain beneficial cargo. With more viable bacteria available after low pH challenge and supporting increased adherence to intestinal epithelial cells, the resulting expansion of probiotic bacteria available within the host should have an increased potentially beneficial effect. Further, we are able to deliver targeted nutrients and substrates directly to the bacteria adhered on the DM surface, which has broad-reaching implications for the type of compounds that can be co-delivered with orally consumed *L. reuteri,* which to date have been limited to carbohydrates that are indigestible by the host. Taken together, our novel delivery system provides an exciting framework for future probiotic development and deployment.

AUTHOR CONTRIBUTIONS

SG, LM, and JN designed the study. JN and LM performed the experimental work. JN, LM, and SG analyzed the data. JN prepared the manuscript; and LM, SG, MB, and LB contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00489/full#supplementary-material

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Lactobacillus fermentum Postbiotic-induced Autophagy as Potential Approach for Treatment of Acetaminophen Hepatotoxicity

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The aim of this study was to investigate the potential of postbiotics originated from *Lactobacillus fermentum* BGHV110 strain (HV110) to counteract acetaminophen (APAP)-induced hepatotoxicity in HepG2 cells. This strain was selected according to its autophagy inducing potential, based on previous studies reporting protective role of autophagy in APAP caused cellular damage. Cell viability was assessed using MTT and LDH assays, while autophagy was monitored by qPCR analysis of *BECN1*, *Atg5*, *p62/SQSTM1,* and *PINK1* mRNA expression and by Western blot analysis of p62/SQSTM1 and lipidated LC3 accumulation. Our results showed that detrimental effect of APAP on cell viability was suppressed in the presence of HV110 which was linked with increased conversion of LC3 protein and p62/SQSTM1 protein degradation. Additionally, higher *p62/SQSTM1* and *PINK1* mRNA transcription were noticed in cells co-treated with APAP/HV110, simultaneously. In conclusion, this study suggests that HV110 enhances activation of PINK1-dependent autophagy in HepG2 cells and its eventual co-supplementation with APAP could be potentially used for alleviation of hepatotoxic side effects caused by APAP overdose.

Keywords: autophagy, *Lactobacillus fermentum*, postbiotics, acetaminophen, hepatotoxicity

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INTRODUCTION

Acetaminophen [paracetamol, *N*-acetyl-*p*-aminophenol (APAP)] is widely used analgesic and antipyretic drug which is safe and effective at a therapeutic dose (Lee, 2004). However, acute or cumulative overdose can cause hepatic necrosis and liver failure (Larson et al., 2005). The mechanisms of APAP-induced liver injury described to this moment include generation of reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) and *p*-aminophenol (PAP) (Miyakawa et al., 2015). Given an important role of autophagy in elimination of damaged organelles, including mitochondria, it has been shown that activation of autophagy could serve as a cellular adaptive mechanism to counteract APAP-induced hepatotoxicity (Igusa et al., 2012; Ni et al., 2012).

Autophagy is tightly regulated and highly inducible catabolic cellular process involved in degradation of organelles and long-living proteins. During this process double-membrane vesicles (autophagosomes) are formed and fused with lysosomes while enclosed material is degraded. Literature data suggest that deregulation of autophagy is highly associated with various liver diseases. For example, in liver ischemia reperfusion injury autophagy exhibits prosurvival activity, while in hepatocellular carcinoma autophagy level is decreased (Rautou et al., 2010). Therefore,

therapeutics capable to induce autophagy could be beneficial for liver associated pathological conditions.

In addition to well-known autophagy promoting stimuli (e.g., starvation, rapamycin, hormones), upregulation of autophagy can occur in bacterial, viral, and parasitic infections (Lum et al., 2005; Mizushima et al., 2010). Irving et al. (2014) demonstrated that peptidoglycan derived from *Helicobacter pylori* and *Pseudomonas aeruginosa* promotes autophagy in epithelial cells via NOD1 receptor activation. Moreover, several studies reported potential of some *Bifidobacteria* and *Lactobacillus* species to stimulate or suppress autophagy (Wu et al., 2013; Lin et al., 2014; Motevaseli et al., 2016).

Lactobacilli are beneficial bacteria, commonly used as probiotics. It has been shown that certain *Lactobacillus* strains were associated with suppression of liver injury caused by oxidative stress, pathogens, hepatic encephalopathy, and alcoholic liver disease (Segawa et al., 2008; Forsyth et al., 2009; Rishi et al., 2009). However, novel trends in probiotic supplementation are oriented toward replacement of live bacteria with non-viable bacterial extracts and metabolic by-products, termed postbiotics (Konstantinov et al., 2013; Patel and Denning, 2013). This new approach reduces health risks associated with consumption of live bacteria, especially concerning their high immune stimulating potential (Tsilingiri et al., 2012). Recent data showed that postbiotics can modulate different cellular pathways. Sharma et al. (2011) reported the cyto-protective activity of supernatants obtained from probiotics *Enterococcus lactis* IITRHR1 and *Lactobacillus acidophilus* MTCC447 against APAP induced hepatotoxicity. Particularly, the authors showed that the postbiotics have potential to restore glutathione level, reduce generation of major oxidative stress markers and to enhance production of anti-apoptotic (Bcl-2) protein.

Considering the fact that mitochondrial damage is a critical event in APAP-induced oxidative stress and cellular necrosis, activation of PINK1-Parkin signaling pathway is crucial for upregulation of mitochondrial autophagy. PINK1 is required for Parkin recruitment to damaged mitochondria when mitochondrial membrane potential is impaired, causing recruitment of p62/SQSTM1, an autophagy adaptor molecule, which is essential for final mitochondrial clearance (Williams and Ding, 2015).

In the light of above presented facts, we assessed the potential of autophagy inducing postbiotics originated from *Lactobacillus fermentum* BGHV110 strain to improve the viability of human hepatoma HepG2 cells exposed to APAP. Hence, according to our knowledge the results of this study for the first time suggest on the cyto-protective effect of postbiotics in APAP mediated hepatotoxicity.

MATERIALS AND METHODS

Bacterial Strain and Preparation of the Bioactive Lysate (Postbiotic)

Lactobacillus fermentum BGHV110, a human isolate from Laboratory collection, was used in the study. Determination of the species identity was performed by 16S rDNA sequencing using UNI16SF and UNI16SR primers complementary to 16S rDNA (Jovcic et al., 2009). PCR amplification was performed using KAPA *Taq* DNA polymerase kit (Kapa Biosystems, Wilmington, MA, USA). Reaction mixture contained: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl2, 50 mM each of the dNTPs, 1 U of *Taq* polymerase, 5 pM of each primer (for multiplex PCR 0.25 μM of each primer), and 0.1 μg of template DNA in a final volume of 50 μl. The PCR product was purified with QIAquick PCR Purification KIT (Qiagen, Hilden, Germany) and sequenced by Macrogen (Seoul, South Korea). The BLAST algorithm[1] was used to determine the most related DNA sequences in the NCBI GenBank database.

Bacteria were cultured in MRS broth (Merck, Darmstadt, Germany) at 37°C under anaerobic conditions using Anaerocult A (Merck). In order to obtain bioactive lysate overnight culture was pelleted (5000 rpm, 10 min) and washed twice with phosphate-buffered saline (PBS). Bacterial pellet was 10 times concentrated in PBS followed by homogenization in a French press (three passages). Homogenized bacterial suspension was lyophilized (Alpha 1–4 LSC Plus Freeze dryer, Martin Christ, Germany) and stored at +4°C until further use.

Cell Culture and Treatments

Human hepatoma cell line HepG2 was cultured in low glucose DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin and 2 mM l-glutamine (Gibco, Life Technologies). The cells were maintained in 75 cm^2 flasks at 37°C in a humidified atmosphere containing 5% CO_2 and split at 80% confluence every 5 days. Cells were seeded in 24-well plate (2×10^5 cells) and incubated at 37°C overnight followed by cells pretreatment with complete DMEM containing high glucose concentration in order to downregulate autophagy (Kobayashi et al., 2012). After 6 h, cells were treated with different concentrations of postbiotics obtained from *Lactobacillus fermentum* BGHV110 strain (HV110) in order to select appropriate dose for further experiments. Postbiotic was dissolved in complete DMEM medium and added to the cells in specific final concentration. In all other experiments seeded cells were treated with 50 mM APAP (Sigma-Aldrich, Germany) alone or co-treated with 50 mM APAP and selected dose of lyophilized HV110. To analyze autophagic flux, simultaneously with treatments, cells were exposed to lysosomotropic agent chloroquine (Sigma-Aldrich) at a concentration of 25 μM, to inhibit autophagosome–lysosome fusion. After 16 h of incubation, cells were subjected to following analysis.

Metabolic Activity of HepG2 Cells

Metabolic activity of HepG2 cells was examined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Serva, Electrophoresis GmbH, Heidelberg, Germany) as described by Mosmann (1983). After the treatment, the cells were washed with PBS and MTT dissolved in complete media was added at the final concentration of 0.5 mg/ml. After 4 h of incubation, at 37°C with 5% CO_2, the media was aspirated and 10% SDS-0.01 N HCl was added to dissolve formazan.

[1] http://www.ncbi.nlm.nih.gov/BLAST

The absorbance was measured with a microplate reader (Tecan Austria GmbH, Grödig, Austria) at a wavelength of 570 nm. Results are presented as percentage of metabolic activity of treated cells compared to control.

Cytotoxicity Assay

The level of cytotoxicity in the cell cultures was measured by lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Thermo Scientific) which detects LDH released from dead cells. After treatments, supernatants were collected and LDH activity was determined by following the manufacturer's instructions. The absorbance was measured at 450 nm on a microplate reader (Tecan). Since there is an interference between APAP and LDH assay (Xu et al., 2003) only the treatments where APAP is used in the same dose are compared and results are presented as absorbance at 450 nm.

Western Blotting

Following the different treatments, cells were lysed with RIPA buffer (50 mM Tris-HCl pH = 7.4; 150 mM NaCl; 1% NP-40; 0.25% sodium deoxycholate) containing Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland) and 1 mM phenylmethyl sulfonyl fluoride (Sigma-Aldrich), for 30 min on ice. Cell lysate was centrifuged at 12000 rpm for 15 min at 4°C and the protein concentration was measured using Bradford reagent (Bio-Rad Laboratories). Total cell proteins (20 μg) were separated on 12% SDS-PAGE and transferred to 0.2 μm nitrocellulose membrane (GE Healthcare) using a Bio-Rad Mini *trans*-blot system (Bio-Rad, Hercules, CA, USA). In case of p62 detection, proteins were transferred to 0.45 μm PVDF membrane (Millipore Corporation, Billerica, MA, USA). Immunoblots were blocked in a 10% non-fat dry milk in TBS-Tween (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, and 0.05% Tween-20) overnight at 4°C followed by 2 h incubation at room temperature with the primary antibodies; anti-LC3 (1:2000; Thermo Fischer Scientific), anti-p62 (1:1000; Progen Biotechnik GmbH, Heidelberg, Germany) and anti-β-actin (1:1000; Thermo Fischer Scientific). The membranes were subsequently washed and incubated with appropriate HPR-conjugated secondary antibodies (goat anti-rabbit; 1:10000; Thermo Fischer Scientific and goat anti-guinea pig; 1:10000; Novex Life Technologies) for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence (Immobilon Western, Merck Milipore). The intensity of the bands was quantified using ImageJ software. p62 was normalized to β-actin loading control. Autophagy induction was measured by calculation of LC3-II/LC3-I ratio.

Quantitative Real-time PCR

Total RNA was extracted from HepG2 cells as previously described by Lukic et al. (2013) with slight modifications. Cells were washed with PBS and lysed in denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.1 M β-mercaptoethanol, 0.5% [wt/vol] *N*-lauroylsarcosinate sodium salt) followed by acid phenol (pH 4) extractions and isopropanol precipitation. cDNA was generated from 0.5 μg total RNA according to the reverse transcriptase manufacturer's protocol (Thermo Scientific). Quantitative PCR was carried out on 7500 real-time PCR system (Applied Biosystems, Waltham, MA, USA) using KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) under the following conditions: 3 min at 95°C activation, 40 cycles of 15 s at 95°C and 60 s at 60°C. All used primers (**Table 1**) were purchased from Thermo Fisher Scientific.

Statistical Analysis

All data are presented as mean values ± standard error of the mean (SEM). One-way ANOVA with the Tukey's *post hoc* test were used to compare multiple groups. The differences between control and experimental groups were compared using Student's *t*-test. Values at $p < 0.05$ or less were considered to be statistically significant. All experiments were repeated at least three times. Statistical analysis was carried out using SPSS 20.0 for Windows. Graphs were drawn in the GraphPad Prism software (trial version).

RESULTS

HV110 Exhibits Dose-dependent Effect on Cell Viability

We initially investigated in which way HV110 affects metabolic activity of HepG2 cells. The results of MTT assay showed dose-dependent effect of HV110 on HepG2 metabolic activity. Doses of 5 and 7 mg/ml of HV110 significantly ($p < 0.05$) decreased cell metabolic activity to 88.11 ± 0.43% and 83.06 ± 0.35%, respectively (**Figure 1A**). As detected decrease in metabolic activity could point on cell death, the level of LDH released in the cell culture was examined in order to determine the HV110 cytotoxicity. Only when applied in dose of 7 mg/ml, significantly higher ($p < 0.05$) LDH level in supernatants of the treated cells was detected in comparison to control (**Figure 1B**). Since the dose of 3 mg/ml didn't change cell metabolic activity or cause cell damage, this dose was used in further experiments.

HV110 Protects Cells against APAP-Induced Hepatotoxicity

In order to explore the potential cyto-protective role of selected dose of HV110 in APAP-induced hepatotoxicity, cell viability

TABLE 1 | The list of primers used in this study.

Primer name	Primer sequence 5′–3′	Reference
β-Actin forward	TTGCTGACAGGATGCAGAAGGAGA	Li et al., 2015
β-Actin reverse	TCAGTAACAGTCCGCCTAGAAGCA	
BECN1 forward	CTGGGACAACAAGTTTGACCAT	Liu et al., 2014
BECN1 reverse	GCTCCTCAGAGTTAAACTGGGTT	
ATG5 forward	CACAAGCAACTCTGGATGGGATTG	He et al., 2013
ATG5 reverse	GCAGCCAC GGACGAAACAG	
p62/SQSTM forward	GCCAGAGGAACAGATGGAGT	Sahani et al., 2014
p62/SQSTM reverse	TCCGATTCTG GCATCTGTAG	
PINK1 forward	GGGGAGTATGGAGCAGTCAC	Kim et al., 2013
PINK1 reverse	CATCAGGGTAGTCGACCAGG	

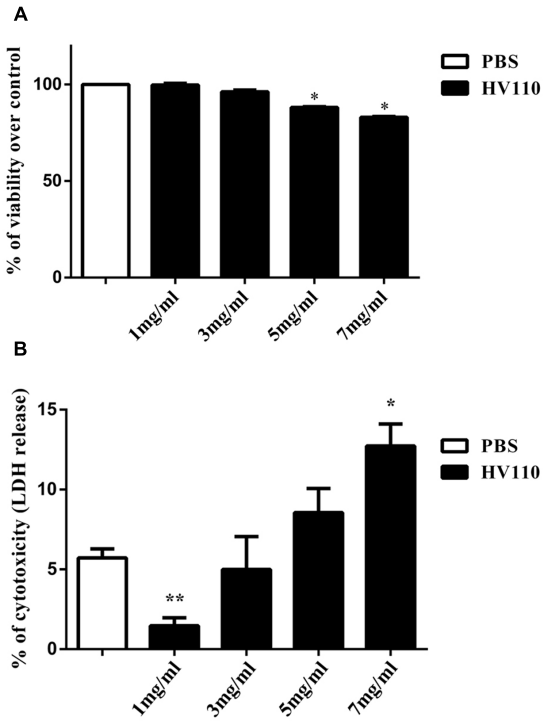

FIGURE 1 | Dose-dependent effect of HV110 on HepG2 cells viability after 16 h of treatment assessed by MTT assay (**A**; $n = 3$) and LDH assay (**B**; $n = 3$). All values are presented as mean ± SEM. Student's *t*-test was used to compare treated groups relative to control (*$p < 0.05$, **$p < 0.01$).

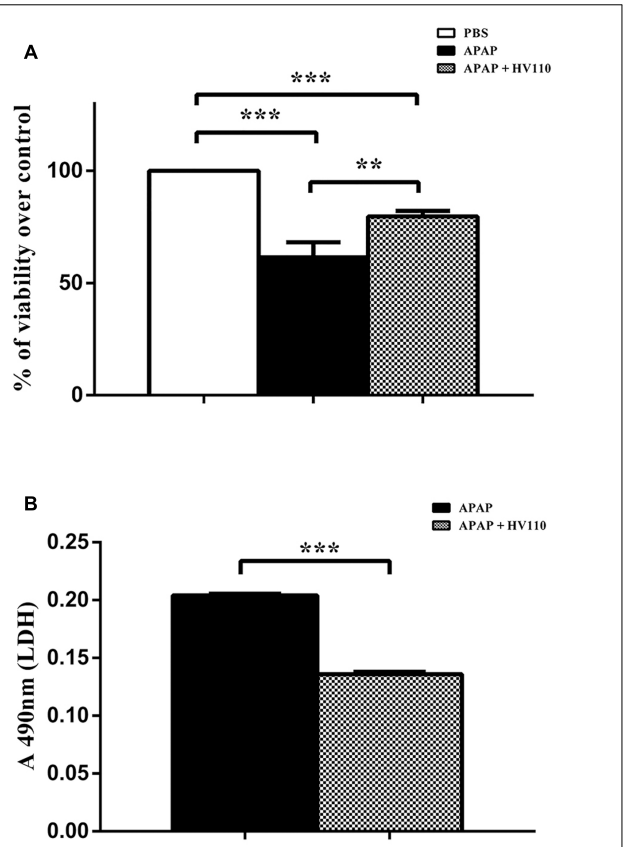

FIGURE 2 | HV110 protects cells against APAP-induced hepatotoxicity. Viability of HepG2 cells treated with 50 mM APAP and 3 mg/ml of HV110 for 16 h was examined by MTT (**A**, $n = 4$) and LDH assay (**B**, $n = 3$). LDH results are presented only as absorbance values indicating that higher absorbance correlates with the elevated cytotoxicity. All values are mean ± SEM. One-way ANOVA with the Tukey's *post hoc* test was used to compare multiple groups (**$p < 0.01$, ***$p < 0.001$).

in the presence of APAP was assessed using MTT and LDH assays. As expected, MTT assay shows that APAP in a dose of 50 mM significantly ($p < 0.001$) reduced cell viability to 61.5 ± 6.65%. Interestingly, the significant ($p < 0.01$) increase in cell viability to 79.7 ± 2.47% was observed in the APAP/HV110 co-treated cells, compared to APAP treated cells (**Figure 2A**). In parallel, LDH release in the media was measured and the results showed significantly lower ($p < 0.001$) toxic effect of APAP in the presence of HV110. Due to interference between APAP and LDH assay, the results of LDH assay are presented only as absorbance values, indicating that higher absorbance correlate with the increased cytotoxicity (**Figure 2B**).

The Influence of HV110 on Autophagy in HepG2 Cells

One of the key cellular adaptive mechanisms involved in attenuation of APAP-induced liver injury is autophagy. To investigate whether autophagy is correlated with protective effect of HV110 on HepG2 cells several factors involved in autophagy process were monitored. At first, conversion of the soluble LC3-I to lipid-bound LC3-II form of LC3 protein, a commonly used marker of autophagosomes formation associated with number of autophagosomes, was assessed by Western blot analysis. The potential of HV110 alone to trigger protective autophagy in HepG2 cells was investigated. Results revealed the significant increase ($p < 0.05$) of LC3-II/LC3-I conversion compared to the control cells (**Figures 3A,B**). In addition, the expression of *BECN1* a gene involved in nucleation step, and *Atg5* gene, involved in elongation step of autophagy process, were determined. The results showed that the expression of *BECN1* was significantly increased ($p < 0.05$) in cells treated with HV110, while the expression of *Atg5* remained unchanged, in comparison to control untreated cells (**Figure 3C**).

Further, the LC3-II/LC3-I ratio in APAP/HV110 co-treatment of HepG2 cells was followed. The results showed significant increase of LC3-II/LC3-I conversion in APAP/HV110 treated cells compared to cells treated with APAP alone ($p < 0.01$) and untreated control cells ($p < 0.001$), respectively (**Figures 4A,B**). Interestingly, although the treatment with APAP alone induced conversion of LC3 protein, it should be noted that this induction was not statistically significant in comparison to untreated cells.

Next, we investigated p62/SQSTM1 protein degradation by autophagy machinery. As a cargo receptor, p62/SQSTM1 binds to LC3 protein and contributes to clearance of ubiquitinated proteins. Consistent with the above mentioned results, APAP treatment as well as APAP/HV110 co-treatment caused

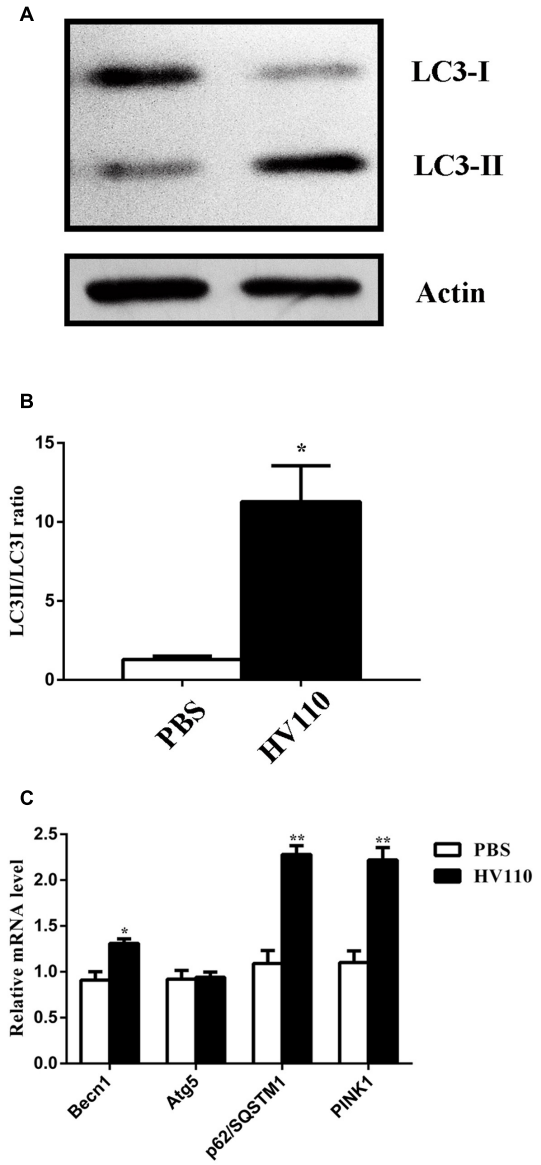

FIGURE 3 | The influence of HV110 on autophagy in HepG2 cells. Representative western blot **(A)** and densitometric analysis (**B**, $n = 4$) of LC3 conversion in HepG2 cells. Quantification of *BECN1*, *Atg5*, *p62/SQSTM1* and *PINK1* mRNA levels (**C**, $n = 3$). HepG2 cells were treated with 3 mg/ml of HV110 for 16 h. Student's *t*-test was used to compare experimental group relative to control (*$p < 0.05$, **$p < 0.01$).

significant degradation of p62/SQSTM1 protein compared to control, respectively ($p < 0.01$, $p < 0.001$). However, difference in p62/SQSTM1 degradation between APAP treatment and APAP/HV110 co-treatment is visible on western blot, but didn't reach statistically significance (**Figures 4C,D**).

Autophagosomes' accumulation could be a consequence either of autophagy activation or inhibition of downstream autophagy steps. Therefore, assessment of autophagy flux, which reflects the dynamics of the process, is essential. Results of autophagy flux monitoring showed significant increase conversion of LC3 marker and accumulation of LC3-II in the APAP/HV110 co-treated cells compared to chloroquine treated control ($p < 0.01$; **Figures 5A,B**), supporting the above mentioned evidence of autophagy activation.

However, levels of mRNA of *BECN1* and *Atg5* genes were decreased after 6 h and reached statistical significant decrease after 16 h of treatment ($p < 0.05$, $p < 0.01$) with no differences between APAP and APAP/HV110 co-treated groups (**Figures 4E,F**).

Autophagy Inhibition Decreases the Protective Effect of HV110

With the aim of the final confirmation of involvement of HV110-induced autophagy in protective effect on HepG2 cells against APAP, the autophagy was inhibited by chloroquine. The results of MTT assay showed that chloroquine added to the APAP/HV110 co-treated HepG2 cells decreased cell survival compared to the same treatment without chloroquine. More precisely, the difference between bars representing the percentage of viable cells after APAP/HV110 co-treatment and the percentage of viable cells after APAP treatment, without added chloroquine, was significantly higher (27.42 ± 1.86%) than in the presence of chloroquine (15.07 ± 1.21%) ($p < 0.01$; **Figure 5C**). This result supports our assumption of autophagy involvement in survival of cells treated with HV110. Additionally, LDH levels in supernatants of the cells treated with APAP, HV110 and chloroquine were much higher compared to treatment with no chloroquine added, but statistical significance wasn't achieved (**Figure 5D**). Considering the fact, that APAP/HV110 co-treated cells in the presence of chloroquine, still exhibit significantly higher viability rate compared to only APAP treated cells (MTT; $p < 0.05$ and LDH; $p < 0.001$) in the presence of chloroquine, the additional mechanism(s) involved in protective effect of HV110 on APAP-induced hepatotoxicity could be assumed.

Gene Expression Profile Revealed the Activation of PINK1 Autophagy Pathway

To analyze whether the PINK1-Parkin signaling pathway could be responsible for autophagy activation, we followed the expression of *PINK1* and *p62/SQSTM1* genes by qPCR. Results revealed that *PINK1* and *p62/SQSTM1* mRNAs were significantly induced in cells treated only with HV110 ($p < 0.01$) (**Figure 3C**).

Next, the expression profile of *PINK1* and *p62/SQSTM1* in APAP-induced hepatotoxicity was studied. The expression of the analyzed genes was not changed after the APAP treatment, regardless of the exposure time. Interestingly, the levels of *PINK1* and *p62/SQSTM1* mRNAs were increased in cells co-treated with both APAP and HV110 for 6 h, compared to control and APAP treated cells ($p < 0.01$; **Figure 4E**). After 16 h of APAP/HV110 treatment, the genes' expression returned to the control level (**Figure 4F**).

DISCUSSION

The literature data regarding the involvement of probiotics in autophagy activation are still limited. Most of the research in this

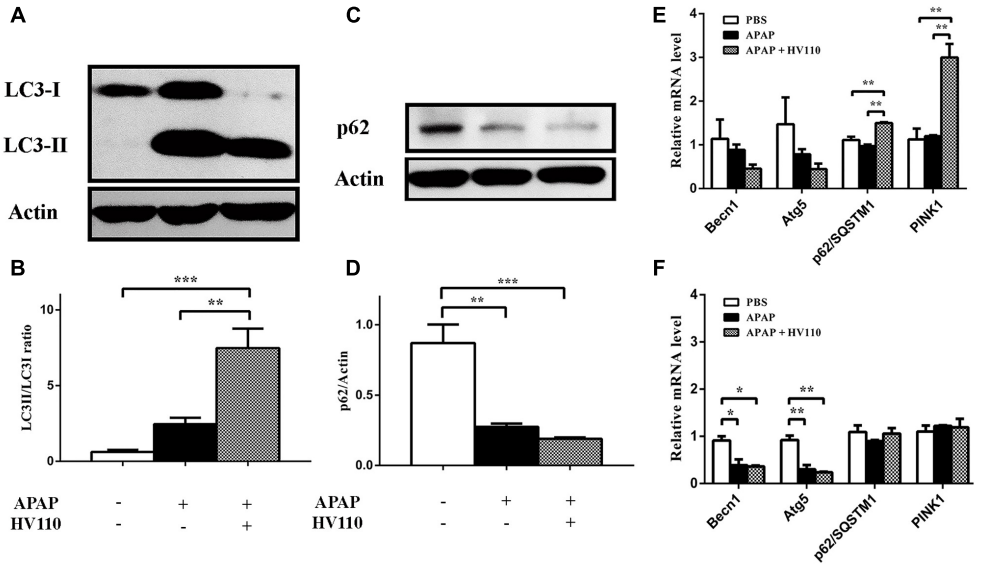

FIGURE 4 | Correlation between autophagy and HV110 protective effects. Evaluation of LC3 conversion and p62 degradation by immunoblot **(A,C)** and densitometric analysis (**B**, $n = 5$; **D**, $n = 4$) in HepG2 cells treated with 50 mM APAP and 3 mg/ml of HV110 for 16 h. The mRNA expression levels of *BECN1*, *Atg5*, *p62/SQSTM1* and *PINK1* after 6 h (**E**, $n = 3$) and 16 h (**F**, $n = 3$) of treatments with APAP and HV110. Values are expressed as mean ± SEM. One-way ANOVA with the Tukey's *post hoc* test were used to compare multiple groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

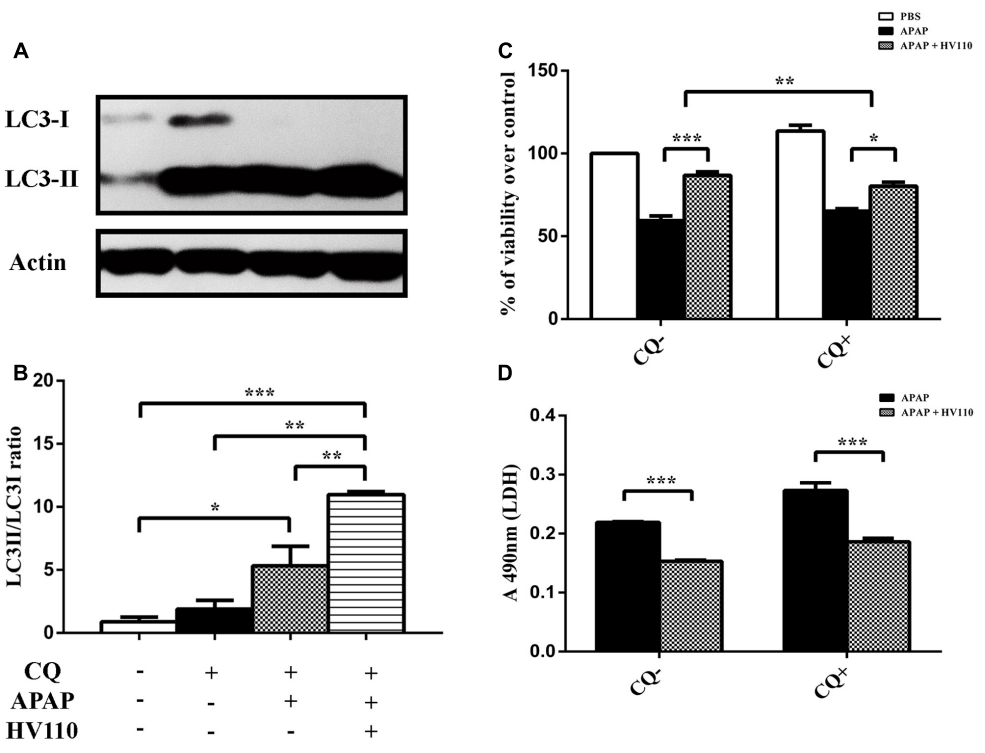

FIGURE 5 | Autophagy flux assessment. Representative western blot **(A)** and densitometric analysis (**B**, $n = 3$) of LC3 conversion in HepG2 cells treated for 16 h with 50 mM APAP and 3 mg/ml of HV110 in the absence or presence of chloroquine (CQ, 25 μM). In parallel, cell viability of HepG2 cells were evaluated using MTT assay (**C**, $n = 3$) and LDH assay (**D**, $n = 3$). All values are presented as mean ± SEM. For comparison of multiple groups, one-way ANOVA with the Tukey's *post hoc* test were used (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

field has been focused on the influence of pathogenic bacteria on autophagy machinery, while little space was given to the research on potential of beneficial bacteria to stimulate autophagy (Escoll et al., 2016; Zhang et al., 2016). This is the first report indicating that postbiotic HV110, originated from *Lactobacillus fermentum* BGHV110 strain, is potent inducer of autophagy in HepG2 cells, as demonstrated by increased LC3 lipidation and mRNA expression of *BECN1*, *PINK1,* and *p62/SQSTM1*. Bacterial lysates are rich in different pathogen associated molecular patterns (PAMPs) which can trigger autophagy through Toll-like receptor (TLR) signaling (Delgado et al., 2008). TLRs play an important role in liver physiology and pathophysiology due to the liver's exposure to gut-derived bacterial products and they are expressed in all cells present in the liver (Seki and Brenner, 2008; Mencin et al., 2009). Also, research over the last few years identified another class of pattern recognition receptors (PRRs), NOD-like receptors, involved in autophagy (Oh and Lee, 2014). On the other side, it has been described that overstimulation of PRRs can lead to induction of apoptosis. For example, TLR2 and TLR4 ligands present in the mycobacterial cell wall were identified as active ingredients of BCG treatment of superficial bladder tumors (Salaun et al., 2007; Subramaniam et al., 2016). This could be a reason for dose dependent decreased of cell viability caused by HV110 which was obtained in this study. However, involvement of PRRs in HV110 induced autophagy and impact on cell viability should be tested in further experiments.

Our finding that HV110 exhibits potential to induce protective autophagy served as the starting point to examine its cyto-protective effects against APAP-induced hepatotoxicity, which was shown that could be alleviated by autophagy induction (Ni et al., 2012). APAP exerts its toxic effects by two mechanisms: by CYP450-dependent NAPQI generation and by formation of PAP, as the result of APAP deacetylation (Miyakawa et al., 2015). CYP450-dependent pathway is activated immediately after APAP exposure and it lasts for the first several hours of exposure. However, after prolonged exposure to APAP, PAP-mediated pathway is activated with higher impact on cell viability compared to CYP450-dependent pathway. We thus assume that, in spite of lower expression of CYP450 enzymes in HepG2 cells (Westerink and Schoonen, 2007), our experimental setup provided enough and sustainable damage in HepG2 cells.

Though numerous studies investigated the effects of cytoprotective agents applied to hepatocytes before or after the addition of cytotoxic agents, our study was concerned with simultaneous HV110/APAP application. According to the results presented by Sharma et al. (2011) post-treatment with cytoprotective probiotics could not effectively reduce hepatocyte damage after APAP exposure, though pre and co-treatment were shown to be effective in the same study. This indicates that cell damage inflicted by high APAP doses is irreversible, as also evident from case studies reporting liver failure after intake of high APAP doses. Eventually, incorporation of postbiotics in formulations containing hepatotoxic drugs could significantly reduce the side effects and the degree of intoxication.

HV110 succeeded to alleviate APAP induced cell damage, as evidenced from MTT and LDH assays. Although APAP exposure *per se* induced protective autophagy in HepG2 cell line, induction of autophagy was threefold higher in APAP/HV110 co-treated cells, according to the degree of LC3 protein conversion and 1.5-fold higher based on the p62/SQSTM1 protein degradation. Chloroquine also favored the formation of autophagic vesicles containing cellular components in cells stimulated with HV110. Moreover, presence of chloroquine resulted in decreased survival of the cells exposed to APAP/HV110, suggesting the role of HV110-induced autophagy in the cells' protection. However, after chloroquine treatment, percentage of viable cells exposed to APAP/HV110 has not decreased to the viability level of those treated only with APAP, suggesting involvement of other protective mechanisms.

According to the results of mRNA expression, APAP/HV110 treated cells elevated mRNA levels of *p62/SQSTM1* and *PINK1* after 6 h. The main role of PINK1 in cells is to promote Parkin-mediated mitophagy by recruiting Parkin to damaged mitochondria (Williams and Ding, 2015). Along with its protective function and the potential to activate mitophagy, it was shown that PINK1 may also have an important role in activation of basal and starvation-induced autophagy by interacting with Beclin-1 protein (Michiorri et al., 2010). On the other hand, p62/SQSTM1 is a molecular adapter between degradation substrates and molecules involved in autophagosome formation and antioxidant defense (Lamark et al., 2009; Rautou et al., 2010; Ichimura et al., 2013). Elevated *p62/SQSTM1* mRNA synthesis in cells co-treated with APAP/HV110 implies activation of NF-E2-related factor 2 (Nrf-2), an important transcription factor that regulates the expression of antioxidant specific genes. Recent data showed that p62/SQSTM1 also plays important role in regulation of Nrf2 activity. The p62 binds to Kelch-like ECH-associating protein 1 (Keap1) causing release of Nrf2 and consequent transcription of genes, including those involved in xenobiotic and ROS (reactive oxygen species) detoxification (Ichimura et al., 2013). Jones et al. (2015) provided evidence that lactobacilli can elicit their beneficial influences in the gut through direct contact with NADPH oxidases on a cell surface, resulting in ROS generation and consequential activation of Nrf2 signaling. However, according to our knowledge this is the first study that reports link between postbiotics and p62/SQSTM1 as possible new mechanism of postbiotics activation of Nrf2 pathway in hepatocytes. *PINK1* gene upregulation suggests that HV110 treatment triggers a low sub-lethal level of oxidative stress that could be reflected at the level of transcription of *PINK1* which is highly sensitive to subtle changes in intracellular environment.

Although APAP/HV110 co-application induced changes in *PINK1* and *p62/SQSTM1* mRNA expression after 6 h treatment, the values returned to basal levels after prolonged treatment (16 h). It was shown that antioxidants produced by cells as response to oxidative damage, in this case caused by APAP, inhibit Nrf2 dependent gene transcription, via negative feedback (Murata et al., 2015). Considering the fact that *PINK1* regulation, as well as regulation of *p62/SQSTM1* transcription is dependent on Nrf2 protein, this could explain downregualtion of *PINK1* and *p62/SQSTM* mRNA expression after 16 h treatment, which was obtained in this study (Jain et al., 2010; Murata et al., 2015). Interestingly, we did not detect any changes in *PINK1*

and *p62/SQSTM1* expression, in cells treated only with APAP, neither after 6 h nor after 16 h of treatment. This suggests that mechanisms which lead to acute cellular response were more intensively activated in the presence of HV110. It could be assumed that rapid activation of these mechanisms might have elevated cellular defensive system, including autophagy response which aided cellular survival, as demonstrated in MTT and LDH assays. All above mentioned results are consistent with the concept of "hormesis," a response to xenobiotic and environmental stimuli, where low stress levels are protective against more destructive stimuli. This concept has generally been given as an explanation for beneficial effects of microbes upon the host (Jones et al., 2015).

In spite of their well-known role in autophagy induction, we surprisingly noticed a decrease of *BECN1* and *Atg5* transcription in APAP treated cells, for which increased LC3 lipidation was obtained. This was irrespective of HV110 presence. Beclin-1, in complex with class III phosphatidylinositol 3-kinase (PI3K) is crucial for the nucleation phase of autophagosome formation, while Atg5 conjugates to Atg12 and facilitates the lipidation of LC3 protein (Rautou et al., 2010; Otomo et al., 2013). Aside from being indispensable for autophagy, Beclin-1 has additional roles not related to autophagosomes formation. Beclin-1 is known as haplo-insufficient tumor-suppressor and accumulating evidence of data suggests its down-regulation in cancers (Li et al., 2013; Huang et al., 2014). Findings of Li et al. (2013) show that down-regulation of Beclin-1 triggered autophagy, decreased apoptosis and stimulated proliferation of cells exposed to gemcitabine in Miapaca2 tumor cells. Therefore, lower levels of *BECN1* mRNA in APAP-treated cells could be explained as a cellular attempt to overcome toxicity caused by very high APAP dose. In the case of *Atg5* gene downregulation, the results could be explained by the role of Atg5 protein to regulate its own transcription through a feedback inhibition loop, as demonstrated by Hu et al. (2011).

CONCLUSION

Our study demonstrated protective effect of autophagy activated by postbiotic HV110 originated from *Lactobacillus fermentum* BGHV110 strain in APAP induced cytotoxicity in HepG2 cells. To the best of our knowledge, this is the first study to correlate autophagy inductive potential of lactobacilli to their protective effects against drug-induced toxicity. Taken together, this could be of special relevance for designing of new analgetic drug formulations with added postbiotic for prevention of possible hepatotoxic side effects, although the safety and health promoting efficacy of such drugs should be further tested.

AUTHOR CONTRIBUTIONS

MD: performed main work, analyzed, interpreted the data and draft the work; JL: conception and design of experiments, performed part of the experiments, analyzed, interpreted the data and critically revised the manuscript; JD: performed part of the experiments, analyzed and interpreted the data; MM: supervised the work, analyzed the data and critically revised the manuscript; IS: analyzed, interpreted and critically revised the manuscript; NG: supervised the work, analyzed and interpreted the data, draft the work; JB: conception and design of the work, supervised the work, analyzed and interpreted the data and critically revised the manuscript. All authors finally approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Development of a Synbiotic Beverage Enriched with Bifidobacteria Strains and Fortified with Whey Proteins

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The objective of this study was to develop a new synbiotic beverage evaluating the ability of some bifidobacteria strains to grow in this beverage which was fortified with whey proteins up to 20 g L^{-1}, and enriched with 10 g L^{-1} of prebiotic inulin or resistant starch. The ability of *Bifidobacterium* strains to survive for 30 days at 4°C was evaluated in two synbiotic whey protein fortified beverages formulated with 2% of whey proteins and 1% of inulin or resistant starch. Microbial growth was significantly affected by the whey protein amount as well as by the kind of prebiotic fiber. Resistant starch promoted the growth of the *Bifidobacterium pseudocatenulatum* strain and its viability under cold storage, also conferring higher sensory scores. The development of this new functional beverage will allow to carry out *in vivo* trials in order to validate its pre- and probiotic effects.

Keywords: *Bifidobacterium*, inulin, resistant starch, whey proteins, fortified beverage

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INTRODUCTION

Functional foods are defined as foods or food ingredients that may provide a health benefit beyond the basic nutrition (IOM/NAS, 1994; Gibson and Williams, 2000). In the last century, a huge amount of research describes the role of *Bifidobacterium* genus in promoting human and animal health (Preising et al., 2010; Fukuda et al., 2011; Russell et al., 2011). Bifidobacteria have been also proposed to promote the correct microbial equilibrium of the intestinal microbiota in preterm infants (Szajewska et al., 2010) due to their beneficial effects on human health and predominance in the intestinal microflora. Furthermore, it has been demonstrated that the consumption of indigestible carbohydrates, such as resistant starch (RS), can enhance positive biological effects induced by natural occurring bifidobacteria in the human gastrointestinal tract and usually defined as prebiosis (Saulnier et al., 2009).

Indeed, bifidobacteria, exploiting their unique glycosidases, transporters, and metabolic enzymes for sugar fermentation, are able to activate fermentable molecules in an environment poor in nutrition and oxygen (Fushinobu, 2010).

Resistant starch, naturally present in many vegetables (e.g., plantains, green bananas, roots, and legumes) or obtained from processed cereals (Quintieri et al., 2012b), withstand the upper gastrointestinal digestive enzymes; in the distal colon, RS, can be preferentially and specifically hydrolyzed by bifidobacteria displaying bifidogenic effects (Nugent, 2005; Queiroz-Monici et al., 2005; Regmi et al., 2011).

Inulin, a linear D-fructose polymer containing small amounts of branched fructose by β(2-1)-glycosidic bonds with a terminal glucose moiety, is naturally present in several plants (Van Loo et al., 1995); it is partially fermented in the distal colon by bifidobacteria with a consequent increase in their population (De Vuyst and Leroy, 2011).

The microbial fermentation of both inulin and RS in the colon produces short chain fatty acids (SCFA; acetate, propionate, and butyrate, lactate), succinate, hydrogen and carbon dioxide; these metabolites, and in particular butyrate, are considered to improve water absorption in the large bowel, to modulate colonic muscular activity, to inhibit the growth of cancer cells, to stimulate the growth of normal cells, and to promote DNA repair in damaged cells, as recently reviewed by Topping et al. (2008). Based on these studies, World Health Organization (WHO) recommends to supplement the diet with low-carb fiber-rich meals in order to maximize colonic disease prevention and reduce body weight (WHO, 2015); thus, inulin and RS are commonly used to enrich different kinds of foods (Crittenden R.G. et al., 2001; Brown, 2004; Topping et al., 2008; Fushinobu, 2010; Pokusaeva et al., 2011). In addition to indigestible fibers, milk proteins also showed bifidogenic effects. In particular, α-lactalbumin was found to promote the growth of *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, as well as caseinomacropeptide, isolated from whey protein concentrates, and supplemented in milk was reported to increase *Bifidobacterium lactis* counts in probiotic fermented milks (Pihlanto-Leppälä et al., 1999; Janer et al., 2004). The hydrolysates of whey proteins also boosted the growth of *Bifidobacterium animalis* subsp. *lactis* BB12 in yogurt preparation (Tian et al., 2015). Moreover, whey protein hydrolysates are rich in peptides endowed with hypotensive, anticancer, opioid antagonistic, and immunomodulatory properties (Morris and Fitzgerald, 2009). Antimicrobial peptides released from many whey proteins (Naidu, 2000) were also found useful for improving food safety and quality (Quintieri et al., 2012a, 2013; Baruzzi et al., 2015; Caputo et al., 2015). The nutritional and physiological aspects of whey protein based foods are well known as previously reported (Walzem et al., 2002; Morris and Fitzgerald, 2009) and, due to their high amount of digestible proteins and branched amino acids, they are usually consumed by sportsmen to strengthen the muscle anabolism (Walzem et al., 2002; Tang et al., 2009; Pennings et al., 2011; Deutz et al., 2014). Moreover, whey proteins could be also useful for debilitated subjects or people under restrictive diets (such as elderly people, gastrectomized or immunocompromised patients). The consumer demand for a daily intake of fibers and molecules with high nutritional value (as reported by WHO, 2003; National Health and Medical Research Council [NHMRC] and New Zealand Ministry of Health, 2006; Fungwe et al., 2007; EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA), 2010) has driven this work toward the optimization of a beverage endowed with these features.

Therefore, in this work three bifidobacteria strains (*B. animalis* subsp. *lactis* BI1 and BB12 and *B. breve* BBR8) also applied from the production of probiotic dairy foods (such as BB12) and the *Bifidobacterium pseudocatenulatum* M115 endowed with promising pro-healthy features were chosen in order to develop a new synbiotic beverage. Firstly, the influence of inulin and RS on the viability of bifidobacteria in a fermented whey protein medium was evaluated under laboratory conditions. Then, a synbiotic whey protein beverage enriched with prebiotic fibers was set up and characterized for microbial viability, residual fiber content, and organoleptic properties throughout cold storage period.

MATERIALS AND METHODS

Experimental plan was arranged on three levels (**Figure 1**) that included the optimization of a whey based medium (WBM) able to sustain bifidobacteria growth (1), the selection of bifidobacteria strains fermenting fibers in WBM (2), then the set up of the synbiotic beverage (3) which was evaluated for its shelf life and sensory profile over 30 days of cold storage.

Microorganisms and Media

The strains *B. animalis* subsp. *lactis* BI1 and *B. breve* BBR8 were a gift of the Centro Sperimentale del Latte S.r.l. (Lodi, Italy), the *B. pseudocatenulatum* M115 was from the Dairy Research Institute of Asturias (IPLA, Villaviciosa, Spain, Department of Science and Food Technology of the Spanish National Research Council, CSIC) and *B. animalis* subsp. *lactis* BB12 was previously isolated and included in the ISPA-CNR bacterial collection.

Fresh microbial cultures of bifidobacteria strains from frozen cultures (−80°C) were routinely grown in MRS (MRS Agar ISO Formulation, Biolife Italiana srl, Milan, Italy) amended with 0.5 g L^{-1} of L-cysteine (MRSC) for 48h at 37°C under anaerobic conditions (ANAEROGEN, AN0025, Oxoid S.p.A., Milan, Italy).

Growth of Bifidobacterium Strains in Whey-Based Medium (WBM)

Bifidobacteria strains were evaluated for their ability to grow in whey-based media (WBM). Whey protein isolate (WPI, Mirabol® Whey Protein Natural 97, Volchem s.r.l., Grossa di Gazzo, Italy), containing *ca.* 97 g of whey proteins on 100 g of powder, was diluted in distilled water in order to obtain two WBM at 10 and 20 g L^{-1} of total protein (WBM10 and WBM20). Based on Mirabol®'s nutritional fact, the raw composition per liter of WBM10 included 5 g of β-lactoglobulin, 1.7 g of α-lactalbumin, 1.8 g of other whey proteins, 0.02 g of simple sugars, 0.04 g of lipids; the protein concentration in WBM10 allowed to estimate a content 2.3 and 0.5 g L^{-1} of branched chain and sulfur-containing amino acids, respectively. In the case of WBM20 the concentration of nutrients doubled. In addition, WBM were supplemented with 0.5 g L^{-1} of L-cysteine and 10 g L^{-1} of lactose. Fresh bifidobacteria cultures were diluted in sterile saline solution in order to read an absorbance at 600 nm of 0.4 ± 0.05 (*ca.* 8 log cfu mL^{-1}), further diluted 1000 times in WBM10 and WBM20 and incubated as above described; MRSC was used as positive control. Then, bifidobacteria were enumerated on Bifidus Selective Medium (BSM) agar plates (Sigma–Aldrich SRL, Milan, Italy) recording pink-purple colored colonies. WBMs were also enriched with prebiotic fiber by the addition of 10 g L^{-1} of

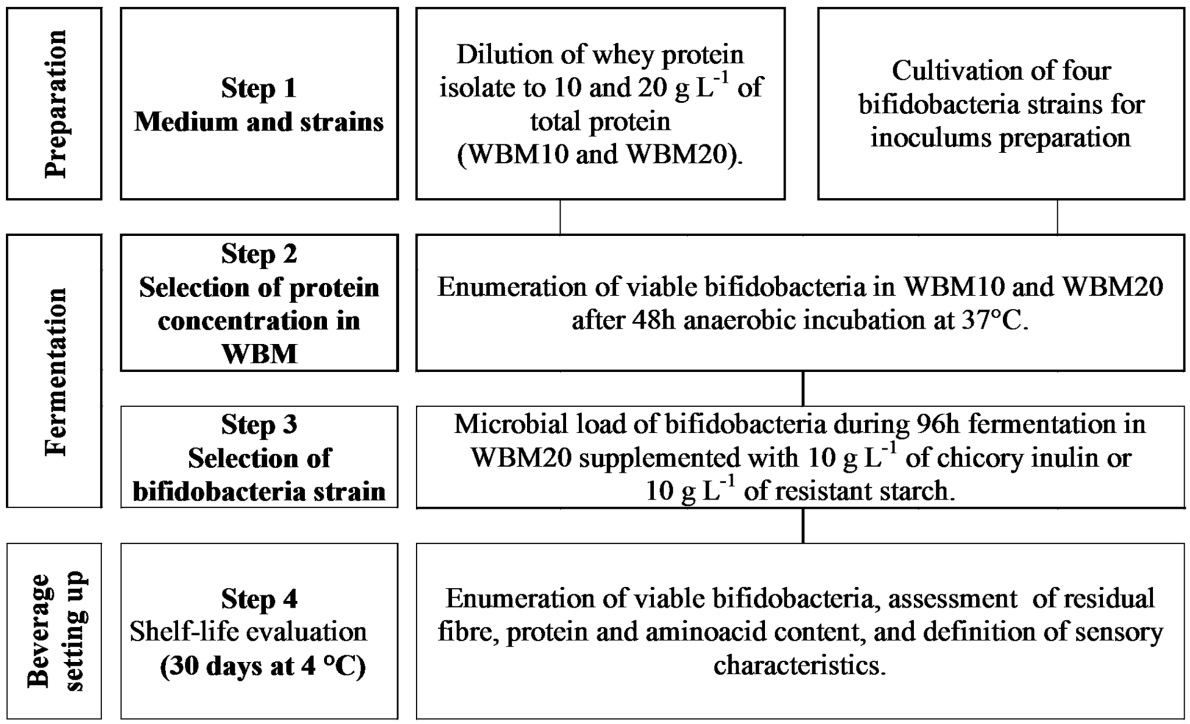

FIGURE 1 | Graphical scheme of the experimental plan carried out in the present work.

chicory inulin with a degree of polymerization > 23 (Orafti HP, BENEO-Orafti, Tienen, Belgium) or 10 g L^{-1} of retrograded RS (Novelose® 330, Ingredion Incorporated, Westchester, IL, USA) and then pasteurized at 110°C × 5 min. In order to reduce whey protein precipitation, thermal treatment parameters were set up by preliminary assays; however, the efficacy of pasteurization was preserved. Prebiotic WBMs were cooled at room temperature and then inoculated with fresh cultures of bifidobacteria, as described above. Strains were incubated under anaerobic conditions at 37°C up to 96 h. Viable cell evaluations were carried out in triplicate every 24 h, as described above.

Set Up of a Whey Protein Fortified Beverage Enriched with Prebiotic Fibers (WPF Beverage)

Two synbiotic whey protein fortified beverages enriched with prebiotic fiber (500 ml) were obtained, in triplicate, by supplementing WBM, at the protein concentration selected in the section "Growth of Bifidobacterium Strains in Whey-based Medium (WBM)," with inulin or with RS (1%). Glass bottles were filled with WPF beverages leaving less than 5 ml empty space on the top of the fluid, under the cork. After pasteurization and cooling, WPF beverages were inoculated with the bifidobacteria strains M115 or BBR8 (**Figure 1**, Step 3) at approximately 4 log cfu mL^{-1} by using a fresh bifidobacteria culture.

WPF beverages were incubated at 37°C under anaerobic conditions in order to obtain a concentration of bifidobacteria of at least 8 log cfu mL^{-1}; depending on the results achieved in the fermentation assays, the incubation ranged from 48 to 72 h. After fermentation, WPF beverages were refrigerated at 4°C for 30 days. WPF beverages without bifidobacteria were also prepared as negative controls.

Chemical and Microbiological Analyses of WPF Beverage

During cold storage (at days 0, 15, and 30), each WPF beverage was evaluated for pH (Model pH50 Lab pHMeter XS-Instrument, Concordia, Italy), concentration of viable bifidobacteria, residual fiber, protein and aminoacid content, and sensory characteristics. The percentage of resistant starches and inulin were determined with the KR-STAR kit and with the K-FRUCHK kit (Megazyme International Ltd., Wicklow, Ireland), respectively, following the manufacturer's instructions.

Protein concentration was determined by the Bradford Method whereas small peptide and aminoacid concentrations were determined after derivatization with *o*-phthaldialdehyde as previously described (Baruzzi et al., 2012).

Sensory Evaluation of WPF Beverage

Sensory evaluation was performed recruiting three groups of five habitual consumers of milk beverages. The samples (30 mL) of the different beverages, stored at 4°C for 0, 15, and 30 days, were served at 12 + 1°C in white plastic cups coded with random three-digit numbers, and mineral water at room temperature was provided for mouth-rising. The overall acceptability of each sample was calculated using a 9-point hedonic scale ranging from 1 ("dislike extremely") to 9 ("like extremely") and the level

of suitability of taste, sweetness, milky appearance, flavor, and mouthfeel, using a 5-point just about right (JAR) scale (1 = too weak, 3 = just about right; 5 = too strong), was followed for each sample, according to Villegas et al. (2010).

Statistical Analysis

The concentration of viable cells in samples was calculated as the average number of colonies found for each decimal dilution, corrected by the dilution factor and expressed as the log cfu mL^{-1} ± standard deviation. Bifidobacteria viable cell counts, RS and inulin content were analyzed by one-way analysis of variance (ANOVA) carried out using IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA). Tukey's test and Fisher LSD *post hoc* test were applied in order to evaluate significant differences ($P \leq 0.05$) among means of viable cell counts and dietary fibers, respectively. In order to compare inoculum levels as well as viable cell load recorded after the same period of incubation among different experiments, the Mann–Whitney non-parametric test was applied.

The independent effects and interactions of the main factors (times of storage, beverages and sensory attributes) on sensory scores were evaluated applying a three- and two-way ANOVA ($P \leq 0.05$); multiple comparisons among individual means of the same strain were made by Fisher's LSD *post hoc* test after rejecting the homogeneity of their variances with the Levene's test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Effect of Whey Proteins on Bifidobacteria Growth

The viable cell counts of Bifidobacteria, grown for 48 h at 37°C, in MRSC and in WBM supplemented with different amount of whey proteins (10 or 20 g L^{-1}; WBM10, WBM20), were reported in **Table 1**. WBM20 cultures of M115, BBR8, and BI1 showed cell counts significantly higher ($P < 0.05$) than those retrieved in WBM10, although all cultures reached the highest load values in MRSC (9.08 log cfu mL^{-1} on average). This result was in accordance with preliminary experiments carried out using different amounts of whey protein concentrate at 80% of proteins (Baruzzi et al., 2014). Furthermore, BB12 cell counts (7.75 log cfu mL^{-1}, on average) in WBM10 and WBM20 were consistent with results of Matijević et al. (2008), who also reported no significant difference in cultures of this strain grown in medium containing 1.5 or 3% of whey protein concentrate. On the basis of these results, the 20 g L^{-1} concentration was chosen for the subsequent analyses.

TABLE 1 | Viable cell counts (mean values ± standard deviation of three independent experiments, expressed as log cfu mL^{-1}) of *Bifidobacterium* strains in MRSC and WBM at 10 and 20 g L^{-1} protein concentration after 48h of anaerobic fermentation at 37°C.

Strain	MRSC	WBM10	WBM20
B. animalis ssp. *lactis* BB12	9.37 ± 0.38^{a}	7.40 ± 0.27^{b}	8.09 ± 0.27^{b}
B. animalis ssp. *lactis* BI1	8.61 ± 0.34^{a}	4.37 ± 0.31^{c}	6.73 ± 0.33^{b}
B. breve BBR8	9.55 ± 0.08^{a}	4.65 ± 0.19^{c}	7.43 ± 0.22^{b}
B. pseudocatenulatum M115	8.80 ± 0.73^{a}	4.50 ± 0.46^{c}	7.40 ± 0.38^{b}

Initial average microbial log cfu mL^{-1} 4.57 ± 0.05. Different letters indicate significant differences ($P < 0.05$) for a least significant difference (LSD) of: BB12, 0.97; BI1, 1.03; BBR8, 0.56; M115, 1.69.

Effect of Dietary Fibers on Bifidobacteria Growth

Aimed at promoting bifidobacteria growth at the similar levels of MRSC, used as a reference medium, within the same period of incubation, WBM enriched with total whey proteins to 20 g L^{-1} (WBM20) was amended with additional carbon sources different from lactose.

Therefore, the WBM20 was supplemented with 10 g L^{-1} of inulin (WBM20-I) or RS (WBM20-RS), as already assayed by Crittenden R. et al. (2001) and Rossi et al. (2005). Inulin and RS were selected as indigestible for humans but favor bifidobacteria growth. **Figure 2** showed that the addition of 10 g L^{-1} of inulin or the addition of 10 g L^{-1} of RS to WBM20 (containing 10 g L^{-1} of lactose) allowed to obtain microbial growth values similar to those occurred in MRS containing 20 g L^{-1} of glucose only when time of incubation was extended.

However, bifidobacteria growth kinetics demonstrated to be different after WBM20 was supplemented with inulin or RS; in particular, BI1 was negatively affected by both probiotic fibers, whilst M115 was favored by RS. Inulin caused a significant and faster growth increase of both M115 and BBR8 strains. Mann–Whitney analysis also showed the viable cell concentrations of all strains at T0 and T48 throughout the experiments of steps 2 and 3 were similar ($P > 0.05$) at experimental steps 2 and 3 (**Table 1** and **Figure 1**).

In accordance with previous studies, our results showed the bifidobacteria growth in presence of glucose (herein included in MRS) or other monosaccharides was improved in presence of complex carbohydrates (Palframan et al., 2003; Rossi et al., 2005; Rada et al., 2008).

Since amylolytic activity is present in approximately 60% of bifidobacteria species (Crittenden R. et al., 2001) only BB12 and M115 strains increased their viable cell loads after the addition of 10 g L^{-1} of RS to WBM20. This finding agrees with Wronkowska et al. (2006) demonstrating either a good growth and an acidifying activity of bifidobacteria in fermented heat-treated starch. With regards to inulin supplementation, the higher cell loads were found only for the M115 and BBR8 strains in WBM20-I, compared to WBM20, and results were in accordance with Selak et al. (2016) reporting the heterogeneously inulin hydrolysis spread among Bifidobacteria species.

Moreover, in this work the growth of *B. pseudocatenulatum* M115 was favored by inulin-type fructans at each time of sampling over 72 h incubation suggesting the presence and expression of β-fructofuranosidase genes as already reported (Janer et al., 2004; Omori et al., 2010). In addition, M115 was the only strain able to grow better also in presence of RS; this behavior could be attributed to the occurrence of glycosyl transferase, starch, and glycoside hydrolase genes generally found in the

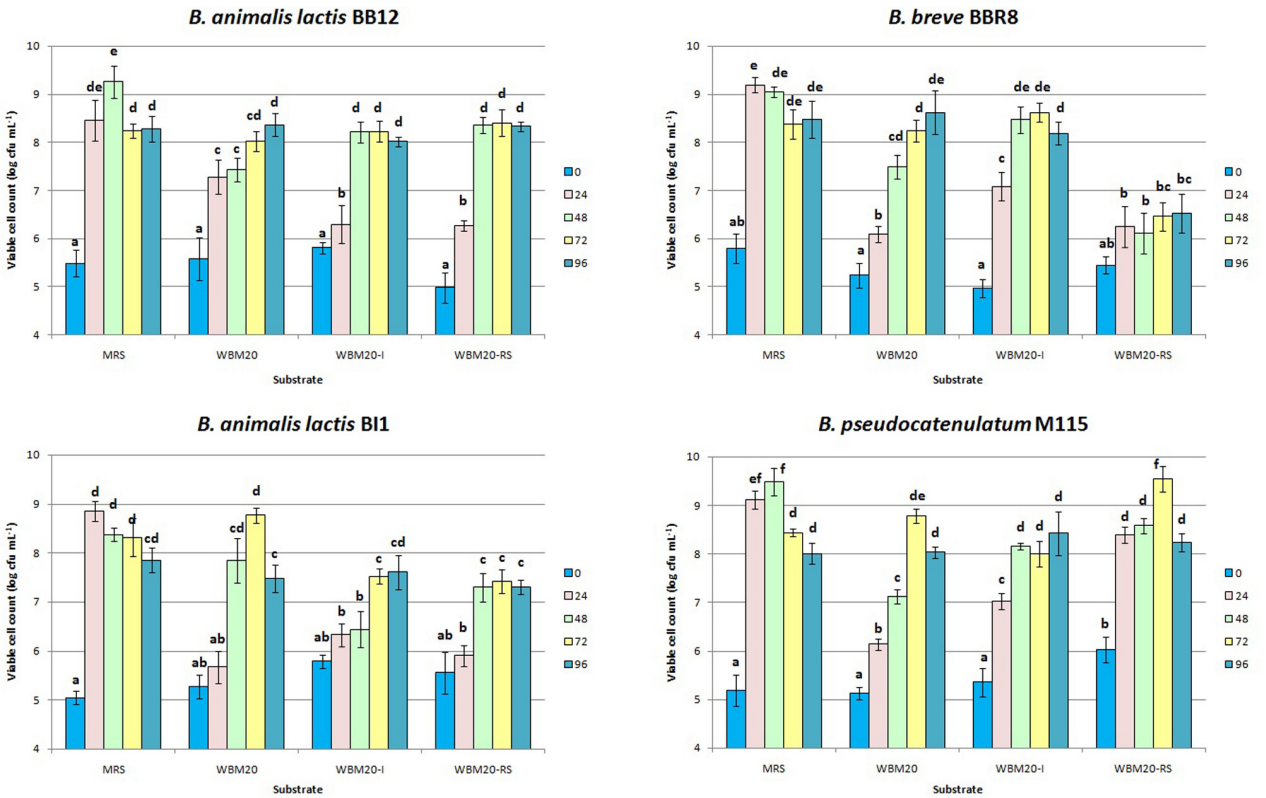

FIGURE 2 | Viable cell concentration of bifidobacteria strains throughout 96 h of anaerobic incubation at 37°C in MRS and in medium containing 20 g L^{-1} of whey proteins (WBM20), in WBM20 supplemented with 10 g L^{-1} of chicory inulin (WBM20-I) or 10 g L^{-1} of resistant starch (WBM20-RS). Bars represent mean value of viable cell count of three independent experiments (± standard deviation); values with different lowercase letters are significantly different ($P < 0.05$) according Tukey's test, within the same strain comparison.

genome of bifidobacteria (Liu et al., 2015) and particularly in the genome of the probiotic strain *B. pseudocatenulatum* IPLA 36007 (Alegría et al., 2014). These results increase the knowledge about the *B. pseudocatenulatum* M115 already proposed as a powerful probiotic strain thanks to its interesting pro-healthy features (Delgado et al., 2008; Losurdo et al., 2013).

Effect of Cold Storage on Viability of Bifidobacteria in WPF Beverages

In the light of previous results, the strain BI1 was excluded as both fibers reduced its viable cell load, conversely, the BBR8 was considered for beverage production only when WBM20 was supplemented with inulin as this fiber promoted its growth within 48 h.

As concerns BB12 and M115, the viable cell loads of both strains increased in WBM20-RS in comparison with WBM20 after 48 and 72 h, respectively. However, due to higher value reached by M115, this strain was selected and BB12 was excluded.

Thus, two synbiotic beverages fortified with whey proteins (at 20 g L^{-1}; WPF) were manufactured: the first beverage contained inulin and the strain BBR8, whilst the second one contained RS and it was fermented with the strain M115. After fermentation (48 h for the BBR8 and 72 h for the M115), WPF beverages were stored at 4°C evaluating viable cell count and pH (**Figure 3**) until day 30.

TABLE 2 | Fiber content in WPF beverages calculated before and after fermentation and throughout 30 days of cold storage.

	Inulin (g L^{-1})[1]	RS (g L^{-1})[2]
Pre-pasteurization	6.77 ± 0.36[a]	6.53 ± 1.08
Post-pasteurization	5.77 ± 0.53[ab]	5.10 ± 0.83
T0f	6.52 ± 0.42[a]	6.73 ± 0.75
T48f/T0cs	4.74 ± 0.43[b]	5.46 ± 1.99
T15cs	3.24 ± 0.51[c]	5.16 ± 0.94
T30cs	2.65 ± 0.43[c]	7.26 ± 1.55

f, fermentation; cs, cold storage. Different letters represent average values statistically different ($P < 0.05$) according to Tukey's test within column. [1]WPF beverage fermented with B. breve BBR8. [2]WPF beverage fermented with B. pseudocatenulatum M115.

Confirming the results of preliminary fermentation assays, bifidobacteria reached a load by means of 8.5 log cfu mL^{-1}; then a reduction of *ca.* 2 log cycles was found in beverages within 15 days of cold storage. Subsequently, cell concentration remained stable for *B. pseudocatenulatum* M115, whereas a further reduction was observed in *B. breve* BBR8 reaching *ca.* 4.9 log cfu mL^{-1} (**Figure 3**).

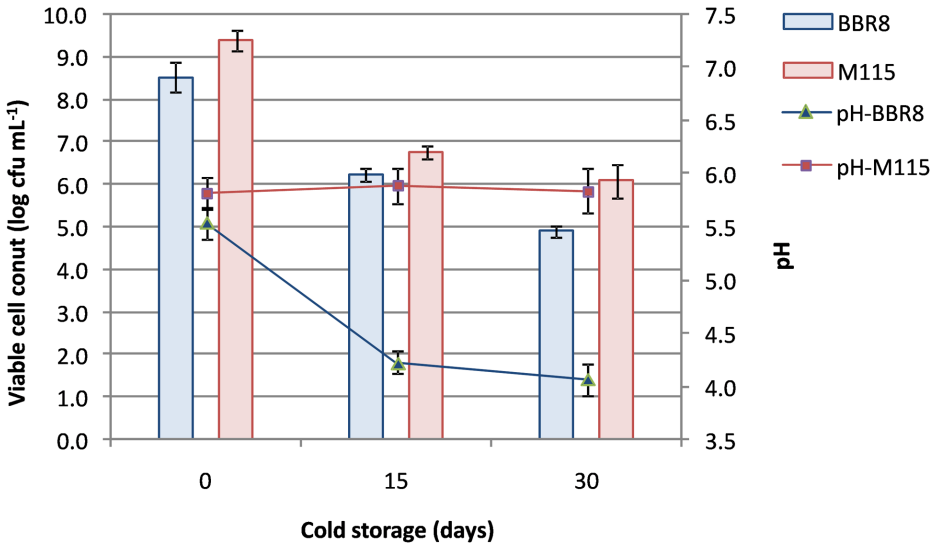

FIGURE 3 | Changes in pH values and viability of *Bifidobacterium breve* BBR8 and *B. pseudocatenulatum* M115 in whey beverages supplemented with inulin and resistant starch, respectively, throughout 30 days of cold storage.

The fiber concentration in WPF beverages during 30 days of cold storage is reported in **Table 2**.

In the case of inulin, after its supplementation in WPF beverage, its concentration was 6.77 ± 0.36 g L^{-1}, far below the estimated amount reported on the product label (10 g L^{-1} of Orafti® HP, corresponding to *ca*. 9.9 g of pure inulin); this difference could be attributed to the different methods used to determine fructans, AOAC 997.08, as stated by the BENEO-Orafti, and the AOAC 999.03 of the Megazyme kit used in this work. At the end of cold storage inulin concentration halved (**Table 2**).

At contrary, the amount of RS remained quite stable (*ca*. 6 g L^{-1}) during both the manufacturing process and the first 15 days of cold storage (**Table 2**). The large standard deviation found in the concentration of RS could be ascribed to their low solubility in water phase; in addition, as reported by the K-RSTAR kit manufacturers, higher errors are expected for samples with RS content lower than 2%.

Since the concentration of RS did not change during preparation, fermentation, and cold storage, it is possible to argue that the growth improvement recorded for M115 (**Figure 2**) could be due to non-resistant polysaccharides supplied with Novelose® 330.

At the end of fermentation, the total protein content of BBR8- or M115-fermented beverages, did not change significantly ($P < 0.05$) remaining stable at their initial average value of 4.19 ± 0.13 mg mL^{-1}. Conversely, the concentrations of small peptides and amino acids dropped from 161.6 ± 4.8 (control samples) to 11.99 ± 0.36 and 8.58 ± 0.26 μg mL^{-1} for BBR8 and M115 strains, respectively. No changes in total protein content as well as in small peptide and amino acid contents were found in both WPF beverages during cold storage (data not shown).

As concerns the reduction of viability of BBR8 and MM115 strains during refrigerate period, our result agreed with those of Donkor et al. (2006) and Jayamanne and Adams (2009) reporting the reduction in viable cells of *B. lactis* LAFTI® B94 or of *B. longum* and *B. animalis* ssp. *lactis* strains in yogurt over cold storage, respectively. Part of this decrease could be also attributed to the absence of L-cysteine that did not protect bifidobacteria cells against deleterious effects of cold storage, as demonstrated in refrigerated milk by Bolduc et al. (2006). We removed L-cysteine to avoid a negative influence on flavor. Refrigeration temperature hampered the increase in microbial load; in addition, cell viability was negatively affected by the post-acidification phenomenon as recently reported for six bifidobacteria cold stored for 21 days in milk (Djelled et al., 2016).

In our beverages the survival of bifidobacteria reached the same levels reported by Kailasapathy (2006) after cell microencapsulation, and was higher than that found by Antunes et al. (2005) in a probiotic yogurt containing a *B. longum* strain and enriched with both whey protein concentrate and skim milk powder. It is possible to argue that the occurrence of fibers in WPF beverages helped the survival of bifidobacteria as demonstrated by Crittenden R.G. et al. (2001) in yogurt supplemented with both RS and inulin, throughout 5 weeks of cold storage period. The inulin supplementation (40 mg g^{-1} of reconstituted skim milk) has been already reported by de Souza Oliveira et al. (2011, 2012), to cause an increase in the viable cell load of a *B. animalis* subsp. *lactis* strain or in the biomass of lactic acid bacteria; however, no data were produced about potential inulin consumption. The decrease in the inulin content found during 30 days of cold storage (to about 40% of the initial amount) suggests that inulin was consumed by the strain. The reduction in the inulin content together with the drop in pH values could be consistent with the production of SCFA from this fiber as already demonstrated either by single strains (Marx et al., 2000) and fecal cultures (Pompei et al., 2008; De Vuyst and Leroy, 2011).

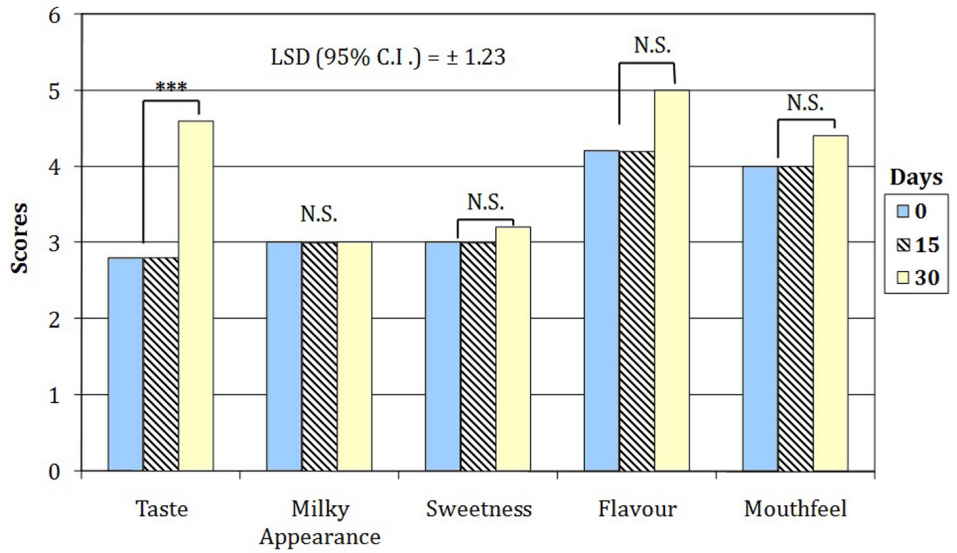

FIGURE 4 | Sensory attributes of WPF beverages fermented by bifidobacteria throughout the cold storage. Scores attributed with a 5-point just about right (JAR) scale (1 = too weak, 3 = just about right; 5 = too strong). NS, values are not significantly different; ***values were found significanlty different for $P < 0.01$.

The bifidogenic effect of RS has been widely reported (Wang et al., 2002; Bouhnik et al., 2004; Lesmes et al., 2008); however, to the best of our knowledge, no studies were reported about the effect of RS on the growth of bifidobacteria monocolture and their fate in milk-based beverage under the experimental conditions reported in this work. It is interesting to note that the M115 strain reached a viable cell load in WPF amended with RS close to that found in different milk based foods and considered sufficient to provide therapeutic benefits (Shin et al., 2000; Pérez-Conesa et al., 2005). Differently from the WPF beverage amended with inulin, the stable content of RS during storage suggests that this WPF beverage could contribute to the adult dietary fiber daily intake.

As concerns protein content, the results of this work confirm the well known low proteolytic activity of *Bifidobacterium* strains, in particular when compared with that of lactic acid bacteria (Klaver et al., 1993), that in our case assimilated preferentially free amino acids for their metabolisms during fermentation.

Sensory Evaluation of WPF Beverages

Sensory evaluation showed the overall acceptability decreased from 7.8 to 4.6 and from 6.8 to 4.2 for WPF with inulin and RS, respectively. As concerns sensory descriptors, the three-way interaction between beverage, time of storage and sensory attributes was not statistically significant ($p = 0.657$); thus, the scores of attributes changed only in relation to the beverage and throughout the time of cold storage. Therefore, in order to examine the effect of the attributes in relations to the time on sensory score only two-way ANOVA analyses were statistically allowed ($P < 0.00001$) for each beverage. Indeed, the simple main effect analysis showed the sensory scores of the beverage M115 were significantly ($P < 0.00001$) higher than those registered by BBR8 only in relation to the mouthfeel notes (data not shown). By contrast, both beverages showed, on average, a highly significant ($P < 0.01$) increase in the taste score only after 30 days of cold storage (**Figure 4**).

The overall acceptability of WPF beverages, at the early stage of cold storage, showed values similar to those previously found in a probiotic yogurt containing skim milk powder and WPC (Antunes et al., 2005). The worsening in sensory attributes was previously found in a yogurt containing microencapsulated bifidobacteria cells (Kailasapathy, 2006). The sensory scores registered for the WPF beverages including RS and fermented with the M115 strain could be partially attributed to the positive influence of RS in accordance with results of Kailasapathy (2006) suggested as positive effect of capsulant and filler materials (alginate and Hi-Maize^{TM} Starch) in masking the grittiness (mouthfeel) of the yogurt fermented with bifidobacteria.

CONCLUSION

The present work demonstrates that a whey-based substrate sustains the growth of bifidobacteria monocultures allowing to obtain a fermented beverage containing 20 g L^{-1} of whey proteins that are rich in branched chain and sulfur-containing amino acids.

The supplementation with prebiotic fibers improves the growth rate of some *Bifidobacterium* strains leading to set up a synbiotic food. In addition, after fermentation, the strains assayed resulted able to survive in these beverages under cold storage without being freeze dried, spray dried, or microencapsulated (McMaster and Kokott, 2005; Kailasapathy, 2006; Yeung et al., 2016).

Under the experimental conditions used in this study, the best result was obtained fermenting a whey protein medium, made up with 20 g L^{-1} of whey proteins and 10 g L^{-1} of RS, with the *B. pseudocatenulatum* M115. Further work will be addressed to improve sensory attributes of this new synbiotic whey protein fortified beverage.

AUTHOR CONTRIBUTIONS

SdC carried out experiments and evaluated viable cell loads of all Bifidobacteria strains; LQ evaluated dietary fibers and protein content throughout fermentation and cold storage; LC was responsible for sensory evaluation and carried out statistical analysis for all experiments; FDL cooperated with SdC in particular for experiment related to the M115 strain; FB being the lead investigator, designed the study and supervised the research team, drafted the manuscript and final proofreading. All authors contributed to the manuscript draft.

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Bile-Salt-Hydrolases from the Probiotic Strain *Lactobacillus johnsonii* La1 Mediate Anti-giardial Activity *in Vitro* and *in Vivo*

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Giardia duodenalis (syn. *G. lamblia, G. intestinalis*) is the protozoan parasite responsible for giardiasis, the most common and widely spread intestinal parasitic disease worldwide, affecting both humans and animals. After cysts ingestion (through either contaminated food or water), *Giardia* excysts in the upper intestinal tract to release replicating trophozoites that are responsible for the production of symptoms. In the gut, *Giardia* cohabits with the host's microbiota, and several studies have revealed the importance of this gut ecosystem and/or some probiotic bacteria in providing protection against *G. duodenalis* infection through mechanisms that remain incompletely understood. Recent findings suggest that Bile-Salt-Hydrolase (BSH)-like activities from the probiotic strain of *Lactobacillus johnsonii* La1 may contribute to the anti-giardial activity displayed by this strain. Here, we cloned and expressed each of the three *bsh* genes present in the *L. johnsonii* La1 genome to study their enzymatic and biological properties. While BSH47 and BSH56 were expressed as recombinant active enzymes, no significant enzymatic activity was detected with BSH12. *In vitro* assays allowed determining the substrate specificities of both BSH47 and BSH56, which were different. Modeling of these BSHs indicated a strong conservation of their 3-D structures despite low conservation of their primary structures. Both recombinant enzymes were able to mediate anti-giardial biological activity against *Giardia* trophozoites *in vitro*. Moreover, BSH47 exerted significant anti-giardial effects when tested in a murine model of giardiasis. These results shed new light on the mechanism, whereby active BSH derived from the probiotic strain *Lactobacillus johnsonii* La1 may yield anti-giardial effects *in vitro* and *in vivo*. These findings pave the way toward novel approaches for the treatment of this widely spread but neglected infectious disease, both in human and in veterinary medicine.

Keywords: *Giardia duodenalis*, lactobacilli, *Lactobacillus johnsonii*, bile salt hydrolases, BSH, conjugated bile salts, anti-giardial activity

INTRODUCTION

Giardia duodenalis (syn. *Giardia lamblia* and *Giardia intestinalis*) is a flagellated protozoan parasite responsible for giardiasis, an intestinal zoonotic disease infection that may cause acute or chronic diarrhea, weight loss, malabsorption, abdominal pain, and nausea (Ankarklev et al., 2010; Cotton et al., 2011). It is one of the most common intestinal parasites and one of the most frequent causes of diarrhea, with over 280 million human symptomatic cases worldwide (Lane and Lloyd, 2002; Platts-Mills et al., 2015). Infections occur mainly by the ingestion of cysts present in contaminated food and water. After ingestion, infectious cysts differentiate into trophozoite stages, which in turn colonize the upper small intestine. Included in the "Neglected Disease Initiative" of the World Health Organization (WHO) in 2004, giardiasis has a significant public health impact in both developed and developing countries (Savioli et al., 2006; Platts-Mills et al., 2015). Metronidazole is the most frequently used drug for treating *G. duodenalis* infections, whereas albendazole, tinidazole, and nitazoxanide may also be used with efficacy (Gardner and Hill, 2001; Petri, 2005). Although these drugs have different modes of action, there is an increasing incidence of parasite resistance, and treatment failure is relatively common (Ansell et al., 2015). Moreover, these standard treatments are commonly associated with undesirable side effects in both medical and veterinary usages (Barr et al., 1994; Gardner and Hill, 2001). A successful vaccine has proven elusive, and *Giardia* is able to escape host immunity by switching its variant-specific surface proteins (Singer et al., 2001). Together, these observations underscore the need for new therapeutic alternatives for the treatment of giardiasis.

In the last decade, some probiotics (i.e., live microorganisms which, when administered in adequate amounts, confer a health benefit on their hosts, WHO 2001), in particular several species belonging to the genus *Lactobacillus*, have shown anti-giardial efficacy in various murine models (see Travers et al., 2011 for review). The mechanisms remain incompletely understood but may involve host immunomodulation and/or extracellular compounds released by the bacteria (Perez et al., 2001; Humen et al., 2005; Shukla et al., 2008; Shukla and Sidhu, 2011; Goyal et al., 2013). In this context, we have recently shown that unconjugated bile salts, generated by secreted or released enzymes by the probiotic strain of *Lactobacillus johnsonii* La1 (also known as *L. johnsonii* NCC533), may contribute to the inhibition of *Giardia* trophozoite growth *in vitro* (Perez et al., 2001; Travers et al., 2016). BSH (also called cholylglycine hydrolase, EC 3.5.1.2) are enzymes that hydrolyze the amide bond of conjugated bile salts, liberating the amino acid moiety from the steroid core and generating deconjugated bile salts (i.e., cholic acid, deoxycholic acid and chenodeoxycholic acid) (Begley et al., 2006). Conjugated-bile salts are synthesized in the liver where conjugation to either glycine or taurine occurs, and these conjugated-bile salts play an important role in the solubility and absorption of lipids and cholesterol in the intestinal tract (Eyssen, 1973; Kim et al., 2005; Begley et al., 2006). Moreover, glyco- and tauro-conjugated bile salts exert detergent and antimicrobial properties (Ruiz et al., 2013). BSH activities lead to bile salt detoxification and confer a competitive advantage to the microbial communities that express them, such as lactobacilli in the upper part of the small intestine (Ridlon et al., 2006; Ruiz et al., 2013).

In this study, we cloned and expressed each one of the three-*bsh* genes (i.e., *bsh12*, *bsh47,* and *bsh56*) from *L. johnsonii* La1 (Pridmore et al., 2004) in *Escherichia coli* in order to evaluate their substrate specificities and to assess their anti-*Giardia* activities, both *in vitro* and *in vivo*. A comparative structural analysis of the three BSHs was also performed using *in silico* approaches to explore whether structural differences could explain possible differences in substrate specificities. The three recombinant BSHs, rBSH12, rBSH47, and rBSH56, were tested against two different strains of the human assemblage A of *G. duodenalis* (WB6 and NF) *in vitro*. Then, rBSH47 was selected to be tested *in vivo* on OF1 suckling mice infected with the *G. duodenalis* strain WB6.

MATERIALS AND METHODS

In Silico Analysis of BSHs

Bile salt hydrolases amino acid sequences from different bacterial species were retrieved from databases using BLASTP program from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and analyzed *in silico*. Multiple sequence alignments of BSH amino acid sequences were performed using CLUSTALO 1.2.1 (http://www.ebi.ac.uk/Tools/msa/clustalo/) to identify the conserved motifs between the different enzymes. Phylogenetic relationships and phylogenic clustering of BSHs from different species were established by neighbor-joining methods using MEGA5 software (http://www.megasoftware.net/; Tamura et al., 2011). Three-dimensional modeling of *L. johnsonii* La1-BSHs was performed using I-TASSER from University of Michigan (http://zhanglab.ccmb.med.umich.edu/I-TASSER/; Roy et al., 2010). According to C-score results, the BSH from *Bifidobacterium longum* (Kumar et al., 2006) was chosen as template for modeling BSH56, whereas the BSH from *Clostridium perfringens* was used as template for modeling both BSH12 and BSH47 (Rossocha et al., 2005). Models for structure predictions were selected according to the highest values of their C-score (measured for evaluating global and local similarity between query and template protein). Protein structure analysis was performed using Pymol (PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

Bacteria and Growth Conditions

Lactobacillus johnsonii La1 strain (Pridmore et al., 2004) was cultured in Man Rogosa Sharpe broth (MRS, Difco) and grown at 37°C in an anaerobic jar using BBL GasPak Anaerobic System (BD), incubated overnight (ON). *E. coli* TOP10 chemically competent cells (Invitrogen) were used for the subcloning of PCR fragments. *E. coli* CYS21 and SE1 chemically competent strains (DelphiGenetic, Belgium) were used, respectively, for the cloning and expression of BSHs. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C ON with vigorous shaking at 180 rpm. All bacterial strains were stored at −80°C with 15% (v/v) glycerol for cryoprotection.

Cloning of *bsh* Genes from *L. johnsonii* La1

Genomic DNA of *L. johnsonii* La1 was extracted from 2 mL of an ON culture using Wizard Genomic DNA Purification Kit Protocol (Promega) and used as template to amplify the 3 *bsh* genes: *bsh12* (Gene ID: 2743525), *bsh47* (Gene ID: 2743183), and *bsh56* (Gene ID: 2743142) (Pridmore et al., 2004). The coding sequences of *bsh*12, *bsh*47, and *bsh*56 genes (excluding the putative signal sequences) were amplified by PCR (Phusion Taq, Thermo Fisher Scientific) using primers described in **Table 1**. These primers were designed to incorporate two restriction sites: *Nhe*I (forward primer) and *Xho*I (reverse primer). The amplified PCR fragments were purified using SV Gel and PCR Clean-Up System (Wizard) and were subcloned into the vector pCR®2.1-TOPO® (Invitrogen). The resulting constructions (pLB487, pLB488, and pLB489) were validated by sequencing (MWG-Genomic Company, Germany) before recovering the *bsh* genes with *Nhe*I and *Xho*I restriction enzymes and cloning them into pStaby 1.2 vector (DelphiGenetics) previously digested with the same enzymes. The pStaby 1.2 plasmid was used for intermediate cloning to introduce a C-terminal six-Histidine tag (His-tag), allowing subsequent purification of rBSHs using affinity chromatography. The resulting plasmids were transferred into *E. coli* CYS21 strains and transformants were grown at 37°C ON in 10 mL of LB containing ampicillin (Amp, 100 μg/ml) with shaking at 180 rpm. Plasmid DNA were extracted from positive clones, sequenced to confirm identity, and subsequently transformed into *E. coli* SE1 expression strain. Bacterial strains, plasmids, and primer sequences used in this study are described in **Table 1**. Immunoblotting experiments were performed on *E. coli* SE1 (pLB490), *E. coli* SE1 (pLB491), and *E. coli* SE1 (pLB492) strains lysates (see below) using mouse monoclonal 6x-His Epitope Tag Antibody (Thermo Fisher Scientific) to detect recombinant BSHs.

TABLE 1 | Bacterial strains, plasmids, primers used in this study.

Material	Relevant properties	Source/ reference
STRAINS		
L. johnsonii		
La1 (NCC533)	Wild type strain	Nestlé collection center NCC533
E. coli		
TOP10	Cloning strain	Invitrogen
CYS21	Cloning strain	DelphiGenetics
SE1	Expression strain	DelphiGenetics
PLASMIDS		
pCR2.1-TOPO	*Amp^r, subcloning vector	Invitrogen
pLB487	*Amp^r, TOPO harboring *bsh12* gene	This study
pLB488	*Amp^r, TOPO harboring *bsh47* gene	This study
pLB489	*Amp^r, TOPO harboring *bsh56* gene	This study
pStaby express	Amp^r, subcloning vector	DelphiGenetics
pLB490	*Amp^r, pStaby harboring *bsh12* gene	This study
pLB491	*Amp^r, pStaby harboring *bsh47* gene	This study
pLB492	*Amp^r, pStaby harboring *bsh56* gene	This study
Primers	Sequence	
bsh12Fw	5′CCGCTAGCTGTACCTCAATTGTTTATAGTTC 3′	This study
bsh12Rev	3′GGCTCGAGATTTTGATAATTAATTGTTTGC 5′	This study
bsh47Fw	5′CCGCTAGCTGTACTGGTTTAAGATTCAC 3′	This study
bsh47Rev	3′GGCTCGAGGTAAGTCACAAGACTGGTTG 5′	This study
bsh56Fw	5′CCGCTAGCTGTACATCAATTTTATATAGTCC 3′	This study
bsh56Rev	3′GGCTCGAGATTTTCAAATTTAATGGCTTG 5′	This study

Expression and Purification of Recombinant BSH12, BSH47, and BSH56 in *E. coli*

E. coli SE1 strains harboring pLB490 (*bsh12*), pLB491 (*bsh47*), and pLB492 (*bsh56*) were grown at 37°C ON in 10 mL of LB supplemented with ampicillin (100 μg/mL) with vigorous shaking at 180 rpm and subsequently grown in 1.5 L of LB/ampicillin (100 μg/mL) at 37°C. When an optical density ($OD_{600\,nm}$) = 0.6–0.8 was reached, gene expression was induced by the addition of 1 mM of Isopropyl β-D-1-Thiogalactopyranoside (IPTG), and cultures were incubated at 21°C ON with shaking at 180 rpm. Bacteria were harvested by centrifugation and cell pellets were washed with PBS and resuspended in 15 ml of Tris-KCl buffer (Tris 50 mM, KCl 100 mM, $MgCl_2$ 10 mM, pH 7.5) supplemented with Triton-X-100 (Sigma-Aldrich) to a final concentration of 1% and protease inhibitors 1X (Roche). Cells were subsequently sonicated for 4 min with alternated pulses on ice (on: 5 s, off: 30 s). The lysed cells were then placed in ultracentrifuge tubes and spun at 220,000 × g at 4°C for 45 min to separate soluble supernatants from pellets.

The soluble fractions containing the recombinant BSH (rBSH) were then collected and rBSHs were purified using affinity chromatography. Briefly, columns kept in nickel-nitrilotriacetic acid (Ni-NTA; Qiagen) agarose were first washed with milliQ water and equilibrated with 50 mM Tris-KCl buffer pH 7.5 according to the supplier's protocol. Soluble lysates were passed through Ni-NTA columns and washed with 50 mM Tris-KCl buffer pH 7.5 to remove unbound proteins. Finally, rBSHs were eluted by increasing imidazole concentrations (25, 75, and 500 mM) as recommended by the supplier. The eluted proteins were desalted using Sephadex G-25 columns (Amersham Biosciences). All fractions were analyzed on Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue.

Bile Salt Hydrolase Activity Assays

The substrate specificity of each rBSHs was assessed on plates using an agar test. *E. coli* strains harboring pLB490, pLB491, and pLB492 were cultured in LB broth in presence of ampicillin (100 μg/ml). Overnight cultures were then spotted on LB agar plates supplemented with either 0.5% taurodeoxycholic acid (TDCA, Sigma-Aldrich) or 0.5% glycodeoxycholic acid (GDCA, Merck Millipore) and incubated at 37°C for 48 h.

The BSH hydrolyzing activities were also monitored using purified recombinant enzymes in presence of conjugated bile salts, in solution, by measuring the liberation of amino acids

(glycine or taurine) as previously described (Grill et al., 2000). A volume of 100 µl of rBSH (20 µg) was mixed with 100 µl of 2.4 g/L of each conjugated bile salts (GDCA, TDCA, glycocholic acid, or taurocholic acid) and incubated for 30 min at 37°C. BSH from *C. perfringens* (Sigma-Aldrich, reference C4018) was used as a positive control. A solution without bile salts was used as a negative control. The hydrolysis of bile salts was stopped by adding 200 µl of 15% trichloroacetic acid (TCA) (v/v%) and the mixture was spun at 10,000 g for 15 min to remove precipitated proteins. The supernatant (80 µl) was subsequently collected and added to 680 µl of 0.3 M borate buffer, 1% SDS (pH 9.5), and 80 µl of 0.3% picrylsulfonic acid solution (Sigma-Aldrich). Mixtures were incubated for 30 min in the dark at room temperature and 800 µL of 0.6 mM HCl was added to stop the colorimetric reaction. The amount of glycine or taurine released was measured at 416 nm using a spectrophotometer and standard curves were established with free glycine and taurine.

Giardia duodenalis Cultures

Two different isolates of assemblage A were used in this study: *G. duodenalis* strains WB clone 6 (WB6, ATCC50803), isolated from a patient with chronic giardiasis, and *G. duodenalis* NF (kindly provided by Dr. André Buret, University of Calgary), obtained from an outbreak of human giardiasis. Trophozoites were cultured in axenic conditions grown in Keiser's modified TYI-S-33 medium (KM) adjusted at pH 6.0 and supplemented with heat-inactivated fetal calf serum (10%) (FCS, reference A15-101, PAA laboratories, GE Healthcare) as recently described (Travers et al., 2016). *In vitro* experiments were performed with or without bovine bile (Difco, DB Diagnostic System, reference 212820) supplementation (0.6 g/L).

Anti-giardial Activity Assays

Increasing concentrations of rBSHs were co-incubated with fresh cultures of *G. duodenalis* WB6 trophozoites (2×10^5 parasites/ml) in KM medium supplemented with 10% FCS in a final volume of 480 µl, at 37°C in anaerobic conditions for 22 h. Experiments were performed with or without bovine bile (0.6 g/L) supplementation. BSH from *C. perfringens* (1U, Sigma-Aldrich, reference C4018) was used as a positive control. Trophozoites were detached from tubes by chilling on ice for 10 min and the parasite load was measured by using hemocytometer (flagella mobility was used as viability criteria). The inhibition levels were determined in comparison with values of non-treated trophozoite cultures (percentage of growth). Three biological replicates were performed, each in duplicates. The half maximal inhibitory concentrations (IC_{50}) were calculated using Prism 5 software (GraphPad).

G. duodenalis Viability Assays on Cell Cultures

Caco-2 epithelial cells (human colonic adenocarcinoma, ATTC HTB-37) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 200 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 10% fetal bovine serum (FBS) (Gibco, reference 12484-028) at 37°C and 5% CO_2. Caco-2 cells were cultured (passage 28–32) at 80% confluence with trypsin-EDTA and seeded at 10^5 cells/mL onto 12-wells plates (Caco-2 growth medium). Cells were cultured until the monolayer was confluent (3–4 days) with medium changes every 48 h. 3 days prior to co-incubation, cultures of *G. duodenalis* NF strain trophozoites were axenically cultured in KM medium supplemented with 10% heat-inactivated FBS at 37°C to confluence. Parasites were ice-chilled for 15 min, harvested by centrifugation for 10 min at 1,300 × *g* (4°C), and resuspended in Caco-2 growth medium supplemented with bovine bile (0.6 g/L). For co-culture experiments, trophozoites were seeded at a multiplicity of infection (MOI) of 10:1. Recombinant BSHs were then added to co-cultures at different concentrations and the plates were incubated at 37°C and 5% CO_2. After 20 h of incubation, trophozoites were collected after chilling of plates on ice and the parasite load was determined using hemocytometer (flagella mobility was used as viability criteria).

Scanning Electron Microscopy

For scanning electron microscopy (SEM), fresh cultures of *G. duodenalis* trophozoites WB6 strain were treated with either rBSH47 (0.5 µg/ml), rBSH56 (0.08 µg/ml), or DCA (0.1 g/L and 0.2 g/L) in KM supplemented with 10% heat-inactivated FCS (10%), with or without bovine bile (0.6 g/L) supplementation. Cultures of treated and untreated *Giardia* trophozoites were subsequently seeded in 12 wells plates on poly-lysine glass coverslips placed at the bottom, and parasites were let to settle on the glass coverslips. After 16 h incubation, the supernatants were removed gently and cells were fixed on the glass coverslips with cacodylate 0.1 M and glutaraldehyde 2.5% (pH 7.2) overnight at 4°C. After two washing steps with 0.1 M cacodylate (pH 7.2), cells were dehydrated in a graded ethanol series (50, 70, 90, and 100%) and critical point-dried in liquid CO_2 (Emitech K850, Quorum Technologies). Coverslips were then mounted onto holders and coated with 20 nm of gold (JEOL Fine Coater JFC-1200). The samples were then examined with a Hitachi Scanning Electron SU3500 Premium.

Experimental Infection Model

OF1 mice were obtained from Charles River (Saint-Germain-Nuelles, France). Mice were housed in pathogen-free conditions and all experiments were performed under a laminar flow hood. Neonatal (suckling) mice were challenged with 10^5 *G. duodenalis* WB6 trophozoites at day 10 by intragastric gavage (100 µl). Recombinant BSH47 was diluted in DMEM with $NaHCO_3$ 16.4% (vehicle) and daily administered by intragastric gavage to neonatal mice from days 10 to 15. Control animals received vehicle instead of rBSH47. Animals were sacrificed by cervical dislocation at day 16 (peak of infection, as determined in parallel assays) and assayed for the presence of *G. duodenalis* trophozoites in the small intestine. Small intestines were resuspended in 5 ml of cold PBS, incubated on ice for 10 min, and mixed thoroughly. The parasite load was estimated using hemocytometer chambers. Mice with no detectable trophozoites (threshold: $<10^3$ parasites/5 ml intestine suspension) were considered as parasite-free. All protocols were carried out in accordance with the institutional ethical guidelines

of the ethics committee ANSES's Animal Health Laboratory at Maisons-Alfort on the campus of the French National Veterinary School of Alfort (ENVA), which approved this study.

Statistical Analysis

Data analysis was performed with Prism 5 software (GraphPad). One-way ANOVA, Mann-Whitney, and *t*-test were used to evaluate difference between means. Results were presented as means ± standard error of the mean (SEM). Statistical significance was calculated at a P value of 0.05 and 95% confidence interval.

RESULTS

In Silico Analysis of *L. johnsonii* La1-BSHs Protein Sequences

The amino acid sequences of *L. johnsonii*-BSH12, BSH47, and BSH56 enzymes were blasted against reported sequences from several Gram-positive bacteria using Blastp. For the three *L. johnsonii* La1 BSH, results indicated high identity levels with BSH of different *Lactobacillus* species ranging from 54 to 100% but lower levels of identity (less than 54%) with BSH from *Bifidobacterium* and *Clostridium* species. In particular, the *L. johnsonii*-BSH12 shared 54, 57, 60, 79–84, and 60–100% identities with BSHs from *C. perfringens*, *L. acidophilus*, *L. reuteri*, *L. gasseri*, and *L. johnsonii*, respectively. *L. johnsonii*-BSH47 shared 54–55, 56–58, 60–66, 57, 70, and 97–100% identities with BSHs from *L. crispatus*, *L. reuteri*, *L. gasseri*, *L. acidophilus, L. amylovorus,* and *L. johnsonii*, respectively. Finally, *L. johnsonii*-BSH56 showed 94 and 99% identity with BSHs from *L. gasseri* and both *L. acidophilus* and *L. johnsonii*, respectively.

The 3D structures of CBAH-1 from *C. perfringens* (Rossocha et al., 2005) and BlBSH from *B. longum* (Kumar et al., 2006) have been determined (PDB: 2BJF and PDB: 2RF8, respectively), revealing the presence of key residues in the enzymatic active site (Cys-2, Arg-18, Asp-21, Asn-82, Asn-172, and Arg-225; numbering referring to CBAH-1). In addition, experimental studies validated the importance of Arg-18 in the catalytic site (Fang et al., 2009; Lin, 2014; Lin et al., 2014). Therefore, multiple amino acid sequence alignments of BSH12, BSH47, and BSH56 with CBAH-1 and BlBSH were performed using ClustalO program (GONNET PAM 250 matrix), thereby indicating that these key residues were indeed highly conserved in all three *L. johnsonii* La1-BSHs (**Figure 1**). Moreover, motifs surrounding these key amino acid positions were also found to be well conserved such as ^{16}FGRNXD, ^{72}NEXGLXXAGLNF, ^{170}VXXLTNXPXF, and ^{213}GXGXGXXGXPGD, a point that has been also reported in other studies (Elkins et al., 2001; Kim and Lee, 2008). However, residues, which are predicted to be involved in the substrate-binding site based on the 3D structure of *C. perfringens*, did not appear to be conserved in either *L. johnsonii* BSH enzyme (Ridlon et al., 2006).

Predicted tridimensional structures of *L. johnsonii* BSH12, BSH47, and BSH56 were modeled with I-TASSER software, using existing 3D structures (**Figure S1**, BSH47 and BSH56 and **Figure S2**, BSH12). *Clostridium perfringens* CBAH-1 was used as a template for BSH47 modeling (RMSD: 0.64; TM-score: 0.991; Identity, 35.4%) and BSH12 (RMSD: 1.5; TM-score: 0.967; Identity: 37.3%). *Bifidobacterium longum* BlBSH was used as a template for BSH56 modeling (RMSD: 1.16; TM-score: 0.966; Identity: 43.8%). A superimposition of BSH47 and BSH56 models revealed very similar structures. In particular, the distinctive αββα-folding pattern is conserved in both proteins (Patel et al., 2010; Lin et al., 2014). In addition, the residues involved in the catalytic site are superimposed which confirms a conservation of 3D structure despite a high variability of amino acid sequence among BSHs. Similar results were obtained with BSH12 (**Figure S2**).

Heterologous Expression and Purification of BSHs in *E. coli*

To study the biochemical and enzymatic characteristics of *L. johnsonii* La1-BSHs, *bsh* genes (i.e., *bsh12*, *bsh47*, and *bsh56*) were cloned in *E. coli* CYS21 strain and expressed in *E. coli* SE1 strain. Western blot analysis from *E. coli* SE1 cells expressing His-tagged BSHs showed an efficient production of BSH12, BSH47, and BSH56, respectively (**Figure 2A**). High yields of heterologous proteins were produced from 1.5 L of recombinant *E. coli* cultures upon 1 mM IPTG induction. No cytotoxic effects were observed during bacterial growth. C-terminal His-tagged rBSHs were subsequently purified using Ni-NTA agarose affinity chromatography and desalted. The purity of BSHs was assessed by Coomassie-blue staining of SDS-PAGE (**Figure 2B**). The molecular weights observed on SDS-PAGE corresponded with those expected (based on theoretical predictions) for rBSH 47 (37.1 kDa) and rBSH56 (35.8 kDa). However, the molecular weight for rBSH12 appeared slightly higher than theoretically expected (37.4 kDa). Nanodrop quantifications of desalted proteins showed that 35 mg of rBSH47 and 28 mg of rBSH56 were successfully purified from 1.5 L of culture. However, only 3 mg of rBSH12 could be recovered.

L. johnsonii La1 BSH Activities and Substrate Specificities

The substrate specificities of the *L. johnsonii* BSHs were assayed by two approaches. Enzymatic activities were monitored in solution, using recombinant enzymes, by measuring amino acids released from the hydrolysis of conjugated bile salts as described in materials and methods. These enzymatic activity assays revealed a slight activity toward glycocholic acid, but a much higher level of activity toward taurocholic acid, for both rBSH47 and rBSH56 (**Table 2**). No significant enzymatic activity was detected with the purified rBSH12 with any substrate (**Table 2**). In parallel, the substrate specificities of the three *L. johnsonii* BSHs (produced in *E. coli*) were determined using LB agar supplemented with either taurodeoxycholic (0.3%) or glycodeoxycholic (0.3%) acids. A white and iridescent precipitate around colonies is indicative of BSH hydrolytic activity. *E. coli* SE1 strain expressing rBSH47 efficiently hydrolyzed tauro-conjugated bile salts, whereas no BSH activity was detected for glyco-conjugated bile salts (**Figure 3A**). *E. coli* SE1 strain expressing rBSH56 efficiently hydrolyzed both tauro- and glycol-conjugated bile salts (data not shown). A slight deconjugation

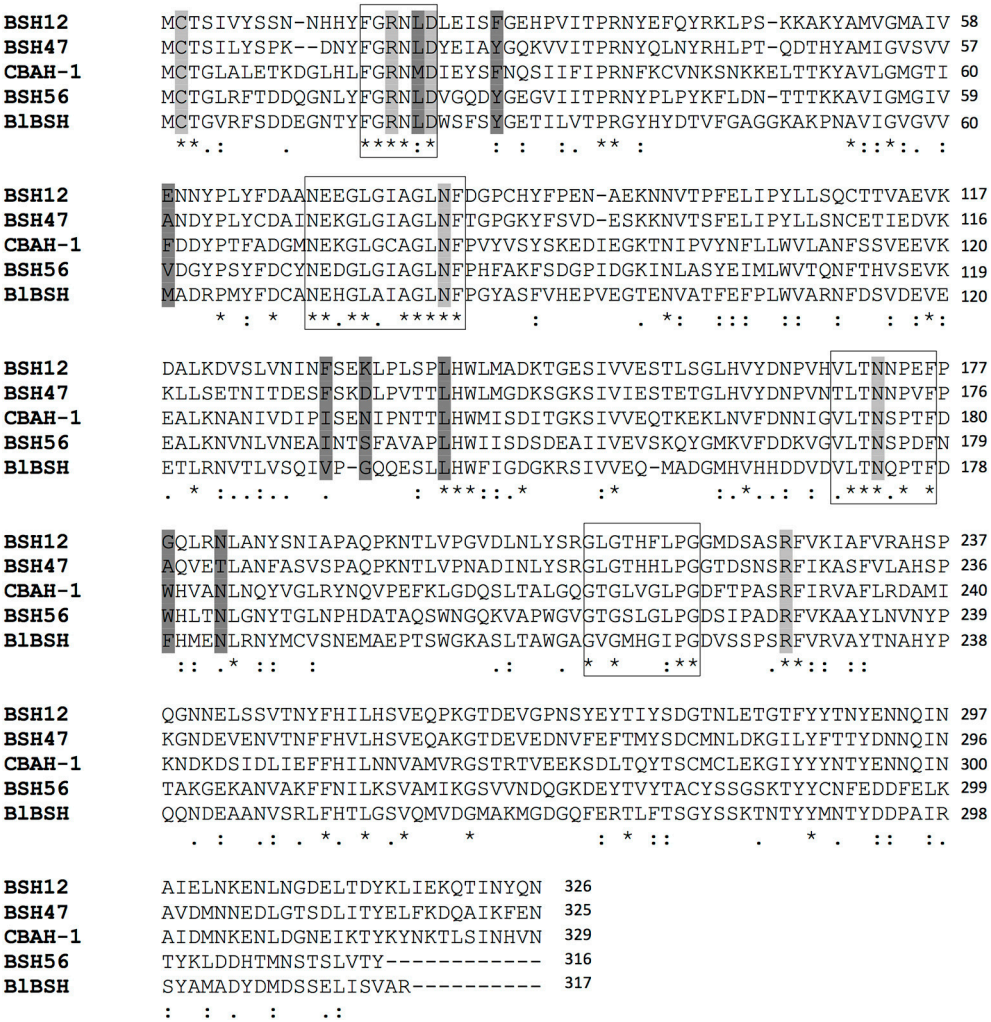

FIGURE 1 | *L. johnsonii* La1 BSH possess conserved key amino acids in their predicted active sites. Multiple sequence alignments of BSH were performed with the ClustalO program (http://www.ebi.ac.uk/Tools/msa/clustalo/) using GONNET 250 matrix. An "*" (asterisk) indicates positions of fully conserved residues in all five sequences; a ":" (colon) indicates conservation of amino acid with strongly similar chemical properties (Gonnet PAM 250 matrix score > 0.5); a "." indicates conservation of amino acid with similar chemical properties (Gonnet PAM 250 matrix score ≤ 0.5). Residues highlighted in light gray correspond to the predicted key active site amino acids, based on the 3D structures of BSHs from both *C. perfringens* (CBAH-1, PDB: 2BJF) (Rossocha et al., 2005) and *B. longum* (BlBSH, PDB: 2RF8) (Kumar et al., 2006). Residues highlighted in dark gray indicate amino acids putatively involved in substrate binding based on CBAH-1 3D structure (Rossocha et al., 2005; Ridlon et al., 2006). Boxes indicate conserved signatures, i.e., ^{16}FGRNXD, ^{72}NEXGLXXAGLNF, ^{170}VXXLTNXPXF, and ^{213}GXGXGXXGXPGD (CBAH-1 numbering).

was observed with *E. coli* strain producing rBSH12 against glyco-conjugated bile salts. However, BSH12 was not further tested in this study.

Phylogenetic relationship among selected BSH sequences and related substrate predictions were represented on a neighbor-joining tree, constructed using amino acid sequences of BSHs whose substrate specificities have been previously characterized, with 500 bootstrap replications using MEGA5 software (http://www.megasoftware.net/). Such a phylogenetic analysis showed that *L. johnsonii* La1-BSH12 is more closely related to *L. johnsonii* 100-100-BSH-α (**Figure 3B**), an enzyme which is able to hydrolyze both tauro- and glyco-conjugated bile salts. The *L. johnsonii* La1-BSH56 is phylogenetically related to a compact cluster including *L. johnsonii* PF01-BSHB (LjBSHB), *L. acidophilus* PF01-BSH (LaBSH), and *L. johnsonii* 100-100-BSH-β (LjBSH-β). Both BSH56 and LjBSH-β display broad substrate specificity, with a slight preference for tauro-conjugated over glyco-conjugated bile salts, whereas LaBSH and LjBSHB exclusively hydrolyze tauro-conjugated bile salts (Chae et al., 2013). Finally, *L. johnsonii* La1-BSH47 was more closely related to *L. johnsonii* PF01-BSHC (LjBSHC) hydrolyzing only glyco-conjugated bile acids, whereas BSH47 displays a preference for tauro-conjugated substrates. These observations suggest that substrate specificities are not systematically conserved among lactobacilli BSHs, despite a good conservation of their 3D-structures and of key amino acids in their active sites, which makes substrate specificity prediction based on phylogenetic analysis not straightforward for the moment.

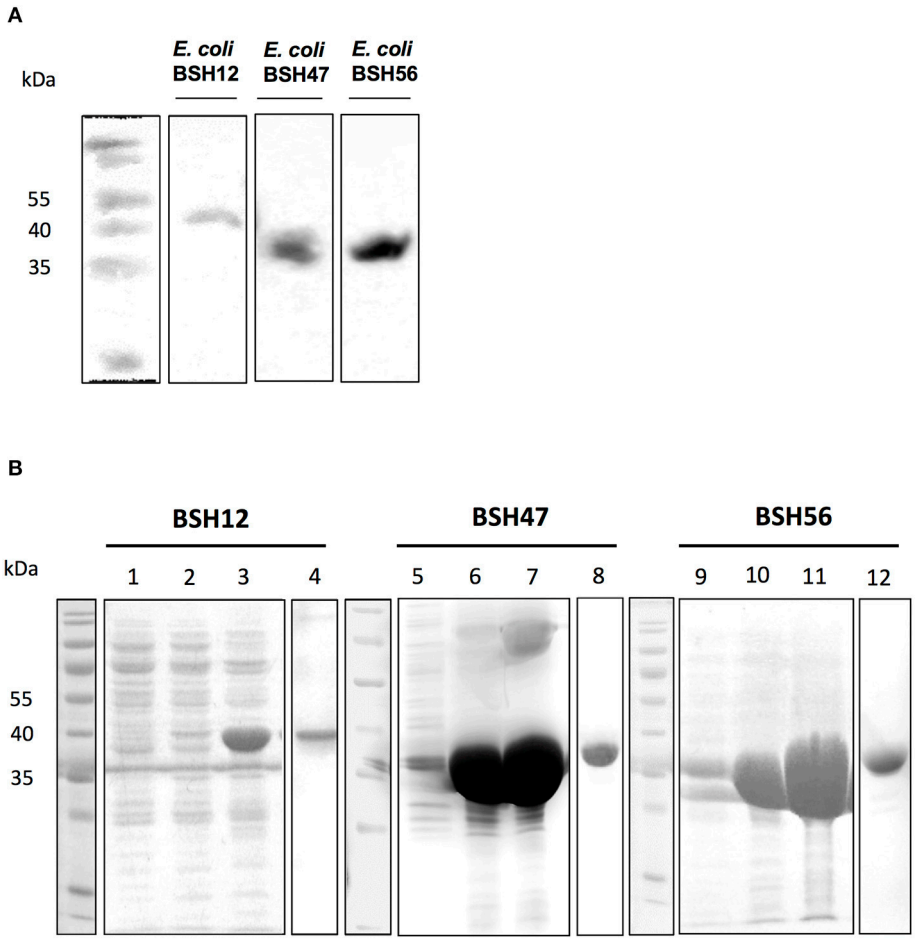

FIGURE 2 | Expression and purification of *L. johnsonii* La1 BSHs. **(A)** Detection of heterologous production of BSH in *E. coli* by Western Blotting using anti-His tag antibody. Production of BSH was done under the control of pStaby system in *E. coli* (induced with 1 mM IPTG). Cytoplasmic fractions were extracted from *E. coli* SE1 harboring pLB490 plasmid (*E. coli* BSH12), pLB491 plasmid (*E. coli* BSH47) or pLB492 plasmid (*E. coli* BSH56). **(B)** SDS–PAGE analysis of purified BSHs. Purification steps of rBSH12, rBSH47, and rBSH56 with Ni-NTA affinity chromatography. Lanes 1–3 correspond to eluted fractions of rBSH12 with imidazole gradient (25, 75, and 500 mM). Lane 4 is rBSH12 after desalting. Lanes 5–7 correspond to eluted fractions of rBSH47 (imidazole gradient: 25, 75, and 500 mM); lane 8 is rBSH47 after desalting step. Lanes 9–11 correspond to eluted fractions of rBSH56 (imidazole gradient: 25, 75, and 500 mM); lane 12 is rBSH56 after desalting step.

TABLE 2 | Activities of *L. johnsonii* BSH against tauro- and glyco-conjugated bile salts.

Enzyme	TDCA hydrolase activity[a]	Relative activity[b]		GDCA hydrolase activity[c]	Relative activity[d]	
		U/g*	%		U/g*	%
BSH12	–	–	Nd	+	–	Nd
BSH47	++	720	100	+	65	9
BSH56	++	2600	100	++	530	21
BSH C. perf	Nd	150	26	Nd	580	100

**μmol/5 min per g of protein.*
[a]Based on activity on taurodeoxycholic acid (plate assay).
[b]Based on activity on taurocholic acid (enzymatic assay).
[c]Based on activity on glycodeoxycholic acid (plate assay).
[d]Based on activity on glycocholic acid (enzymatic assay).
Nd, Non-detected. One unit of BSH activity was defined as the amount of enzyme that can liberate 1 μmol of amino acid from a given substrate in 5 minutes.

Besides, enzymatically active BSHs from *L. johnsonii* La1 that could be measured here showed a higher activity for tauro-conjugated than glyco-conjugated substrates, whereas a clear preference has been observed for glyco-conjugated bile salts among other lactobacilli-BSHs characterized so far (Tanaka et al., 1999).

Enzymatic Activities of *L. johnsonii* La1 BSH47 and BSH56 Display Anti-giardial Effects

To evaluate the anti-giardial potential of purified *L. johnsonii* La1-BSHs, *G. duodenalis* WB6 trophozoites were incubated for 22 h in the presence of increasing concentrations of rBSH47 and rBSH56 from 2×10^{-5} to 17.4 μg/ml (rBSH12 was not tested for anti-giardial activity due to weak BSH-activity). Positive controls with *C. perfringens* BSH (Sigma-Aldrich) and negative experimental controls were set up in each independent inhibition

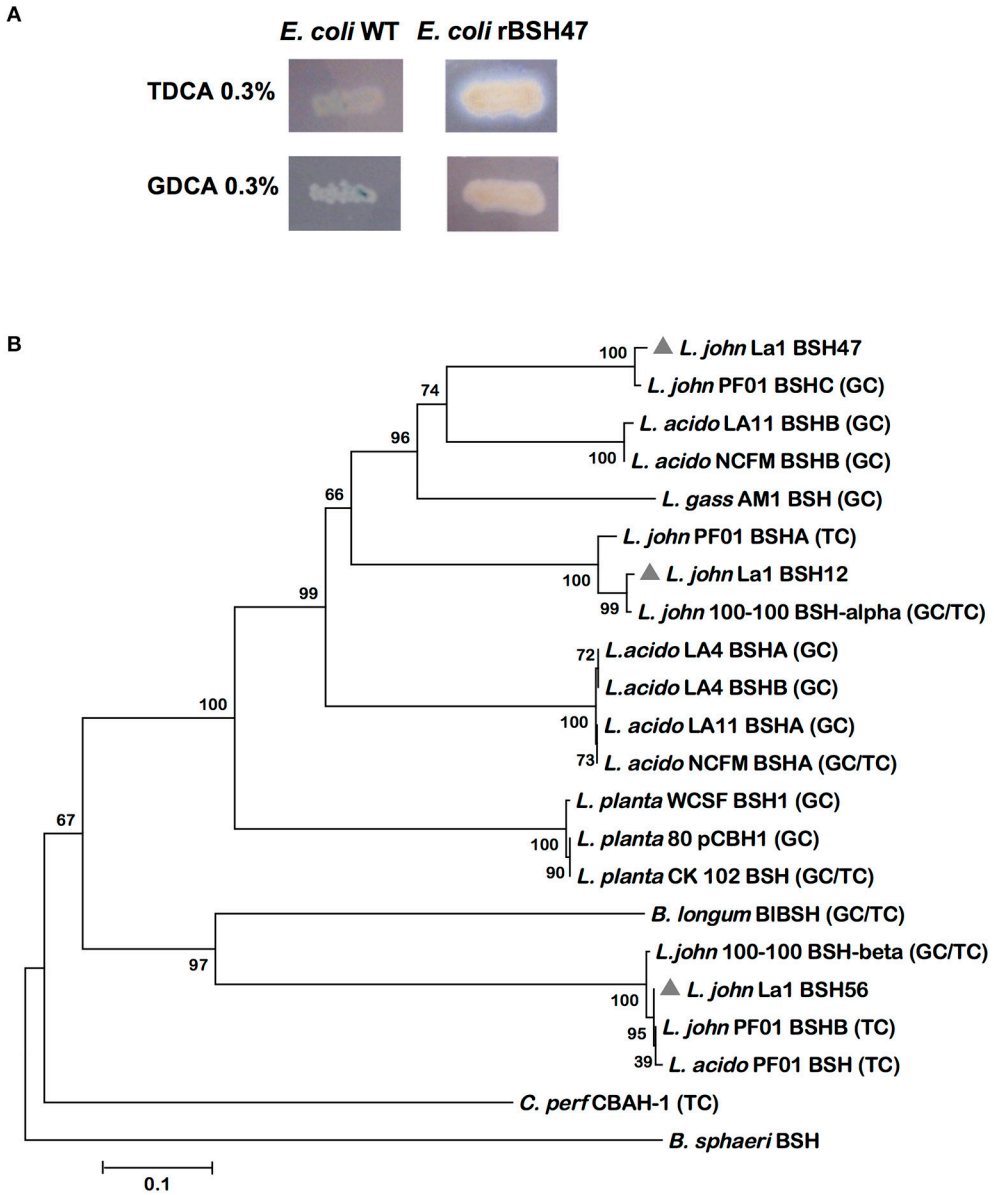

FIGURE 3 | Substrate specificities of *L. johnsonii* La1 BSHs. **(A)** Hydrolase activity of rBSH47 produced in *E. coli* tested on LB plates. *E. coli* SE1 wild type or *E. coli* SE1 expressing rBSH47 were plated in the presence of taurodeoxycholic acid 0.3% (TDCA) or glycodeoxycholic acid 0.3% (GDCA) and incubated for 48–72 h. Activity is detected when a whitish halo forms around colonies. Data with *E. coli* SE1 expressing rBSH12 and rBSH56, respectively, are not shown. **(B)** Phylogenetic relationship among selected BSH sequences and substrate prediction. Programme MEGA5 was used for the phylogenetic tree and for the 500-replication bootstrap analysis. The following predicted amino acid sequences were obtained from UniProtKB/Swiss-Prot databases: BSH12, *L. johnsonii* La1 (Q74IV4_LACJO); BSH47, *L. johnsonii* La1 (Q74JG0_LACJO); BSH12, *L. johnsonii* La1 (Q74LX7_LACJO); CBAH-1, *C. perfringens* strain 13 (P54965.3); BSHA, *L. acidophilus* LA4 (ACL98173.1); BSHB, *L. acidophilus* LA4 (ACL98173.1); BSHA, *L. acidophilus* LA11 (ACL98175.1); BSHB, *L. acidophilus* LA11 (ACL98176.1); BSHA, *L. acidophilus* NCFM (YP_193782.1); BSHB, *L. acidophilus* NCFM (AAV42923.1); BSH, *L. gasseri* AM1 (ACL98172.1); BSH-α, *L. johnsonii* 100-100 (AAG22541.1); BSH-β, *L. johnsonii* 100-100 (AAC34381.1); BSHA, *L. johnsonii* pf01 (EGP12224.1); BSHB, *L. johnsonii* pf01 (EGP13287.1); BSHC, *L. johnsonii* pf01 (EGP12391.1); pCBH1, *L. plantarum* 80 (AAB24746.1); BSH1, *L. plantarum* WCSF (CCC80500.1); BIBSH, *B. longum* subsp. *longum* (2HF0); BSH, *L. acidophilus* PF01 (ABQ01980.1); BSH, *L. plantarum* CK 102 (Ha et al., 2006); Penicillin V Acylase (PVA), *Bacillus sphaericus* (3PVA). Substrate specificity, when known (see text for referenced literature) is indicated for each BSH enzyme (in brackets): (TC), specificity for tauro-conjugated bile salts; (GC), specificity for glyco-conjugated bile salts; (TC/GC), specificity for both tauro and glyco-conjugated bile salts.

assay. Treatments of *Giardia* trophozoites with BSHs showed a dose-dependent inhibition of the parasite growth in presence of bile, when compared to the controls without bile (**Figures 4A,B**). The IC_{50} of rBSH56 ($IC_{50BSH56} = 0.018 \pm 0.002\,\mu g/ml$) was slightly lower than that of rBSH47 ($IC_{50BSH47} = 0.030 \pm 0.003\,\mu g/ml$). Concentrations higher than $1\,\mu g/ml$ of either

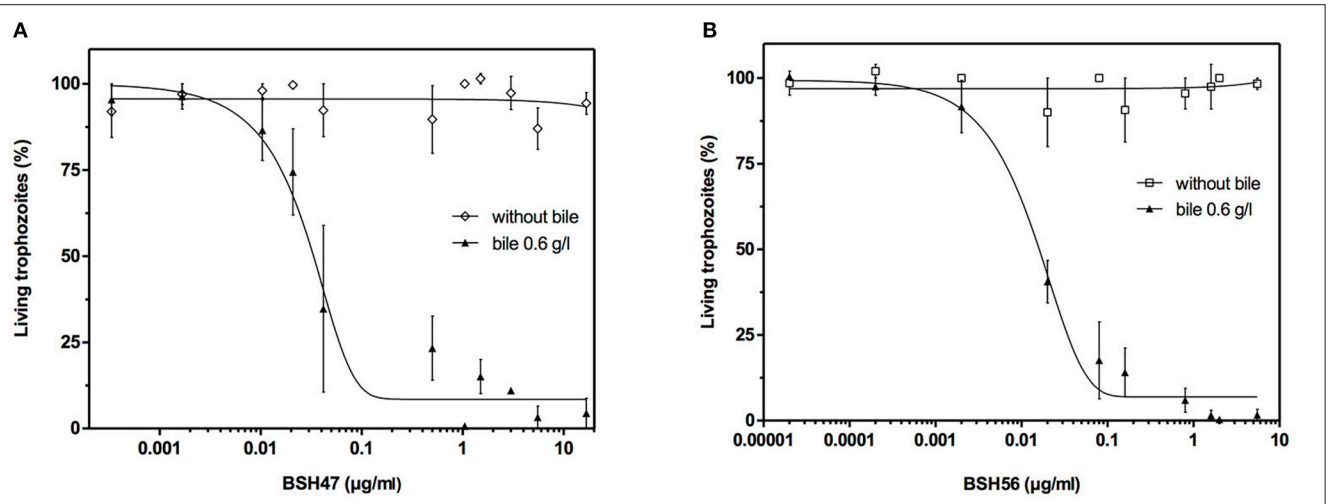

FIGURE 4 | Anti-giardial activity of *L. johnsonii* La1 BSHs *in vitro*. Inhibition of *G. duodenalis* WB6 strain growth by enzymatically active rBSH47 **(A)** and rBSH56 **(B)** from *L. johnsonii* La1 with and without bovine bile supplementation (0.6 g/L) after 22 h of incubation. Increasing doses of BSH were tested ranging from 2×10^{-5} to 17.4 µg/ml. Values are in percentage ± SEM (standard error of the mean). Growth inhibition curves of *G. duodenalis* were calculated using Prism 5 software (GraphPad). $IC50_{BSH56} = 0.018 \pm 0.002$ µg/ml; $IC50_{BSH47} = 0.030 \pm 0.003$ µg/ml.

rBSH47 or rBSH56, respectively, were sufficient to kill 100% of trophozoites in 22 h. Co-cultures of trophozoites and rBSHs in a growth medium without bile supplementation did not exhibit any toxic effects, further supporting the fact that the anti-giardial effect is mediated by BSH activity and requires the presence of an appropriate substrate (bile).

To better characterize the damages induced by BSHs activity, the morphology of *G. duodenalis* trophozoites WB6 was analyzed by SEM after 16 h of treatment with either rBSH56 (0.08 µg/ml), rBSH47 (0.5 µg/ml), or deoxycholic acid (DCA, 0.1 and 0.2 g/L), which is a major product of bile hydrolysis. SEM analysis of non-treated trophozoites showed the characteristic giardial tear-drop shape with no apparent sign of morphological alteration (**Figures 5a,b**). Trophozoites treated with either DCA, or rBSH56 or rBSH47 in presence of bile revealed significant structural damage when compared to controls (**Figures 5c–h**). BSH-treated parasites displayed several alterations such as protrusions and perforations at the surface of their plasma membrane (**Figures 5d,f–h**). In DCA-treated trophozoites, membrane and median body were dramatically disrupted (**Figures 5g,h**). In contrast, with both treatments, the ventral disk microtubule array was still observable.

Assessment of *in Vivo* Anti-giardial Activities of *L. johnsonii* La1 BSH47

Numerous studies have reported that bile acids conjugated to taurine are predominant in mice (Claus et al., 2011). Recombinant BSH47 efficiently hydrolyzed tauro-conjugated bile acids, and its efficacy against *Giardia* has been demonstrated *in vitro*. Since this enzyme was available in larger quantities compared to rBSH56 and rBSH12, rBSH47 was selected to evaluate the potential of rBSH to treat giardiasis in a murine model (**Figure 6A**). OF1 suckling mice were divided into four groups ($n = 7$–12). Mice were challenged with *G. duodenalis* WB6 trophozoites (1×10^5) at day 10 by intragastric gavage. Increasing doses of rBSH47 corresponding to 0.5, 5, and 50 µg (50 µl, diluted in $NaHCO_3$ 16.4%) were thawed and daily administered by intragastric gavage to neonatal mice from day 10 to 15. The control group received vehicle (PBS + $NaHCO_3$ 16.4%). Animals were sacrificed at day 16, corresponding to the peak of trophozoite colonization, and small intestinal contents were sampled and analyzed. Six days after inoculation, trophozoites were able to efficiently colonize and persist in the small intestine with a parasite load 20-fold higher than the inoculum (**Figure 6B**). In groups treated with rBSH47, the parasite burden decreased in a dose-dependent manner (**Figure 6B**). Interestingly, the highest dose of rBSH47 (50 µg daily for 5 days) induced a significant reduction of 68.8% of *G. duodenalis* trophozoites compared to the control group.

DISCUSSION

L. johnsonii La1 is a probiotic strain with pathogen inhibition and host immunomodulation properties (Vidal et al., 2002; Cruchet et al., 2003; Pridmore et al., 2008). The activity of BSH and lactobacilli's bile resistance have been widely accepted as key factors for gut persistence and colonization by these bacteria (Tannock et al., 1994; Tanaka et al., 2000; Begley et al., 2006). Three *bsh* and two bile acid transporters genes were identified in the genome of *L. johnsonii* La1 (Pridmore et al., 2004). In this study, we cloned, purified, and characterized these 3 BSH enzymes in order to assess their antiprotozoal effect on *Giardia*. Nucleotide homology comparisons previously highlighted the similarities between *L. johnsonii* La1 *bsh*12 and *bsh*56 with *cbsH*α and *cbsH*β from *L. johnsonii* 100-100, respectively (Elkins and Savage, 1998; Elkins et al., 2001; Pridmore et al., 2004). A

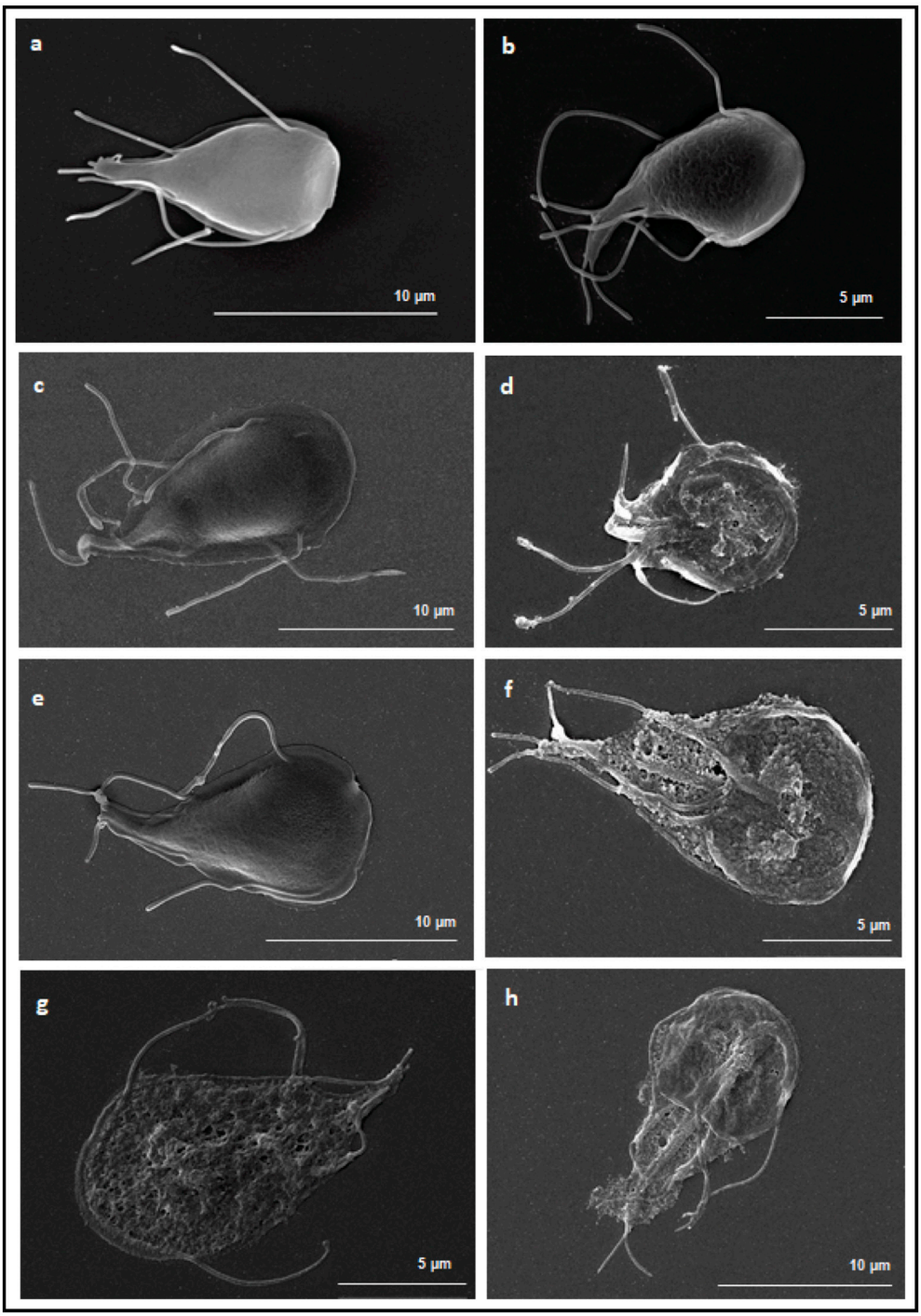

FIGURE 5 | Morphological alterations following *in vitro* treatments of *G. duodenalis* by BSH or deoxycholic acid (DCA). Scanning electron microscopy of *G. duodenalis* trophozoites WB6 strain treated with either BSH47 (0.5 µg/ml), BSH56 (0.08 µg/ml) or DCA (0.1 and 0.2 g/L). **(a)** KM control (KM+ 10% FCS) and **(b)** KM control with bile (bovine bile 0.6 g/l) show the characteristic pear-shaped of trophozoites. **(c)** *G. duodenalis* treated with rBSH47and **(d)** *G. duodenalis* treated with rBSH47 with bile reveal altered morphology and plasma membrane disruption in presence of bile. **(e)** *G. duodenalis* treated with rBSH56 and **(f)** *G. duodenalis* treated with rBSH56 with bile showed similar cell lysis. **(g,h)** DCA-treated (0.1 and 0.2 g/l, respectively) *Giardia* present similar alterations and a disruption of plasma membrane exposing cell interior. Scale bar = 5 µm **(b,d,f,g)** or 10 µm **(a,c,e,h)**.

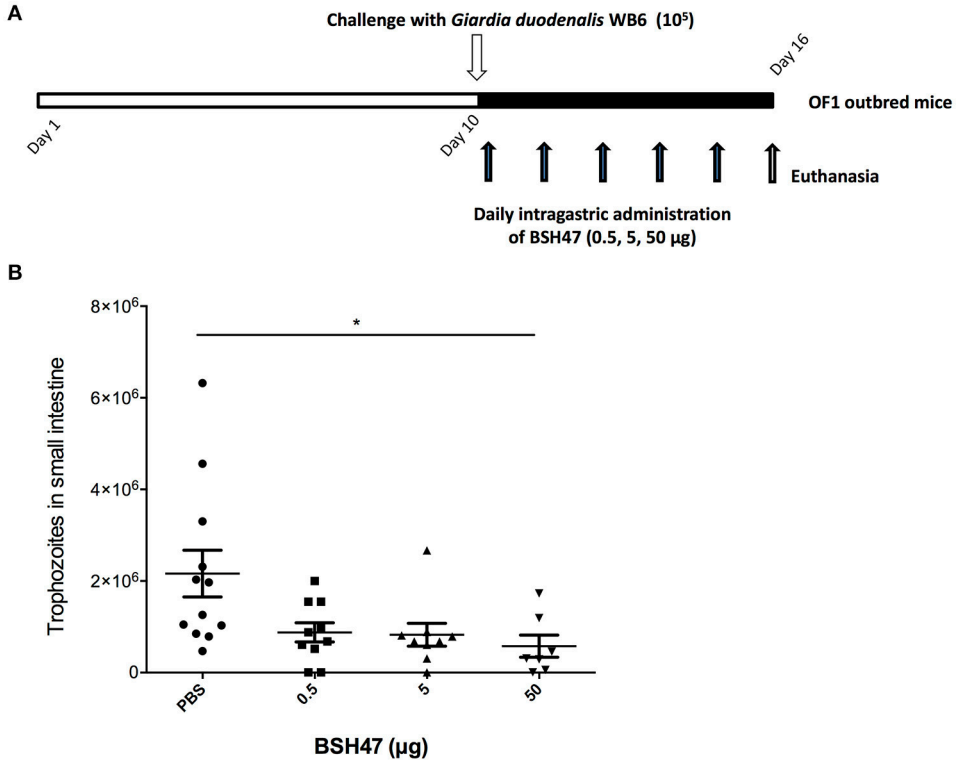

FIGURE 6 | Anti-giardial activity of rBSH47 *in vivo*. **(A)** Experimental design of *G. duodenalis* infection in OF1 suckling mice model. Increasing doses of rBSH47 were daily administered by intragastric gavage (0.5, 5, 50 µg) to OF1 suckling mice from day 10 to 15. Control animals received PBS+ $NaHCO_3$ 16.4% (vehicle). Mice were challenged with *G. duodenalis* WB6 trophozoites (10^5) at day 10 by intragastric gavage. Animals were euthanized by cervical dislocation at day 16. **(B)** *G. duodenalis* trophozoites enumeration in small intestine after rBSH47-treatement. Group I (control): mice received vehicle ($n = 12$); Group II: mice received 0.5 µg of BSH47 daily for 5 days ($n = 10$); Group III: mice received 5 µg of BSH47 daily for 5 days ($n = 9$); mice received 50 µg of BSH47 daily for 5 days ($n = 7$). Small intestines were resuspended in PBS and trophozoites were counted using a hemocytometer. Values are mean ± SEM; $p < 0.0001$.

neighbor-joining tree of various BSHs protein sequences from several lactic acid bacteria confirmed that *L. johnsonii* La1-BSH enzymes are phylogenetically related to BSHs from other species. In addition, they share a high degree of similarity to various subgroups of BSHs; for instance, BSH12 shares 99% identity with *L. johnsonii* 100-100 cbsHα and *L. johnsonii* PF01 BSHA at amino acid level, and BSH56 shares 99% identity with *L. johnsonii* 100-100 cbsHβ and 98% with *L. johnsonii* PF01 BSHB at amino acid level. *L. johnsonii* La1 and *L. johnsonii* PF01 both possess a third BSH gene, *bsh*47 and *bsh*C, respectively, which is absent from *L. johnsonii* 100-100. Besides, the close relationship among *L. johnsonii* La1 BSH47, *L. johnsonii* PF01 BSHC, and *L. acidophilus* NCFM BSHB, at amino acid level, suggests that these enzymes likely share a common ancestor. These observations contribute to the idea that BSH might have been acquired through horizontal gene transfer from microorganisms sharing the same intestinal environment (Corzo and Gilliland, 1999; Franz et al., 2001; McAuliffe et al., 2005; Begley et al., 2006), although this latter hypothesis remains to be tested.

Multiple amino acid sequence alignment of *L. johnsonii* La1 BSHs indicated a high variability among characterized BSHs; however, well-conserved motifs were observed around residues involved in the active site (Cys-2, Arg-18, Asp-21, Tyr-82, Asn-172, and Arg-225). These observations are in agreement with previous studies (Rossocha et al., 2005; Fang et al., 2009; Lin et al., 2014) and highlight that the biological functions of BSHs are strictly conserved despite sequence/phenotypic variabilities. BSHs (EC 3.5.1.24) belong to N-terminal nucleophilic (Ntn) hydrolases, a superfamily of enzymes containing N-terminal cysteine residue involved in the catalytic site (Suresh et al., 1999; Kim et al., 2004). Penicillin V acylases (EC 3.5.1.11), which are closely related to BSH, also belong to Ntn hydrolases and share similar structures. *In silico* modeling, BSH47, BSH56, and BSH12 showed that the typical αββα tertiary structure arrangement, which is characteristic of Ntn superfamily, is conserved (Oinonen and Rouvinen, 2000; Patel et al., 2010; Lin et al., 2014). Taken together, these structural observations indicate that the folding of BSHs remained stable during evolution despite low sequence identity, which suggest a high evolutionary pressure to maintain their functionality (Chothia and Lesk, 1986; Sander and Schneider, 1991; Krieger et al., 2003).

Experimental determination of BSH activities showed that at least 2 of the 3 BSHs from *L. johnsonii* La1 have broad substrate specificities. The enzyme BSH56 exhibited high hydrolysis activities toward tauro- and glyco-conjugated bile salts, with a preference toward tauro-conjugated substrates.

Interestingly, BSH56 belongs to a phylogenetic cluster of BSH enzymes exhibiting activity exclusively directed toward tauro-conjugated bile acids. Similarly, BSH47 was more efficient at hydrolyzing tauro-conjugated bile salts and exhibited a very low relative activity toward glyco-conjugated bile salts. Surprisingly, the amino acid sequence of BSH47 is more closely related to that of *L. johnsonii* BSHC that has been reported to hydrolyze glyco-conjugated bile salts (Chae et al., 2013). Our results, therefore, contrast with previous studies reporting that most BSH enzymes isolated from lactobacilli are very efficient at hydrolyzing glyco-conjugated bile salts (Coleman and Hudson, 1995; Liong and Shah, 2005). In addition, these observations point out the limits of substrate predictions based on phylogenetic relationships considering whole enzyme sequences. The mechanisms responsible for substrate specificity of BSH enzymes are still unclear. Putative amino acids have been associated with substrate binding pockets in *C. perfringens* (Rossocha et al., 2005; Ridlon et al., 2006), but these residues are different in all 3 BSHs from *L. johnsonii*. Several studies emphasized that BSH preferably recognize conjugated substrates at amino acid moieties (Coleman and Hudson, 1995; Tanaka et al., 2000; Kim et al., 2004; Rossocha et al., 2005), whereas others still suggest that steroid moieties are recognized in priority (Moser and Savage, 2001; Begley et al., 2006; Patel et al., 2010) which could explain broad substrate specificities. Experimentally solving the 3D structures of rBSH47 and rBSH56 harboring appropriate substrates would certainly provide invaluable information regarding this important question.

The third BSH isolated from *L. johnsonii* La1, BSH12, was successfully expressed in *E. coli* and clearly observed in SDS-PAGE and by Western blotting, but no specific activity could be detected toward either substrate in liquid tests. However, a slight positive signal was observed with *E. coli* strain-expressing rBHS12 on agar plates supplemented with glyco-specific bile salts, suggesting a weak glyco-specific activity. Similar difficulties to express recombinant BSHs have been observed with BSHs from *L. plantarum* JPP2 (Ren et al., 2011) and BSH2 from *L. plantarum* WCFS1 (Lambert et al., 2008). Proteolytic degradation and misfolding of the recombinant protein produced in *E. coli* may have affected the enzyme's function (Baneyx and Mujacic, 2004). The functionality of BSH12 is, therefore, still under investigation.

As mentioned previously, the putative natural role of BSHs is to decrease the toxicity of conjugated bile salts for bacterial cells (De Smet et al., 1995). In this work, we assessed the anti-parasitic activity of recombinant BSHs and we demonstrated that rBSH47 and rBSH56 were highly active against viable trophozoites of *G. duodenalis* strains WB6 and NF. The minimum concentration found to kill 100% of *G. duodenalis* WB6 trophozoites *in vitro* was 1 μg/mL for both rBSH47 and rBSH56. When tested on human enterocyte Caco-2 cells, both rBSH47 and rBSH56 inhibited the growth of *G. duodenalis* NF in the presence of bile (0.6 g/L) in a dose-dependent fashion and prevented the attachment of the parasites to the cell monolayers (**Figure S3**). The rBSH56 was more effective than rBSH47 in killing both *G. duodenalis* WB6 and NF strains, although it induced more cell damages to Caco-2 cells at high concentrations (**Figure S3**). Recombinant BSH47 was, therefore, chosen to assess the *in vivo* effectiveness of BSH to treat *Giardia* infection in a suckling murine model. Moreover, given that bile acids are predominantly conjugated to taurine in mice (Claus et al., 2011), the ability of BSH47 to preferentially hydrolyze tauro-conjugated bile salts seemed more appropriate. We observed that rBSH47 inhibited *Giardia* growth in a dose-dependent manner *in vivo*, in keeping with the data obtained *in vitro*. At the highest dose (50 μg/mice/day) administered daily for 5 days, the parasite (trophozoite) burden was significantly reduced by 68.8% in the small intestine of the mice. Despite this important decrease of the parasite load, none of the treated mice were free of parasites at 16 days post-inoculation. The anti-giardial effects mediated by rBSH47 *in vivo* were, therefore, modest compared to their efficacy in the *in vitro* assays. This can be explained by a partial degradation of BSHs by acidic proteases and peptidases in the stomach. The activity of BSHs correlates with the amount of conjugated bile salts released in the duodenum, which is highly variable in neonates (Heubi et al., 2007). The production of deconjugated bile salts at inhibiting levels is, therefore, dependent to the bioavailability of BSH in the small intestine.

It has been reported that bile salts, and more specifically conjugated bile salts, have growth promoting effects on *G. duodenalis in vivo* (Halliday et al., 1988). Indeed, bile uptake contributes to cholesterol and exogenous phospholipids needs, which are essentials for parasite growth (Yichoy et al., 2011). So far, to our knowledge, there is no evidence showing that *Giardia* is able to deconjugate bile salts. Conjugated bile salts are, thus, directly consumed by *Giardia* without being detoxified (Farthing et al., 1985). In contrast, bacterial deconjugation aims at reducing the detergent properties of conjugated bile salts, which are more toxic for bacterial cells than secondary bile salts, i.e., cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA) (De Boever and Verstraete, 1999). It is likely that detoxification of bile salts by the duodenal microbiota has a collateral effect on parasite survival. Earlier work carried out in our lab showed that DCA and CDCA exerted cytotoxic effects on *G. duodenalis* trophozoites *in vitro* at non-micellar concentrations (Travers et al., 2016). Therefore, we investigated the morphological perturbations induced by DCA and BSHs on trophozoite cultures and we noticed both induced degenerations and perforations of the parasite plasma membrane. It has been established that DCA perturbs eukaryotic membranes structure by altering the membrane lipid microdomains (Jean-Louis et al., 2006). Furthermore, secondary bile salts induced a redistribution of cholesterol and decrease membrane fluidity. Hence, we hypothesized that secondary bile salt, and more specifically DCA, would kill *Giardia* trophozoites by damaging the cell structure. In the upper parts of the small intestine, where bile salt deconjugation occurs at high rate, the Gram-positive bacteria might be protected against secondary deconjugated bile salts by cell wall peptidoglycan.

A major side effect of BSH-based treatment would be a shift of bile salt balance in the gut. It has been reported in previous studies that an enhancement of BSH activity might impact host physiology by disturbing fat digestion and lipid metabolism (Begley et al., 2006; Lin et al., 2014). Moreover, secondary bile

acids resulting from the deconjugation of bile salts have also been linked to DNA damage in bacterial and host cells, colon cancer, and inflammation (Cheah and Bernstein, 1990; Moser and Savage, 2001; Bernstein et al., 2005). On the other hand, BSH activity is a natural process that plays a central role in the reduction of cholesterol (Jones et al., 2013).

The aim of this study was to evaluate the anti-giardial potential of BSHs against *G. duodenalis*. We expressed for the first time the BSHs isolated from the probiotic strain *L. johnsonii* La1 and showed that rBSH47 and rBSH56 exhibited high specific activity and broad substrate specificities. Antiprotozoal assays demonstrated that BSHs were highly effective against *G. duodenalis in vitro* and *in vivo* and represent a promising therapeutic strategy based on their natural catalytic activity. Future studies will determine whether such treatment approaches should be of short duration in order to avoid putative side effects related to the enhancement of bile salt deconjugation. Besides, this anti-giardial effect can be extended to any BSH activity as long as it efficiently converts conjugated bile salts into their deconjugated counterparts. However, further works are still needed to investigate the positive impact of such treatment on the pathophysiology of giardiasis, including protective effects on epithelial permeability, mucosal injury, and malfunction. Moreover, newer galenic formulations are needed in order to provide a better persistence of rBSHs *in vivo*, which can improve health outcomes in routine clinical and veterinary usages.

AUTHOR CONTRIBUTIONS

IF, PG, BP, TA, and LB-H conceived and designed the study. TA, IF, and LB-H produced and isolated the recombinant BSH, performed the biochemical characterizations with SC and the *in silico* analyses. TA, SC, AB, and IF performed the *Giardia* assays *in vitro* and SEM. TA, MT, and BP performed the *Giardia* assays in the suckling mice model. IV, PL, and PG discussed the experiments and results. TA, IF, and LB-H wrote the manuscript with contributions from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02707/full#supplementary-material

Figure S1 | Protein structure prediction of *L. johnsonii* La1 BSHs. Protein structures were modeled for *L. johnsonii* BSH56 **(a)** and BSH47 **(b)** using I-TASSER software (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). CBAH-1 (PDB: 2BJF) from *C. perfringens* (Rossocha et al., 2005) and BIBSH from *B. longum* (Kumar et al., 2006) (PDB: 2HF0) were used as templates for modeling BSH47 and BSH56, respectively. Best values of the C-score were chosen for model structure prediction. Visualization of predicted structures for BSH47 (red, **a**) and BSH56 (blue, **b**) and superimposition **(c,d)** were performed using Pymol software (https://www.pymol.org/).

Figure S2 | Protein structure prediction of *L. johnsonii* La1 BSH12**.** Protein structure was modeled for *L. johnsonii* BSH12 using I-TASSER software (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). Bile salt hydrolase (PDB: 2RF8) from *C. perfringens* was used as a template for modeling. Best value of the C-score was chosen for model structure prediction. Visualization of predicted structure was performed using Pymol software (https://www.pymol.org/).

Figure S3 | Anti-giardial effect of BSH in co-culture with Caco2 cells. To further investigate the effect of *L. johnsonii* La1-BSHs on the adherence of *Giardia* enterocytes, differentiated Caco-2 cells were co-incubated with fresh trophozoites cultures of *G. duodenalis* NF strain and treated with either rBSH47 or rBSH56, over a range of concentrations from 0.005 to 1 μg/ml. *G. duodenalis* NF strain trophozoites were inoculated to Caco2 cells at a multiplicity of infection (MOI) of 10:1 in the presence of increasing doses of rBSH47, rBSH56 or DMEM. Anti-giardial activity assays were performed with bovine bile (0.6 g/l) added to the medium. The results are expressed as relative percentage of growth compared to untreated trophozoites as negative control. Data are presented as mean ± SEM and correspond to triplicates. As expected from previous experiments done with the *G. duodenalis* WB6 strain, both BSHs triggered a growth inhibition of *G. duodenalis* NF trophozoites, in a dose-dependent fashion, after 20 h of exposure (this figure). In contrast, high concentrations of rBSH in assays induced cell damages on Caco-2 monolayer (data not shown). This phenomenon might be due to a putative cytotoxicity of deconjugated bile salt. Interestingly, neither rBSH47 nor rBSH56 displayed cytotoxic effects when tested in absence of bile (data not shown).

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Bile Salt Hydrolase Activities: A Novel Target to Screen Anti-*Giardia* Lactobacilli?

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Giardia duodenalis is a protozoan parasite responsible for giardiasis, a disease characterized by intestinal malabsorption, diarrhea and abdominal pain in a large number of mammal species. Giardiasis is one of the most common intestinal parasitic diseases in the world and thus a high veterinary, and public health concern. It is well-established that some probiotic bacteria may confer protection against this parasite *in vitro* and *in vivo* and we recently documented the implication of bile-salt hydrolase (BSH)-like activities from strain La1 of *Lactobacillus johnsonii* as mediators of these effects *in vitro*. We showed that these activities were able to generate deconjugated bile salts that were toxic to the parasite. In the present study, a wide collection of lactobacilli strains from different ecological origins was screened to assay their anti-giardial effects. Our results revealed that the anti-parasitic effects of some of the strains tested were well-correlated with the expression of BSH-like activities. The two most active strains *in vitro*, La1 and *Lactobacillus gasseri* CNCM I-4884, were then tested for their capacity to influence *G. duodenalis* infection in a suckling mice model. Strikingly, only *L. gasseri* CNCM I-4884 strain was able to significantly antagonize parasite growth with a dramatic reduction of the trophozoites load in the small intestine. Moreover, this strain also significantly reduced the fecal excretion of *Giardia* cysts after 5 days of treatment, which could contribute to blocking the transmission of the parasite, in contrast of La1 where no effect was observed. This study represents a step toward the development of new prophylactic strategies to combat *G. duodenalis* infection in both humans and animals.

Keywords: *Giardia duodenalis*, lactobacilli, *Lactobacillus johnsonii*, *Lactobacillus gasseri*, probiotics, bile salt hydrolases

INTRODUCTION

Giardia duodenalis (also known as *G. lamblia* or *G. intestinalis*) is the etiologic agent of the zoonotic disease giardiasis, one of the most common waterborne parasitic infections globally. *G. duodenalis* is a flagellate protozoan (Excavata, Diplomonad) that infects a broad diversity of animals such as humans, mammals, reptiles and birds (Thompson and Monis, 2004, 2012). Transmission can occur by ingestion of viable cysts from contaminated water and soil, or directly by contact with an infected animal's feces (Gardner and Hill, 2001). After ingestion,

exposure to gastric acid and proteases in the stomach leads to excystation and liberation of replicative trophozoite stages that adhere transiently to the proximal small intestine and persist for several days (for up to several months in some cases) (Reiner et al., 2003). After this replicative and pathogenic stage, trophozoites are carried into the colon and progressively encyst. Cysts, released through host feces, may then survive in the environment for several months and remain infectious at low doses (Thompson et al., 1993).

The main clinical symptoms of giardiasis are acute or chronic diarrhea, abdominal pain, intestinal malabsorption, steatorrhea, and weight loss (Farthing, 1996; Buret, 2007). Considered as a public health threat and a veterinary concern worldwide, giardiasis is responsible for many waterborne diarrhea outbreaks in developed countries (Roxstrom-Lindquist et al., 2006; Buret, 2007). In humans, it affects mainly children, and undernourished or immunosuppressed individuals. Even though giardiasis can be self-limited, standard antibiotic therapies including 5-nitroimidazole and benzimidazole drugs are needed to treat long-term infections (Savioli et al., 2006). In mammals, *G. duodenalis* infections are common in companion animals, small ruminants and cattle, for which alternative treatments are very limited (Harris et al., 2001).

The increasing number of clinical trial failures and the emergence of resistant *Giardia* strains in both medical and veterinary applications have encouraged the development of new therapeutic strategies (Harris et al., 2001; Upcroft and Upcroft, 2001). In this context, it has been established that gut microbiota plays a pivotal role in the protection against enteropathogens through the production of antimicrobial compounds and by competition for nutrients and attachment to the mucosal surface (Travers et al., 2011; Bengmark, 2013). In particular, probiotic bacteria such as some strains of lactobacilli have been shown to confer host health benefits by enhancing innate and adaptive immune responses (Sanders, 2008; Martin et al., 2013; Sokol, 2014). Moreover, pre-clinical studies suggest that colonization of the small intestine by *G. duodenalis* trophozoites depends on the composition of host gut microbiota (Singer and Nash, 2000; Barash et al., 2017; Bartelt et al., 2017). In recent years, probiotic-based therapies have been explored to treat giardiasis (Allain et al., 2017). For instance, some lactobacilli strains such *Lactobacillus johnsonii* La1 (also known as NCC533 but hereafter named La1), *Lactobacillus casei* MTCC1423 and *Lactobacillus rhamnosus* GG (LGG), have been shown to display anti-giardial properties by (i) antagonizing the proliferation of trophozoites and, (ii) reducing the severity of infection in several murine models (Humen et al., 2005; Shukla et al., 2008; Goyal et al., 2011; Travers et al., 2011). While the molecular mechanisms remain poorly understood, we have recently discovered the involvement of deconjugated bile salts, generated by the hydrolysis of conjugated bile salts from bile by La1 strain, as one of the mechanisms contributing to the inhibition of *Giardia* trophozoite growth *in vitro* (Perez et al., 2001; Travers et al., 2016). Our hypothesis was that these deconjugated bile salts were produced by bile salt hydrolase (BSH)-like enzymes released or secreted by this strain (Travers et al., 2016).

In the current study, we screened a wide collection of lactobacilli strains from various environmental origins in order to determine their ability to display an anti-giardial effect. *In vitro* analysis from supernatant-lactobacilli samples led to the identification of several strains having a strong inhibitory activity against *G. duodenalis* growth (up to the level previously reported for La1). In parallel, we showed that the *in vitro* anti-*Giardia* activity displayed by some of these lactobacilli strains is clearly correlated with their BSH-like activities *in vitro*. The two strains displaying the strongest inhibitory effects: La1 and *Lactobacillus gasseri* CNCM I-4884, were then evaluated *in vivo* in a suckling mice model challenged with the WB6 strain of *G. duodenalis*. Our results showed that *L. gasseri* CNCM I-4884 displayed a higher anti-giardial effect *in vivo* than La1, with an almost complete clearance of *Giardia* infection in suckling mice. These findings show that a screening based on the detection of BSH activities is a promising tool to identify new anti-*Giardia* lactobacilli strains. Overall, our study opens new therapeutic strategies for both preventing and treating giardiasis in humans.

MATERIALS AND METHODS

Giardia duodenalis Culture Conditions

Giardia duodenalis was grown *in vitro* as recently described (Travers et al., 2016). Trophozoites of *G. duodenalis* strain WB clone 6 (WB6) assemblage A1 (ATCC 50803) were grown in Keiser's modified TYI-S-33 medium (KM) (Morrison et al., 2007). KM medium was supplemented with 10% heat-inactivated fetal calf serum (FCS, reference A15-101, PAA Laboratories, GE healthcare), adjusted to pH 6.0, and sterilized with a 0.22 μm filter. *G. duodenalis* WB6 trophozoites were subcultured in anaerobic conditions at 5×10^4 cells per ml after chilling on ice for 10 min and centrifuged at 700 × *g*, 5 min. Aliquots were frozen in liquid nitrogen and stored at −80°C until further use. For *in vitro* and *in vivo* experiments, 48 h old cultures of confluent trophozoites were pelleted at 700 × *g*, 5 min after chilling on ice for 10 min, and resuspended at the needed concentrations.

Bacterial Strains and Culture Conditions

The bacteria collection used in this study comprises 29 lactobacilli isolated from different biotopes (**Table 1**). The strains were grown in Man Rogosa Sharpe (MRS, Difco) medium on agar plates over night at 37°C in anaerobic conditions (GasPack Plus, BBL). Bacteria were then subcultured for 16 h at 37°C in MRS broth and were subsequently grown in Keiser's modified TYI-S-33 medium supplemented with 10% heat-inactivated FCS (see above) to stationary phase. An intermediary subculture in modified TYI-S-33 medium was used for some strains as previously described (Perez et al., 2001). Bacterial supernatants were then collected after centrifugation at 10,000 × *g* for 10 min, sterilized with a 0.22 μm filter and the pH was adjusted to 6.2 with 5N NaOH.

Bile Salt Hydrolase Assays

The bacterial strains were tested for Tauro-deoxycholic acid (TDCA) and Glyco-deoxycholic acid (GDCA) hydrolase activities on MRS-agar plates supplemented with either 0.5%

TABLE 1 | List of tested lactobacilli strains with their characteristics and bile salt hydrolase activity specificities.

Lactobacillus species	Strain[1]	Origin	TDCA (0.5%) hydrolase activity	TDCA hydrolase activity score[2]	GDCA (0.5%) hydrolase activity	GDCA hydrolase activity score[2]
L. acidophilus	ATCC4356	Feces, human	P	+	N	–
L. brevis	CNRZ1845	Malt (beer)	P	+	P	+
L. casei	ATCC393	Cheese	N	–	N	–
L. crispatus	CIP103606	Unknown	N	–	P	++
L. curvatus	CNRZ1335	Fermented sausage/mea	N	–	N	–
L. gasseri	ATCC33323	Vaginal tract, human	N	–	N	–
L. gasseri	CNCM I-4884 ATCC29601	Cariouth tooth, human	P	+++	P	++
L. helveticus	CNRZ1109	Lactic starter	N	–	N	–
L. helveticus	ATCC11977	Lactic starter (cheese)	N	–	P	++
L. johnsonii	CNRZ217	Feces, rat	N	–	P	+
L. johnsonii	CNRZ218	Feces, rat	P	+	P	++
L. johnsonii	CNRZ1897	Fermented milk	P	+++	ND	ND
L. johnsonii	CIP103614	Vaginal tract, human	P	++	N	–
L. johnsonii	CIP103786	Cheese	P	+	P	++
L. johnsonii	CIP103652	Unknown	P	++	N	–
L. johnsonii	CIP103653	Unknown	P	++	N	–
L. johnsonii	CIP103654	Pharmaceutical preparation	P	+	P	+
L. johnsonii	CIP103781	Unknown	P	+++	P	++
L. johnsonii	CIP103782	Urethran, human	P	+	N	–
L. johnsonii	ATCC33200	Blood, human	P	++	P	++
L. johnsonii	La1 (NCC533)	Feces, human	P	+++	P	++
L. paracasei	CNRZ315	Unknown	N	–	N	–
L. pentosus	CNRZ1218	Cheese	N	–	N	–
L. plantarum	CNRZ738	Silage	N	–	P	+
L. rhamnosus	CNRZ317	Unknown	N	–	N	–
L. rhamnosus	CNRZ2084	Fermented milk	N	–	N	–
L. reuteri	CNRZ431	Sourdough	N	–	N	–
L. sakei	CNRZ1332	Moto, starter of sake	N	–	N	–
L. zeae	CNRZ2269	Corn steep liquor	N	–	N	–

P, positive; N, negative; ND, non-determined. [1]Strains were obtained from different collections: CIP, Collection de l'Institut Pasteur, France; CNRZ/CIRM, Centre International de Ressources Microbiennes; CNCM, Collection Nationale de Cultures de Microorganismes, Institut Pasteur, France; ATCC, American Type Culture Collection, United States. [2]TDCA/GDCA hydrolase activity score were determined on the size of the halo zone (diameter): (–): no detected halo; (+): <8 mm; (++): 9 mm to 12 mm; (+++): >13 mm.

TDCA (Sigma–Aldrich) or 0.5% GDCA (Merck) following the protocol described by Moser and Savage (2001). MRS plates with 0.5% TDCA were first incubated at 37°C in anaerobic conditions for 24 h prior to inoculation. Strains were grown anaerobically for 16 h at 37°C in MRS broth, then streaked on MRS-agar plates, supplemented or not, with 0.5% TDCA or 0.5% GDCA and incubated at 37°C in anaerobic conditions for 48–72 h. BSH activity can be easily detected when unconjugated deoxycholic acid precipitates in the MRS-agar plate, forming an iridescent halo below and around active colonies. These assays were performed in duplicates, and included two biological replicates. BSH activity score was determined as indicated in **Table 1**.

In Vitro Anti-giardial Assays

Filtered bacterial supernatants (500 µl) were co-incubated with fresh cultures of *Giardia* trophozoites (1.33×10^5 parasites/ml in KM medium, pH 6.0, supplemented with 10% heat-inactivated FCS) at a volumetric ratio of 1–3, in the presence or absence of bovine bile (0.6 g/L) at 37°C in anaerobic conditions for 22 h. BSH from *Clostridium perfringens* (1 U) (reference C4018, Sigma–Aldrich) was used as a positive control for *Giardia* growth inhibition as previously described (Travers et al., 2016). Samples were then ice-chilled for 10 min and trophozoite load was determined using hemocytometer (flagella mobility was used to screen parasite viability). The inhibition rates were determined by counting the number of living trophozoites and establishing ratios compared to the controls (in percentage). Experiments were conducted in duplicates and included three biological replicates.

Assessment of Anti-giardial Activity *in Vivo*

Cultures of *G. duodenalis* trophozoites WB6 were grown in Keister's modified TYI-S-33 medium with 10% heat-inactivated FCS. Lactobacilli strains (La1, *L. gasseri* CNCM I-4884 and *L. curvatus* CNRZ1335) were cultured in MRS broth for 16 h

at 37°C in anaerobic conditions from precultures. Bacteria were then harvested by centrifugation at 7000 × *g* for 15 min, washed two times and resuspended in a corresponding volume of sterile PBS/Glycerol 15% to obtain a final concentration of 2.5×10^{10} CFU/ml for intragastric administrations (5×10^8 CFU per neonatal mouse). Mice used for reproduction were non-inbred mice of the OF1 strain (Charles River, Saint-Germain-Nuelles, France). All experiments were conducted in a filtered air chamber and manipulations were performed under a laminar flow hood to prevent contamination of the environment by cysts of *G. duodenalis* and other pathogens.

Lactobacilli strains were administered daily by intragastric gavage in a volume of 20 µl (5×10^8 CFU) to 5 days old neonatal mice from day 5 to day 15 (n = 9–12 per group) (**Figure 2**). Control animals (n = 8) received PBS with glycerol 15% instead of bacterial suspensions. Neonatal mice were then challenged with trophozoites at day 10 by intragastric gavage in a volume of 100 µl (10^5 trophozoites). Mice were sacrificed by cervical dislocation at day 16. The small intestine and colon contents were examined for the presence of trophozoites and cysts, by counting the parasite burden, using a hemocytometer. The counting of trophozoites was performed from the small intestine (resuspended in 5 ml of ice-chilled sterile PBS), while cysts were enumerated in the colon and caecum (resuspended in 5 ml of ice-chilled 2.5% Potassium dichromate). The minimum threshold of counting values for statistical analysis was set at 10^3 trophozoites.

Statistical Analysis

Results were expressed as means ± standard error of the mean (SEM). Comparison between groups was assessed by one or two-way analysis of variance (ANOVA), *t*-test, Mann–Whitney and Kruskal–Wallis. Correlation tests were performed using Spearman's Rank Correlation Test. Statistical significance was calculated at *p*-values under 0.05 at 95% confidence interval.

Ethics Statement

All protocols were carried out in accordance with the institutional ethical guidelines of the ethics committee ANSES's Animal Health Laboratory at Maisons-Alfort on the campus of the French National Veterinary School of Alfort (ENVA), which approved this study.

RESULTS

BSH Activities of Lactobacilli Strains

The 29 strains tested in this study were able to grow in MRS-agar supplemented with 0.5% TDCA whereas only 25 grew in MRS-agar supplemented with 0.5% GDCA, supporting that GDCA has a higher bactericidal activity than TDCA *in vivo* (Begley et al., 2006). TDCA/GDCA hydrolase activity is a well-recognized indicator of BSH activity in bacteria (Bustos et al., 2012). A semi-quantitative method was used to monitor the BSH activity by measuring the halo zone of positive strains after 48–72 h of incubation. Among the 29 strains, 6 exhibited TDCA hydrolase activity, 4 exhibited GDCA hydrolase activity and 8 displayed both TDCA and GDCA hydrolase activities (**Table 1**). No halo was detected for 11 strains. Interestingly, all *L. johnsonii* strains tested displayed at least one type of BSH activity. Among the strains harboring both tauro and glyco-specific BSH activities, La1, *L. gasseri* CNCM I-4884 and *L. johnsonii* CIP103782 exhibited the highest BSH scores (**Table 1** and **Figure 1A**).

Characterization of Anti-giardial Lactobacilli Strains

We screened the 29 lactobacilli strains for their inhibitory abilities against *G. duodenalis*. For this, bacterial supernatants were co-incubated for 22 h at 37°C with *Giardia* trophozoite cultures in KM growth medium supplemented, or not, with bovine bile (0.6 g/L). Living trophozoites were enumerated using hemocytometer. Parasite cultures that were grown without bile did not display major differences when compared to trophozoites grown in KM supplemented with bile, showing that cholesterol and lipids from fetal bovine serum are sufficient to fulfill the lipid uptake requirements of *Giardia* and that bile components are not toxic for *Giardia* (Travers et al., 2016).

When co-incubated with *Giardia* in KM supplemented with bovine bile, 19 bacterial supernatants exhibited significant antagonistic effects on *Giardia* growth, with a wide range of inhibition levels (**Figure 1A**). Six strains showed growth inhibition levels from 15 to 30%, 10 exhibited growth inhibition levels from 30 to 70%, and 12 strains displayed a strong reduction of trophozoites by 70–100% ($p < 0.001$). Only supernatant of *L. gasseri* CNCM I-4884 were as efficient as that of La1 to antagonize *Giardia* growth *in vitro* (100%). Using Spearman's Rank Correlation Test, we observed a positive correlation between inhibition levels and BSH activity score ($r = 0.86$; $p < 0.0001$) of bacterial strains (**Figure 1B**). Inhibition assays were then divided into two groups: (i) BSH negative (strains with no detected BSH activity) and (ii) BSH positive (strains displaying either TDCA, GDCA, or both BSH activities). We observed that *Giardia* growth inhibitory activity of BSH positive strains were twofold higher relative to BSH negative strains (Student's *t*-test; $p < 0.0001$) (**Figure 1C**). Moreover, the cytotoxic effect on *Giardia* was lost when trophozoites were co-incubated with bacterial supernatant in the absence of bile (**Figure 1D**). However, a very weak inhibition was still observed for a majority of the strains (range between 5 and 15%), which can be explained by the presence of other extracellular compounds released by lactobacilli (e.g., bacteriocins) that are also potentially deleterious to *Giardia* growth. Taken together, these *in vitro* results strongly suggest that the anti-giardial effect displayed by lactobacilli strains tested in this study is mostly bile-dependent.

In Vivo Effect of Orally Administered Lactobacilli against *G. duodenalis*

In vivo experiments were performed in order to assess the potential of newly identified anti-giardial lactobacilli strains to antagonize *G. duodenalis in vivo*. Bacterial suspensions, or PBS/glycerol were daily administered by intragastric gavage (5×10^8 CFU) to neonatal mice from day 5 to day 15 (**Figure 2**). The persistence of lactobacilli in suckling mice and administration route were determined in previous

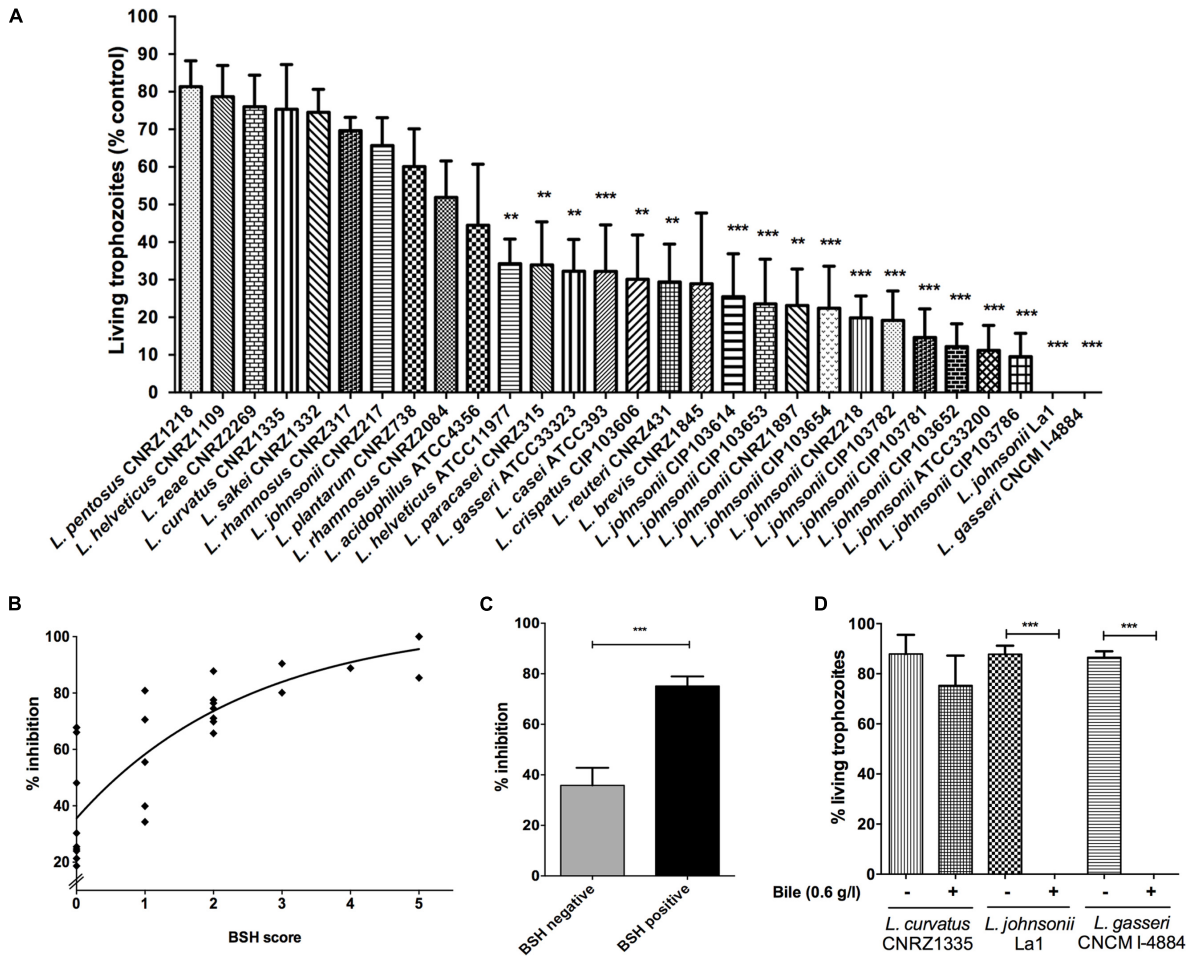

FIGURE 1 | Bile-salt hydrolase (BSH) and anti-*Giardia in vitro* activities of different lactobacilli strains. **(A)** Percentage of living *Giardia duodenalis* trophozoites when cultivated 22 h with lactobacilli supernatants. *G. duodenalis* trophozoites were enumerated after 22 h of co-incubation at 37°C in anaerobic conditions (values are represented with bile supplementation). Values are mean ± SEM. **(B)** Spearman's rank correlation test between percentage of inhibition of lactobacilli strains and their BSH activity score. BSH score was determined depending on the size of the halo zone (+ = 1; ++ = 2; +++ = 3, **Table 1**) and the ability to deconjugate either tauro-conjugated bile salts or glyco-conjugated bile salts or both (addition of BSH score for each substrate specificity). A positive correlation is observed ($r = 0.86$; $p < 0.0001$). **(C)** Inhibition assays according to Bile Salt Hydrolase activities. Lactobacilli strains were divided into two groups: (i) BSH negative (strains with no detected BSH activity), (ii) BSH positive (strains exhibiting either TDCA, GDCA, or both BSH activities). *G. duodenalis* trophozoites were enumerated after 22 h of co-incubation at 37°C in anaerobic conditions (values are represented with bile supplementation). Values are in mean ± SEM. **(D)** Percentage of living *G. duodenalis* trophozoites when co-cultivated 22 h with lactobacilli strains supernatants, with or without bile supplementation (bovine bile 0.6 g/L). *G. duodenalis* trophozoites were enumerated after 22 h of co-incubation at 37°C in anaerobic conditions. Values are mean ± SEM. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

experiments (**Supplementary Figure S1**). Mice were challenged with *G. duodenalis* WB6 trophozoites at day 10 by intragastric gavage (1×10^5 trophozoites) and sacrificed at day 16 (**Figure 2**). In preliminary experiments, we showed that this parasite strain was able to persist and colonize in OF1 suckling mice model, the peak of trophozoite load being reached at day 16 (i.e., 6 days post-inoculation) (**Supplementary Figure S2**). On the other hand, we demonstrated that lactobacilli strains can persist in the gut up to 3–4 days after intragastric gavage.

Mice were divided into four groups with a minimum of eight animals per group. The parasite burden in mice treated with PBS/glycerol was 20-fold higher than the initial dose showing that trophozoites were able to colonize, multiply, and persist in the small intestine. As shown in **Figure 3A**, mice treated with *L. curvatus* CNRZ1335 were not protected against *G. duodenalis* challenge as they present a similar profile in trophozoite colonization and proliferation. In contrast, mice treated with *L. gasseri* CNCM I-4884 exhibited an 18-fold reduction in the trophozoites load in the small intestine (∼93%) ($p < 0.001$) compared to the PBS/glycerol group (**Figure 3A**). Mice fed with the probiotic strain La1 exhibited a twofold reduction in the trophozoites load compared to controls (∼43%). Interestingly, *L. gasseri* CNCM I-4884 was more efficient than La1 in preventing *G. duodenalis* proliferation *in vivo*.

Cysts enumerations were performed in both colon and caecum. We observed a significant reduction (−81%) ($p < 0.01$) in cyst formation in groups treated with *L. gasseri* CNCM I-4884 compared to both PBS/glycerol and *L. curvatus*-treated groups.

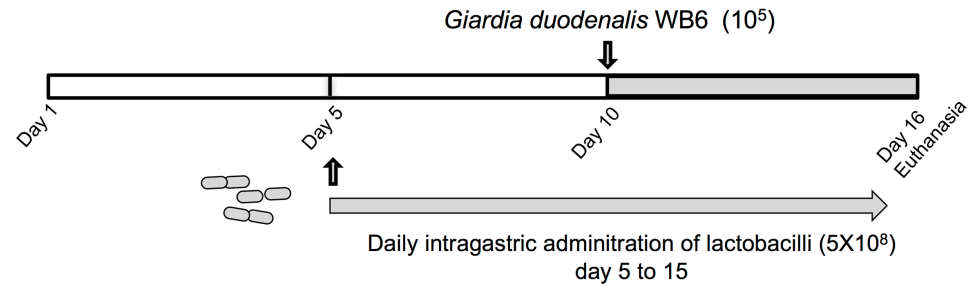

FIGURE 2 | Experimental design of *G. duodenalis* infection in OF1 suckling mice model. Lactobacilli strains were administered daily by intragastric gavage (5×10^8 CFU) to 5 days old OF1 suckling mice from day 5 to day 15. Control animals received PBS/glycerol 15%. Mice were challenged with *G. duodenalis* WB6 trophozoites (10^5) at day 10 by intragastric gavage. Mice were sacrificed by cervical dislocation at day 16.

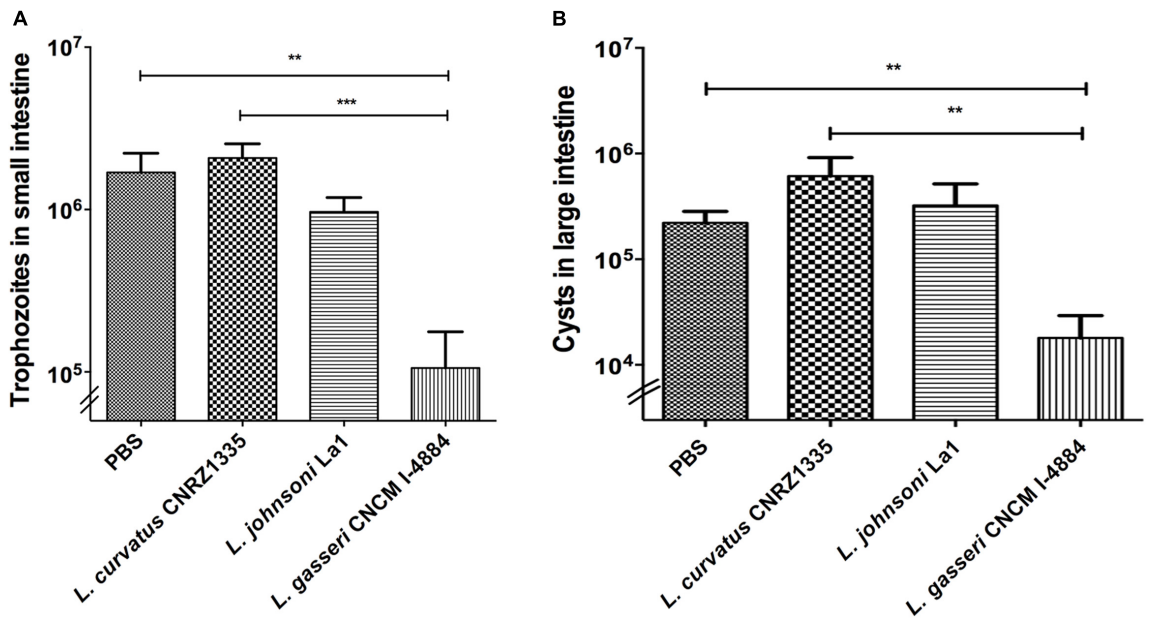

FIGURE 3 | *In vivo* activities of *Lactobacillus gasseri* CNCM I-4884 and La1 lactobacilli strains against *G. duodenalis*. **(A)** *G. duodenalis* trophozoites enumeration after administration to animals. Suckling mice received either PBS ($n = 8$), La1 ($n = 12$), *L. gasseri* CNCM I-4884 ($n = 10$) or *L. curvatus* CNRZ1335 ($n = 9$) by intragastric gavage (5×10^8 CFU/mice) daily from day 5, before inoculation with *G. duodenalis* WB6 trophozoites (10^5 trophozoites per animal) at day 10. Gavages were performed until day 15. Small intestines were resuspended in PBS and trophozoites were counted using a hemocytometer. Values are mean ± SEM; $p < 0.05$. **(B)** *G. duodenalis* cysts counting in colon and caecum. Suckling mice received either PBS/glycerol 15%, La1, *L. gasseri* CNCM I-4884 or *L. curvatus* CNRZ1335 by intragastric gavage (5×10^8 CFU/mice) daily from day 5 before inoculation with *G. duodenalis* WB6 trophozoites (10^5 trophozoites per animal) at day 10. Gavages were performed until day 15 ($n = 8$–12/group). Colons and caeca were resuspended in 5 ml of ice-chilled 2.5% potassium dichromate and cysts were counted using a hemocytometer. Values are mean ± SEM. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

No significant reduction was observed in the group treated with either La1 or *L. curvatus* CNRZ1335 compared to control groups treated with PBS/glycerol (**Figure 3B**).

DISCUSSION

Several studies have demonstrated that probiotics might play key roles against enteropathogens including intestinal prototozan parasites (see Travers et al., 2011, for a review). In the last 10 years, several lactobacilli strains have been studied for their ability to prevent the establishment of *G. duodenalis in vivo* and to reduce the severity of giardial infections (Humen et al., 2005; Shukla et al., 2008; Goyal et al., 2011; Goyal and Shukla, 2013). In some instances, *in vitro* assays were designed to decipher the molecular mechanisms involved in these protective effects. For the probiotic strain La1 of *L. johnsonii,* previously known to antagonize *Giardia* growth *in vitro* and *in vivo,* Perez and collaborators established the presence of an active principle in the supernatant of this probiotic strain (Perez et al., 2001; Humen et al., 2005). More recently, we undertook the molecular characterization of this active principal from La1 and discovered at least one possible mechanism of action: the involvement of bacterial BSH-like activities, that would mediate anti-giardial effect by generating

deconjugated bile salts (toxic for the parasite) from non-toxic conjugated bile salts (Travers et al., 2016). BSH are common in *Lactobacillus* and *Bifidobacterium,* playing an important role for colonization and persistence in the gut (Tanaka et al., 1999; Begley et al., 2006; Denou et al., 2008). Therefore, we looked for other probiotic strains (closely related or not to La1) exhibiting anti-giardial activities. We thus selected 29 lactobacilli strains from diverse origins and screened their supernatants for their putative anti-giardial activities *in vitro* while, in parallel, we analyzed their BSH activities and properties. Among the supernatants issued from the 29 analyzed strains, 19 were found to display anti-parasitic activities on fresh trophozoites *Giardia* cultures. Interestingly, these 19 strains also displayed the highest BSH scores, indicating a correlation between these two properties. These results thereby suggest that a screening method based on BSH activities may predict potential anti-giardial activity in lactobacilli. Interestingly, *L. gasseri* CNCM I-4884 and La1 strains (which killed 100% of trophozoites *in vitro*) displayed both TDCA and GDCA specific BSH activities. We therefore propose that this dual or combined BSH activity would contribute to reinforce the release of deconjugated bile salt toxic for *Giardia,* from a larger panel of substrates. However, no clear relationships appeared between the observed anti-giardial activity and the environmental origins of the lactobacilli isolates (**Table 1**).

Lactobacillus gasseri CNCM I-4884 and La1 were subsequently administrated to OF1 suckling mice to assess their antagonistic effects *in vivo*. *L. curvatus* CNRZ1335, which displayed neither anti-giardial activity nor BSH activities *in vitro*, was used as a negative control. High trophozoites burden in controls showed efficient colonization and proliferation of the *G. duodenalis* WB6 in the OF1 mice (Shukla et al., 2008). Mice treated with *L. gasseri* CNCM I-4884 exhibited a dramatic reduction of viable trophozoites in their small intestines, 6 days post-challenge. La1 strain supplementation also lead to a reduction of trophozoites in mice intestines, but to a much lesser extent than *L. gasseri* CNCM I-4884. Previous studies have reported anti-giardial properties of lactobacilli strains. For instance, *L. casei* MTCC1423 and LGG strains reduced the duration and the severity of *G. duodenalis* infection (Portland strain) in C57BL/6 mice in 7–14 days (Shukla et al., 2008; Goyal et al., 2011) and La1 antagonized *Giardia* (WB6) in Mongolian gerbils in 7–21 days (Humen et al., 2005). However, no correlation between the anti-giardial effects and a BSH-like activity was established in these studies. Strikingly, in the current study, administration of *L. gasseri* CNCM I-4884 led to a stronger reduction of trophozoites load in a short period of time. Cyst formation is another important indication of the control of infectious parasite development (Lujan et al., 1997). A treatment capable of preventing trophozoite differentiation into cysts is indeed essential to minimize their spreading in the environment via animals' feces. We observed that mice fed with *L. gasseri* CNCM I-4884 exhibited a dramatic reduction of cyst excretion 6 days post-challenge compared to untreated infected mice. This is in concordance with previous results showing that other lactobacilli such as *L. casei* MTCC 1423 and LGG were effective in eliminating cysts after 7 days of treatment. In contrast, no cyst reduction was observed in mice treated with La1 although its ability to eliminate cysts has been previously reported in mongolian gerbils (Humen et al., 2005). The differences in terms of *in vivo* efficiency between *L. gasseri* CNCM I-4884 and La1, both BSH-positive strains, can be explained by either a better hydrolysis of conjugated bile salts *in vivo*, a higher ability for competing for biological niches in the small intestine, and a better persistence in the gut.

To date, the underlying mechanisms involved in the antagonistic effect of lactobacilli against *Giardia* is an emerging field. Besides the role of BSH-like activities that we have previously reported for La1 (Travers et al., 2016), and now for other *Lactobacillus* strains, other mechanisms have been identified. Among them, the role of anti-microbial peptides in the anti-giardial activity of lactobacilli has been explored. Indeed, when administered orally, P106, a derived bacteriocin isolated from *L. acidophilus*, was shown to reduce the trophozoite burden in mice at high concentrations (Amer et al., 2014). However, the ability to produce anti-microbial peptides is strain dependent and highly variable and this cannot be extrapolated to all anti-giardial strains. Also, probiotic lactobacilli are known to enhance the mucosal immune system, which participate in clearing enteropathogens from the gut (Howarth and Wang, 2013). The immunomodulatory properties of lactobacilli have also been proposed to explain their anti-giardial properties (Goyal and Shukla, 2013; Allain et al., 2017; Barash et al., 2017; Bartelt et al., 2017; Fink and Singer, 2017). In our study, the local and systemic response to *Giardia* infection has not been assessed due to the fact that suckling mice have an immature immune system (Basha et al., 2014).

While the anti-giardial properties of lactobacilli appear to be multifactorial, there is a clear contribution of BSH-activities. However, further experiments are necessary to investigate the potential of BSH in treating giardiasis, including the direct effect of BSHs *in vitro* and *in vivo*. Knockout mutant strains for all BSH genes (several BSH genes are commonly found in lactobacilli) are still needed to evaluate the relative contribution of each one of these enzymes. To summarize, this study shows BSH activity as a key screening parameter to identify anti-*Giardia* lactobacilli strains. In addition, *L. gasseri* CNCM I-4884, which displayed both high anti-giardial and BSH activities *in vitro*, antagonizes *Giardia* survival in OF1 mice. This study represents a significant step toward the development of new prophylactic strategies, with both human and veterinary applications.

AUTHOR CONTRIBUTIONS

IF, BP, PG, TA, and LB-H conceived and designed the study. TA, BP, SC, and MT performed all the experiments. M-AT, IV, and PL discussed the experiments and results. TA, IF, and LB-H wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00089/full#supplementary-material

FIGURE S1 | Persistence of *L. johnsonii* La1 strain (Ery^r) in OF1 suckling mice. Each mouse received a single administration of 5×10^8 CFU of *L. johnsonii* La1 Ery^r either by oral gavage (days 1, 2, and 3; $n = 4$) or intragastric gavage (days 1, 2, 3, and 4; $n = 4$). *L. johnsonii* La1 was transformed with a plasmid harboring an erythromycin (Ery)-resistance gene as described previously (Allain et al., 2016). Values are in mean ± SEM.

FIGURE S2 | Kinetics of infection of *G. duodenalis* strain WB6 in OF1 suckling mice. **(A)** *G. duodenalis* trophozoites enumeration in small intestine after single gavage (day 0). Trophozoite burden was measured at days 3, 6, 8, 10, 12, 15, and 18 ($n = 10$). Small intestines were resuspended in PBS and trophozoites were counted using a hemocytometer **(B)** *G. duodenalis* cysts enumeration in large intestine after single gavage (day 0). Cysts were measured at days 3, 6, 8, 10, 12, 15, and 18 ($n = 10$). Large intestines were resuspended in 2.5% Potassium dichromate and cysts were counted using a hemocytometer. Values are in mean ± SEM.

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Characterization of Bile Salt Hydrolase from *Lactobacillus gasseri* FR4 and Demonstration of Its Substrate Specificity and Inhibitory Mechanism Using Molecular Docking Analysis

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Probiotic bacteria are beneficial to the health of poultry animals, thus are used as alternative candidates for antibiotics used as growth promoters (AGPs). However, they also reduce the body weight gain due to innate bile salt hydrolase (BSH) activity. Hence, the addition of a suitable BSH inhibitor along with the probiotic feed can decrease the BSH activity. In this study, a BSH gene (981 bp) encoding 326-amino acids was identified from the genome of *Lactobacillus gasseri* FR4 (*Lg*BSH). The *Lg*BSH-encoding gene was cloned and purified using an *Escherichia coli* BL21 (DE3) expression system, and its molecular weight (37 kDa) was confirmed by SDS–PAGE and a Western blot analysis. *Lg*BSH exhibited greater hydrolysis toward glyco-conjugated bile salts compared to tauro-conjugated bile salts. *Lg*BSH displayed optimal activity at 52°C at a pH of 5.5, and activity was further increased by several reducing agents (DTT), surfactants (Triton X-100 and Tween 80), and organic solvents (isopropanol, butanol, and acetone). Riboflavin and penicillin V, respectively, inhibited *Lg*BSH activity by 98.31 and 97.84%. A homology model of *Lg*BSH was predicted using *Ef*BSH (4WL3) as a template. Molecular docking analysis revealed that the glycocholic acid had lowest binding energy of −8.46 kcal/mol; on the other hand, inhibitors, i.e., riboflavin and penicillin V, had relatively higher binding energies of −6.25 and −7.38 kcal/mol, respectively. Our results suggest that *L. gasseri* FR4 along with riboflavin might be a potential alternative to AGPs for poultry animals.

Keywords: bile salt hydrolase, *Lactobacillus gasseri* FR4, antibiotics used as growth promoters, homology modeling, docking analysis

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INTRODUCTION

In recent years, probiotics have been considered as alternative candidates for antibiotics used as growth promoters (AGPs). Basically, AGPs are group of antibiotics (i.e., bambermycin, lincomycin, tylosin, etc.) used in animal feed at sub-therapeutic levels to improve the growth performance and average body mass gain of food animals (Wang et al., 2012). However, negative impacts of AGPs

include the emergence of antibiotic-resistant bacteria which may be transmitted to humans and cause food safety threats and public health issues (Marshall and Levy, 2011; Lin, 2014). European Union member countries have banned use of all AGPs for food animals (Marshall and Levy, 2011). However, banning AGPs has severely affected the health and productivity of poultry animals in several countries (Casewell et al., 2003). Hence, the use of probiotics has emerged as an alternative for AGPs and several health-promoting effects were observed (Lin, 2011). Although probiotic feed supplements improve the growth performance of poultry animals, substantial losses or no significant increases in body weight were observed in probiotic (especially *Lactobacillus* strains) treated animals (Million et al., 2012; Angelakis et al., 2013). This is due to the production of higher amounts of bile salt hydrolase (BSH) enzymes. Generally, BSH activity is considered as a functional probiotic biomarker due to its health-protective effects (i.e., cholesterol reduction, bile tolerance, antimicrobial activity, etc.) (Miremadi et al., 2014). BSH activities may contribute to microbial bile resistance, colonization of the gastrointestinal tract (GIT), host metabolism and energy harvest (Begley et al., 2006; Lin et al., 2014). Probiotic microorganisms have the ability to transform bile salts to a great extent through a bile salt deconjugation mechanism. Bile salts are synthesized from cholesterol, conjugated with glycine or taurine in the liver, stored in the gall bladder and secreted into the small intestine (Kim and Lee, 2005). Bile salts play a significant role in lipid digestion and act as a biological detergent (Hofmann and Eckmann, 2006). The BSH enzyme has the ability to hydrolyze conjugated bile salts into a deconjugated form and release free amino acids. Deconjugated bile salts are much less soluble, hence are not absorbed by intestinal cells and are excreted in feces. This mechanism results in higher utilization of cholesterol for the *de novo* synthesis of bile acids, thereby lowering the serum cholesterol levels (Begley et al., 2006).

To be used as an alternative for AGPs, BSH activities of probiotic bacteria should be inhibited by a specific inhibitor, which could be supplemented along with the probiotic feed. This will dramatically decrease the BSH activity and increase fat deposition in poultry animals (Joyce and Gahan, 2014). Recently several researchers have identified potential BSH inhibitors including gossypetin, caffeic acid phenethyl ester (CAPE), epicatechin monogallate, riboflavin and demonstrated the inhibition of BSH enzyme activity (Lin et al., 2014; Smith et al., 2014). However, BSHs of different microorganism have different protein structures and substrate specificities. Identification of potential BSH inhibitors relies on the availability of defined crystal structures of BSH enzymes (Smith et al., 2014). Hence modern computational strategies, such as homology modeling and molecular docking studies can be used to identify safe, potent and cost-effective BSH inhibitors using *in silico* analysis. The identified novel BSH inhibitors can then be used alongside BSH-positive probiotic microorganisms to decrease host fat digestion in food animals and in turn enhance the profitability of feed-additive industries (Knarreborg et al., 2004; Wang et al., 2012; Lin, 2014). In our previous study, a probiotic *Lactobacillus gasseri* FR4 from the GIT of free-range chickens (*Gallus gallus* subsp. *domesticus*) was isolated and efficiently hydrolyzed taurodeoxycholate (TDC) to deoxycholate on de Man Rogosa Sharpe (MRS)-TDC agar plate (Parveen Rani et al., 2016). We hypothesize that this hydrolysis activity is due to the production of a BSH enzyme. To use this probiotic bacteria as an alternative for AGPs, this BSH enzyme activity should be inhibited. Therefore, we sought to find a potential candidate to inhibit BSH activity.

In this study, we have identified a gene responsible for BSH activity of the probiotic *L. gasseri* FR4, which was cloned and the enzyme overexpressed, purified and characterized. Several compounds were screened to identify a potential BSH inhibitors and riboflavin exhibited higher percentage of inhibition. The protein structure of newly identified BSH enzyme was modeled using homology modeling. Molecular docking analysis was performed to identify the substrate specificity and inhibitory mechanism of identified inhibitors.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The probiotic *L. gasseri* FR4 was previously isolated from the GIT of free-range chickens (Parveen Rani et al., 2016). The strain was routinely subcultured in MRS media at 37°C under anaerobic conditions. *Escherichia coli* DH5α and BL21 (DE3) strains were, respectively, used for the cloning and expression of the BSH enzyme. The *E. coli* expression vector, pET21b (+), was used for expression of His-tagged (6x) recombinant BSH. The cloning and expression hosts were maintained in Luria-Bertani (LB) broth supplemented with ampicillin (100 μg/ml) at 37°C under aerobic conditions.

Identification of the BSH Gene

To identify the putative BSH gene, we mined the available whole-genome sequence of *L. gasseri* ATCC 33323 = JCM 1131 (NC_008530.1) using the Basic Local Alignment Search Tool (BLAST) algorithm from the National Center for Biotechnology Information (NCBI)[1]. The putative BSH gene was aligned with other bacterial BSHs using the Clustal omega multiple sequence alignment tool[2], and the secondary structure was predicted using ESpript 3.0[3] (Robert and Gouet, 2014). The phylogenetic relationship of the identified putative BSH of *L. gasseri* FR4 was confirmed by an unweighted pair group method with arithmetic mean (UPGMA) phylogenetic tree using MEGA6 software (Tamura et al., 2013).

Genomic DNA Extraction and Cloning of the BSH Gene

Genomic DNA of *L. gasseri* FR4 was extracted using a DNeasy Blood & Tissue Kits (Qiagen, Germany) according to the manufacturer's instructions. The putative BSH gene (975 bp) was amplified from the purified genomic DNA using *Lg*BSH-F

[1]http://www.ncbi.nlm.nih.gov/

[2]http://www.ebi.ac.uk/Tools/msa/clustalo/

[3]http://espript.ibcp.fr/ESPript/ESPript/

(5′-gcgGGATCCTGTACCTCAATTATTT-3′) and *Lg*BSH-R (5′-gagCTCGAGATTTTGATAGTTAATATG-3′) primer pairs, respectively, flanked with *Bam*HI and *Xho*I (the underlined sequences) (Supplementary Figure S1). The amplified polymerase chain reaction (PCR) product was purified using a QIAquick PCR purification kit (Qiagen, Germany). pET21b (+) vector DNA and the amplified *Lg*BSH-encoding gene were digested with *Bam*HI and *Xho*I (New England Biolabs), resolved in a 1% agarose gel, extracted from the gel and ligated using T4 DNA ligase (ThermoFisher Scientific). The resulting plasmid was transformed into *E. coli* DH5α-competent cells and plated on LB agar plates with ampicillin (100 μg/ml). The plasmid was extracted and sequenced using T7-F and T7-R universal primers and no mutations in the coding sequence of *Lg*BSH-encoding gene were detected (Supplementary Figure S2).

Expression and Purification of Recombinant *Lg*BSH

To express the recombinant *Lg*BSH, the pET21b-BSH plasmid was purified from *E. coli* DH5α and transformed into the expression host *E. coli* BL21 (DE3). Protein expression and purification was performed as described in Kumar et al. (2013). Briefly, *E. coli* BL21 (DE3) cells harboring the pET21b-BSH plasmid were cultured overnight and cells with an optical density (OD) of 0.15 were inoculated into 50 ml of LB broth with ampicillin. When the culture reached 0.6 OD, 500 μl of 100 mM isopropyl-β-d-thiogalactopyranoside (IPTG) was added and incubated for 3 h to allow protein expression. Then, centrifugation was performed at 8000 rpm for 10 min at 4°C. Cells were resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol (DTT), and 1x SIGMAFAST^{TM} protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, United States). Then, sonication was performed at six cycles of 20 s on/off with a 60% amplitude and 50% duty cycle on ice using a 3-mm probe (Sonics Vibracell-VCX130, United States). Sonicated cells were centrifuged at 8000 rpm for 10 min (at 4°C) and the cell pellets and lysate were separated. *Lg*BSH from the cell lysate was purified using a Ni^{2+}-NTA agarose column, and the purity was analyzed using 12% (wt/vol) sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The molecular weight of purified *Lg*BSH was further confirmed by a Western blot analysis, using a primary antibody against His-Tag (1:5000) and a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:10000). The protein concentration was estimated using the Bradford method (Protein Assay Kit, Bio-Rad).

BSH Activity

Bile salt hydrolase activity was measured by the method described in Wang et al. (2012) with some modifications. Briefly, a 200-μl reaction mixture containing 178 μl of sodium-phosphate buffer (0.1 M, pH 6.0), 10 μl of purified recombinant *Lg*BSH, 2 μl of 1 M DTT, and 10 μl of 100 mM conjugated bile acid were mixed and incubated 37°C for 30 min. The reaction was terminated by mixing of 50 μl of the reaction mixture with an equal volume of 15% (w/v) trichloroacetic acid, and subsequently the precipitates were removed by centrifugation at 13,000 × *g* for 10 min. To estimate concentrations of the liberated amino acids (glycine or taurine) from the conjugated bile acids, 50 μl of the above supernatant was mixed with 950 μl of Ninhydrin reagent and 100 μl of sodium-citrate buffer (0.5 M, pH 5.5). The mixture was kept in a water bath for 15 min and cooled in ice. Absorbance was measured at 570 nm and the amino acid concentration was estimated using a standard curve of either glycine or taurine based on the conjugated bile salts used. The enzyme activity was expressed as micromoles of amino acid released per minute per milligram of BSH. All experiments were performed in triplicate.

Biochemical Characterization of *Lg*BSH

Substrate Specificity of *Lg*BSH

The substrate specificity of purified recombinant *Lg*BSH was determined using different glyco- or tauro-conjugated bile salts [glycocholic acid (GCA), glycodeoxycholic acid (GDCA), taurocholic acid (TCA), and taurodeoxycholic acid (TDCA)] as well as non-substrate compounds (penicillin V and ampicillin) as a substrate. The assay was performed as described above.

Effect of pH and Temperature on *Lg*BSH Activity

To identity the optimum temperature for *Lg*BSH activity, a standard reaction mixture (200 μl) was incubated at different temperature ranges (20–90°C). Similarly, a suitable pH was optimized by performing the enzyme assay at various pH values (pH 3–9). The pH was adjusted by replacing the buffer in the reaction mixture, i.e., sodium acetate buffer (pH 3–6), phosphate buffer (pH 7), and glycine-NaOH buffer (pH 8 and 9). Each experiment was performed in triplicate.

Effects of Reducing Agents, Surfactants, Solvents, and Enzymes on *Lg*BSH Activity

The effects of various reducing agents (DTT, β-ME, and EDTA; 10 mM each), surfactants (Triton X-100 and SDS; 10 mM each and 0.5% Tween 80), and solvents (chloroform, ethanol, acetone, methanol, isopropanol, and butanol; 10% v/v each) on *Lg*BSH activity were demonstrated. Briefly, 200 μl of the reaction mixture was separately added to various reducing agents, surfactants, and solvents and incubated for 30 min at 25°C. After incubation, an enzyme assay was performed using GCA as a substrate, and each experiment was performed in triplicate.

The effects of different enzymes (i.e., lysozyme, proteinase K, pepsin, α-amylase, lipase, and catalase) on *Lg*BSH activity were studied. The reaction mixture (200 μl) was treated with 2 mg/ml of each enzyme and incubated for 30 min at 25°C. The enzyme activity was evaluated after incubation as described earlier and untreated *Lg*BSH served as a control. Each experiment was performed in triplicate.

Inhibitory Effects of Feed Additives on *Lg*BSH Activity

To identify suitable candidates for a BSH inhibitor, various commonly used animal feed additives were screened, which including metal ions, AGPs, and other recently identified BSH

inhibitors. *Lg*BSH was pre-incubated with various metal ions (i.e., $CuCl_2$, $CuSO_4$, $MnCl_2$, $MnSO_4$, $MgCl_2$, $MgSO_4$, $ZnCl_2$, $ZnSO_4$, $CaCl_2$, $NaHIO_3$, and KIO_3) at a concentration of 5 mM for 30 min at 37°C (Wang et al., 2012). Similarly, AGPs, including penicillin V, ampicillin, oxytetracycline, doxycycline hydrochloride, neomycin, erythromycin, and lincomycin, were used at a concentration of 5 mM (Wang et al., 2012; Smith et al., 2014). Apart from these feed additives, several other compounds were also recently screened as BSH inhibitors which included riboflavin (Smith et al., 2014) and ascorbic acid; hence we also tested their inhibitory efficiencies against *Lg*BSH at the concentration of 5 mM. The inhibitory efficiency of inhibitors on *Lg*BSH activity was tested and calculated by dividing the mean activity of the control (without inhibitor) by the treatment group (with inhibitor).

Homology Modeling of *Lg*BSH

To determine the 3D structure of *Lg*BSH, the protein sequence was blasted against the protein data bank (PDB) database. The online software, Protein Homology/analogY Recognition Engine V 2.0[4] (Phyre2), was used to predict the homologous structure of *Lg*BSH using the intensive mode (Kelley et al., 2015). Bumps were removed from the modeled protein and missing side chain atoms were added using the 'WHAT IF Web Interface.'[5] The predicted protein structure was validated using protein structure validation (PSVS) tool[6]. Ramachandran plot was used to analyze the structure quality. The modeled *Lg*BSH protein structures were visualized, and images were rendered using UCSF-Chimera software (Pettersen et al., 2004). Residues involved in substrate binding were identified using the computed atlas of the surface topography of proteins (CASTP) (Dundas et al., 2006) and the SiteHound-web server[7] (Hernandez et al., 2009). Then, predicted residues were compared with the templates. These residues were used to predict putative binding sites for ligands during the docking analysis.

Docking Analysis

To perform the molecular docking studies, SwissDock, a small protein molecule docking web service based on EADock DSS (Fast docking using the CHARMM force field with EADock DSS) was used (Grosdidier et al., 2011). Ligands including GCA ($C_{26}H_{43}NO_6$), GDCA ($C_{26}H_{43}NO_5$), TCA ($C_{26}H_{45}NO_7S$), penicillin V (PenV: $C_{16}H_{18}N_2O_5S$), and riboflavin ($C_{17}H_{20}N_4O_6$) were obtained from the ZINC database[8] and were used in the docking analysis. Ligand structures are shown in Supplementary Figure S3. The root mean square deviation (RMSD) was used to identify the best docked complexes. The predicted docking clusters were analyzed using UCSF-Chimera.

[4]http://www.sbg.bio.ic.ac.uk/phyre2

[5]http://swift.cmbi.ru.nl/servers/html/index.html

[6]http://psvs-1_5-dev.nesg.org

[7]http://sitehound.sanchezlab.org

[8]http://zinc.docking.org/

RESULTS

Identification of the BSH Gene from *L. gasseri* FR4

In our previous study, BSH activity of *L. gasseri* FR4 was demonstrated using a direct plate assay method which exhibited halos of precipitated deoxycholate on MRS-TDC agar due to the hydrolysis of TDC (Parveen Rani et al., 2016). Based on the BLAST results, a putative BSH gene (981 bp) encoding a 326-amino acid (aa) choloylglycine hydrolase family (EC 3.5.1.24) protein was identified from the genome of *L. gasseri* (NC_008530.1). The theoretical molecular weight and isoelectric point of the putative BSH were, respectively, estimated to be 36.69 kDa and 5.18 using the ExPASy analysis tool[9]. The deduced amino acid sequence of the putative *Lg*BSH was aligned with amino acid sequences of available BSH crystal structure sequences from different bacterial species. *Lg*BSH shared a sequence identity of 54% with *Enterococcus faecalis* BSH (*Ef*BSH; 4WL3), 47% with *L. salivarius* BSH (*Ls*BSH; 5HKE), 38% with *Clostridium perfringens* BSH (*Cp*BSH; 2RLC), and 37% with *Bifidobacterium longum* BSH (*Bl*BSH; 2HEZ). *Lg*BSH also shared the lowest identity of 30% with penicillin V acylase (PVA) of *Lysinibacillus sphaericus* (2PVA), since though both enzymes belong to the choloylglycine hydrolase family.

The multiple sequence alignment results revealed similarities of conserved amino acid residues among all of the selected BSHs, which included a catalytic nucleophile residue, Cys1, and other conserved amino acids, such as Arg16, Asp19, Asn79, Asn171, and Arg224 (amino acids were numbered from cysteine) (**Figure 1**). Rossocha et al. (2005), Kumar et al. (2006), and Xu et al. (2016) reported that Cys1 plays an important role in the activity of BSH. This further confirmed that the identified gene belonged to BSH.

The evolutionary relationship of *Lg*BSH with other bacterial choloylglycine hydrolase family proteins (BSH/PVA) was inferred using a UPGMA phylogenetic analysis. Evolutionary distances were computed using the Poisson correction method and are in units of the number of amino acid substitutions per site. The analysis involved 37 aa sequences of BSH and PVA from various bacterial taxa. All positions containing gaps and missing data were eliminated. There were 266 positions in total in the final dataset. The phylogenetic tree showed that BSHs of *Lactobacillus* spp. were distinct from the PVA, which has an evolutionary relationship with BSH. *Lg*BSH was closely related to BSHs of *E. faecalis, C. perfringens,* and *L. salivarius* (Supplementary Figure S4).

Expression and Purification of Recombinant *Lg*BSH

The expression vector pET21b (+) harboring the *Lg*BSH-encoding gene was transformed into *E. coli* BL21 (DE3) to overexpress *Lg*BSH. A recombinant *Lg*BSH protein band was observed after IPTG induction on SDS–PAGE at

[9]http://web.expasy.org/compute_pi

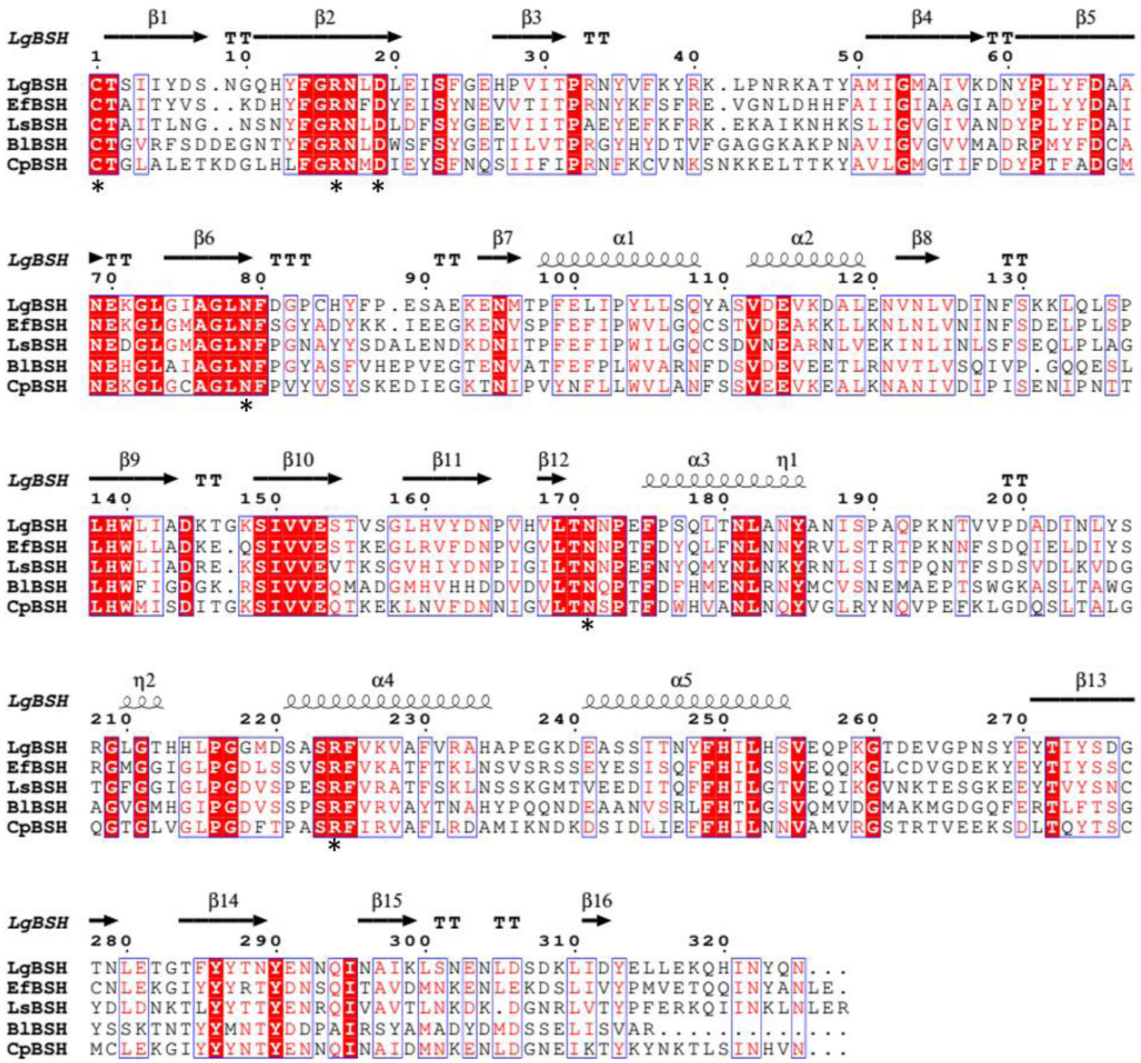

FIGURE 1 | Multiple sequence alignment of bile salt hydrolase (BSH) amino acid sequences from different bacterial species. The protein sequences were aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) software and identical residues were identified by ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/). The identical residues are shown in red background and the similar residues are shown as red color. The conserved residues (Cys-1, Arg-16, Asp-19, Asn-79, Asn-171, and Arg-224) were marked as black asterisk (*).

around 37 kDa, which is the calculated molecular mass of *Lg*BSH (**Figure 2A**, lane 3). A single band was observed at 37 kDa after purification with Ni^{2+}-NTA agarose column, thus confirming the homogeneity and purity of *Lg*BSH (**Figure 2A**, lane 4). The Western blot analysis using anti-His antibodies also confirmed the molecular weight of purified *Lg*BSH (**Figure 2B**).

Biochemical Characterization of *Lg*BSH

Substrate Specificity of *Lg*BSH

To determine the substrate specificity of purified *Lg*BSH, four major glyco- or tauro-conjugated bile salts were used along with two non-substrate compounds (i.e., penicillin V and ampicillin). The highest *Lg*BSH activity was observed with GDCA as a substrate and was set as 100% activity. *Lg*BSH showed greater hydrolysis toward glyco-conjugated bile salts (GCA and GDCA) than to tauro-conjugated (TCA and TDCA) bile salts (**Figure 3**). However, very weak activity (4.78%) was observed when using penicillin V as a substrate, and no activity was detected with ampicillin (**Figure 3**). These results further confirmed that the identified *Lg*BSH was not PVA.

Effects of pH and Temperature on *Lg*BSH Activity

To determine the optimal pH for maximal *Lg*BSH activity, various pH values (pH 3–9) were used and optimal *Lg*BSH activity was observed in the range of pH 5.5–6.5. The maximal BSH activity (18.68 ± 1.15 μmol/min/mg) was observed at pH 5.5. Activities decreased at lower and higher pH values (**Figure 4A**). The optimal temperature for *Lg*BSH activity was determined using various temperature values (20–90°C). The

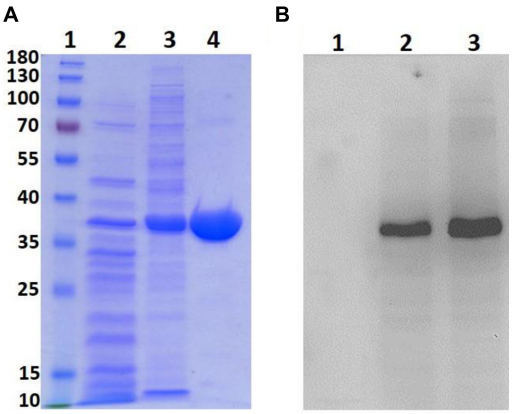

FIGURE 2 | Expression and purification of *Lg*BSH. SDS–PAGE analysis of *Lg*BSH **(A)**. Lane1: Marker (PageRuler^{TM} Prestained Protein Ladder, 10–180 kDa), Lane2: cell lysate of *Escherichia coli* BL21 wild type, Lane3: cell lysate of *E. coli* BL21 expressing *Lg*BSH, Lane4: purified *Lg*BSH. Western blotting analysis of *Lg*BSH using anti-His antibody **(B)**. Lane1: cell lysate of *E. coli* BL21 wild type, Lane2: cell lysate of *E. coli* BL21 expressing *Lg*BSH, Lane3: purified *Lg*BSH.

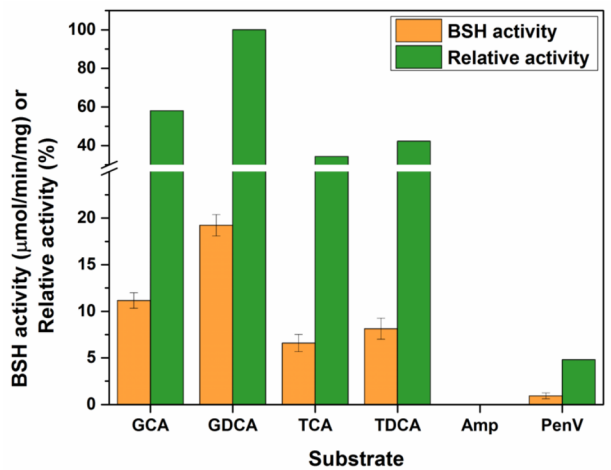

FIGURE 3 | Determining the substrate specificity of *Lg*BSH using various bile salts as well as non-substrate compounds. The BSH activity was expressed as the μmol/min/mg. All the experiments were performed in triplicate and mean ± SD values are represented.

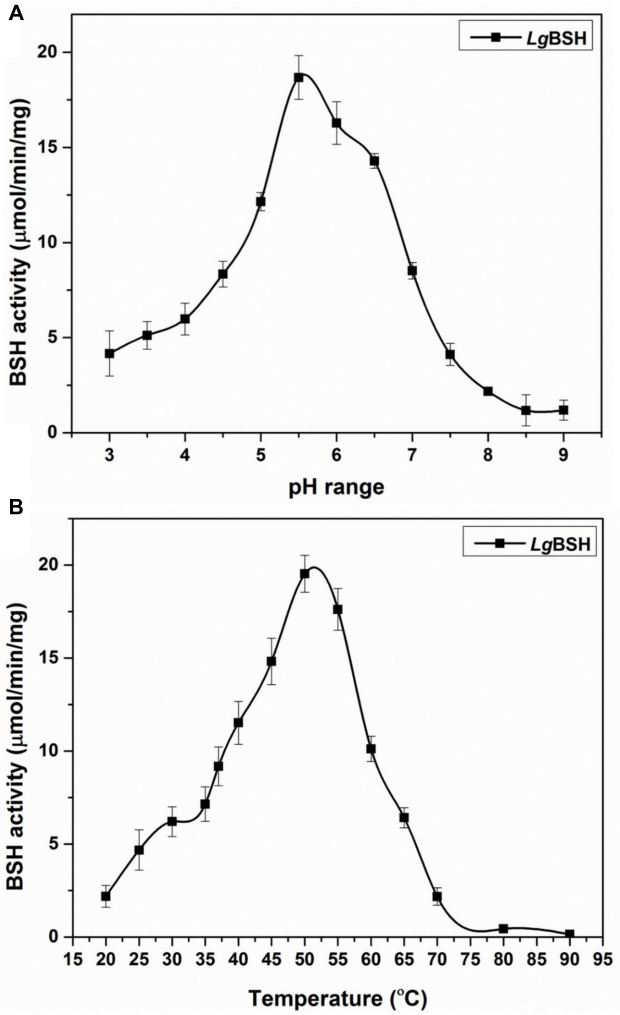

FIGURE 4 | Effect of various pH **(A)** and temperature **(B)** on *Lg*BSH activity. The assay was performed using glycocholic acid (GCA) as a substrate and activity was expressed as the μmol/min/mg. All the experiments were performed in triplicate and mean ± SD values are represented.

maximal BSH activity was observed at 52°C and enzyme activity sharply declined with increasing temperatures (**Figure 4B**).

Effects of Various Reducing Agents, Surfactants, Solvents, and Enzymes on *Lg*BSH Activity

The effects of various reducing agents on *Lg*BSH were analyzed. *Lg*BSH activity increased by about 18% when the enzyme was treated with 10 mM DTT (118.06%). However, the activity reduced by 2 and 4%, when treated with β-mercaptoethanol (98.18%) and EDTA (96.14%), respectively (**Table 1**). Surfactants such as Triton X-100 and Tween 80 significantly increased *Lg*BSH activity by 43 and 28%, respectively. Generally, surfactants or detergents possibly interact with the surfaces of enzymes, thereby enhancing the solubility and stability of proteins (Avinash et al., 2015). On the other hand, SDS dramatically reduced BSH activity by 93% at a concentration of 10 mM.

Organic solvents such as isopropanol, butanol, and acetone, respectively, enhanced *Lg*BSH activities by 41, 18, and 8% when the enzyme was treated with a 10% (v/v) concentration. Several solvents such as methanol, ethanol, and chloroform significantly decreased the enzyme activity. Among them, treatment with methanol drastically decreased the enzyme activity by 31% (**Table 1**).

The influences of various enzymes on *Lg*BSH activity was investigated by treating BSH with appropriate enzymes. Proteinase K and pepsin, respectively, decreased *Lg*BSH activity by 97 and 85%, thus confirming that proteinase K might degrade the BSH enzyme. However, enzymes like lysozyme, α-amylase,

TABLE 1 | Effects of various reducing agents, surfactants, solvents, and enzymes on purified *Lactobacillus gasseri* bile salt hydrolase (*Lg*BSH) activity.

Treatment	Compound	Relative activity (%)[a]
Control		100%
Reducing agents	DTT[b]	118.06 ± 0.31
(10 mM)	β-Mercaptoethanol	98.18 ± 0.83
	EDTA[b]	96.14 ± 1.16
Surfactants	Triton X-100	143.12 ± 1.47
(10 mM or 0.5%)	Tween 80	128.19 ± 0.82
	SDS[b]	2.86 ± 0.19
Solvents	Chloroform	98.91 ± 1.16
(10% [vol/vol])	Ethanol	86.16 ± 1.67
	Acetone	108.71 ± 1.05
	Methanol	69.15 ± 2.15
	Isopropanol	141.21 ± 1.26
	Butanol	118.20 ± 2.19
Enzymes	Lysozyme	91.35 ± 0.92
	Proteinase K	3.49 ± 0.19
	Pepsin	15.16 ± 1.68
	α-Amylase	98.41 ± 1.24
	Lipase	101.19 ± 0.62
	Catalase	98.13 ± 0.53

[a]*The relative activity percentage was calculated based on the control with no treatment, and the assay was performed using glycocholic acid as a substrate.*
[b]*DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecylsulfate.*

TABLE 2 | Inhibitory effects of various compounds on *L. gasseri* bile salt hydrolase (*Lg*BSH) activity.

Compound type	Compound name	Inhibition %[a]
Metal ion or feed additive	$CuCl_2$	98.13
	$CuSO_4$	94.36
	$MnCl_2$	76.42
	$MnSO_4$	73.98
	$MgCl_2$	36.14
	$MgSO_4$	32.69
	$ZnCl_2$	54.21
	$ZnSO_4$	39.64
	$CaCl_2$	18.64
	$NaHIO_3$	98.26
	KIO_3	97.64
Antibiotics used as growth promoters (AGPs)	Penicillin V	97.84
	Ampicillin	96.39
	Oxytetracycline	95.97
	Doxycycline hydrochloride	97.51
	Neomycin	82.93
	Erythromycin	41.62
	Lincomycin	18.58
Potential inhibitors	Riboflavin	98.31
	Ascorbic acid	32.96

[a]*The inhibition percentage was calculated by dividing the mean activity of the control (without an inhibitor) and treatment (with an inhibitor). The assay was performed with 5 mM of each inhibitor, and glycocholic acid was used as a substrate.*

lipase, and catalase did not affect *Lg*BSH activity. This was due to the inefficiencies of these enzymes on *Lg*BSH (**Table 1**).

Inhibitory Effects of Feed Additives on *Lg*BSH Activity

Various commonly used feed additives and AGPs were screened to identify a suitable *Lg*BSH inhibitor. Among the tested compounds, more than 95% inhibition was observed with riboflavin, $NaHIO_3$, $CuCl_2$, penicillin V, KIO_3, doxycycline hydrochloride, ampicillin, and oxytetracycline (**Table 2**). The lowest inhibitory percentage (<50%) was observed with erythromycin, $ZnSO_4$, $MgCl_2$, ascorbic acid, $MgSO_4$, $CuCl_2$, and lincomycin. Among all of the tested inhibitors, riboflavin might be a good candidate for application in animal feed.

Homology Modeling of *Lg*BSH

Protein BLAST search of *Lg*BSH showed highest identity of about 54% with the BSH of *E. faecalis* (4WL3), and 47% identity with the BSH of *L. salivarius* (5HKE). The homology model of *Lg*BSH was predicted with the Phyre2 algorithm using *Ef*BSH (4WL3) as a template (**Figure 5A**), since it had a 100% confidence level (Supplementary Figure S5). The superimposed structure of *Ef*BSH, *Ls*BSH with the *Lg*BSH revealed similarities among their structures and their catalytic active sites contains nucleophile residue (Cys1) (**Figure 5B**). Residues involved in catalysis were identified based on the superimposed structure, including Cys1, Arg16, Asp19, Asn79, Asn171, and Arg224. However, residues in the substrate-binding pocket were conservatively replaced. Two loops were identified near the substrate-binding pockets: loop I contained 8 aa from 20 to 27 (LEISFGEH), and loop II contained 15 aa from 124 to 138 (LVDINFSKKLQLSPL). The secondary structure prediction of *Lg*BSH using PSIPRED V 3.3 revealed that *Lg*BSH contains 14.11% of α-helix, 60.12% of random coil, and 25.76% of extended strand (Supplementary Figure S6). The secondary structural pattern of *Lg*BSH was similar to *Ef*BSH.

The PSVS analysis results of predicted *Lg*BSH structure suggested that the model was of a good quality. The Ramachandran plot analysis showed that 91.6% of residues lies in the most favored regions; 8.1 and 0.4% of residues lies in allowed and disallowed regions, respectively (Supplementary Figure S7). The Procheck G-factor (all dihedral angles) was found to be −0.19, and the VERIFY-3D results showed that 85.54% of residues had an average 3D-1D score of >0.2, thus confirming that the predicted *Lg*BSH structure contained no conformational errors (Supplementary Figure S8). The overall *Z*-score of the ProSA analysis was −6.57 (Supplementary Figure S9), which is in the range of scores typically found for native proteins of similar sizes (Wiederstein and Sippl, 2007). The overall quality factor of the predicted *Lg*BSH model was calculated by ERRAT, and results showed 90.033, which further confirmed the quality of the predicted structure (Supplementary Figure S10). The RMSDs of bond angles and bond lengths were 2.2° and 0.019 Å, respectively. The modeled *Lg*BSH protein structure was submitted to Protein Model Data Base[10] (PMDB) with the model id PM0080976.

[10]https://bioinformatics.cineca.it/PMDB/

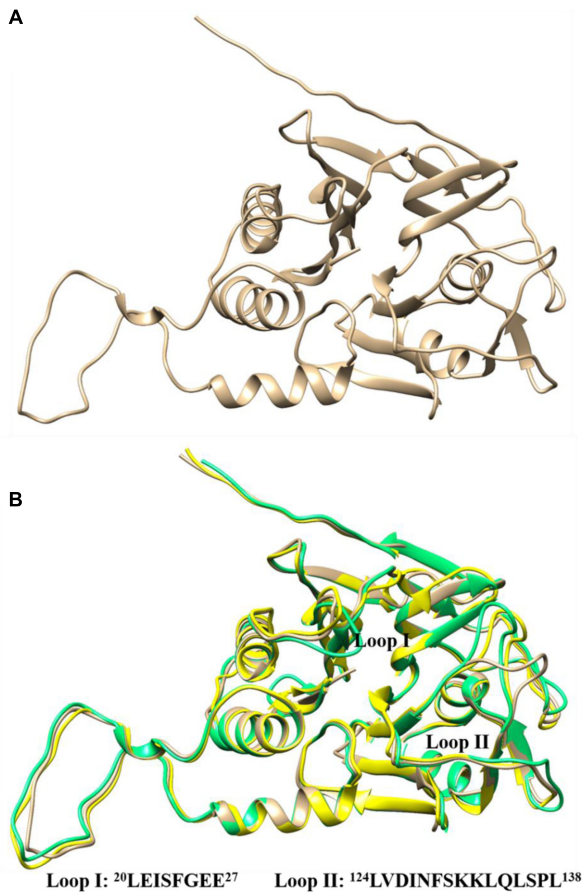

FIGURE 5 | Three-dimensional structure of *Lg*BSH predicted by homology modeling using *Enterococcus faecalis* BSH (4WL3) as template **(A)**. Three-dimensional superimposed structures of *Lg*BSH (tan) with their closest homology structures *Ef*BSH (4WL3) (yellow) and *Ls*BSH (5HKE) (spring green) using UCSF-Chimera. The sequences of loops (I and II) present in the substrate-binding pockets were represented in the figure **(B)**.

The CASTp analysis revealed residues involved in substrate binding. On the whole, 19 possible residues were identified and compared to binding pocket residues of the template protein (Supplementary Figure S11). The approximate binding site volume of *Lg*BSH was found to be 551.7 Å^3. The SiteHound analysis revealed that the total interaction energy (TIE) of the substrate-binding pocket was −480.02 kcal/mol with the lowest and highest interaction energies of −22.34 and −8.96 kcal/mol, respectively.

Molecular Docking Studies to Identify the Substrate Specificity and Inhibitors

*Lg*BSH had a substrate preference toward glyco-conjugated bile salts (GCA and GDCA), compared to tauro-conjugated bile salts. Hence, to demonstrate the substrate specificity, GCA, GDCA, and TCA were docked with *Lg*BSH, and their hydrogen bonding interactions with the enzyme were identified (**Figure 6**). Cys2 and Lys59 (amino acids were numbered from methionine) residues in the substrate-binding pocket of *Lg*BSH formed hydrogen bonds with GCA, with respective bond lengths of 2.637 and 2.252 Å (**Figures 6A,B**). Similarly, Cys2, Phe25, and Lys59 interacted with GDCA via hydrogen bonding with respective bond lengths of 2.384, 1.864, and 2.031 Å (**Figures 6C,D**). On the other hand, TCA formed hydrogen bonds with Lys59 and Gln135 with respective bond lengths of 2.224 and 2.123 Å, (**Figures 6E,F**). However, the binding energy of GCA was found to be lower (−8.46 kcal/mol) than that of GDCA (−6.81 kcal/mol) and TCA (−7.91 kcal/mol), thus further confirming the binding strength of GCA over other substrates.

Based on previous experimental data, we found that riboflavin and penicillin V, respectively, inhibited *Lg*BSH activity by 98.31 and 97.84%. To further investigate the mechanism behind this inhibition and support our experimental data, both substrate (GCA) and inhibitors (riboflavin and penicillin V) were docked with the modeled protein structure using the SwissDock algorithm. All ligands showed favorable binding energies, among which the substrate GCA had the lowest binding energy of −8.46 kcal/mol, while on the other hand, inhibitors, i.e., penicillin V and riboflavin, had relatively higher binding energies of −7.38 and −6.25 kcal/mol, respectively (**Figure 7**).

DISCUSSION

Bile salt hydrolase enzymes of several LAB species play a central role in the lipid metabolism of hosts. During the past several decades, probiotic bacteria with BSH activity were used to alleviate cholesterol levels in humans and animals (Jones et al., 2004). Several authors have previously identified and crystallized BSH enzymes from various bacterial species including *C. perfringens* (Rossocha et al., 2005), *B. longum* (Kumar et al., 2006), *L. salivarius* (Xu et al., 2016), and *E. faecalis*. Kumar et al. (2013) identified and cloned a gene encoding the BSH enzyme from *L. fermentum* NCDO394. The nucleotide sequence of the putative BSH gene contained an open reading frame (ORF) of 978 nucleotides encoding a predicted protein of 325 aa. They reported that the deduced BSH protein had significant similarity to the penicillin V amidases of other *Lactobacillus* spp. Wang et al. (2012) identified a BSH gene from the genome of *L. salivarius* NRRL B-30514, a chicken isolate. Similarly, we have identified a putative BSH gene (981 bp) encoding 326 aa from the genome of *L. gasseri* FR4. The molecular mass of purified *Lg*BSH was found to be 37 kDa on SDS–PAGE and was further confirmed by a Western blot analysis (**Figures 2A,B**). This result is further supported by several publications, where the molecular mass of the BSH enzyme was reported as 37 kDa on SDS–PAGE (Wang et al., 2012; Kumar et al., 2013; Jayashree et al., 2014).

Generally, BSH enzymes have a broad range of substrate specificity toward glyco- and tauro-conjugated bile salts (Lambert et al., 2008; Oh et al., 2008). Purified *Lg*BSH exhibited substrate specificity toward glycine-conjugated bile salts (GCA and GDCA) and in particular, maximal activity was observed on GDCA (**Figure 3**). However, the activity was inhibited when using non-substrate compounds (i.e., ampicillin and

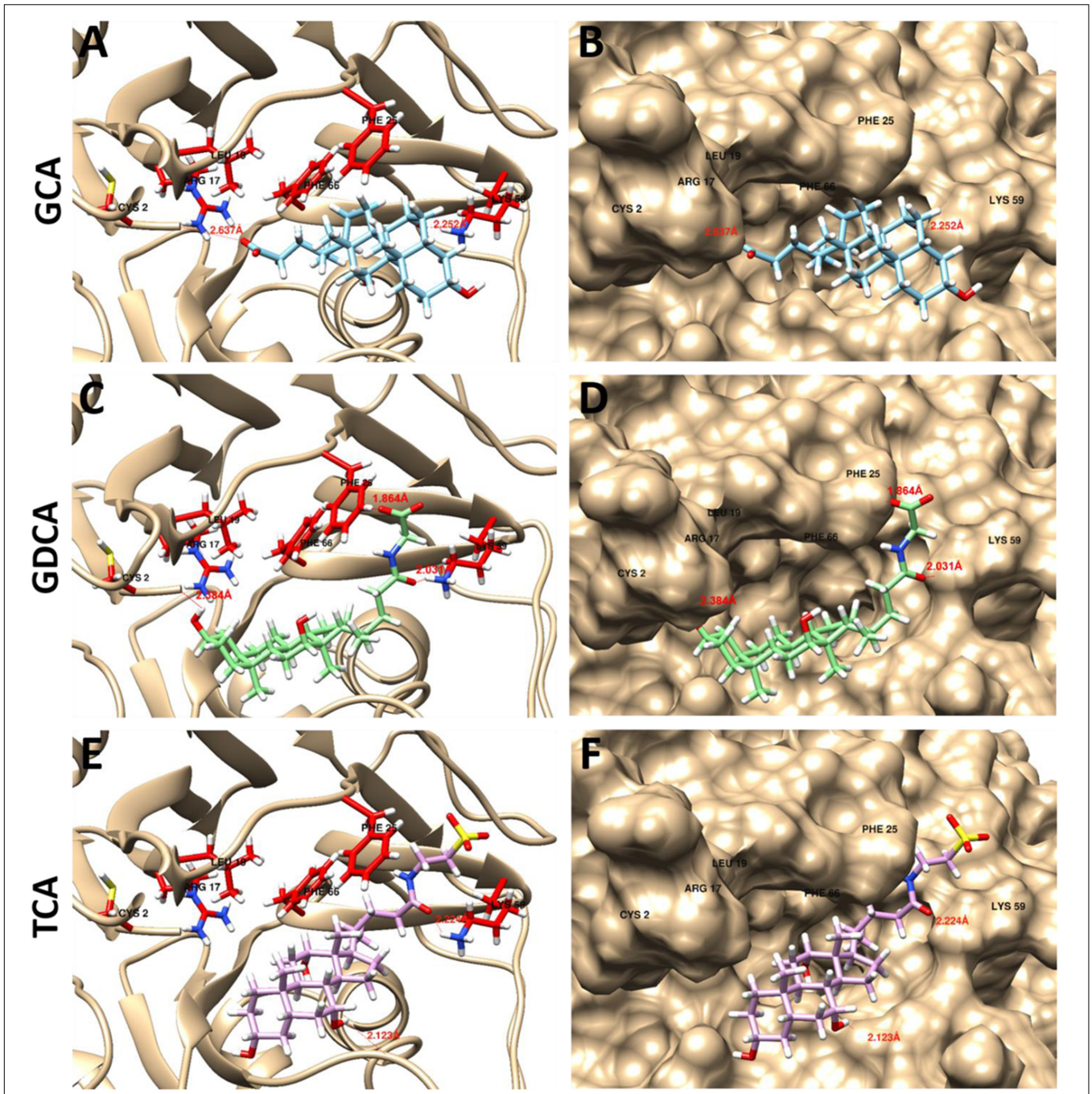

FIGURE 6 | Docking analysis of GCA **(A,B)**, glycodeoxycholic acid (GDCA) **(C,D)**, and taurocholic acid (TCA) **(E,F)** with *Lg*BSH to determine the substrate specificity and identify the hydrogen bonding between enzyme and substrate. Docking was performed using SwissDock and the outputs were analyzed using UCSF-Chimera.

penicillin V) as a substrate. Kumar et al. (2013) studied the characteristics of recombinant (r) BSH of *L. fermentum* NCDO394 and identified that purified rBSH enzyme hydrolyzed six major bile substrates, with a special preference toward glycine-conjugated bile salts and exhibited maximal activity against GCA. Previous studies also confirmed that BSHs from *Lactobacillus* spp., including *L. salivarius* (Wang et al., 2012), *L. plantarum* CGMCC 8198 (Gu et al., 2014), and BSH-C of *L. johnsonii* PF01 (Chae et al., 2013), had a substrate specificity toward glyco-conjugated bile salts. Biochemical characterization studies revealed that the pH and temperature optima of *Lg*BSH were, respectively, found to be 5.5 and 52°C. Our results are in accordance with the results of Wang et al. (2012), in which the BSH from *L. salivarius* showed maximal activity

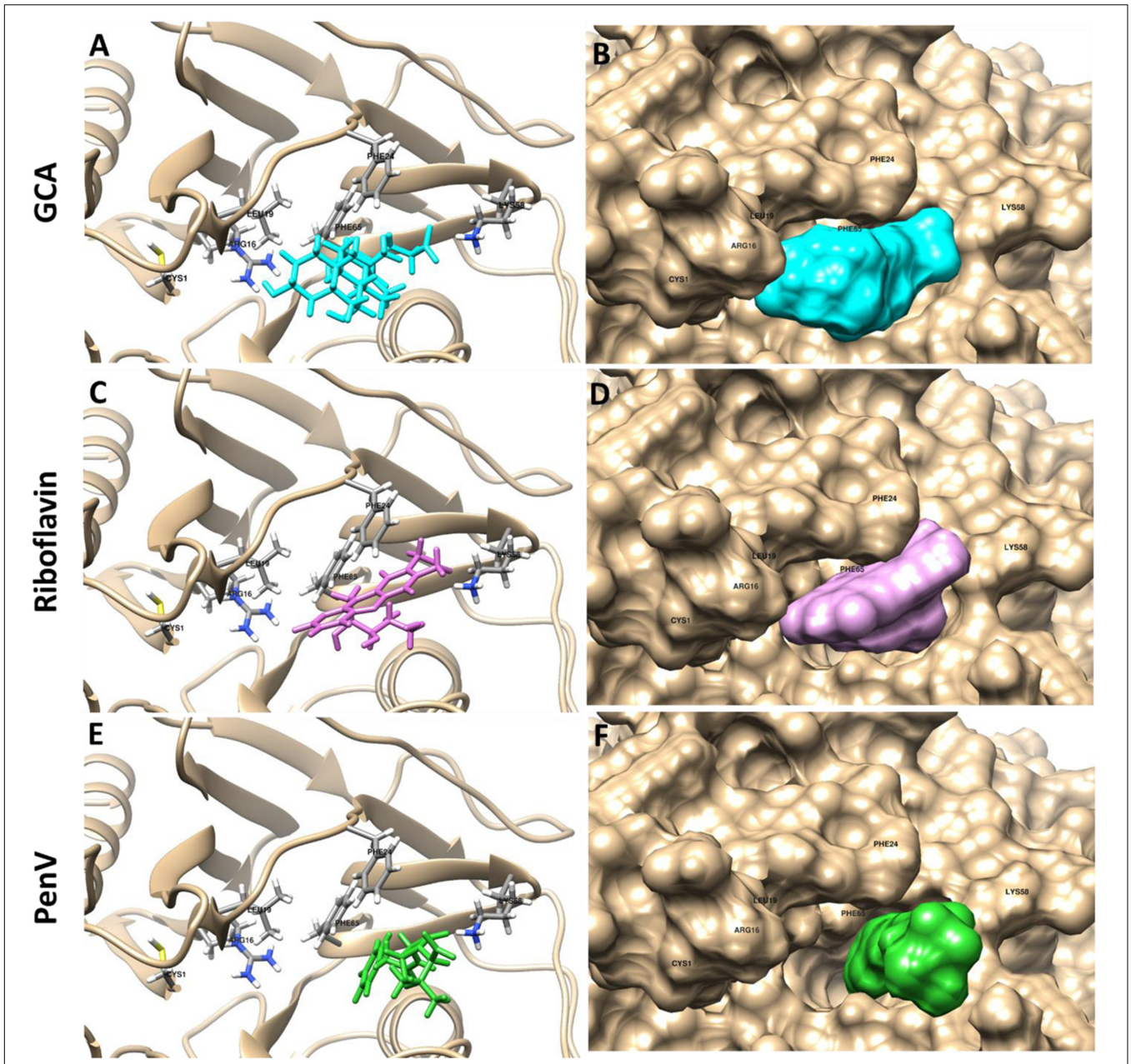

FIGURE 7 | Docking analysis of GCA (**A,B**: cyan), riboflavin (**C,D**: pink), and penicillin V (**E,F**: green) with *Lg*BSH to determine the inhibitory effects. Docking was performed using SwissDock and the outputs were analyzed using UCSF-Chimera.

at 41°C and pH 5.4. Kumar et al. (2013) also found that the optimal pH and temperature for enzymatic activity of BSH from *L. fermentum* NCDO394 were pH 6.0 and 37°C, respectively.

The evolution of protein structures is relatively slower than the evolution of nucleic acids, thus providing stability to protein structures. Proteins with similar sequences have retained identical structure during evolution, and proteins from distantly related organisms with less sequence similarity also have similar protein structures and retain similar functions (Kaczanowski and Zielenkiewicz, 2010; Chae et al., 2013). Although BSHs of various species had the highest sequence divergence, the protein structures were still very similar. Hence, homology modeling is an ideal approach to obtain the putative structure of the identified BSH enzyme, thus helps to understand the characteristics of the substrate-binding pocket and other functions of the enzyme. Superimposing the structure of *Lg*BSH onto that of *Ef*BSH revealed similarities of protein structures and binding pockets. Since BSH and PVA evolved from the same origin, both enzymes have the same catalytically important

active site residues (Cys1, Arg16, Asp19, Asn79, Asn171, and Arg224) except for Asn79, where PVA uses tyrosine instead of asparagine (Rossocha et al., 2005). Recently, Xu et al. (2016) resolved the crystal structure of the BSH enzyme from *L. salivarius,* which had 47% sequence similarity with *Lg*BSH. Hence, we used this crystal structure to characterize our *Lg*BSH. Two loops (loops I and II) were identified from the substrate-binding pocket of *Lg*BSH. According to Xu et al. (2016), residues Leu133 (Leu133 in *Ls*BSH and Leu132 in *Ef* BSH) and Phe129 (Phe129 in *Ls*BSH and Phe128 in *Ef* BSH) in loop II might contribute to limiting the spatial configuration via condensing the entrance of the substrate-binding pocket. Similarly, the Phe24 (Tyr23 in *Ls*BSH and Tyr23 in *Ef* BSH) residue in loop I along with the Phe64 (Phe64 in *Ls*BSH and Phe64 in *Ef* BSH) residue might allow the substrate to intensely dock into the substrate-binding pocket, and thus may also be involved in enzyme–substrate interactions (Rossocha et al., 2005; Xu et al., 2016). The mode of substrate binding is dependent on the loops surrounding the active site, which also define the volume of the site (**Figure 5B**). Polar complementarity toward conjugated bile acids was also proposed as an important facet of substrate specificity (Chand et al., 2017). *Lg*BSH exhibited substrate specificity toward glyco-conjugated bile salts and had the lowest binding energy. Similarly, BSHs from most bacterial species had substrate preferences toward glyco-conjugated bile salts rather than tauro-conjugated ones. The major reasons for these phenomena include, steric hindrance caused by a sulfur atom of taurine and an abundance of glyco-conjugated bile salts in nature (Chand et al., 2017).

Recent epidemiological studies suggests that the use of AGPs is related to the emergence of antibiotic-resistant bacteria which may be transmitted to humans and cause significant threats to food safety and public health (Lin, 2014). However, a ban on AGP usage would create challenges for the animal feed and feed-additive industries. Several products, viz., probiotics, prebiotics, and organic acids, are used to change the intestinal microbiota to improve animal health and production. Studies using swine and poultry fostered understanding of the relationships between AGP supplementation and GIT bacterial compositions. Results of several studies proved that AGPs have created bacterial shifts, have altered the microbial diversity of the intestines, and suggested that certain GIT populations might be more related to animal growth (Danzeisen et al., 2011; Kim et al., 2012; Lin, 2014). However, probiotic feed supplements lead to decreased body mass gains in treated animals. This can be overcome by feeding animals with suitable BSH inhibitors along with the probiotic feed. A high purity of BSH enzymes is necessary to screen BSH inhibitors and study the substrate specificity of the enzyme. Hence, we have purified the BSH enzyme using His tag and used screened various compounds to identify potential BSH inhibitors. The results suggested that $CuCl_2$ decreased BSH activity by 98.13% among all of the tested metal ions (**Table 2**). Wang et al. (2012) demonstrated that copper ($CuCl_2$) and zinc ($ZnSO_4$) inhibited BSH activities by 98.1 and 89.5%, respectively. Conversely, we found that inhibition of *Lg*BSH activity by zinc ($ZnCl_2$ or $ZnSO_4$) was not significant. We also tested the inhibitory effects of currently used AGPs and found that all tested antibiotics (except erythromycin and lincomycin) had significant inhibitory effects on *Lg*BSH. However, the long-term use of these metal ions and AGPs might affect animal health, increase production costs and accumulation of metal ions in treated animals. Recently, Smith et al. (2014) performed high-throughput screening (HTS) to identify safe and cost-effective BSH inhibitors and reported that riboflavin and phenethyl caffeate can act as potential BSH inhibitors. These results were further supported by Lin et al. (2014), who reported that riboflavin and CAPE could inhibit BSH activity. Hence, we used riboflavin as a novel inhibitor and found that *Lg*BSH activity was reduced by up to 98.31% (**Table 2**). Riboflavin (or vitamin B2) play key roles in various intracellular (i.e., energy metabolism) and extracellular (i.e., quorum sensing and extracellular electron transfer) processes in bacteria. Hence, riboflavin has been used as a feed additive for several animals to overcome vitamin B2 deficiencies (Smith et al., 2014; Gutiérrez-Preciado et al., 2015). The Food and Drug Administration (FDA) of United States of America (USA) has already approved riboflavin as a feed additive, since it has well-known metabolic functions (Smith et al., 2014). The addition of riboflavin along with the probiotic *L. gasseri* FR4 can serve as a BSH inhibitor and also increase survival rates of probiotic bacteria. Gutiérrez-Preciado et al. (2015) performed extensive analyses on riboflavin transporters across all bacterial strains and found that *L. gasseri* has its unique transporter for riboflavin. Stahly et al. (2007) conducted a swine study and reported that dietary supplementation with riboflavin (20 mg/kg feed) significantly enhanced the body weight and feed efficiency in pigs. The current studies suggested this might be due to inhibition of the BSH enzyme produced by probiotic microorganisms present in the swine's intestines. To further unravel the mechanism behind the inhibitory effect of riboflavin, we performed a molecular docking analysis using the predicted *Lg*BSH structure. Results showed that both substrate and inhibitors (riboflavin and penicillin V) could bind to the substrate-binding pocket of *Lg*BSH with almost the same binding affinity/energy (**Figure 7**). This might have been due to the inverse mode of binding of riboflavin and penicillin V. This study provides some new insights into the inhibitory effects of several potential BSH inhibitors, and these might serve as alternatives for AGPs.

CONCLUSION

In this study, we identified and characterized a novel BSH enzyme from probiotic *L. gasseri* FR4. *Lg*BSH showed greater hydrolysis toward glyco-conjugated bile salts than to tauro-conjugated bile salts, and no hydrolysis was observed on penicillin V. The homology modeling studies revealed the 3D structure of *Lg*BSH, which has a structural similarity with previously identified BSH enzymes. *Lg*BSH activity was dramatically inhibited by riboflavin and confirmed by molecular docking analysis. Riboflavin had almost the same binding

energy as GCA, thus helping in the inhibition of *Lg*BSH. Hence, supplementation of *L. gasseri* FR4 along with riboflavin might be used as an alternative for AGPs for poultry animals. However, detailed animal studies are necessary to further confirm the efficiency of BSH inhibitors on animal growth and performance.

AUTHOR CONTRIBUTIONS

AR, RR, and MA contributed with the conception and experimental design. RR, carried out all experiments. MA and RR performed the homology modeling and docking analysis. MA and RR analyzed and interpreted the results. RR written the manuscript and corrected by AR and MA. All authors performed a critical revision of the manuscript and approved the final version.

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ORIGINAL RESEARCH
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Propionibacterium freudenreichii Surface Protein SlpB Is Involved in Adhesion to Intestinal HT-29 Cells

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Propionibacterium freudenreichii is a beneficial bacterium traditionally used as a cheese ripening starter and more recently for its probiotic abilities based on the release of beneficial metabolites. In addition to these metabolites (short-chain fatty acids, vitamins, and bifidogenic factor), *P. freudenreichii* revealed an immunomodulatory effect confirmed *in vivo* by the ability to protect mice from induced acute colitis. This effect is, however, highly strain-dependent. Local action of metabolites and of immunomodulatory molecules is favored by the ability of probiotics to adhere to the host cells. This property depends on key surface compounds, still poorly characterized in propionibacteria. In the present study, we showed different adhesion rates to cultured human intestinal cells, among strains of *P. freudenreichii*. The most adhesive one was *P. freudenreichii* CIRM-BIA 129, which is known to expose surface-layer proteins. We evidenced here the involvement of these proteins in adhesion to cultured human colon cells. We then aimed at deciphering the mechanisms involved in adhesion. Adhesion was inhibited by antibodies raised against SlpB, one of the surface-layer proteins in *P. freudenreichii* CIRM-BIA 129. Inactivation of the corresponding gene suppressed adhesion, further evidencing the key role of *slpB* product in cell adhesion. This work confirms the various functions fulfilled by surface-layer proteins, including probiotic/host interactions. It opens new perspectives for the understanding of probiotic determinants in propionibacteria, and for the selection of the most efficient strains within the *P. freudenreichii* species.

Keywords: adhesion, immunomodulation, surface proteins, probiotic, SlpB

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INTRODUCTION

Propionibacterium freudenreichii is a GRAS (Generally Recognized As Safe) actinobacterium consumed in high amounts in fermented dairy products. It is a beneficial bacterium used in the food industry for the production of vitamins, for cheese ripening, and for its probiotic properties (Cousin et al., 2010). Probiotics are defined as "living microorganisms which when administered in adequate amounts confer a health benefit on the host" (Food and Agriculture Organization of the United Nations and World Health Organization, 2002). *P. freudenreichii* indeed revealed probiotic

traits including modulation of intestinal inflammation (Mitsuyama et al., 2007; Foligné et al., 2010, 2013), as well as properties linked to the production of beneficial metabolites such as short-chain fatty acids (Jan et al., 2002; Lan et al., 2007; Cousin et al., 2012b), vitamins and the bifidogenic compound 1,4-dihydroxy-2-naphthoic acid (DHNA) (Bouglé et al., 1999; Kaneko, 1999; Hojo et al., 2002; Ouwehand et al., 2002; Seki et al., 2004; Mitsuyama et al., 2007).

Microorganisms that live in or transit through the digestive tract of humans may establish a symbiotic relationship with the host, thus promoting intestinal homeostasis (de Souza and Fiocchi, 2016). Consumption of *P. freudenreichii* selected strains can enhance human complex intestinal microbiota through the increase of other beneficial bacteria populations, such as bifidobacteria (Bouglé et al., 1999; Kaneko, 1999; Hojo et al., 2002; Ouwehand et al., 2002; Seki et al., 2004; Mitsuyama et al., 2007). In contrast, out of normal physiological conditions, the digestive microbiota may be involved in a variety of immune and inflammatory disorders (Vitetta et al., 2014). One example is inflammatory bowel diseases (IBD), chronic inflammatory disorders that severely affect the digestive tract and may lead, in the long term, to the irreversible deterioration of their structure and function (Belkaid and Hand, 2014; Vitetta et al., 2015). Cheese containing *P. freudenreichii,* in conjunction with *Lactobacillus delbrueckii* (Plé et al., 2016) or as a single strain (Plé et al., 2015), was recently shown to exert immunomodulatory effects, to protect mice against TNBS-induced colitis, to alleviate the severity of symptoms and to modulate local and systemic inflammation markers. Such cheese is currently tested in a pilot clinical trial (ClinicalTrials.gov, 2017). Interestingly, removal of propionibacteria surface-layer (S-layer) proteins, which are non-covalently anchored to the cell surface via an S-layer homology (SLH) domain, suppressed the induction of anti-inflammatory cytokines (Foligné et al., 2010). By contrast, some *P. freudenreichii* strains that possess an extracellular polysaccharide capsule fail to immunomodulate, while mutagenetic suppression of this capsule confers immunomodulatory activity (Deutsch et al., 2012).

Surface proteins of *P. freudenreichii* ITG P20 [*Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire* (CIRM-BIA) 129], which is used as a cheese ripening starter (Richoux et al., 1998; Thierry et al., 2004), were investigated by a combination of proteomic methods previously developed for bacteria and eukaryotic cells (Lortal et al., 1993; Mayrhofer et al., 2006; Rodríguez-Ortega et al., 2006; Berlec et al., 2011; Bøhle et al., 2011; Bensi et al., 2012; Ythier et al., 2012; Michaux et al., 2013). This investigation demonstrated the involvement of certain S-layer proteins in immunomodulation (Bryson et al., 2006; Le Maréchal et al., 2015). Surface proteins, susceptible to enzymatic shaving and to guanidine extraction, were shown to be involved in the ability of *P. freudenreichii* to modulate the release of cytokines by human immune cells (Le Maréchal et al., 2015). However, the respective role of the different bacterial S-layer proteins was not fully elucidate. Immunomodulation is favored by the ability of specific strains to adhere to the host cells and mucus (Tuomola et al., 1999; Ouwehand et al., 2000; Huang and Adams, 2003; Thiel et al., 2004; Le Maréchal et al., 2015). Indeed, the local action of metabolites and of immunomodulatory molecules is favored by the ability of probiotics to adhere to the host cells. Dairy propionibacteria were shown to adhere to mice intestinal epithelial cells both *ex vivo* and *in vivo* (Zarate, 2012) as well as to cultured human intestinal cell lines *in vitro* (Huang and Adams, 2003; Moussavi and Adams, 2010). However, the precise mechanisms are poorly characterized in *P. freudenreichii*. Adhesion moreover constitutes a key criterion in strain selection and is described as the initial step for colonization of the host (Havenaar et al., 1992, havenar; Riedel et al., 2006; Preising et al., 2010), depending on crucial surface compounds, including surface proteins (Lebeer et al., 2010).

The identification of adhesion mechanisms and molecules is a fundamental step in the elucidation of the bacterium/host cross-talk (van de Guchte et al., 2012). This was lacking in probiotic dairy propionibacteria. The aim of our study was thus to identify *P. freudenreichii* protein(s) involved in adhesion to human intestinal epithelial cells.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *P. freudenreichii* wild-type (WT) strains, genetically modified strain and plasmids used in this study are listed in **Table 1**. All strains in this study were obtained from the collection of the CIRM-BIA (STLO, INRA Rennes, France). All *P. freuderenichii* WT strains were grown at 30°C in YEL broth (Malik et al., 1968) without agitation or in cow's milk ultrafiltrate supplemented with 50 mM of sodium L-lactate (galaflowSL60, Société Arnaud, Paris, France) and 5 g/L of casein hydrolysate (Organotechnie, La Courneuve, France), sterilized by 0.2 μm filtration (Nalgene, Roskilde, Denmark) as described previously (Cousin et al., 2012a). For genetically modified strains, YEL and Milk Ultrafiltrate culture media were supplemented with chloramphenicol (10 $\mu g\ ml^{-1}$). The growth of *P. freudenreichii* strains was monitored spectrophotometrically by measuring the optical density at 650 nm (OD650), as well as by counting colony-forming units (CFUs) in YEL medium (Malik et al., 1968) containing 1.5% agar. *P. freudenreichii* strains was harvested in a stationary phase (76 h, 10^9 CFU/mL, determined by plate counts) by centrifugation (6,000 × *g*, 10 min, 4°C). *Escherichia coli* strain DH5α was grown in Luria–Bertani medium at 37°C, and cells carrying DNA plasmid were selected by addition of ampicillin (100 $\mu g\ ml^{-1}$).

Enzymatic Shaving of Surface Proteins

One hundred microliter of propionibacteria stationary phase culture (see above) were harvested by centrifugation (6,000 × *g*, 10 min, 4°C) and washed in an equal volume of PBS [pH 8.5] containing 5 mM DTT before resuspension in 1/10 volume of the same buffer. Sequencing grade modified trypsin (V5111, Promega, Madison, WI, United States) was dissolved in the same buffer (qsp 0.2 g/L) and added to the bacterial suspension. "Shaving" was performed for 1 h at 37°C in a 0.5 mL reaction volume containing 5 × 10^9 bacteria and 4 μg of trypsin,

TABLE 1 | *Propionibacterium freudenreichii* wild-type strains, their genetically modified derivatives and plasmids used in the study.

Strains and plasmids	Relevant genotype and phenotype	Source or reference
Strains *P. freudenreichii*[a]		
CB 118	Wild-type; SlpA, SlpB, SlpE, and InlA proteins detected in guanidine extract[b]	CIRM-BIA
CB 121	Wild-type; InlA and LspA proteins detected in guanidine extract	CIRM-BIA
CB 129	Wild-type; SlpA, SlpB, SlpE, InlA, and LspA proteins detected in guanidine extract	CIRM-BIA
CB 134	Wild-type; SlpA, SlpE, InlA, and LspA proteins detected in guanidine extract	CIRM-BIA
CB 136	Wild-type; SlpA, SlpB, InlA, and LspA proteins detected in guanidine extract	CIRM-BIA
CB 508	Wild-type; SlpA, SlpB, SlpE, InlA, and LspA proteins detected in guanidine extract	CIRM-BIA
CB 527	Wild-type; Absence of surface layer proteins in guanidine extract	CIRM-BIA
CB 129Δ*slpB*	Cm^r; CIRM-BIA 129 with chromosomal insertion of pUC:Δ*slpB*:CmR in the *slpB* sequence; SlpB protein absent in guanidine extract	This Study
Plasmids		
pUC:slpB	pUC18; Amp; harboring *slpB* partial gene sequence for inactivation	This Study
pUC:ΔslpB:CmR	pUC18 carrying a chloramphenicol resistance gene and harboring *slpB* partial gene sequence	This Study

[a]*CB, CIRM-BIA, Centre International de Ressources Microbiennes–Bactéries d'Intérêt Alimentaire, INRA, UMR 1253, Science et Technologie du Lait et de l'Oeuf, Rennes, France.* [b]*Guanidine Hydrochloride treatment used to extract surface layer associated proteins non-covalently bound to the surface is described in "Materials and Methods."*

with gentle agitation (180 rpm). Bacteria were removed by centrifugation (8,000 × *g*, 10 min, 20°C) and subjected to three washes in PBS prior to adhesion assay.

Cell Line and Culture Conditions

The human colon adenocarcinoma cell line HT-29 was obtained from ATCC (American Type Culture Collection, Rockville, MD, United States). These cells was cultured under conditions of 37°C, 5% CO_2, and 90% relative humidity in DMEM High Glucose with L-Glutamine with Sodium Pyruvate (PAN, Dominique Dutscher, Brumath, France) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAN, Dominique Dutscher, Brumath, France) and antibiotics or not (for adhesion assays).

Electroporation and Inactivation of the *slpB* Gene in *P. freudenrenichii* CIRM-BIA 129 by Suicide Vector

Inactivation of *P. freudenreichii* gene was adapted from Deutsch et al. (2012) with some modifications. For insertional inactivation of a *slpB* gene, a 520-bp DNA fragment homologous to nucleotides 30–550 of the 5′ region of the slpB coding region in *P. freudenreichii* CIRM-BIA 129 genome was synthesized by Genscript Inc.[1] with restriction sites *Xba*I-*slpB*-5′ and *BamH*I-*slpB*-3′ resulting in pUC:Δ*slpB* plasmid. The pUC:Δ*slpB* plasmidic DNA was digested with *XbaI* and *BamH*I, purified, and cloned in plasmid pUC:CmR digested by the same enzymes, which resulted in the suicide vector pUC:Δ*slpB*:CmR, which was confirmed by sequencing. See **Supplementary Figure S2**.

Electrocompetent *P. freudenreichii* CIRM-BIA 129 cells was prepared as previously described (Deutsch et al., 2012) with slight modifications. They were cultured in YEL medium supplemented with 0.5 M sucrose and 2% glycine until the early exponential growth phase (OD = 0.1), harvested (6,000 × *g*, 10 min, 4°C). The pellet was washed extensively in ice-cold 0.5 M sucrose and resuspended in electroporation buffer containing 0.5 M sucrose with 10% glycerol and 1 mM potassium acetate (pH 5.5). For electroporation, a 100-µl aliquot of the electrocompetent cells was mixed with 3 µg of pUC:Δ*slpB*:*Cm*R plasmid DNA in a cooled electroporation cuvette. The electroporation of *P. freudenreichii* CIRM-BIA 129 was performed with a Gene Pulser Xcell™ (Bio-Rad Laboratories, Richmond, CA, United States) at 20 kV/cm, 200-Ω resistance, and 25-µF capacitance. Immediately after the pulse, 900 µL of YEL containing 0.5 M sucrose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$ were added before incubation, 24 H at 30°C under microaerophilic conditions. Cells were plated, and incubated 7 days at 30°C under anaerobic conditions, on YEL medium containing 1.5% agar (YELA) supplemented with 10 $\mu g \cdot ml^{-1}$ of chloramphenicol in order to select *P. freudenreichii* mutants harboring inserted pUC:Δ*slpB*:*Cm*R. The *P. freudenreichii* CIRM-BIA 129 Δ*slpB* (CB129Δ*slpB*) mutant strain was further checked by proteomics for the absence of intact SlpB surface proteins as indicated in the "Results" section. Moreover, the stability of the insertion was checked after three independent cultures in YEL and Milk Ultrafiltrate media without chloramphenicol.

In Vitro Adhesion Assays

Adhesion of *P. freudenreichii* (WT and mutant) to the human colon adenocarcinoma cell line HT-29 was examined by adding 10^8 live propionibacteria (washed twice in PBS, numerated by CFU conting, ratio 100 bacteria:1 HT-29 cell, MOI 100) to 10^6 cells in DMEM culture medium without antibiotics. Adhesion assay was conducted by incubation of bacteria/cell at 37°C for 60 min under conditions, 5% CO_2 and 90% relative humidity. Cells were washed twice with prewarmed PBS pH 7.4, and the subsequently supernatant was removed, and 400 µL of trypsin-EDTA (Invitrogen) was added to each well, before incubation for 5 min at 37°C and to trypsin inactivation by adding 800 µL of DMEM culture medium without antibiotics. Cells were harvested (3,000 × *g*, 3 min) and lysed in 0.1% Triton X-100 before serial dilutions and plating on YELA. Finally, plates were incubated at 30°C for 5 days under anaerobic conditions. A rate of adhesion was calculated as follows: (bacterial count after adhesion experiment/bacterial population added on to HT29 cells). The CIRM-BIA 129 WT strain was then used as a reference in this work, with a % adhesion of 100, and used

[1]www.genscript.com

to normalize all other adhesion rates as a percentage of CIRM-BIA 129 WT adhesion. Each adhesion assay was conducted in technical and biological triplicates. To test involvement of surface proteins in adhesion, propionibacteria were subjected (or not) to enzymatic shaving (see section "Enzymatic Shaving of Surface Proteins") before adhesion assay. To confirm this hypothesis, propionibacteria were incubated 60 min at 37°C with 50 μg of *P. freudenreichii* CIRM-BIA 129 guanidine-extracted S-layer associated proteins, in solution in PBS, under agitation, before adhesion. This amount (50 μg) was determined after preliminary experiments to determine amounts efficient in restoring adhesion. For specific inhibition of adhesion by antibodies directed against SlpB, propionibacteria were incubated in PBS pH 7.4 with immunoglobulins purified from rabbit anti-SlpB serum (AGRO-BIO, France) in 1:10.000 dilution, under agitation, 60 min at 37°C. Propionibacteria were washed twice with PBS pH 7.4 before adhesion assay.

The adhesion ratio of CB 129 strain alone was used as a reference to calculate the adhesion rates of different strains and treatments.

Internalization of bacteria was determined as previously described (Bouchard et al., 2013) 2-h post contact following an additional 2-h incubation step with DMEM supplemented with gentamicin (100 μg/ml) to kill extracellular bacteria. Subsequently, HT-29 cells monolayers were washed three times with PBS, treated with trypsin, centrifuged for 5 min at 800 × *g*, and lysed in 0.01% Triton to allow the numeration of internalized propionibacteria population only.

Bacterial Cell Adhesion Observation by Microscopy

Observation of *P. freudenreichii* adhesion to cultured human colon epithelial cells was as described previously for lactobacilli (Tiptiri-Kourpeti et al., 2016), with modifications for propionibacteria. Briefly, propionibacteria, cultured as described above, were washed and resuspended in PBS, prior to the addition of 20 μM CFSE (carboxyfluorescein succinimidyl ester, cell trace proliferation kit for flow cytometry ref C34554 Thermo Fisher Scientific, Waltman, MA, United States), freshly prepared as a 1,000× solution in DMSO and kept in the dark. Incorporation of CFSE was allowed for 30 min at 30°C in the dark, prior to washing and resuspension of propionibacteria in YEL medium, 30°C. Hydrolysis of CFSE by intracellular esterase activity was allowed 30 min at 30°C in the dark to generate intracellular fluorescence. Fluorescence was checked on an epifluorescence microscope (BX-51, Olympus, equipped with a U-MWB2 fluorescence filter cube). HT-29 cells were cultured in Lab-Tek chamber slide (Thermo Fisher Scientific) and labeled bacteria were added to a 1/100 ratio (1×10^6 cells, 1×10^8 bacteria, in 1 mL of DMEM) before incubation, 1 h, 37°C. After two washes with PBS, the plasma membrane of cells was then labeled with the exogenous head-labeled phospholipid fluorescent probe *N*-(Lissamine rhodamine B sulfonyl) dioleoyl phosphatidylethanolamine (Rh-DOPE, Avanti Polar Lipids Inc., Birmingham, England) used at a concentration of 1 μg/mL in DMEM, for 10 min. Cell layer was then washed twice with PBS and slides mounted in DAPI (4,6-diamidino-2-phenylindole)-containing mounting medium (Vectashield mounting medium for fluorescence Vector ref *H-1200*). Cells were observed using a confocal Leica SP8 and a 63/1.4 oilHC PL APO CS objective. Images were acquired using LAS-AF (Leica, Wetzlar, Germany) software.

For scanning electron microscopy, HT-29 cells were cultured in Corning® Transwell® polycarbonate membrane cell culture inserts on polycarbonate 0.4 μm pore size filtration membrane. Adhesion was conducted as described above. Membranes were then removed, washed in PBS, fixed 48 h by 2% (wt/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer [pH 6.8] and rinsed in the same buffer. Samples were dehydrated with ethanol (10, 25, 50, 75, 95, and finally 100%), critical-point dried by the CO_2 method and coated with gold. Cells were examined and photographed with a Philips XL 20 scanning electron microscope operating at 10 kV.

Bacterial Cell Adhesion Determination by Cytometric Analysis

Determination of *P. freudenreichii* adhesion to cultured human colon epithelial cells was performed as described previously for lactobacilli (Tiptiri-Kourpeti et al., 2016). Cells were cultured in DMEM as described above to confluence. CFSE-labeled bacteria were added as described above before a 1-h incubation at 37°C. Cells were trypsinized and analyzed by fluorescence cytometry using an excitation wavelength of 488 and emission at 585 nm (Accuri C6 Becton Dickinson, Le Pont-de-Claix, France). Data were collected from 50,000 cells and analysis was performed with CFlow software.

Guanidine Extraction of Surface Layer Associated Proteins Non-covalently Bound to the Cell Wall

Propionibacteria cultures in stationary phase (76-h) were collected by centrifugation (8,000 × *g*, 10 min, 4°C) for extraction of S-layer proteins by Guanidine Hydrochloride (GuaHCl) (Le Maréchal et al., 2015). The bacterial pellet was washed two times with an equal volume of PBS buffer pH 7.4. This pellet was resuspended in 5 M GuaHCl to a final OD_{650} of 20 then incubated 15 min at 50°C, and subsequently, the suspension was centrifuged (21.000 × *g*, 20 min, 30°C). The cells were eliminated, and the supernatant was dialyzed extensively against PBS buffer pH 7.4 (for adhesion assays) or 0.1% SDS (for SDS–PAGE analysis) for 24 h at 4°C using Slide-A-Lyer® Dialysis Cassette (ThermoScientific, Rockford, IL, United States). This procedure was applied in three independent cultures.

One-Dimensional SDS–Polyacrylamide Gel Electrophoresis (1-DE) and Western Blotting

Extracts of S-layer proteins in 0.1% SDS were diluted in SDS sample buffer and then heat-denatured 10 min at 95°C. One-dimensional polyacrylamide gel electrophoresis (10.0%) was conducted according to Laemmli (Laemmli, 1970) on a Mini-PROTEAN® Tetra Cell (Bio-Rad, Hercules, CA, United

States) and the gels were stained using Coomassie Blue Bio-Safe reagent (Bio-Rad). Alternatively, S-layer protein associated extracts were separated by 10% SDS–PAGE and transferred to PVDF membranes (GE Healthcare). After blocking with 3% non-fat dry milk diluted in TBS (Tris 10 mM, NaCl 0.15 M, 0.3% tween 20), the membranes were incubated overnight at 4°C with primary antibodies purified from rabbit sera (AGRO-BIO, France). These were obtained by injecting the following slpB peptide to rabbits: IDATVDKQNSKGGFGWGG and used at the dilution 1:10,000. After washing, membranes were incubated with secondary antibodies: anti-rabbit IgG conjugated with horseradish peroxidase (1:15,000, AGRO-BIO, France) for 2 h at room temperature. Bound antibodies were visualized with ECL Plus system (GE Healthcare, Vélizy, France) and blots were scanned using the Syngene GBox (Ozyme, Saint-Quentin-en-Yvelines, France). The specificity of anti-SlpB western blotting was checked (**Supplementary Figure S1**). A single band was observed only in strains expressing SlpB and the labeling pattern was distinct from that of anti-SlpA and anti-SlpE western blotting.

Data Analysis

All the experiments were performed with three technical replicates and three biological replicates, and the results were expressed as means ± standard deviations (SD). Statistical analyses were performed in R Statistical Software (Foundation for Statistical Computing, Vienna, Austria) using ANOVA with Tukey *post hoc* analyses for multiple comparisons.

RESULTS

Surface Layer Associated Proteins and Adhesion to Cultured Human Colon Cells Are Variable among Strains of *P. freudenreichii*

Seven strains of *P. freudenreichii* from the CIRM-BIA collection (**Table 1**), CB 118, CB 121, CB 129, CB 134, CB 136, CB 508, and CB 527, have been selected based on preliminary proteomic screening as they all displayed different surface proteomes as shown by their S-layer associated protein pattern after guanidine treatment (**Figure 1A**). The five proteins, previously identified in CB 129 (SlpA, SlpB, SlpE, InlA, and LspA, see Le Maréchal et al., 2015), and thought to play a role in interactions with the host, are indicated in the figure. Preliminary results pointed out SlpB as a potential key surface protein in *P. freudenreichii*. We thus developped antibodies in order to confirm this. Western blot analysis using these antibodies further confirmed variability of surface proteins (**Figure 1B**). SlpB was detected in four strains out of seven, with different intensities. The variability of S-layer associated proteins suggested possible variations regarding interactions with host cells. The seven strains were further compared with respect to adhesion to HT-29 cultured colon cells (**Figure 1C**). The CB129 strain, exhibited the highest adhesion rate (6.44 CFU/1 HT-29 cell) and was used as the reference (100% adhesion) for comparison with the other strains (100.0% ± 17). Indeed, CB129 showed a significant difference ($p < 0.001$) with the other *P. freudenreichii* strains tested under the same experimental conditions. The CB118 strain exhibited a

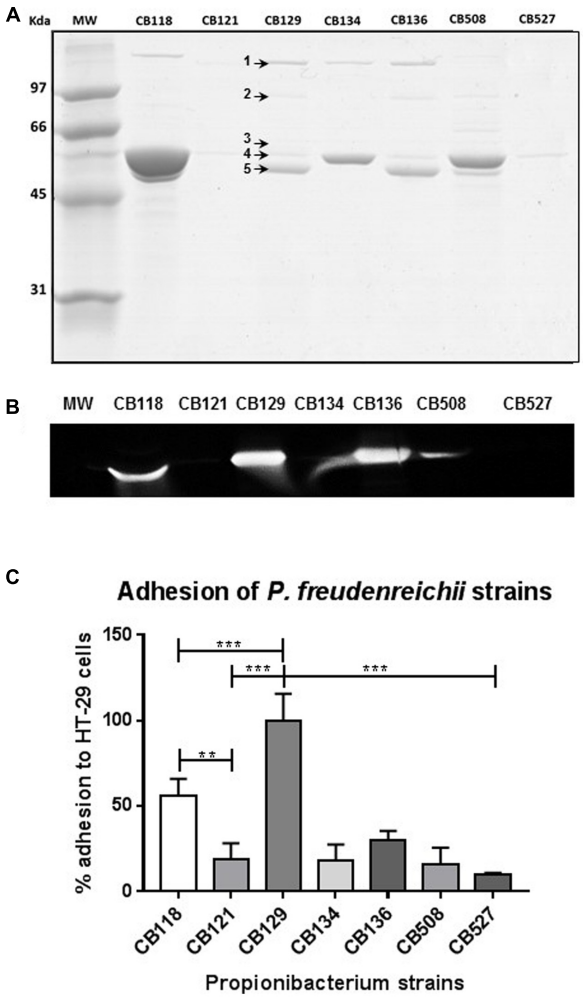

FIGURE 1 | Variability of surface proteome and of adhesion among strains of *Propionibacterium freudenreichii*. **(A)** Guanidine-extracted surface layer associated proteins are variable. Seven strains of *P. freudenreichii* were cultured in milk ultrafiltrate and subjected to guanidine-extraction followed by SDS–PAGE (10%) gel electrophoretic analysis of the extracts. Gels were either Coomassie-Blue-stained **(A)** or transferred to a PVDF membrane. Surface proteins previously identified by mass spectrometry as InlA, LspA, SlpE, SlpA and SlpB in strain CB129 are indicated by 1, 2, 3, 4, and 5, respectively. **(B)** Western Blotting detection of surface layer protein SlpB. PVDF membranes were treated using rabbit antibodies raised against *P. freudenreichii* surface layer protein SlpB. **(C)** Adhesion to cultured human colon epithelial cells is variable. HT-29 cells were cultured to confluence in DMEM prior to co-incubation. Each well (1×10^6 HT-29 cells) was added with 1×10^8 colony-forming unit (CFU) of *P. freudenreichii*. Co-incubation was 60 min at 37°C in DMEM. After thorough washing with PBS, adhered bacteria were enumerated by CFU plate counting in trypsinized cells. Numbers of the strains used are indicated. Asterisks represent statistically significant differences between strains and were indicated as follows: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Adhesion is presented as a percent of the reference CB129 *P. freudenreichii* strain. Original gels and western blots, uncropped, are provided in **Supplementary Figure S1**.

lower but significant adherence percentage of 56.0% ± 10.0 and also displayed SlpB. All the other strains exhibited low adhesion rates without significant differences among them, although CB136 (30.0% ± 5.0), which also displays SlpB, tended to be more adhesive than the rest of this subset. Finally, the lowest adhesion rate was recorded for CB527, 10.0% ± 1.0, for which no surface protein was detected, in accordance with (Deutsch et al., 2017). Different propionibacteria: HT-29-cells ratios were tested for adhesion (100:1, 500:1, and 1,000:1, in technical and biological triplicates) with similar results in adhesion rates ranking. At the MOI of 100:1 used in this study, no internalization of *P. freudenreichii* was observed (data not shown) using the gentamicin method used by our team to monitor staphylococci internalization (Bouchard et al., 2013).

P. freudenreichii CB129 Interacts with Cultured Human Colon Cells

Adhesion of *P. freudenreichii* to HT-29 cells being demonstrated, we further looked at such an interaction, using three-dimensional confocal microscopy. As seen in **Figure 2A**, the sections close to the bottom of the slide culture chamber mainly exhibited the blue fluorescence of the HT-29 nuclei, stained with DAPI, a poorly fluorescent cytoplasm, surrounded by a red-stained plasma membrane (lowest images in **Figure 2A**). Ascending within this "z-stack," higher sections showed dots with intense red fluorescence, corresponding to cell membranes, indicative of colonocytes microvilli constituting the brush border. Higher sections showed co-localization of these red dots with green-fluorescent propionibacteria, caused by CFSE metabolization within propionibacteria. More precisely, propionibacteria appeared as aggregates, in the intercellular space of the epithelial HT-29 monolayer. This localization of propionibacteria in contact with cells is further illustrated in the reconstituted 3-D view (**Figure 2B**). Interaction of propionibacteria with cultured human colonocytes was further illustrated by scanning electron microscopy of co-cultures on cell culture inserts (**Figure 2C**). This revealed localization of propionibacteria at the surface of cells, in contact with the brush border.

P. freudenreichii CB129 Adhesion to Cultured Human Colon Cells Involves Surface Proteins

To determine whether the presence of surface proteins is involved in the adhesion of *P. freudenreichii* to HT-29 cells, the method

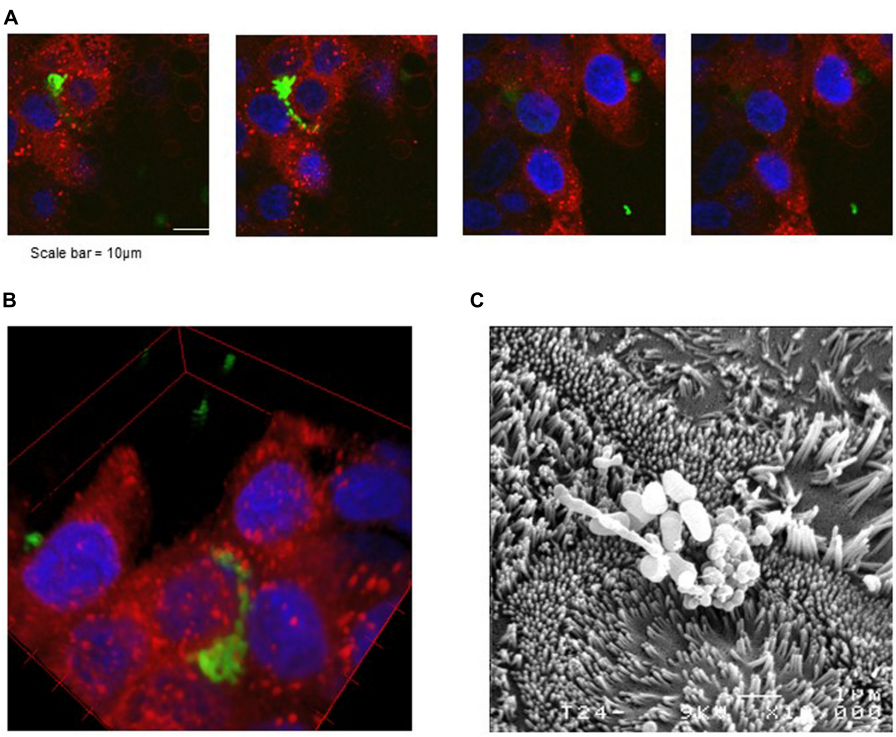

FIGURE 2 | Microscopy imaging of *P. freudenreichii Centre International de Ressources Microbiennes–Bactéries d'Intérêt Alimentaire* (CIRM-BIA) 129 adhesion to cultured human colon epithelial cells. HT-29 cells were cultured to confluence in DMEM on a slide chamber prior to interaction. *P. freudenreichii* was cultured in fermented milk ultrafiltrate prior to intracellular labeling of live bacteria using CFSE. Labeled bacteria were then co-incubated with colon cells in a slide chamber prior to washing with PBS and to staining of plasma membrane using Rh-DOPE and mounting in DAPI-containing mounting medium. **(A,B)** Blue fluorescence evidences colon cells nuclei, red fluorescence their plasma membrane and green fluorescence CFSE-labeled propionibacteria. **(A)** Z-stack, i.e., confocal images acquired at different "z" altitudes in the labeled preparation. **(B)** Reconstituted 3-D image showing a cluster of propionibacteria at surface of cells. **(C)** Scanning electron microscopy observation of propionibacteria adhesion to cultured colon epithelial cells. The same co-incubation was performed in a polycarbonate membrane cell culture insert prior to fixation and scanning electron microscopy observation.

of enzymatic shaving using trypsin was applied, before adhesion assay. A significant reduction ($p < 0.001$) was observed in the adhesion rate: 21.77 ± 8.10% for shaved bacteria, compared to the positive control consisting of propionibacteria (**Figure 3A**). Western blot analysis also indicated absence of SlpB at the surface of *P. freudenreichii* as a result of shaving (**Figure 3B**). To further confirm the role of surface proteins in adhesion, *P. freudenreichii* CB129 cells, shaved or not, were incubated with 50 μg of extracted surface proteins. This guanidine extract from the CB129 strain was previously dialyzed against PBS and quantified by Bradford assay. It contained the five proteins (SlpA, SlpB, SlpE, InlA, and LspA, see **Figure 1A**) in PBS buffer pH 7.4. Adhesion assay was then conducted. This incubation increased the rate of adhesion of *P. freudenreichii* CB129 to HT-29 cells, from 100.00% ± 8.93 to 317.07% ± 46.68. Furthermore, adhesion rate, which was strongly diminished by enzymatic shaving (33.99% ± 14.30), was restored by this incubation (157.44% ± 18.31, **Figure 3C**). This further experiment confirmed the key role of at least one of these surface proteins in adhesion.

Surface Protein SlpB Plays a Key Role in Adhesion to Cultured Human Colon Cells

In a second approach to inhibit adhesion and to precise the role of specific surface proteins, *P. freudenreichii* was incubated with antibodies raised against SlpB, at a dilution of 1:10,000, before adhesion assay. This resulted in a significant reduction following incubation with the anti-SlpB antibodies 39.95% ± 6.92 ($p < 0.001$), (**Figure 4A**). We then further focused on SlpB and inactivated its gene in *P. freudenreichii* CB129. The mutant *P. freudenreichii* CB129Δ*slpB* was obtained by insertion of the pUC:Δ*slpB*:*Cm*R suicide plasmid as described in the "Materials and Methods" section (**Supplementary Figure S2**). The stability of the mutant was validated after growth in the presence or absence of chloramphenicol by checking for the absence of SlpB production. As shown in **Figure 4B**, one protein band (about 55 kDa in size) was lacking in the mutant S-layer associated proteins guanidine extract (line 2), when compared to the WT parental strain (line 1). Western Blot analysis using antibodies raised against the SlpB protein (**Figure 4C**) confirmed that this protein was effectively mutated in the mutant (line 2) when compared to the parental strain (line 1). Efficient and specific inactivation of the *slpB* gene was further established by mass spectrometry analysis of guanidine-extracted S-layer proteins. Indeed, the SlpA, SlpB, and SlpE proteins were clearly identified in the WT CB129 strains, while only SlpA and SlpE were detected in the mutant *P. freudenreichii* CB129Δ*slpB* strain (**Table 2**).

Adhesion to HT-29 cells was then assessed by CFU counting and the mutant CB129Δ*slpB* strain was impaired in adhesion (20.66% ± 8.32) when compared to the WT control (100.00% ± 7.37) (**Figure 4A**, $p < 0.001$). To confirm this result, adhesion of *P. freudenreichii* to HT-29 cells, using CFSE-stained propionibacteria, was quantified by flow cytometry. Cells were treated with CFSE-labeled propionibacteria, WT or CB129Δ*slpB* mutant, for 1 h, before cytometric monitoring of cell fluorescence (**Figures 4D–F**). A shift in fluorescence intensity

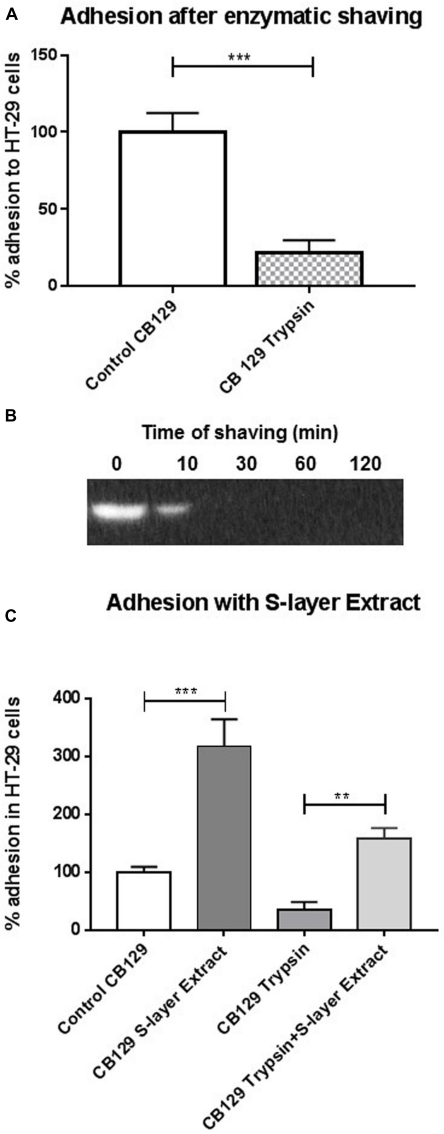

FIGURE 3 | Involvement of *P. freudenreichii* surface proteins in adhesion. **(A)** Trypsin shaving reduces *P. freudenreichii* CIRM-BIA 129 adhesion. Human colon cells were cultured in DMEM prior to co-incubation with propionibacteria. Used propionibacteria were either untreated (control) or submitted to trypsin shaving of surface proteins for 60 min (trypsin). Adhered bacteria were enumerated by CFU plate counting in trypsinized cells. **(B)** Trypsin shaving reduces presence of SlpB protein in *P. freudenreichii* CIRM-BIA 129 in different times of incubation. *P. freudenreichii* CIRM-BIA 129 show after different incubations time with trypsin (T_{zero} min, T_{30} min, T_{60} min, and T_{120} min) a decreased amount of SlpB in Western Blot analysis with anti-SlpB antibodies. **(C)** Addition of extracted surface layer proteins enhances *P. freudenreichii* CIRM-BIA 129 adhesion. Human colon cells were cultured prior to co-incubation with propionibacteria. Used propionibacteria were either shaved for 60 min (trypsin) or untreated (control). They were then added with surface layer guanidine extract (50 μg of proteins) or not. Adhesion was quantified by plate CFU counting of propionibacteria after trypsinization of colon cells. Adhesion is presented as a percent of the reference CIRM-BIA 129 *P. freudenreichii* strain. Asterisks represent statistically significant differences between strains and were indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Adhesion is presented as a percent of the reference CB129 *P. freudenreichii* strain.

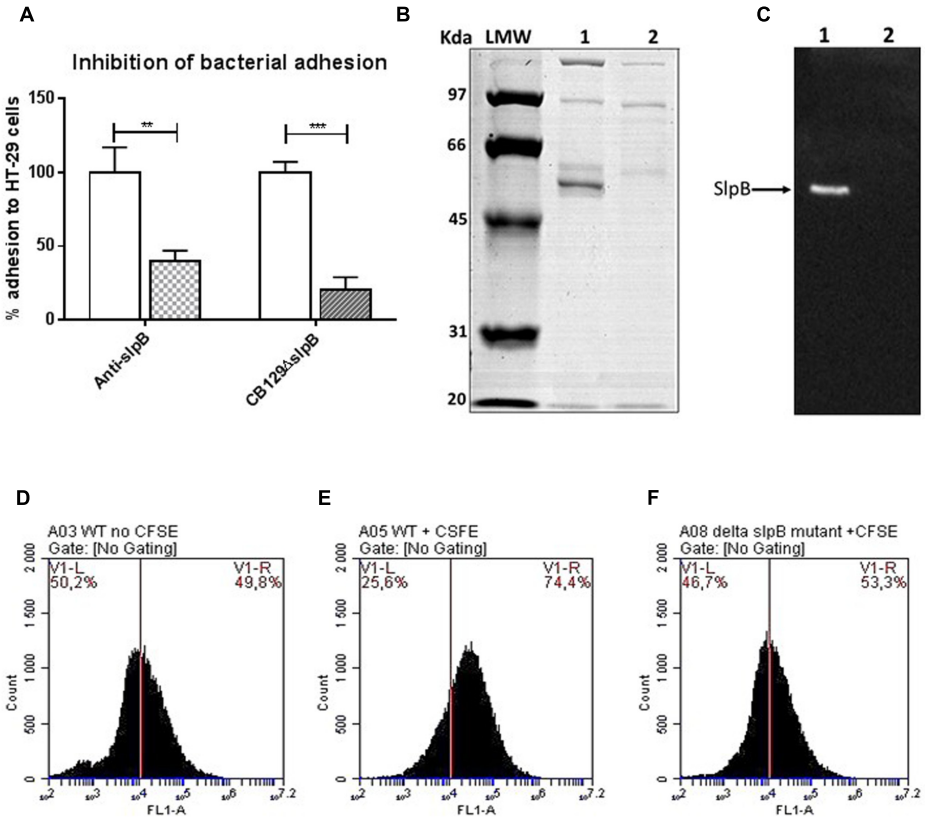

FIGURE 4 | Key role of surface layer protein SlpB in adhesion. **(A)** Antibodies reduce *P. freudenreichii* adhesion and so does *slpB* gene inactivation. Human colon cells were cultured in DMEM prior to co-incubation with propionibacteria. *P. freudenreichii* CIRM-BIA 129 was treated with antibodies raised against SlpB prior to adhesion assay. As an alternative, the *slpB* gene was inactivated in *P. freudenreichii* CIRM-BIA 129 prior to adhesion assay. Adhesion is presented as a percent of the reference CB129 *P. freudenreichii* strain. **(B)** Guanidine-extracted surface layer associated proteins were compared by SDS–PAGE in *P. freudenreichii* CIRM-BIA 129 wild-type (line 1) and in the corresponding mutant CB129Δ*slpB* (line 2). **(C)** The corresponding PVDF membrane was subjected to Western Blotting using rabbit antibodies raised against *P. freudenreichii* surface layer protein SlpB **(B)**. **(D,E)** Fluorescently labeled live *P. freudenreichii* CIRM-BIA 129 adheres to cultured human colon epithelial HT-29 cells. The adhesion of CFSE-labeled propionibacteria was detected by the shift in FL1 intensity **(E)**, compared to HT-29 cells with unlabelled propionibacteria **(D)**. Cells (10^6) were co-incubated with 10^8 CFU of CFSE-stained propionibacteria for 1 h. At least 50.000 cells per sample were analyzed. As an alternative, labeled *P. freudenreichii* mutant CB129Δ*slpB* was co-incubated with HT-29 cells **(F)**. Original gels and western blots, uncropped, are provided in **Supplementary Figure S1**. Asterisks represent statistically significant differences between strains and were indicated as follows: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Adhesion is presented as a percent of the reference CB129 *P. freudenreichii* strain.

TABLE 2 | Surface-layer proteins identified after Guanidine Hydrochloride extraction.

		Wild-type strain			Delta SlpB strain		
Gene name	**Locus tag**	**Log (*e*-value)[a]**	**Cover (%)[b]**	**Peptides[c]**	**Log (*e*-value)[a]**	**Cover (%)[b]**	**Peptides[c]**
SlpB	PFCIRM129_00700	−263.2	74	34	0	0	0
SlpE	PFCIRM129_05460	−125.6	50	19	−138.8	55	18
SlpA	PFCIRM129_09350	−174.3	75	24	−143.5	68	22

[a]*The e-value is the probability that a given peptide score will be achieved by incorrect matches from a database search. Protein e-value is the product of individual peptide e-value. Protein identifications were automatically validated when they showed at least two unique peptides with an e-value below 0.05 corresponding to log (e-value) < −1.3.* [b]*The percentage of the protein amino acid sequence covered by tandem mass spectrometry identification of peptides.* [c]*Number of unique peptide sequence identified with an individual e-value < 0.01 for this protein.*

(FL1) was observed as a result of fluorescent *P. freudenreichii* CB129 adhesion to cells (**Figure 4E**) when compared with control cells without bacteria (**Figure 4D**). This indicates an increase of fluorescence emission at 488 nm, corresponding to 6-carboxyfluorescein succinimidyl harbored by adhering bacteria, as described previously for lactobacilli (Tiptiri-Kourpeti et al., 2016). By contrast, the mutant CB129Δ*slpB* strain failed to reproduce this fluorescence shift in HT-29 cells, and the pattern (**Figure 4F**) was similar to that of HT-29 without bacteria (**Figure 4A**). Altogether, these results confirm the key role of the

SlpB surface protein in adhesion of *P. freudenreichii* to HT-29 cells.

DISCUSSION

Adhesion is a key determinant of host/bacterium interactions, whether pathogenic or probiotic. Adhesion of probiotic bacteria to host intestinal cells may favor important effects including modulation of mucus secretion (Mack et al., 2003), of defensin production (Schlee et al., 2007, 2008), or the local action of beneficial metabolites. It can improve competitive exclusion of pathogens by adhesion competition (Servin, 2004; Lebeer et al., 2008) and constitutes a key factor for several clinical applications of probiotics in the prevention and treatment of gastrointestinal disorders and of IBD. It may involve, on the bacterial side, various microorganism-associated molecular patterns (MAMPs) including flagellin, fimbriae (also called pili) or other surface proteins including moonlighting proteins and S-layer proteins (Lebeer et al., 2010).

Surface-layer proteins constitute a field of research that deserves further investigation. Although anchored to the cell wall via conserved SLH domains, their extracellular protruding part is highly variable, poorly conserved amongst bacterial species and strains. A previous paradigm described S-layers as a macromolecular paracrystalline network formed by the self-assembly of numerous copies of one monomeric protein or glycoprotein and constituting an extracellular S-layer in many bacteria (Sleytr, 1997; Sára and Sleytr, 2000). This was later challenged by studies on *Lactobacillus acidophilus* showing that a S-layer can contain various S-layer proteins or SLPs (Hymes et al., 2016). These proteins are in fact versatile molecules that may play an important role in growth and survival, maintenance of cell integrity, enzyme display, molecular sieving, co-aggregation, immunomodulation, as well as adhesion and persistence within the animal host (Lebeer et al., 2010; Fagan and Fairweather, 2014). In *P. freudenreichii*, such proteins were shown to be involved in immunomodulatory interactions with the host (Le Maréchal et al., 2015), a property highly strain-dependent (Mitsuyama et al., 2007; Foligné et al., 2010, 2013). Indeed, a functional role in immunomodulation by *P. freudenreichii* was recently attributed to a set of proteins: SlpB, SlpE, two putative S-layer proteins with SLH domains, and HsdM3, predicted as cytoplasmic (Deutsch et al., 2017).

Variability of *P. freudenreichii* surface proteins may thus be related to variability in functional properties. In this context, we have selected in the present work seven *P. freudenreichii* strains with different patterns evidenced in a preliminary study.

We confirm here that *P. freudenreichii* S-layer proteins are variable, and so is its ability to adhere to cultured human epithelial cells, as determined by quantitative culturing (Mack et al., 1999), which suggests a functional link between variations in the surface protein pattern. *P. freudenreichii* CIRM-BIA 129, shown to alleviate symptoms of acute colitis in mice, displays S-layer associated proteins and the highest adhesion ability, whatever the bacteria/cell ratio (100:1; 500:1; and 1,000:1). Moreover, at a ratio of 100/1, no internalization was observed. This suggests that propionibacteria either do not internalize into cultured HT-29 cells, or do not suvive within the cells. Cultured colon epithelial HT-29 cells do not produce mucus in our conditions. This suggests that *P. freudenreichii* interacts with epithelial cell surface compounds rather than mucins, a property previously reported for the probiotic *L. acidophilus* (Johnson et al., 2013). Interestingly, CB129 was shown to restore expression of ZO-1, a key protein of tight junctions which expression was impaired in colitis (Plé et al., 2015), as part of its anti-inflammatory effect. Adhesion close to these junctions may favor the local action of propionibacteria via local release of propionate, the major metabolite of propionibacteria, which was shown to improve intestinal barrier function and to restore expression of ZO-1 in DSS-treated mice (Tong et al., 2016). Accordingly, protection toward inflammation-induced barrier defects was reported for the probiotic product VSL#3 (Krishnan et al., 2016).

Enzymatic shaving of surface proteins reduced adhesion and was previously shown to hydrolyze at least 16 distinct proteins (Le Maréchal et al., 2015). Dramatic inhibition of adhesion was observed following blockage with antibodies raised against SlpB. Interruption of the *slpB* gene in CB129 strain also resulted in a drastic reduction ($P < 0.01$) in adhesion. Moreover, addition of purified S-layer proteins restored the adhesion that was suppressed in *P. freudenreichii* by enzymatic shaving. Altogether, these results indicate a role of *P. freudenreichii* S-layer protein, including SlpB, in adhesion, as was reported for the SlpA protein in *L. acidophilus* NCFM (Buck et al., 2005).

This study evidenced a key role of one of the *P. freudenreichii* S-layer proteins in adhesion to human intestinal cells. Understanding determinants of probiotic action is a key challenge. It opens new avenues for the screening of most promising propionibacteria strains, by monitoring their expression, and for the development of new functional products containing them. It is particularly relevant in the context of pathogens competitive exclusion and inflammation remediation.

AUTHOR CONTRIBUTIONS

GJ and FdC designed the research. GJ, YL, and VA supervised the work. FdC, HR, SH, FG, MD, SD, and JJ took part to the experiments. FdC and GJ wrote the manuscript. YL and VA corrected the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01033/full#supplementary-material

FIGURE S1 | Specificity of the anti-SlpB antibodies. The whole gels (I, II) and whole blots (II, IV) corresponding to **Figures 1**, **4** are shown. A single band reacting with anti-SlpB antibodies is evidenced. Moreover, specific inactivation of slpB gene leads to disappearance of this reactive band (IV). Finally, western blot using anti-SlpB antibodies reveals the SlpB protein only in strains which harbor the corresponding slpB gene, as indicated by the Table (V). In supplemental western blots of the same extracts (VI), sera directed against SlpA and SlpE evidence a distinct pattern. In particular, the two close Coomassie-stained bands, 58 and 56 kDa, were identified by western blot (this work) and by mass spectrometry (Le Maréchal et al., 2015) as SlpA and slpB, respectively.

FIGURE S2 | Interruption of *slpB* gene using suicide vector pUC:Δ*slpB*:*CmR*. **(A–C)** Schematic view of homologous recombination producing a mutant CB 128Δ*slpB*. Disruption of *slpB* gene in CB 129 WT by suicide vector pUC:*CmR* harboring 520-bp of *slpB*. Mutant strain show a chloramphenicol resistance by insertion of cassette containing *CmR*. **(D)** Targeting sequence used to inactivate. Partial sequence of *slpB* gene in CB 129 WT and sequence used to homologous recombination (red). The primers annealing site are indicated as underlined bases and oligonucleotides sequence are shown in figure.

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Gene Replacement and Fluorescent Labeling to Study the Functional Role of Exopolysaccharides in *Bifidobacterium animalis* subsp. *lactis*

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An extracellular layer of exopolysaccharides (EPS) covers the surface of some *Bifidobacterium animalis* subsp. *lactis* strains, which could be of relevance for its probiotic performance. In order to understand the functional characteristics of *B. animalis* subsp. *lactis*, two isogenic strains that differ in their EPS-producing phenotype, due to a single mutation in the gene Balat_1410, were studied. By means of a double crossover recombination strategy, successfully used for the first time in bifidobacteria, Balat_1410 in the type strain *B. animalis* subsp. *lactis* DSM10140 was replaced by a mutated gene containing a non-synonymous mutation previously associated with the appearance of a mucoid-ropy phenotype. Nuclear magnetic resonance and SEC-MALS analyses showed that the novel strain harboring the mutation acquired a ropy phenotype, due to the production of a high molecular weight (HMW)-EPS that is not produced in the wild-type strain. Fluorescence labeling of both strains with two fluorescent proteins, m-Cherry and Green Fluorescent Protein, was achieved by expressing the corresponding genes under the control of a native selected promoter (the elongation factor Tu promoter). Remarkably, qualitative and quantitative fluorescence analyses demonstrated that the ropy strain displays a lower capability to adhere to human intestinal epithelial cells. In addition, the presence of the HMW-EPS reduced the capability of the producing strain to form biofilms upon three different abiotic surfaces. This work also highlights the fact that different EPS confer variable functional characteristics to the bifidobacterial surface, which may be relevant for the performance of *B. animalis* subsp. *lactis* as a probiotic. The construction of molecular tools allowing the functional characterization of surface structures in next generation probiotics is still a challenging issue that deserves further attention, given the relevant role that such molecules must play in the interaction with the host.

Keywords: *Bifidobacterium*, exopolysaccharide, gene replacement, fluorescent proteins, NMR, SEC-MALS, biofilms

INTRODUCTION

The definition of a probiotic, proposed in 2001 by FAO/WHO, states that is "live microorganisms which when administered in adequate amounts confer a health benefit on the host." The most commonly commercialized probiotics are some species from *Bifidobacterium* and *Lactobacillus* genera that have been accepted as safe due to their long history of use and they are often delivered into food formulations (Hill et al., 2014). Nowadays, it is becoming more evident that certain intestinal commensal microorganisms could be beneficial to correct microbial dysbioses that have been related with some health disorders; however, they have not been used to promote health yet and, in case they will be applied in this context, they would be treated as novel drugs more than food supplements. These microorganisms can be considered as "next generation probiotics" (NGP) and they are termed as "live biotherapeutic products" (LBP) in the new regulatory framework of the Food and Drug Administration (FDA) of United States of America (O'Toole et al., 2017). Some of the proposed NGP belong to genera *Akkermansia*, *Bacteroides,* and *Faecalibacterium*.

Orally delivered probiotics establish the main contact point with the host at the intestinal mucosa level; in this location, the probiotic-microbiota-cell interplay will drive positive physiological benefits. Despite vast research efforts made into the mechanisms behind the beneficial effects, the manner of probiotic action still remains unclear (Gareau et al., 2010; Wan et al., 2015). The (transitory) contact between bacteria and intestinal epithelial cells might be relevant to initiate this intercellular dialog; the surface microbial associated molecular patterns (MAMPs) interacting with the host pattern recognition receptors (PRR) are involved in triggering the cellular response (Lebeer et al., 2010b; Westermann et al., 2016). One of the most external layers covering the bacterial surface is constituted by exopolysaccharides (EPS), which are carbohydrate polymers whose genetic determinants are present in intestinal bacteria, including most species of the genus *Bifidobacterium* (Ferrario et al., 2016). In fact, some of the beneficial properties attributable to the producing bifidobacteria have been associated with their EPS and their physical-chemical characteristics (Hidalgo-Cantabrana et al., 2014, 2016; Schiavi et al., 2016). Additionally, it has been proven that EPS produced by some NGP, such as *Faecalibacterium prausnitzii*, also has anti-inflammatory properties *in vivo*, thus this bacterium is being proposed as therapeutic agent to treat intestinal inflammatory processes (Rossi et al., 2015).

The development of tools allowing the study of the mechanisms of action, either for well recognized probiotics or NGP, is essential in order to choose those strains which are more valuable for each target population and health benefit. One of the approaches is the use of vectors containing different labeling systems; those applied to lactic acid bacteria and bifidobacteria have recently been reviewed (Landete et al., 2016). A luciferase-based reporter system was developed to monitor the performance of *Bifidobacterium breve* UCC2003 under *in vivo* conditions (Cronin et al., 2008). Different fluorescent proteins have been successfully used as well to label bifidobacteria. In this way, *B. breve*, *B. longum* subsp. *Longum,* and *B. bifidum* were labeled with cyan fluorescent protein (CFP), green fluorescent protein (GFP), yellow fluorescent protein (YFP) or mCherry under the promoter of the *gap* gene (P_{gap}) of *B. bifidum* (Grimm et al., 2014). Additionally, a GFP fluorescent protein containing a flavin-mono-nucleotide-based cofactor (evoglow-Pp1), which emits fluorescence in the presence/absence of oxygen, was included in a vector under control of the elongation factor Tu (P_{tuf} from *B. longum*) that replicates in *B. longum* and *B. breve* (Landete et al., 2014). This system, which supposes an advantage to study bifidobacteria in strict anaerobic conditions, was latterly validated under the control of other promoters (Montenegro-Rodríguez et al., 2015).

Bifidobacterium animalis subsp. *lactis* is one of the most widely used probiotics and there are several human intervention studies supporting its beneficial effects (Tojo et al., 2014). From an industrial point of view, it is one of the most robust bifidobacterial species which facilitates its inclusion in foods or food supplements (Bogsan et al., 2014). The aims pursued in the current work were: (i) to obtain an EPS-producing variant by means of a double crossover marker-less strategy, which produces a chromosomally stable new variant, (ii) the construction of EPS-producing *B. animalis* subsp. *lactis* strains harboring fluorescent proteins, which have not been reported in literature to date, and (iii) the demonstration that different EPS have an influence on the interaction of the producing strain with biotic and abiotic surfaces. To achieve these goals, a model of *B. animalis* subsp. *lactis* strains, which produced EPS with different physical-chemical characteristics, was initially used. This model was previously developed to demonstrate that a single mutation in the gene Balat_1410, coding for a protein involved in the elongation of the polymer chain, was directly related to a higher abundance of the high molecular weight (HMW)-EPS fraction (about 10^6 Da) that conferred a ropy-mucoid phenotype to the producing strain (Hidalgo-Cantabrana et al., 2015). Indeed, strains producing HMW-EPS are able to attenuate the immune response (López et al., 2012) and they have been proposed for their application in reducing intestinal inflammatory states (Hidalgo-Cantabrana et al., 2016).

MATERIALS AND METHODS

Bifidobacteria Strains, Plasmids and Culture Conditions

The *B. animalis* subsp. *lactis* and *Escherichia coli* strains, as well as the plasmids and oligonucleotides used in this study, are listed in **Table 1**. *E. coli* DH11S (Invitrogen™, ThermoFisher Scientific Inc., Waltham, MA, United States) was grown in Luria-Bertani (LB) broth at 37°C under shaking conditions (200 rpm). Bifidobacterial strains were cultivated in MRSc [MRS (Biokar Diagnostics, Beauvais, Francia) supplemented with 0.25% L-cysteine-HCl (Sigma-Chemical Co., St. Louis, MO, United States)] at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) in a MG500 chamber (Don Whitley Scientific, West Yorkshire, United Kingdom). Bacterial cultures and competent cells were prepared under standardized conditions

TABLE 1 | Bacterial strains, plasmids, and oligonucleotide primers used in this study.

Strains	Description	Reference
E. coli DH11S	*mcrA*Δ(*mrr-hsdRMS-mcrBC*) Δ(*lac-proAB*) Δ(*rec1398*) *deoR rpsL srl-thi*-F' *proAB*$^+$ *lacI*^{q}ZΔM15	Invitrogen
B. animalis subsp. *lactis*		
DSM10140^T	Type strain, Plasmid free, EPS$^+$, no ropy phenotype	DSMZ collection (yogurt isolated)
IPLA-R1	Plasmid free, EPS$^+$, ropy-mucoid phenotype	IPLA collection (bile-salt adapted)
DSM10140-ΔBalat_1410	DSM10140 lacking the gene Balat_1410, no ropy phenotype	Hidalgo-Cantabrana et al., 2015
DSM10140-Balat_1410^{S89L} (=S89L)	DSM10140 mutant obtained after gene integration of Balat_1410^{S89L}, EPS$^+$, ropy-mucoid phenotype	This work
DSM10140-mCherry	DSM10140 harboring pCAS-mCherry	This work
DSM10140-GFP	DSM10140 harboring pCAS- GFP	This work
S89L-mCherry	DSM10140-Balat_1410^{S89L} harboring pCAS-mCherry	This work
S89L-GFP	DSM10140-Balat_1410^{S89L} harboring pCAS- GFP	This work
Plasmids	**Description**	**Reference**
pJL74[a]	*E. coli-Bifidobacterium* cloning vector; Amp^r Sp^r; integrative, non-replicative in *Bifidobacterium*	Hidalgo-Cantabrana et al., 2015
pJL-upst/Balat_1410^{S89L} /dst/ (=pCHC3)	pJL74 containing Balat_1410^{S89L} together with the upstream (3 kb) and downstream (2.7 kb) regions	This work
pAM1[a]	*E. coli-Bifidobacterium* cloning vector; Amp^r Em^r	Alvarez-Martín et al., 2008
pCAS-mCherry	mCherry fluorescent protein gene fused to the elongation factor Tu promoter (P_{tuf}) of *B. animalis* subsp. *lactis* in pAM1	This work
pCAS- GFP	GFP fluorescent protein gene fused to the elongation factor Tu promoter (P_{tuf}) of *B. animalis* subsp. *lactis* in pAM1	This work
Oligonucleotides	**Sequence (5′–3′)**	**Reference**
Balat_1410 -GR-F[b]	TATATAGGGCCCGTCACCTCGTCACCATGAGC[b]	Hidalgo-Cantabrana et al., 2015
Balat_1410 -GR-R[b]	TATATAAGATCTCCACGAGAGCACACGAAGAC	Hidalgo-Cantabrana et al., 2015
In-Balat_1410 -F	GGTATGATGTGCAGATTCGGCTTC	This work
In-Balat_1410 -R	TACATGGCCGAGAACGAGGTAAACC	This work
Spec-F	GGAGAAGATTCAGCCACTGC	Hidalgo-Cantabrana et al., 2015
Spec-R	TTAGTCGTCGTATCTGAACC	Hidalgo-Cantabrana et al., 2015
P_{Tu}_F[b]	TATATAAAGCTTACATCCGTTACGAATCACGC	This work
mCh_R[b]	TATATATCTAGATTATTACTTGTACAGCTCGTCC	This work
GFP_R[b]	TATATATCTAGATTATTATTTGTATAGTTCATCC	This work
P_{Tu}_mCh_F[c]	GTCCAGGAGGACAAAAACAT**ATGGTGAGCAAGGGCGAGG**	This work
mCh_P_{Tu}_R[c]	**CCTCGCCCTTGCTCACCAT**ATGTTTTTGTCCTCCTGGAC	This work
P_{Tu}_GFP_F[c]	GTCCAGGAGGACAAAAACAT**ATGCGTAAAGGAGAAGAAC**	This work
GFP_P_{Tu}_R[c]	**GTTCTTCTCCTTTACGCAT**ATGTTTTTGTCCTCCTGGAC	This work

[a]*Amp^r, Em^r, and Sp^r, resistance to ampicillin, erythromycin, and spectinomycin, respectively.* [b]*Restriction enzyme sites are underlined.* [c]*Complementary sequences for splicing overlap extension PCR are bold marked.*

(Hidalgo-Cantabrana et al., 2015) and ampicillin (100 μg/ml), spectinomycin (100 μg/ml) or erythromycin (2.5 μg/ml) were added when required (**Table 1**).

Molecular Techniques

Isolation of Chromosomal and Plasmid DNA, and Plasmid Manipulation

Chromosomal DNA from *B. animalis* subsp. *lactis* was isolated using the GeneElute^{TM} Bacterial Genomic DNA kit (Sigma–Aldrich, Dorset, United Kingdom). Plasmid DNA was isolated from *E. coli* using the Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany), whereas the plasmid isolation from recombinant bifidobacteria was performed by means of the GeneElute^{TM} Plasmid Miniprep kit (Sigma–Aldrich). Manufacturer's recommendations were followed in both cases. For bifidobacterial strains, lysozyme (9 mg/ml, Merck, Darmstadt, Germany) and mutanolysin (5U, Sigma–Aldrich) were added during the lysis step followed by incubation at 37°C

for 1 h. DNA concentration was measured in Gene5^{TM} Teck3 Module (BioTek, Vermont, United States).

For plasmid constructions, PCRs were performed using Platinum® Pfx DNA Polymerase (Invitrogen^{TM}). Digestions and ligations were made with restriction endonucleases from Takara (Takara Bio Group, Otsu, Japan) and with T4 DNA ligase from Invitrogen^{TM}, respectively. All reagents were used according to the manufacturers' instructions. PCR products were checked by electrophoresis in TAE buffer [40 mM TRIS, 20 mM acetic acid, 1 mM EDTA (pH 8)] on 1% agarose gels and then stained with ethidium bromide (0.5 μg/ml). DNA purification from the agarose gels was performed using QIAquick Gel Extraction Kit (QIAgene) and sequenced at Macrogen Inc. (Seoul, South Korea). BLAST algorithm was used for sequence similarity analysis. Finally, Eurx-Taq DNA Polymerase from Roboklon GmbH (Berlin, Germany) was used to check plasmid constructions in *E. coli* and plasmid integration in the bifidobacterial chromosome.

Gene Replacement: Plasmid Construction and Double Crossover Events

The chromosomal DNA from *B. animalis* subsp. *lactis* IPLA-R1 was used as a template for PCR amplification using the specific primers Balat_1410-GR-F/R (**Table 1**) for the construction of the plasmid for gene replacement. These primers amplify 7.1 kb which contain the Balat_1410^{S89L} gene (Hidalgo-Cantabrana et al., 2015) and its flanking regions: upstream (3 kb) and downstream (2.7 kb). The PCR product was digested with *Apa*I and *Bgl*II and cloned into pJL74 previously digested with the same enzymes. Ligation was performed overnight at 16°C and the ligation mixture was purified and transformed into *E. coli* DH11S electrocompetent cells. The resulting plasmid was named pCHC3 (pJL-upst/Balat_1410^{S89L}/dst) and was introduced into *B. animalis* subsp. *lactis* DSM10140-ΔBalat_1410 electrocompetent cells prepared as previously reported (Hidalgo-Cantabrana et al., 2015). After transformation, bifidobacterial cells were immediately recovered in 2 ml of MRSc and incubated at 37°C under anaerobic conditions for 4–6 h before plating onto the same agar-medium containing spectinomycin (100 μg/ml). Plates were then incubated for 48 to 72 h at 37°C in anaerobic conditions. Transformants were checked for plasmid integration into the chromosome (single crossover) by PCR using the specific primers In-Balat_1410-F/R and Spec-F/R (**Table 1**). At this point of the experiment, two of the checked colonies acquired the visually recognizable ropy phenotype (having the integrated plasmid) and they were selected to be grown in 10 ml MRSc without antibiotic. Two subcultures (per day) were made for 5 days to force the loss of the plasmid (second crossover) and, afterward, these bacterial cultures were plated onto agar-MRSc without antibiotics for 48 h. Several colonies were picked up and each of them was grown in agar-MRSc, with and without spectinomycin, to select the non-antibiotic resistant colonies due to the loss of the plasmid. These colonies were checked by PCR using the specific primers In-Balat_1410-F/R and Spec-F/R to analyze the presence of Balat_1410^{S89L} and the absence of spectynomicin-resistance genes into the chromosome; besides, the ropy phenotype, associated with the presence of Balat_1410^{S89L}, was useful for the selection of the right colonies. Thus, one strain with a ropy character, then putatively carrying the Balat_1410^{S89L} gene, and being sensitive to spectynomicin, was selected and named DSM10140-Balat_1410^{S89L}, or S89L in its abbreviated form (**Table 1**). To confirm its genetic background, the chromosomal DNA from S89L was obtained and an inner fragment (1 kb) of Balat_1410^{S89L} gene, containing the mutation (C to T transition) responsible for the ropy trait (Hidalgo-Cantabrana et al., 2015), was amplified using the In-Balat_1410-F/R primers. The genetic background of the *eps* cluster surrounding the insertion and the complete insert was also checked with a set of primers that amplify 1 kb overlapping fragments (data not shown). All these PCR products were sequenced at Macrogen Inc. to confirm the absence of undesirable mutations.

Strain Labeling Using Fluorescence Plasmids

Plasmids harboring fluorescent proteins under the control of the elongation factor Tu promoter from *B. animalis* subsp. *lactis* were constructed using splicing overlap extension PCR strategy to fuse the DNA sequences (Vallejo et al., 1994). The "elongation factor Tu" promoter was amplified from the chromosomal DNA of DSM10140 strain using the specific primers P_{Tu}_F/mCh_P_{Tu}_R (**Table 1**). The gene encoding mCherry fluorescent protein was amplified from the pVG-mCherry plasmid (Grimm et al., 2014) with specific primers P_{Tu}_mCh_F/mCh_R. The PCR products that have complementary tails, between each other, were size-checked in 1% agarose gel. Then, splicing overlap extension PCR was performed to fuse both fragments. The same protocol was followed to fuse the elongation factor Tu promoter to GFP gene: the Tu promoter was amplified using specific primers P_{Tu}_F/GFP_P_{Tu}_R, and GFP gene was obtained from the pVG-GFP plasmid (Grimm et al., 2014) using specific primers P_{Tu}_GFP_F/GFP_R. Fused DNA fragments were checked by electrophoresis and purified from agarose gels. The fused DNA fragments were digested with *Hind*III and *Xba*I at 37°C for 3 h, as well as the plasmid pAM1 (Alvarez-Martín et al., 2008) which was also dephosphorylated. Digestions were also purified from agarose gels and used to perform overnight ligations at 4°C. Electrocompetent *E. coli* DH11S cells were transformed with the ligation mixtures and selection of clones was performed by adding ampicillin (100 μg/ml) to the culture medium. The resulting plasmids were named pCAS-mCherry and pCAS-GFP (**Table 1**). The strains *B. animalis* subsp. *lactis* DSM10140 and S89L were transformed with these plasmids and four fluorescent clones were selected by adding erythromycin (2.5 μg/ml) to the culture medium; the recombinant strains were named as DSM10140-mCherry, DSM10140-GFP, S89L-mCherry and S89L-GFP (**Table 1**).

Qualitative and Quantitative Fluorescence Detection

Fluorescence Scanning

The fluorescent bifidobacterial strains were grown onto the surface of agar-MRSc containing erythromycin, for 3 days at 37°C under anaerobic conditions. The fluorescence of the

colonies was checked in the Typhoon 9400 scanner (GE Healthcare, Biosciences, Uppsala, Sweden). GFP was excited with blue laser (488 nm) and emission was detected with 526 nm band-pass filter. In the case of mCherry, excitation was performed with red laser (633 nm) and emission was acquired with 580 nm band-pass filter. These plates were scanned at a resolution of 100 μm pixel size.

Fluorescence Microscopy and Confocal Scanning Laser Microscopy (CSLM)

To visualize the bifidobacteria expressing the fluorescent proteins, overnight grown cultures (in MRSc + erythromycin) were washed, placed on a slide covered with coverslip No.1 (0.13–0.16 mm thick). These preparations were observed with the Leica DMi8 inverted microscope (Leica Microsystems GmbH, Heidelberg, Germany), using a 100× oil immersion objective. The FITC filter cube (excitation 480/40, emission 527/30) and RHOD filter cube (excitation 546/10, emission 585/40) were used for visualization of the bifidobacteria harboring GFP or mCherry proteins, respectively.

Fluorescent (mCherry) bifidobacteria adhered on the top of the intestinal cell line HT29 or into glass slides (as will be described next) were visualized with the Leica TCS AOBS SP8 X confocal inverted microscope [External Service Unit (ESU) of the University of Oviedo, Asturias, Spain]. To visualize DAPI fluorochrom, samples were excited at 405 nm, by a blue-violet laser diode, whereas to detect the mCherry they were excited at 587 nm by a white light laser. Z-stacks of HT29 monolayers or bifidobacterial biofilms upon glass μ-slides were acquired with a 63×/1.4 oil objective. When needed, a 2.50 optical zoom was used to acquire images of detailed regions. Image captures were reordered and processed with the Leica Application Suit X software (version 1.8.1.13759, Leica).

Fluorescence Spectrometry

Fluorescence quantification was performed with overnight cultures of the four fluorescent bifidobacteria, as well as the two parental DSM10140 and S89L strains used as negative controls. Cells were washed with PBS and standardized to an equal $OD_{600\ nm}$; additionally, they were plated in the corresponding (with and without antibiotic) agar-MRSc media. Afterward, the standardized bacterial suspensions were 10-fold concentrated and from them, serial (half) dilutions were prepared in PBS. 96-well Lumitrack™ 600 white polystyrene plates (VWR, Radnor, PA, United States) were filled with 200 μl (per well) of each bifidobacterial dilution. Fluorescence was measured on the Cary Eclipse (Varian Ibérica, S.A. Madrid, Spain) fluorescence spectrometer using the following conditions: 470 nm excitation/525 nm emission, for GFP quantification, and 585 nm excitation/610 nm emission for mCherry quantification. The corresponding fluorescence background, determined from the control samples (parental, non-labeled bifidobacterial suspensions), was subtracted from data obtained for the fluorescent strains. This experiment was performed with three biological replicates. Finally, linear regression equations between the fluorescence emitted and the number of bacteria (Log CFU/ml) were calculated, as well as the corresponding coefficient of determination (R^2) that shows how well the data fits to the linear regression.

Flow Cytometry

Fluorescence of bifidobacterial suspensions, obtained as previously described, were also quantified in the Cytomics FC500 (Beckman Coulter, Barcelona, Spain) located in the ESU from the University of Oviedo. A fix acquisition time of 90 s with "hi" acquisition speed was used. The 488 nm laser was applied for the excitation of both fluorochromes and the selection of the bifidobacterial population was made by means of FSC log/SSC log (size/complexity). This gate was used to plot the FL1 (for GFP detection) vs. FL3 (for mCherry detection) histograms; the filters 525/40 and 620/30 were used for the detectors FL1 and FL3, respectively. The absence of auto-fluorescence was checked in the corresponding non-labeled bacteria (Supplementary Figure S1). In the labeled strains the recorded fluorescence was compensated to avoid the overlapping of both fluorochromes. Finally, serial dilutions of the samples (from 1/2 to 1/100) were measured and the linear regression equations between the "fluorescence emitted" (total number of events multiplied by the mean fluorescence intensity) and the number of bacteria (CFU/ml); the R^2 coefficients were calculated as well (Supplementary Figure S2).

Chemical Analysis of Purified EPS

EPS Purification

The EPS from strains DSM10140, S89L and IPLA-R1 were isolated from the bifidobacterial biomass collected with water from the surface of agar-MRSc plates as previously described (Ruas-Madiedo et al., 2010). Each bacterial suspension was mixed with 1 volume of 2 M NaOH and kept overnight at room temperature under mild shaking. Bacteria were eliminated by centrifugation and the EPS from the supernatant was precipitated with two volumes of chilled absolute ethanol for 48 h at 4°C. Precipitated sediment was collected with ultra-pure water and dialyzed against water, using dialysis tubes of 12–14 kDa molecular mass cut off (Sigma), at 4°C for 3 days with a daily change of water. Finally, each dialyzed sample was freeze-dried in order to obtain the crude-EPS material from each strain. To isolate the HMW EPS-fraction from strains S89L and IPLA-R1, the crude-EPS (25 mg) was dissolved in ultra-pure water (10 ml), dialysed (against water, for 72 h at 4°C) using Spectra/Por Float-A-Lyser 100 kDa MWCO tubes (Sigma), and the content of these dialyzed tubes was freeze-dried (Leivers et al., 2011).

SEC-MALLS Analysis

The molar mass distribution of the crude-EPS and HMW-EPS, as well the quantification of the relative amount of the different size-fractions, were performed by means of size exclusion chromatography (SEC); a chromatographic system (Waters, Milford, MA, United States) coupled in series with a refractive index (RI) detector (Waters) and with a multi-angle laser light scattering detection (MALLS, Dawn Heleos II, Wyatt Europe GmbH, Dembach, Germany) was used as previously described (Nikolic et al., 2012).

NMR Analysis

The HMW-EPS fractions were analyzed by nuclear magnetic resonance (NMR) at the facilities of the University of Seville (Seville, Spain). A sample of 10 mg was deuterium-exchanged several times by freeze-drying from D_2O and then examined in solution (10 mg/750 mL of 99.96% D_2O, Sigma–Aldrich). Spectra were recorded on a Bruker AV500 spectrometer (Bruker BioSciences, Madrid, Spain) operating at 500.13 MHz (^{1}H). Chemical shifts were given in ppm, using the HDO signal (4.31 ppm at 343 K) as reference (Gottlieb et al., 1997). The 2D heteronuclear one-bond proton-carbon correlation experiment was registered in the ^{1}H-detection mode via single-quantum coherence (HSQC). A data matrix of 256 × 1K points was used to digitize a spectral width of 5208 in F2 and 22522 Hz in F1. ^{13}C decoupling was achieved by the GARP scheme. Squared-cosine-bell functions were applied in both dimensions, and zero-filling was used to expand the data to 1K × 1K.

Adhesion to HT29

The intestinal epithelial cell line HT29 (ECACC 91072201, European Collection of Cell Cultures, Salisbury, United Kingdom) was used to test the adhesion capability of the fluorescence-labeled and non-labeled bifidobacterial strains; *B. animalis* subsp *lactis* BB-12 was used as reference strain. HT29 was maintained under standard conditions using McCoy's medium (MM, Sigma) supplemented with 10% fetal bovine serum (Sigma) and with a mixture of antibiotics (50 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin and 1.25 μg/ml amphotericin B, Sigma). The adhesion experiments were performed upon 11-day old HT29 monolayers grown in microtiter plates. Bifidobacterial suspensions, prepared in MM (without antibiotics), were added to each well at ratio 10:1 (bifidobacteria: HT29) and incubated for 1 h at 37°C/5% CO_2 (Nikolic et al., 2012). For the non-labeled strain the number of bacteria added and bacteria adhered was determined by plating on agar-MRSc and the adhesion percentage was calculated as the ratio between the bacteria adhered with respect to the bacteria added (Nikolic et al., 2012). For the fluorescence-labeled bacteria, the fluorescence was measured by means of flow cytometry, as previously described, and values of absolute fluorescence were used to present the adhesion results. In addition, experiments of competition between the "fluorescence-labeled, ropy" strain and the "non-labeled, non-ropy" strain (or "fluorescence-labeled, non-ropy" vs. "non-labeled, ropy") for adhesion to HT29 were carried out; in this case, both bacteria were added in equal amounts to the cell line (10:1, ratio bacteria: HT29) and the absolute fluorescence was measured.

Bifidobacterial Biofilm Formation upon Abiotic Surfaces

The capability of the ropy and non-ropy EPS-producing bifidobacterial strains to form biofilms was determined using different procedures and abiotic surfaces. A method, based on impedance measurement, recently described by Gutiérrez et al. (2016) to monitor in real time the formation of bacterial biofilms was used. In short, the real time cell analyzer (RTCA) equipment xCelligence RTCA-DP (ACEA Bioscience Inc., San Diego, CA, United States) was introduced in an incubator, at 37°C with 5% CO_2, at least 2 h before the experiments. Bifidobacterial cultures were washed twice with PBS to prepare standardized suspensions in fresh MRSc (∼10^9 cfu/ml) which were placed in the wells (100 μl/well) of specific E-plates (ACEA Bioscience Inc.) coated with gold-microelectrodes that are able to transmit the impedance signal. The RTCA software 2.0 (ACEA Bioscience) was used for data collection and the biofilm formation was followed for 46 h, using three biological replicates for each strain; finally, the wells were stained with crystal violet, as will be described next. Biofilms were also formed upon polystyrene plates (96-well microplates Nunc, Thermo-Fisher Scientific Inc.), upon microscope cover glasses (No. 1, 18 mm diameter, Marienfeld GmbH, Lauda-Königshofen, Germany) previously sterilized by autoclaving (121°C, 20 min) which were placed into 6-well microplates (Thermo Fisher Scientific Inc.), and upon the surface of μ-slide-2-well glass bottom (Ibidi GmbH, Martinsried, Germany). After 24-h incubation at 37°C in anaerobic chamber, these biofilms were also stained with crystal violet. Additionally, the fluorescence-labeled bifidobacterial biofilms (incubated in darkness) formed upon cover glasses were visualized under the epifluorescence microscope and those formed upon the surface of μ-slide-2-well glass bottom were detected with the CSLM.

Crystal Violet Staining

The end-point crystal violet method was used to quantify the bifidobacterial biofilm formation upon the three abiotic surfaces used (Gutiérrez et al., 2016). In brief, supernatants from different abiotic-material wells were removed and biofilms washed twice with PBS, dried for 15 min at room temperature and stained with a solution (0.1% w/v) of crystal violet for 15 min. Then, biofilms were gently washed with water, de-stained with a solution (33%) of acetic acid for at least 15 min and, finally, the absorbance of the supernatants was measured at 595 nm in a Microplate Benchmark Plus (Bio-Rad, Hercules, CA, United States) spectrophotometer.

Statistical Analysis

The statistical package IBM SPSS Statistics for Windows Version 22.0 (IBM Corp., Armonk, NY, United States) was used to assess differences among strains by means of one-way ANOVA followed, when needed, by SNK (Student-Newman–Keuls, $p < 0.05$) mean comparison test. The legend of each figure indicates the analysis performed. Finally, the R^2 coefficients, that reflect the adjustment to linear regression equations between different parameters, were calculated.

RESULTS AND DISCUSSION

Generation of a Ropy Strain with a Non-synonymous Mutation in the Gene Balat_1410

In a previous work, an isogenic mutant derived from the type strain *B. animalis* subsp. *lactis* DSM10140 by removing the gene Balat_1410, using a knockout mutation system based

on the integrative plasmid pJL74, was constructed (Hidalgo-Cantabrana et al., 2015). Our first aim in the present study was to reintroduce into the genome of *B. animalis* subsp. *lactis* DSM10140-ΔBalat_1410 a mutated Balat_1410 gene containing a non-synonymous, single nucleotide mutation previously associated with the appearance of a mucoid-ropy phenotype in the strain DSM10140-ΔBalat_1410-pAM1-Balat_1410^{S89L} (Hidalgo-Cantabrana et al., 2015). To do that, a double-crossover marker-less strategy previously used for the deletion of the gene, was followed. The strain DSM10140-ΔBalat_1410 was transformed with the plasmid pCHC3 (**Table 1**) containing a fragment of approximately 7 kb amplified from the genome of the strain IPLA-R1 (**Table 1**), that includes the regions immediately located upstream and downstream of the gene Balat_1410 as well as the mutated gene Balat_1410^{S89L} between those regions. Gene integration was achieved as previously described (Hidalgo-Cantabrana et al., 2015), resulting in *B. animalis* subsp. *lactis* DSM10140-Balat_1410^{S89L} (abbreviated as S89L), a strain that has exactly the same genetic background as *B. animalis* subsp. *lactis* DSM10140 which underwent a gene replacement that resulted in a C to T transition in the gene Balat_1410 at position 266, causing a codon change in position 89 (a serine is substituted by a leucine). Although there are several works that have reported gene deletion and interruption systems in bifidobacteria, including *B. breve* (Ruiz et al., 2012), *B. longum* (Fukuda et al., 2011; Hirayama et al., 2012) and *B. animalis* subsp. *lactis* (Arigoni and Delley, 2008; Hidalgo-Cantabrana et al., 2015), to our knowledge this is the first report of a successful gene replacement strategy in bifidobacteria. Due to the lack of genetic tools to introduce specific point mutations in bifidobacterial genomes, our methodology represents a suitable alternative to overcome this limitation.

Analysis of EPS Synthesized by the Recombinant Ropy Strain

In the EPS synthesized by the two ropy strains *B. animalis* subsp. *lactis* IPLA-R1 (parental) and S89L (recombinant) the HMW-EPS fraction (about 1×10^6 Da) was present in a higher proportion than in the non-ropy DSM10140 (parental) strain (**Figure 1A**). It should be noticed that the polymer material purified from the three strains, with the procedures used in this study, is the cell-associated EPS but not that liberated into the medium. Previously, the production of the HMW-EPS was correlated with the occurrence of the mucoid-ropy appearance in a recombinant strain harboring the mutated gene in a multi-copy plasmid (Hidalgo-Cantabrana et al., 2015); thus, currently this finding was reinforced with the acquisition of the ropy phenotype in the novel S89L having the single mutation stabilized into the chromosome. After purification of the HMW-EPS fraction from polymers synthesized by IPLA-R1 and S89L strains, one- and two-dimensional NMR analyses revealed an identical

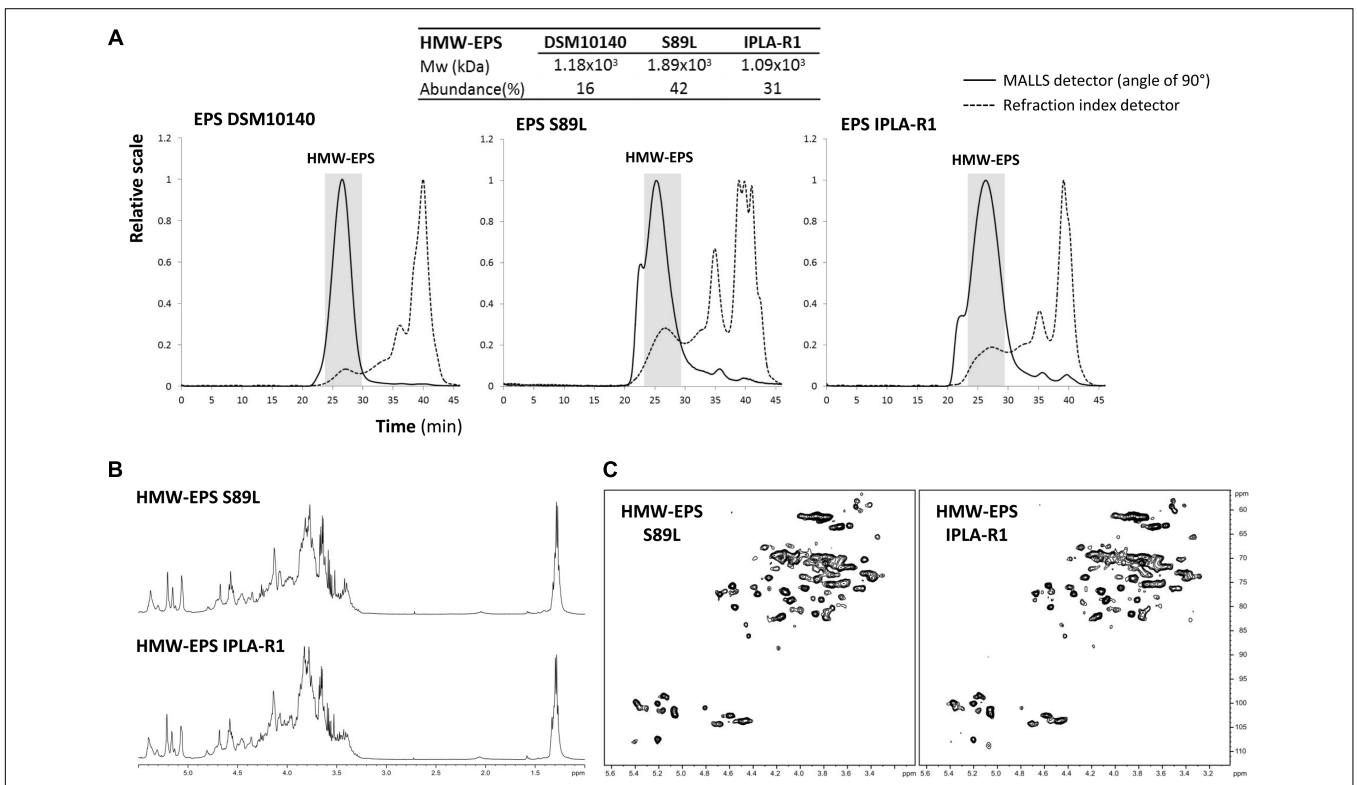

FIGURE 1 | Physical-chemical analysis of EPS isolated from three *B. animalis* subsp. *lactis* strains. SEC-MALLS analysis of the EPS-DSM10140, EPS-S89L and EPS-IPLA-R1 showing the molecular weight (Mw) and relative abundance of the high molecular weight (HMW) fraction in each polymer **(A)**. ^{1}H-NMR (500 MHz, 343 K) spectra **(B)** and 500-MHz ^{1}H-^{13}C- HSQC spectra **(C)** of HMW-EPS purified from EPS-IPLA-R1 and EPS-S89L polymers.

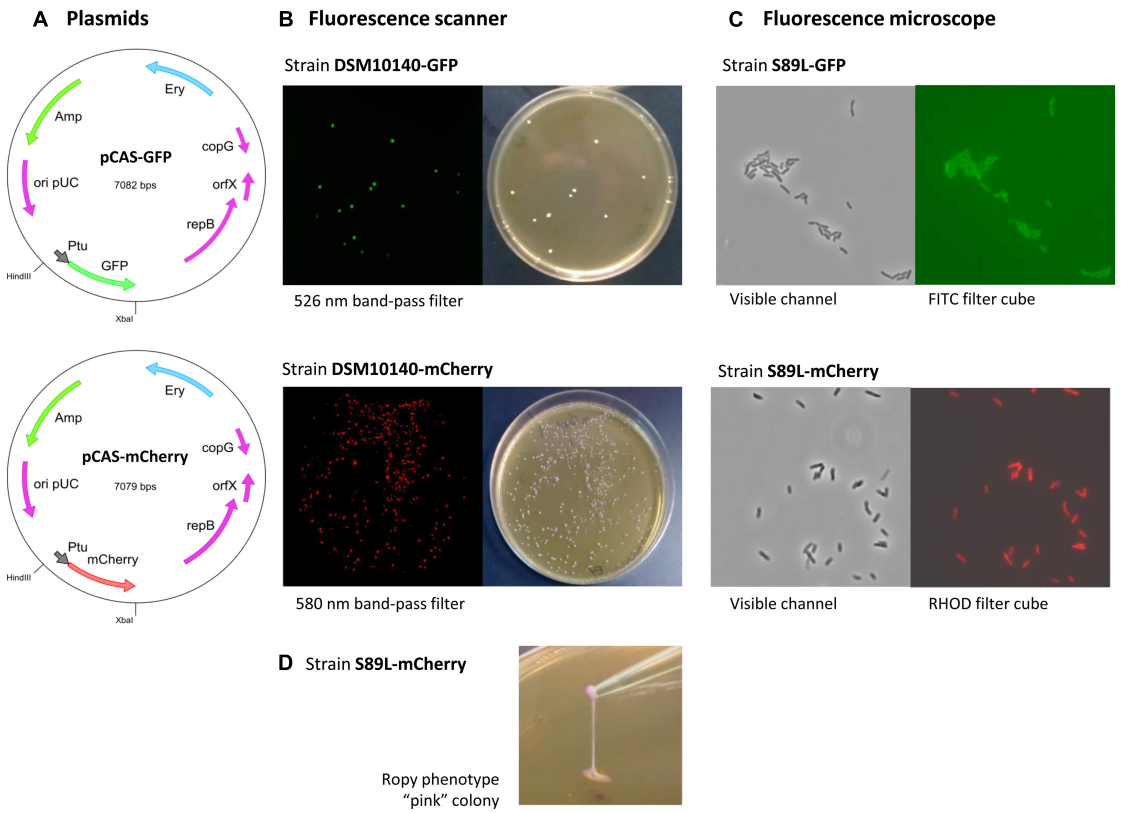

FIGURE 2 | Physical map of pCAS-GFP and pCAS-mCherry, in which the GFP and mCherry genes are located downstream from the elongation factor Tu promoter (P_{tuf}) from *B. animalis* subsp. *lactis* DSM10140 **(A)**. Detection of fluorescence green (top) or red (bottom) colonies of *B. animalis* subsp. *lactis* DSM10140-GFP and DSM10140-mCherry, respectively, using the Typhoon 9400 scanner fluorescence scanner (left hand photographs) and aspect of the colonies visualized with a conventional camera (right hand photographs) **(B)**. Fluorescent *B. animalis* subsp. *lactis* S89L, transformed with pCAS-GFP (top) and pCAS-mCherry (bottom), visualized with the Leica DMi8 inverted microscope using a 100× oil immersion objective **(C)**. Strain *B. animalis* subsp. *lactis* S89L-mCherry showing a colored pink colony with a ropy phenotype denoted for the formation of a long, unbreakable filament **(D)**.

chemical composition (**Figures 1B,C**). These physical-chemical analyses undoubtedly prove that the gene replacement strategy applied to obtain the recombinant S89L strain was successful to introduce the mutation linked to the production of the HMW-EPS. The structural repeating unit of the HMW-EPS was already described for strain IPLA-R1 (Leivers et al., 2011); it is an hexapolysaccharide with 50% rhamnose content, and it is very similar to that reported for strain *B. animalis* subsp. *lactis* LKM512 (Uemura and Matsumoto, 2014).

Expression of Fluorescent Proteins in *B. animalis* subsp. *lactis* and Fluorescence Detection

Bifidobacterium animalis subsp. *lactis* DSM10140 and S89L were transformed with the plasmids pVG-GFP and pVG-mCherry, containing the genes coding for the proteins GFP and mCherry, respectively. It was reported that these plasmids were successfully used for the fluorescent detection of *B. longum*, *B. breve,* and *B. bifidum* strains, grown in similar conditions to those used in our study (Grimm et al., 2014). However, no fluorescence was detected in our *B. animalis* subsp. *lactis* strains using fluorescence spectrometry techniques, suggesting that either the genes are not expressed or the proteins do not emit fluorescence under our experimental conditions. Since gene expression in the plasmids pVG-GFP and pVG-mCherry is under the control of the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene of *B. bifidum* (P_{gap}), a specific promoter of *B. animalis* subsp. *lactis* was investigated which could be suitable to trigger the expression of the GFP and mCherry genes in our strains. Our previous work on bifidobacteria allowed us to depict a detailed protein map of the most abundant cytoplasmic proteins in their soluble proteome (Sánchez et al., 2005, 2007). Indeed, one of the most abundant proteins in the soluble proteome of some bifidobacteria is the elongation factor tu (the product of the *tuf* gene; Sánchez et al., 2005; Wei et al., 2016). Furthermore, previous reports have shown that the P_{tuf} of *B. longum* is a suitable strong and constitutive promoter able to trigger the expression of fluorescent proteins in *B. longum* and *B. breve* (Landete et al., 2014). These previous findings indicate that the *tuf* promoter could be a good candidate for the expression of heterologous genes in *B. animalis* subsp. *lactis*. With this in mind, the promoter of the glyceraldehyde-3-phosphate dehydrogenase, originally present in the plasmids

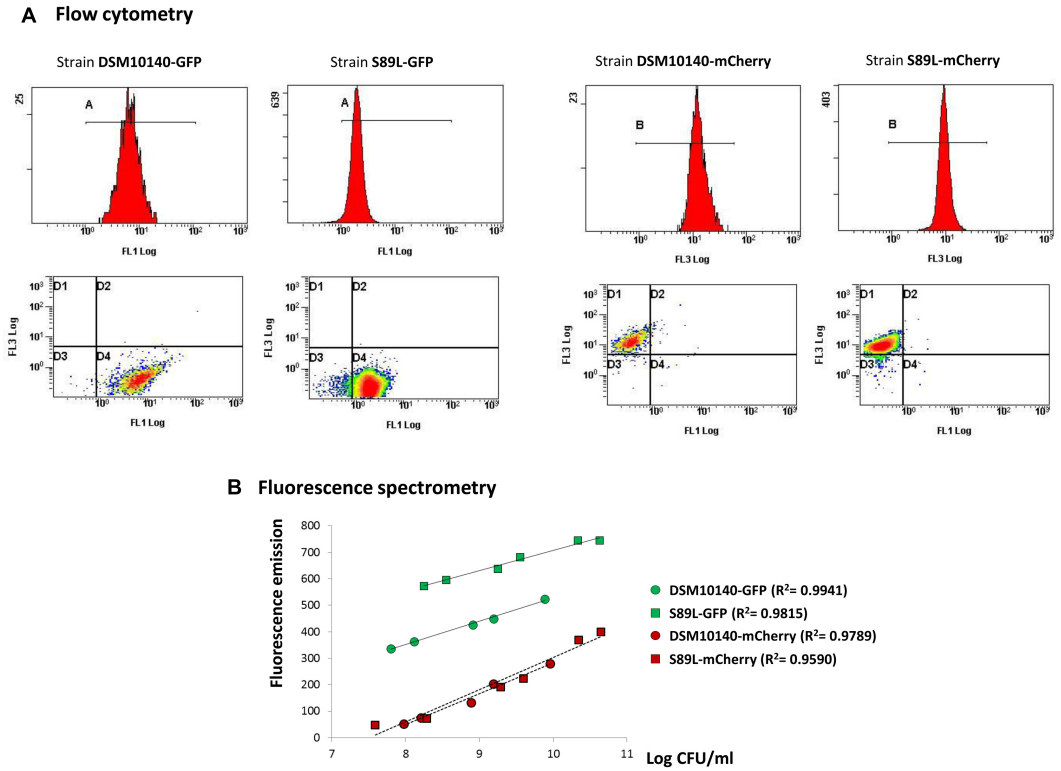

FIGURE 3 | Fluorescence quantification of GFP (the two left panels) and mCherry proteins (the two right panels) of the bifidobacterial strains harboring the corresponding plasmids using the Cytomics FC500 flow cytometer; see Supplementary Figure S2 for correlation with bacterial enumeration in agar-MRSc **(A)**. Linear regression between the fluorescence emitted, recorded by the CaryEclipse fluorescence spectrometer, and the bacterial counts (Log CFU/ml) of seriated dilutions of the fluorescent bifidobacterial suspensions; the coefficients of determination (R^2), showing how well data fit to the regression line equations, are indicated in bold letters **(B)**.

pVG-GFP and pVG-mCherry, was replaced by the upstream region of the *tuf* gene of *B. animalis* subsp. *lactis* DSM10140, containing P_{tuf}, yielding the plasmids pCAS-mCherry and pCAS-GFP (**Table 1** and **Figure 2A**). These plasmids were used to transform *B. animalis* subsp. *lactis* DSM10140 and *B. animalis* subsp. *lactis* S89L; green and red fluorescence in the resulting strains (DSM10140-mCherry, DSM10140-GFP, S89L-mCherry and S89L-GFP) were detected by fluorescence scanning (**Figure 2B**) and fluorescence microscopy (**Figure 2C**). The ropy phenotype, denoted by the formation of a filament, was detected in the strain S89L-mCherrey which also acquired a pink color in the colony (**Figure 2D**). Furthermore, a quantitative analysis of the fluorescently labeled bifidobacteria was performed using flow cytometry (**Figure 3A**), and the fluorescence signal was correlated with bacterial counts by a linear regression analysis (Supplementary Figure S2). Our results showed that the fluorescence quantification of the strains labeled with the two fluorescent proteins correlate with the bacterial counts in agar plates. The coefficients of determination (R^2) obtained in these analysis were in all cases higher than 0.95, indicating the suitability of our approach to quantify fluorescently labeled bifidobacterial populations in a range of 2 log count units (**Figure 3A** and Supplementary Figure S2). Very good fits to the linear regression were also obtained in a range of 3 log count units when the fluorescence quantification was carried out using spectrometric techniques (**Figure 3B**). Previous works have shown that other species, such as *B. longum*, *B. bifidum,* and *B. breve*, can be fluorescently labeled with a variety of proteins, including GFP, m-Cherry, Yellow Fluorescent proteins and Cyan Fluorescent proteins (Grimm et al., 2014; Landete et al., 2014). However, it is worth highlighting that, to the best of our knowledge, this is the first report describing the fluorescent labeling of *B. animalis* subsp. *lactis*, which opens new possibilities to track the functional properties of this *Bifidobacterium* under *in vitro* and *in vivo* conditions.

Regarding the stability of the fluorescence emission of GFP and mCherry, the fluorescence signal of GFP was quite unstable compared with that of m-Cherry. Once the cell growth was stopped, the cells were not able to emit fluorescence for longer than 1 h, independently of the technique used to measure the GFP fluorescence. A quick decrease in the fluorescence in the two *B. animalis* subsp. *lactis* strains analyzed was observed. However, m-Cherry fluorescence was more intense and stable for longer periods of time (a significant decrease of the fluorescent signal after 24 h at RT was not observed) (data not shown). Therefore, the strains labeled with m-Cherry were selected to perform the experiments described in the following sections of this work.

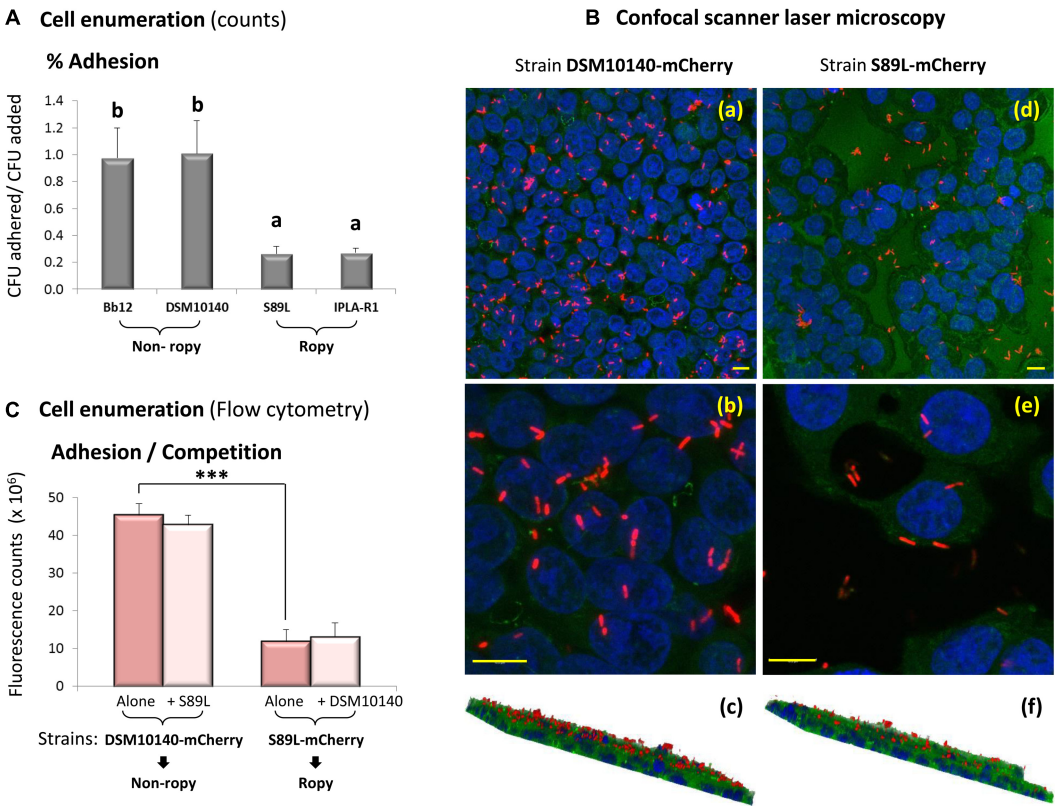

FIGURE 4 | Adhesion to the intestinal cell line HT29 of four *B. animalis* subsp. *lactis* strains: DSM10140 and Bb12 display a non-ropy phenotype and S89L and IPLA-R1 display a ropy phenotype. Bars that do not share a common letter are statistically ($p < 0.05$) different **(A)**. Visualization of DSM10140-mCherry (a, b, and c) and S89L-mCherry (d, e, and f) strains adhered to HT29 using the TCS SPE confocal module of the Leica DMi8 inverted microscope (bars 10 μm). The nucleus was DAPI-stained in blue (excited at 405 nm), the green color corresponds with the auto-fluorescence emitted by cytoplasmic molecules of the cell line, and the fluorescent bifidobacteria were detected in red (excited at 561 nm). The bottom photographs (c and f) represent a rotated 3D-image obtained from the Z- projection (10-XY slides, thickness about 13–15 μm) showing the integrity of the HT29 monolayer covered by a "grass" of fluorescent *B. animalis* subsp. *lactis* strains **(B)**. Adhesion to HT29 of strains DSM10140-mCherry and S89L-mCherry (red bars) quantified by means of flow cytometry; statistical difference between both strains are indicated with asterisks ($p < 0.01$). The pink bars show the adhesion of the fluorescent strains in competition with the non-fluorescent counterpart strains **(C)**.

Application of Fluorescence Labeling to Assess the Role of Ropy EPS in the Adhesion to HT29

In the last consensus statement in the probiotics field revised in 2014, it was considered that the FAO/WHO Guidelines proposed in 2002 (Food, and Agricultural Organization of the United Nations, and World Health Organization [FAO/WHO], 2002) still provide a useful approach to search for and validate probiotic candidates (Hill et al., 2014). Among the *in vitro* tests carried out to study new strains, the capability to adhere to mucus and/or intestinal epithelial cells is extensively analyzed before undertaking *in vivo* studies. In this study, conventional (culturing) techniques have been used to test the adhesion to the human intestinal cell line HT29 of the recombinant *B. animalis* subsp. *lactis* S89L strain in comparison with their isogenic parental strains. The ropy S89L and IPLA-R1 strains, both synthesizing in higher abundance the HMW-EPS, adhered significantly less to HT29 than DSM10140 and Bb12 (a probiotic reference) strains (**Figure 4A**). This result was contrary to that obtained in a previous work with the recombinant (ropy) DSM10140-ΔBalat_1410-pAM1-Balat_1410^{S89L} strain harboring the mutated Balat_1410 gene in the multicopy plasmid pAM1 (Hidalgo-Cantabrana et al., 2015). In fact, the three recombinant strains carrying pAM1 plasmid showed higher adhesion values (above 3%) to HT29 than the values obtained in the current work for the better adherent (around 1%) strains (DSM10140 and Bb12). Thus, in order to clarify this, apparently, contradictory result, the fluorescently labeled strains were used to address their capability to adhere to HT29 by means of different techniques and using the new experimental models in which there is a single copy of Balat_1410 (or Balat_1410^{S89L}) in the chromosome. The CSLM visualization corroborated that the ropy, HMW-EPS producing S89L-mCherry strain adhered to HT29 in lower proportions than the non-ropy DSM10140-mCherry strain (**Figure 4B**). Similarly, the quantitative flow cytometry technique showed about a fivefold reduction ($p < 0.05$) in the adhesion of the first strain with respect to the second (**Figure 4C**). Additionally, the simultaneous addition of a fluorescent strain in combination with

its non-labeled counterpart (e.g., strain DSM10140-mCherry and S89L) did not modify the adhesion capability of both fluorescent ropy and non-ropy strains (**Figure 4C**); this indicates that the higher abundance of the HMW-EPS did not represent a disadvantage for *in vitro* competition to adhere to the epithelial cell line. Thus results obtained in the current study support the generalized finding that EPS of high molecular mass make difficult the adhesion of the producing strain to the intestinal epithelium because they could hinder the accessibility of other molecules acting as adhesins (Lebeer et al., 2009; Nikolic et al., 2012; Horn et al., 2014). However, the role of bacterial EPS on the adhesion capability of the producing strain has not been clearly established since it is highly dependent on the producing strain, the intrinsic characteristics of the polymer, as well as on the biological model of study used. As an illustrative example, *in vitro* studies showed that the EPS surrounding *Lactobacillus rhamnosus* GG reduced the adhesion of the strain to the intestinal cell line Caco2 (Lebeer et al., 2009) because the accessibility of the pili acting as strong adhesin to enterocytes was hindered (Lebeer et al., 2012). However, *in vivo* studies showed that this EPS forms a protective shield against host-antimicrobial secreted factors which helps the persistence of strain GG in the gut (Lebeer et al., 2010a). Thus, production of EPS by *L. rhamnosus* GG is relevant to keep an optimal performance, i.e., a balance between protection and adhesion.

Role of Ropy EPS in the Biofilm Formation upon Abiotic Surfaces

Another important role that EPS synthesized by probiotic bacteria could play in the gut ecosystem is the capability of reducing the activity of pathogens in the intestine, including their adhesion to the intestinal epithelium. This has been proved with *in vitro* (Ksonzeková et al., 2016; Zivkovic et al., 2016) as well as *in vivo* studies (Fanning et al., 2012; Chen et al., 2014; Nácher-Vázquez et al., 2015) using different intestinal cell lines and animal models, respectively. It was postulated that the mechanism behind this EPS protection could be the formation of a protective "biofilm-like" layer covering the intestinal epithelium and, therefore, preventing the adhesion of the pathogen or its toxins (Ruas-Madiedo et al., 2010; Fanning et al., 2012). Thus, to test the biofilm-formation ability of our EPS-producing bacteria different methodologies and abiotic surfaces were used as an initial approach before demonstrating this capability in biotic surfaces which, currently, is a challenging issue (**Figure 5**). The recently developed impedance-based method to monitor in real time the bacterial biofilm formation upon gold-microelectrodes of RTCA E-plates (Gutiérrez et al., 2016) showed that the ropy S89L strain presented a lower adhesion ability than the non-ropy DSM10140 parental one during the incubation period (**Figure 5A**). To discard any influence of the abiotic material on the adhesion ability, 24 h-old biofilms were formed upon glass-cover or polystyrene and compared with 46 h-old biofilms made of RTCA E-plates; all biofilms were stained with crystal violet and this method also supported the same tendency between both strains ($p < 0.05$) regardless of the abiotic surface considered

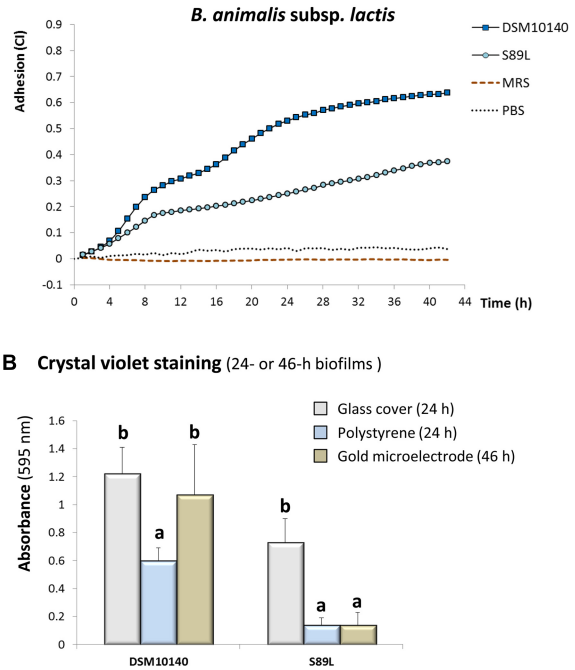

FIGURE 5 | Monitoring in real time the formation of biofilms by strains *B. animalis* subsp. *lactis* DSM10140 and S89L upon (gold-microelectrodes) E-plates in the RTCA (real time cell analyzed) equipment **(A)**. Biofilms formed by the same strains in three abiotic surfaces and stained, at different end-point times, with the crystal violet method; within each strain, bars that do not share a common letter are significantly ($p < 0.05$) different **(B)**.

(**Figure 5B**). However, statistical differences were noted when the behavior of each strain in the three abiotic surfaces was compared; for strain S89L the lowest biofilm formation capability was upon gold and polystyrene, whereas strain DSM10140 showed better adherence to gold (**Figure 5B**). This variability could be related to variations in the hydrophilic/hydrophobic nature of both bacterial surfaces that could suppose variable affinities for materials with different physical properties. In fact, it has been proved that the polar and non-polar characteristics of abiotic surfaces are involved in the differential capability to form biofilms of some pathogens (Meira et al., 2012).

The visualization by epifluorescence microscopy of 24 h-old biofilms formed with the fluorescent-labeled strains showed a higher bacterial density on the glass covered with the non-ropy DSM10140-mCherry strain, in comparison with the ropy S89L-mCherry ones (**Figure 6A**); a quantitative test performed with the same strains confirmed this difference ($p < 0.05$). Similarly, when intact bifidobacterial biofilms formed upon μ-slides were visualized with CSLM a very thin 3-D structure was detected only for strain DSM10140-mCherry whereas only residual bacteria remained attached for strain S89L-mCherry (**Figure 6B**). As far as we could note, this is the first report showing the biofilm formation by *Bifidobacterium* spp. and depending on the type of polymer that is synthesized it could favor or prevent the

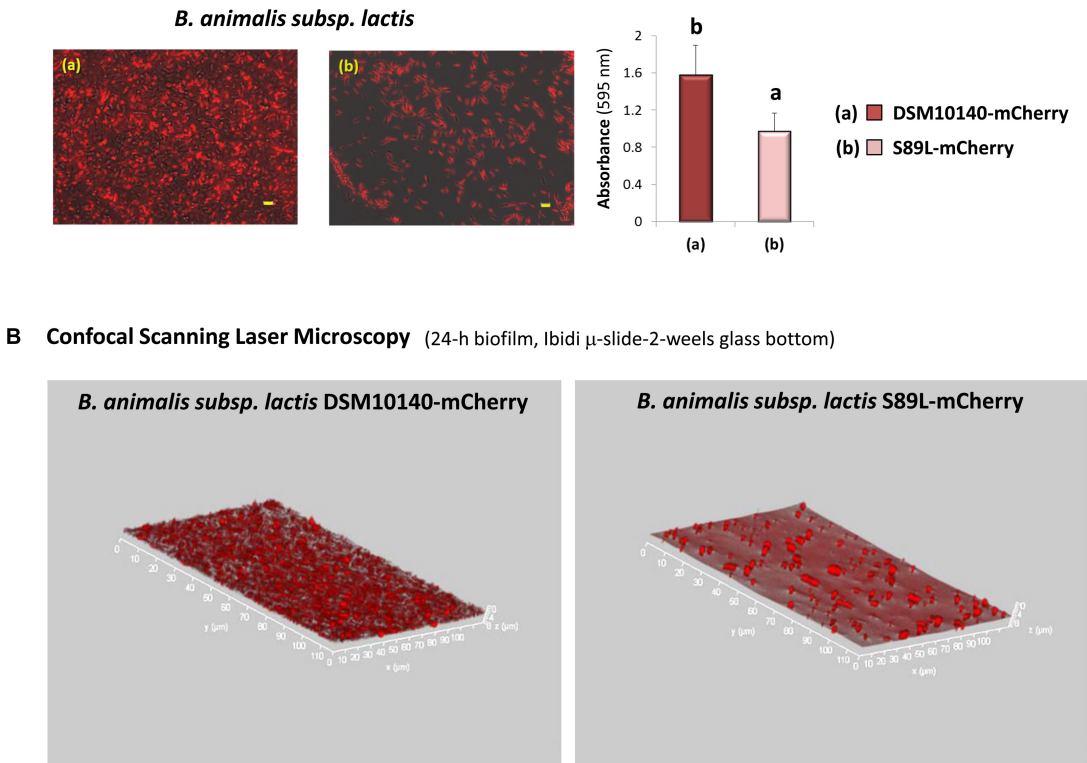

FIGURE 6 | Detection of fluorescence-labeled bifidobacterial DSM10140-mCherry and S89L-mCherry strains upon glass covers using the epifluorescence microscope (bar 5 μm) and crystal violet quantification of the biofilms formed upon the glass covers; bars that do not share a common letter are significantly ($p < 0.05$) different **(A)**. Rotated 3D-images obtained from the Z- projection (thickness about 2–4 μm) showing the biofilm formed by *B. animalis* subsp. *lactis* DSM10140-mCherry upon glass μ-slides and the absence of biofilm by strain S89L-mCherry **(B)**.

adhesion of the bifidobacteria to different abiotic surfaces. As it was indicated above, the presence of a HMW-EPS fraction could reduce the contact of other surface adhesins with the abiotic surface, thus avoiding the biofilm formation.

Therefore, all approaches used showed a reduced capability of the HMW-EPS-producing strains to form biofilms upon abiotic surfaces, as was also observed in the biotic (HT29) surface. Our observation confirms that previously reported with the high molecular mass EPS synthesized by *L. rhamnosus* GG (38) and *L. johnsonii* FI9785 (Dertli et al., 2015). The molecular weight of the HMW-EPS synthesized by these lactobacilli as well as our strain S89L was equal or higher than 1×10^6 Da; whereas, the most abundant polymer fractions in the DSM10140 had lower molecular weight (less than 3×10^4 Da). Therefore, although it cannot be discarded that some specific EPS could form a biofilm layer on the gut surface, other mechanisms could be involved in the capability of EPS-producing bacteria to counteract the adhesion of pathogens. In this regard, our previous observations with polymers purified from lactobacilli and bifidobacteria suggest that these polymers could also act as analogs of the eukaryotic PRR for pathogens, i.e., having a "lectin-like" activity, and therefore reducing their adhesion to the gut mucosa (Ruas-Madiedo et al., 2006). In any case, the contribution of EPS to counteracting microbial dysbiosis caused by infections deserves future research (Reid et al., 2011).

To sum up, our results show that fluorescent labeling of *B. animalis* subsp. *lactis* can be achieved by using green and mCherry fluorescent proteins but that these genes need to be under the control of a promoter from this species. It was demonstrated that the introduction of Balat-1410 mutation into the chromosome of parental DSM10140 strain was linked to the production of a larger abundance of a HMW-EPS, which in turn confers a ropy phenotype. Tagging bifidobacteria with mCherry allowed us to determine that some relevant functional characteristics of the strains, such as the adhesion to human intestinal cells or the capacity to form biofilms, are associated with the presence/absence of this ropy phenotype. The novel recombinant strains obtained in this work are a valuable tool to study the cross-talk mechanisms between bifidobacteria and host cells, and can make possible the development of further approaches to elucidate the role of these bacteria in complex *in vivo* systems. Furthermore, similar methodological approaches are required to demonstrate the functional properties of NGP bacteria which must be used to decipher the role of surface molecules involved in the beneficial properties attributable to beneficial microorganisms.

AUTHOR CONTRIBUTIONS

CH-C, PR-M, and AM contributed with the conception, experimental design and results interpretation of this study. NC-B carried out all experiments, some of them performed with the collaboration of MR-C. PR-M was in charge of the statistical analyses. AM and PR-M were in charge of writing the drafted manuscript. All authors performed a critical revision of the manuscript and approved the final version.

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Isolation of Human Intestinal Bacteria Capable of Producing the Bioactive Metabolite Isourolithin A from Ellagic Acid

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Urolithins are intestinal microbial metabolites produced from ellagitannin- and ellagic acid-containing foods such as walnuts, strawberries, and pomegranates. These metabolites, better absorbed than their precursors, can contribute significantly to the beneficial properties attributed to the polyphenols ellagitannins and ellagic acid (EA). However, both the ability of producing the final metabolites in this catabolism (urolithins A, B and isourolithin A) and the health benefits associated with ellagitannin consumption differ considerably among individuals depending on their gut microbiota composition. Three human urolithin metabotypes have been previously described, i.e., metabotype 0 (urolithin non-producers), metabotype A (production of urolithin A as unique final urolithin) and metabotype B (urolithin B and/or isourolithin A are produced besides urolithin A). Although production of some intermediary urolithins has been recently attributed to intestinal species from *Eggerthellaceae* family named *Gordonibacter urolithinfaciens* and *Gordonibacter pamelaeae*, the identification of the microorganisms responsible for the complete transformation of EA into the final urolithins, especially those related to metabotype B, are still unknown. In the present research we illustrate the isolation of urolithin-producing strains from human feces of a healthy adult and their ability to transform EA into different urolithin metabolites, including isourolithin A. The isolates belong to a new genus from *Eggerthellaceae* family. EA transformation and urolithin production arisen during the stationary phase of the growth of the bacteria under anaerobic conditions. The HPLC-DAD-MS analyses demonstrated the sequential appearance of 3,8,9,10-tetrahydroxy-urolithin (urolithin M6), 3,8,9-trihydroxy-urolithin (urolithin C) and 3,9-dihydroxy-urolithin (isourolithin A) while 3,8-dihydroxy-urolithin (urolithin A) and 3-hydroxy-urolithin (urolithin B) were not detected. For the first time isourolithin A production capacity of pure strains has been described. The biological activity attributed to urolithins A and B and isourolithin A (anti-inflammatory, anti-carcinogenic, cardioprotective, and neuroprotective properties)

explains the relevance of identifying these urolithin-producing bacteria as potential novel probiotics with applications in the development of functional foods and nutraceuticals. Their human administration could improve the health benefits upon ellagitannin consumption, especially in metabotype 0 individuals. However, further research is necessary to probe well-established beneficial effects on the host and safety requirements before being considered among the next-generation probiotics.

Keywords: urolithin, ellagitannin, bioconversion, metabotype, novel probiotic, gut bacteria, polyphenols

INTRODUCTION

Ellagitannins and EA produce some health benefits through the consumption of walnuts, strawberries, and pomegranates among other fruits (Tomás-Barberán et al., 2016a). Their bioavailability is low but it is now well-established that they are transformed by the intestinal bacteria to urolithins that are better absorbed (Cerdá et al., 2004). Identification of the urolithins released from dietary ellagitannins by intestinal microbiota is a present tendency in phenolic investigation because of the health implication of these microbial metabolites as potential anti-inflammatory, antioxidant, cardioprotective, neuroprotective, and cancer preventive compounds (Larrosa et al., 2010; Espín et al., 2013). However, both the health benefits associated with ellagitannin consumption and the ability of producing urolithins in this catabolism differ considerably among individuals. Population has been categorized into three ellagitannin-metabolizing phenotypes, i.e., 'urolithin metabotypes,' depending on the quantitative percentage and type of the urolithins formed. Thus, metabotype A is distinguished by the production of urolithin A, metabotype B individuals produce isourolithin A and urolithin B besides urolithin A, and those with metabotype 0 do not produce the final metabolites urolithin A, isourolithin A, or urolithin B. This interindividual variability has been related with dissimilarities in the intestinal microbiota (Tomás-Barberán et al., 2014; Romo-Vaquero et al., 2015; Selma et al., 2015). Therefore, the identification of the microbial species able to transform the ellagitannins, EA and other polyphenols is also an important goal because of the possible development of functional foods with health benefits on low producers of urolithins (Tomás-Barberán et al., 2016b; Espín et al., 2017). The bacterial species responsible for urolithin production are scarcely known. Only two urolithin-producing species *Gordonibacter pamelaeae* (DSM 19378^T) and *Gordonibacter urolithinfaciens* (DSM 27213^T) have been identified as producers of intermediary urolithins (Selma et al., 2014a,b). Therefore, other still unknown bacteria are necessary for the complete transformation of EA into the final metabolites (urolithin A, isourolithin A, or urolithin B). In the present research we illustrate for the first time the isolation of an urolithin-producing bacteria from human feces from a healthy adult, its phylogenetic analysis and its ability to transform EA into different urolithin metabolites including the final metabolite isourolithin-A.

Abbreviations: ABB, anaerobic basal broth; EA, ellagic acid.

MATERIALS AND METHODS

Isolation of Urolithin-Producing Bacteria

A healthy male donor (aged 41), who previously demonstrated to produce urolithins *in vivo,* provided the stool samples. The study was conformed to ethical guidelines outlined in the Declaration of Helsinki and its amendments. The protocol (included in the project AGL2015-64124-R) was approved by the Spanish National Research Council's Bioethics Committee (Spain). Donor gave written informed consent in accordance with the Declaration of Helsinki. Urolithins were identified in feces and urine after walnut consumption as explained elsewhere (Selma et al., 2016). The feces were prepared for isolation of microorganisms following the isolation protocol previously described with some modifications (Selma et al., 2014a,b). Briefly, after 1/10 (w/v) fecal dilution in nutrient broth (Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with 0.05% L-cysteine hydrochloride (PanReac Química, Barcelona, Spain), the filtrated was homogenized and further diluted in ABB (Oxoid). In order to first evaluate the metabolic activity, 15 μM urolithin C (Dalton Pharma Services, Toronto, ON, Canada) was dissolved in propylene glycol (PanReac Quiìmica SLU, Barcelona, Spain) and added to the broth. After anaerobic incubation, a portion of the culture, having metabolic activity, was seeded on ABB agar. Approximately 200 colonies were collected, inoculated into 5 ml of ABB containing EA (Sigma–Aldrich, St. Louis, MO, United States) at 15 μM and after incubation; their capacity to convert EA into urolithins was assayed. Urolithin-producing colonies were sub-cultured until urolithin-producing strains were isolated. The isolation procedure and plate incubation was achieved under anoxic environment with an atmosphere consisting of $N_2/H_2/CO_2$ (85/5/10) in an anaerobic chamber (Concept 400, Baker Ruskin Technologies, Ltd, Bridgend, South Wales, United Kingdom) at 37°C. Samples (5 ml) were prepared for HPLC-DAD-MS analyses of urolithins as explained below.

Phylogenetic Classification of the Urolithin-Producing Bacteria

The almost-complete 16S rRNA gene sequence of the isolated strains was obtained by PCR amplification on a AG 22331 thermocycler (Eppendorf), followed by direct sequencing using primers 616V (forward) and 699R (reverse), as described before (Arahal et al., 2008) to target about 1000 nt close to the 5′ end and primers P609D and P1525R to target positions 785–802 and 1525–1541, respectively, as previously described (Lucena et al., 2010). The resulting amplicons were analyzed

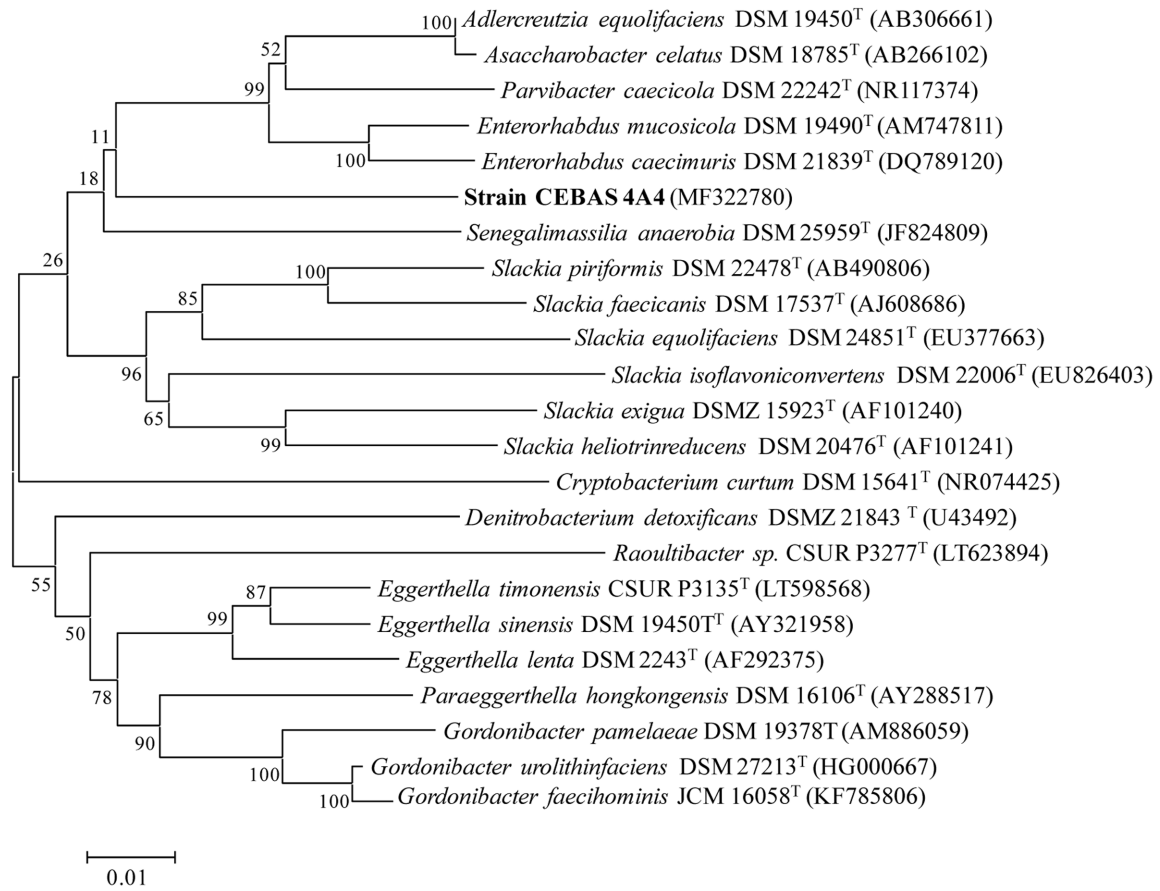

FIGURE 1 | Phylogenetic tree showing the relationship between the strain CEBAS 4A4 and other representatives of the family *Eggerthellaceae*. The tree was constructed by using the neighbor-joining method based on 16S rRNA gene sequences. Distance matrix was calculated by the Jukes and Cantor method. GenBank accession numbers are presented in parentheses. Bar, 1% nucleotide sequence difference. Numbers at nodes (≥70%) indicate support for internal branches within the tree obtained by bootstrap analysis (percentages of 500 re-samplings).

by Sanger sequencing. Sequencing data were assessed using Lasergene (DNASTAR), were manually corrected and compared with public sequences in the EMBL database using the BLAST program (National Center for Biotechnology Information[1]). The phylogenetic analysis was performed with MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar et al., 2016). Neighbor-joining treeing method was used and distance matrix was calculated by the Jukes and Cantor method (Jukes and Cantor, 1969). The data subsets were performed using the appropriate MEGA 7 tools.

Growth Kinetics and Time-Course Transformation of Ellagic Acid and Urolithin C

An isolated strain with the ability to produce isourolithin A, preserved frozen, was incubated on ABB agar plate for 6 days. A single colony was cultivated in 5 ml ABB tube. Two milliliters of diluted inoculum were transferred to ABB (200 ml) obtaining an initial load of 10^4 cfu ml^{-1}. Separately, EA or urolithin C, dissolved in propylene glycol, were added to the 200 ml cultures to obtain a final concentration of 15 μM. During incubation in anoxic environment at 37°C, aliquots (5 ml) were taken for HPLC analyses as described below. Plate counts in ABB agar were carried out. Growth curves were made in triplicate and the experiment was repeated three times.

HPLC-DAD-MS Analyses

Aliquots collected during the incubation of isolated strains, were extracted and analyzed by HPLC-DAD-ESI-Q (MS) as previously described (García-Villalba et al., 2016). Briefly, fermented medium (5 ml) was extracted with ethyl acetate (5 ml) (Labscan, Dublin, Ireland) acidified with 1.5% formic acid (Panreac), vortexed for 2 min and centrifuged at 3500 *g* for 10 min. The organic phase was separated and evaporated and the dry samples were then re-dissolved in methanol (250 μl) (Romil, Barcelona, Spain). An HPLC system (1200 Series, Agilent Technologies, Madrid, Spain) equipped with a photodiode-array detector (DAD) and a single quadrupole mass spectrometer detector in

[1]http://ncbi.nlm.nih.gov/

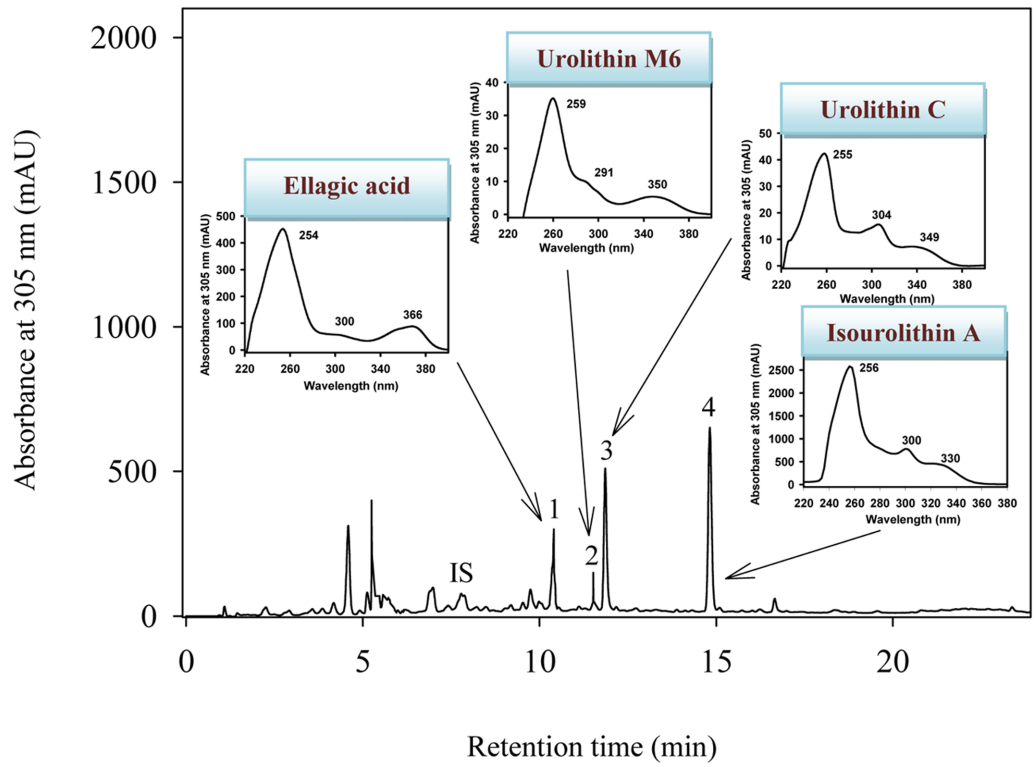

FIGURE 2 | HPLC-DAD-MS elution profile of *in vitro* metabolism of EA by the strain CEBAS 4A4 under anaerobic conditions. The insets show the UV spectra of EA and its metabolites. IS (internal standard; 6,7-dihydroxycoumarin), (1) EA, (2) 3,8,9,10-tetrahydroxy-urolithin (urolithin M6), (3) 3,8,9-trihydroxy-urolithin (urolithin C), (4) 3,9-dihydroxy-urolithin (isourolithin A).

series (6120 Quadrupole, Agilent Technologies, Madrid, Spain) was used as previously described (García-Villalba et al., 2016). Calibration curves were obtained for EA, urolithin C as well as urolithin M6 and isourolithin A (Villapharma SL, Murcia, Spain) with good linearity ($R^2 > 0.998$). Isourolithin A and urolithin C were quantified at 305 nm, while urolithin M5, urolithin M6, and EA were quantified at 360 nm, all with their own standards.

Data Modeling of Growth Curves

Bacterial growth curves and main growth parameters (lag time, maximum specific growth rate, and estimated correlation coefficient) were fitted with the function of Baranyi et al. (1993).

RESULTS

Identification of Urolithin Producing Bacteria

Enrichment cultures resulted in the isolation of four pure bacterial cultures (strains CEBAS 4A1, 4A2, 4A3, 4A4) which showed the capacity to convert EA into isourolithin A as final metabolite under anaerobic conditions. The 16S rRNA gene sequence of the isolates showed 100% similarity and one of the isolates (strain CEBAS 4A4) has been deposited in the NCBI nucleotide sequence database under accession number MF322780. The sequence of the 16S rRNA gene and phylogenetic characteristics from more closely related species showed that strain CEBAS 4A4 belonged to the family *Eggerthellaceae* (**Figure 1**). Strain CEBAS 4A4 has been deposited in two public culture collections (= DSM 104140^T = CCUG 70284^T).

Analysis of Urolithins Produced by the Strain CEBAS 4A4

The HPLC-DAD-MS analyses showed that urolithin M6, urolithin C, and isourolithin A were produced from EA by all the isolated strains (CEBAS 4A1, 4A2, 4A3, 4A4) (**Figure 2**). Identification of metabolites was carried out by direct comparison (MS and UV spectra) with pure standards and confirmed by their molecular mass and spectra (García-Villalba et al., 2016).

Growth Kinetics and Time-Course Catabolism of EA and Urolithin C by the Strain CEBAS 4A4

The lag phase of growth for strain CEBAS 4A4 was 1.5 ± 0.6 h while the growth rate was 0.15 ± 0.01 h^{-1} with and without EA or with and without urolithin C at 15 μM. EA and urolithin C catabolism and isourolithin A production took place during the stationary phase of the growth (**Figure 3**). EA disappeared

simultaneously that urolithins appeared (**Figure 3A**). Urolithin M5 was not detected while urolithin M6 was only observed in the sample obtained at day 6, being the first metabolite detected. Urolithin C arrived to a maximum at day 7, and then diminished progressively whereas isourolithin A was formed. The full conversion of EA into urolithin C (35%) and isourolithin A (65%) was reached at day 13 while urolithin A was not detected (**Figures 3A,B**). A longer incubation up to 15 days did not produce further hydroxyl removals from isourolithin A. Therefore, 3-hydroxy-urolithin (urolithin B) was not detected. In contrast to *in vitro* catabolism of EA, the complete transformation of urolithin C into isourolithin A was not obtained during incubation with the strain CEBAS 4A4 (**Figure 3C**). After 15 days, the maximum conversion of EA into isourolithin A was 33%.

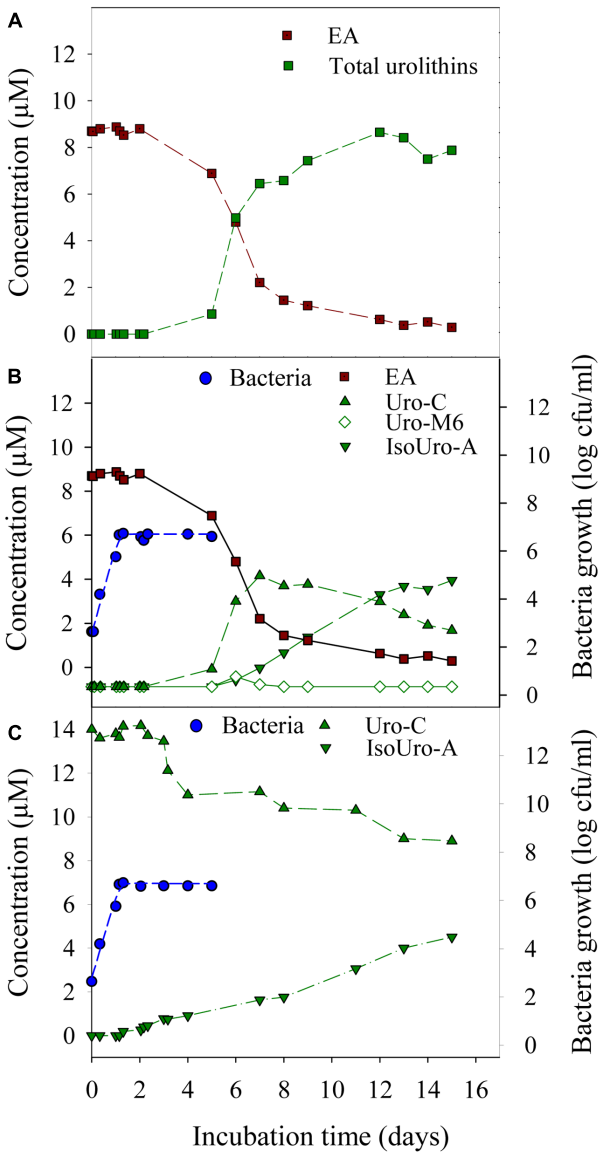

FIGURE 3 | Bacterial growth and time course production of urolithins by the strain CEBAS 4A4. Metabolism of EA to total urolithins **(A)** and to isourolithin A (IsoUro-A) via urolithin M6 (Uro-M6) and urolithin C (Uro-C) **(B)**. Metabolism of urolithin C (Uro-C) to isourolithin-A (IsoUro-A) in absence of EA **(C)**.

DISCUSSION

The percentage of metabotype B in adult healthy population ranges from 20 to 30%, and it is mainly characterized by isourolithin A and/or urolithin B production as final urolithins (Tomás-Barberán et al., 2014). However, the gut bacteria able to produce these metabolites have not been described so far. Four gut bacteria strains (CEBAS 4A1, 4A2, 4A3, 4A4) isolated from a healthy donor and able to produce urolithins M6, C and isourolithin A, are described in the present study. Strains were found at high concentrations ($\geq 10^7$ cfu g^{-1} feces). Comparison of the 16S rRNA gene sequences of the strains showed that the four isolates belong to the same species and that they are phylogenetically members of the family *Eggerthellaceae*. Recently, the class Coriobacteriia containing the family *Coriobacteriaceae* has been divided into the Eggerthellales ord. nov. (including the family *Eggerthellaceae* fam. nov.) and the emended order Coriobacteriales (including the emended family *Coriobacteriaceae* and *Atopobiaceae* fam. nov.) (Gupta et al., 2013). Based on 16S rRNA gene sequence, the closest relatives of isolated strain CEBAS 4A4 from the *Eggethellaceae* family includes *Enterorhabdus musicola* DSM 19490^T and *Enterorhabdus caecimuris* DSM 21839T (93.0% identity), *Adlercreutzia equolifaciens* DSM 19450^T (93.0% identity), *Asaccharobacter celatus* DSM 18785^T (92.0% identity), and *Parvibacter caecicola* DSM 22242^T (91.0% identity). Even if it is not possible to differentiate species by reason of 16S rRNA sequence differences only, at the present is in general established that bacteria showing > 5% 16S rRNA gene sequence difference are of different genus (Stackebrandt and Goebel, 1994; Fournier et al., 2015). Results of the phylogenetic analysis suggest that isolated strains belong to a novel genus and further analyses describing these bacteria as novel genus and species are being carried out for publication.

Previous studies have identified bacterial species able to transform different polyphenols such as flavan-3-ols and isoflavones to simpler bioactive molecules (Braune and Blaut, 2016). *Eggerthella lenta* and *A. equolifaciens* are able to dehydroxylate flavan-3-ols and their C-ring cleavage products at the B-ring. Transformation of isoflavones to equol have been also associated to *E. musicola, A. equolifaciens, A. celatus, Slackia isoflavoniconvertens, Slackia equolifaciens* (Matthies et al., 2008; Thawornkuno et al., 2009; Braune and Blaut, 2016). There is much less information in relation to the bacteria responsible for the transformation of ellagitannins. Only urolithin C-producing bacteria have been described so far (*G. urolithinfaciens* and *G. pamelaeae*) (Selma et al., 2014a,b). Similarities of 16S rRNA gene sequence of strain CEBAS 4A4 are 91% (76% cover) with *G. pamelaeae* (DSM 19378^T) and 90% (73% cover) with *G. urolithinfaciens* (DSM 27213^T). All these bacteria are members of the *Eggerthellaceae* family although they were previously considered from *Coriobacteriaceae* family. Therefore,

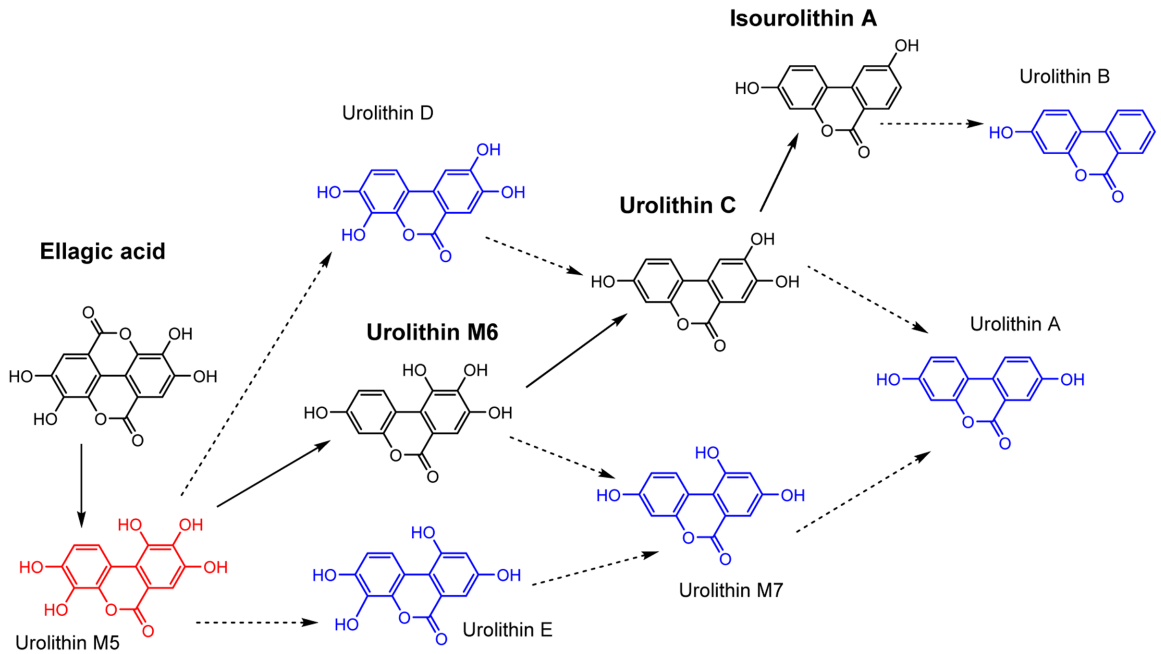

FIGURE 4 | Catabolic pathway for EA by intestinal bacteria. Proposed metabolic pathway of EA by strain CEBAS 4A4 (black color). Other urolithins which are not produced by strain CEBAS 4A4 (blue color).

as a result of the recent division of the class Coriobacteriia, bacterial species described as polyphenol transformers have been regrouped within the Eggerthellales ord. nov. while phylogenetic neighbors, not associated with the transformation of polyphenols (*Collinsella, Atopobium,* and *Olsenella* genera), are grouped in the emended order Coriobacteriales. Further studies should be performed to elucidate the potential health benefits of bacteria from *Eggerthellaceae* family due to their capacity to produce bioactive molecules from dietary polyphenols.

In the current research, the chronological production of urolithins M6, C and isourolithin A by isolated strains (CEBAS 4A1, 4A2, 4A3, 4A4) has been shown (**Figure 4**). Although isolated strains share with *G. urolithinfaciens* and *G. pamelaeae* (Selma et al., 2014a,b) the ability to produce urolithin C, strains CEBAS 4A1, 4A2, 4A3, 4A4 are able to produce a further dehydroxylation to yield the final metabolite isourolithin A. Previous studies identified urolithins A, B, C and isourolithin A in human urine, plasma and target tissues such as the colon and prostate (González-Sarrías et al., 2010, 2016; Nuñez-Sánchez et al., 2014). Production of urolithins M5, M6 and M7, E, C, A, B and isourolithin A by human fecal microbiota has also been described *in batch culture* (García-Villalba et al., 2013) and in a intestinal simulator (TWIN-SHIME®) (García-Villalba et al., 2017). However, it is in the present study where one bacterial species is identified as producer of isourolithin A. Accordingly, other intestinal species are needed for producing urolithins A and B. Additional research should be performed to find out if the deficiency of the isolated bacteria described in the present study is the restrictive factor in the formation of isourolithin A *in vivo*. In the case of the bacteria involved in the formation of isourolithin A, and according to the catabolic pathway of EA to yield urolithins (**Figure 4**), it is remarkable the specific *o*-dehydroxylase activity displayed by this microorganism. Whereas *G. urolithinfaciens* has been reported to *o*-dehydroxylate urolithin M6 to yield urolithin C (Selma et al., 2014b) however, it was not able to catalyze further dehydroxylations. In this case, the strain CEBAS 4A4 selectively and sequentially can *o*-dehydroxylate both urolithins M6 and C to yield isourolithin A but it was not able to catalyze the dehydroxylation of the hydroxyl group at the *para* position (**Figure 4**). This is somehow paradoxical as this hydroxyl group, located at the 9-position, has been reported to be more reactive than the hydroxyl group at the 8-position (González-Sarrías et al., 2017). Indeed, the ionization of the phenolic hydroxyl at 9-position is preferential against the hydroxyl at 8-position in urolithin A, which indicates that the hydroxyl at 9-position is more acidic and thus can perhaps be a better substrate for a number of enzymes (González-Sarrías et al., 2017).

Complete transformation of EA (8 μM) into urolithins by isolated strain CEBAS 4A4 (13 days) or *G. urolithinfaciens* was produced after 7 days, being urolithin C the major metabolite. However, 5 more days were needed to achieve the highest concentration of isourolithin A in the case of isolated strain CEBAS 4A4 while *G. urolithinfaciens* did not produce further dehydroxylations from urolithin C. Urolithins produced by strain CEBAS 4A4 or by *Gordonibacter* species are secondary metabolites without role in the bacterial growth because the stationary phase of growth is achieved before urolithin production. In opposition to synthetic chemistry, metabolite

production by microorganisms is often more convenient (Craney et al., 2013). Bacterial secondary metabolites, such as antibiotics, antitumorals, cholesterol-lowering agents, immunomodulating agents are being progressively used more in diseases treated only by synthetic medicines in the past. In addition to the classical probiotics (*Lactobacillus* and *Bifidobacterium*), other beneficial microbiota such as the butyrate-producing *Faecalibacterium prausnitzii* (Eppinga et al., 2016) and the mucin-degrading *Akkermansia muciniphila* (Derrien et al., 2016) have recently been described. The urolithin-producing bacteria described herein, in addition to other dietary polyphenol-transforming bacteria, could also have potential as novel probiotics as well as in the industrial manufacture of bioactive metabolites to develop new ingredients, beverages, nutraceuticals, pharmaceuticals, and/or functional foods. However, further studies should be carried out to demonstrate these emerging points.

CONCLUSION

We report here the isolation of human gut bacterial strains that belong to a new genus from *Eggerthellaceae* family. This is the first description of bacterial strains capable of converting *in vitro* EA into the final metabolite isourolithin-A. The human health benefits associated with urolithins explain the relevance of identifying the responsible gut bacteria potentially useful for the development of novel probiotics, functional foods, and food complements. This is especially relevant in those individuals with metabotype 0, who are not able to produce bioactive urolithins. However, further research is necessary to probe well-established health effects on the host as well as safety requirements before being considered among the next-generation probiotics.

AUTHOR CONTRIBUTIONS

DB, ML, and RG-V performed most of the laboratory work including the isolation of the isourolithin producing bacteria. MR-V and AM performed the phylogenetic analysis of the isolated bacteria. MS established anaerobic experiment conditions and wrote the manuscript. FT-B and JE established HPLC experimental conditions. MS, FT-B, and JE were the project leaders who formed the idea and supervised the work.

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Microbial Anti-Inflammatory Molecule (MAM) from *Faecalibacterium prausnitzii* Shows a Protective Effect on DNBS and DSS-Induced Colitis Model in Mice through Inhibition of NF-κB Pathway

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Faecalibacterium prausnitzii and its supernatant showed protective effects in different chemically-induced colitis models in mice. Recently, we described 7 peptides found in the *F. prausnitzii* supernatant, all belonging to a protein called Microbial Anti-inflammatory Molecule (MAM). These peptides were able to inhibit NF-κB pathway *in vitro* and showed anti-inflammatory properties *in vivo* in a DiNitroBenzene Sulfate (DNBS)-induced colitis model. In this current proof we tested MAM effect on NF-κB pathway *in vivo*, using a transgenic model of mice producing luciferase under the control of NF-κB promoter. Moreover, we tested this protein on Dextran Sodium Sulfate (DSS)-induced colitis in mice. To study the effect of MAM we orally administered to the mice a *Lactococcus lactis* strain carrying a plasmid containing the cDNA of MAM under the control of a eukaryotic promoter. *L. lactis* delivered plasmids in epithelial cells of the intestinal membrane allowing thus the production of MAM directly by host. We showed that MAM administration inhibits NF-κB pathway *in vivo*. We confirmed the anti-inflammatory properties of MAM in DNBS-induced colitis but also in DSS model. In DSS model MAM was able to inhibit Th1 and Th17 immune response while in DNBS model MAM reduced Th1, Th2, and Th17 immune response and increased TGFβ production.

Keywords: ***Faecalibacterium prausnitzii*, inflammation, colitis models, MAM (Microbial Anti-inflammatory Molecule), *Lactococcus lactis*, NF-κB**

INTRODUCTION

Intestinal microbiota homeostasis contributes to the protective mechanism of intestinal mucosa against the development of chronic inflammation. In mice, some commensal bacteria such as segmented filamentous bacteria (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009), *Bacteroides fragilis* (Round and Mazmanian, 2017) and Clostridia members as *Faecalibacterium prausnitzii* are able to shape gut immune responses (Sokol et al., 2008; Atarashi et al., 2013). The *F. prausnitzii* bacterium accounts for 3–5% of total fecal bacteria and is one of the predominant bacterial groups

in human feces. Decreased gut levels of *F. prausnitzii* can result in reduced capacity of self-defense against inflammatory reactions. This protective mechanism may involve the inhibition of pro-inflammatory cytokines and stimulation of anti-inflammatory cytokines secretion by active molecules (Zhang et al., 2014; Quévrain et al., 2016a). One of the mechanisms used by *F. prausnitzii* to inhibit the inflammation is the secretion of bioactive molecules that are able to block nuclear factor kB (NF-κB) activation (Sokol et al., 2008). We recently demonstrated the NF-κB blockage by a bioactive molecule, named Microbial Anti-inflammatory Molecule (MAM), from *F. prausnitzii* in epithelial cells culture (Quévrain et al., 2016a). Years of studies demonstrated some others bioactive molecules able to reduce intestinal inflammation. For example, there are two proteins secreted by *Lactobacillus rhamnosus* GG, p75, and p40, which are able to inhibit epithelial cells apoptosis induced by pro-inflammatory cytokines (Yan et al., 2007). In other hand, one of the best characterized bioactive molecule produced by commensal bacteria is Polysaccharide A (PSA) from *B. fragilis*, a commensal bacterium exhibiting anti-inflammatory properties (Mazmanian et al., 2005, 2008). PSA, which can be found at the surface of vesicles secreted by *B. fragilis*, induce the conversion of CD4 (+) T cells into Foxp3 (+) Treg cells reducing thus the intestinal inflammation (Round and Mazmanian, 2017). These findings highlighted that the searching for such bioactive molecules remains challenging scientifically but could open the door to innovative therapeutic strategies.

Since we described that the loss of *F. prausnitzii* is predictive of Crohn's Disease (CD) relapse after surgery (Sokol et al., 2008) lots of progresses have been made in the comprehension of *F. prausnitzii* role and mechanisms of action (Miquel et al., 2013). Inflammatory Bowel Diseases (IBD), CD and Ulcerative Colitis (UC), remains big issues for public health because only suspensive treatment are available. *F. prausnitzii* and its supernatant showed protective effects in various chemically-induced colitis models in mice (Sokol et al., 2008; Martín et al., 2014, 2015). Recently, we showed that MAM has anti-inflammatory properties *in vivo* in a DNBS-induced colitis model (Quévrain et al., 2016a). In order to do so, we used a food grade bacterium, *Lactococcus lactis*, modified to contain a plasmid with an expression cassette carrying the cDNA coding for MAM under the control of a eukaryotic promoter (pCMV). We have demonstrated that such recombinant *L. lactis* strains are able to transfer fully functional plasmids to eukaryotic cells *in vitro* and *in vivo* resulting in production of protein of interest by the host (Chatel et al., 2008; Del Carmen et al., 2013; Aubry et al., 2015; Souza et al., 2016). *In vitro*, the percentage of cells expressing GFP after co-incubation with *L. lactis* carrying GFP cDNA could reach 1% (Innocentin et al., 2009). As described with MAM or GFP, plasmid transfer occurred in small intestine but also in colon (Almeida et al., 2014; Quévrain et al., 2016a). The number of enterocytes transfected was two times more in colon than small intestine (Almeida et al., 2014) but we have shown recently that plasmid transfer targeted also 5% of the DCs in small intestine or colon (Michon et al., 2015).

Here we wanted to go further in the description of the anti-inflammatory properties of MAM. In order to do so, we used a transgenic mice model where luciferase expression is under the control of NF-κB promoter to test the inhibitory effect of MAM on NF-κB pathway *in vivo*. We also characterized the effect of MAM on DSS-induced colitis model.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactococcus lactis MG1363 containing pILEMPTY (LL-pILEMPTY) plasmid and *L. lactis* MG1363 containing pILMAM plasmid (LL-pILMAM) (Quévrain et al., 2016a) were grown in M17 medium (Difco) supplemented with 1% glucose and erythromycin (10 μg/mL) at 30°C without agitation overnight. The next day, the cultures were diluted 1/20 in M17 medium and grown up at 30°C without agitation. Based in our knowledge, at OD = 1 the bacteria concentration is around 5×10^8 CFU/mL. Mice were gavaged with 5×10^9 CFU/mouse. For all gavages, aliquots 1OX concentrate were previously prepared as described and frozen at −80°C. To use, aliquots were gradually taw on ice bath to preserve all structures and diluted with PBS.

Mice Experiment

This study was carried out in accordance with the guidelines of the local ethics committee. Housing conditions and procedures were specified by the French Law regarding the protection of laboratory animals (authorization #78–149 of the French Veterinary Services). Sets of 6-week-old C57BL/6J mice ($n = 8$ per group) (Janvier, France) were housed in groups of 4 mice per plastic cage, under standard environmental conditions with free access to food and water in the Animal Facility of National Institute of Agronomic Research.

DNBS-Induced Colitis

Colitis was induced in mice under light anesthesia by a single intra-rectal instillation of DNBS (100 mg. kg^{-1} of body weight) diluted in 30% ethanol. During all long experiment, from 7 days before the DNBS (MPBio) instillation to the sacrifice of the mice, mice were fed daily by intragastric gavage with recombinant strains (LL-pILEMPTY and LL-pILMAM 5×10^9 CFU/200 μL/mouse) or with PBS.

Mice were monitored daily for weight loss. Mice were sacrificed at day 4, the abdomen was opened by midline incision and the descending colon and Mesenteric Lymphatic Node (MLN) were removed. MLN were stocked in RPMI medium on ice and after mashed and cells counting to stimulate with anti-CD3 and anti-CD28 for 48 h in 37°C and 5% CO_2. Colon was rinsed in PBS, opened along the anti-mesenteric border, cleaned and cut for cytokines assays.

DSS-Induced Colitis

Colitis was induced in mice by oral administration of 2.5% (w/v) of Dextran Sulfate Sodium Salt (DSS) at 36,000–50,000 of molecular weight (MPBio) dissolved in drinking water from day 0 to day 7. During all long experiment, from 7 days before the DSS administration to the sacrifice day (D14), mice were fed by

intragastric gavage with recombinant strains (LL-pILEMPTY and LL-pILMAM–5 × 10^9 CFU/200 μL/mouse) or with PBS.

Mice were monitored daily for weight loss, stool consistency, and fecal occult blood (Hemoccult, Beckman Coulter). Disease Activity Index (DAI) has been calculated according to the protocol by Cooper et al. (1993). The mice were sacrificed at day 14 and MLN and colon were collected as described above.

Protein Extraction in Colon

One centimeter of colonic tissue was mashed by GentleMax™ (Miltenyl Biotec) in 1 mL of PBS plus anti-protease (Roche). The lysate was centrifuged and the supernatant as used to measure cytokine level by ELISA (Mabtech). The cytokines tested were Th1- related cytokine (IFNγ and IL12); Th2-related cytokines (IL4 and IL5); Th17-related cytokine (IL17) and Treg–related cytokines (IL10 and TGFβ), Th22- related cytokine (IL22) and IL6 (NF-κB pathway).

Interleukin Secretion by Stimulated Lymphocytes

Mesenteric Lymph Nodes (MLN) isolated from mice were mashed and filtered (70 μm, BD biosciences). Lymphocytes were counted by flow cytometry (Accuri C6) and suspended in RPMI (Lonza) with 100 Unit of Streptomycin, Penicillin, PAA Laboratories and 10% Fetal Calf Serum (FCS) (Lonza) at a concentration of 2,5.10^6 cells/mL in 24 wells plate (Costar) pre-incubated with anti-CD3 and anti-CD28 antibodies, 4 μg/mL of each antibody (eBioscience) in PBS with 0.5% FCS. Plates were incubated 48 h at 37°C, 5% of CO_2 and cytokine level was assessed by ELISA (Mabtech). The cytokines tested were Th1- related cytokine (IFNγ); Th2-related cytokines (IL5); Th17-related cytokine (IL17) and Treg–related cytokines (IL10 and TGFβ) and Th22- related cytokine (IL22).

IVIS (Analysis *In vivo*)

NF-κB-luciferase mice, transgenic mice model where luciferase expression is under the control of NF-κB promoter, were used to test the effect of MAM on NF-κB pathway *in vivo*. Mice were anesthetized with a cocktail of 0.1% ketamine (Imalgene 1000, Merial, France) and 0.06% xylazine (Rompun, Alcyon, France) and luciferine 15 mg/mL were administrated by intraperitoneal injection (50 μl). The luminescence of NF-κB recombinant mice was evaluated by IVIS (*In vivo* Imaging System) 200 (Perkin Elmer) at D2 and D4 after DNBS challenge. Region of interest (ROI) measurements were accessed by photon quantification.

Statistical Analysis

All statistics and graphics have been performed on Prism-GraphPad®. Results represent means ± s.e.m. Statistical significance was determined by the Mann-Whitney test. It has been considered that $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

RESULTS

MAM Shows a Protective Effect on DNBS-Induced Colitis by Decreasing NF-κB Activation and Increasing Regulatory and Repairing Pathways

Colitis was induced in NF-κB-luciferase mice treated or not with LL-pILMAM or LL-pILEMPTY. NF-κB activation was monitored directly on live animals using IVIS. At D4, the luminescence was lower in DNBS- induced colitis mice treated with LL-pILMAM than in LL-pILEMPTY or control mice (PBS). Moreover, the NF-κB signal didn't increase from D2 to D4 in LL-pILMAM treated mice while it increased for LL-pILEMPTY and control mice. This result suggests a protective effect by LL-pILMAM compared with the other groups by inhibiting NF-κB pathway. The region of interest (ROI) measurement confirms these evidences (**Figure 1**).

Pro and anti-inflammatory cytokines secreted by lymphocytes from MLN were monitored 4 days after DNBS administration. As shown in **Figure 2**, a significant decrease in IL17, IFNγ and IL5 was found in supernatant of lymphocytes from LL-pILMAM treated mice compared to LL-pILEMPTY and control mice. The concentration of IL10 was significantly increased in LL-pILMAM mice compared to LL-pILEMPTY mice, but there is no statistically significant difference compared to control animals. However, no differences in TGFβ and IL22 concentrations were observed among these groups of mice (**Figure 2**).

In parallel, proteins were extracted from colon tissue and cytokines concentrations were assayed. We observed a strong increase of TGFβ in LL-pILMAM treated mice compared to LL-pILEMPTY and control mice. Moreover, the IL5 and IL17 cytokine levels decreased in LL-pILMAM and control mice compared to LL-pILEMPTY mice (**Figure 3**). We could not observe any differences in IL4 and IL10 production among all groups of mice. It has to be noticed that treatment with LL-pILEMPTY induced an increase of IL5, IL17 and IFNγ compared to control mice.

Mam Protects Mice from DSS-Induced Colitis

To determine the impact of local production of MAM on another chemically-induced colitis model, we performed a DSS-induced colitis model on mice orally administered with LL-pILMAM or LL-pILEMPTY or PBS. Recombinant bacteria were administered 7 days before, during and after colitis induction. We did not observe any difference in the weight loss among these groups of mice (**Figure 4A**). Oral administration of LL-pILMAM decreased the DAI at D5 compared to pILEMPTY or control mice. At D8 and D9 the DAI difference was only significant compared to pILEMPTY treated mice. At this stage of the colitis daily administration of bacteria seems to increase the DAI (**Figure 4B**). The effect of MAM is particularly significant on bleeding (**Figure 4C**).

After 7 days of inflammation followed by 5 days of recovery, MLN and colon tissues were removed and immune response was analyzed. In MLN supernatant, INFγ and IL17 were both

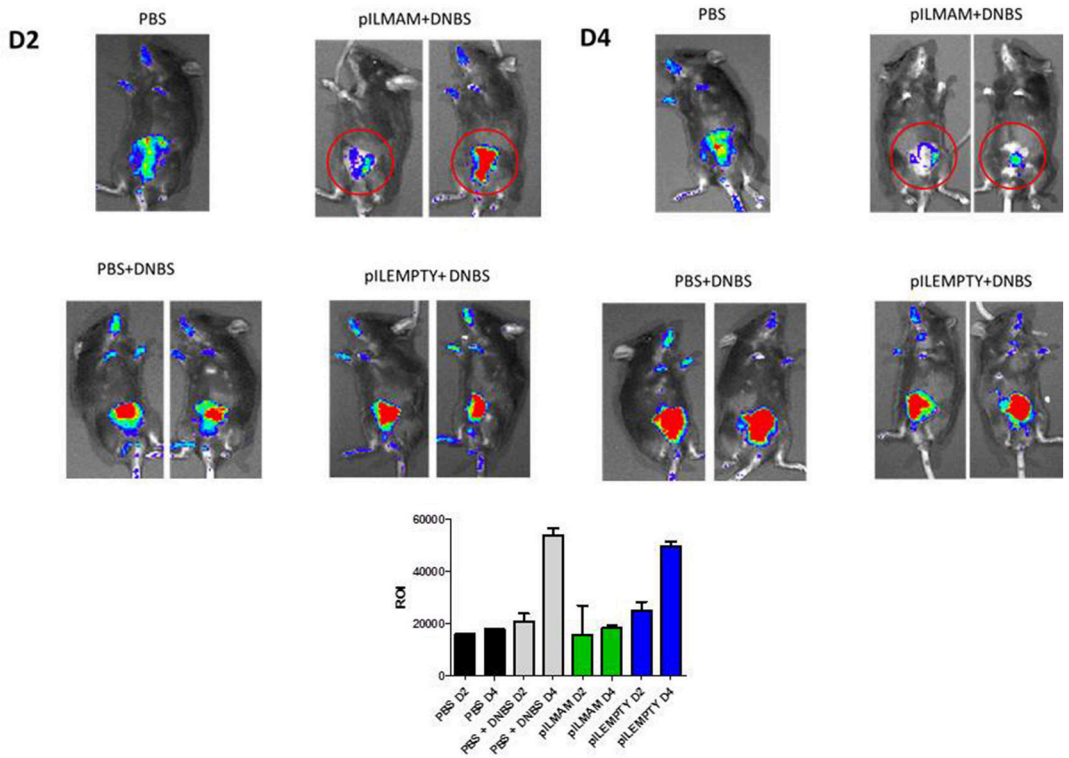

FIGURE 1 | Inhibition of NF-κB pathway *in vivo* after LL-pILMAM administration. NF-κB luciferase mice, transgenic mice expressing luciferase under the control of NF-κB, were orally administered with PBS, LL-pILEMPTY or LL-pILMAM 7 days before DNBS intrarectal injection (D0) and until sacrifice (D4). NF-κB activation was monitored by luminescence *in vivo* on whole animal using IVIS Spectrum. ROI (Regions of interest) measurements were used to determinate how many photons are radiating from the source.

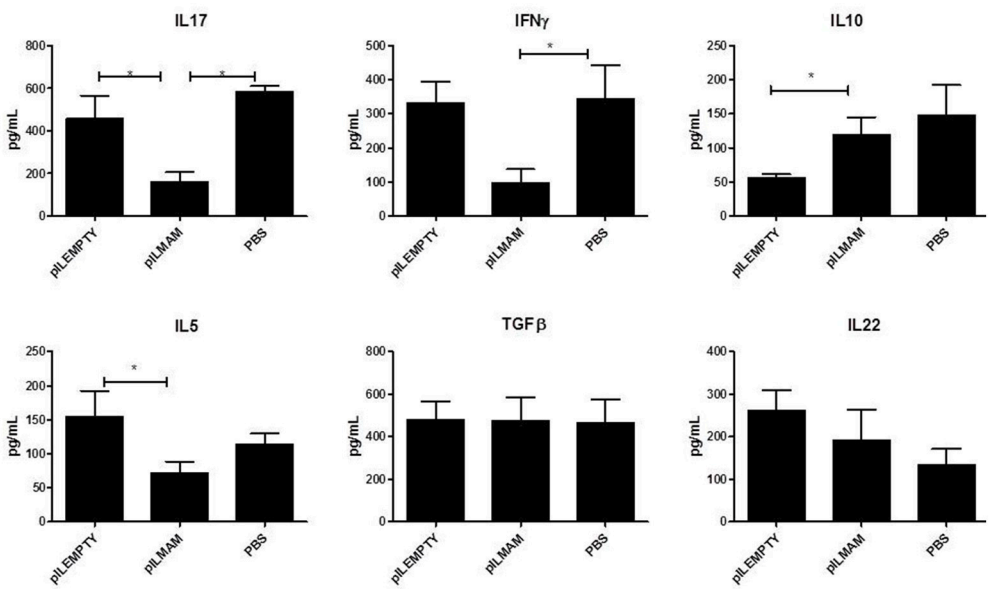

FIGURE 2 | Cytokines secreted by reactivated lymphocytes from MLN in a DNBS-induced colitis model. NF-κB luciferase mice were orally administered with PBS, LL-pILEMPTY or LL-pILMAM 7 days before DNBS intrarectal injection (D0) and until sacrifice (D4). At D4 MLN were withdrawn and isolated lymphocytes reactivated by anti-CD3/anti-CD28 antibodies. Cytokine concentration in medium was monitored 48 h after reactivation by ELISA. *$P < 0.05$.

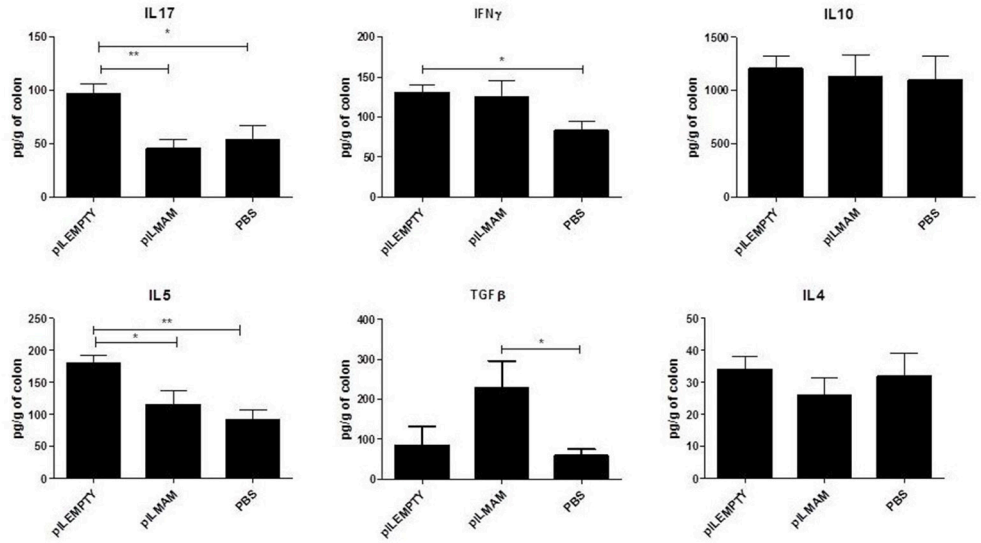

FIGURE 3 | Cytokines produced by colon tissue in DNBS-induced colitis model. NF-κB luciferase mice were orally administered with PBS, LL-pILEMPTY or LL-pILMAM 7 days before DNBS intrarectal injection (D0) and until sacrifice (D4). At D4 colon was withdrawn and proteins extracted. Cytokine concentration in protein extracts was monitored by ELISA. *$P < 0.05$, **$P < 0.01$.

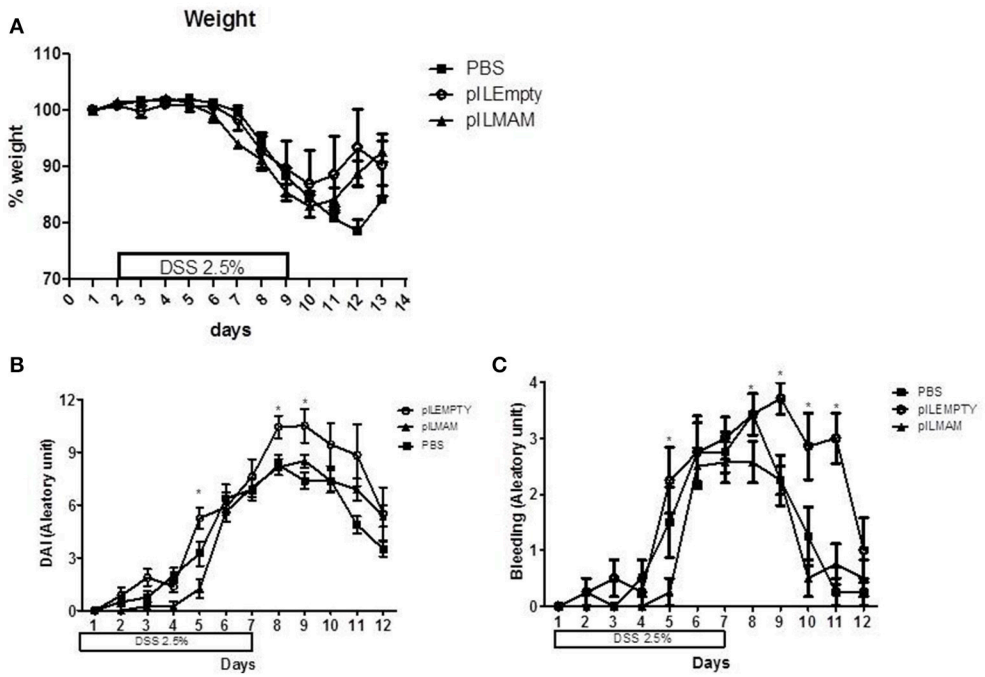

FIGURE 4 | Effect of MAM on macroscopic scores in a DSS-induced colitis model. Mice were orally administered with LL-pILMAM, LL-pILEMPTY or PBS 7 days before and during colitis induction. Colitis was induced by adding DSS in drinking water during 7 days (D0-D7). Then DSS was removed from drinking water and mice allowed to recover during 5 days (D7-D12). Weight **(A)** and DAI **(B)** including bleeding **(C)** were monitored daily. *$P < 0.05$.

decreased in LL-pILMAM group compared with LL-pILEMPTY (**Figure 5**) as observed previously in DNBS experiment. TGFβ was also decreased whereas no differences in IL5 and IL10 concentrations were observed among the mice groups. In proteins extracts from colon tissues we could observe only a decrease of IL-6 in LL-pILMAM compared with the other groups (**Figure 6**). No differences in IFNγ, IL-10, IL-12, TGFβ, and IL-22 concentrations were monitored among the mice groups.

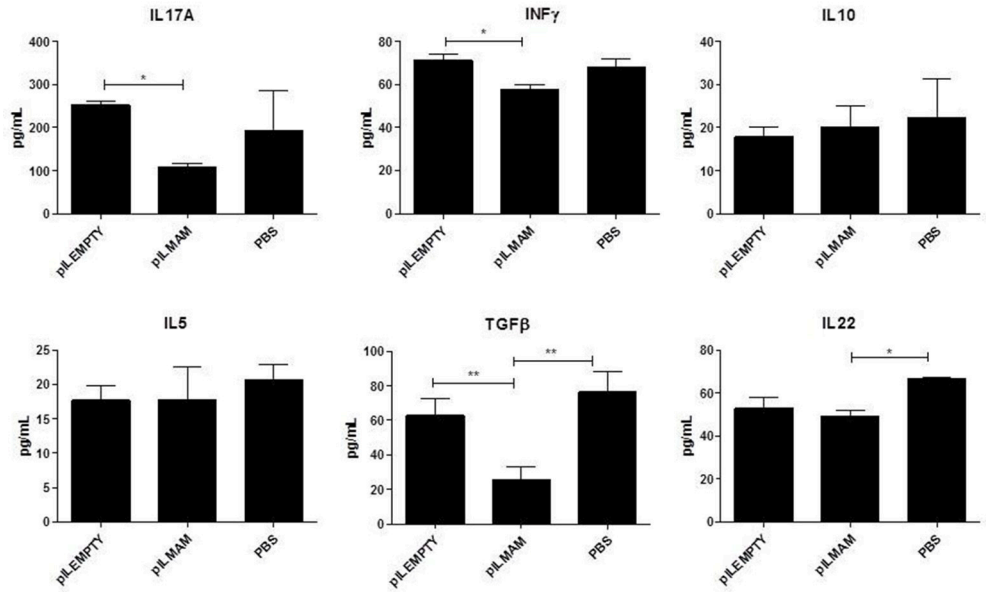

FIGURE 5 | Cytokines secreted by reactivated lymphocytes from MLN in DSS-induced colitis model. Mice were orally administered with LL-pILMAM, LL-pILEMPTY or PBS 7 days before and during colitis induction. Colitis was induced by adding DSS in drinking water during 7 days (D0-D7). Then DSS was removed from drinking water and mice allowed to recover during 5 days (D7-D12). At D12 mice were killed, MLN withdrawn, cells were isolated and lymphocytes reactivated with antiCD3/anti-CD28 antibodies. Cytokine concentration in medium was monitored 48 h after reactivation by ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$.

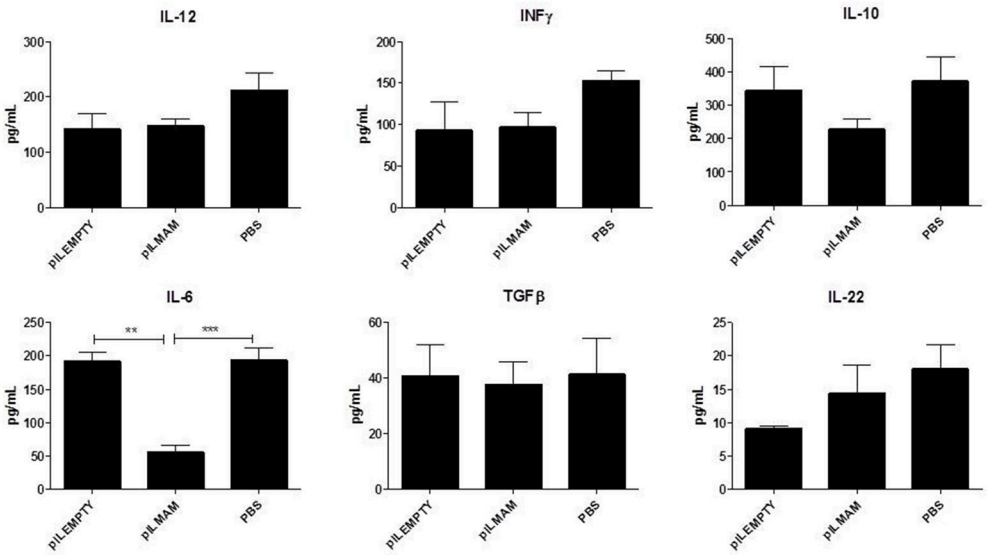

FIGURE 6 | Cytokines produced by colon tissue in DSS-induced colitis model. Mice were orally administered with LL-pILMAM, LL-pILEMPTY or PBS 7 days before and during colitis induction. Colitis was induced by adding DSS in drinking water during 7 days (D0-D7). Then DSS was removed from drinking water and mice allowed to recover during 5 days (D7-D12). At D12 mice were killed, colon was withdrawn and proteins extracted. Cytokine concentration in protein extracts was monitored by ELISA. $^{**}P < 0.01$, $^{***}P < 0.001$.

DISCUSSION

Anti-inflammatory properties of MAM have been characterized by an inhibition of NF-κB *in vitro* and a protective effect in DNBS-induced colitis model on weight loss, macroscopic score and a decrease in IL-17A and IFNγ secreted by activated lymphocytes from MLN (Quévrain et al., 2016a). Here we showed for the first time that MAM inhibits NF-κB pathway *in vivo* by using NF-κB-luciferase transgenic mice. Ours results clearly indicated that delivery of MAM cDNA

at intestinal mucosa decreased NF-κB pathway activated by DNBS.

In DSS-induced colitis model, MAM administration didn't modify the weight loss significantly like in DNBS-induced colitis model (Quévrain et al., 2016a) but showed a positive effect on Disease Activity Index (DAI) at several days and especially on bleeding. DAI, which is one of the main macroscopic markers, is established by summing different scores, bleeding, stool consistency, and weight loss reflecting the global physiological state of the digestive tract. These results suggest that MAM is probably able to restore tissue integrity but the mechanism is unclear as IL22, a cytokine involved in tissue repair is slightly decreased. It has to be noticed that this difference is significant considering only pILEMPTY group and not the control PBS group.

We wanted also to characterize more precisely the mechanisms of action of MAM by going deeper in the immune response description and by using a DSS-induced colitis model, both models being commonly used and having different characteristics. Despite the shortcomings, DNBS model is often described to be a good model of CD mainly driven by Th1 and Th17 biased-immune response but also very useful to study the role of adaptive immune response in IBD (Kiesler et al., 2015). One of the other main chemical used to induce colitis models is DSS. DSS model has been described to be closer to UC with a Th2 exacerbated immune response. But DSS inflammation is also mainly driven by innate immune response (Chassaing et al., 2014).

First we confirmed our results by showing that IL-17A (Th17) and IFNγ (Th1) are decreased in MLN from LL-pILMAM group in DNBS-induced colitis. Suppression of Th1 and Th17 cytokine is correlated with beneficial anti-colitis effect (Reyes et al., 2016). We could observe also a decrease of IL-5 and an increase of IL-10 both Th2 cytokines. It has to be noticed that these differences are only significant regarding LL-pILEMPTY group. In DSS model, MAM decreased by 2-fold IL17 secreted by reactivated MLN lymphocytes as in DNBS model. IFNγ is also reduced but less than in DNBS. Surprisingly, TGFβ and IL22 secretion was lower in MAM treated group than in pILEMPTY or PBS group. Thus, MAM has a robust inhibition effect on IL17 and IFNγ secretion in MLN in both models.

We enlarged our investigation field and looked for the cytokines production in colon. In DNBS model, we observed that, like with MLN, IL17, and IL5 are reduced in colon but only regarding pILEMPTY. More striking, we found that TGFβ, which is involved in Treg development, is increased by MAM in colon tissue. In healthy conditions, Treg cells play an important role in controlling immune homeostasis. In IBD, Th1, and Th17 responses overwhelm the control mechanisms of Treg cells. This imbalance in the intestinal immunity of IBD patients, shifting toward the pro-inflammatory side, leads to intestinal inflammation. In DSS model, we observed only a decrease in IL6 production in the colon from mice treated with MAM. Nuclear factor-kB participates in controlling the activation of various pro-inflammatory cytokine genes such as IL6, IFNγ or IL17 suggesting that NF-κB pathway is also turned off in MAM treated mice in DSS colitis model.

As noticed above we observed particularly in DNBS-induced colitis an increase of IL-5, IL-17 and IFNγ in colon tissue of mice treated with LL-pILEMPTY compared to control group and a tendency to decrease IL10 even if the difference is not statistically significant. This profile of response describes *L. lactis* as a pro-inflammatory bacterium. This is in accordance with previous results where we showed that *L. lactis* co-incubated with PBMC give a very low IL10/IL12 ratio (Sokol et al., 2008; Kechaou et al., 2013) or a slight increase of IL-8 secreted by HT-29 after TNFα activation (Kechaou et al., 2013). These two criteria are characteristic of a pro-inflammatory strain. Nevertheless, this slight pro-inflammatory effect of our bacterial vector didn't counterbalance the anti-inflammatory properties of MAM as we have less weight loss or a macroscopic score lower in MAM treated group compared to pILEMPTY treated group (Quévrain et al., 2016a).

Our strategy of plasmid transfer results in MAM expression by epithelial cells. We have no proof that MAM or its peptides produced naturally by *F. prausnitzii* (Quévrain et al., 2016b) have to enter inside the cells of the intestinal membrane to show their protective effect. We tried to produce MAM or its peptides by heterologous protein production or by chemical synthesis without success (Quévrain et al., 2016a). Nevertheless, our strategy of producing MAM by epithelial cell mimics the results obtained with *F. prausnitzii* or its supernatant containing MAM as inhibition of NF-kB, protection against weight loss, increase of IL10 (Sokol et al., 2008), decrease of IL17 (Zhang et al., 2014), decrease of IL6 or IFNγ (Martín et al., 2014) validating our approach.

Our observations suggest that MAM was able to decrease Th1 and Th17 pro-inflammatory cytokines in MLN and colon tissue in both DNBS and DSS colitis model and to enhance TGFβ in DNBS model promoting thus host protective effect and attenuating intestinal inflammation through mechanisms affecting NF-κB activation. Nevertheless, MAM was not able to decrease the Th2 immune response, typically induced in DSS colitis, which results in a less effective protection.

These results can be considered as a new step in the characterization of MAM mechanism of action opening thus the development of innovative therapeutic strategies based on the use of this bioactive molecule. The challenge is to define the immunological regulation associated with this protein or part of this, the location of this protein and the vector used to deliver able to suppress inflammation and determine the optimal means to translate this knowledge to the treatment of inflammatory disease.

AUTHOR CONTRIBUTIONS

This work was drafted by JC and PL and they received a support financial from INRA transfer to apply in this study. NB and CM were the responsible to perform the experiments, analysis and interpretation of data. CD, PV and FC helped them to develop some key experiments and they participated also in the interpretation of those data. All data were discussed with all authors to establish an integral and coherent analysis. JC, PL, and VA gave the final approval of the version to be published.

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In Silico Screening of the Human Gut Metaproteome Identifies Th17-Promoting Peptides Encrypted in Proteins of Commensal Bacteria

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Scientific studies focused on the role of the human microbiome over human health have generated billions of gigabits of genetic information during the last decade. Nowadays integration of all this information in public databases and development of pipelines allowing us to biotechnologically exploit this information are urgently needed. Prediction of the potential bioactivity of the products encoded by the human gut microbiome, or metaproteome, is the first step for identifying proteins responsible for the molecular interaction between microorganisms and the immune system. We have recently published the Mechanism of Action of the Human Microbiome (MAHMI) database (http://www.mahmi.org), conceived as a resource compiling peptide sequences with a potential immunomodulatory activity. Fifteen out of the 300 hundred million peptides contained in the MAHMI database were synthesized. These peptides were identified as being encrypted in proteins produced by gut microbiota members, they do not contain cleavage points for the major intestinal endoproteases and displayed high probability to have immunomodulatory bioactivity. The bacterial peptides FR-16 and LR-17 encrypted in proteins from *Bifidobacterium longum* DJ010A and *Bifidobacterium fragilis* YCH46 respectively, showed the higher immune modulation capability over human peripheral blood mononuclear cells. Both peptides modulated the immune response toward increases in the Th17 and decreases in the Th1 cell response, together with an induction of IL-22 production. These results strongly suggest the combined use of bioinformatics and *in vitro* tools as a first stage in the screening of bioactive peptides encrypted in the human gut metaproteome.

Keywords: bacterial peptides, Th17 response, CD4 cytokines, flow cytometry, microbiome, gut metaproteome

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INTRODUCTION

The composition of the human microbiome has revealed strong associations with human health. Alterations in the intestinal microbial populations when compared to control groups, denominated dysbiosis, are nowadays associated with several diseases from intestinal and extraintestinal nature (Shreiner et al., 2015). Modulation of the human microbiome in order to balance the microbial populations toward a healthy situation is a promising intervention to improve human health in the framework of many diseases (Drew, 2016).

The human microbiome performs key metabolic roles such as production of vitamins, short-chain fatty acids and other essential compounds, digestion of several components of the diet such as fibers, gut homeostasis maintenance and modulation of the host immune response (O'Toole et al., 2017). Gut microbiota and human cells communicate with each other through surface-associate proteins or extracellular components, in a dialog denominated molecular cross-talk (Hevia et al., 2015). Moreover, bacteria structural components have their own receptors in the innate immune system; in this way, the constant fraction of the surface-associated flagellin is recognized by Toll-like receptor 5 (TLR-5) and the cell-wall component lipopolysaccharide by TLR-4 (Sanchez et al., 2011). These ligands are denominated microbial associated molecular patterns (MAMPs) and their recognizing counterparts on the human host are termed pattern recognition receptors (PRRs). Recognition and binding of MAMPs to PRRs triggers different intracellular signalization pathways that led to changes on the surface molecules of the immune cells and in their effector secretion profiles, among interleukins and other cytokines (Ley et al., 2006). The type, amount and temporal pattern of MAMP-PRR interaction will finally determine the type of immune response that can be roughly divided into proinflammatory (mainly Th1, Th2 and Th17) or regulatory responses (Treg) (Ruiz et al., 2016a).

Extracellular and surface-associated proteins of the gut microbiota have been studied in the last years as candidates responsible for part of the immunomodulatory action of intestinal microorganisms, given that these proteins performs important roles in the interaction with the host and the environment (Ruiz et al., 2016b). Many of this research has been focused on the identification of molecules responsible for the expansion of Treg cells, a Tcell subset essential in the maintenance of a controlled and low-grade inflammation status of the gut mucosa (Levy et al., 2017). Few proteins involved in gut homeostasis maintenance and produced by gut bacteria have been identified so far. *Faecalibacterium prausnitzii* is a bacterium whose absence from the human gut microbiome is associated with Crohn's Disease, and which produces a small protein with anti-inflammatory properties by inhibiting the proinflammatory NF-κB pathway (Quevrain et al., 2016). Lactic acid bacteria are transitory members of the human gut microbiota in individuals with fermented product intake within their diets. It has been shown that some lactobacilli species are able to produce immunomodulatory peptides encrypted in larger extracellular proteins. One of these peptides is STp, which when recognized by dendritic cells isolated from Ulcerative Colitis patients induced a switch toward an anti-inflammatory cytokine profile (Bernardo et al., 2012; Al-Hassi et al., 2014).

Therefore, the protein content of an intestinal microorganism may play a key role in the way it modulates the immune system functions in the host (Furusawa et al., 2015). Identifying the molecules responsible for all the molecular crosstalk mechanisms as well as the immune receptors/signalization pathways induced/repressed by the different MAMPs are needed for understanding the immune mechanism of action of a gut microbiota taken as a whole. With these limitations in mind, our research group has developed the Mechanism of Action of the Human Microbiome (MAHMI) database[1] (Blanco-Miguez et al., 2017). MAHMI is an online resource for the prediction of peptide bioactivity based on their amino acid sequence which is fuelled by public Metahit project metagenomes[2]. This database is a unique resource for the organization and processing of potential immunomodulatory peptides encrypted in the human gut metaproteome.

In this work, we have identified 15 potential bioactive peptides through MAHMI pipeline, to test whether this database was able to identify potential immunomodulatory peptides. The *in silico* test was completed with an *in vitro* test where 15 peptides were incubated with human peripheral blood mononuclear cells (PBMCs), and coupled to a multiplex secreted cytokine assay. Main results are discussed next.

MATERIALS AND METHODS

Ethics approval for this study (reference code AGL2013-44039-R) was obtained from the Regional Ethics Committee for Clinical Research (*Comité de Ética de la Investigación del Principado de Asturias*) in compliance with the Declaration of Helsinki. Samples used in this study were obtained from anonymous donors of our blood donation system.

Bioactivity Prediction

The bacterial peptides used in this study were previously selected regarding their theoretical bioactivity based on *in silico* analyses performed through the MAHMI database[1] (Blanco-Miguez et al., 2017). Briefly, the pipeline starts with the *in silico* digestion of the proteins obtained from the human gut microbiome (gut metaproteome). Potential bioactivity of the encrypted peptides is performed by comparison of their amino acid sequence to a curated database of immunomodulatory peptides. Selected peptides (**Table 1**) were synthesized at GeneCust facilities (Ellange, Luxemburg). Peptides were resuspended in phosphate buffered saline (PBS) (Oxoid Limited, Hampshire, United Kingdom) at a concentration of 2 mg mL^{-1} and stored at −80°C until their use.

[1]http://www.mahmi.org

[2]http://gigadb.org/dataset/100064

TABLE 1 | Potential immunomodulatory peptides used in this work and information associated.

Peptide	Peptide sequence	Uniprot ID	Uniprot protein	Uniprot organism	Uniprot gene	Bioactivity prediction
QE14	QHVENNKSTPLPME	P54355	ENTM_BACFG Fragilysin	*Bacteroides fragilis*	bftP	60
LR17	LPLAFFVLTFLWALILR	Q3V815	MTGA_BACFR Monofunctional biosynthetic peptidoglycan transglycosylase	*Bacteroides fragilis* YCH46	mtgA	80
DW15	DEILENVDEVLGNDW	Q45119	TNPA_BACFG Transposase for insertion sequence element IS21-like	*Bacteroides fragilis*	tnpA	75
KF14	KETILEPVEGVGHF	Q08425	TETQ_BACFG Tetracycline resistance protein TetQ	*Bacteroides fragilis*	tetQ	66
KE13	KKMTPANEARPIE	D4QFE7	BGAL_BIFBI Beta-galactosidase BbgII	*Bifidobacterium bifidum*	bbgII	75
SW14	SASLVDGIARKDPW	D4QFE7	BGAL_BIFBI Beta-galactosidase BbgII	*Bifidobacterium bifidum*	bbgII	66
EY12	EMVEDILTGQCY	C6H178	BGAL2_LACAI Beta-galactosidase LacA	*Lactobacillus acidophilus*	lacA	60
GR16	GTSFEGNEYFEGPSIR	P48790	XYLA_CLOSR Xylosidase/arabinosidase	*Clostridium stercorarium*	xylA	100
AR15	AIQDEIDQLSTEIDR	P80583	FLA_CLOTY Flagellin (Fragment)	*Clostridium tyrobutyricum*	fla	75
FR16	FAIVDEVDSILIDEAR	B3DR89	SECA_BIFLD Protein translocase subunit SecA	*Bifidobacterium longum* DJO10A	secA	100
WK19	WIEAVGYSLTQHPDPELEK	E8MGH8	HYBA1_BIFL2 Non-reducing end beta-L-arabinofuranosidase	*Bifidobacterium longum subsp. longum* ATCC 15707	hypBA1	100
VF10	VPSEKKAELF	P38059	SLAP_LACHE S-layer protein	*Lactobacillus helveticus*	slpH	80
HK11	HIGVHSDPVDK	P30269	AMY_BUTFI Alpha-amylase	*Butyrivibrio fibrisolvens*	amyA	75
AF16	ASPEDEAASMEKAKAF	A6L1Z2	G1095_BACV8 Glycosyl hydrolase family 109 protein 5	*Bacteroides vulgatus* ATCC 8482	BVU_2041	80
NR15	NAALASYSLASDLDR	Q9XD84	TIBA_ECOH1 Adhesin/invasin TibA autotransporter	*Escherichia coli O78:H11* (H10407)	tibA	100

Peripheral Blood Mononuclear Cells (PBMC) Isolation

The capability of synthetic bacterial peptides to induce immune modulation *in vitro* was assessed using a PBMC model. PBMCs were isolated from the buffy coat of 5 healthy donors from the Community Center for Blood and Tissues of Asturias (Oviedo, Spain). Then, 5 mL of each buffy were diluted with one volume of PBS and added on the top of a 5 mL of Ficoll-Hypaque (Lymphoprep; Nycomed, Oslo, Norway) for gradient separation. Cells were separated by centrifugation (1,800 rpm, 30 min) with two further washes with PBS; the first one at 1,200 rpm, 10 min (to discard platelets) and the second one at 1,500 rpm, 5 min. The number of PBMCs was estimated using a Neubauer chamber (Brand, VWRI Eurolab, Barcelona, Spain) and adjusted to a final concentration of 2×106 mL^{-1} in RPMI 1640 broth (Lonza, Basilea, Switzerland) supplemented with 10% (v/v) fetal bovine serum and antibiotics (Sigma-Aldrich, San Luis, MO, United States).

Co-cultivation of Peptides and PBMCs

Peripheral blood mononuclear cells were cultivated in round bottom 96 wells microplates using 200 μL of the cell suspension described above. Bacterial peptides were added at a final concentration of 50 μg mL^{-1}, based on previous studies of the immunomodulatory peptide STp (Bernardo et al., 2012). PBMCs were activated with 100 ng mL^{-1} of anti-CD3 added to the RPMI media. For each donor positive (LPS, 1 μg mL^{-1}) and negative controls of PBMCs stimulation were included. Microplates were incubated for 5 days at 37°C with 5% CO_2.

Cytokine Quantification

After 5 days, supernatant was collected and stored at −80°C for multiplexed cytokine analyses. The production of 18 different cytokines (GM-CSF, IFNγ, IL-1β, Il-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, and TNFα) were quantified using the Th1/Th2/Th9/Th17/Th22/Treg Cytokine 18-Plex Human ProcartaPlex™ Panel (Affymetrix eBioscience, San Diego, CA, United States) and the Luminex® xMap Technology equipment following manufacturer's settings. The results for each cytokine were represented using box plot diagrams and differences between peptides were statistically analyzed.

In the case of peptides FR-16 and LR-17, the following ratios were calculated for each sample: IL10/TNFα, IL10/IFNγ, IL10/IL17, IL10/IL4, IFNγ/IL17, Th1/Th2, Th17/Th1, Th17/Th2, Th1/Treg, Th2/Treg, Th1 + Th2 + Th17/Treg, Th17/Treg, Th22/Th1, Th22/Th2, Th22/Th17, Th22/Treg and Th22 + Th17/Th1 + Th2. These ratios were calculated considering the T-cell response cytokines, either signature or induced cytokines (**Figure 1**) and represented using boxplots. Cytokines were included in each ratio according to the review of Wan and Flavell with the addition of the Th22 subset (Wan and Flavell, 2009).

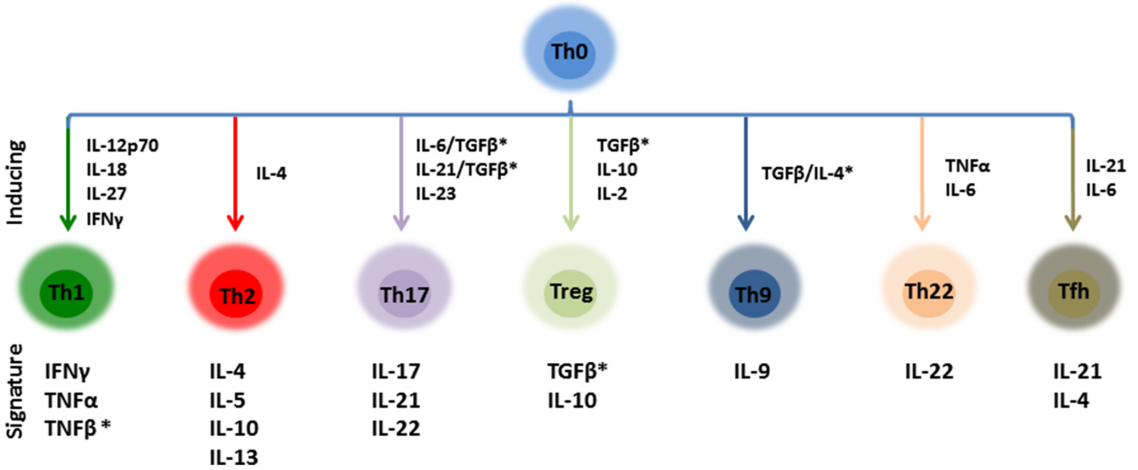

FIGURE 1 | Schematic representation of the main CD4+ effector T cells and their inducing/signature cytokines. Ratios calculated in this work were calculated taken into account this distribution. *These cytokines were not included in the ratios as TGFβ and TNFβ have not been quantified.

Statistical Analyses

All experiments were performed in independent biological quintuplicates. Data distribution did not follow normality, so initial comparisons were performed with the non-parametric Wilcoxon and Tukey pairwise tests. Differences in the value ranks between two conditions were assessed with the Mann–Whitney *U* tests for equal medians with Monte Carlo permutations ($n = 99{,}999$). Comparisons with a p-value ≤ 0.05 were considered statistically significant. Whisker and boxes plots were generated using ggplot2 in R environment, Past3 v3.15 (Hammer et al., 2001) and the IBM SPSS Statistics package (SPSS Inc., Chicago, IL, United States).

RESULTS AND DISCUSSION

In the present work we have synthesized 15 peptides (**Table 1**) encrypted in proteins produced by the human gut microbiome and selected due to their high potential immune modulatory properties predicted through the MAHMI pipeline[3]. To determine the immune response elicited by these bacterial peptides we selected an *in vitro* PBMC model from human healthy donors, as monocyte-derived dendritic cells have been shown as not suitable for these purposes (unpublished data). Eighteen cytokines related to T-cell response were quantified in the PBMC supernatants of five healthy donors after 5 days of co-culture with the peptides. Cytokine levels were measured basally (PBMCs without anti-CD3), in the activation control (PBMCs with anti-CD3), in the positive control (LPS + activated PBMCs) and for each peptide of study (peptide + activated PBMCs) (**Supplementary Figure S1**).

In general every peptide was sensed by PBMCs, which reacted with changes in their secreted cytokine profiles compared to the activation control. All the peptides displayed an effect modulating the production of at least one cytokine, although few of them induced consistently the production of interleukins from single T-cell response pathways, represented in **Figure 1**. For instance, the production of IL-2 and IL-6 were statistically induced ($p < 0.05$) by peptides FR-16, LR-17, AR-15, EY-12, KE-13 when compared to the control, as well as the LPS (**Supplementary Figure S1**). Peptides FR-16 and LR-17, with an immunomodulatory bioactivity prediction in MAHMI database of 100 and 80% respectively (**Table 1**), showed the higher immune modulation capability as reflected in the cytokine secretion induced over PBMCs (**Figure 2**).

FR-16 and LR-17 are bacterial peptides encrypted in proteins from *Bifidobacterium longum* DJ010A and *Bacteroides fragilis* YCH46, species that are amongst the more abundant members of the human microbiome (Arboleya et al., 2016; Lloyd-Price et al., 2016). As shown in **Figure 2**, both peptides increased the production of IL-6, IL17a, IL12p70, IL22, IL23 and TNFα either significantly or at the same levels than LPS did (with p-values very close to 0.05). All these cytokines are important in the Th17 and Th22 differentiation pathways (Wan and Flavell, 2009).

As the T cell response in our model seemed to be modulated by FR16 and LR17, different ratios were calculated taken into account the inducing and signature cytokines for each pathway and also key cytokines such as IL10, TNFα or IFNγ (**Supplementary Figure S2**). As it can be shown in **Figure 3**, the Th17/Th1 and the Th17/Th2 ratio was consistently higher for both peptides compared with the anti-CD3 activation control. When Th22/Th1 and Th22/Th17 ratios were computed, the basal conditions were displaced toward the Th22 pathway and addition of the anti-CD3 antibody induced production of both Th1 and Th17 cytokines. Interestingly, increase in the Th22 pathway was significantly higher for both peptides whatever the pathway used for calculate the ratio, and always compared to the activation control.

Th17 and Th22 response develop important roles in the maintenance of gut homeostasis. In general, it has been reported

[3]http://www.mahmi.org

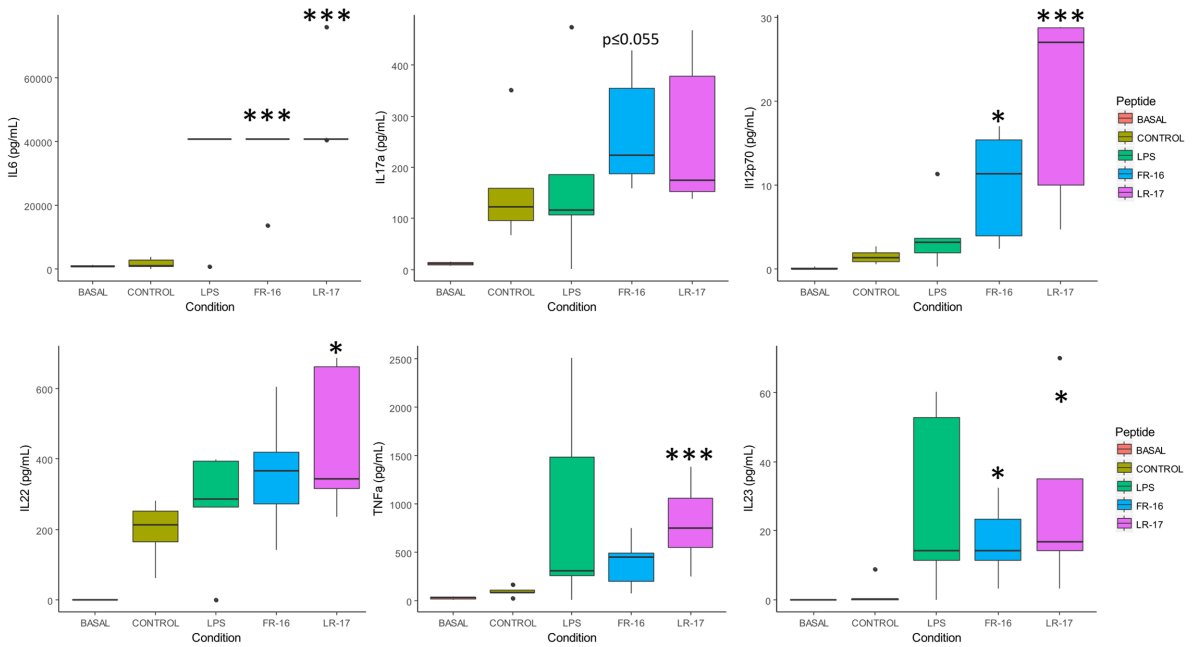

FIGURE 2 | Main cytokine levels affected by the presence of peptides LR17 and FR16. Boxplots represent median and interquartile range for the selected cytokines (see all in **Supplementary Figure S1**). These were quantified in the supernatants after 5 days of *in vitro* co-culture of human PBMCs and the bacterial peptides FR-16 and LR-17, from *B. longum* DJO10A and *B. fragilis* YCH46, respectively. Significant differences were assessed by the non-parametric Mann–Whitney *U* test and are represented with *, **, or *** ($p < 0.05$, 0.01, or 0.001 respectively). Significant differences refer to control conditions (PBMCs + anti-CD3).

that commensal microbiota influence the balance between Th1/Th2 (Ventura et al., 2012). Some probiotic bacteria possess immunomodulatory properties either by acting over immune cell populations and epithelial cells. This is the case of the yogurt starter *Lactobacillus delbrueckii* subsp. *bulgaricus* 8481 which reduced the concentration of cytokine IL-8 in serum following yogurt consumption. IL8 is an important pro-inflammatory chemokine produced by epithelial cells (and also macrophages) with induces chemotaxis in many immune cells, attracting them to infection sites (Moro-Garcia et al., 2013).

In bifidobacteria, the capability to induce different immune responses is a strain-dependent trait (Lopez et al., 2010), and for instance *B. bifidum* LMG13195 is able to induce Treg response (Lopez et al., 2011). On the other hand, surface proteins like the sortase-dependent pili from *B. bifidum* PRL2010 induced a Th1 response (Turroni et al., 2013). Other surface-associated protein are more related in regulating inflammatory responses such as *Lactobacillus acidophilus* S-layer A, which has been shown to protect mouse against experimentally induced colitis through a specific interaction with SIGNR3 (Lightfoot et al., 2015). Treg cells have become one of the main focuses of study in immune modulation properties of probiotics, and the balance between Treg/Th responses plays a key role in the maintenance of gut homeostasis (Maloy and Powrie, 2011). Treg induction has been previously demonstrated for where the surface associated proteins may play a key role. In our study, no significant differences were found regarding Treg cell differentiation induction by our selected peptides. However, interleukin 2 was upregulated by peptides FR-16 and LR-17 (and other peptides) (**Supplementary Figure S1**). IL-2 is directly related with Treg proliferation and maintenance of the subset population, and indeed Treg cells express the receptor for IL-2 (which is CD25) (Boyman and Sprent, 2012).

Expansion of Th17 cells is characteristic of segmented filamentous bacteria, which inhabits the murine gut and whose presence is directly linked with this specific T cell response (Ivanov et al., 2009; Farkas et al., 2015). Indeed presence of these bacteria confers resistance to the intestinal pathogen *Citrobacter rodentium* (Ivanov et al., 2009). There is strong scientific evidence of the key role of the gut microbiota in Th17 differentiation to promote a balance in the immune response to ensure a healthy status (Ivanov et al., 2008). In general, the Th17 pathway serves as protection against extracellular pathogens through recruitment of neutrophils (IL-17) and induction of anti-microbial peptides (IL-22). Th17 cells contribute to the resistance and the clearance of different enteropathogens such as *Salmonella* or *Listeria*, but also for airways pathogens such as *Klebsiella pneumoniae* (Happel et al., 2005; Kleinschek et al., 2006).

IL-22 is the signature cytokine of the Th22 cell subset and it has been related with the maintenance of the intestinal epithelium (tissue regeneration and cell proliferation) and contributing to the mucosal integrity ensuring the intestinal barrier function against pathogens (Rutz et al., 2014). It has also a role regulating CD4+ T cell response to commensal bacteria (Parks et al., 2016). One of the main roles of Th22 cells is to keep the epithelial barrier and reinforce it against potential pathogens such as *Clostridium difficile* (Hasegawa et al., 2014). IL-22 develops also important roles in intestinal epithelial cell proliferation and survival, two

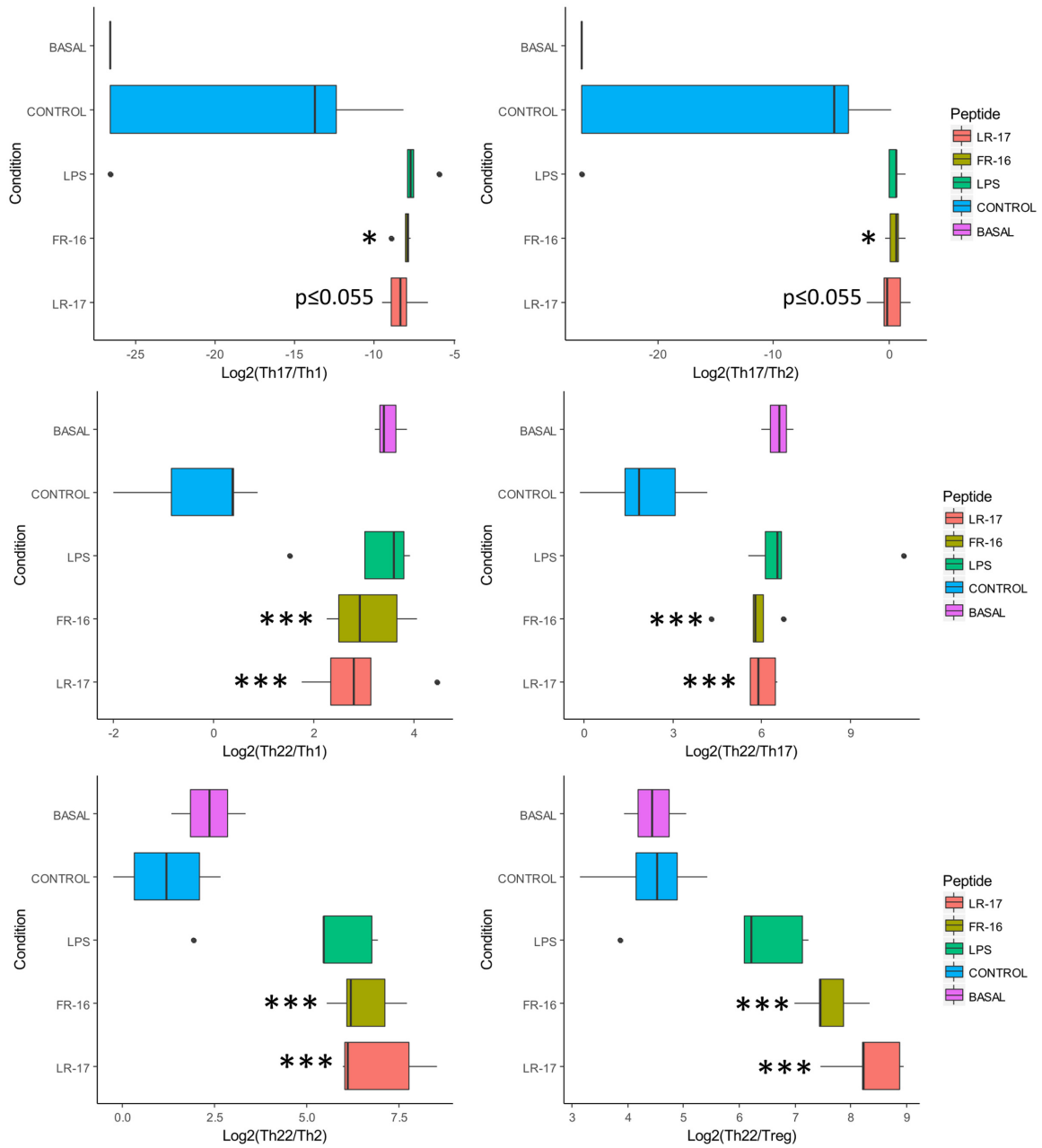

FIGURE 3 | Ratios affected by the presence of peptides LR17 and FR16. Boxplots represent median and interquartile range for the ratios (see all in **Supplementary Figure S2**). Significant differences were assessed by the non-parametric Mann–Whitney *U* test and are represented with *, **, or *** ($p < 0.05$, 0.01, or 0.001 respectively). Significant differences refer to control conditions (PBMCs + anti-CD3).

factors that are compromised in the course of an infection, and IL-22 is mainly produced by Th17 cells, Innate Lymphoid Cells, γδ T cells, and Natural Killer T cells (Ouyang et al., 2011). Human Th22 cells are characterized by producing low amounts of IL-17 and differenciate from naïve CD4+ T cells with IL-6 and without TGF-β (Zheng et al., 2007).

LR-17 but not FR-16 induced production of GM-CSF and IL-1β, very similar to the action of LPS over PBMCs (**Figure 4**). These suggests that LR-17 action could be related to macrophage activation, which are important cells in inflammation with roles including elimination of pathogens and debris clearance. Macrophages show high degree of functional plasticity and may acquire different roles depending on the activation signals. It is well known that IFNγ-activated macrophages (Th1) are pro-inflammatory and antimicrobial, and their dysregulation is key in development of several inflammatory disorders, whereas IL4/IL13-activated macrophages (Th2) have anti-inflammatory roles (Arnold et al., 2015). Macrophages are very abundant in

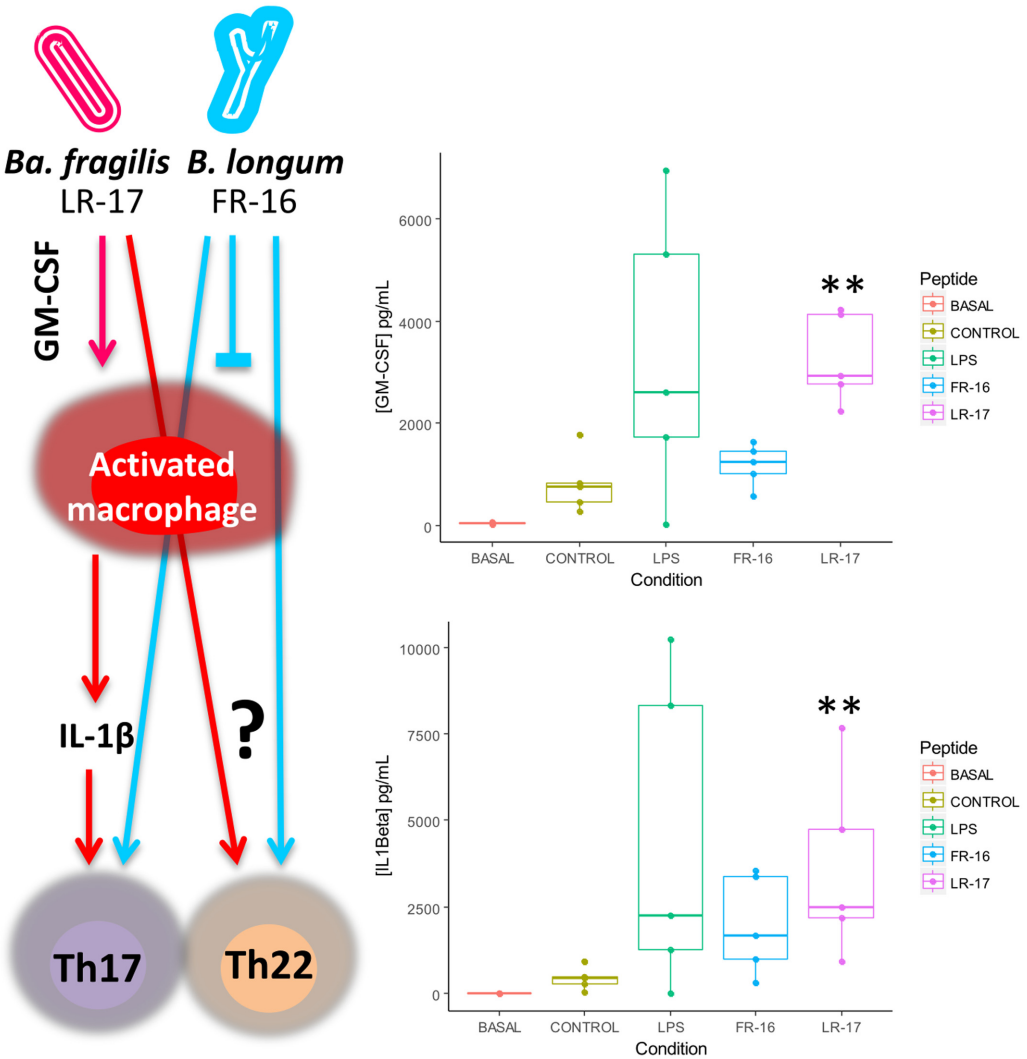

FIGURE 4 | Proposed mechanism of action of LR17 and FR16 over the human immune system as supported by cytokine measurement. Significant differences were assessed by the non-parametric Mann–Whitney *U* test (**$p < 0.01$). Significant differences refer to control conditions (PBMCs + anti-CD3).

the inflammation sites, and indeed their numbers correlate with Th17 activity, contrarily to other antigen presenting cells such as dendritic cells (Allam et al., 2011). High amounts of IL-1β were produced in the presence of LR-17, but not in the presence of FR-16. IL-1β is produced by activated macrophages and is an important mediator of the inflammatory response and for Th17 expansion (Lasiglie et al., 2011), supporting Th17 expansion through macrophage activation as the mechanism of action of LR-17. In fact, the higher concentration of IL-1β induced by LR-17 correlates with the higher amounts of IL-22 detected, as the former induce Th17 cells to produce IL-22 (Monteiro et al., 2013). Finally this suggests the existence of a specific LR-17 receptor on antigen presentation cells, although this hypothesis deserves further research.

Why LR-17 and not FR-16 activated macrophages is out of the scope of our experimental setup. Strains of the species *Bacteroides fragilis* are known for the production of Polysaccharide A (PSA), an extracellular glycan with anti-inflammatory properties (Mazmanian et al., 2008), but many other surface components have been shown to possess immunomodulatory activity (Wexler and Goodman, 2017). On the contrary, higher abundances of commensal bifidobacteria are linked to Treg promoting pathways (Lopez et al., 2012), and many of the probiotic products targeting intestinal inflammatory disorders contain bifidobacteria (Ng et al., 2010). The cellular type targeted by bifidobacterial FR-16 peptide remains a mystery.

It must be considered that dysregulation of both Th17 and Th22 pathways are linked to inflammatory and autoimmune diseases. For instance higher production of IL-22 in the colonic mucosa has been observed in patients suffering from inflammatory bowel disease (Parks et al., 2016), and a pathogenic role for the overproduction of this cytokine has been proposed in rheumatoid arthritis (Rutz et al., 2014). However, increasing the Th17 response may be protective in other autoimmune diseases

such as type 1 diabetes, so this type of encrypted peptides could have an application in the remission of these disorders (Sanchez et al., 2015). In addition, *in vivo* studies suggest that infection by *Citrobacter rodentium* in IL-23 -/- mice can be prevented by transferring Th22 but not Th17 cells (Basu et al., 2012). Further research will elucidate precisely the mechanism of action of this type of peptides over CD4+ effector T cells.

CONCLUSION

All bacterial peptides analyzed in this study were able to modulate, *in vitro*, the immune response of human PBMCs, based on the cytokine pattern production. These results corroborated the *in silico* prediction performed with MAHMI database showing the usefulness of this tool to make accurate prediction for a screening in the selection of bioactive peptides. Results obtained for these peptides using human PBMCs strongly suggested that these Th17-promoting peptides might be detected by specific receptors, although this is a speculative statement deserving further experiments. The peptides FR-16 and LR-17, encrypted within the human gut metaproteome, were able to induce a higher immune response that is related with Th17 and Th22 responses. Incubation of these peptides with PBMCs allowed us to test the hypothesis of whether our gut microbiota is able to interact with the immune system through peptides encrypted in larger proteins. We propose the possibility to use bacterial peptides encrypted in the human microbiome proteins, as key inductors to modulate the human immune response for several immunological disorders, as recently reviewed (Blanco-Míguez et al., 2016).

AUTHOR CONTRIBUTIONS

BS conceived the experiments and wrote the manuscript. RA-A and MM-G designed the immunological study, AB-M, FF-R, and AL participated in the design of the MAHMI database and peptide retrieval, and CH-C and MM-G performed the experiments and drafted the first version of the manuscript. All authors reviewed the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01726/full#supplementary-material

FIGURE S1 | Boxplots represent median and interquartile range of cytokines (pg/mL) quantified in the supernatants after 5 days of *in vitro* co-culture of human PBMCs and bacterial peptides.

FIGURE S2 | Ratios among the different cytokines taken into account the distribution depicted in **Figure 1**.

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ORIGINAL RESEARCH
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Novel Aggregation Promoting Factor AggE Contributes to the Probiotic Properties of *Enterococcus faecium* BGGO9-28

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The understanding of mechanisms of interactions between various bacterial cell surface proteins and host receptors has become imperative for the study of the health promoting features of probiotic enterococci. This study, for the first time, describes a novel enterococcal aggregation protein, AggE, from *Enterococcus faecium* BGGO9-28, selected from a laboratory collection of enterococcal isolates with auto-aggregation phenotypes. Among them, *En. faecium* BGGO9-28 showed the strongest auto-aggregation, adhesion to components of ECM and biofilm formation. Novel aggregation promoting factor AggE, a protein of 178.1 kDa, belongs to the collagen-binding superfamily of proteins and shares similar architecture with previously discovered aggregation factors from lactic acid bacteria (LAB). Its expression in heterologous enterococcal and lactococcal hosts demonstrates that the *aggE* gene is sufficient for cell aggregation. The derivatives carrying *aggE* exhibited the ten times higher adhesion ability to collagen and fibronectin, possess about two times higher adhesion to mucin and contribute to the increase of biofilm formation, comparing to the control strains. Analysis for the presence of virulence factors (cytolysin and gelatinase production), antibiotic resistance (antibiotic susceptibility) and genes (*cylA*, *agg*, *gelE*, *esp*, *hylN*, *ace*, *efaA*^{fs}, and *efaA*^{fm}) showed that BGGO9-28 was sensitive to all tested antibiotics, without hemolytic or gelatinase activity. This strain does not carry any of the tested genes encoding for known virulence factors. Results showed that BGGO9-28 was resistant to low pH and high concentrations of bile salts. Also, it adhered strongly to the Caco-2 human epithelial cell line. In conclusion, the results of this study indicate that the presence of AggE protein on the cell surface in enterococci is a desirable probiotic feature.

Keywords: enterococci, auto-aggregation, AggE, adhesion, biofilm, probiotic

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INTRODUCTION

Enterococci are an important group of lactic acid bacteria (LAB) which have the ability to survive various environmental conditions, allowing them to inhabit different ecological niches, such as water, soil (Giraffa, 2003), human, and animal gastrointestinal (GI) tracts (Bhardwaj et al., 2011) and food products, especially fermented dairy products (Gomes et al., 2008; Martín-Platero et al., 2009). It has been shown that enterococci have positive properties, such as enterocin production

and aroma producing components (Foulquié-Moreno et al., 2006). Many enterococci isolated from fermented dairy products have proven to be great natural probiotics and are generally considered to be beneficial and safe to the host (Eaton and Gasson, 2001; Pieniz et al., 2014).

To evaluate the microorganism as novel food constituent with a health claim, several essential characteristics should be considered, including the survival throughout the GI passage, adhesion, metabolic activities and its effect on intestinal homeostasis (Miquel et al., 2015).

One of the important criteria for probiotic selection is the capability to adhere to the host's intestinal epithelium, according to Guidelines for the Evaluation of Probiotics in Food published in 2006 by the FAO/WHO working group. It is believed that adherence ability of probiotic bacteria to intestinal cells is important for successful colonization and consequently may lead to exclusion of pathogens and/or immunomodulation; expected to provide long time lasting beneficial effects for health (McNaught and MacFie, 2001; Kravtsov et al., 2008). There are also scientists who have a controversial opinion on this issue (or that it is still questionable) because proximity to the intestinal mucosa and a long transit time in the gut are not sufficient to maximize the beneficial effects of a strain (Miquel et al., 2015). Moreover, microorganisms do not have to permanently colonize the system to be achieved the effect on the host. The mechanism by which the consortium of strains from fermented milk product elicit this response is still unclear, but it seems that the effect is rapid (occurring within the first 24 h after application) and it lasts regardless of whether the consortium was introduced during a 1 day period, or with subsequent repeated applications over a several weeks (McNulty et al., 2011).

The precise mechanisms of host-microbe interaction remain still unclear, although there is growing evidence that adherence to the components of the extracellular matrix (ECM) such as mucin, fibronectin, fibrinogen, collagen or laminin (Lorca et al., 2002; Yadav et al., 2013) depends on bacterial cell-surface composition. The expression of ECM-binding proteins on the surface of pathogenic bacteria provide adherence to distinct components of the ECM. The presence of enterococcal surface proteins has been shown to increase the persistence of bacteria in the urinary bladders of mice (Shankar et al., 2001). Collagen binding protein from *Enterococcus faecalis* has been shown to be a putative virulence factor involved in the colonization of renal tissue (Lebreton et al., 2009). On the other hand, probiotic microorganisms express cell-surface adhesins that mediate microbial adhesion to ECM components of host tissue. The expression of aggregation factors on the cell surface of bacteria could induce cell aggregation, visible as auto-aggregation, an important property for colonization of the oral cavity, human gut or urogenital tract.

In LAB and bifidobacteria strains, the ability of adhesion and auto-aggregation has been reported to be significantly related (Del Re et al., 2000). Correlations between the adhesion, aggregation and surface charges were observed among the *Lactobacillus fermentum* and *Lb. paracasei* strains (Piwat et al., 2015). Aggregation promoting factors from LAB differ in molecular weight and primary structure. The best characterized aggregation factors of high molecular weight (proteins of molecular mass >170 kDa), which are responsible for forming large cell aggregates, causing strong auto-aggregation and directly involved in adhesion to collagen and fibronectin, are from *Lactococcus lactis* subsp. *lactis* BGKP1 (Kojic et al., 2011), *Lb. paracasei* subsp. *paracasei* BGNJ1-64 (Miljkovic et al., 2015) and *Lb. paracasei* subsp. *paracasei* BGSJ2-8 (Lozo et al., 2007).

This paper, for the first time, describes the occurrence of novel enterococcal aggregation protein AggE from *En. faecium* BGGO9-28, selected from a laboratory collection as a strain, which belongs to a group with strong aggregation ability. The objectives of this study were to assess the adhesion and aggregation abilities of *En. faecium* BGGO9-28 and to determine a possible correlation between the adhesion of cells and aggregation ability. The novel plasmid-located *aggE* gene was cloned, sequenced and expressed in homologous and heterologous enterococcal and lactococcal hosts, showing that AggE protein is sufficient for cell aggregation in all tested hosts. AggE aggregation factor, protein of 178.1 kDa shares the highest identity with AggL protein from lactococci (81.4%). In addition to its adherence property, BGGO9-28 fulfills several essential criteria required to be considered as a potential probiotic strain: absence of genes coding for virulence factors, the ability to survive in the presence of gastric juices, bile salts and intestinal juices, and strong adhesion ability to host intestinal cells.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions

The *Enterococcus* and *Lactococcus* strains used in this study (**Table 1**) were grown in M17 broth (Merck, GmbH, Darmstadt, Germany) supplemented with glucose (0.5% w/v) (GM17) at 30°C. Enterococcal and lactococcal transformants were grown in GM17 medium supplemented with erythromycin (10 μg/ml). *Escherichia coli* DH5α was grown in Luria-Bertani broth (LB) at 37°C aerobically. Solid medium plates were prepared by adding 1.7% agar (Torlak, Belgrade, Serbia) into each medium broth.

Identification of Strains

Primary identification of enterococcal isolates was done by catalase testing, Gram staining and cell morphology, hydrolysis of arginine, CO_2 production from glucose and formation of black zones on bile esculin agar (Himedia, Mumbai, India). Sequencing of genes for 16S rRNA of BGGO9-28 was performed using primers: UNI16SF (5′-GAGAGTTTGATCCTGGC-3′) and UNI16SR (5′-AGGAGGTGATCCAGCCG-3′) (Jovcic et al., 2009).

The Polymerase Chain Reaction (PCR) amplicons were purified using a GeneJet PCR Purification Kit (Thermo Scientific, Lithuania) and sequenced (Macrogen Europe, Amsterdam, The Netherlands). The Basic Local Alignment Search Tool (BLAST) algorithm was used to determine the most related sequences in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/BLAST). Based on 16S rRNA gene sequencing results

TABLE 1 | Bacterial strains and plasmids used in this study.

Strains	Source	Strain characteristics	Source or references
***Enterococcus* sp.**			
BGGO9-27	Semi-fat 30 days artisanal old cheese	Agg^+	Terzic-Vidojevic et al., 2014
BGGO9-28		Agg^+	
BGGO9-30		Agg^+	
BGGO9-32		Agg^+	
BGGO9-33		Agg^+	
BGGO9-56		Agg^+	
BGGO10-37		Agg^+	
BGGO10-38		Agg^+	
BGGO11-27	Semi-fat 10 days artisanal old cheese	Agg^+	Golić et al., 2013
BGGO11-29		Agg^+	
BGGO11-33		Agg^+	
BGGO11-41		Agg^+	
BGDU4-1	Semi-had, semi-fat, 15 days old cheese	Agg^+	Laboratory collection
BGDB23-11	Semi-had, full-fat, 12 days old cheese	Agg^+	Laboratory collection
BGZLS10-27	Semi-fat 10 days artisanal old cheese	Agg^-	Valenzuela et al., 2009
BGZLS10-27/pAggES10	Derivative of BGZLS10-27 carrying pAggES10	Agg^+, Em^r	This study
BGZLS10-27/pAggEPX48	Derivative of BGZLS10-27 carrying pAggEPX48	Agg^+, Em^r	This study
Lactococcus lactis* subsp. *lactis			
BGKP1		Agg^+	Kojic et al., 2011
Lactococcus lactis* subsp. *cremoris			
MG7284	Derivative of MG1363	Fus^r, Spc^r, Agg^-	Gasson, 1983
MG7284/pAggES10	Derivative of MG7284 carrying pAggES10	Agg^+, Em^r	This study
MG7284/pAggEPX48	Derivative of MG7284 carrying pAggEPX48	Agg^+, Em^r	This study
Escherichia coli			
DH5α		*supE44 ΔlacU169 (ø80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
PLASMIDS AND CONSTRUCTS			
pAZIL	Shuttle cloning vector	Em^r	Kojic et al., 2011
pAggES10	Derivative of pAZIL vector	Carrying *Stu*I fragment of 8,944 bp from BGGO9-28	This study
pAggEPX48	Derivative of pAZIL vector	Carrying *Pvu*I-*Xba*I fragment of 7,115 bp subcloned from pAggES10	This study

Agg^+, aggregation-positive; Agg^-, aggregation-negative; Em^r, resistance to erythromycin; Fus^r, resistance to fusaric acid; Spc^r, resistance to spectinomycin.

the BGGO9-28 strain was classified as *En. faecium* (submitted to the European Nucleotide Archive (ENA) under accession No: LT222049).

Auto-Aggregation Assay

The auto-aggregation ability of the selected enterococci strains was tested according to the method of García-Cayuela et al. (2014). Percentage of auto-aggregation was determined using the equation: $[1 - (At/A0) \times 100]$ where At represents the absorbance at different time points (1, 2, 3, 4, and 5 h) and A0 is absorbance at time 0 of three independent measurements.

In Vitro Adhesion Ability to Components of ECM

The collagen binding ability of all the selected enterococci strains and derivates was tested according to Miljkovic et al. (2015). The wells were coated with 100 μg/ml of type I collagen from rat tail (BD Bioscience, New Jersey, USA). The ability of the tested strains to bind to fibronectin was assessed as previously described by Ahmed et al. (2001). The wells were coated with 100 μg/ml human fibronectin (Serva, Heidelberg, Germany). The ability of the tested strains to bind to mucin was tested according to Muñoz-Provencio et al. (2009) with modification. The wells were coated with 100 μg/ml mucin from porcine stomach (Sigma,

St. Louis, USA). After coating with collagen/fibronectin/mucin, wells were washed with PBS and blocked with bovine serine albumin (BSA) (2% in PBS). Also, empty wells were coated with BSA (control wells). Upon removal of BSA solution and washing of wells with PBS, the test cultures (100 μl, 10^8 CFU/ml) were added and plates were incubated on an orbital platform shaker for 2 h at 37°C. Non-adhered cells were removed by washing of the wells three times with 200 μl of PBS. The adhered cells were fixed at 60°C for 20 min and stained with crystal violet (100 μl/well, 0.1% solution) for 45 min. Wells were subsequently washed tree times with PBS to remove the excess stain. The stain bounded to the cells was dissolved by 100 μl of citrate buffer (pH 4.3) and absorbance was measured at 570 nm. The results are presented as the average of absorbance values per each strain (normalized with respect to absorbance values of BSA-coated wells) from three independent experiments per strain.

Biofilm Formation Assay

The ability of the enterococci to form biofilm was assessed as previously described by Peter et al. (2013) with minor modifications. The culture (10^8 CFU/mL) was diluted to 1:40 in GM17 and 200 μl of dilution was used to inoculate sterile 96 well-polystyrene microtiter plates. After incubating for 24 h at 37°C, the excess bacterial cells were removed (the removal of "planktonic bacteria") and wells were gently washed tree times with PBS, dried in an inverted position and stained with crystal violet (100 μl/well, 0.1% solution) for 45 min. The wells were rinsed again with PBS and retained crystal violet was solubilized in 200 μl of ethanol - acetone (80:20) mixture. The absorbance was measured at 570 nm. The results were presented as average of absorbance values per each strain (normalized with respect to absorbance values of empty wells) from three independent experiments.

Southern Blot Hybridization

Transfer of DNA from agarose gel to membrane, labeling of probe and detection of hybrids was performed as recommended by the manufacturer of the DIG DNA Labeling and Detection Kit (Roche, Mannheim, Germany). Hybridizations were carried out at 65°C.

Cloning and Expression of Novel Enterococcal Aggregation Promoting Factor AggE

The procedure described by O'Sullivan and Klaenhammer (1993) was applied for plasmid isolation from enterococci and lactococci.

For preparation of plasmid libraries, plasmid DNA from *En. faecium* BGGO9-28 was digested separately with *Xba*I and *Stu*I restriction enzymes. The resulting DNA fragments were cloned into the pAZIL vector and plasmid libraries were screened in *E. coli* DH5α. All derivatives carrying different fragments were transferred into non-aggregating derivatives *Lc. lactis* subsp. *cremoris* MG7284 (Agg^-) and *En. faecalis* BGZLS10-27 (Agg^-).

All relevant constructs were sequenced (Macrogen, Amsterdam, The Netherlands). The BLAST program was used for sequence annotation and analysis of sequence similarities. ORF prediction was obtained by DNA Strider. Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and Superfamily 1.75 (http://supfam.org/) programs were used to analyse AggE protein.

Comparative analyses of enterococcal (AggE) and lactococcal (AggL) aggregation factors were performed by PCR reactions. The primers used in PCR amplification were: AggE-1DF (5′-GAC TGCTAAGTCAACGGGGG-3′) and AggE-1DR (5′-GATAGGT AATATTTGCTGG-3′). Total DNA (1 ng) was mixed with 17.75 μl of bidistilled water, 2.5 μl of 10 × PCR buffer (Fermentas, Lithuania), 1 μl dNTP mix (10 mM), 1.5 μl of $MgCl_2$ (25 mM), 1 μl (10 pmole) of each primer and 0.25 μl of Taq polymerase (Fermentas, Lithuania). Performed using the GeneAmp 2700 PCR Cycler (Applied Biosystems, Foster City, California, USA), the PCR program consisted of 5 min at 96°C, 30 cycles of 96°C for 30 s, 40°C for 30 s and 72°C for 60 s, and an additional extension step of 5 min at 72°C.

Haemolytic and Gelatinase Activities

Haemolytic activity of enterococcal strain BGGO9-28 was tested on Columbia Blood Agar (Oxoid LTD, Basingstoke, Hampshire, England) containing 5% (v/v) defibrinated horse blood after 48 h of incubation at 37°C. Production of β-haemolysin was detected as the appearance of clear zones around colonies. Also, production of gelatinase of enterococcal strain BGGO9-28 was tested on agar plates containing 30 g of gelatine (Difco, Detroit, MI, USA) per liter as described by Lopes et al. (2006). After filling the Petri plate with 550 g/l ammonium sulfate, the appearance of clean zones around colonies indicated gelatinase production.

Susceptibility Testing

The antibiotic susceptibility of enterococcal strain BGGO9-28 was done by determining the minimum inhibitory concentrations (MICs) by microdilution testing following The Clinical and Laboratory Standards Institute (CLSI), 2015 criteria. Susceptibility was tested against: ampicillin (4–16 μg/ml), vancomycin (4–32 μg/ml), erythromycin (0.5–8 μg/ml), tetracycline (4–16 μg/ml), ciprofloxacin (1–4 μg/ml), nitrofurantoin (32–128 μg/ml), chloramphenicol (8–32 μg/ml), linezolid (2–8 μg/ml) and gentamicin (>500 μg/ml). After 24 h incubation at 30°C, cell density was determined spectrophotometrically by measuring absorbance at 595 nm using a Plate Reader Infinite 200 pro (MTX Lab Systems, Austria). MIC values were determined as the lowest concentration of antibiotic that inhibits visible growth of bacteria.

PCR Detection of Virulence Determinants

In order to test for the presence of genes which indicate virulence, determinants cytolysin (*cylA*), aggregation factor (*agg*), gelatinase (*gelE*), enterococcal surface protein (*esp*), cell wall adhesions ($efaA^{fs}$, and $efaA^{fm}$), collagen adhesin (*ace*), and hyaluronidase (*hylN*) (Eaton and Gasson, 2001; Vankerckhoven et al., 2004) the total DNA of *En. faecium* BGGO9-28 was used.

Survival in Simulated GI Tract

Survival of enterococcal strain BGGO9-28 in a simulated GI tract was performed according to Nikolic et al. (2012). Strain was grown in GM17 for 24 h and culture was harvested by centrifugation (10,000 g for 10 min), washed twice with 0.85% NaCl and concentrated 10-times in reconstituted (10%) sterile skimmed-milk (Difco, Becton Dickinson, Franklin Lakes, NJ, USA). Afterwards, bacterial suspensions were diluted 10-times with gastric juice (125 mM NaCl, 7 mM KCl, 45 mM NaHCO3, 0.3% pepsin (Sigma, St. Louis, MO, USA) adjusted to pH 2.0 with HCl), incubated for 90 min at 37°C in aerobic conditions under shaking. Then, bacterial suspensions were centrifuged (2,050 g, 15 min), resuspended in duodenal juice [1% bovine bile (Sigma, St. Louis, MO, USA) adjusted with 10 M NaOH to pH 8.0] and incubated for 10 min at 37°C in anaerobic conditions. Finally, harvested cell suspensions were resuspended in intestinal juice (0.3% bovine bile, 0.1% pancreas acetone powder porcine type I (Sigma, St. Louis, MO, USA), pH 8.0) and incubated for 120 min at 37°C in anaerobic conditions. Determination of viable counts was carried out in the initial cultures and after each of the challenges (gastric, bile salt, and intestinal juices). Serial dilutions of the samples were made and inoculated on the surface of GM17 agar using spread plate technique. Viable colony counts were determined and survival rate was presented as means of log units of BGGO9-28 growth. Experiments were carried out in triplicate.

Adhesion to Caco-2 Cell Lines

The colonocyte-like cell line Caco-2, was used to determine the adhesion ability of enterococcal strain BGGO9-28. A Caco-2 cell line was purchased from the European Collection of Cell Cultures (ECACC No. 86010202). Adhesion to the Caco-2 cell line was done according to Sánchez et al. (2010). The results of adhesion were expressed as percentage (%) (colony forming units (CFU) adhered bacteria/CFU added bacteria × 100) from two replicated plates. In each plate three wells were used per sample.

Statistical Analysis

Differences between treatments were examined for significance by a Student's *t*-test after analysis of variance (Kirkman, 1996).

RESULTS AND DISCUSSION

The important goals of this study were to investigate the aggregation ability of *Enterococcus* sp. in general, and to examine the possible role of *Enterococcus* aggregation factor as a potential probiotic feature.

Identification and Auto-Aggregation of Selected Enterococci

Considering the importance of aggregation phenomena for human health, the laboratory collection of enterococci was screened for strains exhibiting strong aggregation ability for further analysis. Fourteen *Enterococcus* strains were selected from among 636 examined enterococcal isolates (Terzić-Vidojević et al., 2015) from a laboratory collection, based on their ability to cause auto-aggregation of the cells, using an aggregation visual assay. All of them were isolated from artisanal homemade cow's milk cheeses, produced in households of the Golija and Valjevo mountain regions of the Republic of Serbia (**Table 1**). All enterococcal isolates were Gram positive and catalase negative cocci, in pairs or short chains. Hydrolysis of arginine and formation of black zones on bile esculin agar were positive. None of the enterococci chosen were able to produce CO_2 from glucose. It was found that aggregation ability is a rare phenotype among enterococci (2.2% in our laboratory collection) similarly as in other LAB (Miljkovic et al., 2015).

Some LAB, including enterococcal species, represent beneficial microbiota existing in the human and animal GI and urogenital tracts (Bhardwaj et al., 2011). One beneficial property of bacteria with auto-aggregation ability is the prevention of colonization of pathogenic bacteria by the formation of a barrier via auto-aggregation to intestinal mucosa (O'Toole and Cooney, 2008; Prince et al., 2012).

Based on a visual assay, auto-aggregation ability was further confirmed by monitoring the changes in absorbance (OD_{600}) of cultures during 5 h of sedimentation at 30°C. All analyzed enterococci strains showed varying levels (1 h: 5.34%–81.09%; 5 h: 29.825–89.86%) of auto-aggregation ability and can be classified in three groups: (*i*) strong auto-aggregation (strains BGGO9-27, BGGO9-28, BGGO9-32, BGGO9-33, BGGO9-56, BGGO10-37, BGGO11-27, BGGO11-41, BGDU4-1, and BGDB23-11; with more than 80% of precipitated cells for 5 h); (*ii*) medium auto-aggregation (strains BGGO10-38 and BGGO11-29; with about 60% of the precipitated cells for 5 h); and (*iii*) low auto-aggregation (strains BGGO9-30 and BGGO11-33; with about 40% of the precipitated cells for 5 h) (**Figure 1A**).

Adhesion of Selected Enterococci to Components of ECM

One of the important goals of this study was to investigate the possible role of *Enterococcus* aggregation factor in its adherence to the main components of ECM, such as collagen (**Figure 1B**), structural glycoprotein fibronectin (**Figure 1C**) and mucin (**Figure 1D**), in order to assess its probiotic potential. The strains most adhesive to all tested components of ECM were BGGO9-27, BGGO9-28, BGGO9-32, BGGO9-33, and BGGO9-56. The rest of the strains showed a difference in affinity for various components of ECM. Interestingly, strains BGGO11-29 and BGGO11-41 exhibited the ability to bind collagen (absorbance value 1.17 and 1.97, respectively), while binding to fibronectin and mucin was absent (absorbance value is less that 0.4). Also, it was noted that strain BGDB23-11 binds to collagen and fibronectin (absorbance value 2.82 to collagen and 2.16 to fibronectin), but binding to mucin was very poor (absorbance value 0.28).

The results obtained for auto-aggregation and adhesion to components of ECM among the selected enterococcal strains indicate that they possess a different kind of molecules present on their surface that are involved in cell-to-cell interaction. However, the selected enterococci can be classified into at least four groups: group I (consisting of strains BGGO9-27,

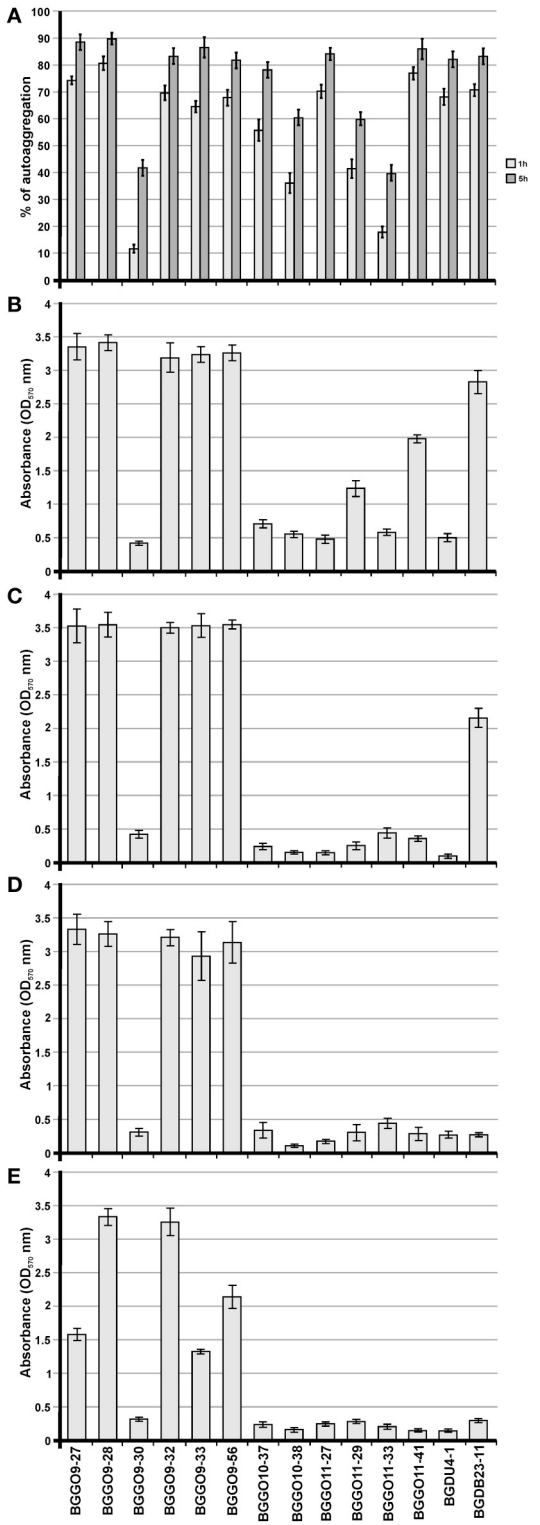

FIGURE 1 | Adhesion capability of selected enterococal strains. Graphical presentation of results obtained in **(A)** auto-aggregation assay (auto-aggregation ability is expressed as percentages); **(B)** collagen-binding assay; **(C)** fibronectin-binding assay; **(D)** mucin-binding assay; **(E)** biofilm formation assay of selected strains (results were expressed as average of normalized A_{570} values). The error bars show the standard deviations.

BGGO9-28, BGGO9-32, BGGO9-33, and BGGO9-56) showed strong ability to bind to collagen, fibronectin and mucin, while at the same time exhibiting very powerful auto-aggregation ability; group II (consisting of strains BGGO9-30, BGGO10-37, BGGO10-38, BGGO11-27, BGGO11-33, and BGDU4-1) showed a moderate to high auto-aggregation ability, but poor adhesion to all ECM components; group III (consisting of strains BGGO11-29 and BGGO11-41) showed a moderate to high auto-aggregation ability and binding to collagen, but poor adhesion to other ECM components; and group IV (consisting of strain BGDB23-11) showed high auto-aggregation ability and binding to collagen and fibronectin, but poor adhesion to mucin.

Biofilm Formation of Selected Enterococci

Excluding many environmental factors, the number of genetic factors known to be involved in biofilm production has increased in recent years (Mohamed and Huang, 2007). Different surface molecules, such as polysaccharides, have been implicated in biofilm formation. An *En. faecalis* gene mutation which encodes a putative glycosyltransferase that is involved in polysaccharide synthesis showed a 73% reduction in biofilm formation (Xu et al., 2000). In this context, we evaluated biofilm formation by the selected enterococcal strains and investigated the possible correlation between the presence of aggregation factor on the cell surface and the ability to form biofilm.

Cells of the tested strains showed differing abilities to form biofilm. Among the tested enterococci, strains BGGO9-28 and BGGO9-32 showed the strongest ability to form biofilms on the plates (plastic surface), and three strains (BGGO9-27, BGGO9-33, and BGGO9-56) showed moderate biofilm formation ability (**Figure 1E**). Two strains, BGGO9-28 and BGGO9-32, belong to the aforementioned group I of strains (among the tested enterococci), which also showed strong auto-aggregation ability and adhesion to components of ECM. This is the first study that indicates the existence of a relationship between biofilm formation and aggregation in non-pathogenic enterococcal strains.

Localization of *aggE* Gene Responsible for Auto-Aggregation Phenotype

The autochthonous plasmids of the selected enterococcal strains were analyzed to confirm the location of genes correlated with auto-aggregation ability (**Figure 2**). To determine the relationship between the presence of plasmids and the auto-aggregation phenotype, which was very similar to that obtained for lactococci and lactobacilli (Kojic et al., 2011; Miljkovic et al., 2015), Southern blot hybridizations with *aggL* and *aggLb* gene probes from *Lc. lactis* BGKP1 and *Lb. paracasei* subsp. *paracasei* BGNJ1-64 were performed (separately) using plasmid DNA isolated from all selected enterococci. The results indicate that the gene(s) similar to *aggL* determining auto-aggregation in all selected enterococcal strains are plasmid located (on the largest common plasmid; **Figure 2**), which allows fast horizontal transfer among bacteria of the same niche and most probably a selective advantage for carriers. It is interesting that in enterococci, like in lacococci and lactobacilli, genes encoding

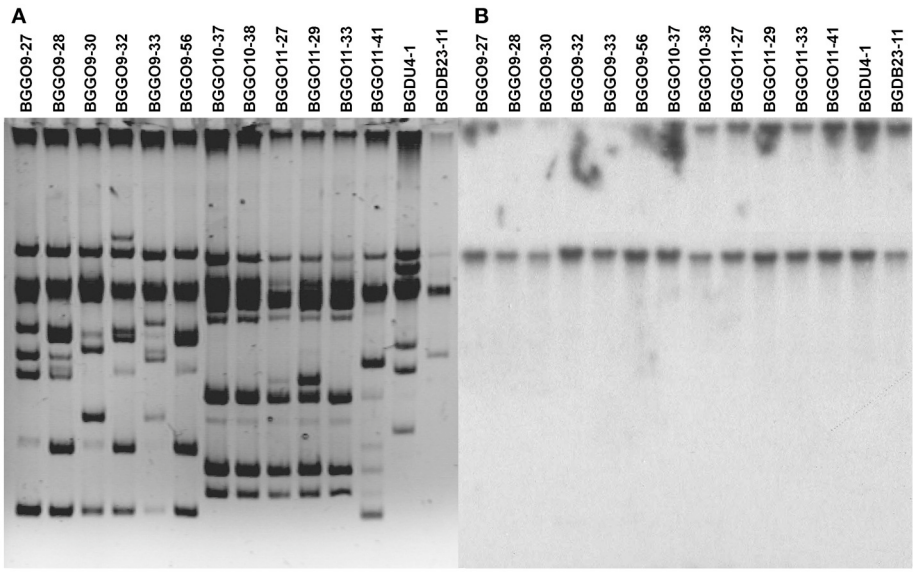

FIGURE 2 | Localization of genes correlated with auto-aggregation ability. **(A)** The electrophoresis of total plasmid DNA of selected enterococcal strains expressing aggregation ability from laboratory collection on 1% agaroze gel; **(B)** Southern blot hybridization with *aggL* gene probe.

for this type of aggregation factor are located on plasmids of different size indicating possible common plasmid origin. In selected enterococci it seems that plasmids carrying *aggE* gene are almost the same size even aggregation positive strains were isolated from different locations. According to plasmid profiles of selected strains it is possible to noted four groups of strains: two big groups (consisted of seven and five strains) with many common plasmids and two groups each consisted of one strain. Unfortunately, it is not possible to give final conclusion regarding plasmids carrying *aggE* gene since all analyzed strains belong to the same collection isolated from narrow distance; additional data from other groups are necessary. During propagation of selected enterococcal strains no loss of aggregation phenotype was noticed, indicating that plasmids are stable (structurally and segregationally) without any selection indicating that it could contributes to the fitness of carrier strains.

Cloning and Heterologous Expression of AggE Aggregation Factor from Strain BGGO9-28

Since the strain BGGO9-28 showed the strongest adhesion capability among selected enterococal strains, it was chosen for the cloning of *aggE* by constructing a plasmid library in pAZIL vector. All plasmid library constructs, carrying different fragments of the total plasmids of strain BGGO9-28, amplified in *E. coli*, were transferred into non-aggregating heterologous plasmid-free lactococcal strain MG7284 and enterococcal strain BGZLS10-27. Only the construct pAggES10 (carrying a *Stu*I fragment of 8,944 bp) provided auto-aggregation ability to strains MG7284 and BGZLS10-27. After the auto-aggregation capacity of construct pAggES10 was confirmed, the cloned fragment was completely sequenced. On the fragment was found an ORF with 70.6% identity with *aggL* from *Lc. lactis* BGKP1 (Kojic et al., 2011), responsible for aggregation in lactococci, and was named *aggE*. In addition, an upstream promoter region of *aggE* showed very high identity (96%) with the promoter region of *aggL* gene from lactococci. The DNA sequence of the region carrying the *aggE* from strain BGGO9-28 was submitted to the European Nucleotide Archive under accession No: LT222050.

Based on the sequence analysis, a shortened *Pvu*I/*Xba*I fragment of 7,115 bp that contained only the *aggE* was subcloned into the pAZIL vector. The resulting construct, named pAggEPX48, successfully restored aggregation phenotype in strains MG7284 and BGZLS10-27, after transformation (**Figure 3**). It was noted that *aggE* is sufficient for the expression of aggregation phenotype in closely related LAB species (lactococci and enterococci). The result supports the conclusion that aggregation ability was stronger in transformants of enterococcal strain BGZLS10-27 compared to that of lactococcal MG7284, in contrast to results obtained with lactococcal *aggL* indicating that there are structural adaptations specific to each group of bacteria. It was found that *aggE* is surrounded by two integrase genes; upstream of *aggE* gene are located genes for RepA protein and for an integrase with high identity with the same genes on an unnamed plasmid from *En. faecium* strain UW8175, and downstream are located genes for integrase, magnesium and cobalt transport protein CorA, and a gene for protein OrfX. A constellation of genes on the sequenced fragment (RepA and OrfX separated by a cassette) and the presence of the genes for integrase on both sides of *aggE* gene indicate a possible horizontal gene transfer.

In Silico Analysis of AggE Protein

Primary structural analysis of AggE protein revealed that it is a 178 kDa (1,637 amino acids) membrane-anchored protein rich

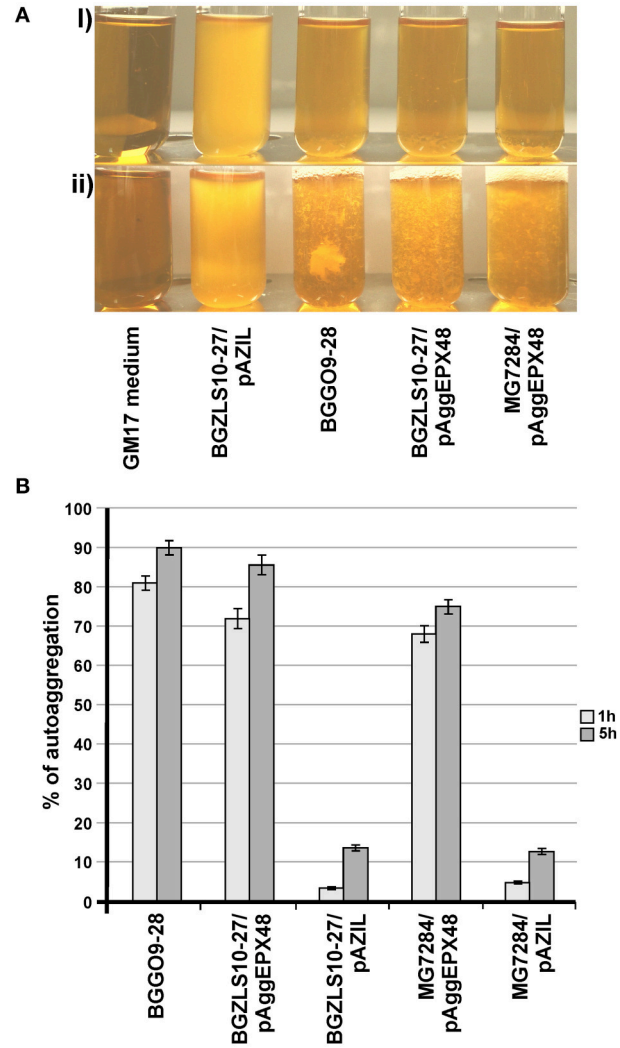

FIGURE 3 | Heterologous expression of AggE aggregation factor from BGGO9-28. Aggregation ability of *En. faecium* BGGO9-28 and transformants (carryng construct pAggEPX48) in growth medium after **(A-i)** overnight cultivation and **(A-ii)** vigorous mixing; **(B)** graphical presentation of results obtained in auto-aggregation assay; auto-aggregation ability is expressed as percentages; the error bars show the standard deviations of three independent observations.

with amino acids threonine (12.6%) and lysine (9.3%). A BLAST search showed that AggE shares the highest identity with AggL from lactococci (81.4%) and a recently discovered hypothetical protein from *En. faecium* (WP_058138517.1) (69.6%), which is shorter by 386 amino acids. It was shown that AggE contains several motifs in relation to cell adhesion, like collagen-binding (three heterologous domains with less than 25% identity at positions 411–534, 829–944, and 964–1,089), collagen-binding B domains (four almost identical domains with consensus sequence TSVSGQKTWSDHDNQDGVRPDEITVNLLADGK KVDSKTVTAKDGWKYEFNDLDKFKGQEIKYTVAEAAVD GYKTTYDGNNIVNTH at positions 1,214–1,299, 1,304–1,389, 1,394–1,479, and 1,484–1,567), a CnaB-like domain (at position 1,132–1,200) as well as a cell wall anchoring domain (LPXTG) at the C-terminus (**Figure 4**).

Aggregation substance (AS) of *En. faecalis*, a sex pheromone plasmid encoded cell surface proteins (Asp1, Asa1 and Asc10; these proteins share more than 90% identity throughout of the protein), mediates the formation of bacterial aggregates, thereby promoting plasmid transfer. The aggregation ability encoded by *asp1* gene was first characterized as a virulence factor of 142 kDa (Galli et al., 1990). It was proposed that amino acid motifs Arg-Gly-Asp-Ser (RGDS) and Arg-Gly-Asp-Val (RGDV) in that aggregation protein play a crucial role in adherence to eukaryotic cells. In this study we have discovered a novel aggregation promoting factor, AggE, responsible for strong aggregation of enterococcal cells. Both types of the enterococcal aggregation proteins (AS and AggE) are high molecular mass proteins rich by amino acids threonine and lysine. The difference between them is that novel aggregation factor AggE from enterococci shows different architecture compared to that characterized as a virulence factor and does not contain RGDS or RGDV amino acid motifs. In addition these two types of aggregation proteins exhibit different binding affinity to ECM proteins: the presence of AS increased enterococcal adherence to fibronectin more than eight-fold and to type I collagen more than two-fold (Rozdzinski et al., 2001) while AggE protein provides an almost identical affinity for both the collagen and the fibronectin, which has been increased more than 10 times. AggE enterococcal aggregation promoting factor showed a structure similar to aggregation factors discovered in other LAB composed of collagen binding and CnaB-like domains (Kojic et al., 2011; Miljkovic et al.,

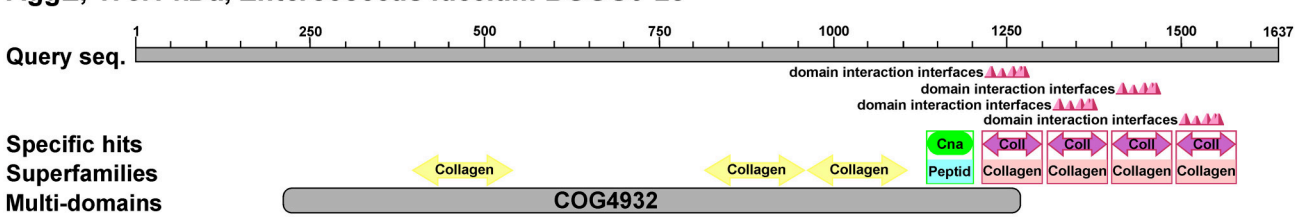

FIGURE 4 | Schematic representation of AggE protein, 178.1 kDa *Enterococcus faecium* BGGO9-28. Prediction of putative conserved domains in AggE protein was analyzed by protein BLASTP search. COG4932-Uncharacterized surface anchored protein, Collagen, Collagen binding domain; Peptid, peptidase associated domain; Coll, repeat unit of collagen binding protein domain B; Cna, Cna protein B-type domain.

2015). The highest level of identity, 81.4%, was detected with an aggregation protein of lactococci (AggL). Comparative analysis of these two proteins indicated their common origin. Differences between these two proteins were seen only in two regions: a region between amino acids 171 and 228 of AggE (that region is deleted in AggL since it is composed of multiple repeated SSTSTT

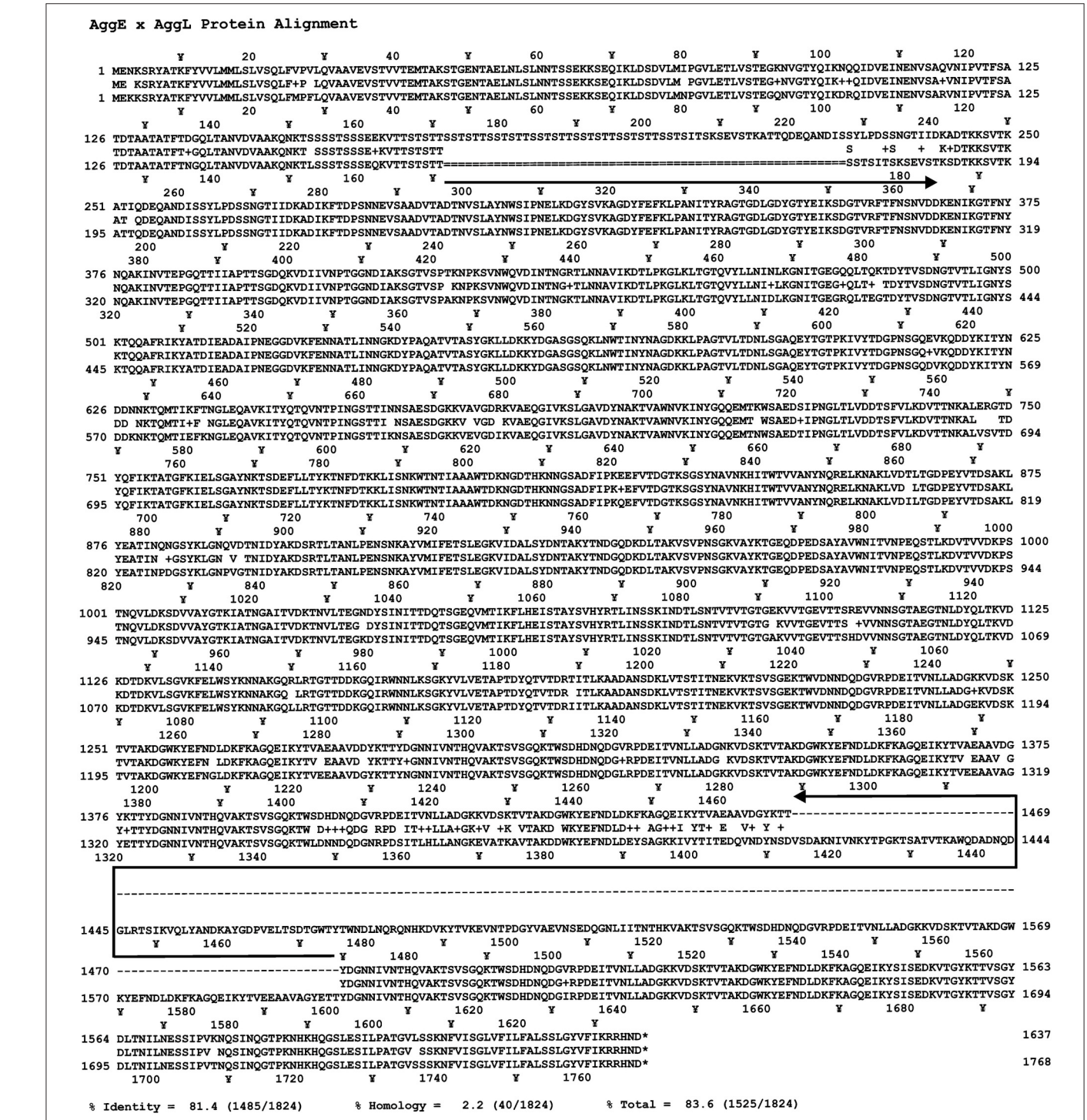

FIGURE 5 | Comparison of the amino acid sequence of AggL and AggE aggregation factors using Strider software. Black arrow oriented to the right indicates the absence of amino acids in AggL protein corresponding to the region between amino acids 171 and 228 of AggE. Black arrow oriented to the left indicates the absence of amino acids in AggE protein corresponding to the region between amino acids 1,413 and 1,600 of AggL. +, − Indicates for amino acids belonging to the same physicochemical group.

sequences), and a second region between amino acids 1,400 and 1,600 of AggL that is not present in AggE and contains two collagen binding B domains (**Figure 5**). These two differences may be the result of an independent selection in each organism and are most likely responsible for the different expression of aggregation proteins in lactococci and enterococci (AggL is better expressed in *Lactococcus* while AggE is better expressed in *Enterococcus*). The hypothesis of a common origin of these two genes is supported by two additional facts; an almost identical promoter region and the presence of the integrase genes close to *agg* gene. In order to determine whether the differences in *aggE* (from BGGO9-28) are a feature of all Agg^+ enterococci, the region (which surrounds the first deletion in *aggL*, positioned between nucleotides 511–680 of *aggE*) was amplified using primers AggE-1DF (positioned in both, *aggL* and *aggE* genes between nucleotides 129–148) and AggE-1DR (positioned in *aggL* between nucleotides 817–835 and in *aggE* between 985 and 1,003), resulting in amplified fragments for *aggL* of 707 bp and for *aggE* of 875 bp (**Figure 5**). For the second deleted region, since it is located in a repeated region composed of sequences completely identical to each other, it was not possible to design primers with a unique position. The results of PCR amplification support the conclusion that this region is variable, not only between lactococci and enterococci, but also among enterococci, or is still under stabilizing selection (**Figure 6**). A comparison of the ability of aggregation and the size of the deleted region in *aggE* gene/protein indicates that there is no corelation between the size of deletion and agregation, collagen, fibronectin and mucin binding ability, and biofilm formation. It seems that the structure and the presence of the respective domains, not the length of the peptide, is responsible for the expression of certain auto-aggregation phenotypes or adhesion ability, as demonstrated previously for AggLb (Miljkovic et al., 2016).

The Role of AggE in Auto-Aggregation, Adhesion to Components of ECM, and Biofilm Formation

The auto-aggregation ability of the wild-type strain BGGO9-28, derivatives of which carry *aggE* gene (BGZLS10-27/pAggEPX48 and MG7284/pAggEPX48) and their non-aggregating derivative carrying an empty plasmid (BGZLS10-27/pAZIL and MG7284/pAZIL) was measured for a period of 5 h and the results are presented in **Figure 3B**.

Earlier it was shown that isolates carrying *aggL* (the aggregation promoting factors from lactococci, AggL) or *aggLb* gene (the aggregation promoting factors from lactobacilli, AggLb) exhibited a direct correlation between auto-aggregation and their collagen binding ability, we tested the binding abilities of derivatives which carry *aggE* gene (BGZLS10-27/pAggEPX48 and MG7284/pAggEPX48) and their non-aggregating derivatives (BGZLS10-27/pAZIL and MG7284/pAZIL) to collagen. The different extents of adhesion to immobilized collagen (**Figure 7A**) and fibronectin (**Figure 7B**) were observed. BGGO9-28 exhibited the highest adhesion ability (absorbance value 3.54 to collagen and 3.48 to fibronectin), while the derivatives MG7284/pAggEPX48 (absorbance value 2.32 to collagen and 2.95 to fibronectin) and BGZLS10-27/pAggEPX48 (absorbance value 2.95 to collagen and 2.47 to fibronectin) possess slightly lower adhesion ability. Significant differences in the adherence to immobilized collagen and fibronectin were apparent between the mentioned aggregation-positive strains (BGGO9-28, MG7284/pAggEPX48, and BGZLS10-27/pAggEPX48) and their aggregation-negative controls, which showed almost no ability to bind to collagen or fibronectin (MG7284/pAZIL and BGZLS10-27/pAZIL). These results indicate a role for AggE in the specific interaction of BGGO9-28 with collagen and fibronectin on the cell surface. The higher adhesion ability of strain BGGO9-28 compared to the

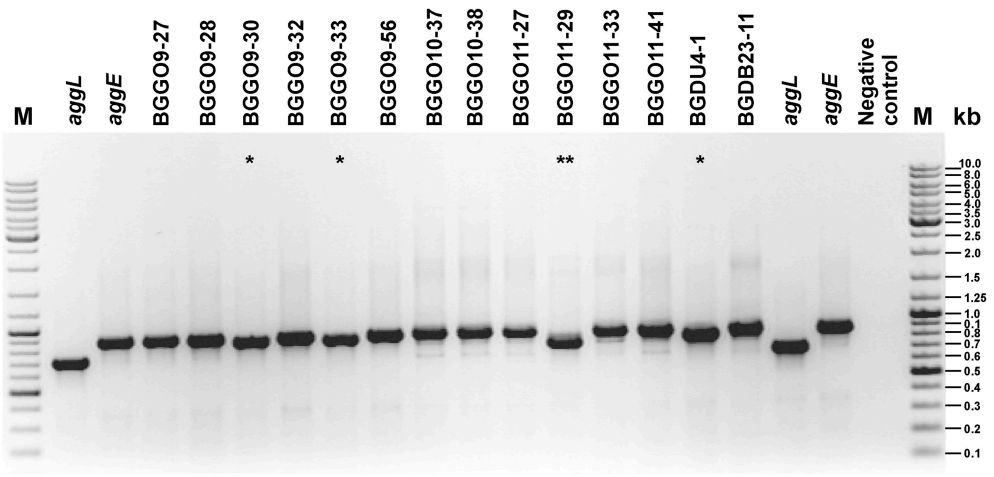

FIGURE 6 | Comparation of *aggE* and *aggL* genes by PCR analyses. The electrophoresis of PCR fragments amplified using primers AggE-1DF and AggE-1DR on plasmid DNA of enterococcal strains and clones (*aggE* and *aggL*) on 1% agarose gel. *Little shorter fragment than obtained for *aggE*; **Fragment thereof according to the size of between *aggE* and *aggL*; M, GeneRuler DNA Ladder Mix (Thermo Scientific); kb, kilobase.

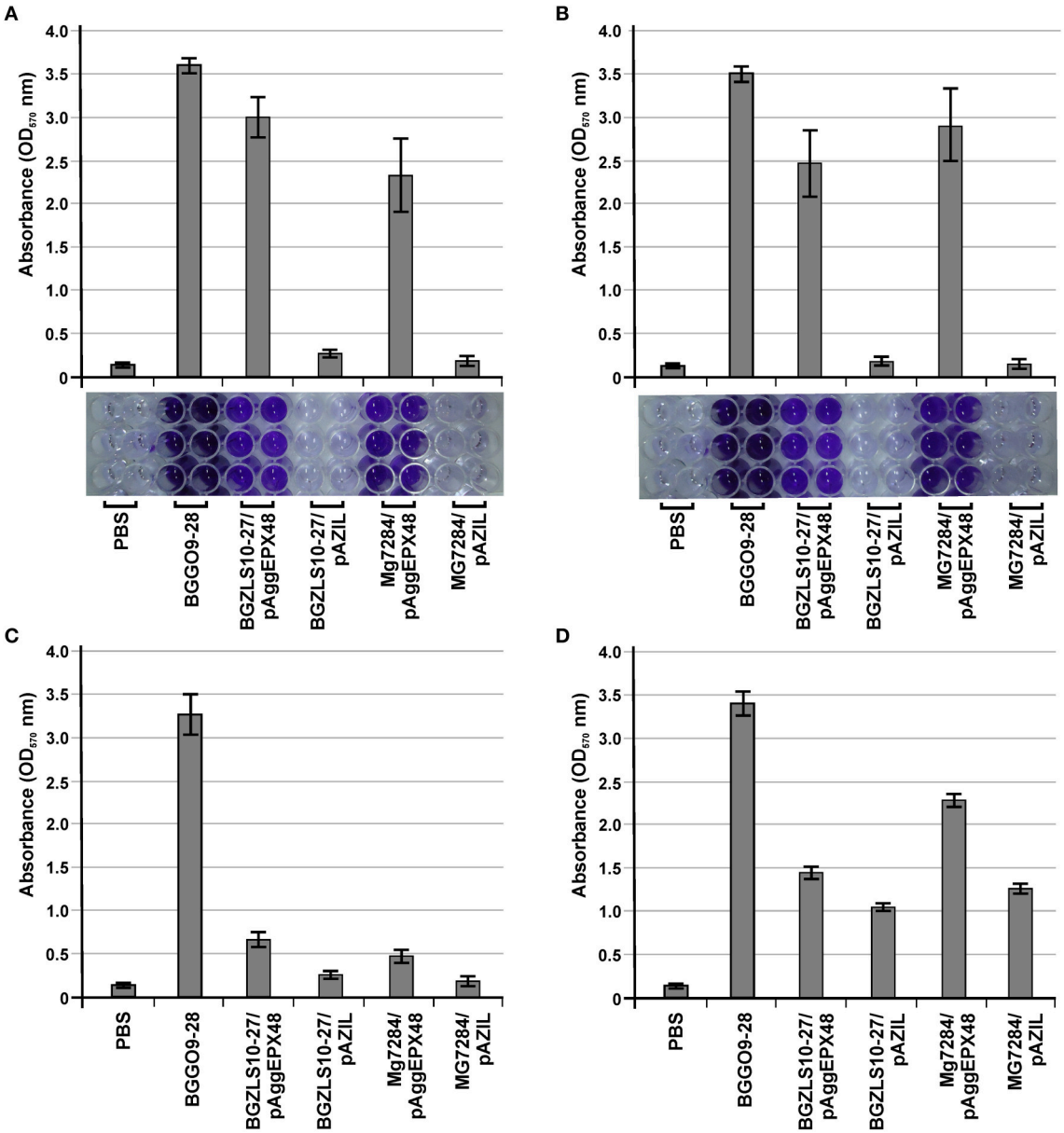

FIGURE 7 | The role of AggE in adhesion to components of ECM and biofilm formation. Graphical presentation and photo of microtiter plates of results obtained in **(A)** collagen-binding assay; **(B)** fibronectin-binding assay; Graphical presentation of results obtained in **(C)** mucin binding assay and **(D)** biofilm formation assay. Results were expressed as average of normalized A_{570} values. The error bars show the standard deviations of three independent observations.

transformants indicates that some other factors in addition to AggE, present on the cell surface of the strain, are involved in the binding interaction to collagen and fibronectin.

Strain BGGO9-28 possesses a very high adhesion to mucin, while derivatives carrying *aggE* gene (BGZLS10-27/pAggEPX48 and MG7284/pAggEPX48) and their non-aggregating derivatives (BGZLS10-27/pAZIL and MG7284/pAZIL) showed lower adhesion abilities to mucin, with a small difference between them (clones showed only about two times higher binding ability) in contrast to binding to collagen and fibronectin (**Figure 7C**). It can be assumed that a direct correlation between the presence of the *aggE* gene and binding ability to mucin has not been determined (since AggE gives little contribution to mucin binding), confirming the specificity of the AggE binding factor for only certain components of the ECM. Interaction between bacterial cells is a complex process that involves a number of factors. It seems that for some processes such as auto-aggregation the most important is *aggE*/AggE, but other cell surface properties or environmental factors can contribute to the modulation of the expression of a given phenotype.

Evaluation of the formation of biofilms and their interaction with the bacterial surface molecules is a complementary approach

to better understanding the mechanisms by which bacteria colonize a niche as a consequence of adaptation to environmental stresses (Peter et al., 2013). Aggregation and biofilm formation are multicellular processes that allow a community to be more resistant to stress conditions. Given that these are similar processes, it is not surprising that the same protein could be involved in both functions (Miljkovic et al., 2016). It is considered that the expressed *aggE* gene contributes to the increase of biofilm formation in a heterologous host, but it is not entirely (clones carrying AggE increased only about two times formation of biofim), indicated that additional factors/genes in enterococci are responsible for the full expression of this phenotype (**Figure 7D**).

Probiotic Characteristics of BGGO9-28 Strain

The susceptibility of dairy isolate *En. faecium* BGGO9-28 to antibiotics according to CLSI (2015), and the presence of putative virulence genes, were determined in this study in the interest of safety considerations. Strain BGGO9-28 is sensitive to all of the antibiotics tested, without hemolytic or gelatinase activity, and does not carry any of the tested virulence determinants. Our results are in accordance with data that report the absence of these virulence factors in food-isolated *En. faecium* strains (Zheng et al., 2015); although previously, several starter and food-originated enterococci were shown to harbor some of the virulence factors, suggesting the importance of strain-specific properties for carrying certain virulence factors (Eaton and Gasson, 2001).

The main desirable characteristic required for probiotic bacteria is their ability to survive during GI tract passage, in which low pH, bile salts, and digestion conditions are the main factors affecting this ability (Tan et al., 2013). The cells of strain BGGO9-28 were strongly maintained, with 8.30 log units in artificial gastric juice, 8.39 log units in bile salt juice and 8.22 log units for artificial intestine juice, respectively (**Figure 8**). It has been revealed that *En. faecium* BGGO9-28 was stable in acidic conditions (pH 2.0 for 3 h) and high bile salt, and had a high level of tolerance to pancreatin. BGGO9-28 has a high survival ability in the GI tract, which is similar to previous observations (Nueno-Palop and Narbad, 2011) reporting high levels of survival for enterococci.

Like the ability to survive under harsh conditions, adhesion is also an important functional characteristic of probiotic strains. The adhesion ability of *En. faecium* BGGO9-28 to the epithelial intestinal cell line Caco-2 was determined in order to define a percentage of attachment of the potential probiotic strain. The results obtained in this study clearly indicate that the strain possesses high adhesion abilities, about 80% adhesion to the Caco-2 cell line (data not shown).

The capability of probiotic strains to neutralize the negative effects of pathogens is their important health promoting property (FAO-WHO, 2006). One of the mechanisms of action is antimicrobial activity related to the production of bacteriocins, and colonization competition, or pathogen exclusion (Gareau et al., 2010; Satish-Kumar et al., 2011). Since strain BGGO9-28 does not exhibit antimicrobial activity, it has been proposed

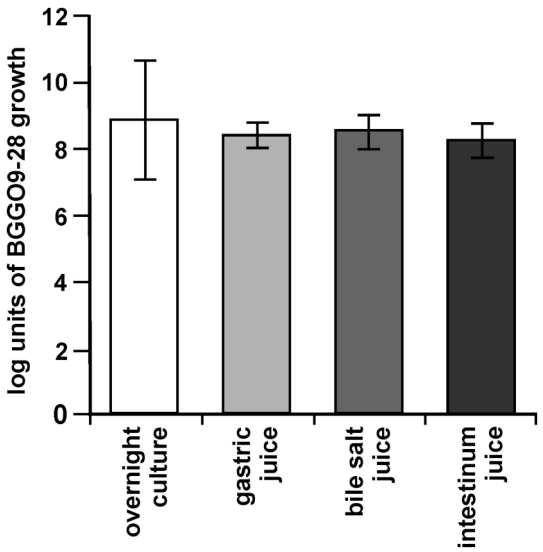

FIGURE 8 | Survival of BGGO9-28 strain in GI tract conditions. Bacterial counts in suspensions made in 10% skimmed-milk of the *En. faecium* BGGO9-28 after chemically simulating the GI tract: in gastric, bile salt and intestinal juices. The error bars show the standard deviations of three independent observations.

that the cell surface components involved in desirable probiotic features could be cell surface associated proteins, S-layer macromolecules built from proteins (Zhang et al., 2010) and auto-aggregation factors (Miljkovic et al., 2015).

It is important to emphasize that *in vitro* tests have many limits, because adhesion properties and mucus production depend on the cell line used in the study (Turpin et al., 2012). Additionally, some *in vitro* studies that estimated the degree of adhesion also indicate the cytotoxicity of tested strains to the target cells (Miquel et al., 2015). This suggests that adhesion and/or long-term colonization of probiotic agents usually is not without consequences.

This paper describes a new aggregation factor in enterococci, AggE, which belongs structurally to those of the other LAB, responsible for auto-aggregation and binding of carrier cells to specific components of the ECM. In conclusion, our findings indicate that there is a relationship between the auto-aggregation and adhesiveness of BGGO9-28 that is mediated by AggE on the cell surface. In particular, it should be noted that despite the fact that the adhesion of enterococci is generally extremely high, specificity of interaction with certain components of ECM is determined by AggE protein. The results of this study emphasize the direct link between AggE protein and auto-aggregation and binding to components of ECM, but also note the importance of other host and environmental factors on the overall adhesion of the bacterial cells.

AUTHOR CONTRIBUTIONS

MK conceived, designed and coordinated this study, interpreted all of results and wrote this paper. KV, NP, and MM designed,

performed, analyzed the experiments and wrote this paper. MT and AT provided experimental assistance, contributed to the preparation of the figures, provided technical assistance and contributed to the preparation of this paper. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Intra-species Genomic and Physiological Variability Impact Stress Resistance in Strains of Probiotic Potential

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Large-scale microbiome studies have established that most of the diversity contained in the gastrointestinal tract is represented at the strain level; however, exhaustive genomic and physiological characterization of human isolates is still lacking. With increased use of probiotics as interventions for gastrointestinal disorders, genomic and functional characterization of novel microorganisms becomes essential. In this study, we explored the impact of strain-level genomic variability on bacterial physiology of two novel human *Lactobacillus rhamnosus* strains (AMC143 and AMC010) of probiotic potential in relation to stress resistance. The strains showed differences with known probiotic strains (*L. rhamnosus* GG, Lc705, and HN001) at the genomic level, including nucleotide polymorphisms, mutations in non-coding regulatory regions, and rearrangements of genomic architecture. Transcriptomics analysis revealed that gene expression profiles differed between strains when exposed to simulated gastrointestinal stresses, suggesting the presence of unique regulatory systems in each strain. *In vitro* physiological assays to test resistance to conditions mimicking the gut environment (acid, alkali, and bile stress) showed that growth of *L. rhamnosus* AMC143 was inhibited upon exposure to alkaline pH, while AMC010 and control strain LGG were unaffected. AMC143 also showed a significant survival advantage compared to the other strains upon bile exposure. Reverse transcription qPCR targeting the bile salt hydrolase gene (*bsh*) revealed that AMC143 expressed *bsh* poorly (a consequence of a deletion in the *bsh* promoter and truncation of *bsh* gene in AMC143), while AMC010 had significantly higher expression levels than AMC143 or LGG. Insertional inactivation of the *bsh* gene in AMC010 suggested that *bsh* could be detrimental to bacterial survival during bile stress. Together, these findings show that coupling of classical microbiology with functional genomics methods for the characterization of bacterial strains is critical for the development of novel probiotics, as variability between strains can dramatically alter bacterial physiology and functionality.

Keywords: probiotics, intra-Species variability, bacterial stress, *Lactobacillus rhamnosus*, bile salt hydrolase (BSH)

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INTRODUCTION

The gastrointestinal tract is host to one of the densest and most diverse microbial communities on the planet (Gill et al., 2006; Li et al., 2012). The composition and function of the gut microbiota is critical to maintenance of host gastrointestinal health (Jones, 2016). In fact, diseases including diabetes (Tilg and Moschen, 2014), obesity (Carding et al., 2015), and colorectal cancer (Sobhani et al., 2011; Borges-Canha et al., 2015) have been shown to have gut dysbioses associated with progression. Conversely, modulation of the gut microbiota can alleviate or even eliminate disorders like *Clostridium difficile* infections (Petrof et al., 2013; Allegretti et al., 2014), inflammatory bowel disease (D'Haens et al., 2014), and lactose intolerance (Azcarate-Peril et al., 2017).

Intra-species genetic polymorphisms constitute the majority of the diversity within the microbiota of the human gut (Greenblum et al., 2015; Zhang and Zhao, 2016). Coupling classical microbiology approaches with next generation sequencing provides an opportunity to study physiological characteristics of individual microbial strains, and to identify unique genomic elements associated with those phenotypes. Moreover, advances in cultivation technologies have improved isolation of novel microorganisms from human subjects, provided the ability to study physiology of difficult to grow microbes (Faith et al., 2010), and ultimately develop advanced microbiota-derived treatments for gastrointestinal diseases (Forster and Lawley, 2015).

Use of probiotics, live microbes that convey a benefit to their host when administered in adequate amounts, as interventions for gastrointestinal disorders has gained increasing support (Grover et al., 2012; Vandenplas et al., 2015); however, intra-species variations can impact their functionality as effective probiotics (Chapman et al., 2012). Isolation and characterization of novel intestinal organisms are important steps toward the development of new and improved probiotics. Moreover, high throughput sequencing technology allow for cost-effective whole genome sequencing of bacterial isolates, providing a wealth of genomic data. Nevertheless, identification and characterization of novel probiotics also require careful examination of microbial physiology (Papadimitriou et al., 2015).

One essential physiological characteristic of probiotics is their ability to survive the environmental conditions of the gastrointestinal tract. Gut microorganisms have evolved highly conserved mechanisms for tolerance to gastrointestinal stresses, which include variations in pH and the antimicrobial effects of bile (Azcarate-Peril et al., 2004; Bruno-Bárcena et al., 2004). These mechanisms are controlled by well-regulated genetic systems (Azcarate-Peril et al., 2004), often with rapid response times to acute stresses, allowing for immediate resistance to environmental changes (Anderson et al., 2010; Bove et al., 2013). Bacterial responses to environmental stress occur in a highly regulated manner. First, stress-specific genes aim to alleviate the immediate stress (maintenance of cytoplasmic pH homeostasis, transport/degradation of toxic compounds, and others), followed by universal stress response systems aimed to repair DNA, protein, membrane, and cell wall damage.

If exposed to an acidic environment, H^+ pump of ATPases (F_0F_1-ATPases) maintain pH homeostasis within the cytoplasm by pumping excess H^+ ions back into the environment (Wang et al., 2013; Cusumano and Caparon, 2015). Other mechanisms involved in resistance to acid stress in *Lactobacillus* are arginine deiminases (ArcABC) (Fulde et al., 2014) that synthesize ammonia from arginine and free H^+ within the cytoplasm (Casiano-Colón and Marquis, 1988) and amino acid decarboxylases coupled with amino acid/biogenic amines antiporter systems (Azcarate-Peril et al., 2004; Papadimitriou et al., 2016). Upon surviving the acidic conditions in the stomach and passing into the duodenum, the pH of the environment shifts from highly acidic to alkaline. Alkaline Shock Proteins (Asp) are encoded by lactic acid bacteria including *Lactobacillus rhamnosus* (Arnold et al., 2017), and have been implicated in resistance to sudden pH shifts (Kuroda et al., 1995; Seetharaaman, 2008; Anderson et al., 2010).

Entry into the duodenum not only involves a change in pH, but also exposes microorganisms to bile salts, which act as detergents, causing cell damage and cytotoxicity (Andreichin, 1980; Begley et al., 2005). Lactic acid bacteria including *Lactobacillus* and *Bifidobacterium* species encode bile salt hydrolases (*bsh*), which have been shown in some cases to provide resistance to bile toxicity (Grill et al., 2000; Patel et al., 2010; Lin et al., 2014). *Lactobacillus acidophilus* and *Bifidobacterium* encode multiple *bsh* genes, which exhibit substrate specificity for different conjugated bile salt targets (McAuliffe et al., 2005; Jarocki and Targonski, 2013). As different bile salts have varying toxicity, individual *bsh* genes may or may not provide survival advantages against bile (Fang et al., 2009). In addition to providing survival advantages in the gastrointestinal environment, bile salt hydrolases encoded by lactic acid bacteria (Jarocki and Targonski, 2013; Jarocki et al., 2014; Pithva et al., 2014) have been implicated in modulation of host cholesterol and lipid metabolism (Patel et al., 2010; Joyce et al., 2014), and as a mechanism for microbe-host signaling (Ridlon et al., 2006).

Universal bacterial stress-response systems encode mechanisms to cope with the deleterious consequences of exposure to environmental stresses, including DNA damage, protein misfolding, and loss of cell wall/membrane integrity. Bacterial DNA damage is repaired by MutS and RecA-like recombinases (Lee and Pi, 2010; Rossi et al., 2016; Calderini et al., 2017; Overbeck et al., 2017). Protein misfolding is reduced and repaired by molecular chaperones including heat-shock proteins (Ruiz et al., 2013; Calderini et al., 2017). Cell wall integrity of gram positive bacteria is maintained through lipoteichoic acid (LTA), exopolysaccharide, and fatty acid metabolism/synthesis genes (Koskenniemi et al., 2011). Membrane proteins including metalloproteases provide additional stress resistance in lactic acid bacteria (Bove et al., 2012). *Lactobacillus* also encodes two component regulatory systems (2CRS) that promote rapid responses to environmental stresses. These systems usually consist of a histidine kinase and a response regulator gene that work to sense and react to changes in the environment (Yu et al., 2014; Monedero et al., 2017), facilitating stress resistance (Morel-Deville et al., 1998; Alcántara et al., 2011). Together, universal

stress-response genes work with specific stress resistance systems to ensure bacterial survival to gastrointestinal conditions.

Douillard et al. (2013) in their analysis of 100 *L. rhamnosus* genomes, showed that strains had unique genomic elements coupled with physiological traits that were influenced by the strain origin. In particular, bile resistance was higher in *L. rhamnosus* isolates derived from the gastrointestinal tract as opposed to dairy, oral, or vaginal isolates. In this study we analyzed genomic, transcriptomic, and physiological characteristics of two novel human *L. rhamnosus* strains of probiotic potential for which our group recently generated genomic sequence information (Thompson et al., 2015; Arnold et al., 2017), and compared them with an established probiotic strain of human origin (LGG). *Lactobacillus rhamnosus* GG (LGG) is a well-characterized probiotic lactic acid bacteria isolated from a healthy human host in 1983. The strain provides a number of benefits to its host including immunomodulation (Lebeer et al., 2012; Segers and Lebeer, 2014), pathogen exclusion (De Keersmaecker et al., 2006; Makras et al., 2006), and modulation of host gene expression (Kankainen et al., 2009; Yan et al., 2013; Segers and Lebeer, 2014). LGG sets a precedent for *L. rhamnosus* as a functional probiotic as well as for the isolation and identification of novel probiotics from healthy human hosts. Additionally, the strains were compared with *L. rhamnosus* of dairy origin (Lc705, HN001; Ceapa et al., 2015) to demonstrate the impact of intra-species genetic polymorphisms on tolerance to gastrointestinal stress.

MATERIALS AND METHODS

Strains and Culture Media

Bacterial strains used in this study are presented in **Table 1**. Strains were propagated in MRS broth (Pronadisa, Madrid) at 37°C without agitation, or on MRS agar plates containing 1.5% agar. For growth assays, MRS supplemented with 0.1–1.0% Oxgall was used to test resistance to bile. MRS was titrated with HCl–pH 4 to test growth in acidic conditions, and titrated with NaOH to pH 8 to test growth under alkaline stress.

Bacterial Growth Assays

Lactobacillus rhamnosus strains were grown statically for 16 h in MRS broth at 37°C. Freshly harvested cells were washed with MRS without glucose and diluted 1:100 in MRS (pH 6.6) containing either bile (0, 0.1, 0.3, 0.5, and 1.0% w/v oxgall), or adjusted to pH 4 (HCl) or pH 8 (NaOH). 200 μl of bacterial suspensions were placed into each well of a 96 well plate, sealed and placed into Tecan Infinite 200 Pro spectrophotometer (Tecan, Switzerland), where they were grown for 24 h at 37°C. Measurements of optical density at 600 nm ($OD_{600\,nm}$) were taken every 15 min during this 24-h period. Maximum specific growth rates (μ_{max}) were then calculated for each treatment type and plotted in Origin2016 (OriginLab, Northampton, MA).

Bacterial Survival Assays

Bacterial cells grown to mid-log growth phase ($OD_{600\,nm}$ = ∼0.6) were centrifuged, washed twice with sterile PBS and diluted 1:10,000 in simulated gastric juice (0.5% pepsin, 0.5% NaCl, pH3), simulated intestinal juice (0.3% pancreatin, 0.5% NaCl, pH8), or bile (0.5% Oxgall in MRS + 0% Glucose). Cell suspensions were incubated for 2 h at 37°C and then plated onto MRS agar plates by WASP Spiral Plater (Don Whitley Scientific, West Yorkshire, UK) at 1 and 2 h post inoculation. Plates were incubated at 37°C for 48 h. Survival was measured using ProtoCOL colony counter (Synbiosis, Frederick, MD) to count colonies present on MRS plates of treated and untreated cells. Three biological replicates with three technical replicates were included in survival experiments. Pairwise comparisons using a two-tailed Student's *t*-test were performed to determine statistically significant differences between treatments.

TABLE 1 | Bacterial strains used in this study.

Strain	Origin	References
L. rhamnosus GG	Healthy human isolate	Kankainen et al., 2009
L. rhamnosus Lc705	Fermented dairy product	Kankainen et al., 2009
L. rhamnosus HN001	Fermented dairy product	Tannock et al., 2014
L. rhamnosus AMC010	Human infant stool	Arnold et al., 2017
L. rhamnosus AMC010::*bsh*		This study
L. rhamnosus AMC143	Human infant stool	Arnold et al., 2017

RNA Isolation

AMC010, AMC143, and LGG were grown to early log phase ($OD_{600\,nm}$ = ∼0.4) in MRS prior to harvesting. Cells were then washed twice with sterile PBS and re-suspended in simulated gastric juice, simulated intestinal juice, or bile (0.5% w/v Oxgall in sterile PBS) and incubated for 10 min at 37°C. Three biological replicates were included in expression experiments. After incubation, cells were centrifuged, flash frozen in RNAlater (Thermo Fisher Scientific, Waltham, MA), and stored in −80°C until RNA isolation was performed. RNA isolation was performed using the Qiagen RNeasy PowerMicrobiome kit (Qiagen, Valencia, CA) as directed. RNA was eluted in 50 μl RNase free water and quantified using the 2200TapeStation (Agilent Technologies, Santa Clara, CA).

Real Time Quantitative PCR

Five ng of total RNA isolated from bacterial cultures exposed to bile (0.5% w/v Oxgall in sterile PBS for 30 min at 37°C) were reverse transcribed using qScript cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD). To generate a standard curve, genomic DNA from AMC010 was amplified using primer set BSHqPCR-F (TTGGCGCTGACGACTTGC), BSHqPCR-R (AATCTTGACGCCTTGACC) (this study) and purified via gel extraction in 1.5% agarose gel. The purified PCR product was serially diluted and used in RT–qPCR experiments. The qPCR master mix included 10 μl of Power SYBR® Green 2x PCR Master Mix (Applied Biosystems, Foster City, CA), 2 μl of each primer (BSHqPCR-F, BSHqPCR-R) (1 μM stock), 1 μl PCR grade water, and 5 μl of template cDNA (1 ng/μl). The reaction was run for 40 cycles of melting (95°C) 15 s, annealing/extension (65°C) 45 s followed by SYBR detection was carried out on the 7,500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA)

after 10 min denaturation step at 95°C. Samples and standards were run in triplicate.

mRNA Sequencing

Ribosomal RNA was depleted from total RNA using the Ribo-Zero Gold Bacterial rRNA Removal Reagent (Epidemiology Kit) (Illumina, San Diego, CA) according to manufacturer's instructions. Briefly, the rRNA-specific magnetic beads were removed from storage buffer and mixed with 500 ng of total sample RNA. Subsequently, rRNA removal solution was added and samples were incubated for 10 min at 65°C. Finally, samples were placed on magnetic stand for 15 min at 22°C and mRNA was removed and immediately processed with TruSeq Stranded mRNA HT kit (Illumina, San Diego, CA) according to manufacturer's instructions. Briefly, RNA was mixed with Fragment-Prime mix and incubated at 94°C for 8 min. Samples were immediately subject to first strand and second strand cDNA synthesis reactions, respectively, followed by 3′ end repair, adenylation and adapter ligation. After adapter ligation, the libraries were enriched by PCR using the following thermal cycling conditions: 98°C for 30 s followed by 15 cycles of 98°C for 10 s, 60°C for 30 s and 72°C for 30 s. Final extension step of 70°C for 5 min was carried out following the last cycle. After enrichment, libraries were purified with Beckman Coulter magnetic beads (Brea, CA), washed with 80% ethanol and eluted in Tris pH 8.5. Following enrichment, cDNA was barcoded for multiplexing via PCR, using dual-index barcodes [index 1(i7) and index 2(i5)] (Illumina, San Diego, CA) in a combination unique to each sample. Final ds cDNA was purified with Beckman Coulter magnetic beads (Brea, CA). Library concentrations and quality were measured via TapeStation2200 (Agilent Technologies, Santa Clara, CA). Barcoded libraries were pooled at equimolar concentrations and sequenced on the Illumina HiSeq platform (Illumina, San Diego, CA).

mRNA Sequencing Data Analysis

Sequencing output from the Illumina HiSeq platform was converted to FASTQ format and demultiplexed using Illumina BclFastq 2.18.0.12 (Illumina, San Diego, CA). Quality control was performed via FastQC on both raw and processed sequencing reads (Babrahm Institute, Cambridge, UK). Demultiplexed FASTQ sequence files were uploaded to Geneious software (Biomatters, New Zealand). Sequencing reads from each treatment were mapped against LGG genome in Geneious software using "Geneious for RNA Seq" mapper, a minimum mapping quality of 30 (99.9% confidence), allowing gaps with a maximum of 10% per read, 20% maximum mismatches per read, with word length of 20 bases. Expression levels were calculated in Geneious and compared between treatment types. Genes identified as significantly differentially regulated between treatments ($p \leq 0.05$) were further filtered to include only genes with 2-fold expression differences. Genes identified as significantly differentially regulated at ≥2-fold in at least one treatment type for each isolate were plotted as heat maps in OriginLab software (Origin Lab, Northampton, MA), and compared between strains to show differences.

Preparation of Electrocompetent Cells of *L. rhamnosus*

Electrocompetent *L. rhamnosus* cells were generated using a combination of previously described methods (Kim et al., 2005; Welker et al., 2015), with minor adjustments. Briefly, an overnight culture of *L. rhamnosus* AMC010 was diluted to 10^6 cells/ml in pre-warmed MRS broth supplemented with 2% glycine and was incubated overnight at 37°C without agitation. After incubation, 5 ml of culture was inoculated in 100 ml of freshly prepared, pre-warmed MRS medium supplemented with 2% glycine. The culture was incubated at 37°C without agitation until it reached and $OD_{600\,nm}$ of 0.2. Ampicillin was then added to the culture at a final concentration of 10 μg/ml, and incubation at 37°C continued until the culture reached an $OD_{600\,nm}$ of 0.4. Cells were then harvested by centrifugation at room temperature (10 min at 6,000 g), washed twice at room temperature with electroporation buffer (0.5 M sucrose, 7 mM potassium phosphate pH7.4, 1 mM $MgCl_2$), resuspended in 1 ml of the same buffer, and placed on ice. The electrocompetent cells were used immediately for electroporation.

Generation of the *bsh* Insertional Mutant Strain

Insertional inactivation of the *bsh* gene in *L. rhamnosus* AMC010 was done as described (Welker et al., 2015). Briefly, primers BshFHindIII (TATTAAGCTTTTCAGACAGAGGCGGCTTTGC) and BshREcoRI (AATAGAATTCAATCTTGACGCCTTGACCAC) were designed to amplify a 652 bp region of the *bsh* gene in AMC010. The primers included EcoR1 and HindIII restriction sites for cloning into the pFAJ-5301 vector (Lebeer et al., 2012). The resulting vector (pFAJ-BSHi) was used to transform AMC010 by electroporation using an Bio-Rad Gene Pulser (peak voltage, 1.7 kV; capacitance, 25 μF; resistance, 200 Ω with time constant of 2–4 ms). Electroporated AMC010 cells were allowed to recover for 24 h in MRS at 37°C without agitation, and subsequently plated on MRS containing erythromycin at a final concentration of 2 μg/ml. Individual erythromycin resistant colonies were selected after 48 h of growth at 37°C and sub-cultured in MRS containing 2 μg/ml erythromycin overnight without agitation at 37°C. Disruption of the *bsh* genes was verified by PCR amplification using primers Bsh1753F (ATTGCCTGACCTAGATGCAGG) and Bsh1753R (AACACCGGCGACAGGTCCATC). Genomic integration of the erythromycin resistance cassette was also verified by PCR amplification with the primers Ery625F (CTACTTAATCTGATAAGTGAGC) and Ery625R (TCAGCACAGTTCATTATCAACC). AMC010::*bsh* mutants were cultivated in MRS broth containing 2 μg/ml erythromycin and stored at −80°C in 15% glycerol.

RESULTS

Comparative Analysis of the *L. rhamnosus* Stress Genomic Complement

The genomes of *L. rhamnosus* AMC 143 and AMC010 isolated from infant stools (Thompson et al., 2015; Arnold et al., 2017) were aligned to the genome of the well-characterized probiotic

L. rhamnosus GG (Segers and Lebeer, 2014) for comparison (**Figure 1A**). Our study focused on stress genes and systems encoded by AMC143 and AMC010 in comparison with other *L. rhamnosus* strains of dairy and human origin.

When exposed to environmental pH fluctuations, proton translocating F_0F_1-ATPases are the primary genes responsible for maintaining cytoplasmic pH homeostasis (Azcarate-Peril et al., 2004). Each strain in this study encoded ATPase genes with high nucleotide identity to one another (>98%), however the genomic architecture surrounding the ABC-F Family ATPase varied between strains (**Figure 1B**). In addition to ATPases, cytoplasmic pH homeostasis can be maintained by amino acid decarboxylases and their corresponding antiporters (Azcarate-Peril et al., 2004). Both AMC010 and AMC143 encoded the arginine/ornithine antiporter *arcD* gene; however, the 12 kb region that included this unique *arc*D gene was absent in LGG and HN001 (**Figure 1C**). This region encompassed *arc*D as well as an NADH oxidase, two diguanylate cyclases, cellulose synthase, glycosyl transferase, glycosyl hydrolase and a hypothetical protein. An ornithine decarboxylase was identified in all strains with over 97% nucleotide identity with no architectural deviations between strains. Another amino acid decarboxylase and antiporter system, the glutamate decarboxylase/glutamate gamma-aminobutyrate antiporter (*gad*C) has been shown to reduce the impact of acid stress in *Lactobacillus* (Azcarate-Peril et al., 2004); however, the *gad*C gene, as well as the corresponding decarboxylase gene were absent in both AMC010 and AMC143. Finally, an uncharacterized amino acid decarboxylase gene was present in LGG (LGG_RS12751) with 97.2% nucleotide identity to its homolog in AMC010, but this gene was absent in AMC143.

Similarly to acid exposure, cytoplasmic pH homeostasis is critical to maintain when cells are exposed to alkaline stress. Sodium-proton antiporters and potassium-proton antiporters maintain cytoplasmic pH in alkaline extracellular environments (Lee et al., 2011; Nyanga-Koumou et al., 2012). The strains in this study encoded 3 sodium-proton antiporters (98.2, 98, and 97.1% nucleotide identity). LGG encoded a unique sodium-proton antiporter absent in AMC010 and AMC143. Each strain also encoded a single potassium transporter, with 97.3% nucleotide identity between strains. Alkaline shock proteins (Asp) have been implicated in providing protection from damage caused by increases in extracellular pH change (Kuroda et al., 1995; Seetharaaman, 2008; Anderson et al., 2010). Each of the strains in this study encoded 4 *asp* genes including *asp23*, each with minimum of 98.3% nucleotide identity between strains.

The anti-microbial/detergent properties of bile salts require cells to respond upon exposure in order to resist cytotoxic effects. Each strain in this study encoded a single copy of a bile salt hydrolase (*bsh*). This gene has been implicated in bile-stress tolerance in other lactobacilli (McAuliffe et al., 2005). The sequence identity of the *bsh* gene exceeded 98.2% between strains, however AMC143 contained a deletion of 208 base pairs upstream of the *bsh* gene, eliminating a putative promoter, ribosome binding site, and inducing a 38 bp truncation of the *bsh*

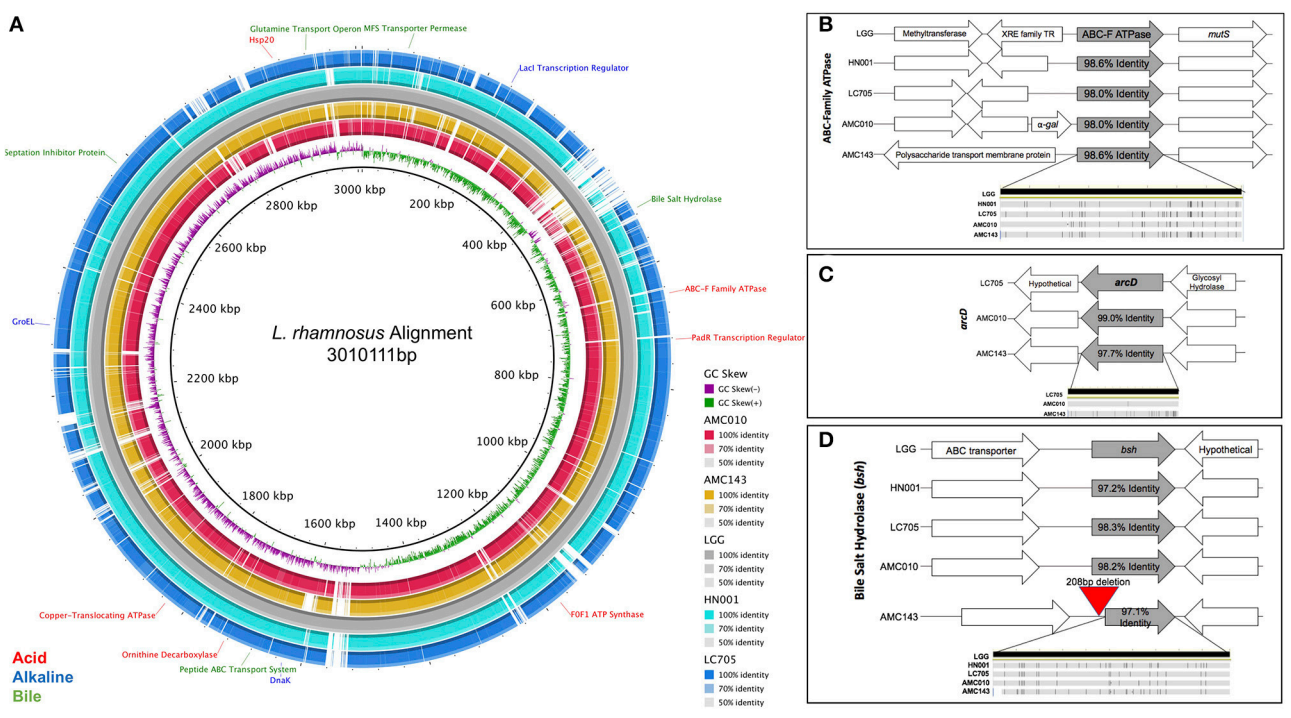

FIGURE 1 | Comparative genomic analysis of *Lactobacillus rhamnosus* isolates. **(A)** Genome alignment of *L. rhamnosus* strains. Strains AMC43, AMC010, Lc705, and HN001 were compared to LGG using BRIG genome alignment software. Relevant stress-response genes are annotated. Comparison of relevant stress-response genes in *L. rhamnosus*, **(B)** ABC-F ATPase, **(C)** *arc*D, **(D)** *bsh* were aligned and compared in Geneious 10.1.3 software. The bottom figure in each graph represents single nucleotide polymorphisms within the compared gene.

gene itself (**Figures 1D**, **5B**). Aside from the deletion in AMC143, the *bsh* upstream non-coding region has 93% nucleotide identity between strains. Each strain also encoded cell wall synthesis/repair pathways including DltABCD (Koskenniemi et al., 2011).

In addition to specific stress-response mechanisms, universal stress response systems were present in AMC143 and AMC010. Genes associated with DNA repair were highly conserved, including *radA*, *recN*, *recO*, *recU*, *rad*C, *recA*, and *mut*S, each of which had over 96.5% nucleotide identity between strains. Additionally, each strain encoded a suite of highly conserved molecular chaperones, which mitigate stress-associated protein misfolding. These genes included *dnaK*, *groEL*, *groES*, *HSP20*, *HSP33, dnaI*, and *clpB*, each exhibiting 98.2% nucleotide identity or higher between strains. Finally, bacteria membrane integrity is often compromised as a result of environmental stress, allowing metal ions otherwise excluded from the cytoplasm to permeate through the membrane, causing further DNA damage and cell death (Pratviel, 2012; Qiao and Ma, 2013). One mechanism that bacteria have to survive declines in membrane integrity is to actively pump cytotoxic metal ions out of their cytoplasm utilizing metal translocating ATPases (Chien et al., 2013). Each strain encoded three different non-specific metal transporting ATPases each with 97.6% nucleotide identity between strains, as well as specific ATPases for transport of copper (97.0% nucleotide identity between strains) and magnesium (97.5% nucleotide identity between strains).

Gene Expression Analysis under Stress Conditions

Differential transcription profiles were generated for AMC010 and AMC143 from mRNA sequencing data obtained from cells exposed to acid (pH 3 adjusted with HCl), alkaline (pH8 adjusted with NaOH), or bile (0.5% w/v oxgall in PBS) stress, and compared to untreated cells. A total 216 genes were identified as differentially regulated (>2-fold change, $p < 0.05$) for AMC010 in response to all treatments, while 79 genes were identified in AMC143. **Figure 2** shows a heatmap indicating genes that were up or down regulated in each strain. We mapped our strains to *L. rhamnosus* GG to provide gene homolog references. Our data confirmed differential regulation of universal stress-response genes and specific stress response genes, although most specific genes were differentially regulated below either the statistical or fold change cutoffs (**Supplementary Table 1**).

Exposure to pH 3 increased expression of a copper-translocating P-type ATPase homologous to LGG_RS08675 by approximately 4-fold in both strains. F_0F_1-ATPases were not differentially regulated between strains, however a metal transporting ATPase homologous to LGG_RS00670 was up-regulated approximately 1.5-fold in both AMC010 and AMC143 upon acid exposure. Amino acid decarboxylases were not differentially regulated between strains. All treatments induced expression of a gene encoding a small heat shock protein Hsp20 homologous to LGG_RS13420 in both AMC143 and AMC010. Exposure of AMC010 to acid induced expression of an additional uncharacterized Hsp20-like molecular chaperone (LGG_RS03170, 11.3-fold), the *padR* transcription regulator (LGG_RS03330, 7.5-fold), and two hypothetical proteins (uncharacterized, *Lactobacillus*-specific LGG_RS11075, 9.2-fold, and *fliK*-like LGG_RS13395, 6-fold). Conversely, a gene cluster including 5 genes involved in purine biosynthesis (LGG_RS08715, LGG_RS08720, LGG_RS08725, LGG_RS08730, and LGG_RS08735) was inhibited in AMC010 across all conditions.

Exposure to alkaline conditions induced expression of the alkaline-shock protein Asp23/Gls24 family envelope stress response protein homologous to LGG_RS01130 in AMC010 by 2.3-fold. This gene also showed a non-statistically significant 2-fold induction in AMC143. Other alkaline shock proteins homologous to LGG_RS01125 and LGG_RS08100 were induced by at least 2-fold in both strains, while the putative alkaline shock protein homologous to LGG_RS01130 was repressed by approximately 2-fold under the same condition, though *p*-values for each of these genes fell below the significance cutoffs set for this study. Alkaline stress also induced the *lacI* transcription regulator (homologous to LGG_RS01785) in both AMC010 (6.1-fold) and AMC143 (12.1-fold), the Hsp20-like molecular chaperone (LGG_RS03170, 19.7-fold in AMC143), which was also induced by exposure to acid in AMC010, and uncharacterized hypothetical proteins homologous to LGG_RS00635 (7-fold) and LGG_RS01080 (5.7-fold) in AMC010. Additionally, exposure to pH 8 resulted in decreased expression of the translation initiation factor IF-3 (LGG_RS08400) and a ribosomal-processing cysteine protease (Prp) homologous to LGG_RS08120, reducing expression levels by 2.6-fold and 4.3-fold respectively in AMC143. Alkaline challenge of AMC010 resulted in reduced expression of the septation inhibitor protein similar to *divIC* (Bennett et al., 2007) (LGG_RS12050) by 3.7-fold, and *minC/ftsL*, genes associated with inhibition of cell division (LGG_RS06095 by 2-fold and LGG_RS06140 by −3.5-fold) ABC transport ATP binding protein (LGG_RS09570) by 4.3-fold and a YbjQ_1 domain-containing hypothetical protein homologous to LGG_RS02955 by 42.2-fold. The galactose-6-phosphate isomerase operon (LGG_RS03135, LGG_RS03140, LGG_RS03145, and LGG_RS03150) was specifically up regulated in AMC143 exposed to pH 8, while the Rpml/translation initiation factor gene cluster (LGG_RS08400 and LGG_RS08395) was down regulated in the same strain by approximately 3-fold.

Exposure to bile resulted in the differential expression of 52 genes in AMC143 and 111 genes in AMC010 with considerable overlap with alkaline treatment. Although the *bsh* gene has been shown to be induced by exposure to bile in *Lactobacillus* (Koskenniemi et al., 2011), in our study, *bsh* (similar to LGG_RS02395) showed a non-statistically significant ($p = 0.2$) 1.2-fold induction in AMC010 while AMC143 showed a marginal repression. The genes more impacted by bile exposure in AMC143 were a HU-family transcription regulator homologous to LGG_RS06660 (induced 2-fold), a septation inhibitor protein (LGG_RS12050), which was down regulated 9-fold, the glutamine ABC transport system operon composed of genes homologus to LGG_RS13875, LGG_RS13880, and LGG_RS13885, which were down regulated

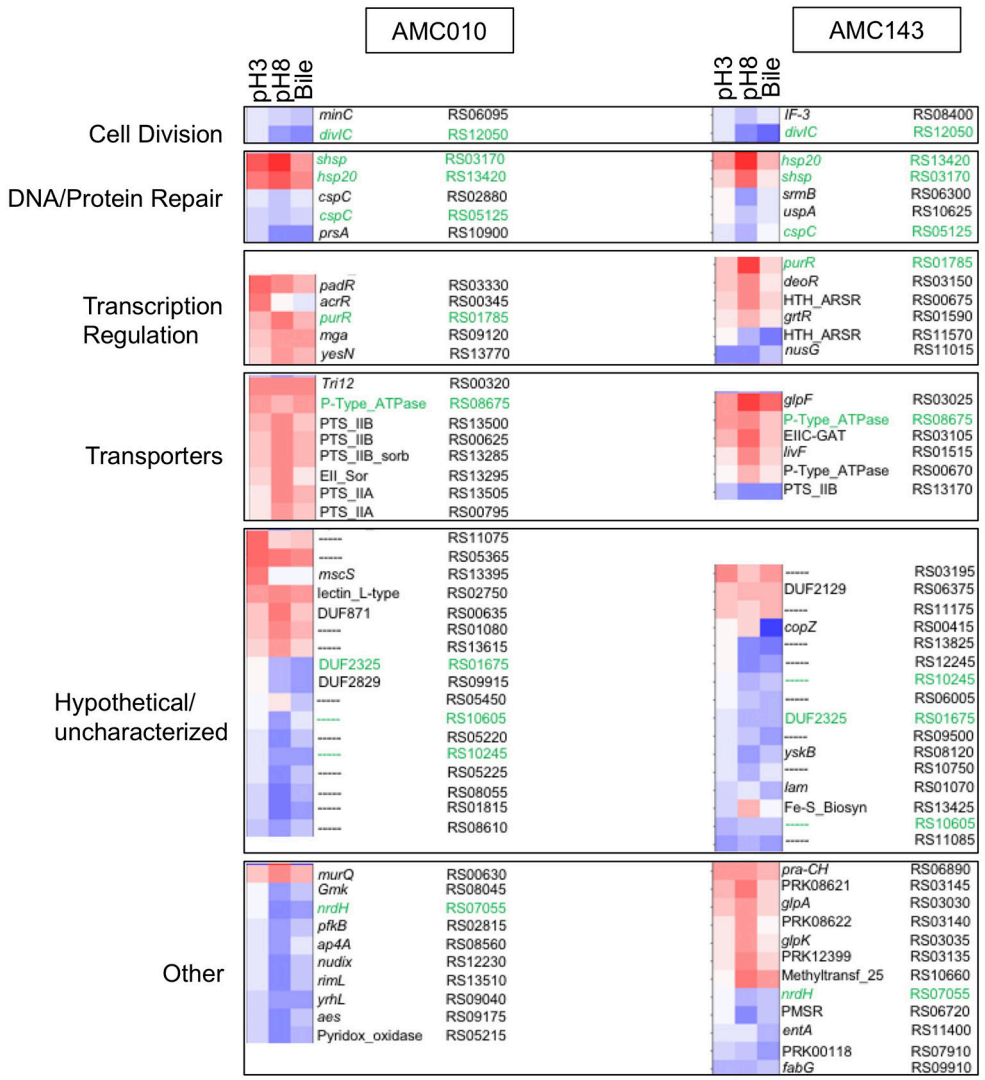

FIGURE 2 | Transcriptomics analysis. mRNA sequencing data from each treatment (pH3, pH8, 0.5%, w/v Bile) was mapped to LGG genome and compared to untreated cells to calculate Log2 differential expression ratio and *p*-value. The 50 genes with the highest differential expression ratio (FDR corrected $p \leq 0.05$) from each strain were categorized by function (cell division, DNA/protein repair, transcription regulation, and molecular transport) and plotted in OriginLab software. Highly differentially expressed hypothetical genes and genes with functions that did not fit into a defined category were also included. Green text highlights genes differentially regulated in both AMC010 and AMC143.

2-fold, and hypothetical proteins homologous to LGG_RS01070, LGG_RS09500, and LGG_RS00415, down regulated 2.8-, 4-, and 13.9-fold respectively.

Exposure of AMC010 to Oxgall resulted in a 2.8-fold induction of a 2CRS response regulator (RR), *dcu*R (homologous to LGG_RS13770), similar to the RR involved in regulation of the anaerobic fumarate respiratory system in *E. coli* (Janausch et al., 2004), as well as a 5.3-fold induction of a major facilitator superfamily (MFS) transporter permease (LGG_RS00320) and 4.3-fold induction of the Hsp20-like molecular chaperone (LGG_RS03170). Conversely, a septation inhibitor protein (LGG_RS12050), *prsA* similar to LGG_RS10900, a molecular chaperone potentially involved in a late stage of protein export (Jakob et al., 2015; Jousselin et al., 2015), the NrdH-redoxin (LGG_RS07055), uracil transport (LGG_RS06995, LGG_RS07000, and LGG_RS07005) and peptide ABC transport system operon (LGG_RS07940, LGG_RS07945, LGG_RS07950, LGG_RS07955, and LGG_RS07960 were down regulated in AMC010 exposed to bile.

In Vitro Growth and Survival of *L. rhamnosus* Exposed to Simulated Gastrointestinal Conditions

Under normal growth conditions (MRS broth, pH 6.6 at 37°C) LGG showed the highest growth rate among strains (0.63 h^{-1}),

while AMC143 had the lowest growth rate (0.48 h^{-1}) with no significant differences between AMC010, Lc705, and HN001 (**Figure 3A**). While growth rates between strains were similar under normal conditions, there were distinct differences in growth phenotypes between strains when exposed to simulated gastrointestinal conditions. Growth rates of all strains were reduced approximately 5-fold at pH 4 (adjusted with HCl); however the strains isolated from dairy products, HN001 and Lc705, showed further reduced rates than the human-derived strains, AMC010, AMC143, and LGG (**Figure 3A**). As expected, survival of early-log cells exposed to simulated gastric juice (pepsin, NaCl, HCl pH3) was minimally impacted. No decrease in survival was observed for LGG, Lc705, or AMC143 after 1 h of exposure, while an approximately 15% decrease was observed for HN001 and AMC010 strains. After 2 h, Lc705 showed close to 100% survival, LGG and HN001 were reduced by approximately 20%, and AMC010 and AMC143 populations were reduced by 40% (**Figure 3B**).

Exposure of *L. rhamnosus* strains to MRS adjusted to pH 8 with NaOH showed nearly a 50% decline in growth rate only in AMC143. HN001 and Lc705 showed increased growth rates when grown in media at higher pH 8, while AMC010 and LGG showed no significant differences in growth rates compared to pH 6.6 (**Figure 3A**). When early-log cells were exposed to simulated intestinal juice (pancreatin, NaCl, NaOH pH8) (Charteris et al., 1998) cell numbers increased after 1 h to then decrease in all strains, except HN001 where survival was stable (**Figure 3C**).

Finally, we sought to evaluate growth and survival of *L. rhamnosus* strains in the presence of bile. At sub-physiological concentrations of bile (0.1% w/v), the only strain that showed growth inhibition was Lc705 (60% growth rate reduction). As the concentration of bile in the growth medium increased to physiological levels (0.3% w/v), growth rates decreased by approximately 50% for AMC010, AMC143, and LGG, while HN001 and Lc705 were further inhibited (58 and 80% respectively) (**Figure 4A**). Lc705 showed significant decreased growth rates in all bile concentrations tested. As bile concentrations exceeded physiological levels (0.5% bile) growth rates decreased by over 60%. The growth inhibition observed at 1.0% w/v bile was greater for HN001 and Lc705 (83–92%) than for the strains of human origin (71–78%).

Exposure of *L. rhamnosus* to 0.5% bile resulted in a significant decrease in survival in all strains except AMC143. AMC143 exhibited nearly 20-fold higher survival rates compared to all other strains after 1 h of exposure to 0.5% bile. After 2 h, no HN001 colonies were detected, and the LGG and AMC010 populations were reduced by >95%. AMC143 however remained resistant to the antimicrobial effects of bile, with over 15% of cells surviving after 2 h of exposure (**Figure 4B**).

The Bile Salt Hydrolase (*bsh*) Gene from *L. rhamnosus* AMC010 and AMC143

Despite a highly conserved chromosomal architecture and gene sequence, the region upstream of the *bsh* gene in AMC143

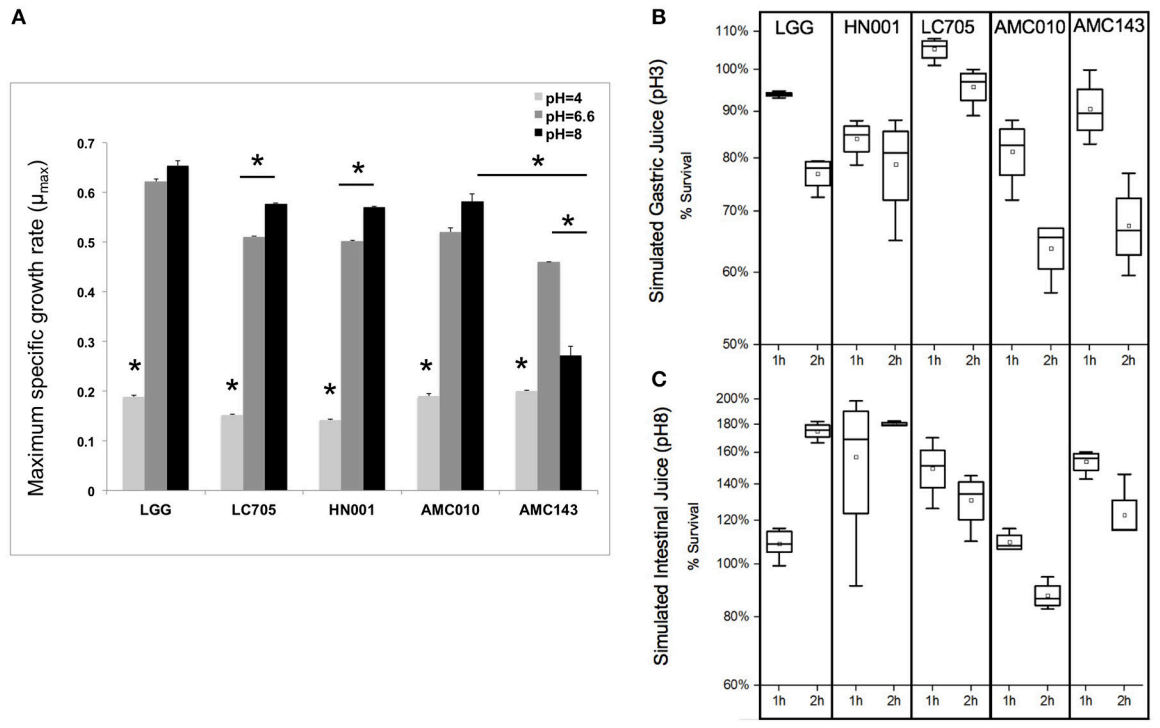

FIGURE 3 | Growth and survival of *L. rhamnosus* under pH-stress conditions. **(A)** Growth rates were calculated for cells grown at pH 6.6 (MRS) pH4 (adjusted with HCl), or pH8 (adjusted with NaOH). (*$p < 0.01$). **(B,C)** Bacterial survival was assayed by plating and enumerating cells exposed to simulated gastric juice (NaCl, HCl, Pepsin pH3) or simulated intestinal juice (NaCl, HCl, Pancreatin pH8) for 1 or 2 h.

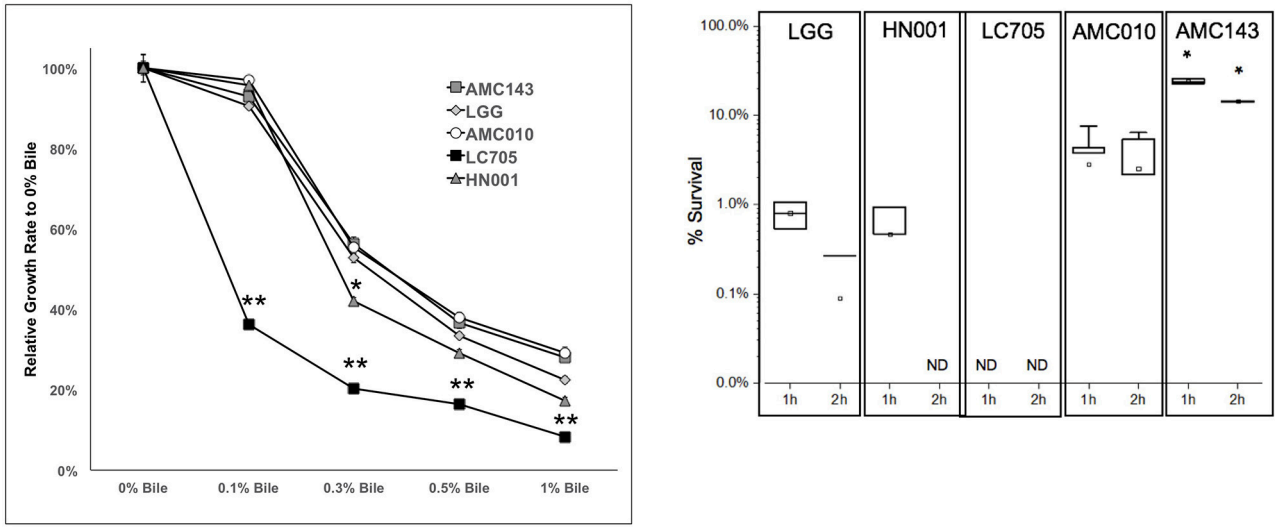

FIGURE 4 | Growth and survival of *L. rhamnosus* under bile-stress conditions. **(A)** Growth rates relative to 0% bile were calculated for cells grown in MRS containing 0.1, 0.3 0.5, and 1.0% w/v oxgall. (*$p > 0.05$, **$p > 0.01$). **(B)** Bacterial survival was determined by plating and enumerating colonies post exposure to 0.5% w/v oxgall in MRS devoid of any carbohydrate source for 2 h. *Indicates significant difference between AMC143 and each other strain tested. (*$p > 0.01$, *ND* = No detected growth).

contained a 208 bp deletion, eliminating a putative promoter region and ribosome binding site that is highly conserved in the other strains in the study (**Figure 5B**). Additionally, this deletion resulted in a truncation of the *bsh* gene. AMC143 showed significantly increased resistance to bile. To investigate if the deletion was responsible for the increased resistance of the strain to bile, we first assessed *bsh* expression levels in *L. rhamnosus* AMC143, AMC010, and LGG by RT-qPCR. Data showed variable expression of the gene in absence of bile. Upon bile exposure the *bsh* gene in AMC010 was induced approximately 4-fold, while expression of *bsh* homologs in AMC143 and LGG did not vary. Moreover, *bsh* expression was significantly lower in the presence or absence of bile in AMC143 (**Figure 5A**).

To determine if inactivation of the *bsh* gene in AMC010 would replicate the bile resistant phenotype observed in AMC143, *bsh* was disrupted by insertion of the pFAJ-BSHi vector. Growth of the AMC010::*bsh* mutant under normal growth conditions was indistinguishable from the wild type strain. Survival and growth rates of the mutant were assayed under each stress condition previously performed in this study and compared to the wild type strain. When exposed to acid stress conditions, growth and survival of AMC010::*bsh* was identical to AMC010. Similarly, growth and survival at high pH was identical between the two strains (**Supplementary Figure 1A**). Growth rates of AMC010::*bsh* and AMC010 in varying concentrations of bile (0–1.0% w/v Oxgall) were similar in the presence of glucose (**Supplementary Figure 1B**); however, when exposed to high concentrations of bile (0.5% w/v Oxgall) without a carbohydrate source, survival of AMC010::*bsh* was enhanced 4–6-fold compared to the wild type strain (**Figure 6**), further suggesting a link between *bsh* expression and bile resistance in *L. rhamnosus*.

DISCUSSION

The future of personalized medicine will employ defined bacterial consortia to treat or prevent diseases according to host genotype, diet, and life stage, and the essential first step to develop such consortia is the extensive characterization of gastrointestinal organisms (Faith et al., 2010).

High-throughput *in silico* and *in vitro* analyses of gene-level variation across a large array of species in the human gut are useful as screening tools; however, detailed characterization of strains is essential to determine functional implications of strain variation in the complex ecosystem of the gut (Greenblum et al., 2015). Our study confirms that strain-level variability contribute significantly to microbial diversity within complex microbial communities (Zhang and Zhao, 2016). We compared genomic and physiological data of two novel strains of *L. rhamnosus* (AMC010 and AMC143) (Thompson et al., 2015; Arnold et al., 2017) to three characterized strains (LGG, Lc705, and HN001) to determine their response to simulated gastrointestinal stress.

One of the most striking physiological differences between the strains in our study was the growth inhibition of AMC143 exposed to alkaline conditions. Comparative genomic analysis revealed no major differences between genes previously identified as alkaline-stress response genes, with nucleotide identities ranging from 98.5 to 99.6% between strains. Despite this growth defect, the strain had comparable survival rates when exposed to simulated intestinal juice, suggesting that the growth defect is caused by arrest of cell division as opposed to cell death. Upon

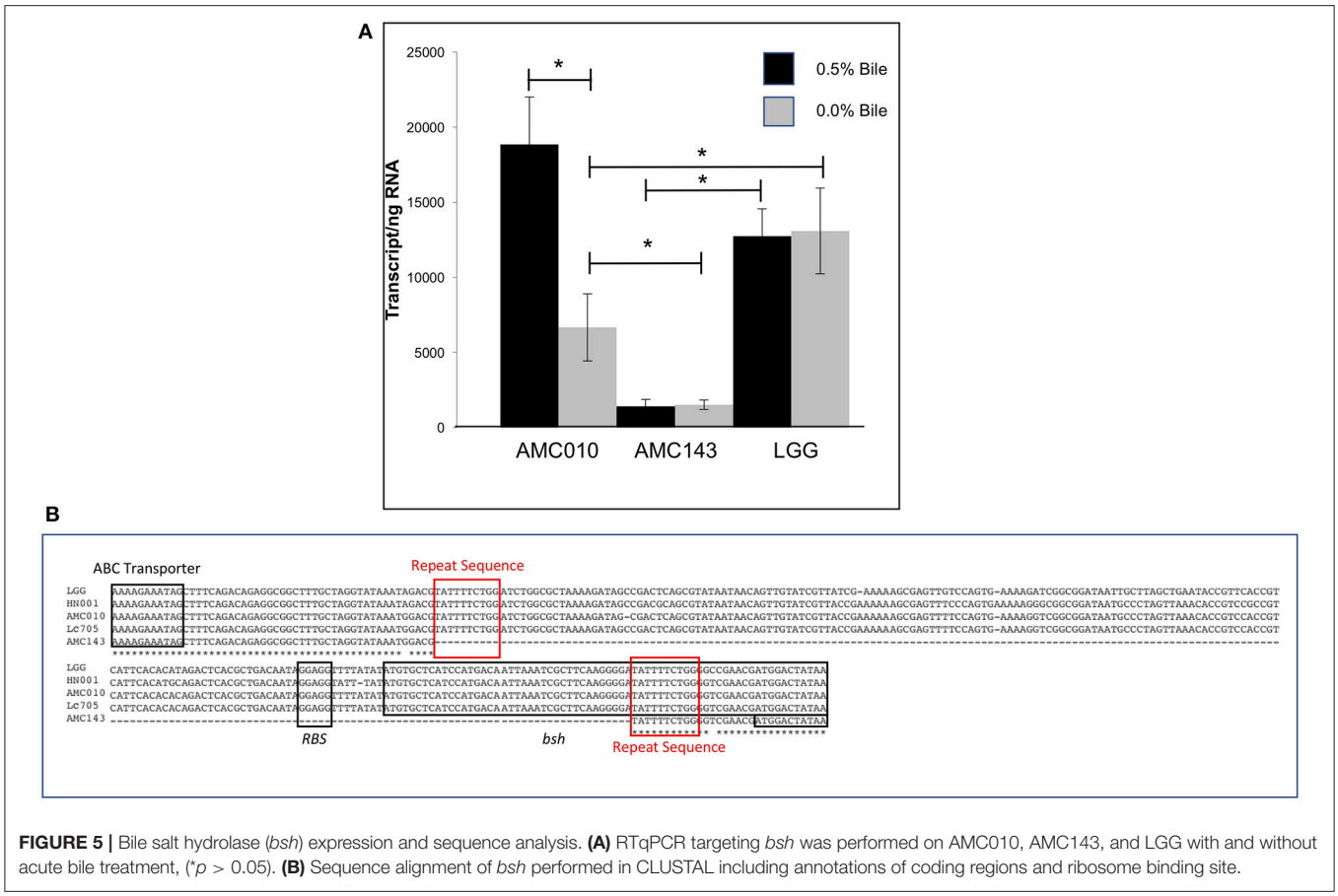

FIGURE 5 | Bile salt hydrolase (*bsh*) expression and sequence analysis. **(A)** RTqPCR targeting *bsh* was performed on AMC010, AMC143, and LGG with and without acute bile treatment, (*$p > 0.05$). **(B)** Sequence alignment of *bsh* performed in CLUSTAL including annotations of coding regions and ribosome binding site.

exposure to alkaline stress, expression levels of genes associated with growth inhibition (*divIC*, *ftsL*, and *minC*) were repressed in AMC010, while expression of the same genes in AMC143 was unaffected. Conversely, expression of the translation initiation factor *IF-3* was downregulated in AMC143 when exposed to alkaline conditions. We could speculate that this differential expression plays a role in the growth inhibition observed for AMC143. A previous study in *Lactobacillus plantarum* showed that expression of two endopeptidases (Accession numbers Q52071 and Q048X8) was reduced under alkaline conditions (Lee et al., 2011); however we failed to identify homologus genes significantly downregulated in AMC143. Further study of cellular response to alkaline pH is required to better understand the reasons for a growth defect in AMC143.

Analysis of genome sequencing data revealed a 208 bp deletion upstream of the *bsh* gene in AMC143 (**Figure 1D**). Sequence comparison between strains identified a repeat of 10 base pairs flanking the deleted region, suggesting that this deletion may have occurred in AMC143 through homologous recombination. Physiological assays showed that AMC143 was more resistant to bile-induced toxicity than the other strains tested, which led us to investigate the impact of *bsh* expression on bile toxicity. RTqPCR expression data showed a significantly lower *bsh* transcript count in AMC143 compared to LGG or AMC010, both in absence or presence of bile. A non significant induction of the *bsh* gene in AMC010 was also observed by mRNA sequencing data. Bile salt hydrolases have been associated to bile stress resistance in *Lactobacillus* and *Bifidobacterium* (Grill et al., 2000; McAuliffe et al., 2005; Lin et al., 2014) and implicated as a mechanism for host-microbe signaling (Zhou and Hylemon, 2014; Song et al., 2015; McMillin et al., 2016). These enzymes hydrolyze conjugated bile salts generating unconjugated bile acids, which often function as a signaling molecules to host cells and have strong anti-microbial effects (Sytnik et al., 1978; Grill et al., 2000; Schmidt et al., 2001; Kong et al., 2011), and an amino acid, which can be utilized by both host and microorganisms for protein synthesis (Ridlon et al., 2006, 2014; Patel et al., 2010). Studies have shown that BSHs do not always provide a survival advantage to bacteria exposed to bile (Fang et al., 2009), suggesting that BSH activity differs between microorganisms. In fact, lactobacilli encode variable *bsh* genes, each with unique substrates and activities (McAuliffe et al., 2005; Ren et al., 2011). To determine if *bsh* expression was correlated to lower survival upon bile exposure in our strains, we generated a mutant strain of AMC010 containing a disrupted *bsh* gene by site directed insertion (Lebeer et al., 2012). AMC010::*bsh* recapitulated the physiological phenotype observed in AMC143. These findings suggest that the unconjugated products of BSH activity were cytotoxic to the strains used in this study. Our survival assays were performed in MRS broth devoided of a carbohydrate

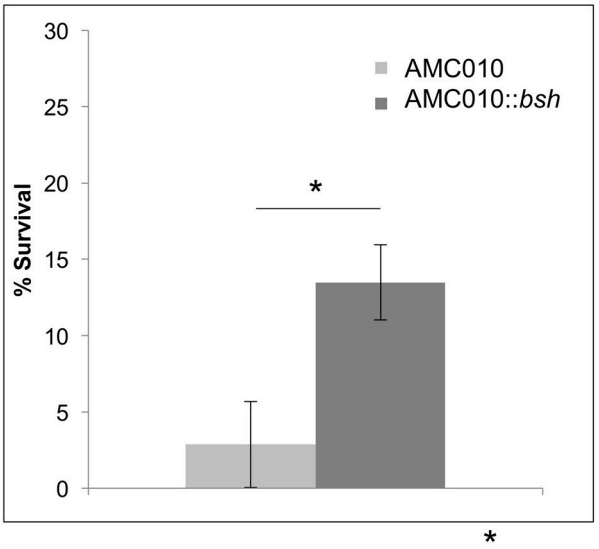

FIGURE 6 | Survival of AMC010::*bsh* under bile-stress. Survival of AMC010::*bsh* was compared to wild type AMC010 by plating and enumerating colonies post exposure to 0.5% w/v oxgall (*$p < 0.01$).

source. In accordance to a previous report (Ziar et al., 2014), when survival experiments were performed in the presence of a carbohydrate source, bile cytotoxity was almost completely abolished for all strains (data not shown).

Strains of the same species derived from different environments are likely to have evolved different abilities to tolerate environmental stress (Douillard et al., 2013). Our data shows distinct physiological differences between strains isolated from human hosts (LGG, AMC010, and AMC143) and strains isolated from fermented dairy products (Lc705, HN001). When grown in alkaline conditions, dairy-derived strains exhibited accelerated growth, which correlates with studies done with other dairy-derived lactobacillus strains (Mojgani et al., 2015), while intestinal strains were either inhibited (AMC143) or unaffected (LGG, AMC010). Additionally, we found that while strains isolated from human hosts were able to grow with limited inhibition at sub-physiological levels of bile, the dairy isolate Lc705 was significantly inhibited even at very low bile concentrations (0.1%w/v). Moreover, survival of dairy-derived strains exposed to high bile concentrations was lower than intestinal strains, suggesting that the strain environmental origin may have driven evolution of their stress response pathways. Strains evolving in environments devoid of bile have no selective pressure to retain bile resistance genes, and these molecular mechanisms are lost over time. This phenomenon has been observed in dairy derived samples as well as in the oral microbiota (Douillard et al., 2013; de Barros et al., 2016).

Analysis of 16S rRNA sequencing data is unable to accurately resolve taxonomy at the species/sub-species level (Janda and Abbott, 2007) as single gene sequencing is insufficient to differentiate between similar species, especially in highly diverse and complex communities. Even with the best of what next generation sequencing has to offer, classical microbiology approaches are absolutely critical in understanding how microbial behavior and physiology correlate to genomic, proteomic, and transcriptomic data. As novel microbiota-derived interventions like new probiotics are being developed, it is important to keep in mind the magnitude to which seemingly minor genomic variability can result in changes to cellular physiology and thus probiotics efficacy.

AUTHOR CONTRIBUTIONS

JA Designed and performed experiments, and wrote the manuscript; JS and JK Performed experiments; JR Performed bioinformatics analysis; MA Designed the experiments, advised JA, contributed to bioinformatic and statistical analyses of data and wrote the manuscript; All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00242/full#supplementary-material

Supplementary Figure 1 | (A) Growth rates of AMC010::*bsh* in MRS at pH 4, 6.6, and 8. **(B)** Relative growth rate of AMC010::*bsh* in bile (0, 0.1, 0.3, 0.5, and 1.0% w/v oxgall).

Supplementary Table 1 | Expression fold change of Lactobacillus rhamnosus genes under stress conditions.

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METHODS
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A Versatile New Model of Chemically Induced Chronic Colitis Using an Outbred Murine Strain

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Murine colitis models are crucial tools for understanding intestinal homeostasis and inflammation. However, most current models utilize a highly inbred strain of mice, and often only one sex is employed to limit bias. This targeted approach, which in itself is biased, means that murine genetic diversity and sex-related differences are ignored, making it even more difficult to extend findings to humans, who are highly heterogeneous. Furthermore, most models do not examine the chronic form of colitis, an important fact taking into account the chronic nature of the inflammatory bowel diseases (IBD). Here, we attempted to create a more realistic murine colitis model by addressing these three issues. Using chemically induced chronic colon inflammation in an outbred strain of mice (RjOrl:SWISS [CD-1]), we (i) mimicked the relapsing nature of the disease, (ii) better represented normal genetic variability, and (iii) employed both female and male mice. Colitis was induced by intrarectal administration of dinitrobenzene sulfonic acid (DNBS). After a recovery period and 3 days before the mice were euthanized, colitis was reactivated by a second administration of DNBS. Protocol length was 24 days. Colitis severity was assessed using body mass, macroscopic scores, and histological scores. Myeloperoxidase (MPO) activity, cytokine levels, and lymphocyte populations were also characterized. Our results show that the intrarectal administration of DNBS effectively causes colitis in both female and male CD-1 mice in a dose-dependent manner, as reflected by loss of body mass, macroscopic scores and histological scores. Furthermore, colon cytokine levels and mesenteric lymph node characteristics indicate that this model involves immune system activation. Although some variables were sex-specific, most of the results support including both females and males in the model. Our ultimate goal is to make this model available to researchers for testing candidate anti-inflammatory agents, such as classical or next-generation probiotics; we also aim for the results to be more easily transferrable to human trials.

Keywords: DNBS, CD-1 mice, gut inflammation, murine IBD model, colitis

INTRODUCTION

Murine colitis models are crucial tools for understanding intestinal homeostasis and inflammation (Martin et al., 2017). Their use over recent years has resulted in an exponential growth of knowledge on host–bacteria interactions. The most common *in vivo* models use rodents; they mimic different types of colitis with the aim of testing how the microbiota affects colon inflammation. Models can be placed into one of four categories based on their disease induction method: (i) chemically induced colitis; (ii) bacterially induced colitis; (iii) spontaneous colitis (including congenital and genetically engineered forms); and (iv) adoptive-cell-transfer colitis (Martin et al., 2017). All these models have advantages and disadvantages. For instance, the intrinsic similarities and differences between mice and humans as well as external factors (e.g., living conditions and diet) might influence the ability of murine models to represent disease-related changes that occur in human microbiota (Nguyen et al., 2015).

Here, we focus on chemically induced colitis models, which recreate the morphological, histopathological, and clinical features of human inflammatory bowel diseases (IBD) by orally or intrarectally administering various chemical compounds (Randhawa et al., 2014). For example, colitis can be induced by giving rodents drinking water containing dextran sodium sulfate (DSS) for several days (Wirtz et al., 2007). DSS is toxic to colon epithelial cells and causes the complete loss of the surface epithelium in the intestine (Randhawa et al., 2014). The integrity of the mucosal barrier is therefore affected—large molecules can pass through, provoking colitis (Ni et al., 1996). Colitis can also be induced by rectally injecting a haptenating agent dissolved in ethanol, which allows the agent to pass through the mucosal barrier. The agent is then thought to act upon autologous or microbial proteins in the colon, which makes them immunogenic to the host immune system (Wirtz et al., 2007). The most commonly used haptenating agents are trinitrobenzene sulfonic acid (TNBS) and dinitrobenzene sulfonic acid (DNBS). Both TNBS and DNBS produce isolated points of inflammation and necrosis, as well as self-antigens that provoke immune responses (Elson et al., 2005). Although the models are similar, they are not identical—model functionality may vary, depending on host species identity and genetic background (Mizoguchi and Mizoguchi, 2008; Mizoguchi, 2012). Traditionally, acute protocols are used, in which the DNBS/TNBS injection or DSS period is performed just once and the recovery phase is optional (for some examples, see **Supplementary Figure S1**). However, because inflammation can be chronic, a more realistic model would employ a protocol in which colitis is reactivated at least once, thus mimicking flare-ups and relapses. Colitis development is evaluated using changes in body mass, clinical symptoms (e.g., diarrhea, constipation, and bloody feces), colon morphology, and histological features. Furthermore, because this form of colitis is clearly tied to the immune system, colon cytokine concentrations, lymphocyte levels, and myeloperoxidase (MPO) activity (indicator of neutrophil infiltration that reflects the local immune response) are helpful markers of colitis severity (Wirtz et al., 2007; Martin et al., 2014a).

Researchers use these models to identify and characterize candidate anti-inflammatory agents and test their effects on different IBD or other forms of intestinal mucosal inflammation. Such anti-inflammatory agents include for instance different type of molecules and also microorganisms known as probiotics. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). At present, thanks to our improved knowledge of the human microbiota, candidate probiotics have been identified from among the dominant members of the gastrointestinal tract (GIT) microbiota found in healthy adults. They are referred to as next-generation probiotics (NGPs) and were originally identified as commensal bacteria species that can reestablish or enhance colonization resistance (Pamer, 2016). However, this definition has been expanded rapidly to include more potential health benefits, overlapping with the emerging concept of live biotherapeutics (O'Toole et al., 2017). NGPs must be shown to be safe for the host; able to survive production, storage, and GIT transit; and elicit a positive host response that confers demonstrable health benefits (Martin et al., 2014b). Since these properties are strain specific, each candidate will have to be tested individually (Pineiro and Stanton, 2007; Hill et al., 2014; Miquel et al., 2015). In the normal sequence of events, these functional analyses involve preliminary *in vitro* testing and then preclinical *in vivo* testing in murine models. The final objective is to perform clinical trials in humans.

There are two main challenges in this process. It is necessary to, first, reproduce the *in vitro* results in the *in vivo* models and, second, reproduce the *in vivo* results in clinical trials. The use of several *in vitro* markers and models has been proposed with the aim of linking *in vitro* results with *in vivo* results. For instance, it was recently suggested that the ratio of anti-inflammatory and pro-inflammatory cytokines (interleukin IL-10 and IL-12, respectively) produced by peripheral blood mononuclear cells (PBMCs) upon *in vitro* exposure to probiotic strains could be a predictor of protective effects *in vivo* in a chemically induced murine colitis model (Foligne et al., 2007). Nevertheless, *in vivo* interactions are much more complex than *in vitro* interactions, and it is difficult to identify the best *in vitro* test for predicting the impact that a candidate anti-inflammatory agent will have *in vivo*. The most widely accepted scientific strategy is to employ a combination of several *in vitro* tests. However, transferring murine results onto a human framework is a separate challenge because success depends upon how well effects in rodents translate into effects in humans. Indeed, past studies found that humans did not experience the beneficial effects of anti-inflammatory agents that were observed in a murine model of colon inflammation mimicking IBD. For example, a *Lactococcus lactis* strain secreting IL-10 was found to decrease DSS-induced colitis by 50%; however, humans treated with a biocontained strain (thyA-/hIL-10+) did not experience beneficial effects in a phase II-A trial (Steidler et al., 2000, 2003, 2009).

Although this discordance in the results obtained in murines vs. humans could be due to their intrinsic differences (Nguyen et al., 2015), it could also be that researchers failed to carefully consider model suitability. At present, most models use an inbred strain of mice (individuals are genetically identical because

of extensive inbreeding); furthermore, often only one sex is utilized to limit bias. However, this targeted approach itself introduces bias because it ignores natural genetic diversity and sex-related differences. As a result, it becomes even more difficult to extrapolate any knowledge gleaned from murine models to human populations. Here, our aim is to describe a versatile model of chemically induced chronic colitis that utilizes an outbred strain of mice and both females and males. The ultimate objective is to establish a more realistic model for effectively testing anti-inflammatory agents, for example probiotics; the model should be able to better translate effects in rodents to effects in humans.

MATERIALS AND METHODS

Animals, Experimental Design, and Sampling Procedure

We performed two trials looking at chronic colitis development in RjOrl:SWISS (CD-1) mice (Janvier, Le Genest Saint Isle, France) using C57BL/6JRj (Black-6) mice as control in the first trial. The general characteristics of the two murine strains are described in **Table 1**. The experiment was carried by duplicate in two different periods for each trial. In each period, 5 weeks-old mice were distributed into eight cages based on strain and sex (five mice/cage) and evenly assigned to control or treatment groups (one cage/experimental group). For each trial a total of 40 females and 40 males were used (two cage/experimental group, n = 10 mice per group) for a total of 160 mice used in all the study including both trials. Mice were maintained in the animal facilities of the French National Institute of Agricultural Research (IERP, INRA Jouy-en-Josas, France) under specific pathogen free (SPF) conditions at 21°C and housed in cages of 5. They were given food and water *ad libitum* and experienced a 12:12 h light-dark cycle. Before the experiments began, animals were kept under these conditions for 1 week to allow them time to acclimate.

The experimental protocol for inducing chronic inflammation is illustrated in **Figure 1**. At week 6, mice were anesthetized using an intraperitoneal (i.p.) injection of 0.1% ketamine (Imalgene 1000, Merial, France) and 0.06% xylazine (Rompun, Bayer, France) (**Figure 1B1**). Colitis was induced using DNBS (Sigma-Aldrich, France) resuspended in 50 µl of 30% ethanol (EtOH) in PBS (see **Table 2**). In the first experiment, we wanted to compare inflammation between the two mouse strains; Black-6 is the classical inbred murine strain typically used in these types of experiments. Animals in the treatment group were injected twice with 200 mg/kg of DNBS, which corresponds to 2.7, 3, 4.1, and 4.3 mg/mouse for Black-6 females and males and CD-1 females and males, respectively. In the second experiment, we wished to obtain different degrees of colitis severity in CD-1 mice. We therefore modulated the DNBS dose in the treatment groups. Doses were fixed at 1.5, 2.5, and 3.5 mg/mice, irrespective of mouse mass or sex. In both experiments, the DNBS solution was administered on day 1 by injection with a tuberculin syringe (Terumo, France) and a flexible plastic probe (model V0104040, ECIMED, France) inserted 3.5 cm into the colon (**Figure 1B2**). Control groups were injected with equivalent amounts of the 30% EtOH solution. All mice received a subcutaneous injection of 1 ml of saline solution (0.9% NaCl) to prevent dehydration (**Figure 1B3**). Mice were kept in a horizontal position until they awoke (**Figure 1B4**). These saline injections were repeated daily for the first 3 days (no anesthesia). In this model, colitis develops in the first 3 days following the DNBS injection. During this activation period, the mice lost significant body weight. Mice were allowed to recover for 18 days and then received a second DNBS injection at day 21, reactivating inflammation. During this reactivation period, mice lost weight until the experiment's endpoint; no saline injections were performed because they could have affected body mass values at the endpoint. Mice were constantly monitored for the duration of the experiment, but especially so during the first 3 days after the DNBS injections. The model we employed in this study is a chronic colitis model because we used two DNBS injections: the first injection induces colitis, a recovery period follows, and then the second injection initiates a reactivation period. Classical acute models utilize a single injection, and colitis induction may or may not be followed by a recovery period. These model types are compared in **Supplementary Figure S1**.

On day 24, blood samples were collected from the submandibular vein, and mice were euthanized by cervical dislocation. The abdomen was then sterilized with 70% EtOH, the abdominal cavity was opened to collect the spleen and the mesenteric lymph nodes (MLNs), and the entire large intestine was removed. Bowel length was measured, and a small portion of distal colon was immediately placed in a 4% paraformaldehyde (PFA, Prolabo, France) PBS solution for later histological analyses. The intestine was then cut open longitudinally, and the tissue was washed with saline solution after removing the contents. Colon sections of 1 cm were collected and immediately frozen in liquid nitrogen.

All procedures were performed in accordance with European Union (EU) rules on ethical animal care (Directive 2010/63/EU)

TABLE 1 | Murine strains used in this study.

Name	RjOrl:SWISS (CD-1)	C57BL/6JRj (Black-6)
Type	Outbred mouse (guaranteed to display less than 1% inbreeding per generation)	Inbred mouse (guaranteed to display autosomal pair homozygosity)
Distributor and origin	Janvier Labs CSAL (Orleans)—1965	Janvier Labs CSAL (Orleans)—1993
Color and related genotype	Albino mouse—Tyrc/Tyrc	Black mouse, an (a/a) non-agouti MHC: Haplotype H2b
Breeding	Good breeder, strong maternal instinct	Good breeder but difficult to rear due to environmental sensitivity, pup cannibalism

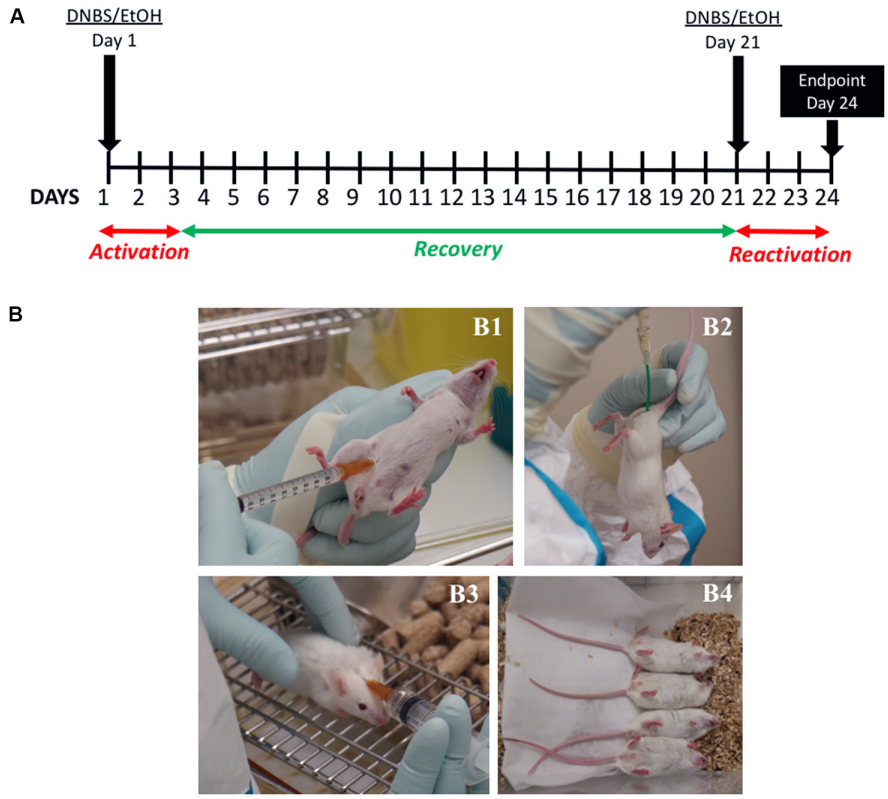

FIGURE 1 | Experimental design **(A)** and key methodological steps **(B)**. Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine and xylazine **(B1)**. The DNBS solution or the control solution was administered by injection, using a tuberculin syringe and a flexible plastic tube inserted 3.5 cm into the colon **(B2)**. Following the treatment, all mice received a subcutaneous injection of 1 ml of saline solution to prevent dehydration **(B3)**; they were kept in a horizontal position until they awoke **(B4)**.

and were approved by the French Ministry of Research and COMETHEA, the animal ethics committee at INRA Jouy-en-Josas (authorization #3445-2016010615159974).

Weight Trend and Survival Rate

In both trials, mice were carefully monitored. Their body mass was measured daily throughout the entire experimental period. Saline solution was administered when there was significant loss of body mass to prevent dehydration. In accordance with EU regulations (Directive 2010/63/EU), if mice lost 20% or more of their initial mass and/or showed signs of severe distress, they were euthanized and their id numbers were recorded. Percentage loss of body mass was calculated 3 days after each DNBS injection to compare results among groups.

Macroscopic Scores

Dinitrobenzene sulfonic acid-induced chronic inflammation is usually visible at the macroscopic level, and inflammation intensity can be evaluated by measuring different parameters, like mucosal damage in colon tissue and stool consistency. In both trials, macroscopic scores were determined using Wallace's score (Wallace et al., 1989), with the following modifications: tissue sections from each mouse were scored by evaluating ulcerations (score of 0–5), adhesions (presence/absence: 0/1), hyperemia (presence/absence: 0/1), altered transit, such as diarrhea or constipation (presence/absence: 0/1), and increases in colon wall thickness (presence/absence: 0/1; measured using an electronic caliper, Control Company, WVR, United States). The macroscopic scoring system is summarized in **Table 3** and **Supplementary Figure S2**. Although colon length is not typically part of the macroscopic score in these types of models, it was also recorded (see above).

Histological Scores

In both trials, the tissues collected for the histological analyses were fixed for 24 h in a 4% paraformaldehyde (PFA) solution

TABLE 2 | Dinitrobenzene sulfonic acid (DNBS) doses employed in this study.

Experiments	DNBS Dose
Trial 1: Inflammation patterns in CD-1 mice vs. Black-6 mice	200 mg/kg
	2.7 mg—Black-6 females 3 mg—Black-6 males
	4.1 mg—CD-1 females 4.3 mg—CD-1 males
Trial 2: Protocol optimization using CD-1 mice	3.5 mg/2.5 mg/1.5 mg

TABLE 3 | Macroscopic score.

Characteristic	Score
Ulcers	Absence: 0 1 ulcer smaller than 0.5 cm: 1 1 ulcer between 0.5 and 1 cm: 2 2 ulcers smaller than 1 cm or 1 ulcer larger than 1 cm: 3 2 ulcers larger than 1 cm: 4 More than 2 ulcers: 5
Adhesions	Presence: 1; Absence: 0
Hyperaemia	Presence: 1; Absence: 0
Altered transit	Presence: 1; Absence: 0
Colon wall thickening	Presence: 1; Absence: 0

and then transferred to 70% EtOH. After 24–48 h, the tissues were gradually dehydrated by soaking for 1 h each in 80% EtOH, 90% EtOH, 100% EtOH, and xylene in an automated tissue processer (Leica Biosystem, Germany). Samples were embedded in paraffin using a tissue embedding system (Leica), cut into 5-μm sections using a microtome (UC6, Reicher E - Leica UC6), and then stained with hematoxylin and eosin (HE) for histological scoring using an automated staining system (Leica). All these procedures were performed following conventional methodologies by the histological platform of the GABI Joint Research Unit (INRA, Jouy-en-Josas). Tissues were visualized using a high-capacity digital slide scanner (3DHISTECH Ltd., Budapest) and Panoramic and Case software (3DHISTECH Ltd.). For each animal, at least three tissue sections were evaluated to characterize alterations in mucosal architecture, the degree of immune cell infiltration, and Goblet cell depletion (Ameho score: 0–6) (Ameho et al., 1997).

Myeloperoxidase Activity and Cytokine Levels

In the second trial, to measure myeloperoxidase (MPO) activity, a 1-cm section of colon tissue from each mouse was weighed and homogenized with Precellys (Bertin Corp., France) in 300 μl of a 0.5% hexadecyltrimethyl-ammonium bromide (HTAB, Sigma-Aldrich) solution in 50 mM potassium phosphate buffer (PPB, pH 6.0); 0.35–0.40 mg of 1.4 and 2.8 mm ceramic beads (Ozyme, France) were added. Each sample was then vortexed for 10 s, centrifuged at 13,000 × *g* and 4°C for 10 min, and then transferred to a 96-well plate. To assay MPO activity, 50 μl of each aliquot was mixed with 200 μl of 50 mM PPB (pH 6.0) containing 0.167 mg/ml of o-dianisidine-dihydrochloride (Sigma-Aldrich, France) and 0.0005% hydrogen peroxide (H_2O_2, Sigma-Aldrich). The colorimetric reaction was measured by reading absorbance at 405 nm with a spectrophotometer (Infinite M200, Tecan, Switzerland) at two-time points: immediately and after 1 h. MPO activity was characterized by comparison with a standard (MPO activity of human polymorphonuclear leukocytes, Merck Chemicals, Germany) and then expressed in units/mg of tissue. One activity unit represents the conversion of 1 μM of H_2O_2 to water in 1 min at room temperature. To measure cytokine levels, 25 μl of each aliquot or 25 μl of serum were transferred to a 96-well plate. We quantified concentrations of IFN-γ, IL-5, TNF-α, IL-2, IL-6, IL-4, IL-10, IL-9, IL-17A, IL-17F, IL-21, IL-22, and IL-13 using a cytometric bead array system, the Mouse Th Cytokine Panel (13-plex; BioLegend, France), in accordance with manufacturer instructions.

Lymphocyte Populations in the Spleen and Mesenteric Lymph Nodes

In the second trial, cell suspensions were obtained by mechanically extruding the spleen and MLNs using the plunger end of a syringe and a 75-μm nylon cell strainer (BD, Switzerland). Cells were washed through the strainer using 1 ml of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, France) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (PS, Lonza, France). The red blood cell lysing buffer Hybri-Max (Sigma-Aldrich) was used to lyse the erythrocytes present in the cell suspension isolated from spleen, in accordance with manufacturer instructions. For each sample, aliquots of 10^6 cells were transferred to two 96-well plates (Greiner, France). Following standard protocols, cells were stained with anti-CD4-FITC, anti-CD3e-PE, anti-T-bet-APC, and anti-Gata3-PerCP as well as with anti-CD4-FITC, anti-CD3e-PerCP, and anti-Foxp3-PE, both stainings were performed in the presence of CD16/CD32 (all products came from eBioscience, France) to avoid unspecific staining. In brief, the cells were washed with PBS and incubated for 30 min with 0.5 μg of purified anti-mouse CD16/CD32 and surface antibodies (anti-CD4, anti-CD3) in PBS with 10% FBS and 1% sodium azide (Sigma-Aldrich). Intracellular staining was performed as follows using the Foxp3 Transcription Factor Staining Buffer Kit (eBioscience) in accordance with manufacturer instructions. Briefly, samples were washed with PBS and incubated for 20 min with a permeabilization/fixation buffer. They were then stained with intracellular antibodies (anti-T-bet-APC and anti-Gata3-PerCP or anti-Foxp3-PE) in permeabilization buffer over a period of 30 min. Samples were subsequently washed in permeabilization buffer, resuspended in PBS, and analyzed using an Accuri C6 cytometer (BD). The data obtained from the cytofluorimetric analysis were processed using CFlow Sampler software (BD).

Gastrointestinal Tract Permeability

In the second trial, 0.6 mg/g of fluorescein isothiocyanate-dextran 4 (FITC-dex 4; Sigma-Aldrich) dissolved in PBS was administered intragastrically to each mouse. Blood samples were collected after 3.5 h as described above, and 80 μl of serum was transferred to a 96-well black plate (Greiner). The concentration of FITC-dex 4 was determined using fluorescence spectrophotometry (excitation: 488 nm; emission: 520 nm; Infinite M200, Tecan); serially diluted FITC-dextran was the standard (range: 0–12,000 μg/ml).

Statistics

Statistical analyses were performed using GraphPad (GraphPad Software, San Diego, CA, United States). Survival curves analyses have been performed by Logrank test (Mantel Cox). For weight curves, a multiple unpaired *T*-test was performed per day

with fewer assumptions corrected for multiple comparison with Holm–Sidak method. Normality and variance analysis were performed using Shapiro–Wilk normality test and one-way ANOVA (Brown-Forsythe test), respectively. For normal samples (Gaussian distribution) with equal variances two-way ANOVA has been performed to compare the effect of the strain and the dose for the first trial and of the sex and the dose for the second trial; multiple comparisons were carried out using Tukey's test. For non-normal samples or/and with unequal variances non-parametric tests have been performed inside the groups (Kruskal–Wallis test); multiple comparisons were carried out using Dunn's test. *P*-values less than 0.05 were considered statistically significant. More statistical details are included in each figure legend.

RESULTS AND DISCUSSION

CD1 Mice Are Susceptible to DNBS-Induced Chronic Colitis

To design a murine model that will better predict results in humans, it is crucial to consider the real-life context of the target disease. IBD, including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by an abnormal activation of the gut immune system, which results in local chronic inflammation. Throughout their lives, patients with these diseases display active and inactive phases of variable duration that result in successive periods of relapse and quiescence. A good murine model must account for these disease dynamics. To trigger immune-mediated inflammation, it is possible to use haptenating agents, chemical compounds typically dissolved in ethanol. The ethanol allows the compounds to pass through the mucosal barrier. They then act upon either autologous or microbial proteins in the colon, rendering them immunogenic and thus provoking the abnormal activation of the immune system (Wirtz et al., 2007). As mentioned above, DNBS is one of the most common haptenating agents (Martin et al., 2017); it consistently induces chronic inflammation (Martin et al., 2014a). Most murine models of colitis use inbred strains, such as C57BL/6JRj (Black-6), and only employ males or females with the purported goal of limiting bias. However, this approach makes it problematic to transfer results to humans because representation of natural diversity in the mouse population is poor. Here, we wished to develop a more realistic murine colitis model, and we thus focused

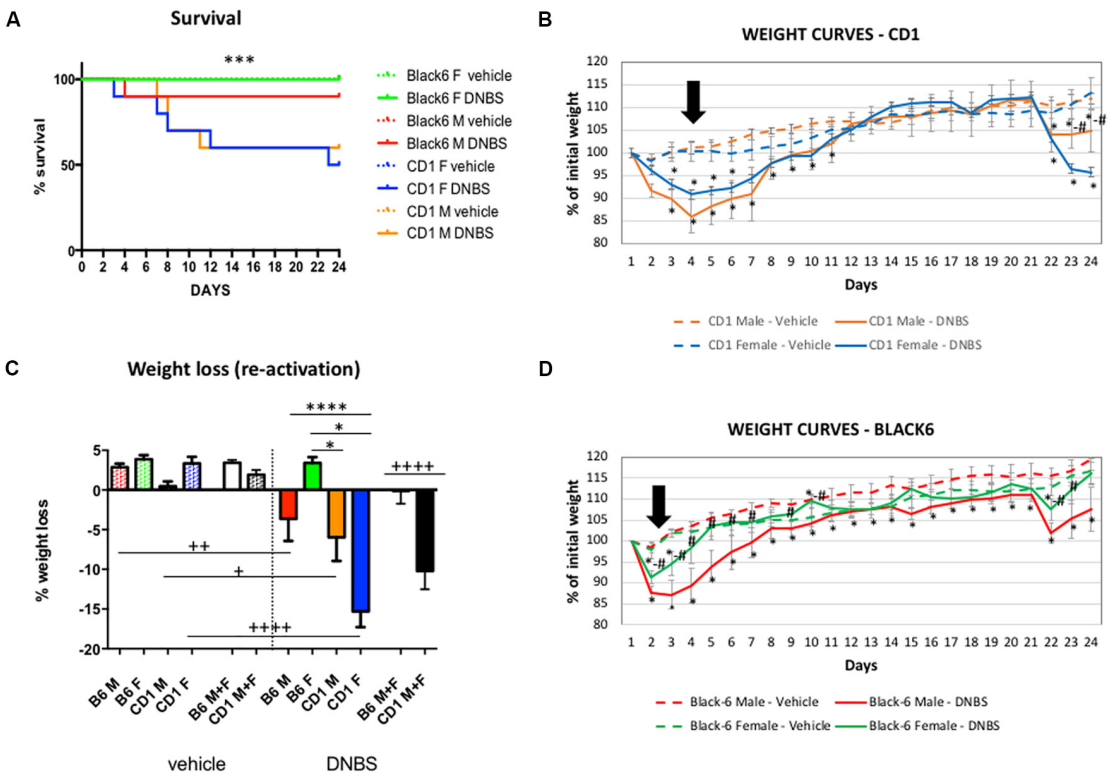

FIGURE 2 | Survival rate **(A)** and body mass trends in CD-1 **(B)** and Black-6 **(D)** mice, and loss of body mass after second DNBS injection **(C)**. For the survival rate analysis Logrank test (Mantel Cox) was performed. For weight curves, a multiple unpaired *T*-test was performed per day with fewer assumptions corrected for multiple comparison with Holm–Sidak method, (*) indicates significance vs. vehicle group and (#) significance between female and male individuals in DNBS treated groups. $n = 10$; $p < 0.05$. For the weight loss analyses, due to the lack of uniform variances when included the vehicle groups, two-way ANOVA was performed only in inflamed groups with strain and sex as factors followed by a Tukey test (results indicated as *). In order to compare the effect of the DNBS vs. the vehicle groups, a non-parametric Kruskal–Wallis test followed by a Dunn's test was performed inside CD-1 and Black 6 groups separately (results indicated as +). $n = 10$; $^{*}p < 0.05$, $^{****}p < 0.0001$, $^{+}p < 0.05$, $^{++}p < 0.01$, $^{++++}p < 0.0001$. The black arrows indicate the moment when mice started to recover weight after the first DNBS injection. B6M, Black-6 males; B6F, Black-6 females; CD1M, CD-1 males; CD1F, CD-1 females; B6 M+F, Black-6 mice; CD-1 M+F, CD-1 mice.

on three improvements to classical models: (i) mimicking the chronic nature of the disease; (ii) accounting for normal genetic variability by using outbred mice; and (iii) employing both female and male mice. More specifically, we used DNBS to chemically induce chronic inflammation in females and males of an outbred murine strain (RjOrl:SWISS [CD-1]) following the protocol described in **Figure 1**.

In our first trial, we compared inflammation patterns in CD-1 and Black-6 mice; the latter is the inbred murine strain traditionally used in colitis models. We induced initial inflammation and then relapse by sequential injections of 200 mg/kg of DNBS; the dosage was thus mass calibrated. We observed that, although CD-1 mice were heavier than Black-6 mice (mean body mass: 29.9 g and 20.1 g, respectively), they were also more sensitive to inflammation in a significant way as observed in the survival curves (Log-rank test $p = 0.0048$). In fact, the mortality rate was 5% for Black-6 mice (0% for females and 10% for males) and 45% for CD-1 mice (50% for females and 40% for males) (**Figure 2A**). Survival curves analyses using Log-rank test also showed that the differences were also significant when sex differences were taken into account ($p = 0.0368$). Nevertheless, no statistical significant sex-related differences were found inside the different strains ($p = 0.6793$ and 0.3173 for CD-1 and Black-6 mice, respectively), supporting the accurateness of pooling female and male individuals. The pattern was the same when evaluating body mass (**Figures 2B–D**). CD-1 mice lost more body mass after the first and second injections than did Black-6 mice, being this effect more persistent during reactivation (**Figure 2C**). This effect was stronger in CD-1 females than in CD-1 males, indicating they are more sensitive to DNBS-induced colitis (**Figures 2B,C**). In Black-6 mice, the pattern was reversed: females lost less body mass than did males (**Figures 2C,D**). Two-way ANOVA analyses of inflamed mice showed the presence of strain effect ($p = 0.0002$) as well as interaction between sex and strain factors ($p = 0.0021$), confirming the differences observed. Furthermore, a delay at the beginning of the recovery period was observed in CD-1 mice, while Black-6 mice started to recover at days 2–3, CD-1 mice began at day 4 (**Figures 2B,D**).

Three days after the second DNBS injection, all the mice were sacrificed, and their colons were recovered for sampling and scoring. The macroscopic scores, which took into account the presence of ulcers, adhesions, hyperemia, altered transit, and colon wall thickness, provided complementary evidence that CD-1 mice were more sensitive than Black-6 mice to inflammation (**Figure 3A**). The sex-specific patterns in macroscopic scores mirrored those seen for body mass: CD-1 females had higher scores than did CD-1 males, indicating greater sensitivity, and Black-6 females had lower scores than did Black-6 males, indicating lesser sensitivity or a failure of colitis induction (see next paragraph). Histological scoring yielded similar results (**Figure 3B**). Of note, levels of eosinophils were higher in CD-1 mice than in Black-6 mice (**Figures 3C,D**).

Traditionally, the dosage of the haptenating substance is based on body mass. However, because Black-6 females and males differed dramatically in mass (mean body mass: 17.8 and 22.3 g, respectively), this approach may have been inappropriate. Black-6 females received lower doses of DNBS because of their lighter mass, and that dose might have been too low to trigger inflammation. However, it is difficult to conclude if the lack of inflammation was due to the low DNBS dose and/or to a possible difference in sensitivity between females and males. However, significant sex-specific differences in body mass were also observed in CD-1 mice (mean body mass for females and males: 27.6 and 32.2 g, respectively), and females were clearly more sensitive than males to inflammation. However, because standard deviation values were not very large, it was possible to pool females and males for most of the characteristics analyzed (**Figures 2C**, **3A,B**).

Ultimately, one of the goals of murine colitis models is to test the efficacy of candidate anti-inflammatory agents, including probiotics. Using the model described here, we would expect effective treatments to result in an improvement in inflammation-related symptoms. More specifically, mortality rates should decline, body mass should recover more quickly, and macroscopic and histological scores should be lower.

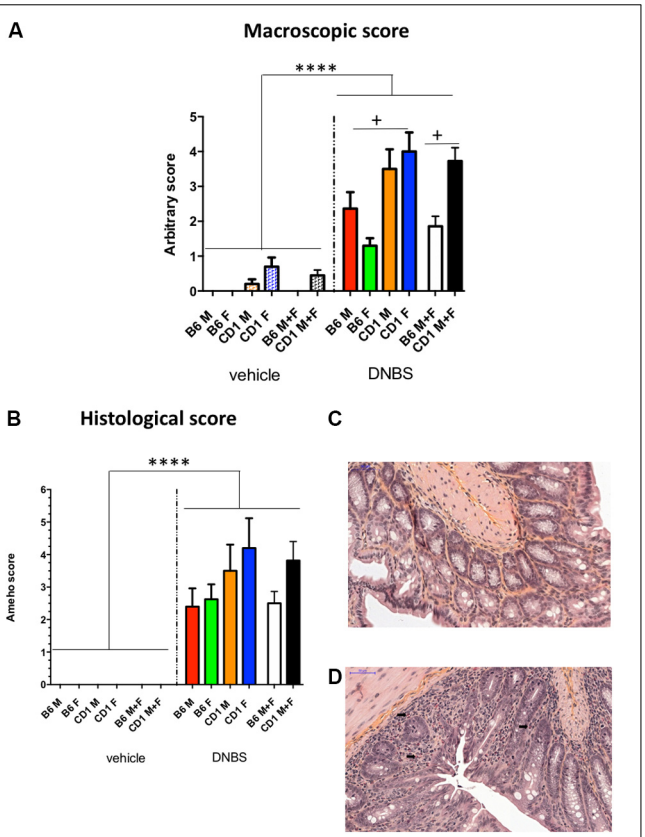

FIGURE 3 | Macroscopic **(A)** and histological scores **(B)** and representative images of Black-6 mice **(C)** and CD-1 mice **(D)**. As both scores do not follow a Gaussian distribution, in order to compare the effect of the DNBS vs. the vehicle groups, a non-parametric Kruskal–Wallis test followed by a Dunn's test was performed inside CD-1 and Black 6 groups separately (results indicated as *). The same test was performed for testing differences among inflamed groups (results indicated as +). $n = 10$; $^{****}p < 0.0001$, $^{+}p < 0.05$. The black arrows indicate eosinophils. B6M, Black-6 males; B6F, Black-6 females; CD1M, CD-1 males; CD1F, CD-1 females; B6 M+F, Black-6 mice; CD-1 M+F, CD-1 mice.

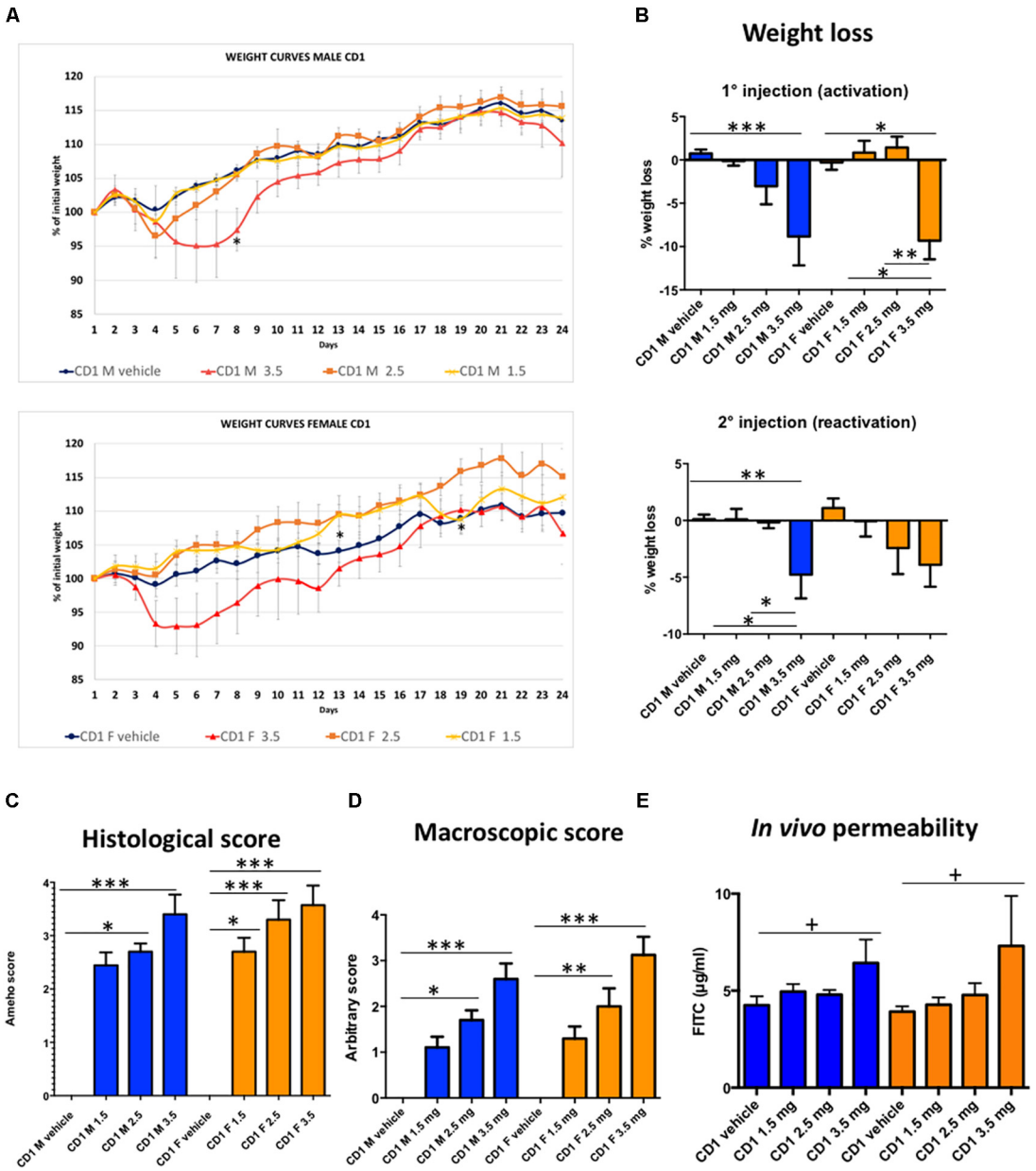

FIGURE 4 | Response of CD-1 mice to colitis induced by different doses of DNBS. Body mass trends **(A)**, loss of body mass after the first DNBS injection and the second DNBS injection **(B)**, macroscopic **(C)** and histological scores **(D)** and *in vivo* permeability **(E)**. For weight curves, a multiple unpaired *T*-test was performed per day with fewer assumptions corrected for multiple comparison with Holm–Sidak method, (*) indicates significance vs. vehicle group. $n = 10$; $p < 0.05$. No Gaussian data comparisons (loss of body mass and macro and histological scores) were performed using a non-parametric Kruskal–Wallis test followed by a Dunn's test (results indicated as *). For the permeability analyses, two-way ANOVA was performed with dose and sex as factors followed by a Tukey test (results indicated as +). CD-1 males (M, in blue) and CD-1 females (F, in orange). $n = 10$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{+}p < 0.05$.

The degree of improvement would be proportional to agent efficacy, but it would not necessarily reveal the mechanisms involved. An important caveat is that the underlying mechanism for DNBS-induced colitis is abnormal stimulation of the immune system. As a result, the model is not suitable for testing certain anti-inflammatory agents. For instance, probiotics, molecules or others that provide functional benefits unrelated to inflammation, such as excluding pathogens or modulating metabolic processes, could not be properly tested using this model.

Chronic Colitis Severity in CD-1 Mice Can Be Modulated Using DNBS Dosage

Once we had verified that CD-1 mice were good candidates for developing a murine model of chronic colitis, we performed a

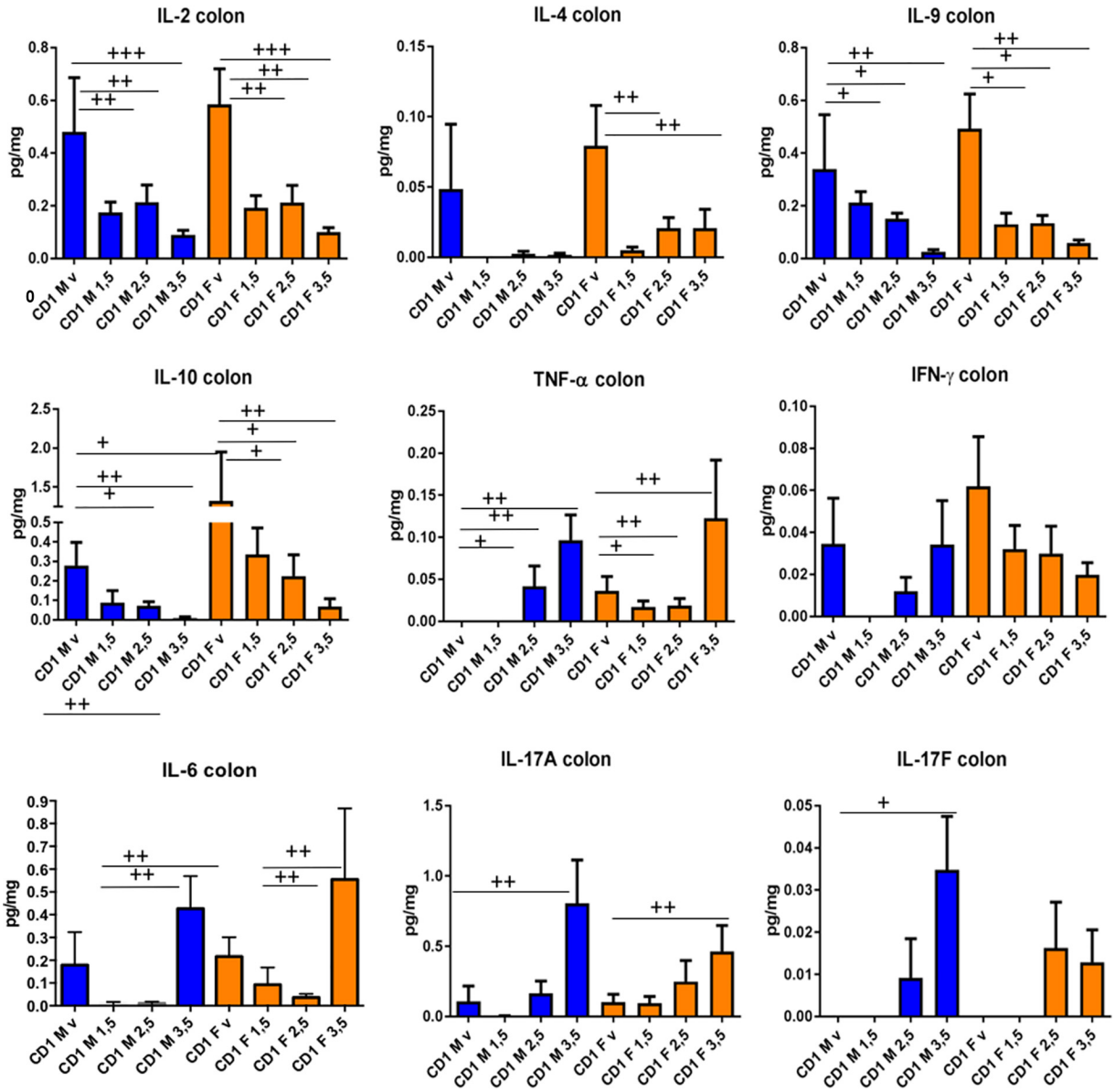

FIGURE 5 | Colon levels of cytokines induced by different doses of DNBS in CD-1 mice. Analyses were performed by two-way ANOVA with dose and sex as factors followed by a Tukey test. CD-1 males (M, in blue) and CD-1 females (F, in orange) injected with vehicle (v), 1.5 mg (1.5), 2.5 mg (2.5), or 3.5 mg (3.5) of DNBS. $n = 10$; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$.

second experiment in which we modulated DNBS dose to obtain different degrees of colitis severity. This experiment allowed us to better characterize the model and to gather data that, in future studies, will clarify the appropriate DNBS dosage depending on agent type, presumed agent efficacy, and target disease. We tested three different doses of DNBS—1.5, 2.5, and 3.5 mg per mouse. We selected these doses using the findings from comparative experiments with Black-6 mice in the first trial, where a dose of around 4 mg per mouse resulted in a high mortality rate. Furthermore, the doses were not calibrated for body mass because the results from the first experiment showed that smaller doses might not produce sufficient inflammation, and that there are probably sex-related differences in DNBS sensitivity.

In the second experiment, mortality rates were lower: only two female mice, given a dose of 3.5 mg, died. As expected, after both DNBS injections, a dose-dependent effect on body mass was observed (**Figures 4A,B**). It is worth noting that loss of body mass was similar in females and males given the same dose. Taken together, these results suggest that CD-1 females are more sensitive to severe and severe-to-moderate inflammation (50% mortality at 4 mg of DNBS and 20% mortality at 3.5 mg); however, this sensitivity was not manifest when inflammation was moderate or low. A similar dose-dependent effect was

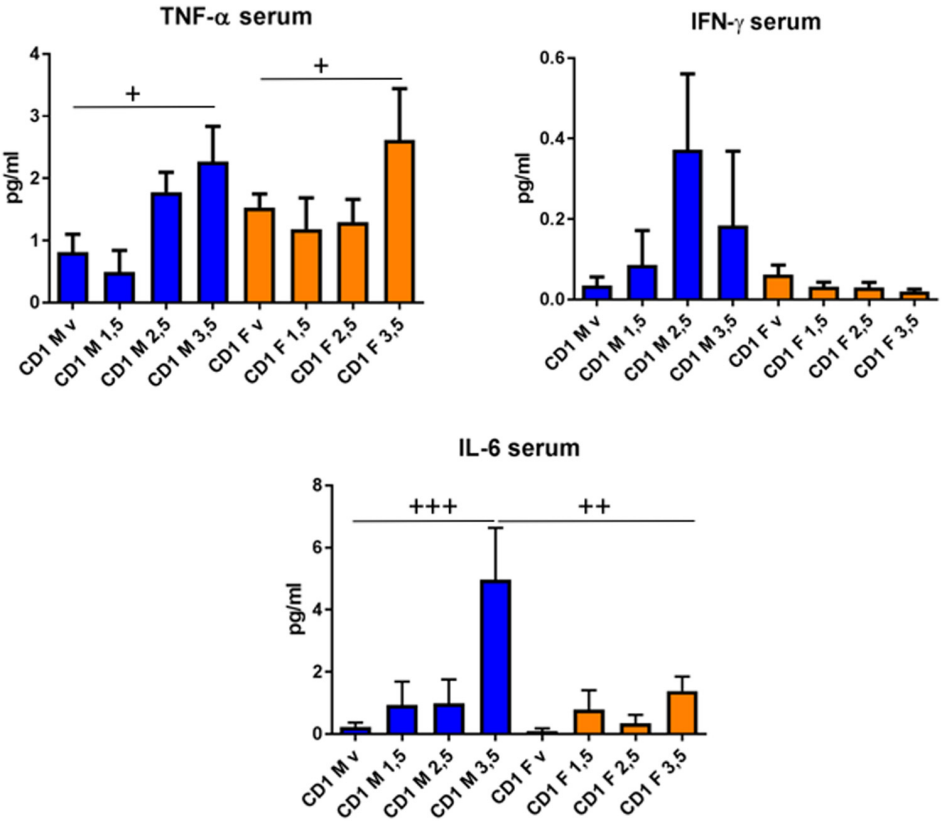

FIGURE 6 | Serum levels of cytokines induced by different doses of DNBS in CD-1 mice. Analyses were performed by two-way ANOVA with dose and sex as factors followed by a Tukey test. CD-1 males (M, in blue) and CD-1 females (F, in orange) injected with vehicle (v), 1.5 mg (1.5), 2.5 mg (2.5), or 3.5 mg (3.5) of DNBS. $n = 10$; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$.

seen in the macroscopic and histological scores (**Figures 4C,D**). Furthermore, the observed standard deviations were small, indicating that both sexes could be pooled in analyses.

DNBS-Induced Chronic Colitis in CD-1 Mice Modifies Intestinal Permeability, Colon Cytokine Levels, and Lymphocyte Populations in the Spleen and Mesenteric Lymph Nodes (MLN)

As mentioned above, this murine model can be useful in two ways. First, the model's general metrics such as loss of body mass, macroscopic scores, and histological scores could reveal the efficacy of potential treatments (e.g., anti-inflammatory compounds or probiotic strains). Second, the model could also help decipher the mechanisms underlying any positive effects. Because our model induces colitis using DNBS, it is best suited for examining immunomodulatory properties. For example, DNBS-provoked inflammation in Black-6 mice appears to arise from such mechanisms as altered gut barrier permeability and the activation of specific immune responses (Martin et al., 2014a). Consequently, this model could be used by researchers to study the specific effects of candidate anti-inflammatory agents on gut permeability and the immune system. Nevertheless, it is not possible to describe the expected results to be obtained when testing an anti-inflammatory agent as they will depend on their mechanisms of action.

Such permeability alterations are also present in CD-1 mice with DNBS-induced colitis (**Figure 4E**). Dysfunction of the epithelial barrier is a hallmark of inflammatory intestinal diseases. GIT permeability can be characterized by orally administering the paracellular tracer FITC-dextran. This technique reveals the degree of colon permeability and has been successfully linked to directly measure alterations in local permeability in colon tissues employing Ussing chambers (Martin et al., 2015). In this study, GIT permeability was modified in CD-1 mice challenged with different doses of DNBS (trial 2). Two-way ANOVA analysis showed that there is a dose effect ($p = 0.0270$), although no sex effect or interaction between both factors have been found ($p = 0.9628$ and $p = 0.8642$, respectively). Even if there was a clear response in males and females at the highest DNBS concentration tested ($p < 0.005$), basal permeability appears to be high, necessitating a strong dose of DNBS to obtain results (**Figure 4E**). These findings suggest that it may be problematic to use CD-1 mice to characterize permeability using moderate or low-grade inflammation models. However, we must interpret the results with caution since there is the possibility that permeability might be altered at other points along the GIT.

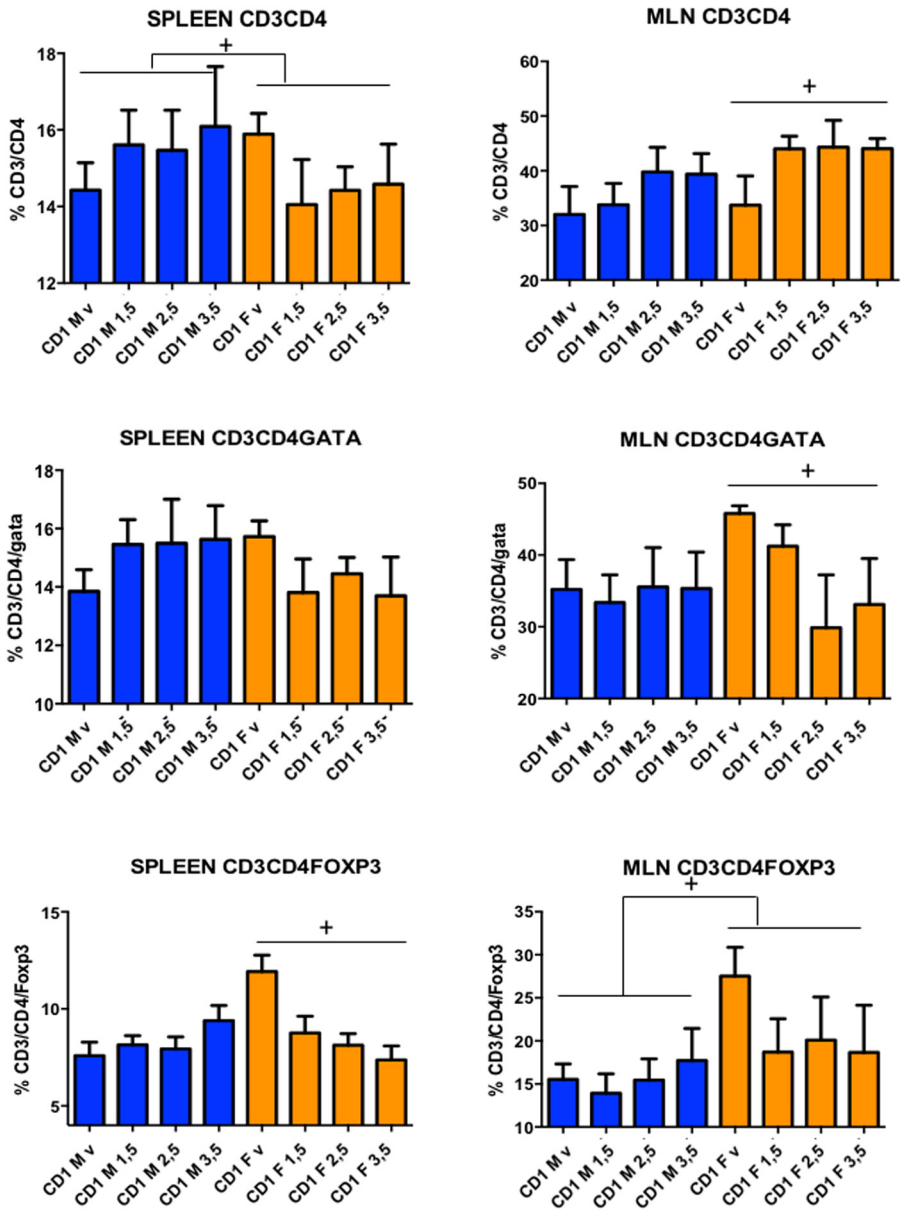

FIGURE 7 | Levels of lymphocytes induced by different doses of DNBS in CD-1 mice. Lymphocyte populations were characterized using flow cytometry. Analyses were performed by two-way ANOVA with dose and sex as factors followed by a Tukey test. CD-1 males (M, in blue) and CD-1 females (F, in orange) injected with vehicle (v), 1.5 mg (1.5), 2.5 mg (2.5), or 3.5 mg (3.5) of DNBS. $n = 10$; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$.

It is apparent that DNBS-induced chronic colitis changes levels of several cytokines in CD-1 mice (**Figures 5**, **6**), confirming the generally dose-dependent nature of the inflammation response. We observed IL-9, IL-10, IL-17A, TNF-α, IL-2, IL-17F, IL-6, and IL-4 changes in the colon samples (**Figure 5**) and TNF-α and IL-6 in the serum samples (**Figure 6**). Two-way ANOVA analyses revealed dose-dependent responses for IL-2, IL-9, IL-10, TNF-α IL-6, IL-17A, and IL-17-B and sex influence on IL-4, IL-10, and serum IL-6 ($p < 0.005$). IL-10 is an important immunoregulatory cytokine—it reduces inflammation by suppressing the exaggerated mucosal immune response in the colon (Schreiber et al., 2000) and thus preserves the mucus barrier (Hasnain et al., 2013). It is a cytokine of reference in almost all murine colitis models. Similarly, we observed a decline in IL-9, IL-2, and IL-4. IL-9 controls intestinal barrier function (Gerlach et al., 2015); IL-2 is a potent inducer of T-cell proliferation and drives T-helper 1 (Th1) and Th2 effector T-cell differentiation (Hoyer et al., 2008); and IL-4 has anti-inflammatory properties. Indeed, IL-4 has the ability to stimulate alternative macrophages (M2s). In contrast to classical macrophages (M1s), M2s participate in a T helper type 1 (Th1)-polarized response and enhance the production of

pro-inflammatory cytokines, which counteracts inflammation and promotes tissue repair (Goerdt et al., 1999; Gordon, 2003; Mantovani et al., 2007). In this sense, neutrophil activation, measured by myeloperoxidase (MPO) activity, reveal a week activation of neutrophils as MPO activity was similar for CD-1 mice that those in Black-6 mice treated with low doses of DNBS [(Martin et al., 2015) and data not shown]. As neutrophils are involved in inflammation, macrophage recruitment and M2s differentiation this result point also for a slight Th1 response in CD-1 mice.

On the other hand, we saw an increase in IL-17A, IL-17F, IL-6, and TNF-α, underscoring that pro-inflammatory responses were occurring as well (Gabay, 2006; Bradley, 2008; Jin and Dong, 2013). The results for IFN-γ highlight that mouse strain matters: IFN-γ is a pro-inflammatory cytokine that plays a central role in DNBS-induced inflammation in Black-6 mice (Martin et al., 2014a), but that seemed to have the opposite effect in CD-1 mice (**Figures 5**, **6**). Consequently, it appears that DNBS and TNBS can elicit a Th1-mediated immune response (Randhawa et al., 2014) but that model functionality may vary depending on the host species and its genetic background (Mizoguchi and Mizoguchi, 2008; Mizoguchi, 2012). For instance, when treated with these compounds, SJL/J mice displayed a major Th1-mediated response (Neurath et al., 1995, 1996), while IFN-$\gamma^{-/-}$ mice with a Balb/c background showed a Th2-mediated response (Dohi et al., 1999). In our study, to identify the major Th cell lines involved in the response of the CD-1 mice, T-cells from the spleen and MLNs were isolated and analyzed using flow cytometry. Several differences were found between male and female mice (**Figure 7**). CD-1 males had a weak response—there was a slight increase in CD3/CD4 cells in both the spleen and MLNs. CD-1 females had a different, stronger response—CD3/CD4 cells decreased in the spleen and increased in the MLNs (**Figure 7**). Furthermore, CD-1 females had diminished Th2 and Treg levels in both the spleen and MLNs (revealed by GATA-3 and Fox-p3 staining, respectively). In contrast, males displayed slightly increased Th2 levels in the spleen alone (**Figure 7**). These results, taken in tandem with the high levels of eosinophils (**Figure 3**) suggest that the Th2 response played a major role in CD-1 mice. The Th1 response, measured using T-bet staining, was not strong enough to be detected (data not shown). These findings indicate that, in future studies, it may be better to use females when testing for immunomodulation by candidate probiotics *in vivo*, especially if *in vitro* trials indicate that the mechanism of action involves changes in IL-10 production; IL-10 is produced by Treg cells, among others. However, it is necessary to broadly examine cytokine production and lymphocyte levels to fully clarify the mechanisms of action of any anti-inflammatory agent.

Overall, our findings allow us to recommend this model to test anti-inflammatory agents, including probiotics. We strongly recommend to perform the experiment at least in duplicate with a minimum of 10 mice per group. Nevertheless, this is a minimum, as the efficacy of the agent tested will determine the number of mice required to have statistical significant results. The use of 3.5 mg seems the better choice, however, as the dose-effect observed is usually animal facility-dependent, a preliminary study to optimize the dose is mandatory.

FINAL REMARKS

Here, we describe a murine model of chronic colitis in which inflammation was induced by the intrarectal administration of DNBS; it is novel because it used an outbred murine strain, CD-1, and employed both female and male mice. Ultimately, we want to make this model available to researchers who are testing the efficacy of anti-inflammatory agents, including probiotics (mainly NGPs), with immunomodulatory properties. The model could also serve to identify the anti-inflammatory agent potential mechanisms of action. Indeed, our aim is to provide the scientific community with a realistic alternative model for testing the efficacy of anti-inflammatory agents, for instance candidate probiotics—a model that can be customized based on agent type and target disease. We showed that it is possible to use an outbred murine strain without having any problems of reproducibility and that females and males can be pooled. Taken together, they yielded more representative results for some characteristics. However, combining results for the two sexes should be done with caution because we observed evidence of sex-specific sensitivity to severe inflammation protocols and sex-specific differences in some of the characteristics measured.

AUTHOR CONTRIBUTIONS

MB, LB-H, PB, PL, and RM designed the project. MB, FC, and RM designed and performed the experiments. MB, HS, and RM analyzed the results. MB and RM drafted the manuscript. MB, FC, HS, LB-H, PB, PL, and RM reviewed and edited the manuscript. All the authors read and approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00565/full#supplementary-material

FIGURE S1 | Our experimental design **(A)** in contrast to other acute models **(B,C)**. TNBS/DNBS **(B)** and DSS **(C)** classical protocols. In contrast to classical

models of acute colitis with or without recovery, in our experimental design we have performed a chronic model in which a reactivation phase is included after recovery for better mimicking colitis flares and relapses.

FIGURE S2 | Macroscopic score description. Representative figure of some of the symptoms: adhesion **(A)**, altered transit **(B)**, hyperemia **(C)**, and ulcers and thickening of colon wall **(D,E)**.

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