ENGINEERING MICROBES FOR THERAPY

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ENGINEERING MICROBES FOR THERAPY

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Microbes can play protective role in human health, and the concepts of probiotics and microbiota have been well established in recent years. Probiotics have an important economic impact in food, food supplement and veterinary industry with increasing market size. Engineering microbes for therapy can lead to selection of new microbial strains and mixtures, or targeted improvement of existing microbial strains, achieved by mutagenesis, genetic engineering and synthetic biology. Engineering of microbes can also encompass the development and improvement of their dosage forms. Possible uses of engineered microbes include antigen delivery, immunomodulation, inflammation, cancer, infectious diseases and metabolic disorders. The eBook represents an up-to-date overview, shows new results, as well as demonstrates future trends in the developing field of therapeutic microbial engineering.

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EDITORIAL
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Editorial: Engineering Microbes for Therapy

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Editorial on the Research Topic

Engineering Microbes for Therapy

The notion that microbes are not just vicious pathogens but can play protective role in human health has been well-established in recent years. The concept of probiotics, "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host," has been introduced, focusing mainly on species of lactic acid bacteria already present in food products. The beneficial activity of selected probiotics has been well-documented and supported in clinical trials. Probiotics have an important economic impact in food, food supplement, and veterinary industry with increasing market size.

The introduction of next generation sequencing techniques has boosted the research of human microbiota and established the importance of its composition. The perturbation of composition of microbiota can lead to imbalance and dysbiosis—a disease characterized by the changes in microbiota. The latter has been demonstrated in inflammatory bowel disease, irritable bowel syndrome, allergy, obesity, colon cancer, and even autism. The ability to restore the balance with microbial intervention was also demonstrated. The stunning example is fecal transplant for the treatment of recurrent *Clostridium difficile* infection.

Engineering microbes for therapy can lead to selection of new microbial strains and mixtures, or targeted improvement of existing microbial strains, achieved by mutagenesis, genetic engineering, and synthetic biology. The new species will exceed the existent probiotic definition for food applications, but may well be adopted by the pharmaceutical industry as "pharmabiotics" or "live biotherapeutic products."

Delivery of antigens for the purpose of vaccination is maybe among the most frequently explored ways of using genetically engineered microbes, although not many examples have actually reached the human clinical stage. Prosperi de Castro et al. have reviewed the use of lactic acid bacterium *Lactococcus lactis* as a mucosal vaccine delivery vehicle. Special emphasis was put on the delivery of DNA vaccines that enable *in situ* translation of the antigen. Novel, advanced and food-grade vectors have been developed for that purpose. Apart from *L. lactis,* Ramos-Vega et al. have reviewed the use of another, much less established microbial host—microalgae from the genus *Schizochytrium.* These were shown as favorable hosts for influenza subunit vaccine production, and were described as safe for human consumption. Apart from delivery of antigens, the microbes can also be engineered to deliver allergens with the final goal of desensitizing allergic individuals, essentially enabling immunotherapy of allergic disease. This field has been thoroughly described by Zahirović and Lunder.

Other possible uses of engineered microbes include immunomodulation, inflammation, cancer, infectious diseases, and metabolic disorders. Some recent examples from this field have been reviewed by Bron and Kleerebezem. Jacouton et al. have developed *L. lactis* secreting IL-17A

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that prevented the formation of tumors in a mouse model. Mauras et al. have developed new plasmid vector for *Bifidobacterium bifidum* and used it to express Interleukin-10. These recombinant bacteria were effective in decreasing intestinal inflammation in mice. Therapeutic proteins, delivered by engineered microbes, can be either secreted or displayed on the surface. Some species from the genus *Lactobacillus* contain surface (S) layers that are made of crystalline arrays of repeating subunits of S layer proteins. Klotz and Barrangou reviewed the possibilities to apply these structures for biotherapeutic applications. Apart from engineering microbes to deliver therapeutic proteins, they can also be used to activate prodrugs at a defined site of action, by delivering appropriate enzymes, thereby minimizing side effects. Aučynaitė et al. report the discovery of deaminases that convert 5-Fluoroisocytosine into 5-Fluorouracil.

Engineering of microbes can also encompass the development and improvement of their dosage forms. Cordeiro et al. have improved the viability of *Lactobacillus casei* BL23 and *Propionibacterium freudenreichii* 138 in fermented skim milk by the addition of whey protein isolate. The latter has increased the protective activity of the beverage against mucositis in mice. Another mode of protection of bacteria, namely incorporation of *L. lactis* in sodium alginate microcapsules, has been reported by Coelho-Rocha et al. Considerably higher expression of the model protein in mice gastrointestinal tract has been achieved.

The studies of engineering of microbes do not focus only on expressing heterologous proteins, but also on the role of intrinsic bacterial proteins. *Propionibacterium freudenreichii* is a beneficial Gram-positive bacterium, currently considered as an emerging probiotic with promising immunomodulatory properties. do Carmo et al. have shown that its S layer protein SlpB has a crucial pleiotropic role in mediating beneficial activity. Inactivating SlpB led to dramatic change of bacterial surface properties, as well as on the entire bacterial proteome. Aucouturier et al. have shown the importance of prophages in the genome of *Lactococcus lactis* ssp. *lactis* IL1403. Their removal contributed significantly to the changes in the lactococcal physiology, and may play a role in the adaptation of bacteria to the changes in their environment.

The use of engineered microbes is not limited to human medicine only, but also includes veterinary applications. Hu et al. reported a standardized preparation for fecal transplantation of microbiota in pigs. Such protocols are crucial for establishing stool banks of appropriate safety, and thereby promoting this approach as an alternative to antibiotics for intestinal health of animals.

The compendium of original and review articles that were assembled in the Research Topic Engineering microbes for therapy represents an up to date overview, shows new results, as well as demonstrates future trends. These and other studies suggest that the therapeutic use of microbes has exciting future. However, the crucial step from successful proof-of-principle reports in animal models to well-executed human clinical trials is, at the moment, still lacking in most of the cases and represents a challenge for the future.

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REVIEW
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Vector Development Timeline for Mucosal Vaccination and Treatment of Disease Using *Lactococcus lactis* and Design Approaches of Next Generation Food Grade Plasmids

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Lactococcus lactis has been used historically in fermentation and food preservation processes as it is considered safe for human consumption (GRAS—*Generally Recognized As Safe*). Nowadays, in addition to its wide use in the food industry, *L. lactis* has been used as a bioreactor for the production of molecules of medical interest, as well as vectors for DNA delivery. These applications are possible due to the development of promising genetic tools over the past few decades, such as gene expression, protein targeting systems, and vaccine plasmids. Thus, this review presents some of these genetic tools and their evolution, which allow us to envision new biotechnological and therapeutic uses of *L. lactis*. Constitutive and inductive expression systems will be discussed, many of which have been used successfully for heterologous production of different proteins, tested on animal models. In addition, advances in the construction of new plasmids to be used as potential DNA vaccines, delivered by this microorganism, will also be viewed. Finally, we will focus on the scene of gene expression systems known as "food-grade systems" based on inducing compounds and safe selection markers, which eliminate the need for the use of compounds harmful to humans or animal health and potential future prospects for their applications.

Keywords: lactic acid bacteria, recombinant *L. lactis*, expression systems, DNA vaccine, food grade vectors

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Lactococcus lactis: THE MODEL LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) constitute a diverse group of Gram-positive microorganisms, including species of genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus Leuconostoc*, and *Oenococcus*, which, among other shared characteristics, have the capacity to convert sugars into lactic acid (Makarova and Koonin, 2007). Because they have long been used in fermentation and food preservation processes, most of these bacteria are considered safe for human consumption, possessing the GRAS (*Generally Recognized As Safe*) status (van de Guchte et al., 2006). Among the representatives of this group, *L. lactis* is the best characterized species and figures as the model organism for its study. This, in turn, is due not only to its evident economic importance, but also to the fact that this bacterium is a microorganism of easy manipulation and has its genome sequenced. A great number of genetic tools have been developed. Thus, *L. lactis* has

also been widely used in the field of biotechnology for the production and delivery of antigens and cytokines to mucosal surfaces (Wells, 2011), and more recently as a vehicle for the delivery of DNA vaccines (Pereira et al., 2014, 2017; Pontes et al., 2014; Zurita-Turk et al., 2014; Souza et al., 2016; Mancha-Agresti et al., 2017).

This microorganism has several characteristics that make it an interesting vector for the production and delivery of biomolecules, especially for oral route, such as resisting stomach acidic environment and being able to survive in the gastrointestinal tract. Another attractive property of *L. lactis* is that it lacks lipopolysaccharides in its cell wall, which eliminates the risk of endotoxin shock. Finally, this bacterium is poorly immunogenic, and for this reason, it can be continuously used in immunization programs (Mercenier et al., 2000). Thus, *L. lactis* offers great versatility, and several vectors to be used in this microorganism have already been constructed, allowing a wide range of biotechnological applications, besides its use in the food industry.

Lactococcus lactis AS A CELL FACTORY—PROTEINS OF BIOTECHNOLOGICAL INTEREST

The use of *L. lactis* as a cell factory for high-level protein production was developed using constitutive and inducible promoters. The history regarding the use of vectors for cloning exogenous genes in the LAB started with two cryptic broad host-range plasmids: pWV01 (Kok et al., 1984) and pSH71 (de Vos et al., 1997). The replicons of these plasmids have been the base for creating useful cloning vectors being able to replicate in a broad range of genera, such as *L. lactis* and also in *Escherichia coli* (recA+strain) (De Vos and Simons, 1994). In general, these small plasmids undergo a rolling-circle replication with chloramphenicol resistance. Another family of vectors derived from pAMbeta1 (from *Enterococcus faecalis*), which can only replicate in Gram-positive host strains, was constructed, pIL252 and pIL253, showing low and high copy plasmids, respectively, as having erythromycin resistance (Simon and Chopin, 1988; Kleerebezem et al., 1997b). These large plasmids replicate via "theta" replication and are equipped with *E. coli* replicons, generating pOri23 (Que et al., 2000), allowing efficient shuttling between Gram-positive and Gram-negative bacteria (O'Sullivan and Klaenhammer, 1993).

Afterwards, several vectors containing constitutive or inducible promoters were developed, and they represent the basis of all expression systems in *L. lactis* and other LAB (Pontes et al., 2011). Although the high production of heterologous proteins in *L. lactis* has been achieved using constitutive promoters (De Vos, 1999), this continuous production of high-level protein could generate intracellular accumulation, which could lead to degradation in the cytoplasm and sometimes could be toxic to the cell. This drawback, together with the fact that inducible promoters offer better handling, makes them the vectors of choice. In 1995, the most promising and frequently used system based on genes involved in biosynthesis and regulation of nisin, an antimicrobial peptide whose biosynthesis is encoded by a cluster of 11 genes, was developed (Kuipers et al., 1995). The NICE system (nisin-controlled expression), as it is called, comprises NIS operon regulatory elements, NisR and NisK code, for the regulator two-component system. The histidine-protein kinase NisK located in the cytoplasmic membrane binds the nisin molecule and activates NisR via phosphorylation, which in turn induces transcription of two promoters in the nisin gene cluster: PnisA and PnisF. In fact, this system has been demonstrated to have high protein production and is also easy to use as it has been extensively used in the industry. However, nisin has a high cost limiting its use. Due to nisin, researchers developed another system that is inducible by xylose sugar (Miyoshi et al., 2004). The xylose-inducible expression system (XIES) has the PxylT promoter, and in the presence of other sugars such as glucose, fructose, and/or mannose, it is tightly repressed. Thus, in the presence of xylose, PxylT is transcriptionally activated (Lokman et al., 1994). Another important characteristic of this system is its capacity to produce cytoplasmic or secreted proteins, and it can be easily switched on or off by adding either xylose or glucose, respectively (Miyoshi et al., 2004). As a sugar is used to operate this system, it is considered cheap and very useful for implementation in biotechnological processes. The secretion of heterologous proteins has advantages over intracellular-expressed ones, in the way in which only a simple purification step is necessary, higher yields are reached, and better target interactions are achieved (Le Loir et al., 2005).

Two other inducible systems for *L. lactis* were described recently. One is the zinc-inducible expression system, called ZIREX. This system allows induction of genes regulated by zinc ions and the pneumococcal repressor SczA (Mu et al., 2013). The other inducible system for *L. lactis* is stress-inducible controlled expression system (SICE). This system uses a promoter of the *L. lactis* groESL operon, whose expression is induced under stress conditions, which does not need exogenous induction, nor the presence of regulatory genes (Benbouziane et al., 2013). Characteristics of all these vectors are summarized in **Table 1**. Many of these vector applications have been described with satisfactory results. A recent report showed the gastrointestinal passage time of bacteria and their spatial localization in the gut. To this end, the authors used a plasmid from the NICE system (pNZ8148) in which IRFP reporters (pNZIRFP713 and pNZ-IRFP682) were cloned, resulting in near-infrared fluorescent proteins being expressed in the LAB (Berlec et al., 2015).

Concerning the XIES system, some therapeutic molecules such as 15-lipoxygenase were cloned in it. *L. lactis* (pXIES:CYT:*15lox-1*)-fermented milk was effective in the prevention of intestinal damage associated with inflammatory bowel disease (IBD) in a trinitrobenzenesulfonic acid-induced IBD mouse model (Saraiva et al., 2015) and, in addition, a decrease in IFN-γ and IL-4 was also observed. Also, an increase in IL-10 was observed in a dextran sodium sulfate-induced (DSS-induced) IBD mouse model, where mice were orally administrated with the culture of this strain (Carvalho et al., 2016). *L. lactis* (pXIES:*hsp65*) expressing the 65-kDa heat

TABLE 1 | Characteristics of constructed *L. lactis* vectors for heterologous protein production.

Plasmid/ System	Characteristics	Cloned genes	Reference
pWV01	Lactococcal cryptic plasmid, rolling circle replication, (RCR) type, Cm^r, 2.1 kb size, P23 promoter.	–	Kok et al., 1984
pSH71	Rolling circle replication (RCR) type, Cm^r.	–	de Vos et al., 1997
pIL252	Low-copy-number, broad-host- range cloning vector, theta-replication, Ery^r, 4.7 kb size, P23/59 promoters.	–	Simon and Chopin, 1988
pIL253	High-copy-number, broad-host-range cloning vector, theta-replication, Ery^r, 5.2 kb size P23/P32 promoters.	–	Kleerebezem et al., 1997a
pORI23	High-copy-number, broad-host-range cloning vector, theta-replication, *oriCol*E1 origin, Ery^r, 5.2 kb size P23 promoter	*Luciferase*	Que et al., 2000
NICE system	Nisin controlled gene expression system, Cm^r, PnisA and PnisF. promoters. Secreted heterologous protein *L. lactis* NZ900 is the only host strain (NisK, nisR in genome). Nisin induced.		Kuipers et al., 1995
		GroEL	Miyoshi et al., 2006
		Catalase	de Moreno de LeBlanc et al., 2008
		HPV-16 E7/IL-12	Bermudez-Humaran et al., 2005
		Human -Leptin	Bermúdez-Humarán et al., 2007
XIES system	Xylose inducible expression system. Cm^r, pXylT promoter. Two versions of heterologous proteins, either cytoplasmic or secreted. Xylose induced.		Miyoshi et al., 2004
		Mirabilis MrpA fimbrial protein	Scavone et al., 2007
		IL10	Marinho et al., 2010
		15-Lox-1	Saraiva et al., 2015; Carvalho et al., 2016
		hsp65	de Azevedo et al., 2012
ZIREX system	Zinc-inducible expression system. Cm^r pczcD promoter with SczA gene (for its regulatory protein).	*gfp*	Mu et al., 2013
SICE System	Stress Inducible Controlled Expression system. Cm^r, groESL promoter, Stress conditions induced.	*IL10* *HPV-16E7*	Benbouziane et al., 2013

Em^r -erythromycin resistance gene, Cm^r - Chloramphenicol resistance gene, hsp65: heat-shock protein coding sequence of Mycobacterium leprae, GroEL: heat-shock protein coding sequence of B. abortus, 15-Lox-1: human 15 lypoxygenase coding sequence, HPV-16/E7: human papillomavirus type 16 cell wall-anchored E7 Antigen.

shock protein from *Mycobacterium leprae* (de Azevedo et al., 2012) was able to prevent the development of experimental autoimmune encephalomyelitis in C57BL/6 mice which received this bacterium by oral administration. The reduction of IL-17 and the increase of IL-10 in mesenteric lymph node and spleen cell cultures were observed (Rezende et al., 2013). The same recombinant strain was tested in the DSS-induced colitis mouse model. In colonic tissue levels, proinflammatory cytokines (IFN-γ, IL-6, and TNF-α) were reduced by increasing IL-10 production. An expansion of regulatory T cells ($CD4^+Foxp3^+$ and $CD4^+LAP^+$) was also observed in the spleen, as well as in mesenteric lymph nodes. Thus, these data indicate that oral pretreatment with genetically modified *L. lactis* Hsp65 protein production is able to prevent DSS-induced colitis in C57BL/6 mice (Gomes-Santos et al., 2017).

DNA VACCINE VECTORS

Since the early 1990s, several studies have been conducted to test the efficiency of DNA vaccines in the prevention or treatment of different diseases (Huang and Gorman, 1990; Galvin et al., 2000). Modern vaccinology employs plasmids obtained from bacteria that encode antigen polypeptide sequences. These constructions are made using classical molecular biology techniques and more recently molecular techniques such as synthetic biology. There are many advantages of using bacterial plasmids as a vaccine strategy, and some of them are related to their stability at room temperature and reduction of production costs once the purification of the protein in interest is not required, which increases safety in administration (Suschak et al., 2017). Since these vaccine techniques began to be developed, many studies have been conducted aiming at empowering the delivery of these plasmids to host cells, and among these different strategies, we can cite, for example, the different routes of administration and use of adjuvants. However, much emphasis has been placed on the design of vectors that will be used as a vaccine platform (Sørensen et al., 2005; Oliveira and Mairhofer, 2013; Williams, 2014; Suschak et al., 2017).

Currently, there are very few groups worldwide working on the development of vectors for DNA vaccines using *L. lactis*

as delivery vehicles, and in recent years, only a few papers have been published in the scientific literature (Guimarães et al., 2009; Tao et al., 2011; Mancha-Agresti et al., 2016; Yagnik et al., 2016, 2017). Vectors generally exhibit some common features such as the presence of a strong eukaryotic promoter, such as cytomegalovirus, multiple cloning sites, a polyadenylation signal in addition to a prokaryotic region that includes a selection marker, usually an antibiotic resistance gene, as well as a prokaryotic origin of replication (Kutzler and Weiner, 2008). In 2009, our research group published a new vector for DNA delivery, pValac (vaccination using lactic acid bacteria), using lactococci. This vector exhibits the characteristics cited above including bovine growth hormone (BGH polyA) polyadenylation sequence, prokaryotic region that allows its replication in both *E. coli* and *L. lactis* strains, and chloramphenicol resistance gene as a selection marker, in addition to presenting a rolling circle origin of replication, which contributes to the relatively small size of the plasmid (3742 bp). *L. lactis* carrying pValac was able to efficiently deliver the vector to eukaryotic cells, allowing expression of green fluorescent protein (GFP) that could be observed by fluorescence microscopy and flow cytometry (Guimarães et al., 2009). Tao et al. (2011) published a paper describing the construction of the pLKV1 vector (4400 bp), which is quite similar to pValac. However, its selection marker is the kanamycin resistance gene, which is seen as an advantage due to the Food and Drug Administration recommending its use in DNA vaccines. The DsRed2 gene, which encodes a red fluorescent protein, was cloned in it. The functionality of this plasmid was verified by visualizing the expression of this protein in epifluorescent microscopy 48 h after the transfection of Caco-2 cells (Tao et al., 2011). However, there are no reports in the literature about *in vivo* studies using this plasmid. More recently, Yagnik et al. (2016) published the construction of the pPERDBY vector (4800 bp), which, in addition to sharing features common to other vectors already mentioned above, shows the polyadenylation signal of SV40 and the enhanced green fluorescent protein, eGFP, linked to the multiple cloning sites. This feature was designed to facilitate other protein expression, since the eGFP is expressed fused to the protein of interest where the presence of immunostimulatory CpG motifs, which could act as adjuvants, has been described in it. In the Chinese hamster ovary cells (CHO) cell transfection assay, it was possible to observe eGFP expression, demonstrating the functionality of the vector. However, in the flow cytometry analysis, the percentage of cells expressing the reporter protein was extremely low, showing only 0.53% of positive cells (Yagnik et al., 2016). In 2016, our research group published a study relating to the construction of the pExu vector (extra chromosomal unit) with 6854 bp, which in comparison with the described vectors presents the "theta" type origin of replication, which gives it both greater structural and segregational stability. Eukaryotic cells were transfected with pExu:*egfp* plasmid and analyzed by confocal microscopy and flow cytometry 48 h after transfection. The flow cytometric analysis showed that 18.7% cells were positive for eGFP expression. In addition, *in vivo* expression kinetics were performed on mice that had received the *L. lactis* (pExu:*egfp*) strain by oral administration. The results demonstrated that intestinal cells, specifically in the duodenal region of the bowel, were able to produce the protein of interest from 12 h to 72 h after treatment (Mancha-Agresti et al., 2016) in high amounts. These interesting results would be beneficial from a therapeutic standpoint because it reduces the need for multiple doses in a short period of time. **Table 2** summarizes some characteristics of major plasmids constructed for DNA vaccine.

TABLE 2 | Main characteristics of vectors constructed for DNA vaccine uses.

Plasmid	Characteristics	Cloned genes	Reference
pValac	Eukaryotic expression vector (pCMV/RCR/RepA/RepC/Cm^r/BGH polyA). Size: 3742 pb	*gfp* *Ag85A* *ESAT-6* *IL-10* *IL-4*	Guimarães et al., 2009 Mancha-Agresti et al., 2017 Pereira et al., 2014 Zurita-Turk et al., 2014 Souza et al., 2016
pLKV1	Eukaryotic expression vector, (pCMV/RCR/RepA/RepC/Km^r/BGH polyA). Size: 4400 pb	*DsRed2* *GFPmut3*	Tao et al., 2011
pPERDBY	Eukaryotic expression vector (pCMV/RCR/RepA/RepC/Cm^r/BGH SV40 polyA/ *eGFP*). Size: 4800 pb	*egfp*	Yagnik et al., 2016
pExu	Eukaryotic expression vector (pCMV/TER/RepD/RepE/Ery^r/BGH polyA). Size: 6854 pb	*egfp* *mCherry* *hsp65*	Mancha-Agresti et al., 2016 (Personal communication Dr. Azevedo) (Personal communication Dr. Azevedo)

pCMV cytomegalovirus promoter; RepA, RepC, RepD, and RepE replication origins; Cm^r-chloramphenicol resistance gene; Ery^r-erythromycin resistance gene; Km^r-kanamycin resistance gene, egfp enhanced green fluorescent protein coding sequence; RCR: a rolling circle origin of replication, TER: a theta origin of replication. Ag85A coding sequence of Mycobacterium tuberculosis, ESAT-6: early secreted antigenic target coding sequence of Mycobacterium tuberculosis, DSRed2: red fluorescent protein coding sequence, GFPmut3: mutant green fluorescent protein coding sequence, egfp; enhanced green fluorescent protein coding sequence, mCherry: red fluorescent protein coding sequence, hsp65: heat-shock protein coding sequence of Mycobacterium leprae.

FOOD-GRADE VECTORS FOR *Lactococcus lactis*

The use of *L. lactis* for the production of heterologous proteins, as well as for the delivery of DNA vaccines, is currently a promising reality. However, for further clinical trials, the assessment and minimization of risks to human health will be necessary. One of the major concerns related to the use of recombinant bacteria in clinical trials is related to the safety of their use by the host. Historically, cloning and expression vectors are designed using antibiotic resistance genes as marker selection; therefore, this feature may make it unfeasible to use extra-laboratories of *L. lactis* in the food and pharmaceutical industry.

Many questions are raised, not only about this specific issue, but also about the long-term adverse effects, as well as questions about possible integration into genome, dissemination and toxicity of these plasmids during human trials. To minimize these concerns, the development of new expression systems composed of safer elements, called food-grade systems, that allow selection, induction, and maintenance in the host is necessary to overcome the use of conventional gene expression systems and preserve the GRAS status of this bacterium (Cotter et al., 2003; Landete, 2017). Thus, some tools can be used, such as optimization of vector design, allowing the construction of food grade plasmids, without antibiotic resistance markers, for example, which could increase the safety in use. In addition, bioinformatics tools can aid in this process, allowing the construction of more robust vectors without, however, compromising the safety in the use to treat diseases in humans (Cho et al., 2018).

Among the main mechanisms of resistance and induction used for the development of food-grade vectors such as auxotrophic complementation, chromosome integration, use of sugars, resistance to heavy metals, and more recently, the use of CRISPR/Cas technology can be mentioned (Berlec and Strukelj, 2009; Landete, 2017; Berlec et al., 2018). As an example of auxotrophic complementation, MacCormick and coworkers developed a food-grade system based on lactose metabolism of the *L. lactis* MG5267 strain. This strain, which has *lac* operon in its chromosome, was subjected to deletion of the *lacF* gene, and when this gene was expressed in a plasmid, its ability to metabolize lactose was restored, presenting potential for industrial use (MacCormick et al., 1995). Another selection system based on threonine complementation was described. For this reason, researchers constructed the pJAG5 vector containing the gene that encodes homoserine dehydrogenase-homoserine kinase protein as a selective marker, which catalyzes two steps of the conversion of the aspartate into threonine. This vector, when used in an *L. lactis* MG1363 strain that presents deletions in two genes encoding threonine biosynthetic enzymes, proved to be quite stable and was able to express green fluorescent protein in eukaryotic cells (Glenting et al., 2002). In another study, a purine auxotrophic *L. lactis* DN209 strain (Dickely et al., 1995) was transformed with replicative food-grade plasmid pFG1 coding for genes producing peptidases from *Lactobacillus helveticus*. This strain was able to reduce the ripening period in cheese manufacturing, as well as producing special varieties of cheese (Joutsjoki et al., 2002). Besides auxotrophic complementation, resistance to heavy metals can also be explored with the aim of developing food-grade expression systems for *L. lactis* (Landete, 2017). The pND302 vector identified in *L. lactis* M71 strain, for example, offers resistance to cadmium (Liu et al., 1996). Even though it seems paradoxical, the use of these markers is safe, because this vector uses a theta origin of replication, thus being stable even in the absence of selective pressure (Emond et al., 2001).

However, it is important to note that some food-grade vectors present structural instability, and to circumvent this problem, some systems based on chromosomal integration have been developed (Wegmann et al., 1999; Gosalbes et al., 2000; Sheng et al., 2015). Perhaps the most promising example of this technique is the work of Steidler et al. (2003). These researchers replaced thymidylate synthase gene (thyA) of *L. lactis* with a synthetic human IL10 gene by double homologous crossover (Steidler et al., 2003). This strain contains no antibiotic resistance markers, and because of its thymidine auxotrophy, it cannot spread in the environment, making it one of the safest genetically modified organisms ever designed. The efficacy of this strain was assessed in a first phase 1 clinical trial in a patient with Crohn's disease, and the results from this study revealed that the use of genetically modified *L. lactis* to deliver therapeutic molecules, such as IL-10, at the mucosal level is a viable strategy in humans with chronic intestinal inflammation (Braat et al., 2006). Another elegant report showed a recombinant strain of LAB, capable to both produce and secrete the mucosal protectant human trefoil factor 1 at the site of the targeted oral mucosa [*L. lactis* (AGO13)]. This strain was engineered based on the same strategy (deficient in the gene encoding thymidylate synthase), being a food-grade strain. Results in patients with oral mucositis (phase 1 clinical trial) demonstrated that this strain was able to ameliorate the symptoms of this disease (Limaye et al., 2013).

Although research works with recombinant LAB show promising results in animal models of human disease, these particular ones were the only studies in which recombinant bacteria were used in human clinical trials, demonstrating its potential as a biotechnological tool for the treatment of diseases, but still remaining as a proof of concept. Concerning the regulation of gene expression in food-grade vectors, sugar, temperature, and pH-induced systems are highlighted. The promoter P170 present in *L. lactis* is inducible by the accumulation of lactic acid at pH 6.0–6.5, when the culture is reaching the stationary phase (Madsen et al., 1999). Therefore, this self-inducible promoter could be considered for the construction of new food-grade expression systems.

P1 and P2 are classic examples of temperature-induced promoters. The repressor of the P2 promoter is inactivated when the medium temperature reaches 40°C. Thus, shifting growth temperature to 24–42°C results in an increase of gene expression controlled by this promoter (Sanders et al., 1998). Sugars can be a safe and inexpensive way to induce gene expression in food-grade systems. Payne and colleagues developed a food-grade expression system induced by lactose; they used the previously mentioned *L. lactis* strain MG5267, which possesses

the lactose operon integrated in the bacterial chromosome. After the lysin gene from *Listeria monocytogenes* bacteriophage LM-4 was integrated into the chromosome, the chromosomal *lacG* gene, which encodes phospho-beta-galactosidase, was inactivated by a double cross-over event. Thus, the expression of the lysin gene was shown to be regulated by growth in the presence of lactose, proving to be an important strategy for controlled protein expression in *L. lactis* (Payne et al., 1996).

PERSPECTIVES FOR DEVELOPMENT OF NEXT GENERATION VECTORS

Lactococcus lactis, the model lactic acid bacteria, has enormous potential to be used as a biofactory for the production of numerous proteins of medical and industrial interests, as well as a carrier vehicle for DNA vaccines. Nowadays, this is a reality on the laboratory scale. As new information concerning this microorganism emerges, new possibilities for its use are being contemplated. Constant discoveries in the areas of genomics, transcriptomics, and proteomics of lactic acid bacteria provide us information for new approaches for the construction of the next generation of vectors. These should include the investigation of *L. lactis* cryptic plasmids, as well as exploration of new selection markers that exclude any type of resistance to antibiotics. In addition, features such as host range, stability, and copy number of plasmids must be considered and also the type of replication origin to be used in vector construction. The theta-type replication mechanism is indicated for reasons already mentioned in previous sections. Moreover, food-grade systems can also be developed by integrating vectors into the host's chromosomal DNA allowing more stability. Finally, mechanisms of gene expression induction that include the use of cheap and non-harmful compounds such as sugar, pH, or temperature should be considered for future biomedical, biotechnological, and industrial applications. Furthermore, the most promising technique in the field of genetic studies is the CRISPR Cas9 (clustered regularly interspaced short palindromic repeats) system. CRISPR is known as the prokaryote adaptive immune system that provides resistance against foreign DNA, such as plasmids and bacteriophage (Bolotin et al., 2005; Deveau et al., 2008). This system has been studied for the last 30 years (Ishino et al., 1987), but only in 2013 the first experiments were conducted, demonstrating its functionality as an editing genome tool (Cong et al., 2013; Mali et al., 2013). This system has been used in different studies to help understand both disease models and therapeutic schemes for several diseases (Jiang et al., 2013; Makarova et al., 2015; Di Bella et al., 2016).

Although several studies have been carried out with bacterial species using this strategy for genomic editing, only few studies are reported to be using LAB (Oh and van Pijkeren, 2014; Sanozky-Dawes et al., 2015; Song et al., 2017; Berlec et al., 2018). In this way, this technique can be explored for the development of food-grade expression systems and opens perspectives for the use of *L. lactis* in clinical routine in the near future.

AUTHOR CONTRIBUTIONS

CP, MD, and PM-A conceptualized the study. CP, MD, PM-A, VB, and AN wrote the original draft of the paper. CP, MD, PM-A, and VA reviewed and edited the manuscript. VA acquired funding and supervised the study.

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REVIEW
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Prospects on the Use of *Schizochytrium* sp. to Develop Oral Vaccines

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Although oral subunit vaccines are highly relevant in the fight against widespread diseases, their high cost, safety and proper immunogenicity are attributes that have yet to be addressed in many cases and thus these limitations should be considered in the development of new oral vaccines. Prominent examples of new platforms proposed to address these limitations are plant cells and microalgae. *Schizochytrium sp.* constitutes an attractive expression host for vaccine production because of its high biosynthetic capacity, fast growth in low cost culture media, and the availability of processes for industrial scale production. In addition, whole *Schizochytrium sp.* cells may serve as delivery vectors; especially for oral vaccines since *Schizochytrium sp.* is safe for oral consumption, produces immunomodulatory compounds, and may provide bioencapsulation to the antigen, thus increasing its bioavailability. Remarkably, *Schizochytrium sp.* was recently used for the production of a highly immunoprotective influenza vaccine. Moreover, an efficient method for transient expression of antigens based on viral vectors and *Schizochytrium sp.* as host has been recently developed. In this review, the potential of *Schizochytrium sp.* in vaccinology is placed in perspective, with emphasis on its use as an attractive oral vaccination vehicle.

Keywords: adjuvant, bioencapsulation, microalgae, oral vaccine, thermostable vaccine, Algevir system

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RELEVANCE AND CHALLENGES IN ORAL VACCINE DEVELOPMENT

Vaccination is a primary intervention against infectious diseases, thus, affordable vaccination campaigns for government budgets, especially in developing countries, are a priority. However, many potentially vaccine-preventable diseases in low-income countries are inadequately prevented due to an insufficient use of the existing vaccines. For instance, there is a lack of efficient distribution and delivery logistics, in addition to the associated high cost of the vaccine (Kochhar et al., 2013; Chen et al., 2014a). Most of the current available vaccines are designed for subcutaneous or intramuscular administration. However, these routes have limitations such as the problems associated with unsafe injections that are consequence of low economic resources and limited trained personnel (Hauri et al., 2004; Wilkhu et al., 2011). In addition, parenteral vaccines generally require "cold chain," which represents further economic and logistical burdens (Kumru et al., 2014). Therefore, oral immunization with thermostable vaccines is highly desired since it avoids the need of cold chain, specialized devices, and trained personnel for administration (Scherliess, 2011).

Since the vast majority of pathogens infect their host through the mucosa, local immune responses at these sites serve as the first line of defense against the pathogen (Hornef, 2015). Interestingly, several vaccines administered via mucosal routes have a proven effective induction of both systemic and local immunity (Lamichhane et al., 2014). However, to achieve efficacy, higher and more frequently administered doses are required in oral immunization schemes when compared to intramuscular or subcutaneous vaccines; which is a consequence of antigen dilution and degradation in the gastrointestinal tract as well as a poor antigen uptake (Doherty, 2015; Truong-Le et al., 2015).

Another important aspect is related to the fact that the mucosal tissues maintain homeostasis by mounting specialized anti-inflammatory immune defenses, including the induction of tolerance against innocuous soluble substances and commensal bacteria (Kweon, 2014). Therefore, oral vaccination must overcome the induction of local and systemic immunological tolerance, known as oral tolerance (Wilkhu et al., 2011). This obstacle can be overridden by antigen encapsulation (Kai and Chi, 2008) and the inclusion of adjuvants to enhance the immunogenic properties of the formulation (Hasegawa et al., 2015; Savelkoul et al., 2015). Although several technologies for antigen encapsulation (Trovato and Berardinis, 2015) and adjuvants are under evaluation (Newsted et al., 2015), there is a clear need to continue in the exploration of new oral vaccination strategies; which is reflected by the limited number of oral vaccines available in the clinic (Yuki and Kiyono, 2009).

MICROALGAE-MADE VACCINES

Among the current trends in biotechnology for the production of biopharmaceuticals in attractive platforms, algae have been used to produce monoclonal antibodies, vaccine antigens, therapeutic enzymes, blood proteins, cytokines, growth factors, and growth hormones. Microalgae-based expression systems are inherently faster to develop, potentially less expensive, and require less space for production. In addition, the biomass is relatively inexpensive to produce. Algae-based vaccines offer antigen protection from proteolytic degradation due to the cell wall. In addition, subcellular compartmentalization may also influence antigen release and thus bioavailability (Gregory et al., 2013). Moreover, algae are capable of performing post-translational modifications (e.g., glycosylation in endoplasmic reticulum and Golgi) that are frequently important for the antigen activity, can be produced relatively fast and some species could be used to formulate vaccines in a straightforward manner since they hold a GRAS status (Specht and Mayfield, 2014). This notion has been primarily explored for the freshwater microalga *Chlamydomonas reinhardtii* with important advances toward the development of low cost orally-delivered vaccines (Bañuelos-Hernández et al., 2015; Rasala and Mayfield, 2015; Dyo and Purton, 2018). Two interesting reports in this area comprise a developed *S. aureus* vaccine based on *C. reinhardtii*, showing antigen yields up to 0.7% TSP. The vaccine was stable at room temperature up to 20 months. Moreover, the mucosal IgA and systemic IgG responses were induced in orally immunized mice subjected to a scheme consisting of priming and 4 boosts administered weekly. Remarkably, an 80% survival rate after a lethal challenge with *S. aureus* was achieved (Dreesen et al., 2010). In the same microalga, a vaccine against malaria was developed. The vaccine was able to induce the systemic IgG responses and conferred protection against *Plasmodium berghei* in terms of reduction of parasitic load in red blood cells from mice treated with a single vaccine dose (Dauvillée et al., 2010). Another case of an oral algae-based vaccine against malaria consisted of a fusion protein comprising the cholera toxin B subunit (CTB) as adjuvant and the antigen of *Plasmodium falciparum* Pfs25. In this case, the oral vaccination of BALB/c mice using algae producing CTB-Pfs25 elicited CTB-specific serum IgG, fecal IgA antibodies, as well as Pfs25-specific IgA antibodies (Gregory et al., 2013). Diatoms have also been applied for the expression of vaccine antigens with promising findings on the expression of IbpA DR2 antigen from *Histophilus somni* (Corbeil et al., 2015; Davis et al., 2017). Although no clinical trials are ongoing for algae-based vaccines, the technology seems promising and these evaluations could begin in the short term (Rosales-Mendoza and Salazar-González, 2014).

APPLICATION OF MARINE MICROALGAE IN VACCINE DEVELOPMENT

Marine organisms are attractive hosts in this field as they are currently produced at industrial levels in culture media based on marine water to produce compounds with pharmaceutical, nutrition, and health applications; among other industrial applications (Mayer et al., 2011; Dewapriya and Kim, 2014). Interestingly, marine microalgae have been used in the production of vaccines. For instance, *Phaeodactylum tricornutum* was used to produce a monoclonal human IgG antibody against the Hepatitis B surface antigen (HBsAg) as well as HBsAg fused to GFP or an ER retention signal. The achieved antibody production was 8.7% of the total soluble protein (TSP; 1.6 mg per liter of culture or 21 mg antibody per gram algal dry weight), whereas HBsAg yields were up to 0.7% TSP (Hempel et al., 2011). Similarly, *Dunaliella salina* was transformed for the expression of HBsAg. In this case, the yields obtained were up to 3 ng/mg soluble protein and the positive clones were grown in non-selective liquid media for at least 60 generations; showing that the HBsAg protein was stably expressed in the transformed cells (Geng et al., 2003). On the other hand, the expression of the viral protein 28 (VP28) from the *White spot syndrome virus* was reported in the marine microalga *Dunaliella salina* with yields up to 780 μg VP28 per liter of culture. This vaccine was able to induce a 41% reduction in shrimp mortality after a lethal challenge experiment in orally immunized animals (Feng et al., 2014).

RELEVANT CHARACTERISTICS OF *Schizochytrium sp.* FOR VACCINE DEVELOPMENT

Schizochytrium sp., a thraustochytrid, is a heterokont marine microalgae with a cell diameter of about 9–14 μm belonging

to the Labyrinthulomycetes class, which is used to produce Docosahexaenoic acid (DHA) that accumulates up to 50% of dry weight lipids. Furthermore, it contains up to 10% of protein and 25% of carbohydrates (Qu et al., 2013; Fedorova-Dahms et al., 2014; Yao et al., 2015). In addition β-carotene is accumulated at significant levels in some species of this genus (Aki et al., 2003; Yokoyama and Honda, 2007; Ren et al., 2010). *Schizochytrium sp.* can be propagated at the industrial scale in heterotrophic conditions in which low cost medium is used and no complex photobioreactors are required since the process does not depend on light irradiation. Since some species of *Schizochytrium sp.* may grow in marine water-based culture media, their industrial use could not interfere with fresh water sources used for agriculture (Barclay, 1992; Chang et al., 2014). This microalga is currently used as food supplement in mammals and poultry (Meale et al., 2014). For instance, the *Schizochytrium sp.* supplementation of the laying hen diet has a beneficial effect on egg production, egg weight, yolk color, and blood lipid profiles of the layer hen (Park et al., 2015). In addition, DHA from *Schizochytrium sp.* is a key component in dietary supplements, cosmetics products, and pharmaceutical formulations (Fedorova-Dahms et al., 2014; Aasen et al., 2016). Moreover, several immunomodulating compounds are also produced by this microalga, which highlights a potential contribution to vaccine efficacy when used as a delivery vehicle (**Figure 1**). For instance, it produces squalene, a compound with adjuvant activity (Hoang et al., 2014).

Schizochytrium sp. is an interesting alternative for vaccine production and delivery due to its capacity for recombinant protein expression, being able to efficiently export proteins toward the extracellular compartment; which is a substantial advantage over the bacterial hosts since the recombinant protein can be easily purified due to the simpler composition of culture supernatants. In addition, a singular advantage of *Schizochytrium sp.* is the capability of exporting full-length complex membrane-bound insoluble proteins in a secreted form while retaining full functionality and a properly active structure (Bayne et al., 2013). Since *Schizochytrium sp.* has singular properties among the algae species, the implications of this species in vaccinology are analyzed.

Bioencapsulation Effects

Since a major challenge in oral vaccine development is antigen degradation by commensal bacteria, proteases, and the acidic stomach environment, it is necessary to protect it and include suitable mucosal adjuvants to enhance antigen bioavailability and recognition by the elements of the gut-associated lymphoid tissues (Hernández et al., 2014). One alternative to address these challenges is antigen encapsulation and among the approaches explored in this regard is the use of nano and microparticles for oral vaccine delivery. There is an array of different polymers suitable for this purpose, which can be either natural or synthetic, in which antigens can be encapsulated within the particles. Although promising, it should be considered that these systems require a synthesis approach that involve strict reaction conditions for synthesis, purification and characterization, which is laborious and costly (Sinyakov et al., 2006).

In contrast, *Schizochytrium* sp. can be used not only as the biofactory of antigens but also as a natural microcapsule (9–14 μm), which is easier and cheaper to obtain than synthetic micro particles. When an antigen is intracellularly accumulated algae biomass can be used as a microencapsulated vaccine not requiring complex processing (i.e., purification). In this manner, the microalgal cell adds its components to the vaccine activity, which could favorably influence: (i) the antigen bioavailability as it is believed to protect the antigen from degradation but at the same time to mediate a proper release of the antigen to make it bioavailable while maintaining its native conformation in the microalgae and therefore the antigenic determinants are preserved (Gregory et al., 2013); and (ii) stimulation of the cells involved in antigen translocation, processing and presentation by the action of algal compounds; improving the response triggered by the antigen. In fact, microalgae have allowed the oral delivery of intact nanobodies in the intestine of mice (Barrera et al., 2015). Therefore, antigens encapsulated into *Schizochytrium* cells offer a cheaper and more practical system compared with conventional micro and nanoparticulated systems.

Presence of Immunostimulatory Compounds

Immunostimulatory compounds, such as adjuvants and immunostimulants, can enhance vaccine efficacy since they support the induction of robust immune responses through several mechanisms (Reed et al., 2013, **Table 1**). The benefits of immunostimulatory compounds include enhanced immunogenicity, antigen-sparing, and achievement of long-lasting immunoprotection (Petrovsky and Aguilar, 2004; Lee and Nguyen, 2015). Therefore, immunostimulatory compounds may contribute to reduce the number and magnitude of antigen doses as well as achieving proper immune polarization (Reed et al., 2013; Ranasinghe, 2014). Using organisms that serve as biofactories but at the same time as delivery vehicles containing immunostimulatory compounds is the ideal case for vaccine design (Rosales-Mendoza and Salazar-González, 2014).

In this context, marine organisms serve as a source of a myriad of potent bioactive compounds; including immunostimulatory molecules of relevance in vaccination. In particular, several algae species have been identified as a source of inflammatory modulators as well as anti-nociceptive and anti-cancer compounds (De Almeida et al., 2011; Farooqi et al., 2014; De Jesus Raposo et al., 2015). To date, the compounds produced by *Schizochytrium sp.* with known anti-nociceptive and anti-cancer effects are DHA and EPA (van Beelen et al., 2007; Mann et al., 2018; Mitome et al., 2018). In addition, *Schizochytrium sp.* contains several bioactive compounds such as flavonoids, β-glucans, β-carotene, polysaccharides, nucleotides and peptides; many of them considered immunostimulants and immunomodulators that, in adequate amounts or in appropriate combination, can improve immune competence (Ibañez and Cifuentes, 2013; Kousoulaki et al., 2015). Interestingly, no signs of toxicity have been observed thus far in the use of bioactive compounds from *Schizochytrium sp.* in humans or animals. Moreover, no intermediary metabolites involved in the synthesis

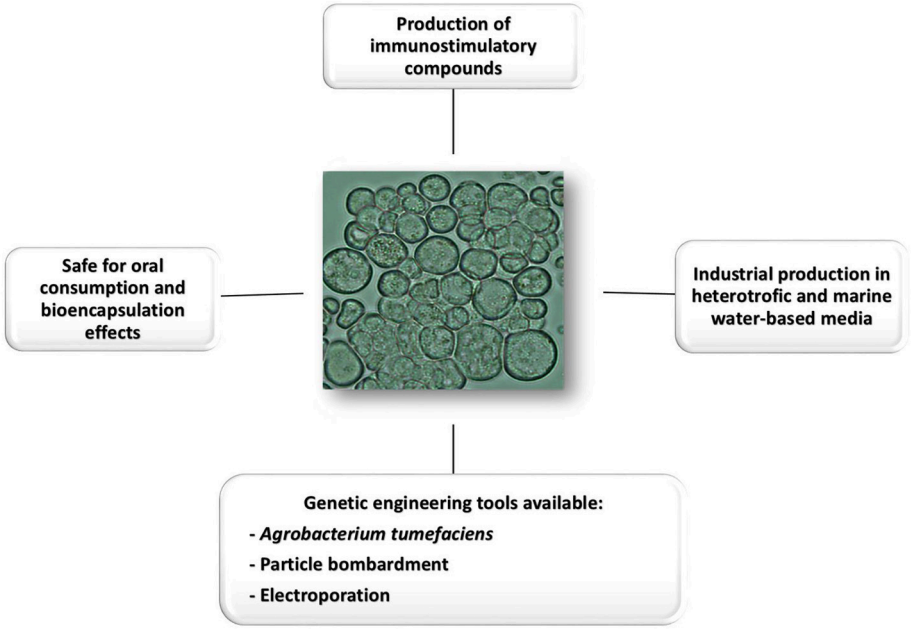

FIGURE 1 | Compilation of the main attributes that make *Schizochytrium* an attractive host for vaccine production and oral delivery.

TABLE 1 | Compounds produced by *Schizochytrium sp.* with known immunostimulatory activity.

Compound	Immunomodulatory activity and/or mechanism	References
DHA and EPA	Anti-inflammatory response through the inhibition of the kinases JNK and ERK of the NFκβ pathway, which leads to reduced production of cytokines such as TNF-α, IFN-γ, IL-1β, IL-6, IL-12, and enhanced systemic IgG and mucosal IgA production	Xue et al., 2006; Weldon et al., 2007; Draper et al., 2011; Siriwardhana et al., 2012; Magee et al., 2012; Tsunoda et al., 2015; He et al., 2017; Dawczynski et al., 2018
Palmitic acid	Pro-inflammatory response through the TLR4/IKKβ/NFκβ pathway, which leads to increased production of monocyte chemoattractant protein-1, nitric oxide, TNF-α, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-15, and IFN-γ; and enhanced mucosal IgA production	Karsten et al., 1994; Medzhitov et al., 1997; Kim et al., 2007; Nguyen et al., 2007; Schaeffler et al., 2009; Kunisawa et al., 2012; Zhou et al., 2013
Squalene	Squalene-based adjuvants exert a proinflamatory response, which involves the production of cytokines and chemokines, such as MCP-1, IL-1β, IL-8 (CXCL-8), CCL3, CCL4 and IL-4; and enhancement of IgG production	Calabro et al., 2013 Vinay et al., 2013
Polysaccharides	Mechanisms involved in their expected immunomodulatory effects are unknown	Laurienzo, 2010 Chang et al., 2013 Liu et al., 2014

of toxic compounds have been reported in *Schizochytrium sp.* Overall, these features make *Schizochytrium sp.* an attractive species to be used for food purposes and as a valuable source of immunostimulatory compounds for animals and humans (Mioso et al., 2014). The relationship between *Schizochytrium sp.* immunostimulatory compounds and the immune system in the context of vaccine development will be briefly discussed in the following sections.

Lipids

Lipids represent up to 56% of the total dry weight of *Schizochytrium* sp. (Yokochi et al., 1998; Ren et al., 2010), with some of them exerting health-promoting effects (Chen et al., 2014b; Raposo and De Morais, 2015). Lipids with immunostimulatory or immunomodulatory activities produced by *Schizochytrium sp.* comprise DHA, EPA, palmitic acid, and squalene (Huang et al., 2010; Taparia et al., 2016). DHA and EPA have proinflammatory and antinflammatory effects (Kelley, 2001; Ramakers et al., 2005) and improve Th1 and Th2 responses following vaccination (Hogenkamp et al., 2011). DHA and EPA immunomodulatory effects have been evaluated *in vitro* in cells derived from human and animals such as macrophages, dendritic cells, neutrophils, lymphocytes, epithelial cell lines, among others. Therefore, DHA and EPA differently modulate immune responses related to phagocytosis, phosphorylation of intracellular signaling molecules, activation of transcription factors, and effector immune-related gene expression; which largely depends of the cell type or target species. The overall

modulatory effects of DHA and EPA are related to a polarized anti-inflammatory response demonstrated by the reduction of cytokines such as TNF-α, IFN-γ, IL-1β, IL-6, IL-12, and the anti-inflammatory cytokine IL-10, through the inhibition of kinases (i.e., JNK, ERK) of the NFκβ pathway (Xue et al., 2006; Weldon et al., 2007; Draper et al., 2011; Magee et al., 2012; Siriwardhana et al., 2012; Tsunoda et al., 2015; He et al., 2017; Dawczynski et al., 2018). These effects could be associated with the eicosanoid synthesis pathway (Lokesh et al., 1988). In addition, it should be taken into account that DHA and EPA enhance neutrophil and macrophage phagocytosis, nitric oxide production (a pro-inflammatory mediator), and lymphocyte proliferation (Omura et al., 2001; Verlengia et al., 2004; Gorjão et al., 2006, 2009). Furthermore, it has been suggested that DHA promotes the production of pro-resolving cytokines from T helper lymphocytes and monocytes, via activation of the PPAR-gamma transcription factor; which finally contributes to adequate pro-resolving inflammatory responses that maintain a healthy status (Jaudszus et al., 2013). Moreover, it has been reported that the administration of DHA and/or EPA regulates the immune response in several animals such as cattle, goats, poultry, and pigs (Moreno-Indias et al., 2012; Bragaglio et al., 2015; Swiatkiewicz et al., 2015). Therefore, DHA and EPA could improve the efficacy of vaccines against inflammatory disorders. Interestingly, supplementation with DHA modulated antigen-specific T cell responses through an IL-10-mediated mechanism in vaccinated pigs (Bassaganya-Riera et al., 2007), and reduced TNF-α and IL-1β production and increased IgG titers against bacterial toxins in vaccinated infants (López-Alarcón et al., 2008; Furuhjelm et al., 2011). Similarly, the supplementation of the maternal diet with DHA positively favors the activation of B cells and the response to a potential food antigen upon challenge in suckled offspring (Richard et al., 2015). On this regard, the evidence suggest that both DHA and EPA promote B cell activation and antibody production, particularly enhancing mucosal IgA responses, which is relevant to protect against infectious diseases (Gurzell et al., 2013; Teague et al., 2013; Whelan et al., 2016). Remarkably, the study of DHA and DHA-derivatives as potential adjuvants seems promising in vaccinology. Using 17-HDHA led to an enhanced serum protective antibody response after OVA and H1N1 vaccination in a mouse model (Ramon et al., 2014). Dietary DHA has also been proposed as a potential adjuvant in cancer treatments (Merendino et al., 2013) and has been tested in children and adolescents with acute lymphoblastic leukemia (Elbarbary et al., 2016). It is clear that dietary fatty acids influence the response of the immune system to vaccination and the potential benefits from marine (n-3) PUFA have been reported (Hogenkamp et al., 2011). Therefore, it can be expected that the fatty acids existing in *Schizochytrium*-based vaccines may account for the efficacy of the formulation (Maroufyan et al., 2012).

Another fatty acid present in high levels in *Schizochytrium sp.* is palmitic acid (PA), which triggers a pro-inflammatory response by the activation of macrophages (Talbot et al., 2014; Tian et al., 2015). In addition, PA is involved in the improvement of antigen presentation by antigen presenting cells (APC); an effect that is partly mediated by TLR4 and TLR2 binding (Weatherill et al., 2005; Huang et al., 2012). In general, it is known that PA exerts immunostimulatory effects through the TLR4/IKKβ/NFκβ pathway. The downstream TLR4 signaling induced by PA leads to the activation of NF-kB and it has been associated with an increase in the secretion of monocyte chemoattractant protein-1 and pro-inflammatory molecules, such as nitric oxide, TNF-α, IL-1β, IL-6, and IL-8 (Medzhitov et al., 1997; Kim et al., 2007; Schaeffler et al., 2009; Zhou et al., 2013). In particular, the production of IFN-γ and IL-2 is enhanced in human peripheral lymphocytes upon PA treatment (Karsten et al., 1994). Additionally, PA was found to enhance secretory IgA responses; which are supported by the production of interleukins such as IL-5, IL-6, IL-10, and IL-15 (Nguyen et al., 2007; Kunisawa et al., 2012). On the other hand, dietary administration of PA in mice stimulates plasma cells to produce antibodies in intestine, highlighting its potential as a diet-mucosal adjuvant (Kunisawa et al., 2012). Particularly, PA-diet supplementation induced higher intestinal IgA responses in orally-immunized mice with OVA antigen and cholera toxin (Kunisawa et al., 2014). Interestingly, the palmitoyl group is also crucial in the approaches to produce immunogenic conjugates able to elicit specific and long lasting humoral immune response without the need of additional adjuvants (Kargakis et al., 2007). Vaccine formulations containing PA and palmitic acid-derivatives as adjuvants resulted in improved efficacy against several diseases including tuberculosis (Gupta et al., 2016), cancer (Rueda et al., 2017), rabies (Liu et al., 2016), canine distemper (Chua et al., 2007), and toxoplasmosis (Tan et al., 2010); an effect that is associated with enhanced pro-inflammatory cytokine production (Moyle, 2017).

Schizochytrium lipids are also the basis of some commercial adjuvants. Squalene is a lipid (polyunsaturated triterpen) of the terpenoid family, typically obtained from animal sources; however, recent advances in purification processes have allowed the use of plants and microalgae as squalene sources (Brito et al., 2011). Interestingly, squalene is particularly produced at high levels in *Schizochytrium* (Yue and Jiang, 2009; Hoang et al., 2014). Squalene is used to produce adjuvants with proven efficacy, such as MF59, AF03, and AS03 (Reddy and Couvreur, 2009; O'Hagan et al., 2012; Kedl and Kedl, 2015; Bonam et al., 2017). Although the activity of such adjuvants is consequence of the combination of squalene with other compounds, it has been reported that neutrophils, dendritic cells and macrophages are the main players involved in the production of proinflamatory cytokines and chemokines (Calabro et al., 2013). Intraperitoneal administration of pure squalene (536.5 ul kg^{-1}) in fish led to safe inflammatory cellular and humoral responses at the site of injection and in immune-relevant tissues (Vinay et al., 2013). It is known that squalene is efficiently absorbed through the intestinal mucosa, rapidly enter into lymphatic circulation and is metabolized (Tilvis and Miettinen, 1983; Gylling and Miettinen, 1994) and therefore it could be active in oral vaccine formulations. For instance, MF59 has been licensed in more than 20 countries for use in an improved influenza vaccine called Fluad® (Frey et al., 2014). Squalene-based adjuvants efficiently enhance immune responses and are safe for humans and animals (O'Hagan et al.,

2012; Fox and Haensler, 2013; Black, 2015; Haensler et al., 2015).

Therefore, lipids produced by *Schizochytrium sp.* might account, in combination with other inmunostimulatory molecules synthesized by the alga, for the immunogenicity of vaccines. The current information in the literature encourages performing more studies to investigate in detail the immunostimulatory effects of individual compounds or mixtures of them in oral immunization prototypes. All of this evidence on the production of lipids with immunomodulatory properties accounts for the potential of *Schizochytrium sp.* as an attractive host for the development of efficient oral vaccines.

Polysaccharides

Polysaccharides derived from marine microorganisms have had great importance in the industry (Sudha et al., 2014) and are also of relevance for the biomedical field having several applications. For instance, they can serve as vaccine vehicles and adjuvants (Petrovsky and Cooper, 2011; Shinchi et al., 2015). In fact, several polysaccharides have anti-tumor and immunomodulatory properties (Yang and Zhang, 2009; Laurienzo, 2010; Na et al., 2010). In particular, sulfated polysaccharides significantly improved the humoral response; an effect associated to the promotion of lymphocyte proliferation and macrophage activation via TLR-4 binding (Huang et al., 2008). In addition, several polysaccharides favor Th1 responses, promoting protection against intracellular pathogens such as mycobacteria (Pi et al., 2014). A remarkable case is the ADVAX ™ adjuvant, made with pectin, which enhances the humoral and cellular responses against hepatitis and influenza vaccines and is currently under clinical evaluation (Saade et al., 2013; Honda-Okubo et al., 2015).

Marine microalgae are known as one of the most abundant sources of polysaccharides (Laurienzo, 2010). Particularly *Schizochytrium sp.* produces high levels of exopolysaccharides (EPS), at rates of around 300 mg per liter of culture (Chang et al., 2013), that are easily isolated from cultures since these are exported to the culture media (Laurienzo, 2010; Liu et al., 2014). EPS synthetized by the *Schizochytrium* species and other members of the Labyrinthulomycetes class are of great biotechnological interest (Jain et al., 2005). Overall, EPS exert immunostimulatory activity and adjuvant effects (Feng et al., 2015; Li and Wang, 2015), although no report exists about the immunostimulatory activity of EPS from *Schizochytrium sp.* and it constitutes an open field of study. Interestingly, it is known that the EPS production can be modulated by certain factors, such as glucose concentration in culture media; therefore, optimization of the EPS synthesis during *Schizochytrium*–based vaccines production could be an important aspect to optimize (Liu et al., 2014).

Schizochytrium sp. also exports many other compounds into the culture media including proteins, lipids, uronic acids, and sulfates (Lee Chang et al., 2014). Moreover, *Schizochytrium sp.* produces high amounts of xanthophylls (Aki et al., 2003) which possess immunomodulatory properties that promote cellular and humoral responses (Park et al., 2011; Ghodratizadeh et al., 2014).

Although no detailed characterization on the cell wall composition is available for *Schizochytrium sp.*, it can be expected that the cell wall of *Schizochytrium sp.* could exert singular inmunostimulatory effects. It is known that microalgae possess a singular cell wall composition: an apparent lack of cellulose and the cell wall components are layers of crystalline Ara-rich, Hyp-rich glycoproteins (Miller et al., 1974; Roberts, 1974). Therefore, it can be speculated that the components from the cell wall of *Schizochytrium sp.* may exert inmunostimulatory activity leading to highly effective oral vaccines when used as delivery vehicle.

GENETIC ENGINEERING TOOLS

The current genetic engineering methods for *Schizochytrium sp.* comprise transgene installation into the nuclear genome (**Table 2**), which has been achieved by the following transformation techniques: particle bombardment (Lippmeier et al., 2009; Metz et al., 2009; Sakaguchi et al., 2012; Bayne et al., 2013); *Agrobacterium*-mediated transformation using protoplasts (Cheng et al., 2012); and electroporation (Cheng et al., 2011).

Selective agents used in these successful methods include paromomycin, zeocin and geneticin. In terms of regulatory sequences, the EF1 promoter has led to convenient protein yields up to 5–20 mg of recombinant protein per liter of culture (Cheng et al., 2013). However, no extensive evaluation of distinct regulatory sequences and a wide diversity of target proteins have been explored in *Schizochytrium sp.* Despite the fact that homologous recombination occurs in *Schizochytrium sp.* and it has been exploited to generate mutants for lipid metabolism studies (Metz et al., 2009), this feature has not been exploited to generate clones with site directed insertions to favor efficient expression of the transgene in vaccine production.

In this context, the knowledge on the genetic engineering approaches explored for the case of the *C. reinhardtii* microalga model is a relevant reference that can be used to expand the developments for *Schizochytrium sp.* According to several reports, *C. reinhardtii* tends to show complex expression patterns under nuclear expression approaches (Mardanova et al., 2015), however improvements on yields have been achieved by a number of approaches. For instance, co-expressing the gene of interest and the gene marker in a transcriptional arrangement where, once under translation, the presence of the picornaviral 2A element between both sequences induces the release of independent proteins. The yields obtained under this approach were up to 0.25% TSP. Nonetheless, the limitations of this approach include the fact that this split mechanism does not occur in all the molecules and thus a fraction of the produced protein corresponds to fusion proteins (Rasala and Mayfield, 2015). Another approach has consisted on generating mutant strains by UV light exposure and the use of codon optimized selectable markers (Barahimipour et al., 2016). A bicistronic arrangement driven by the promoter of the endogenous intraflagellar transport 25 protein has led to attractive protein yields (Dong et al., 2017). Insertions of the first intron of the

TABLE 2 | Summary of the genetic engineering methodologies implemented for the marine microalgae *Schizochytrium* sp.

Objective	Vector	Promoter	Terminator	Transformation method	Selection approach	Findings	References
Generate mutants for polyunsaturated fatty acid (PUFA) synthase to characterize PUFA biosynthesis	Schizochytrium PFA1: (pBSK:A) Resistance to zeocin: (pTUBZEO11-2)	*Schizochytrium* α-tubulin	Simian virus SV40	Particle bombardment	Zeocin resistance (*Sh ble* gene)	Mutants are auxotrophic and required supplementation with PUFAs Transformation efficiency: 10–100 primary transformants per bombardment	Lippmeier et al., 2009
Generate mutant for fatty acid synthase (FAS) by homologous recombination to characterize fatty acid biosynthesis	(pBluescript SK(þ) from Stratagene)	*Schizochytrium* α-tubulin	SV40	Particle bombardment	Zeocin (*Sh ble* gene)	Mutants are lethal and rescued only when grown under supplementation with appropriate saturated fatty acids in combination with methylated cyclodextrins	Metz et al., 2009
Investigate a transgene expression system by 18S rDNA-targeted homologous recombination	pUCT-18S	TEF1	CYC1	Electroporation	Zeocin (*Sh ble* gene)	The majority of the transformants showed similar biomass and total lipid content when compared to the wild type strain Transformation efficiency: 1 μg linearized plasmid yield more than 100 transformants	Cheng et al., 2011
Develop a novel transformation approach using *Agrobacterium tumefaciens* and a binary vector	pCAMBIA2301	*egfp* gene: TEF1 *gus* gene: CaMV35S	*egfp* gene: CYC1 *gus* gene: nos	*A. tumefaciens.*	G418 resistance (*nptII* gene)	*A. tumefaciens* allowed for the stable insertion of the T-DNA Transformation efficiency: 150 transformants of *Schizochytrium sp.* per experiment	Cheng et al., 2012
Introduce the *Escherichia coli* acetyl-CoA synthetase (ACS) gene to reduce the negative impact of acetate accumulation on the fermentation products	pBluescript II SK (+)	TEF1	CYC1	Electroporation	G418 resistance (*nptII* gene)	The genetically modified *Schizochytrium sp.* showed a significantly higher biomass and fatty acid proportion. Transformants produce lower acetate levels (0.84 and 0.66 g/l) than the wild-type strain (1.66 g/l) Transformation efficiency: unspecified	Yan et al., 2013
Produce the recombinant hemagglutinin (rHA) protein derived from A/Puerto Rico/8/34 (H1N1) influenza Virus as a subunit vaccine	pCL0143, pCL0154, pCL0161, pCL0160, pCL0153	Elongation factor 1	PFA3	Particle bombardment	Paromomycin resistance (*aphH* gene)	Protective immunity against a lethal challenge with homologous virus was achieved by immunizing with a single dose of 1.7, 5, or 15 mg rHA with or without adjuvant (survival rates: 80–100%) Full protection (100%) was achieved at all dose levels with or without adjuvant when mice were given a second vaccination	Bayne et al., 2013
Develop a versatile transformation system for thraustochytrids applicable to both multiple transgene expression and gene targeting	pGEM-T Easy (Promega) and pUC18 (TaKaRa Bio)	EF-1α promoter	Ubiquitin terminator	Particle bombardment and electroporation	G418 resistance (*nptII* gene) Hygromicin resistance (*hyg* gene)	A multiple gene expression and gene targeting was achieved Transformation efficiency Microprojectile bombardment: 4.6×10^1 Colonies/μg DNA vector Electroporation: 0 Colonies	Sakaguchi et al., 2012

(Continued)

TABLE 2 | Continued

Objective	Vector	Promoter	Terminator	Transformation method	Selection approach	Findings	References
Increase the levels of docosahexaenoic acid by the heterologous expression of ω-3 fatty acid desaturase from the nematode *Caenorhabditis elegans*. Achieve site-directed mutagenesis of an acetolactate synthase gene	1. pTUBZEO11-2 2. plasmid pMON50201, or ALSmut1-7; pMON50202, or ALSmut2-2; pMON50203, or ALSmut3-5	*Schizochytrium* tubulin gene promoter	SV40 terminator	Particle bombardment and electroporation	Zeocin (*Sh ble* gene)	Expression of the ω-3 fatty acid desaturase increased the levels of docosahexaenoic acid in *Schizochytrium sp*. Homologous recombination occurs in *Schizochytrium sp.*, allowing for the generation of site-directed mutants Transformation efficiency: about 68% of the resulting Zeocin™-resistant clones were PCR positive for the fat-1 gene	http://www.google.com/patents/US7001772

ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit 2 (rbcS2i1) along with codon-optimized coding sequences has proven to enhance the yields of the recombinant protein (Baier et al., 2018).

Looking to develop innovative expression systems, we have reported for the first time the use of a viral vector to efficiently produce biopharmaceuticals in algae, using *Schizochytrium sp.* as the model alga and a geminiviral vector constructed with sequences of the begomovirus *Ageratum enation virus* (Bañuelos-Hernández et al., 2017). The vector mediates the inducible expression driven by the AlcA promoter, which has a fungal origin and is induced by ethanol in a mechanism mediated by the AlcR protein. The proof of concept was provided by expressing in *Schizochytrium sp,.* a complex viral protein, namely GP1 protein from *Zaire ebolavirus*, and a bacterial toxin subunit (B subunit of the heat-labile *E. coli* enterotoxin). High levels of the target antigens were achieved (GP1 yields up to 1.2 mg per gram of fresh weight biomass). **Figure 2** depicts the general methodology comprised by the Algevir system.

In terms of yields, *Schizochytrium sp*. possesses a competitive productivity for recombinant protein production (5–20 mg/L) when compared with other microalgae. For instance, maximum yields observed in photosynthetic algae (*C. reinhardtii*) are 15 mg/l in a system based in the secretion of the target protein using either synthetic glycomodules (tandem serine and proline repeats) that improve secretion or a mutant strain that efficiently expresses heterologous genes (Bayne et al., 2013; Lauersen et al., 2013; Ramos-Martinez et al., 2017). Another promising approach relies in chloroplast genome engineering, by which yields up to 3 mg/L have been achieved (Gimpel et al., 2015). However *Schizochytrium sp*. does not possesses this organelle.

In this context, Algevir is a robust and attractive system as it possesses the following advantages that override the mentioned limitations: nuclear expression offers the possibility to access the complex cellular machinery to perform complex post-translational modifications (e.g., glycosylation); intracellular accumulation at high levels of the biopharmaceutical allows using the alga cell as the delivery vehicle in oral formulations; inducible expression allows a tight control that might lead to an efficient production of recombinant proteins that have toxic effects in algae; transient expression avoids the long time investment required to select stably transformed clones. Therefore, Algevir is a versatile system offering the advantages of transient expression (short production time and high yields) that are ideal for the production of vaccines in response to epidemics.

Conventional systems for recombinant protein production allow overall higher yields than those of the microalgae-based systems. For instance bacterial systems have a productivity in the order of 5 g/l, yeast systems in the order of 30 g/l, whereas mammalian cells are in the range of 5–25 g/l (Jarvis, 2008; Demain and Vaishnav, 2009). However, besides yields it should also be considered that bacterial systems often led to significant losses when refolding is required, the system has limitations for the synthesis of complex proteins and the host produces endotoxins (Feng et al., 2012). In the case of mammalian cells, the limitations are high production cost and possible contamination with human pathogens (Moody et al.,

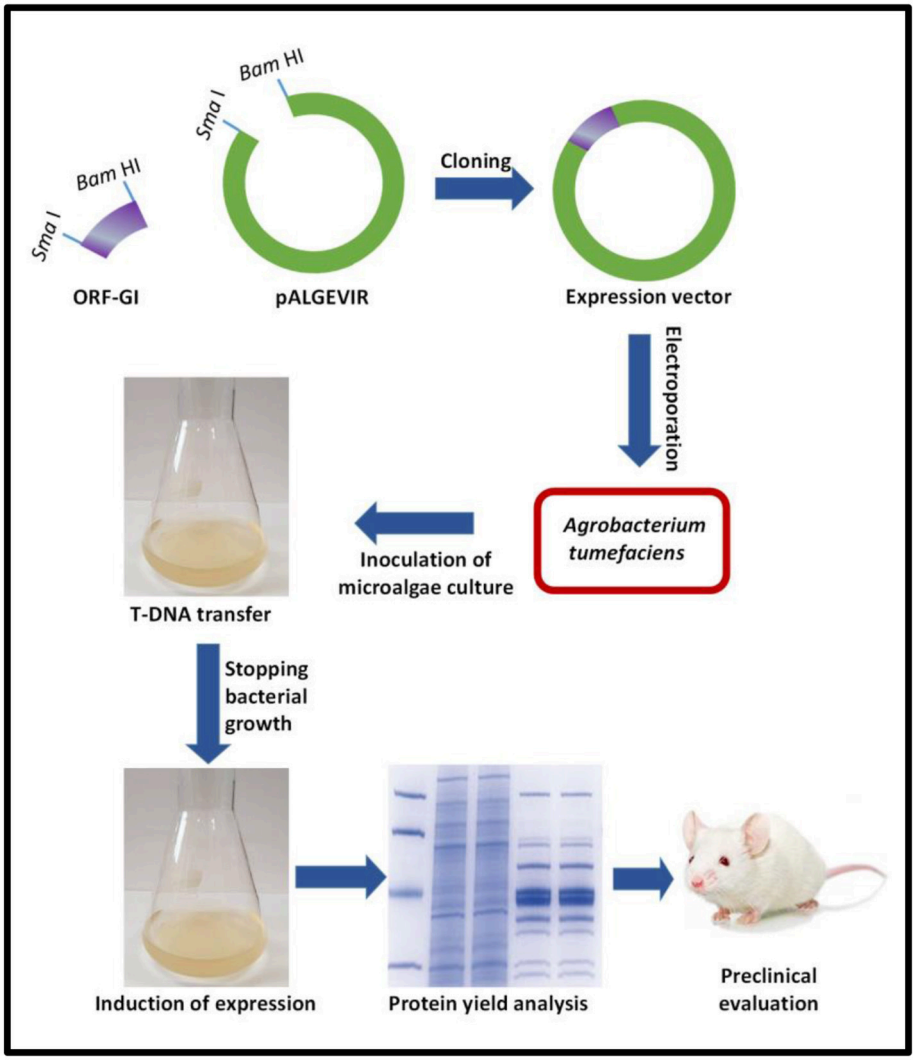

FIGURE 2 | General workflow for the Algevir system. (i) The Open Reading Frame of the Gene of Interest (ORF–GI) is cloned into the pALGEVIR plasmid through the restriction sites *Sma*I and *Bam* HI, (ii) The expression vector is transferred via electroporation to *Agrobacterium tumefaciens*, (iii) Microalgae culture is inoculated with the recombinant *A. tumefaciens* strain carrying the pALGEVIR vector and incubated during 16 h to allow T-DNA transfer, (iv) Agrobacterium growth is stopped by the addition of cefotaxime, (iv) Expression is induced by adding 1% ethanol, (v) biomass is harvested to determine protein yields and perform preclinical evaluation.

2011). Although yeast is also a fast-growing, GRAS, heterotrophic eukaryotic microorganism leading to high yields of recombinant proteins, it is often associated with hyper- glycosylation problems that may impair correct protein folding and functionality (Wildt and Gerngross, 2005). Therefore, although *Schizochytrium sp.* offers modest productivity in this context, the overall features of this organism make it an attractive host for vaccine production.

INITIAL EFFORTS USING *Schizochytrium sp.* IN VACCINE DEVELOPMENT

Bayne et al. (2013) reported a pioneering study on the expression of hemagglutinin (rHA) from A/Puerto Rico/8/34 (H1N1) influenza Virus in *Schizochytrium sp.* The *Schizochytrium sp.* EF-1 promoter, PFA3 terminator, and the aphH gene conferrying paromomycin resistance were used. Clones transformed by particle bombardment were obtained and characterized at the molecular and immunogenic level. The algae-made HA was successfully detected in the extracellular space retaining activity as revealed by hemagglutination activities from 16 to 512 hemagglutination activity units (HAU)/50 μL of cell-free supernatants (CFS).

The algae-made HA antigen was purified from fermentation culture supernatants with average yields of 5–20 mg of HA per liter of culture. The authors explored several immunization schemes in BALB/c mice groups comprising doses of 1.7, 5, and 15 μg of HA alone or co-administered with the AddaVax™ adjuvant. Another variable was a boost administered 3 weeks after priming. The hemagglutination inhibition (HI) activity assays revealed that the adjuvant-formulated vaccine induced

higher HA antibody titers, which significantly increased after the second injection.

The protective immunity against a lethal challenge with a homologous virus was evaluated following an immunization scheme comprising three rHA dose levels (1.7, 5, or 15 μg) with or without adjuvant. In spite of the dose, all mice were fully protected after two vaccinations. In terms of infectious virus titers, the test animals receiving adjuvant showed lower virus titers than adjuvant-free vaccine-treated mice.

The Algevir system has been applied to produce several antigenic proteins with distinct yields, namely the GP1 antigen Zaire ebolavirus (yields: 6 mg/l), the B subunit of the heat labile *E. coli* enterotoxin (yields: 0.4 mg/l), and a chimeric protein targeting the receptor of advanced glycation end products for Alzheimer's disease (RAGE; yields: 0.4 mg/l) (Bañuelos-Hernández et al., 2017; Ortega-Berlanga et al., 2018).

These seminal reports indicate a high potential of *Schizochytrium sp.* in the microalgae-made vaccines field, in particular for the production of antigens requiring complex post-translational modifications in a robust system. Several directions in which this technology can be exploited are identified and discussed in the next section.

FUTURE DIRECTIONS

The positive outcomes derived from the Influenza vaccine prototype developed with *Schizochytrium sp.* indicate a great potential to develop other vaccine candidates. Several directions in which this organism can be exploited in vaccinology are identified. For instance, biomass from transgenic lines expressing the antigen of interest will allow evaluating oral vaccines formulated in a straightforward manner without complex processing (e.g., freeze-dried biomass could be used for oral immunization). In addition, several cellular localizations can be assessed and the implications on immunogenicity and yields determined. For example, proteins can be retained in the endoplasmic reticulum or expressed in the form of amylosomes as has been performed in *C. reinhardtii* (Dauvillée et al., 2010); another possibility is the association to lipid bodies or protein bodies which have been accomplished in seed crops. In plants, the oil body fusion technology consists in fusing the target protein to the N- or C-terminus of oleosin in the oil body surface; since the expression is driven by a seed specific promoter, the protein can be efficiently expressed and rescued from seeds (Stoger et al., 2005; Boothe et al., 2010). This approach also extends protein half-life allowing easier transportation and storage (Bhatla et al., 2010). Given the high accumulation of oil bodies in *Schizochytrium sp.* (Morita et al., 2006), this process is considered viable and proposed as an efficient approach for recombinant antigen production having implications in the immunogenic activity since high molecular size complexes carrying the antigen could be produced in the recombinant algae.

A number of mechanisms may underline the immunomodulatory (pro- and anti-inflammatory) effects of fatty acids present in *Schizochytrium sp.* in prototype vaccines. All of them should be considered case-by-case determining the immunological outcomes required to fight the target disease. Thus, efforts to elucidate the immunological role and impact of the compounds present in *Schizochytrium* sp. are needed to understand and ultimately manipulate the immune responses induced by the vaccine. In terms of antigen design, studying the potential to produce virus like particles, antibody-antigen immunocomplexes, and adjuvant-antigen fusions are relevant, pending goals (Wen et al., 2016; Ding et al., 2018).

Moreover, conventional mutagenesis and genetic engineering along with the currently available *Schizochytrium* sp. genome bring opportunities for implementing strategies to improve the production of recombinant subunit vaccines. For instance, endogenous promoters and signal peptides could be used to improve the expression of the target antigens (Molino et al., 2018). Moreover, modifying the expression of enzymes involved in the synthesis of immunostimulatory compounds could lead to strains with modified metabolite profiles that could lead to improved immunogenicity (Park et al., 2018). Exploring these avenues will reinforce the use of *Schizochytrium sp.* as a convenient host in the production of attractive vaccines. The use of *Schizochytrium sp.* for vaccine production will also require the implementation of Good Management Practices-compliant process and validation of its safety for consumption by humans. It should be considered that *Schizochytrium sp.* is safe as food supplement for animals and thus the veterinary field could be the first to be benefited from the evaluation and commercialization of the *Schizochytrium*-made vaccines (Franklin et al., 1999; Meale et al., 2014; Kousoulaki et al., 2015; Park et al., 2015). This will be highly convenient since aquaculture and poultry intensive farming practices encourage the spread of diseases but the small size and low value of juvenile animals make other vaccination approaches impractical (Charoonnart et al., 2018).

CONCLUSIONS

Safe consumption, bioencapsulation effects, and the presence of immunomodulatory compounds are attributes that account for the potential of *Schizochytrium sp.* as an innovative platform for oral vaccine development. In addition, the well-established industrial process for *Schizochytrium sp.* production and the availability of efficient genetic engineering tools will support the perspectives of this technology.

Therefore, *Schizochytrium sp.* is an interesting alga species with implications in the development of improved algae-made vaccines that will benefit animal and human health. This research path will be particularly relevant in developing countries where heat-stable, low cost, easy to administer, and safe vaccines are urgently needed.

AUTHOR CONTRIBUTIONS

CA and SR-M conceived the manuscript. AR-V wrote most of the sections under supervision of SR-M and CA. BB-H wrote the section on the Algevir system.

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REVIEW
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Microbial Delivery Vehicles for Allergens and Allergen-Derived Peptides in Immunotherapy of Allergic Diseases

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Allergen-specific immunotherapy represents the only available curative approach to allergic diseases. The treatment has proven effective, but it requires repetitive administrations of allergen extracts over 3–5 years and is often associated with adverse events. This implies the need for novel therapeutic strategies with reduced side effects and decreased treatment time, which would improve patients' compliance. Development of vaccines that are molecularly well defined and have improved safety profile in comparison to whole allergen extracts represents a promising approach. Molecular allergy vaccines are based on major allergen proteins or allergen-derived peptides. Often, such vaccines are associated with lower immunogenicity and stability and therefore require an appropriate delivery vehicle. In this respect, viruses, bacteria, and their protein components have been intensively studied for their adjuvant capacity. This article provides an overview of the microbial delivery vehicles that have been tested for use in allergy immunotherapy. We review *in vitro* and *in vivo* data on the immunomodulatory capacity of different microbial vehicles for allergens and allergen-derived peptides and evaluate their potential in development of allergy vaccines. We also discuss relevant aspects and challenges concerning the use of microbes and their components in immunotherapy of allergic diseases.

Keywords: allergy immunotherapy, bacteriophage, delivery vehicle, lactic acid bacteria, S-layers, virus-like particle, viral surface protein

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INTRODUCTION

Allergen-specific immunotherapy is based on the repeated administration of increasing doses of allergen extracts over 3–5 years (Akdis and Akdis, 2014). Although this conventional immunotherapy regimen has proven effective, several important weaknesses such as the high percentage of undesired IgE-mediated adverse effects and long treatment duration are forcing the development of novel immunotherapeutic approaches and preparations (Larsen et al., 2016). One possible approach relies on design of molecular allergy vaccines which include individual allergen proteins (recombinant allergens), allergen-derived peptides carrying relevant epitopes

Abbreviations: AAVLP, adeno-associated virus-like particles; BLPs, bacteria-like particles; CpG, cytosine-phosphate-guanine deoxynucleotides; GRAS, generally regarded as safe; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LAB, lactic acid bacteria; LL-OVA, OVA-secreting *L. lactis*; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; Qβ-VLPs, bacteriophage Qβ derived VLPs; T_H1, type 1 helper cells; VLP, virus-like particle.

or epitope-mimicking peptides (mimotopes) (Szalai et al., 2008; Marth et al., 2014; Tscheppe and Breiteneder, 2017; Valenta et al., 2017). Allergen-derived peptides are designed to contain antigenic determinants of major allergens (epitopes) that are capable of activating the appropriate cellular and humoral responses while avoiding possible allergenic and/or reactogenic responses induced by whole allergens and allergen extracts. T cell epitope peptides are longer synthetic peptide sequences derived from primary allergen structure (Moldaver and Larché, 2011). B cell epitope peptides and mimotopes are short peptides that include or mimic three-dimensional IgE binding sites of allergens, respectively.

Recombinant allergens and particularly allergen-derived peptides are generally inadequately immunogenic and their immunogenicity is usually enhanced with application of an adjuvant (Siskind et al., 1966; Valenta et al., 1999). However, traditional agents and preparations with adjuvant properties are badly tolerated, and only a couple of them are appropriate for human use (Petrovsky, 2015). Aluminum hydroxide is the most widely used adjuvant in allergy immunotherapy with excellent safety record but has some limitations, particularly with regard to its profound T_H2-biasing effects (Hogenesch, 2012). Therefore, current research aims to develop new, potent and effective delivery vehicles for allergens or allergen-derived peptides that are able to induce tolerance and analogous to CpG motifs exhibit T_H1-immunostimulating properties (Johansen et al., 2005). An ideal delivery vehicle should possess the following characteristics: (1) provide targeted delivery and efficient presentation of vaccine components to the specific immune cells in a manner that would induce appropriate immune response (Moingeon et al., 2002; Souza et al., 2005); (2) exhibit low intrinsic immunogenicity to allow readministration in order to boost relevant specific immune response (Chesné et al., 2016); (3) sustain the vaccine release over an extended period of time; (4) protect vaccine components from degradation; and (5) allow large-scale production at low cost (Souza et al., 2005; Jafari and Abediankenari, 2016).

Over the recent years, viruses and bacteria have been intensively studied for their potential as delivery vehicles in allergy vaccines. Here, we discuss different aspects of microbial delivery vehicles of allergens and allergen-derived peptides employed in allergy immunotherapy in the attempt to develop formulations with improved immunogenicity and stability as well as the ability to target specific cells.

WHOLE VIRAL AND BACTERIOPHAGE PARTICLES AS DELIVERY VEHICLES

Despite their inherent ability to induce humoral and cellular immune responses, a major obstacle in using eukaryotic viruses as delivery vehicles in humans is their potential pathogenicity and oncogenic integration into the genome of the host cells (Souza et al., 2005; Bakhshinejad and Sadeghizadeh, 2014; Jafari and Abediankenari, 2016). The presence of pre-existing immunity to the viral vector, which causes fast viral clearance from the body and thereby reduces the dose of the vectored antigen even before it is able to elicit an immune response, is another limitation to their general use (Souza et al., 2005; Saxena et al., 2013). These disadvantages have stimulated the search for novel more adequate vaccine delivery vehicles among non-eukaryotic viruses. **Figure 1** shows a schematic representation of different types of viral delivery vehicles that have been tested for use in allergy immunotherapy.

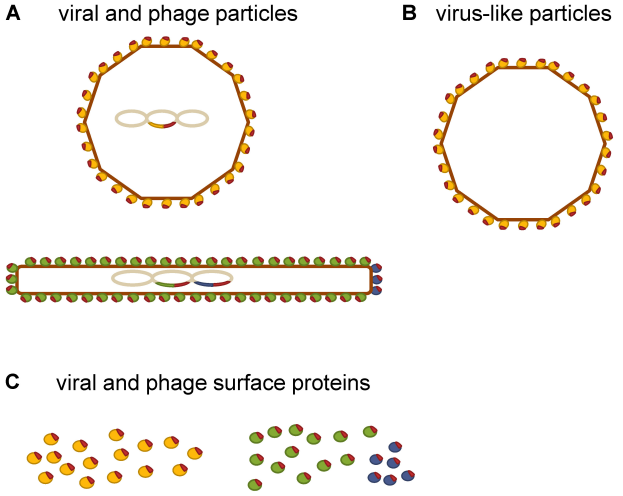

FIGURE 1 | Schematic representation of viral delivery vehicles that have been tested for use in allergy immunotherapy. **(A)** Viral and phage delivery vehicles displaying allergen-derived peptides (red) on their surface. **(B)** Virus-like particles as delivery vehicles of allergens or allergen-derived peptides (red) that are incorporated into the virus-like particle either by chemical coupling or by recombinant expression as fusion proteins. **(C)** Single viral (yellow) and phage surface protein carriers (green and blue) of allergen-derived peptides.

Bacteriophages (or phages for short) are viruses that infect bacteria. They are composed of DNA or RNA genome encapsulated inside a protein shield called capsid. In contrast to eukaryotic viruses, phages propagate in a prokaryotic host, and therefore appear as an attractive alternative for use in humans (Bakhshinejad and Sadeghizadeh, 2014; Jafari and Abediankenari, 2016). They act as inert particulate antigens, which are taken up and processed by antigen-presenting cells, and thus induce specific immune response by targeting delivery to these cells (Gao et al., 2010). The studies demonstrated that phages can induce both humoral and cellular immune responses without the use of an adjuvant (Adhya et al., 2014; Clark and March, 2014; Jafari and Abediankenari, 2016). Protection of the displayed peptides from a variety of harsh environmental conditions by phage particle provides extended degradation time and makes phages appropriate vectors for oral and mucosal applications (Delmastro et al., 1997; Jensen-Jarolim et al., 1998; Jepson and March, 2004). Combination of the feasibility of large-scale, cost-effective production and ease of modification makes them appealing for the industrial development of phage-based vaccines.

Phage-based vaccines are usually developed by recombinant fusion of the antigen to one of the virion surface proteins, of which the pIII and pVIII of M13 phage are used most frequently

(**Figure 1A**). Such phage virions have the antigenic sequence included in their genome, which allows steady production of the vaccine construct through bacterial amplification (Aghebati-Maleki et al., 2016). Alternatively, the antigenic sequence may be conjugated to the phage surface via artificial linkers (van Houten et al., 2010). This allows a broader range of antigens to be displayed. However, the vaccine construct must be prepared by synthesis for each batch. The intensity of immune response depends on characteristics of the displayed peptide and the method of its display. For example, pIII fusions usually exhibit lower immunogenicity than pVIII fusions (Jensen-Jarolim et al., 1998; Scholl et al., 2002). The reason for this probably lies in the copy number difference. There are 2,700 copies of the major coat protein (pVIII) and only five copies of the minor coat protein (pIII) present on the surface of M13 filamentous phage (**Figure 1A**). Namely, an antigen displayed in high copy on the surface of an individual phage virion is more effective in eliciting an immune response than the same antigen displayed in low copy number (Rakonjac et al., 2011). In general, phages possess comparable immunogenicity to that of the traditional carrier proteins such as bovine serum albumin or keyhole limpet hemocyanin and essentially have a small number of their own B cell epitopes to deflect antibody response away from the molecule they display (Luzar et al., 2016b). Indeed, it has been shown that filamentous phage carriers elicit antibody response that is more focused against displayed peptides compared to the traditional carrier protein OVA (van Houten et al., 2006). Although still in early stages of development, phage-based vaccines have been used to induce protection against infectious diseases and cancers in preclinical studies and have also been tested in phase I/II clinical studies (Roehnisch et al., 2014). Both icosahedral phages (such as lambda, T7, T4) and filamentous phages (such as fd, F1, M13) have been used for production of these vaccines (Jafari and Abediankenari, 2016).

In allergy immunotherapy, filamentous phage M13 has been most commonly employed for delivery of allergen-derived peptides (Chen and Dreskin, 2017). In the study performed by Luzar et al. (2016a), the filamentous phage particles displaying mimotopes of major cat allergen Fel d 1 (approximately 150 copies fused to major coat protein pVIII) were constructed and evaluated for their potential as vehicles for a cat allergy vaccine. Even though the mimotopes recognized IgE from sera of cat-allergic patients they did not activate the basophils of these patients. The phage carrier on the other hand caused non-specific stimulation of basophils probably triggering receptors of innate immunity such as Toll-like receptors, which are known to be present on basophil surface (Suurmond et al., 2014). Importantly, phage-displayed mimotopes were able to induce preferentially T_H1 directed response (increased IFN-γ production) in cultures of PBMCs from allergic patients.

The ability of phage-displayed mimotopes to induce antibodies specific for the whole allergen has been demonstrated in animal studies. In a mouse model, intragastric as well as intranasal administration of phages displaying mimotopes of major birch pollen allergen Bet v 1 (approximately 2,700 copies fused to major coat protein pVIII) induced Bet v 1-specific IgG response (Jensen-Jarolim et al., 1998; Scholl et al., 2002). Unfortunately, this IgG failed to induce immune tolerance to Bet v 1 in skin reactivity test emphasizing the importance of peptides' ability to adequately mimic the IgE epitopes on allergen (Knittelfelder et al., 2009). Since epitope specificity of an induced IgG antibody can be decisive for the success of therapy, particular attention must be paid to this issue during the development of peptide-based vaccines.

The studies suggest a possible future for filamentous phages as delivery vehicles in the therapy of allergic reactions. Nevertheless, several important drawbacks currently limit their application in clinical practice. Although the phage therapy has proven safe in healthy human volunteers (Bruttin and Brussow, 2005), some phages have the potential to release endotoxin from lysed Gram-negative bacteria (Young, 1992). This concern can be avoided with the application of non-lytic filamentous phages (e.g., M13 phage). However, when administered via oral route, these phages may transfer virulence factors or genes that confer antibiotic resistance to F-pili positive intestinal microbiota and thereby generate new unwanted traits (Bazan et al., 2012; Colavecchio et al., 2017). Additionally, long-term treatments with phages or phage exposure itself can induce an antibody response against phages, which can decrease their titer and reduce the effectiveness of therapy (Clark and March, 2014). Therefore, substantial evidence acquired in clinical trials, particularly regarding phage safety and effectiveness in subjects, who are positive for anti-phage antibodies, is still missing.

SELF-ASSEMBLING VIRUS-LIKE PARTICLES AS DELIVERY VEHICLES

Virus-like particles are composed of one or several viral structural proteins that have the ability to self-assemble during recombinant expression (Fuenmayor et al., 2017). They resemble and mimic the structure of actual viruses (Zeltins, 2013). A key advantage of VLPs is the lack of viral genomic material, which enhances safety during both manufacture and administration (Klimek et al., 2014). VLPs are composed of many subunits of one or more viral capsid proteins, which can be modified to display short peptide sequences in high-density at their surface either by genetic engineering or by chemical coupling, as schematically represented in **Figure 1B** (Brown et al., 2009; Schmitz et al., 2009). The findings in mouse models showed that an antigen presented to the immune cells in a highly ordered repetitive fashion is capable of eliciting strong antibody response even in the absence of adjuvant, while the same antigen presented as a monomer appears to be non-immunogenic (Feldmann and Easten, 1971; Marth et al., 2013). Examples of such natural repetitive immunogenic structures are surfaces of viruses and bacteria. There is also epidemiologic evidence that the repetitiveness of antigen correlates with its immunogenicity for B cells in human subjects (Jegerlehner et al., 2002a). This was essentially the rationale for the use of VLPs as carriers of allergens and allergen-derived peptides.

First, Jegerlehner et al. (2002b) showed that the antigens displayed on VLPs derived from the 180 coat protein subunits of the bacteriophage Qβ (Qβ-VLPs) are highly immunogenic in mice. A strong IgG2-dominated antibody response was induced by Qβ-VLPs because of the presence of bacterial host RNA, which is a ligand of Toll-like receptors. It was encapsulated into the VLPs during self-assembly process (Forsbach et al., 2007). Similarly, Qβ-VLPs have been shown to greatly enhance the immunogenicity of major cat allergen Fel d 1 chemically coupled to their surface (Schmitz et al., 2009).

In the first human use of VLP-based vaccines, Kündig et al. (2006) generated a construct composed of a peptide sequence from major house dust mite allergen Der p 1 chemically coupled to bacteriophage Qβ coat protein and evaluated its safety and immunogenicity in phase I clinical trial. Twenty-four healthy volunteers were vaccinated by two different routes (subcutaneous and intramuscular) with two different doses (10 μg and 50 μg of total protein) without the use of an adjuvant. The treatment was well tolerated. All immunized subjects developed a significant antibody response to both Der p 1 and bacteriophage Qβ coat protein, even after single injection demonstrating that allergen coupled to highly repetitive VLPs is an efficient approach for rapid induction of high titers of antibodies in human subjects (Kündig et al., 2006). The response depended on the administered dose, while the immunization route had only small influence. These findings were extended in phase I/IIa clinical trial, in which Senti et al. (2009) investigated the safety, tolerability and clinical effectiveness of treatment composed of house dust mite extract and deoxynucleotides with CpG motifs packaged into Qβ-VLPs. CpG motifs are known ligands of Toll-like receptor 9 with T_H1-immunostimulating properties (Johansen et al., 2005). Twenty-one dust mite allergic patients were enrolled in an open monocentric study. The results showed high level of safety and good tolerability. The treatment led to increased allergen-specific IgG and reduced skin reactivity to house dust mite extract. Almost complete tolerance to the allergen in conjunctival provocation testing and a significant reduction of rhinitis and allergic asthma symptoms were observed. After 10 weeks of treatment, patients were almost without symptoms. This alleviation lasted for at least 38 weeks after the treatment (Senti et al., 2009).

Virus-like particles obtained from adeno-associated viruses (AAVLPs) are composed of 60 copies of the VP3 capsid protein, which can be genetically modified to display short peptide sequences. Manzano-Szalai et al. (2014) assessed on a mouse model the immunogenicity and safety of such AAVLPs displaying a B-cell epitope peptide of food allergen OVA. The results showed that the titers of IgG1 specific for OVA in mice immunized with AAVLP-OVA were comparable to those induced by native OVA. However, native OVA elicited high levels of IgE, whereas OVA displayed on AAVLPs produced background IgE values only. Accordingly, OVA-immunized mice, but not mice immunized AAVLP-OVA, developed an anaphylactic reaction upon intravenous allergen challenge, which manifested as a significant drop in body temperature (Manzano-Szalai et al., 2014).

VIRAL AND PHAGE SURFACE PROTEINS AS DELIVERY VEHICLES

Different viral and phage surface proteins have also been tested for delivery of allergen-derived peptides (**Figure 1C**). In a number of preclinical studies, they showed excellent immunomodulatory capacity (Focke et al., 2001, 2010; Niespodziana et al., 2011; Valenta et al., 2016) and proved to be suitable for clinical trials (Zieglmayer et al., 2016).

VP1 Surface Protein From Human Rhinovirus

A study by Edlmayr et al. (2009) reported the construction of a recombinant vaccine for grass pollen allergy using surface protein VP1 from rhinovirus, which plays a major role in viral infection of respiratory cells. Recombinant fusion proteins composed of VP1 and a B cell epitope peptide derived from the major grass pollen allergen Phl p 1 were not recognized by patients' IgE and showed no allergenic activity in basophil activation test. Immunization of mice and rabbits with the fusion proteins resulted in the production of IgG that cross-reacted with group 1 grass pollen allergens. The induced antibodies were able to block recognition of native Phl p 1 by patients' IgE and Phl p 1-induced activation of basophils.

Hemagglutinin A Surface Protein From Influenza A Virus

Hemagglutinin A is a dominant glycoprotein on the envelope of influenza virus and a key antigen in the host response to virus infection. In a study conducted by Mrkić et al. (2016), the immunomodulatory potential of recombinant chimeric protein composed of the major allergen of house dust mite, Der p 2, and hemagglutinin A was tested in a mouse model. Intranasal pretreatment of mice with Der p 2/hemagglutinin A fusion, prior to sensitization with the allergen, significantly decreased IgE levels and markedly increased allergen-specific IgG and IgA levels in sera. Moreover, enhanced proliferation of CD4+CD25+ regulatory T cells was detected in mouse spleens after pretreatment with fusion molecule but not with the native allergen. This indicates that the carrier beneficially affects the immunomodulating properties of the vaccine.

PreS Surface Protein From Hepatitis B Virus

The PreS domain is a part of a large surface protein, which forms the hepatitis B virus envelope along with middle and small surface proteins. It showed good immunogenicity and safety during clinical use as a hepatitis B vaccine (Ilaria et al., 2016). Recently, several recombinant fusion proteins composed of PreS and B cell epitopes derived from allergens Fel d 1 (Niespodziana et al., 2011), Der p 23 (Banerjee et al., 2014), and Bet v 1 (Marth et al., 2013) have been constructed as vaccine candidates. The recombinant fusion proteins showed no relevant IgE reactivity and strongly reduced allergenic activity. Immunization of animals resulted in the production of allergen-specific IgG that inhibited the binding of allergic patients' IgE

to the native allergen as well as allergen-induced activation of basophils to a similar extent or better as did IgG elicited by vaccination with native allergen (Marth et al., 2013). This indicated that some of these fusion proteins have the ability to focus IgG response against the major IgE-reactive sites on allergen better than the allergen itself. In PBMCs from allergic patients, lower T cell proliferation and lower levels of T_H2 cytokine IL-5 were observed compared to the effect of native allergens. This was additionally associated with the secretion of the higher levels of the tolerogenic cytokine IL-10 and the T_H1-specific cytokine IFN-γ (Banerjee et al., 2014).

In the study conducted by Focke-Tejkl et al. (2015), four fusion proteins composed of B-cell epitope peptides from the major timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, and Phl p 6) and the PreS were generated and evaluated as components of the vaccine termed BM32. The BM32 vaccine, whose allergenic activity was almost completely eliminated, showed significantly reduced T-cell proliferation and decrease in production of proinflammatory cytokines in patients' PBMCs compared to grass pollen allergens. The vaccine was capable of inducing specific IgG antibodies directed toward native allergens in mice. Moreover, induced IgG were able to inhibit the binding of patients' IgE to all four major grass pollen allergens as well as inhibit the activation of basophils by the allergens. BM32 is at present farther advanced B-cell epitope-based vaccine. In the most recent multicentered double-blind, placebo-controlled phase 2b clinical trial, BM32 was well tolerated and efficiently relieved symptoms of allergic rhinitis in patients (Zieglmayer et al., 2016; Niederberger et al., 2018).

PIII Surface Protein From M13 Phage

Multivalent display of antigenic epitopes provides high immunogenicity to the therapeutic constructs and is desirable in most cases. However, this may present a limitation in the context of mimotope immunotherapy. Since mimotopes imitate the natural IgE epitopes and normally bind IgE, they might cross-link IgE on effector cells if displayed in high density on a carrier. Therefore, the application of monovalent carriers of allergen mimotopes may be more favorable. We tested the minor coat protein pIII from M13 phage as a delivery vehicle for mimotopes of major bee venom allergen Api m 1. PIII-fused mimotopes were recognized specifically by patients' IgEs, thus demonstrating that they imitate the natural IgE epitope; however, they caused no basophil degranulation in corresponding patients. This confirmed the absence of allergenic activity and demonstrated that the mimotopes bound to a monovalent carrier such as the minor coat protein pIII are not able to cross-link IgE on basophils. In addition, pIII-fused mimotopes exhibited immunomodulatory effects by eliciting secretion of T_H1 cytokine IFN-γ in PBMCs from bee venom-allergic patients, as opposed to bee venom and Api m 1, indicating a shift from T_H2 toward T_H1 immune response. These results suggest that the minor coat protein pIII might be suitable as a delivery vehicle for mimotopes obtained from phage display libraries. By using single coat proteins the problems associated with the application of whole phages in humans such as their potential to transfer antibiotic resistance to F-pili positive microbiota can also be avoided. Furthermore, the mimotopes fused to pIII preserve the conformation they had when displayed on phage. Hence, we can circumvent the problems with the loss of mimicry potential which was observed in the case of chemical coupling of mimotopes to certain traditional protein carriers such as keyhole limpet hemocyanin (May et al., 2003).

LIVE BACTERIA AS DELIVERY VEHICLES

Owing to their immunomodulatory properties, a number of probiotic strains have shown beneficial effects in the treatment of allergic diseases (Repa et al., 2003; Karimi et al., 2009; Costa et al., 2014; Ai et al., 2015b; Yepes-Nunez et al., 2016). In light of this, recombinant LAB engineered to produce and/or deliver allergens or allergen-derived peptides to mucosal surfaces to induce tolerance have emerged. **Figure 2** shows a schematic representation of different types of bacterial delivery vehicles that have been tested for use in allergy immunotherapy. Allergen vaccination via mucosal route is a desirable alternative to subcutaneous injections. It is not only easier but also increases the effectiveness against allergens that enter the body through mucosal surfaces (Wyszyńska et al., 2015). Some LAB strains have adjuvant properties and can enhance the immune response to the carried antigen (Schabussova and Wiedermann, 2008). They are especially suited for human use because of their "GRAS" status (Berlec et al., 2012; Trombert, 2015). Furthermore, LAB ability to withstand the passage through the gastrointestinal tract makes them an ideal oral delivery vehicle (Hynonen and Palva, 2013). Gut colonization by live strains allows the reduction of the number of vaccine doses required and also simplifies the immunization procedure to a great extent. Recombinant LAB serve as production hosts and as protective coatings at the same time. This can result in lower costs, as there is less need for purification of allergen protein and development of formulation. Additionally, lyophilization of LAB increases their stability at room temperature (Berlec et al., 2012). All of these characteristics, particularly, the immunomodulating and adjuvant effects as well as high safety profile, make LAB an attractive delivery vehicle for the construction of allergy vaccines. Live bacterial vehicles with intracellular, cell wall or membrane based display of antigens are schematically represented in **Figure 2A**.

Mucosal Delivery of Allergen-Expressing LAB in Food Allergy

In the context of food allergy, oral pretreatment of mice with *L. lactis* strains secreting β-lactoglobulin, the major cow's milk allergen, induced a shift toward T_H1 immune response and reduced the IgE levels. The best results were obtained with the strains that produced the highest amounts of β-lactoglobulin (Adel-Patient et al., 2005). In another study, similar effects on mice were obtained with *Lb. casei* expressing the same allergen (Hazebrouck et al., 2006).

Oral tolerance is an unresponsiveness of gut immune system to innocuous food antigens ingested by oral route. This process is regulated by multiple mechanisms, in which the dose of the antigen has an important role. High doses of antigen induce

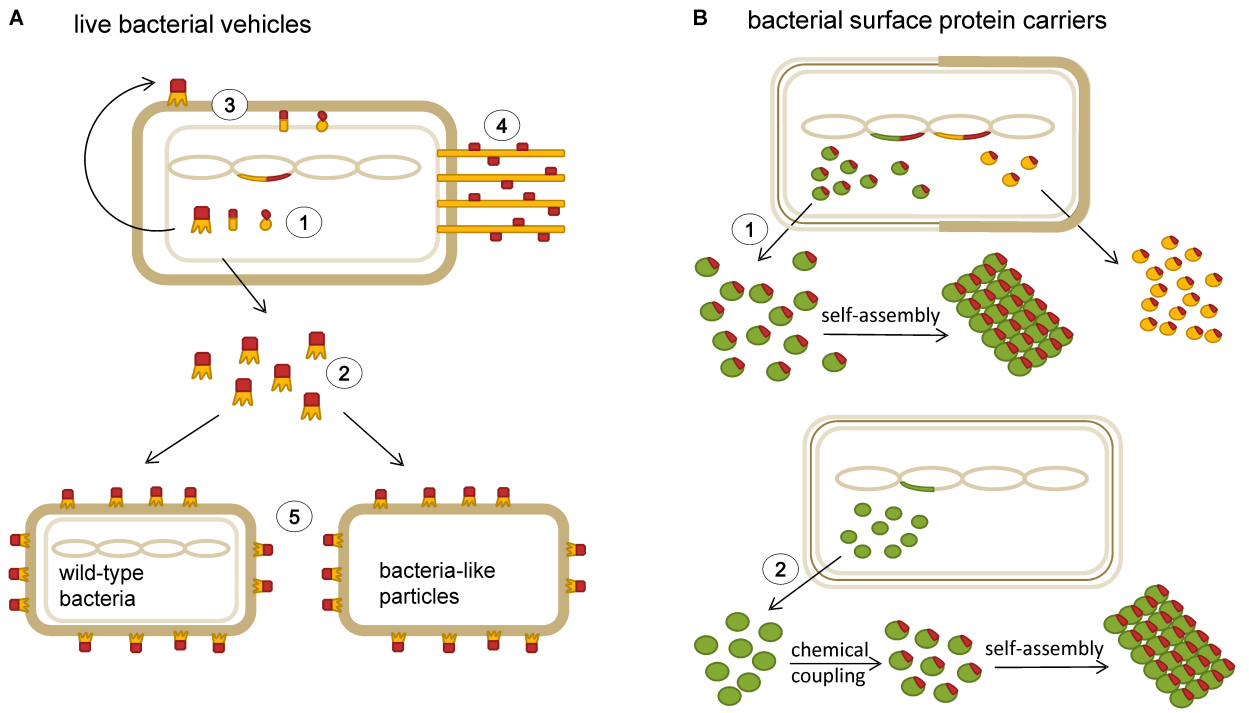

FIGURE 2 | Schematic representation of bacterial delivery vehicles that have been tested for use in allergy immunotherapy. **(A)** Live bacterial vehicles with various modes of delivery of allergens or allergen-derived peptides (red): intracellular expression (1), secretion (2), membrane- and cell wall display (3), pilus-mediated display (4); heterologous display (5). **(B)** Bacterial surface protein carriers; S-layer proteins (green) and albumin binding protein (yellow): recombinant expression of carrier proteins with allergen-derived peptides (1), isolation of surface proteins and chemical coupling to allergens or allergen-derived peptides (2).

deletion or anergy of the T cells recognizing the antigen, whereas low doses induce antigen-specific regulatory T cells, which promote active suppression through secretion of tolerogenic cytokines (such as IL-10 and transforming growth factor β). Induction of regulatory T cells is a major goal for immunotherapy of allergic diseases, and it can be achieved by exposing the mucosal immune system to low doses of antigen (Mowat, 2003). Accordingly, the application of *L. lactis* secreting egg ovalbumin (LL-OVA) to transgenic mice with expressed OVA-specific T cell receptor on CD4+ T cells caused a reduction in delayed-type hypersensitivity responses to OVA. This reduction was mediated by induction of CD4+CD25+ regulatory T cells that secreted transforming growth factor β. Restimulation of splenocytes and gut-associated lymph node tissue from these mice with OVA caused reduced IFN-γ and increased IL-10 production. The effect was achieved with substantially lower doses of OVA (up to 10 μg), secreted in the gut upon repeated oral administration of LL-OVA, compared to that typically used for successful tolerance induction (5 mg). Interestingly, control *L. lactis* carrying the empty vector also suppressed OVA-specific delayed-type hypersensitivity responses albeit to a lesser extent than LL-OVA. Contrary to the control, induction of regulatory T cells was detected only in mice fed with LL-OVA (Huibregtse et al., 2007). The mechanism by which wild-type *L. lactis* suppressed OVA-specific delayed-type hypersensitivity response remains to be explored. Whether administration of *L. lactis* simply mixed with low doses of soluble OVA also induces oral tolerance is worth exploring as it can overcome the need to use recombinant bacteria.

Mucosal Delivery of Allergen-Expressing LAB in Respiratory Allergies

In studies involving inhalational allergies, oral administration of *Lb. plantarum* expressing major dust mite allergen Der p 1 and *Lb. acidophilus* expressing another major dust mite allergen Der p 5 reduced hyperreactivity and inflammation of the airways induced by allergen and decreased the production of specific IgE (Charng et al., 2006; Rigaux et al., 2009). Also, in a murine model of cedar pollinosis *Lb. plantarum* secreting major Japanese cedar pollen allergen Cry j 1 was able to suppress nasal clinical symptoms and allergen-specific IgE response upon oral administrations (Ohkouchi et al., 2012). Similarly, recombinant *L. lactis* and *Lb. plantarum* that produce inhalational allergen Bet v 1 were evaluated for their immunomodulating potential in a murine model of birch pollen allergy. A prophylactic intranasal immunization of mice, caused an increased production of allergen-specific IgA and induced a shift toward T_H1-specific immune response (Daniel et al., 2006). Different routes of application were compared and intranasal application seemed more effective than the intragastric route. Moreover, *Lb. plantarum* was more effective carrier compared to *L. lactis*. This was attributed to lower production of Bet v 1 and shorter intestinal transit time of *L. lactis*

(Daniel et al., 2007). Therefore, the intrinsic immunomodulating capacities of the strain, gut persistence, or both, are important contributing factors. Additionally, cellular location of produced allergen showed influence on the treatment efficacy. The Bet v 1 secreting strain of *Lb. plantarum*, particularly after intranasal pretreatment, led to a stronger reduction of allergen-specific IgE and higher secretion of T_H1-dependent IgG2a compared to the *Lb. plantarum* that produced the allergen intracellularly (Daniel et al., 2007).

Recently, pre-, peri- and/or postnatal periods have been proposed as a critical short-time interval when many factors influence the onset and course of allergic diseases. Several clinical studies have found an association between a reduced number of lactobacilli or bifidobacteria in the early intestinal microbiota of atopic children and the occurrence of allergic diseases later in life (Marschan et al., 2008). Thus, intervening at an early developmental stage seems reasonable targeted preventive strategy to modulate immune responses in a long term. Indeed, this was confirmed in a study conducted by Schwarzer et al. (2011), in which germ-free mice were colonized with the *Lb. plantarum* producing Bet v 1 and did not develop birch pollen allergy later on. The effect was associated with an increased immunoregulatory responses and a shift to a non-allergic T_H1 phenotype. Notably, in this study the wild-type *Lb. plantarum* itself had no suppressive effects on the allergic immune response. The effects depended on the expression of the specific allergen.

Besides allergens, allergen-derived T cell epitope peptides displayed on LAB were evaluated for active vaccination and induction of tolerance in allergy. Mucosal immunization of mice with peptide from the house dust mite followed by treatment with recombinant *Lb. plantarum* expressing an immunodominant T-cell epitope of major dust mite allergen Der p 1 inhibited production of both IFN-γ and IL-5. The effect on IFN-γ was shown to be a non-specific effect of *Lb. plantarum,* while the effect on IL-5 production was observed only when the *Lb. plantarum* expressing Der p 1-derived peptide was used for treatment (Kruisselbrink et al., 2001). Similarly, *L. lactis* was engineered to express peptides containing major T cell epitopes of another major dust mite allergen Der p 2 and their protective effects were evaluated in Der p 2-sensitized BALB/c mice model. Mucosal delivery of these strains reduced specific IgE levels and decreased lung inflammatory responses caused by Der p 2. The study showed an increase of specific IgG2a in serum and a proliferation of regulatory T cells in mesenteric lymph nodes in association with the protective responses (Ai et al., 2015a).

L. lactis produces a cell envelope structural component known as a polysaccharide pellicle, which might restrict accessibility of the heterologous peptide (Chapot-Chartier et al., 2010). This can be circumvented by displaying foreign peptides on the tip of a pilus to expose the peptides at a distance from the cell envelope (see **Figure 2A** for schematic representation) (Quigley et al., 2009). The pilus structure is also highly immunogenic. In the proof-of-principle study, the peptide from the major egg allergen ovomucoid (Ova324–339) was inserted into three different loop regions of the monomeric pilus backbone protein from group A *Streptococcus pyogenes* serotype M1 (PilM1) and expressed in high copy number as a part of pili on the surface of *L. lactis* (LL-PilM1-Ova). Intranasal immunization of mice with LL-PilM1-Ova generated measurable Ova-specific systemic and mucosal responses (IgA and IgG). Notably, Ova-specific IgG or IgA were not detected in serum or mucosal sites when synthetic Ova324–339 was mixed with LL-PilM1. This indicates that the adjuvant property of *L. lactis* alone is not sufficient to induce Ova-specific immunity and suggests that the physical integration of the peptide into the pilus structure is important, probably due to peptide stabilization and prevention of enzymatic degradation (Wagachchi et al., 2018). This method seems to be a promising strategy for display of allergen-derived peptides on the LAB and remains to be compared with other modes of delivery (e.g., intracellular, cell wall anchored).

Taken together, these studies have shown that recombinant LAB pose as efficacious live vehicles that elicit specific and protective immune responses against the allergens or allergen-derived peptides. However, none of these constructs have been tested in human studies thus far. Several disadvantages of the engineered recombinant LAB have prevented their wider use in therapy. In the case of *in vivo* production of therapeutic molecules, the precise dosage is difficult to control. Moreover, the fate of the bacteria in the intestine and pharmacokinetics are difficult to determine (Berlec et al., 2012). Importantly, the major hindrance has been the fear of release of the genetically modified organism into the environment. Even though this has been successfully tackled by the development of containment system for *L. lactis* (Steidler et al., 2003) regulatory authorities will probably prefer the use of killed bacteria or BLPs (Berlec et al., 2012). BLPs are a non-recombinant alternative to live bacteria. They are obtained by treatment with hot trichloroacetic acid, which causes depletion of surface lipoteichoic acids, proteins, and the cytoplasmic content. The remaining intact peptidoglycan layer retains the particle shape similar to that of live cells (van Roosmalen et al., 2006). Because of the lack of recombinant DNA the risk of uncontrolled spreading of genetically modified material into the environment is eliminated. Another interesting approach to avoid the use of recombinant LAB is based on the non-covalent heterologous surface display of fusion proteins on unmodified, wild-type LAB (Hu et al., 2011; Zadravec et al., 2015a,b). In both cases, foreign proteins are produced as fusions with cell-wall binding domains in the recombinant host and are subsequently mixed with either BLPs or unmodified, wild-type LAB (see **Figure 2A** for schematic representation). These platforms allow the simultaneous presentation of several antigens, which may be significant for the production of vaccine candidates composed of several important allergens. Although these non-recombinant display technologies have not yet been tested in allergy immunotherapy, they open up new possibilities for improvement of allergy vaccine formulations.

BACTERIAL SURFACE PROTEINS AS DELIVERY VEHICLES

S-layer Proteins

Bacterial surface S-layers are two-dimensional crystalline arrays of glycoprotein subunits that make up the outermost layer

of many bacteria (**Figure 2B**). S-layers have been shown to possess strong adjuvant properties and represent excellent carrier candidates for immunotherapeutic vaccines (Raha et al., 2005; Sleytr et al., 2014). The general applicability of S-layers as vaccine carriers for treatment of type I allergy was tested using S-layer self-assembly products from *Lysinibacillus sphaericus* or *Thermoanaerobacter thermohydrosulfuricus* chemically conjugated with recombinant Bet v 1 (Jahn-Schmid et al., 1996). In a subsequent study by Jahn-Schmid et al. (1997), T cell lines derived from PBMCs of birch pollen-allergic patients were induced either using recombinant Bet v 1 alone or Bet v 1/S-layer conjugates. After re-stimulation with Bet v 1, T cell lines induced with conjugates showed substantial increase in IFN-γ production compared to T cell lines induced with allergen only. The presence of IFN-γ in the induction phase of T cell lines has been described to lead to a preferential development of T cell clones with a T_H1-like phenotype. Indeed, most of the T cell clones derived from the Bet v 1-induced T cell lines (55%) exhibited a T_H2-like pattern of cytokine production and majority (79%) of the T cell clones established with the Bet v 1/S-layer conjugates revealed T_H1 pattern. In PBMC cultures stimulation with S-layer proteins and Bet v 1/S-layer conjugates but not recombinant Bet v 1 increased production of IL-12, an essential mediator of T_H1 response. This indicates an adjuvant effect of S-layer mediated by IL-12 (Jahn-Schmid et al., 1997).

In the following years, recombinant fusion of the Bet v 1 to S-layer proteins successfully replaced the procedures of chemical coupling. For example, recombinant fusion of Bet v 1 with S-layer proteins, SbpA from *Bacillus sphaericus* and SbsC from *Bacillus stearothermophilus*, yielded two S-layer/allergen recombinant constructs, which showed strongly reduced capacity to bind IgE compared to free Bet v 1 and possessed the ability to induce allergen-specific T_H0/T_H1 and regulatory T cell immune responses (Breitwieser et al., 2002; Ilk et al., 2002; Gerstmayr et al., 2007). Initially, the S-layer/allergen fusion proteins were expressed in Gram-negative host *E. coli* and the associated endotoxin was subsequently removed by purification procedure, which is costly and time-consuming. In a recent study a Gram-positive, non-pathogenic bacteria with naturally high secretory capacity, *Bacillus subtilis*, was tested for expression of the endotoxin-free recombinant protein. The obtained fusion protein consisting of Bet v 1 and S-layer surface protein SbpA from *Lysinibacillus sphaericus* showed excellent recrystallization properties and immune reactivity (Ilk et al., 2011). Bacterial S-layers also proved to be applicable as carriers for the development of a peanut allergen-derived peptides. In a study by Anzengruber et al. (2017), a fusion protein of the S-layer protein SlpB from *Lb. buchneri* and the peptide AH3a42, containing immunodominant B-cell epitopes and one T cell epitope of major peanut allergen Ara h 2, was generated. The fusion protein SlpB-AH3a42 was recognized by IgE from 69% of the allergic patients and did not induce β-hexosaminidase release from sensitized rat basophil leukemia cells. However, IgG antibodies induced by immunization of rabbits with the SlpB-AH3a42 molecule weakly inhibited IgE-binding to the natural Ara h 2 (no more than 30% reduction observed with 20 patient sera) in comparison with the inhibition by anti-Ara h 2 rabbit IgG antibodies (48% reduction). These results indicate that more than one peptide, derived from allergen, would probably be needed to promote wider patient protection.

Albumin Binding Protein

In the study by Ganglberger et al. (2001), the Bet v 1 mimotopes were expressed as fusion proteins with streptococcal albumin binding protein as a monovalent carrier and their antigenicity and allergenicity were examined. The fusion proteins were shown to selectively bind to anti-Bet v 1 human IgE thus demonstrating that the mimotopes fused to albumin binding protein resemble the genuine IgE epitopes. Even though they possess IgE binding structures, the recombinant mimotope-albumin binding protein constructs did not cause skin test reactivity in Bet v 1-allergic mice, indicating that mimotopes of IgE epitopes are safe for immunotherapy when presented in a monovalent form. Furthermore, upon vaccination of BALB/c mice, the constructs were able to induce Bet v 1-specific IgGs that inhibited recognition of Bet v 1 by patients' IgE.

CONCLUSION

Microbial delivery vehicles have been applied in allergy immunotherapy to enhance its efficacy, reduce side effects, and shorten the treatment. Several promising viral and bacterial carriers have been developed and tested. Regarding phage carriers, apart from regulatory constraints, the pre-existing immunity and possible transfer of antibiotic resistance prevent their broader application in spite of the exceptional stability, cost-effectiveness, and ease-of-production. In general, delivery vehicles that are not genetically modified and are not problematic from the regulatory point of view are gaining momentum in today's research. From this perspective, non-recombinant alternatives to GRAS probiotic carriers displaying particularly good performance in mucosal and gastrointestinal delivery are receiving attention. However, none of the proposed whole bacterial or whole viral delivery vehicles have reached the clinical phase of investigation thus far.

Despite numerous applications which have been proposed and their proof-of-principle demonstrated on animal models, only two virus-derived carriers have entered human trials. VLPs (in combination with CpGs) showed suitable clinical tolerance and beneficial immunological and clinical effects. Based on their viral immunomodulatory properties, VLPs in general and CpGs as adjuvants were successfully used in the treatment of allergic rhinitis. However, large controlled studies are needed to collect more extensive clinical experience with this new technology. Hepatitis B virus PreS surface protein is another viral carrier that successfully underwent phase 2b clinical trial, in which it proved

to be safe and effective as a vehicle for subcutaneous delivery of B cell epitope peptides in grass pollen allergy.

In the future, the expression of multiple allergens or allergen-derived peptides on a single carrier will probably be necessary to cover a larger repertoire of the epitopes and to elicit optimal anti-allergic immune responses. This strategy also opens possibilities for patient-specific immunotherapy. Knowledge of the epitopes characteristic for the individual allergic patient, together with technology for development of the appropriate carriers, would allow targeted, personalized therapy. Moreover, the combination of several functional molecules (e.g., delivery vehicle, adjuvant, and allergen; such as VLP/CpG/house dust mite allergens) might be required to maximize vaccine efficiency. Finally, a deeper understanding of cellular and humoral factors involved in immune responses will contribute to optimization of these delivery systems and their faster translation to clinical practice.

AUTHOR CONTRIBUTIONS

ML conceived of the presented idea. AZ reviewed the literature and collected the data. Both ML and AZ contributed to the final version of the manuscript.

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REVIEW
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Lactic Acid Bacteria for Delivery of Endogenous or Engineered Therapeutic Molecules

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Food-grade lactic acid bacteria (LAB) are considered suitable vehicles for the production and/or delivery of health promoting or therapeutic, bioactive molecules. The molecules considered for health-beneficial use include the endogenous effector molecules produced by probiotics (mostly lactobacilli), as well as heterologous bioactives that can be produced in LAB by genetic engineering (mostly using lactococci). Both strategies aim to deliver appropriate dosages of specific bioactive molecules to the site of action. This review uses specific examples of both strategies to illustrate the different avenues of research involved in these applications as well as their translation to human health-promoting applications. These examples pinpoint that despite the promising perspectives of these approaches, the evidence for their effective applications in human populations is lagging behind.

Keywords: lactic acid bacteria, probiotics, *Lactococcus lactis*, *Lactobacillus*, genetic engineering, therapeutic molecules

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LAB FOR FOOD-GRADE BIOACTIVE DELIVERY

Lactic acid bacteria (LAB) have a long history of safe consumption in the form of fermented food products and as a consequence are considered food-grade. LAB encompass various bacterial genera, including *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*. The safety status of these bacteria has inspired their use for the production and delivery of bioactive molecules that intend to improve consumers' health. An important concept in this domain is the use of probiotics that are live microorganisms that when administered in adequate amounts confer a health benefit on the host (Hill et al., 2014), a domain dominated by species of the *Lactobacillus* genus. An alternative approach employs LAB for the engineered production and/or delivery of heterologous bioactive molecules, which mostly employs *Lactococcus lactis* as a production host, because of its extensive genetic toolbox. Both conceptual approaches are illustrated in this review through specific examples that in our opinion are closest to actual application as health promoting concepts. This highlights the impressive potential of using these bacteria for bioactive delivery, but also exemplifies the complexity of translation toward predictable effects in human applications.

THE PROBIOTIC LACTOBACILLI

Lactobacillus species encompass a group of LAB that are encountered in a diverse range of nutrition-rich environments, including the human and animal gastrointestinal tract, plants, as well as food and feed (Duar et al., 2017). Specific *Lactobacillus* strains are marketed as probiotics.

An intrinsic characteristic of these probiotics lactobacilli is that they produce and deliver (specific) health promoting effector molecules *in situ* that modulate host physiology in a health-promoting direction. For many probiotic lactobacilli the molecular mechanism by which they impact host health remains largely unknown. However, in depth molecular research on specific probiotic strains has generated an impressive knowledgebase of the effector molecules of these strains that play a role in the modulation of specific host pathways. Many of the probiotic effector molecules that have been described appear to reside in the bacterial cell-envelope (Bron et al., 2011, 2013; Lebeer et al., 2018). Since the research related to probiotic effector molecule discovery has recently been reviewed, we do not expand this section beyond the exemplary case discussed below. The chosen example is in our opinion among the best-described in this field, and includes technological improvements by encapsulation for *in situ* delivery of the probiotic bacteria and/or their effector molecules (Li et al., 2016).

The major secreted proteins P40 and P75 produced by the extensively studied probiotic strain *Lactobacillus rhamnosus* GG were demonstrated to modulate epidermal growth factor receptor (EGF-R) activity in epithelial cells, thereby modulating Akt activation. This interaction was shown to inhibit cytokine-induced apoptosis in epithelial cells *in vitro*, and to promote epithelial cell growth *ex vivo* in human and mouse colon-tissue explants (Yan et al., 2011; Yan and Polk, 2012). Importantly, EGF-R modulation by P40 protected mice against chemically induced colitis (Yan et al., 2011; Yan and Polk, 2012). Notably, homologues of these two secreted proteins are present in other *L. rhamnosus* strains as well as in the related species *Lactobacillus casei*, implying that the effects reported for *L. rhamnosus* GG could potentially also be achieved with related strains (Bauerl et al., 2010). The same effector molecules probably underlie the observation that *L. rhamnosus* GG is able to stimulate epithelial wound healing in *in vitro* scratch assays using both skin- and gingival- human epithelial-cell lines (Mohammedsaeed et al., 2015; Fernandez-Gutierrez et al., 2017). Interestingly, *in vivo* transcriptome responses in human duodenal tissues following *L. rhamnosus* GG consumption revealed significant modulation of epithelial wound healing pathways in the human intestinal mucosa (van Baarlen et al., 2011), indicating the congruency of these molecular interactions in different model systems. However, the connection of these conserved responses to reliable health benefits in human populations remains highly challenging. This may be due to the extensive degree of human individuality and the corresponding individualized physiological relevance of such cellular modulations, which could predominantly depend on the baseline molecular state of an individual. This conceptual view was previously coined as the "band-width of health" and may represent a principal hurdle in the reliable application of probiotics and/or their effector molecules in human (Bron et al., 2011; van Baarlen et al., 2011; Klaenhammer et al., 2012).

ENGINEERED LACTOCOCCI

For specific diseases it is well established which human proteins are underrepresented in the diseased population and/or have an established role in therapy. LAB engineered to produce such heterologous bioactive and/or therapeutic molecules provide an approach to delivering such molecules *in situ* as live bacterial therapeutics, or may serve as a production host to obtain these molecules in purified form. General genetic engineering capacities and in particular the controlled gene expression toolboxes available for the lactobacilli are lagging behind those that have been developed for the paradigm LAB *L. lactis* (Kok et al., 2017). As a consequence, the majority of these engineering approaches for the production (and delivery) have employed *L. lactis* as a production host. A lot of effort has been invested in the production and delivery of vaccine-antigens and allergens by engineered lactococci, and since this area has recently been reviewed (Wyszynska et al., 2015; Wang et al., 2016; Szatraj et al., 2017) we focus on some specific examples of alternative categories of bioactive molecules, including bacterial enzymes and human factors known to modulate mucosal physiology and/or immune function. The examples presented were chosen on basis of available scale-up and commercialization strategies and employ *L. lactis* as a production host.

LACTOCOCCAL PRODUCTION OF AN ANTIMICROBIAL ENZYME

Staphylococcus simulans harbors *lss* which encodes a 25 kDa antibacterial protein termed lysostaphin that hydrolyzes the Gly–Gly bond present in the cell wall of *Staphylococcus aureus*, thereby lysing this important pathogen (Thumm and Gotz, 1997). Lysostaphin administration in a nasal spray effectively reduces *S. aureus* carriage, supporting the prevention of spreading of this bacterium in hospital environments (Quickel et al., 1971). Biosynexus Inc. is involved in development of protein-based pharmaceutical products aiming to ameliorate staphylococcal infections in infants, including lysostaphin. To provide a suitable production system for this selective antimicrobial enzyme, the encoding *lss* gene was cloned under control of the P_{nisA} promoter and introduced in the expression host-strain NZ3900, allowing food-grade, lactose-based plasmid selection and induction of lysostaphin production through the NICE system (Mierau and Kleerebezem, 2005; Mierau et al., 2005). Following optimization of lysostaphin production at 1 L scale it was shown to be readily scalable to 300 and 3000 L production facilities. In addition, the protein could be purified from the lactococcal culture supernatant via straightforward and industrially scalable chromatography procedures (Mierau et al., 2005). This example

illustrates that *L. lactis* is a suitable host for the production and purification of relevant bioactive enzymes, in this case an anti-*Staphylococcus* agent. Purification of the compound of interest. avoids the consequences of releasing genetically modified *L. lactis* strains and is suitable for bioactive molecules that do not depend on the presence or co-modulatory effects of the production host in the application.

LIVE-BIOTHERAPEUTICS USING ENGINEERED *L. lactis*

Intrexon develops so-called Actobiotics, a class of orally delivered biopharmaceuticals employing engineered and biologically contained *L. lactis* production hosts for the *in situ* production and delivery of various proteins and peptides in the oral and gastrointestinal tract of humans. The selection of the bioactives to be produced within this delivery system aim to provide therapies against oral, gastrointestinal, metabolic, allergic, and autoimmune diseases, focusing on bioactives with known pharmacological activity and that are supported by documented efficacy and safety information. The company's ambition is strongly based on the landmark study that demonstrated that chemically induced colitis in mice could be effectively treated with *L. lactis* strains that produce interleukin-10 (IL-10) (Steidler et al., 2000). Later work illustrated that the *L. lactis* based intestinal IL-10 delivery could also contribute to reduction of food-induced anaphylaxis in mice models, which may support the prevention of IgE-type sensitization to common food allergens (Frossard et al., 2007). Likewise, IL-10 delivery combined with secretion of the type-1 diabetes (T1D) auto-antigen GAD65370-575 by the same *L. lactis* strain was effective in suppressing T1D in a preclinical NOD-mouse model (Robert and Steidler, 2014; Robert et al., 2014). Besides these IL-10 producing *L. lactis* strains and their derivatives, the same platform for production and delivery of bioactives is employed for a variety of other molecules intending to provide live lactococcal therapeutics for a range of human and animal diseases (Vandenbroucke et al., 2004; McLean et al., 2017). Moreover, some comparative work using similar IL-10 producing *Lactobacillus* strains has been reported and may expand or enhance the application potential of these concepts (Steidler, 2003). Besides the bioactive production and delivery technology, the Actobiotics platform is supported by effective strategies for the biological containment of genetically engineered bacteria as well as scalable production and processing procedures for the live biotherapeutic bacteria (Steidler, 2003). However, initial human trials in patients suffering from inflammatory bowel disease established the safety and biological containment of the IL-10 producing lactococcal delivery vehicle (Braat et al., 2006), but failed to deliver convincing clinical efficacy evidence for disease symptom reduction to date, illustrating that translation of the mouse-model outcomes to human applications still requires further investigation.

Aurealis Pharma is a pharmaceutical company that employs genetically modified *L. lactis* strains for the delivery of combinations of therapeutic proteins to diseased tissues. For example, *L. lactis* MG1363 was engineered to allow plasmid-based expression of three genes encoding human fibroblast growth factor 2 (FGF-2), interleukin 4 (IL-4), and colony stimulating growth factor 1 (CSF-1) that were selected based on their functional properties (Yun et al., 2010; Hume and MacDonald, 2012; Sica and Mantovani, 2012) and availability of clinical safety data. An animal study demonstrated the efficacy of the combination therapy provided by the three proteins and the *L. lactis* strain in a delayed wound healing model in diabetic mice[1]. Aurealis Pharma is currently producing a clinical grade product for a clinical phase 1 trial to build a safety dossier for the use of this live bacterial therapeutic in diabetic foot ulcer patients.

PERSPECTIVES

This short review illustrates that significant advances have been made in understanding the mechanism of action of endogenous probiotic *Lactobacillus* effector molecules, and that heterologous production of therapeutic bioactives in engineered lactococci has reached technological maturity. The examples chosen employed in this review are actively pursued by commercial initiatives and involve either purified bioactives or the use of the engineered LAB as live biotherapeutic.

However, in both strategies, the translation of the health-beneficial effects toward reliable human application is lagging behind despite the consistent findings across the different layers of the translational pipeline progressing from molecular insight in molecular interactions involved toward their conservation in *in situ* human response. Lagging efficacy evidence may be largely due to human individuality in health and disease, which implies that careful consideration should be given to selecting the appropriate target population. This may require novel strategies toward subject stratification and responsiveness prediction to better select and treat human subjects that are likely to have a perceivable benefit from the response elicited by the bioactive supplied.

AUTHOR CONTRIBUTIONS

Both authors conceived the review, wrote it together and decided to submit it to this Research Topic.

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Anti-tumoral Effects of Recombinant *Lactococcus lactis* Strain Secreting IL-17A Cytokine

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Interleukin-17A (IL-17A) is a pro-inflammatory cytokine produced by T_{H17} cells that participates and contributes in host defense and autoimmune disease. We have recently reported antitumor properties of the probiotic strain of *Lactobacillus casei* BL23 in mice and T_{H17} cells was shown to play an important role in this beneficial effect. In order to better understand the role of IL-17A in cancer, we constructed a recombinant strain of *Lactococcus lactis* producing this cytokine and we determined its biological activity in: (i) a bioassay test for the induction of IL-6 production by murine fibroblasts 3T3 L1 cells line and (ii) in a mouse allograft model of human papilloma virus (HPV)-induced cancer. Our data show that recombinant *L. lactis* produces and efficiently secretes biologically active IL-17A cytokine. Interestingly, ∼26% of mice intranasally treated with *L. lactis*-IL-17A and challenged with TC-1 cells remained tumor free over the experiment, in contrast to control mice treated with the wild type strain of *L. lactis* which developed 100% of aggressive tumors. In addition, the median size of the ∼74% tumor-bearing mice treated with recombinant *L. lactis*-IL-17A, was significantly lower than mice treated with *L. lactis*-wt. Altogether, our results demonstrate that intranasal administration with *L. lactis* secreting IL-17A results in a partial protection against TC-1-induced tumors in mice, confirming antitumor effects of this cytokine in our cancer model.

Keywords: ***Lactococcus lactis*, lactic acid bacteria, IL-17A, cancer, HPV**

INTRODUCTION

Cancer remains a serious health concern in human society worldwide and colorectal cancer (CRC), prostrate, lung, stomach, liver and breast cancers are among the major types associated with significant mortality every year (Ferlay et al., 2012). Cancer is generally considered to be a disease involving both host genetics and environmental factors; however microorganisms (such as viruses and bacteria) are associated in ∼20% of human cancers (de Martel et al., 2012). Recent studies suggest that probiotics can help to fight cancer. Probiotics are live

microorganism which, when administered in adequate amounts confer a health benefit on the host (Food and Agriculture Organization, 2002). For instance, probiotics can induce dendritic cells (DC) maturation (Delcenserie et al., 2008), enhance natural killer (NK) cells cytotoxicity (Takagi et al., 2001), and upregulate cytokine secretion (Delcenserie et al., 2008; Azcarate-Peril et al., 2011). It has also been reported that some strains of *Lactobacillus* can induce DC maturation and T_{H1} (antiviral and bacterial immunity) and T_{H17} (inflammation and auto-immunity) differentiation (Kemgang et al., 2014; Cai et al., 2016; Lee et al., 2016). However, despite the great number of studies that have demonstrated anti-cancer effects of different strains of *Lactobacillus* (Khazaie et al., 2012; Konishi et al., 2016; Lenoir et al., 2016), the precise host molecular mechanisms of these antitumor properties remain unclear. Next generation probiotics, such as *Akkermansia muciniphila* and *Faecalibacterium* genus as well as genetically modified microorganisms (GMOs) (O'Toole et al., 2017) have demonstrated beneficial effects in the context of cancer, promoting the immune checkpoints inhibitors therapy targeting the programmed cell death protein 1 (PD-1) and cytotoxic lymphocyte-associated antigen (CTLA-4). In addition, other studies support the role of *Bifidobacterium, Bacteroides*, *Faecalibacterium* and *Akkermansia* species in cancer therapy targeting the immune checkpoint blockade (CTLA-4, PD-1), showing a T cell-specific anti-tumor-induced response (Sivan et al., 2015; Vetizou et al., 2015; Gopalakrishnan et al., 2018; Routy et al., 2018).

We previously demonstrated that mucosal administration of the probiotic strain of *Lactobacillus casei* BL23 displays anti-tumor properties in three different murine models of cancer (Lenoir et al., 2016; Jacouton et al., 2017). Interestingly, we showed that this strain was able to modulate a T-cell immune response toward a T_{H17}-biased immune response, accompanied by the expression of regulatory cytokines (e.g., IL-6, IL-17, IL-10, and TGF-β), in a murine model of CRC (Lenoir et al., 2016). In particular we were intrigued by IL-17 induction, since IL-17 seems to be essential for both metastasis and elimination of tumor cells (Murugaiyan and Saha, 2009). Thus, IL-17-producing T_{H17} cells have recently gained considerable importance in cancer (Maniati et al., 2010). Therefore, we hypothesized that IL-17-induced by *L. casei* BL23 could play an important role in the anti-tumor effect of this probiotic strain. We thus decided to use a genetically modified strain of *Lactococcus lactis*, the model lactic acid bacterium (LAB), to produce and deliver exogenous murine IL-17 and to determine its anti-tumor effect in a mouse allograft model of human papilloma virus (HPV)-induced cancer.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

L. lactis MG1363 (Gasson, 1983) was grown in M17 medium (Difco Laboratories, England) supplemented with 0.5% glucose (GM17) and 15 μg/ml of chloramphenicol at 30°C without agitation.

Construction of a Recombinant Strain of *L. lactis* Secreting Murine IL-17

To construct a vector which will allow stress-inducible IL-17 expression in *L. lactis* a DNA fragment encoding IL-17 mature sequence was obtained from a recombinant plasmid containing murine *il-17* gene (synthesized by Geneart, Invitrogen) with *Nsi*I/*Eco*RI enzymes. As previously described (Benbouziane et al., 2013), we used pLB333 vector containing *nucB* gene under the control of the stress inducible *groESL* promotor. pLB333 was digested with the same enzymes to replace *nuc* gene by *il-17* gene. The resulting vector, pSICE:IL-17 (**Figure 1A**), was established into *L. lactis* MG1363 strain to obtain *L. lactis*-IL-17. For detection of IL-17, *L. lactis*-IL-17 strain (*L. lactis-wt* was used as negative control) was grown overnight (ON, optical density $(OD)_{600nm}$ = 2.0–2.5) as described above. Plasmid DNA isolation and general procedures for DNA manipulation were essentially performed as described previously (Sambrook et al., 1989). PCR amplification was performed using High Fidelity PCR Enzyme Mix (Fermentas) with a thermal cycler (Applied Biosystem). DNA sequences were confirmed by sequencing (MWG-Genomic Company, Germany).

In vitro Validation of IL-17 Production and Secretion by Recombinant *L. lactis*

Over-night cultures were washed twice using PBS and culture pursued (after a 1:10 dilution in GM17 medium) until OD_{600nm} ∼0.6. Then, cultures were induced with 2.5% NaCl for 30 min and protein samples prepared from 2 ml of the induced cultures. After centrifugation (10 min, 17500*g*), the cellular pellet (C) and supernatant (S) were treated separately. The S samples were precipitated with 200 μl of trichloroacetic acid (TCA) 100% for 1 h on ice to recover proteins (centrifugation at 17500*g* at 4°C for 30 min) and resuspended in 200 μl of 50mM NaOH. The C fraction was resuspended in 200 μl of PBS plus protease inhibitors (Roche) and sonicated 30 s with alternated pulses on ice (on: 5 s, off: 30 s). Protein samples were diluted 1:1 in Laemmli sample buffer containing 355 mM β-mercaptoethanol and denaturated 5 min at 95°C. Equal amounts of proteins were loaded and separated on a Mini-PROTEAN TGX stain free 4–20% SDS gel at 200 V and further transferred to a PVDF membrane using a Trans-Blot Turbo transfer system (Biorad). Membrane was probed with primary antibody anti-mouse IL-17A (R&D Systems) and secondary anti-rat IgG HRP-conjugated antibody (Abliance) at 1:1000 dilutions. Bound secondary antibody was visualized by the Clarity ECL Western Substrate (Bio-Rad) and Chemidoc imaging system (Biorad). The concentration of IL-17A secreted in the medium was assessed by ELISA (mouse IL-17 ELISA Development Kit, Mabtech).

Determination of the Biological Activity of IL-17 Produced by Recombinant *L. lactis*

Murine fibroblasts 3T3 L1 cells line, grown in DMEM medium (Lonza, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin and 50 U/ml streptomycin (Lonza, Levallois-Perret, France) were cultivated

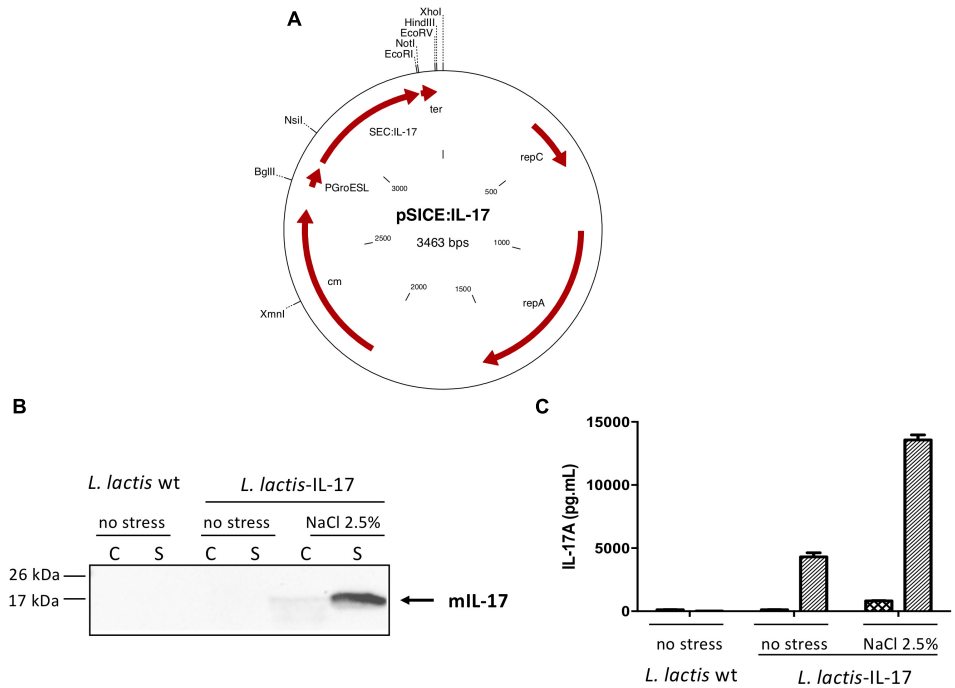

FIGURE 1 | IL-17 expression by *L. lactis*. **(A)** Schematic representation of pSICE:IL-17 plasmid. Protein samples (C and S) were prepared from both non-stressed and stressed *L. lactis*-wt and *L. lactis*-IL-17 cultures and IL-17 production was assessed by **(B)** Western blot and **(C)** ELISA. Position and size of molecular mass markers is indicated on the left. The position of mature murine IL-17 is given by an arrow. Values are mean ± SEM.

at 1×10^5 cells per well during 24 h at 37°C, 5% CO_2. Then, medium was changed and bacterial preparations added at 10% for supernatants, pellet and control medium or MOI 100 for bacteria suspensions during 24 h. Supernatants of co-incubations were collected and stored at −80°C before ELISA analyses (mouse IL-6 DuoSet ELISA, R&D).

Mice and TC-1 Cell Line

Specific pathogen-free C57BL/6 mice (females, 6–8 weeks old; Janvier SAS, St. Berthevin, France) were housed in a pathogen-free isolator ($n = 4$ mice per cage) under sterile conditions in 12-h light cycles in the animal facilities of the French National Institute for Agricultural Research (INRA, IERP, Jouy-en-Josas, France). Animals were supplied with water and fed *ad libitum* (normal chow: R 03-40, SAFE). Temperature and moisture were carefully controlled. Mice were observed once a day to ensure their welfare. All protocols were carried out in accordance with the institutional ethical guidelines of the ethics committee COMETHEA (Comité d'Ethique en Expérimentation Animale of the Centre INRA of Jouy-en-Josas and AgroParisTech), which approved this study.

The mouse (C57BL/6) lung tumor cell line TC-1 (generated by transduction with a retroviral vector harboring HPV-16 E6/E7 genes plus a retrovirus expressing activated human oncogene c-Ha-*ras* (Lin et al., 1996)) was grown in RPMI medium 1640 (Lonza, Switzerland) supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin, 50 U/ml streptomycin (Lonza, Levallois-Perret, France), 0.4 mg/ml G418 and 0.2 mg/ml hygromycin in 5% CO_2 atmosphere.

TC-1 Cell Line Challenge and Bacteria Administration

Groups of mice ($n = 22$ from 3 independent *in vivo* experiments) were intranasally (*i.n.*) administered using a micropipette with 1×10^9 colony-forming units (CFU) of either *L. lactis*-wt or *L. lactis*-IL-17 strain (suspended in 10 µl of PBS). ON cultures were washed two times and finally suspended in PBS at 1×10^{11} CFU/ml. Each mouse received 5 µl of the solution in each nostril on days −35, −21, and −7. Control mice received identical quantities of PBS (i.e., 10 µl). Mice were challenged 7 days after the final bacterial administration (D0) by subcutaneous (*s.c.*) injection in the right rear flank with 5×10^4 TC-1 cells in 100 µl of sterile PBS. The dimensions of the tumor at the site of injection were measured every week in two perpendicular directions with a caliper, and tumor volume was estimated as (length × width^2)/2 (Bermudez-Humaran et al., 2005). Mice were sacrificed by vertebral dislocation at D28.

Analysis of the Immune Response in Mice Treated With Recombinant *L. lactis* and Challenged With TC-1 Cells

Mice were euthanized at D28 and spleens collected and isolated via gentle extrusion of the tissue through a 50-µm-mesh nylon cell strainer (BD). Cells were resuspended in DMEM medium supplemented with 10% FCS, 2 mM L-glutamine, 50 U/mg penicillin and 50 U/mg streptomycin. Erythrocytes

were lysed with red-blood-cell lysing buffer (Sigma-Aldrich). For stimulation experiments, 1×10^6 cells per well were stimulated for 48 h (37°C, 10% CO2) in DMEM medium in P24 plates in presence of PMA (phorbol 12-myristate 13-acetate) ionomycin cocktail 1× (eBioscience). Culture supernatant was frozen at −80°C until processing. Levels of the cytokines IL-6 (mouse IL-6 DuoSet ELISA, R&D), IL-17A, and IFN-γ (ELISA Development Kit, Mabtech) were determined using ELISA according manufacturer's instructions.

Statistical Analysis

All data are expressed as mean values and standard deviations. Data analysis was performed using the GraphPad Prism Software V.5.00. Experiments were analyzed using an unpaired *t*-test. The two-tailed unpaired Mann–Whitney test was used to evaluate differences between two groups. In all experiments, a value of $P < 0.05$ was considered significant ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

RESULTS

Characterization of IL-17 Production by Recombinant *Lactococcus lactis*

Before to test the biological effect of the genetically modified strain of *L. lactis* harboring pSICE:IL-17 plasmid (**Figure 1A**), we first analyzed IL-17 production and secretion from both non-stressed and stressed *L. lactis*-wt and *L. lactis*-IL-17 cultures by Western blot (**Figure 1B**). A band of approximately 15 kDa was detected in the supernatant (S) fraction from induced cultures of *L. lactis*-IL-17 strain, which corresponds to secreted mature murine IL-17. IL-17 secretion and quantification was then determined by ELISA in C and S samples. As shown in **Figure 1C**, a better production of IL-17 was observed (∼3-fold) under stress conditions (i.e., NaCl 2.5%): ∼15,000 pg/ml versus ∼5000 pg/ml. As expected no IL-17 signal was detected in the negative control *L. lactis*-wt.

Recombinant *Lactococcus lactis* Secretes a Biologically Active IL-17 Cytokine

Besides IL-17 detection in S samples of bacterial cultures, we determined the biological activity of this cytokine secreted by recombinant *L. lactis*. IL-17A is known to stimulate several cytokines (including IL-6) in different cell lines (such as fibroblast, epithelial cells and immune cells). Thus, we selected murine fibroblasts 3T3 L1 cells to assess specific IL-6-induction by recombinant *L. lactis*. Our results showed that S samples of *L. lactis*-IL-17 strain and stressed with NaCl 2.5% induced a significant IL-6 secretion in 3T3 L1 cells (**Figure 2A**) in comparison with their respective negative control. No significant IL-6 production was observed in bacterial cultures without stress induction. In parallel we confirmed by ELISA the presence of IL-17 cytokine in S samples of recombinant bacteria (**Figure 2B**).

L. lactis IL-17 Has a Protective Effect Against Tumors in TC-1 Allograft Model of HPV-Induced Cancer

To further evaluate *in vivo* the biological activity of IL-17 produced by recombinant *L. lactis,* and in particular the impact of this cytokine in the TC-1 mouse allograft model of HPV-induced cancer, we analyzed the effect after *i.n.* administration of this strain in the TC-1 tumor mice. Mice were immunized as described in Material and Methods and tumor absence/presence monitored every week. As shown in **Figure 3A**, *L. lactis* IL-17 displayed a protective effect against tumor development at D28 (the end of the experiment) since 77% (5/22) of mice that had been administered *L. lactis* IL-17 developed tumors with a mean tumor size of ∼0.80 cm^3 (**Figure 3B**) compared to 100% (22/22) of mice receiving *L. lactis-wt* control strain (mean tumor size of ∼1.2 cm^3) (**Figures 3A,B**). These results confirm

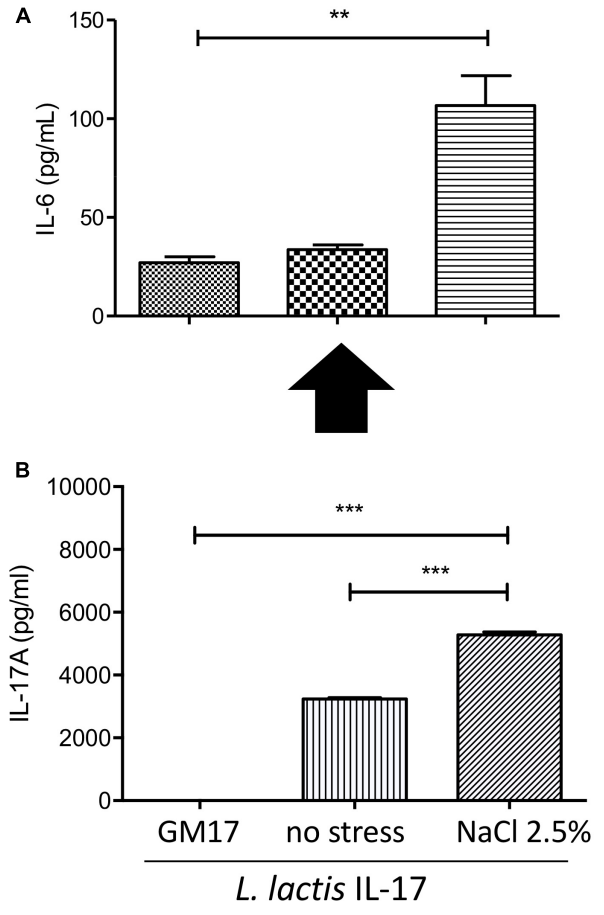

FIGURE 2 | Cellular-based bioassay for *L. lactis*-secreted bioactive IL-17. **(A)** IL-6 secretion by 3T3 L1 cells after exposure to supernatant samples of recombinant *L. lactis*. **(B)** Quantification of IL-17 production by recombinant lactococci by ELISA. Data are represented as mean ± SEM of two independent *in vitro* assays. GM17 was used a control for supernatant conditions. Stress was induced with NaCl 2.5%. Data were treated by ANOVA (Turkey post-test).

that IL-17 cytokine display anti-tumor effects in our cancer model.

L. lactis IL-17 Induces IL-6 and IL-17 Secretion in Reactivated Splenocytes From Mice Challenged With the Tumoral Cell Line

In order to further explore the impact on the immune response of mice treated with recombinant *L. lactis* IL-17 we analyzed cytokines release by reactivated splenocytes from mice 28 days after *i.n* administration of recombinant bacteria and challenged with TC-1 cells. Interestingly, *L. lactis* IL-17 induced a significant IL-6 secretion in splenocytes compared to *L. lactis*-wt (**Figure 4**). This modulation was correlated with a slight IL-17 induction but without reach statistical significance (**Figure 4**). No effect was observed on IFN-γ production by recombinant bacteria (**Figure 4**).

DISCUSSION AND CONCLUSION

A better understanding of the interactions between cancer cells and stromal components in the tumor associated pro-inflammatory microenvironment would be important for the management of this disease (Ferlay et al., 2012). Anti-tumor response involves different components of the immune system, such as NK cells, DC, macrophages and T cells. A growing body of evidence suggests that probiotics can help to combat cancer by either protecting against gastrointestinal infections or enhancing immune response. Indeed, it has been shown that probiotics can induce DC maturation (Delcenserie et al., 2008), enhance NK cell cytotoxicity (Takagi et al., 2001), and upregulate cytokine secretion (Delcenserie et al., 2008; Azcarate-Peril et al., 2011). In addition, recent studies described the role of specific members of microbiota in cancer therapy by targeting the immune checkpoint blockade (CTLA-4, PD-1) (Sivan et al., 2015; Vetizou et al., 2015; Gopalakrishnan et al., 2018; Routy et al., 2018).

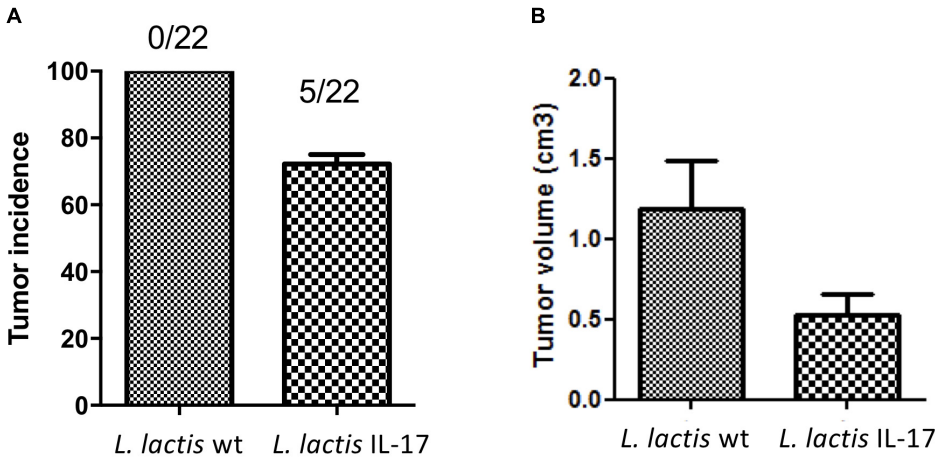

FIGURE 3 | Effect of recombinant *L. lactis* expressing IL-17 against tumors in TC-1 allograft model of HPV-induced cancer. Mice were *i.n.* treated with 1×10^9 CFU resuspended in 10 μl of PBS (5 μl were administered with a micropipette into each nostril) on D-35, -21, and -7. Seven days after the last administration (D0), a challenge with the tumoral cell line TC-1 was performed, and the presence and size of the tumor was monitored once a week. **(A)** Tumor incidence with proportions of tumor-free animals **(B)** Individual tumor volume at the end of week 10. Data represented mean ± SEM from 3 independent *in vivo* experiments.

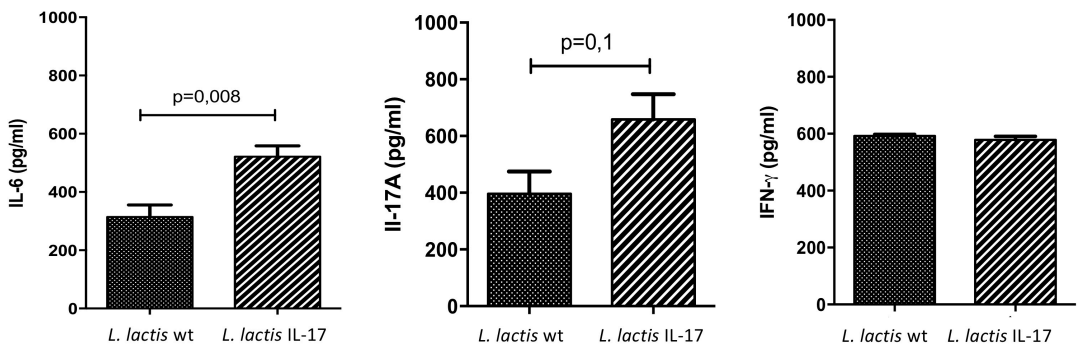

FIGURE 4 | Cytokines production by reactivated splenocytes from mice treated with recombinant bacteria and challenged with TC-1 cells. Splenocytes were stimulated for 48 h with PMA ionomycin before measuring cytokines levels. Data represented mean ± SEM from 4 (*L. lactis* wt) to 8 mice (*L. lactis* IL-17). Data are analyzed with unpaired *t*-test followed by Mann–Withney post-test.

Among the potential anti-tumoral mechanisms of probiotics, two of the most known are the modulation of the immune response and the induction of cellular apoptosis. For instance, two strains of *L. casei* are able to decrease tumor cell proliferation and enhance apoptosis in allograft models of CRC (Lee et al., 2004; Baldwin et al., 2010; Konishi et al., 2016). Similarly, oral administration of a *L. casei* strain reduces the onset of chemically induced tumors via the stimulation of IL-12 or NK-cell cytotoxicity mechanisms (Takagi et al., 2001, 2008). Furthermore, our team recently demonstrated protective effects of the probiotic strain *L. casei* BL23 in three different mouse models of cancer, including CRC (Lenoir et al., 2016; Jacouton et al., 2017) and the TC-1 allograft model (Lenoir et al., 2016). In one of our two CRC models (Lenoir et al., 2016), the anti-tumor effects of *L. casei* BL23 were associated with the reduction of pro-inflammatory cytokines, but the precise molecular and cellular mechanisms involved in tumor prevention of this bacterium remain unclear. Since cancer therapy includes chemotherapy, drug, vaccines, and cytokines, and for instance, current therapies are toward to enhance the immune system as use of pro-inflammatory cytokines (such as IL-2, one of the first cytokines used in cancer therapy) and immune check points inhibitors (CTLA-4, PD-1). In this work, we constructed a recombinant strain of *L. lactis* expressing IL-17. Strikingly, we showed that *i.n.* administration of this strain results in a lower tumor incidence and that tumor size was reduced in comparison to the control *L. lactis-wt*, a LAB strain for which no positive effect has been reported in the HPV-induced cancer model. Our results not only suggest a positive effect of IL-17 but also reinforce the idea that some of the molecular mechanisms of *L. casei* BL23 against cancer could be related to activation of T_H and NK *via* T_{H17}. IL-17 is a pro-inflammatory cytokine, although its role is controversially, some studies report that IL-17 deficiency state may have a protective role or a harmful role in tumorigenesis (Welch et al., 2015; Qian et al., 2017). For example, in IL-17 deficient mice, enhanced lung and subcutaneous tumor growth and metastasis is correlated to a decrease in the number of IFN-γ producing NK cells (Kryczek et al., 2009). Recent research provided substantial insights into the mode of action of IL-17 cytokines in a variety of tumors, suggesting an anti-tumor activity of IL-17 could be achieved by means of a T cell-dependent mechanism increasing generation of specific cytotoxic T lymphocytes (Alshaker and Matalka, 2011). Paradigms are changing, and IL-17 cytokines are double-edged agents acting in a cancer-type depending manner as anti- and pro-tumor cytokines (Fabre et al., 2016). IL-6 is a pro-inflammatory cytokine involved, in part, in a TH17-related immune response with a feedback loop. Thus, we assessed the *in vivo* ability of *L. lactis* secreting IL-17 to stimulate the TH17 pathway. Here, we demonstrated an *in vivo* induction of IL-6 resulting in an increase of IL-17. We hypothesized that the anti-tumoral effect of recombinant lactococci secreting biologically active IL-17 could due to a TH17 immune response even if more experiments are needed to further decipher the precise molecular mechanisms.

In conclusion, our results demonstrate that *i.n.* administration with a genetically modified strain of *L. lactis* secreting IL-17 results in a partial protection against TC-1-induced tumors in mice, confirming antitumor effects of this cytokine in this model.

AUTHOR CONTRIBUTIONS

EJ and LB-H conceived and designed the study. EJ, ETM, and A-SB conducted all experiments. NP, APV, IN, FC, and VA contributed analytic tools. EJ, ETM, PL and LB-H performed the data analysis. ETM and LB-H wrote the manuscript.

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A New Bifidobacteria Expression SysTem (BEST) to Produce and Deliver Interleukin-10 in *Bifidobacterium bifidum*

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In the last years there has been a growing interest in the use of genetically modified bacteria to deliver molecules of therapeutic interest at mucosal surfaces. Due to the well-recognized probiotic properties of some strains, bifidobacteria represent excellent candidates for the development of live vehicles to produce and deliver heterologous proteins at mucosal surfaces. However, very few studies have considered this genus because of its complexity to be genetically manipulated. In this work, we report the development of a new Bifidobacteria Expression SysTem (BEST) allowing the production of heterologous proteins in *Bifidobacterium bifidum*. This system is based on: i) the broad host range plasmid pWV01, ii) a stress-inducible promoter, and iii) two different signal peptides (SPs) one issued from *Lactococcus lactis* (SP_{Exp4}) and issued from *Bifidobacterium longum* (SP_{BL1181}). The functionality of BEST system was validated by cloning murine interleukin-10 (IL-10) and establishing the resulting plasmids (i.e., $pBEST_{Exp4}$:IL-10 and $pBEST_{BL1181}$:IL-10) in the strain of *B. bifidum* BS42. We then demonstrated *in vitro* that recombinant *B. bifidum* BS42 harboring $pBEST_{BL1181}$:IL-10 plasmid efficiently secreted IL-10 and that this secretion was significantly higher (sevenfold) than its counterpart *B. bifidum* BS42 harboring $pBEST_{Exp4}$:IL-10 plasmid. Finally, we validated *in vivo* that recombinant *B. bifidum* strains producing IL-10 using BEST system efficiently delivered this cytokine at mucosal surfaces and exhibit beneficial effects in a murine model of low-grade intestinal inflammation.

Keywords: *Bifidobacterium bifidum*, microbiota, recombinant bacteria, heterologous expression system, IL-10, low-grade intestinal inflammation

INTRODUCTION

Bifidobacteria are strict anaerobic Gram-positive bacteria with irregular forms, belonging to the dominant microbiota in the human gut (especially in young children). Some species of bifidobacteria display beneficial properties for human health and are recognized as GRAS (Generally Regarded As Safe) microorganisms. In addition, some strains are already marketed as

food supplements (with probiotic claims) such as *Bifidobacterium breve* M16-V, *Bifidobacterium longum* BB536, and *Bifidobacterium bifidum* BGN4. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host, as defined by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) (Hill et al., 2014).

While recombinant bacteria are used in industry for the production of insulin or vaccines, researchers are increasingly interested by the direct administration of these recombinant bacteria to humans for either the development of mucosal vaccines or *in situ* delivery of proteins of therapeutic interest such as antioxidants, cytokines and protease inhibitors (Bermúdez-Humarán et al., 2013; Kumar et al., 2016). One of the main benefits in the use of probiotics as live vectors to deliver biologically active cytokines is to combine: (i) the intrinsic beneficial properties of some strains and (ii) the potential effect of the delivered protein. Moreover, several probiotics strains belong to some bacteria already present in our organism (i.e., intestinal microbiota) such as certain lactobacilli and bifidobacteria strains. This feature allows these strains to withstand gut environment wherein they can release biologically active molecules. Most bacteria used to deliver heterologous protein so far are lactobacilli and lactococci. Indeed, several trials have been carried out in animal models using either recombinant lactococci or lactobacilli to treat different human pathologies such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Motta et al., 2012; Martín et al., 2014; Allain et al., 2016). Strikingly, some human clinical trials (phase I and II) have been conducted with *L. lactis* producing either IL-10 or trefoil factor (TTF) to treat, respectively IBD (Braat et al., 2006) and oral mucositis (Limaye et al., 2013), confirming both the feasibility and the potential in the use of recombinant probiotics for human health.

In contrast to lactobacilli and lactococci, *Bifidobacterium* sp. could be a more attractive live vector to deliver proteins. Indeed, *B. breve* has been shown to persist for up to 32 days into mouse digestive tract after 3 days of gavage (Sheehan et al., 2007), in contrast to *Lactobacillus plantarum* which persists only 10 days (Pavan et al., 2003) and *Lactococcus lactis* that did not colonize with a transit time of 24–48 h (Kimoto et al., 2003). Finally, bifidobacteria have a low intrinsic and acquired resistance to antibiotics, showing therefore a safe profile for use in humans (Moubareck et al., 2005). Today, only few strains of bifidobacteria have been proposed as live delivery vehicles. For instance, some strains of *B. longum* have been used as a delivery vector for the development of: (i) oral vaccine against Hepatitis C virus (HVC) and Enterovirus 71 (Yu et al., 2013; Takei et al., 2014), (ii) anti-tumoral treatment (Shirakawa and Kitagawa, 2017), and (iii) to treat metabolic disorders in mice (Zhang et al., 2018). Other reports have described the use of genetically modified bifidobacteria to express diverse heterologous proteins (Khokhlova et al., 2010; Losurdo et al., 2013).

Currently, several expression systems are available for both lactobacilli and lactococci, including constitutive and inducible promoters and the possibility to express the desired protein into different cellular locations (i.e., intracellular, extracellular, or cell wall-attached) (Bermúdez-Humarán et al., 2013). However, as some proteins (such as cytokines) can accumulate in the cytoplasm and consequently be deleterious to bacteria growth, there is a need to develop new controlled-expression systems. These systems could avoid the negative outcomes of the over-production of heterologous proteins by controlling expression through an inductor such as changes in either the pH, temperature or even the addition of bile salts or antimicrobial peptides. One of the best-described expression systems in *L. lactis* is the NICE system (for NIsin Controlled Expression) (Kuipers et al., 1995), where gene expression is turned on by the addition of nisin to the culture medium. However, NICE system requires the presence of some regulatory genes (i.e., *nisRK*) that hampers its further applications in both fed-batch production and *in vivo* models. One alternative is the use of an expression system that does not require either the presence of regulatory genes or induction of bacterial cultures before use. Thus, we developed in our laboratory two *in vivo* stress-inducible expression systems for both lactococci [i.e., the SICE system (Benbouziane et al., 2013)] and lactobacilli [i.e., the LIVE system (Allain et al., 2016)], which allow the direct delivery of heterologous proteins *in situ* by recombinant bacteria. In the current work, we seek to develop a comparable system to deliver proteins of health interest by recombinant bifidobacteria. This system, named BEST for Bifidobacteria Expression SysTem, is based in the use of the broad-host range plasmid pWV01 (Morello et al., 2008), a *dnaK* promoter from *B. longum dnaK* operon, and two different signal peptides (SPs): one issued from the lactic acid bacterium (LAB) model *L. lactis* (SP_{Exp4}) (Morello et al., 2008), and one from *B. longum*: SP_{BL1181}. Furthermore, the functionality of BEST system was validated *in vivo*. For this, we cloned murine interleukin-10 (IL-10) an anti-inflammatory cytokine successfully expressed in different LAB (Braat et al., 2006), under BEST system and we determine its beneficial effects in a model of murine colitis (Martín et al., 2014). Our results show that genetically modified *B. bifidum* BS42 harboring IL-10 under BEST system successfully delivered the recombinant cytokine at mucosal surfaces and display beneficial properties in the murine model of intestinal low-grade inflammation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and DNA Manipulation

Strains and plasmids used in this work are listed in **Table 1**. *Escherichia coli* was grown in Luria-Bertani medium (Oxoid, Dardilly, France) at 37°C, under aerobic conditions with shaking. *L. lactis* was grown in M17 medium (Difco Laboratories, Detroit, MI, United States) supplemented with 1% glucose at 30°C without agitation. Bifidobacteria were cultured in de Man Rogosa Sharpe medium (MRS, Oxoid), supplemented prior to inoculation with cysteine (500 mg/L, Oxoid), at 37°C under anaerobic conditions (N2:CO2:H2; 80:10:10) in an anaerobic chamber (MACS, Chemunex AES Laboratoire, Bruz, France). Recombinant bacteria were selected by the addition of chloramphenicol (10 mg/L) except for *E. coli* harboring TOPO plasmid which was selected with kanamycin (50 mg/L).

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristic(s)[a]	Reference or source
Strains		
E. coli TOP10	Commercial competent cells	TOPOKit Invitrogen
B. bifidum BS42	Infant feces	Ménard et al., 2008
Plasmids		
pCR®2.1	Ap^RK^R subcloning TOPO vector	TOPOKit Invitrogen
pCR-TOPO:*dnaK*	Ap^RK^RpCR-TOPO subcloning vector harboring a DNA fragment containing *dnaK* promoter	This study
pLB141	Cm^R, pGK plasmid (a derivative from the broad host range plasmid pWV01) expressing a secreted form of the Staphylococcal nuclease (Nuc)(cassette SPexp4-NucB) under the control PnisA promoter	Morello et al., 2008
pLB270	Cm^R, pGK plasmid expressing a secreted form of murine IL-10 (cassette SPLcob-IL-10) under the control PnisA promoter	Motta et al., 2012
$pBEST_{Exp4}$:NUC	Cm^R, pGK plasmid expressing a secreted form of the Staphylococcal nuclease (Nuc)(cassette SPexp4-NucB) under the control PdnaK promoter	This study
$pBEST_{Exp4}$:IL-10	Cm^R, pGK plasmid expressing a secreted form of murine Il-10 (cassette SP_{Exp4}-IL-10) under the control PdnaK promoter	This study
pCR-TOPO:SP_{BL1181}	Ap^RK^RpCR-TOPO subcloning vector harboring a DNA fragment containing the signal peptide of *BL1181* hypothetical protein from *B. longum*	This study
$pBEST_{BL1181}$:IL-10	Cm^R, pGK plasmid expressing a secreted form of murine IL-10 (cassette SP_{BL1181}-IL-10) under the control PdnaK promoter	This study

[a]*For strains origin are given; for plasmids and cloned cassette characteristic are given. Ap, ampicillin; K, kanamycin; Cm, chloramphenicol.*

General procedures for DNA manipulation were essentially performed as described previously (Sambrook et al., 1989). Plasmid DNA isolation was performed using the Qiagen Midi-prep kit (Qiagen, Courtaboeuf, France). DNA sequences were confirmed by sequencing (Genewiz, Paris, France). Plasmid constructions were first established in either *E. coli* or *L. lactis* by electrotransformation (Sambrook et al., 1989) and then transferred into bifidobacteria. Electrocompetent *B. bifidum* protocols were adapted from Alvarez (Álvarez-Martín et al., 2008). Briefly, bifidobacteria were grown up to the optical density 0.6 at 600 nm (OD_{600nm}) in MRS supplemented with 0.5 M of sucrose. Cultures were then put on ice for 20 min. After centrifugation for 15 min at 4700 rpm, 4°C, pellets were washed three times with cold 0.5 M sucrose + 10% glycerol. This step allowed a 200-fold bifidobacteria concentration. Aliquots were incubated 1 h on ice, and then frozen at −80°C. Cells (5×10^{10} colony-forming units, CFU/mL) were electroporated at 200 Ω, 2.5 kV/cm, 25 μF (Bio-Rad, Marnes-la-Coquette, France) with 0.1–0.3 μg of DNA and resuspended in MRS for 3 h at 37°C in anaerobic condition before plating on MRS agar plus chloramphenicol (10 mg/L).

Construction of the Bifidobacteria Expression SysTem (BEST)

For the construction of a vector allowing heterologous protein production in *Bifidobacterium* sp., we cloned *dnaK* promoter from a strain of *B. longum* into pLB141 vector (**Table 1**; Morello et al., 2008), a derivative of the broad-host range plasmid pWV01 (GenBank Accession Number: NC_002192). Briefly, a DNA fragment (241 bp) containing *dnaK* promoter was amplified by PCR from genomic DNA of *B. longum* (GenBank Accession Number: NC_004307.2) with primers: *Bgl*II-P*dnaK* (5′-CCA***AGATCT***CAAAAAACBTGAGCCWAMMWYRCTCA ACTT-3′) for the coding strand and *Nhe*I-P*dnaK* (5′-***AGCT AGC***GTTAATAAAGCAAGGTTTATTTTTTTCATAACGTGTG CTCCTTAATTAYTCGTTTGTTCTTACGTTSTTYG-3′) for the complementary strand (with B = C or G or T; W = A or T; M = A or C; Y = C or T; R = A or G). The PCR product was then subcloned into a pCR-TOPO plasmid (Invitrogen, Carlsbad, CA, United States) resulting in pCR-TOPO:*dnaK* (**Table 1**). Then, the DNA fragment containing *dnaK* promoter was recovered from pCR-TOPO:*dnaK* plasmid with *Bgl*II/*Nhe*I enzymes (New England Biolabs, Evry, France) and cloned into purified backbone isolated from *Bgl*II/*Nhe*I-cut pLB141 vector, resulting in pLB141:*dnaK*:Nuc. Nucleotide sequence analysis of this construction revealed a deletion in the SP_{Exp4} (data not shown). To repair this, we performed a directed mutagenesis step by PCR. Briefly, we established pLB141:*dnaK*:Nuc vector in *E. coli* TG1 strain in order to purify large quantities of the plasmid, we then performed a first PCR reaction with forward primer: 5′-GTTATGAAA AAAATAAACCTTGCTTTATTAACGCTA-3′ using *Pfu* turbo DNA polymerase (Promega). Parameters of PCR were a first cycle at 95°C 1 min and 5 cycles at 95°C 30 s, 55°C 1 min, and 68°C 4 min. Afterward, we performed a second PCR reaction on the first PCR product with forward and reverse primers: 5′-TAGCGTTAATAAAGCAAGGTTTATTTTTTTCATAAC-3′). PCR parameters were as follow: first cycle 95°C, 1 min; 20 cycles at 95°C 30 s/55°C 1 min/68°C 4 min; 4°C. Then we performed one digestion with *Dpn*I enzyme (New England Biolabs, Evry, France) before establishment of the plasmid into *L. lactis*. The final construction $pBEST_{Exp4}$:Nuc (**Figure 1** and **Table 1**) was confirmed by sequencing.

Expression of IL-10 Cytokine Using BEST System

For the expression of IL-10 using BEST system, a DNA fragment encoding for IL-10 mature sequence was obtained from a plasmid harboring murine *il-10* gene (cassette SP_{Usp45}:IL-10) under the control of P_{nisA} promoter, also known as pLB270

FIGURE 1 | Schematic representation of plasmids construction.

plasmid (Motta et al., 2012; Benbouziane et al., 2013), with *Nsi*I/*Spe*I enzymes (Thermo Scientific, Courtaboeuf, France) and cloned into pBEST$_{Exp4}$:Nuc vector digested with the same enzymes and replacing the corresponding *nuc* DNA fragment. The resulting vector pBEST$_{Exp4}$:IL-10 was established into *B. bifidum*.

In parallel and in order to determine the impact of SP$_{Exp4}$ (issued from *L. lactis*) for heterologous protein secretion in bifidobacteria, we replaced this SP with a new one issued from *B. longum*: SP of the hypothetical protein BL1181 from *B. longum* NCC2705 (Schell et al., 2002). Indeed, after *in silico* analysis (using SignalP software[1]) of homologous proteins efficiently secreted by bifidobacteria we put a particular interest in this SP since it was one with the highest score of "cleavage site." For the cloning: a DNA fragment containing the SP of BL1181 (SP$_{BL1181}$) was PCR amplified from genomic DNA of *B. longum* strain (GenBank Accession Number: NC_004307.2) with the following primers: *Eco*RV-SP$_{BL1181}$ (5′-G***GATATC***AAGGAGATATAAAA ATGACGAACGTACGTGTGATCAAGCCCGC-3′) for the coding strand and *Nsi*I-SP$_{BL1181}$ (5′-TG***ATGCAT***CTGCCTGGG CAGGCTGTGCCGAGCTGAACG-3′) for the complementary strand. The PCR product (132 pb) was subcloned into a pCR-TOPO kit (Invitrogen, Carlsbad, CA, United States) resulting in pCR-TOPO:*SP*$_{BL1181}$ (**Table 1**). Afterward, the DNA fragment containing SP$_{BL1181}$ was obtained from this plasmid with *Eco*RV/*Nsi*I enzymes (Thermo Scientific) and cloned into purified backbone isolated from blunt-ended-*Afl*III/*Nsi*I-cut pBEST$_{Exp4}$:IL-10 vector. The resulting plasmid pBEST$_{BL1181}$:IL-10 (**Figure 1**) was established into *B. bifidum*.

For IL-10 detection, bacteria were grown until exponential phase (OD$_{600nm}$ = 0.4–0.8) and the centrifuged culture resuspended (after one wash step with PBS) into the culture medium: MRS plus cysteine (500 mg/L) in order to apply different stresses: pH4, pH8, 42°C, NaCl (25 g/L) or bile salts (100 or 500 mg/L, Sigma-Aldrich, Saint-Quentin-Fallavier, France). Induction was performed in anaerobic conditions during 30 min. Culture extraction was performed as followings. Protein samples were prepared from 2 ml of induced cultures. After centrifugation (5 min, 5000 rpm), the cell pellet (C) and supernatant (S) were treated separately. The S samples were treated by filtration on 0.25 μm filter then frozen at −20°C. The C fraction was obtained after treatment in a PBS complemented with anti-protease 1× and lysozyme (100 mg/mL, Roche), at 37°C during 1 h and then sonicated (6 × 10 sec with a 30 s of timeout on ice). IL-10 concentration in S and C was assessed by ELISA (eBioscience, Paris, France) and normalized by the final OD$_{600nm}$.

For western blot experiments, the C fraction was obtained as described for ELISA detection. The S samples were also treated by filtration on 0.25 μm filter plus a 50-fold concentration by the addition and precipitation of 10% trichloroacetic acid (TCA) during 15 min on ice and then centrifuged. Pellets were resuspended in NaOH. Protein concentration was determined by Bradford assay. Ten micrograms of protein were mixed with the blue gel-loading buffer and loaded in pre-casted gel (Bio-Rad). Proteins were then transferred on PVDF membrane and then blocked overnight in 5% milk in TBST. Membranes were stained with a primary rabbit polyclonal antibody against murine IL-10 (Abcam, ab9735) and a second goat HRP-conjugated against Rabbit IgG (BI2407, Abliance). Blots were reveled using Bio-Rad Kit.

Detection of IL-10 *in vivo*

The two strains BS42:BEST$_{Exp4}$:IL-10 and BS42:BEST$_{BL1181}$:IL-10 were administered by oral gavage to 6 weeks old IL-10-KO mice during 11 days. To mimic the context of our standard experiment in WT mice (**Figure 2**), animals received one intra-rectal injection of a low dose of 2,4-DiNitroBenzene Sulfonic acid (DNBS, 2 mg/mouse), 7 days after the beginning of the gavage with bacteria. After sacrifice, colonic content and caecal content were collected. After being weighted, contents were diluted at 1/4 in PBS 150 mM pH7.2 with 1× anti-protease cocktail (Sigma-Aldrich, Saint-Quentin-Fallavier, France), and then placed 30 min at 4°C under mechanic agitation. Supernatants were collected after centrifugation and IL-10 was measured using ELISA (BioLegend, San Diego, CA, United States).

DNBS-Induced Colitis Model

Male C57BL/6 mice (6 to 8-week-old) (Janvier, Le-Genest-Saint-Isle, France) were maintained at the animal care facilities of the National Institute of Agricultural Research (IERP, INRA, Jouy-en-Josas, France). Mice were housed under standard conditions for a minimum of 1 week before experimentation. All experiments were performed in accordance with European Community rules for animal care and were approved by COMETHEA C2EA-45 with authorization n°3445-2016010615159974.

For the induction of colitis, we used an adapted protocol from Martín (Martín et al., 2014; **Figure 2**) based on the intrarectal administration of DNBS. Briefly, mice (∼20 g) were anesthetized by intraperitoneal injection of 150 μL of 0.1% ketamine (Imalgene 1000, Merial) and 0.06% xylazine (Rompun). Then, a 4 cm-long catheter (Ecimed, Boissy-Saint-Léger, France) was attached to a tuberculin syringe, inserted into the colon and a dose of 100 mg/Kg of DNBS solution (Sigma-Aldrich, Saint-Quentin Fallavier, France) in 30% ethanol (EtOH) was injected intra-rectally to induce colitis in all group except control mice. Control mice (without colitis) received only 30% EtOH. Ten days after the so-called DNBS period, 1X10^9 CFU of bacteria in PBS or PBS alone were intragastrically administered daily for 10 days (gavage period). The inflammation was reactivated 21 days after the first DNBS injection (recovery period) with a second injection of 100 mg/Kg of DNBS solution. Weight loss was monitored daily to assess the severity of colitis. Experiment was performed twice and mice were treated as follow: with ETOH-PBS (control mice without colitis, n = 16), with DNBS-PBS (colitis control group, n = 20), with *B. bifidum* BS42 wild-type strain (WT-BS42, n = 16), with *B. bifidum* BS42 harboring pBEST$_{Exp4}$:IL-10 plasmid (BS42:BEST$_{Exp4}$:IL-10, n = 16), and with *B. bifidum* BS42 harboring pBEST$_{BL1181}$:IL-10 plasmid (BS42:BEST$_{BL1181}$:IL-10, n = 16).

[1]http://www.cbs.dtu.dk/services/SignalP/

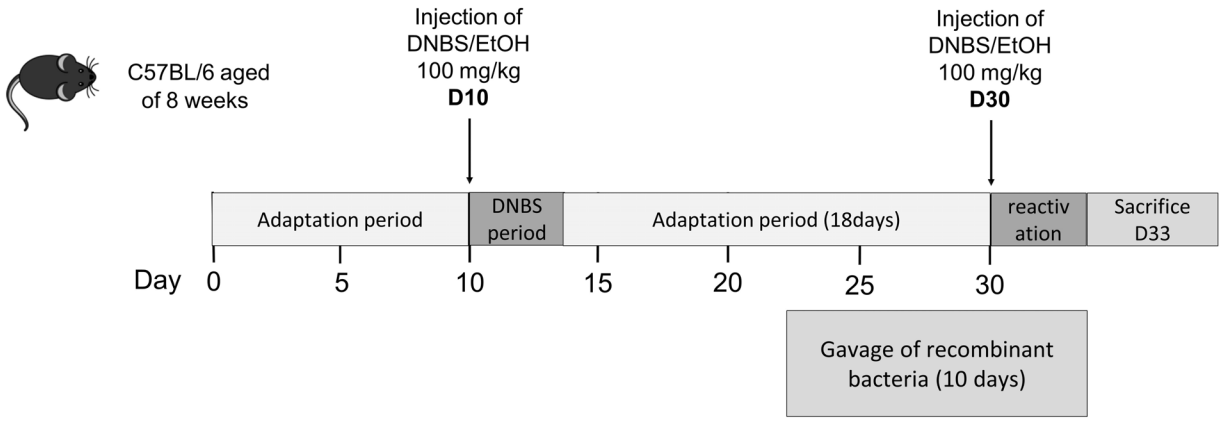

FIGURE 2 | Experimental protocol for the mouse chronic low-grade inflammation model.

Macroscopic Damage Scores

Macroscopic scores were recorded in the abdominal cavity using previously described system (Wallace and Keenan, 1990). Briefly, the macroscopic criteria (assessed on a scale from 0 to 9) include macroscopic mucosal damages such as ulcers, thickening of the colon wall, the presence of adhesions between the colon and other intra-abdominal organs, the consistency of fecal material (as an indicator of diarrhea), and the presence of hyperemia in colon (increase of blood flow).

Histology on Colonic Samples

Fragment of colon were fixed in Formalin then dehydrated with ethanol 70%, and paraffin-embedded. Section of 3 μm-thick sections were stained with hematoxylin and eosin (H&E). As previously described (Cooper et al., 1993), colonic damage was scored according to architectural derangement, goblet cell depletion, edema/ulceration, and degree of inflammatory cell infiltration.

Lipocalin-2 (LCN-2) Levels

Colonic contents were homogenized in FastPrep instrument with cold PBS. Supernatants were collected and stored at −80°C until analysis. LCN-2 was measured using R&D ELISA kit (R&D System, Minneapolis, MN, United States) and following manufacturer instruction.

Cytokine Production by CD3/CD28-Stimulated Splenocytes

The day of sacrifice, spleens were removed and placed in RPMI medium for culture (Breyner et al., 2017). Briefly, spleens were crushed and filtered through a 70 μm nylon filter (BD, Le Pont-de-Claix, France) and were resuspended in RPMI (Lonza, Levallois-Perret, France) completed with 100 Unit of Streptomycin, Penicillin and 10% Fetal Calf Serum (FCS). Red blood cells were removed with buffered Blood Cell Lysis Buffer (Sigma-Aldrich, France). Plates were pre-coated with anti-CD3 and anti-CD28 antibodies, 4 μg/ml of each antibody (eBioscience) in PBS with 0.5% FCS. Splenocytes cells were adjusted to 2×10^6 cells/ml per well and were cultured in 24-well plates at 37°C in a 5% CO2 and 95% air atmosphere. Supernatants were collected after 48 h of culture and were stored at −80°C until further analysis. Splenocytes culture supernatant levels of IL-4, IL-5 (Mabtech, Stockholm, Sweden), IL-6, IFN-γ (eBioscience), and IL-10 (BioLegend, San Diego, CA, United States) were quantified by ELISA, following manufacturer instructions.

Statistical Analyses

Data were analyzed using GraphPad Prism (GraphPad Software Inc, San Diego, CA, United States) by non-parametric Kruskal–Wallis test followed by a Mann–Whitney test. A *P*-value of less than 0.05 was considered significant.

RESULTS

Development of a New Expression System to Produce and Deliver IL-10 Cytokine in *Bifidobacterium bifidum*

To obtain a functional expression plasmid allowing *in vivo* stress-inducible heterologous protein expression in bifidobacteria, we first cloned the promoter from *B. longum dnaK* operon (Sanchez et al., 2005) into pLB270 plasmid (**Table 1**) resulting in pBEST$_{Exp4}$:Nuc vector, replacing P_{nisA} promoter by P_{dnaK} promoter (**Figure 1**). We chose this promoter, because *dnaK* operon encodes heat-shock proteins partially involved in the adaptation and survival of some bacteria (such as Bifidobacteria) in the gut (Sanchez et al., 2005). In addition, in this genus, transcription of heat-shock proteins (and more particularly DnaK), are greatly enhanced by exposure to both bile salts and different pH.

In order to validate the functionality of BEST system *in vivo, nuc* gene was replaced from this vector for murine IL-10 to obtain plasmid pBEST$_{Exp4}$:IL-10. The resulting vector, pBEST$_{Exp4}$:IL-10 (**Figure 1**), was established into *B. bifidum* BS42. As expected, no IL-10 was detected with the BS42 wild-type strain. As shown in **Figure 3**, *B. bifidum* BS42 harboring pBEST$_{Exp4}$:IL-10 plasmid

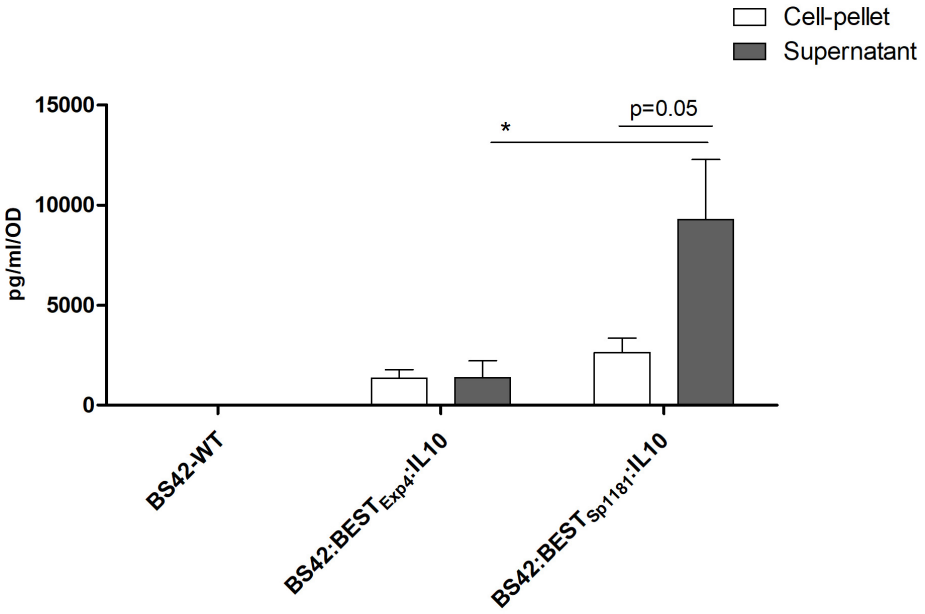

FIGURE 3 | IL-10 production of recombinant Bifidobacteria BS42-WT, BS42:$BEST_{Exp4}$:IL-10 and BS42:$BEST_{BL1181}$:IL-10 grown to OD_{600nm} = 0.6–0.8 in MRS-cysteine at 37°C under anaerobic atmosphere. Cultures were washed once with PBS and placed in fresh medium, for 30 min at 37°C under anaerobic atmosphere. IL-10 was measured by ELISA in both cell-pellet (C) and supernatant filtered samples (S) (n = 3). Comparison between cell-pellet, supernatant and strains involved the non-parametric Mann–Whitney test. *$p < 0.05$.

without any stress revealed IL-10 both in C and S samples reaching a maximal concentration of 2000 ng/L/OD. This phenomenon suggests a basal activity of the BEST system. To further explore if we can improve IL-10 secretion we replaced the SP_{Exp4} which is issued from *L. lactis* by a new one issued from a *B. longum* (SP_{BL1181}). Our results show that there was no significant difference in C samples between the two recombinant strains; however, the constitutive production of IL-10 was sevenfold higher in S samples (9283 ± 2981 ng/L/OD) prepared from recombinant BS42:$BEST_{BL1181}$:IL-10 compared to BS42:$BEST_{Exp4}$:IL-10 strain: S (1377 ± 840 ng/L/OD) ($p < 0.05$) (**Figure 3**). Due to protein concentration and the sensitivity of western blot together with the fact that this cytokine is acid sensitive, only IL-10 produced by BS42:$BEST_{BL1181}$:IL-10 in S samples was detected (**Supplementary Figure S1**). The *in vitro* production of IL-10 from recombinant *B. bifidum* BS42 harboring either p$BEST_{Exp4}$:IL-10 or p$BEST_{BL1181}$:IL-10 plasmid was studied under 6 different stress conditions during 30 min and compared to non-induced cultures in order to validate the inducibility of the BEST system (**Figures 4A,B**).

Regardless of the stress conditions, IL-10 concentration was higher in S fraction from BS42:$BEST_{BL1181}$:IL-10 than BS42:$BEST_{Exp4}$:IL-10 ($p < 0.05$, **Figure 4C**). Heat (42°C), NaCl, and bile salts stresses did not induce a significant difference in IL-10 concentrations neither in C nor S samples in BS42:$BEST_{BL1181}$:IL-10 strain compared to non-induced condition (**Figure 4A**). In contrast, compared to non-induced conditions, IL-10 concentrations were significantly lower in S fraction at pH4 ($p < 0.05$) or higher at pH8 ($p = 0.05$). IL-10 amount was much higher in S than in C under pH8 and bile salt stress conditions ($p < 0.05$). Concerning the analysis in BS42:$BEST_{Exp4}$:IL-10 strain (**Figure 4B**), nonetheless the stress condition, IL-10 levels were similar in both C and S samples even if IL-10 amount decreased in C at pH4 ($p = 0.05$). Finally, the two recombinant strains displayed a different IL-10 secretion profile.

Detection of IL-10 Delivered *in vivo* in IL-10 KO Mice

Our IL-10 KO mice did not develop colitis: percentage of weight loss and macroscopic score were measured but no differences were observed (data not shown) between control and DNBS mice. Our results revealed that IL-10 could be detected in (i) ceacal samples of either 62.5% of mice (5/8) treated with BS42:$BEST_{BL1181}$:IL-10 strain or 50% of mice (4/8) treated with BS42:$BEST_{Exp4}$:IL-10, with a mean level of 0.759 and 0.554 pg/g, respectively (**Supplementary Figure S2A**); and (ii) colonic content samples of either 50% of mice (4/8) treated with BS42:$BEST_{BL1181}$:IL-10 strain or 25% of mice (2/8) treated with BS42:$BEST_{Exp4}$:IL-10, with a mean level of 0.280 and 0.097 pg/g, respectively (**Supplementary Figure S2B**). The presence of IL-10 in caecal and colonic content in IL-10 KO mice shows that the recombinant BS42:$BEST_{BL1181}$:IL-10 and BS42:$BEST_{Exp4}$:IL-10 are able to bring IL-10 *in vivo* within the digestive tract.

Impact of IL-10 Delivery by Recombinant *B. bifidum* Using BEST System in a Low-Grade Inflammation Murine Model

In vivo delivery of IL-10 through administration of BS42:$BEST_{BL1181}$:IL-10 and BS42:$BEST_{Exp4}$:IL-10 strains

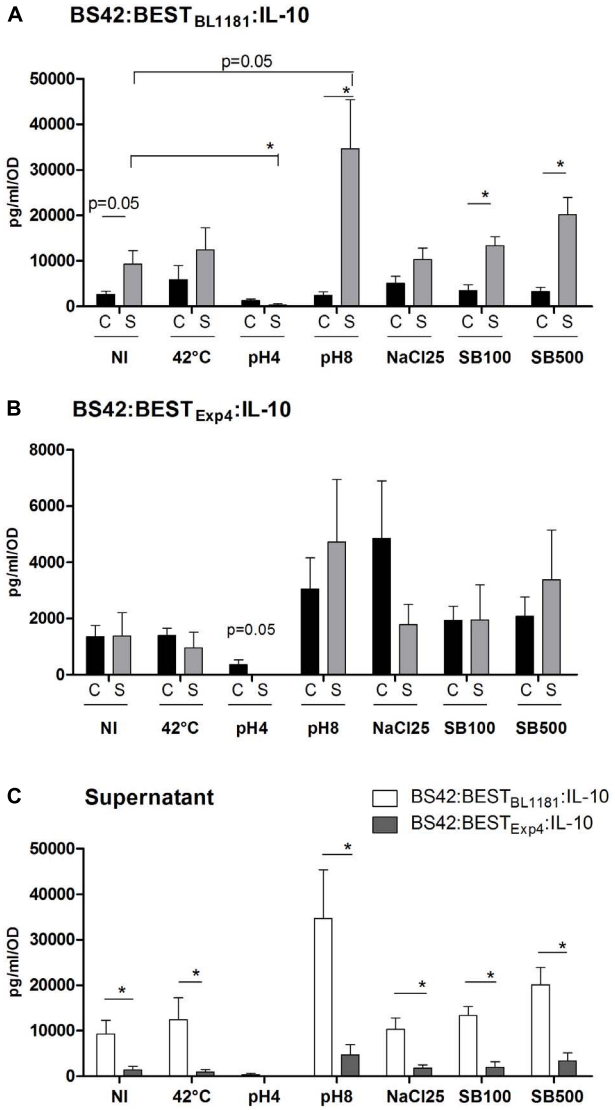

FIGURE 4 | Effect of heat-shock, pH and bile salts on IL-10 production. BS42:BEST$_{BL1181}$:IL-10 **(A)** and BS42:BEST$_{Exp4}$:IL-10 **(B)** were cultured in MRS-cysteine broth under anaerobic condition until OD_{600nm} = 0.6–0.8. Cultures were washed once with PBS and re-cultured in fresh medium for 30 min at 37°C (except for heat-shock). **(C)** Comparison of IL-10 production in supernatant samples of the two recombinant strains. NI, non-induced (pH6); heat-shock: 42°C; acid shocks: pH4 and pH8; salt-shocks: NaCl25 = 25 g/L of NaCl; SB100 = 100 mg/L of bile salts; SB500 500 mg/L of bile salts. IL-10 was measured with ELISA in Cell-pellet and filtered Supernatant. Data (means plus SEMs) are the result of four separate experiments. Comparison involved the non-parametric Mann–Whitney test. *$p < 0.05$.

were then studied in a murine model of low-grade intestinal inflammation in wild type mice (**Figure 2**). As shown in **Figure 5A**, compared to ethanol, DNBS-treatment induced a weight loss (90.6 ± 0.94% at day 31, $p < 0.001$) after the second rectal injection and the next 3 days. Mice treated with IL-10-producing bifidobacterial strains tended to lose less weight, 93.06 ± 1.02% and 92.5 ± 1.18% for BS42:BEST$_{BL1181}$:IL-10 and BS42:BEST$_{Exp4}$:IL-10 groups, respectively, compared to the DNBS-PBS control group, (i.e., colitis control). Nevertheless, only the Ethanol-PBS group is significantly different compared to this colitis control group. The macroscopic score was significantly higher in the DNBS-PBS group compared to Ethanol-PBS group ($p < 0.0001$, **Figure 5B**). Mice treated with BS42 strain showed almost the same score as DNBS-PBS group. In contrast, BS42:BEST$_{BL1181}$:IL-10 treatment significantly decreased this score ($p < 0.05$) and BS42:BEST$_{Exp4}$:IL-10 induced a non-significant slight decrease in macroscopic score.

LCN-2, an early marker of inflammation, was measured in colonic content at day 33 (**Figure 5C**). The level of LCN-2 was significantly higher in the DNBS-PBS group compared to negative control ($p < 0.0001$). LCN-2 level was significantly lower with BS42:BEST$_{BL1181}$:IL-10 group ($p < 0.05$), and, although not significant, tended to be decreased following treatment with BS42 and BS42:BEST$_{Exp4}$:IL-10.

The histological score was significantly lower in BS42:BEST$_{BL1181}$:IL-10 and BS42:BEST$_{Exp4}$:IL-10 groups (respectively $p < 0.05$ and $p < 0.0001$) than in the DNBS-PBS group (**Figures 5D,E**).

The impact on systemic T-helper cells balance was determined by measuring IL-4, IL-5, IL-6, IFN-γ, and IL-10 cytokines in supernatant from mice splenocytes after 48H of CD3/CD28 re-stimulation (**Figure 6**). For all cytokines, there were significant differences between the colitis control group and the negative control group, confirming the inflammatory status of DNBS-PBS group. The intake of BS42:BEST$_{BL1181}$:IL-10 slightly decreased IL-5, IL-6, and IFN-γ (not significant). However, there was no effect on IL-4 and IL-10. The intake of BS42:BEST$_{Exp4}$:IL-10 as well as the BS42 tended to decrease the level of IL-5 and IFN- γ, with no effect on other cytokines.

DISCUSSION

In this work, we sought to construct an inducible expression system for bifidobacteria allowing *in vivo* delivery of proteins of health interest. For this, we developed a *Bifidobacterium* expression system (BEST) regulated by stress that we validated in *B. bifidum*. The functionality of BEST system was validated by cloning murine IL-10 cytokine and confirming both its correct expression and biological activity *in vivo* in a mouse model of low-grade intestinal inflammation. For the expression of IL-10 we confirm that the two recombinant *B. bifidum* strains were able to produce and secrete (even without any stress) the same IL-10 levels than those previously reported in recombinant bifidobacteria (Escogido et al., 2007; Khokhlova et al., 2010). For instance, a recombinant strain of *B. longum* was reported to produce 22 pg/ml of IL-10 (Escogido et al., 2007) whereas a recombinant strain of *B. breve* produced 1900 pg/ml of IL-10 (Khokhlova et al., 2010). In our study, we observed a constitutive production of IL-10 comparable or even greater (up to sevenfold) than those described with another plasmid, with an average yield of 1000 pg/ml with *B. bifidum* harboring pBEST$_{Exp4}$:IL-10 plasmid and reaching 7000 pg/ml with *B. bifidum* harboring pBEST$_{SP1181}$:IL-10. Moreover, at pH 8 (a stress condition under basic pH), we observed an IL-10

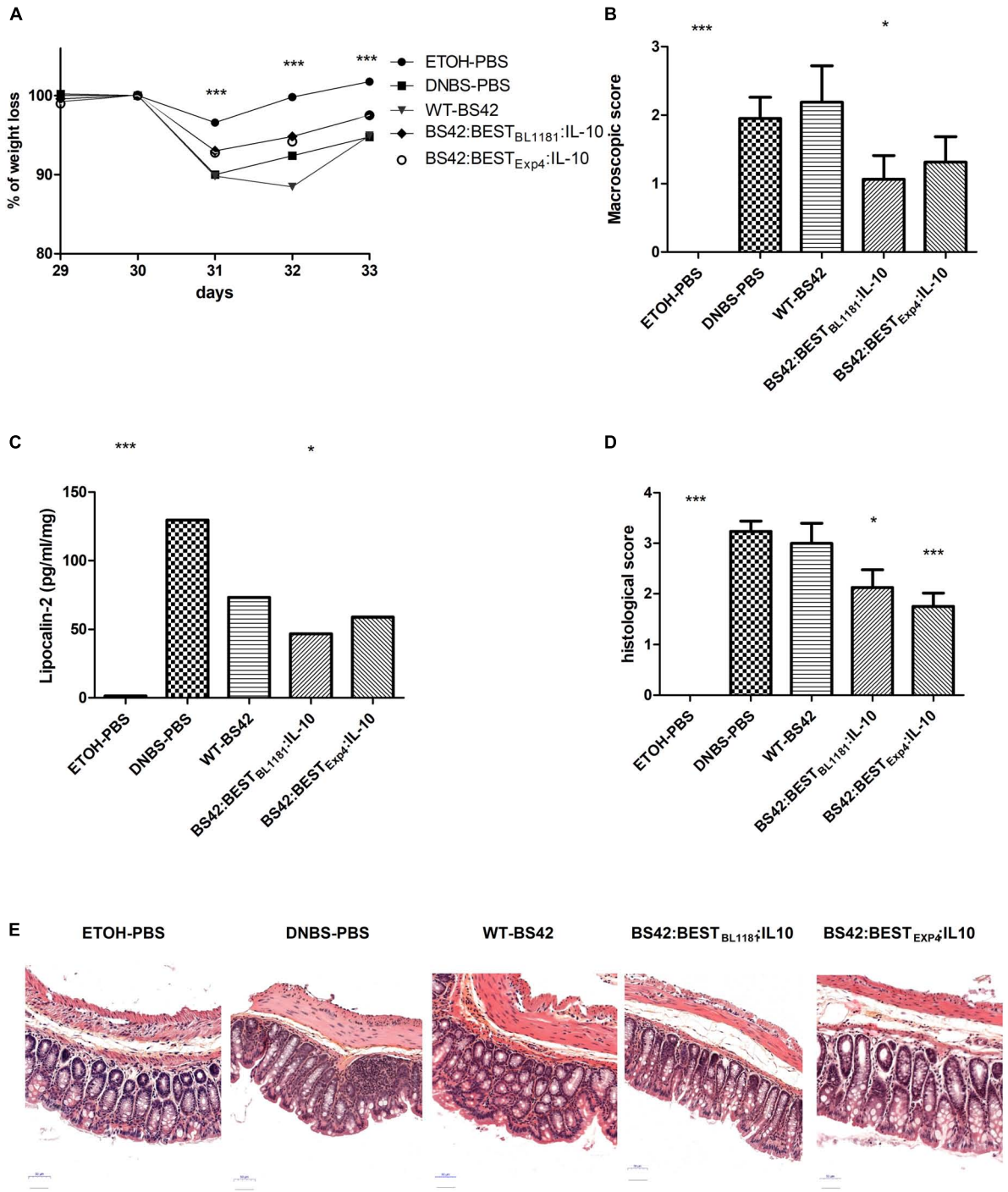

FIGURE 5 | Impact of recombinant *B. bifidum* strains secreting murine IL-10 on a murine model of low grade inflammation induced by two rectal administrations of DNBS. Severity of the colitis reactivation was assessed by **(A)** change in body weight at day 33, **(B)** macroscopic scores and **(C)** fecal lipocalin-2 levels at day 34 and **(D)** histological score on colon and **(E)** Histological representation (H&E staining) (n = 2*8 = 16 mice/group). Statistically significant differences are calculated relative to the DNBS group using non-parametric Mann–Whitney test. *$p < 0.05$, ***$p < 0.0001$.

overproduction reaching 24000 pg/ml. This pH, which could reflect the pH of pancreatic juice when it is spilled into the duodenum, models a condition that *Bifidobacterium* can shortly encounter following oral administration. It shows the inductivity of our system as, in the large intestine where they are mainly located, *Bifidobacterium* will encounter a combination of factors and stresses which is difficult to reproduce *in vitro*. It is also interesting to note that pH8 can also stabilize IL-10. Indeed,

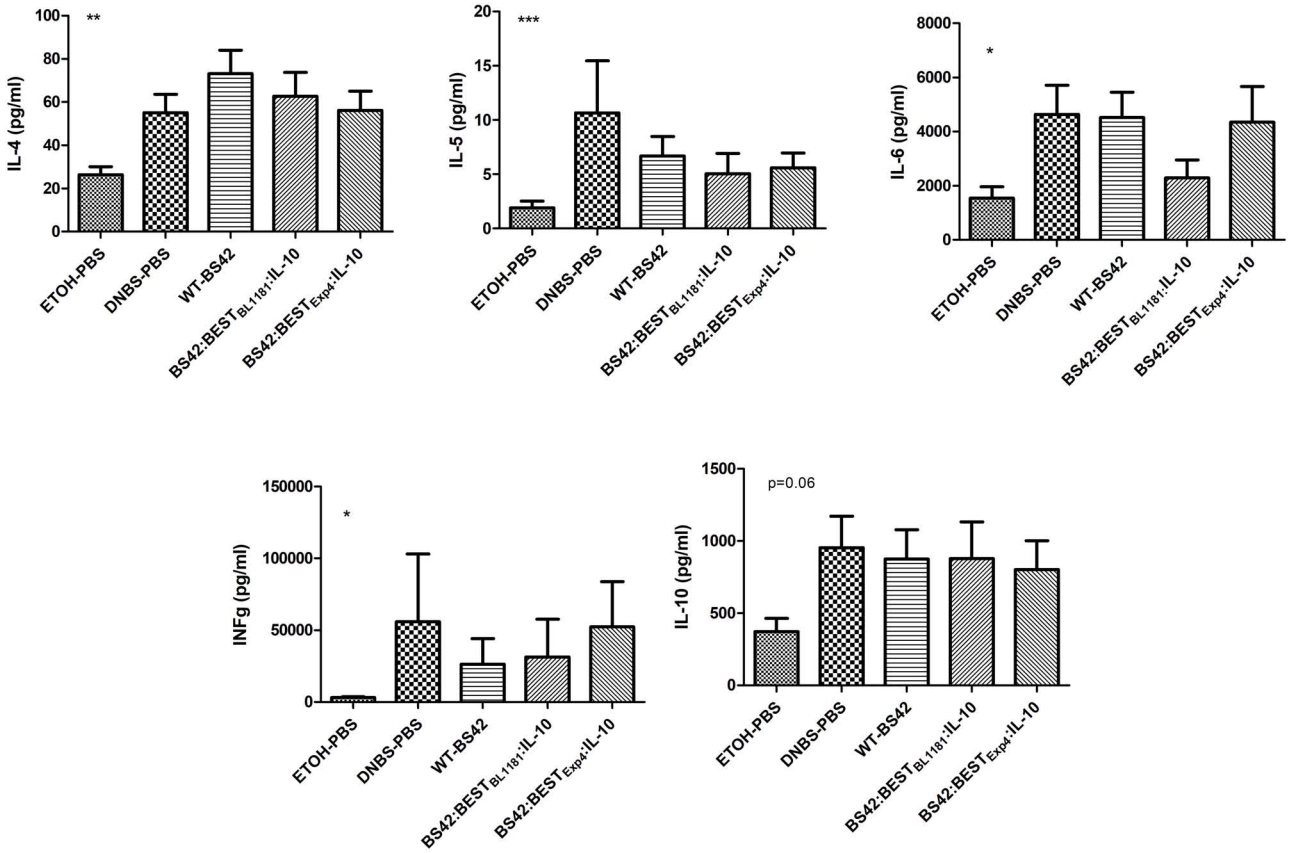

FIGURE 6 | Impact of recombinant *B. bifidum* strains secreting murine IL-10 on the systemic T-helper balance. Cytokines secreted by splenocytes from each mice group, stimulated for 48h with CD3/CD28, were measured using ELISA (n = 2*8 = 16 mice/group). Comparison was performed with the non-parametric Mann–Whitney test. Statistically significant differences were calculated relative to the DNBS group. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

it has been shown that at pH8.5, human IL-10 is under a highly stable dimer form (Syto et al., 1998). In the same study, the authors showed that IL-10 retained high activity between pH6 and 10. Below pH6.0, its activity decreased significantly and at pH2.5 only a small percent of IL-10 activity remained. A comparison analysis with commercial murine recombinant IL-10 allowed us to confirm that IL-10 produced by recombinant Bifidobacteria was stable in all the tested stresses conditions except at pH4 and lower (data not shown). In addition, pH4 can impact bifidobacteria growth. Except for some strains of *B. thermacidophilus*, bifidobacteria can survive (but not grow) at pH4.5–5 (Biavati and Mattarelli, 2015), this can explain that after 30 min at pH4, no IL-10 was detected in the culture medium.

To improve secretion, we included in the BEST system a new signal peptide issued from *B. longum*: SP_{BL1181}. At pH8 (pancreatic juice), IL-10 secretion was 2.7-fold increased, confirming the interest of both SP_{BL1181} and P_{DnaK} promoter. Concentrations of IL-10 in the C fraction were similar for our two recombinant BS42; however, a sevenfold higher concentration was observed in the S of BS42:BEST$_{BL1181}$:IL-10 compared to that of BS42:BEST$_{Exp4}$:IL-10. SP_{Exp4} allows only a small amount of IL-10 to be secreted compared to SP_{BL1181}. This can be easily understandable by the bifidobacterial origin of this SP compared to SP_{Exp4} which is issued from *L. lactis*. Thus, BEST system using SP_{BL1181} seems to be an efficient system to secrete heterologous protein in *B. bifidum*. In addition, this SP (issued from *B. longum*) could also work in other *Bifidobacterium* strains, including *B. longum* itself; and although preliminary data suggest that SP_{1181} is functional in *B. longum* and *B. breve*, this remains to be confirmed.

To assess IL-10 *in vivo* delivery by recombinant bifidobacteria, we used IL-10 KO mice model. In these mice, following oral administration of the recombinant strains, we detected IL-10 in ceacal and colonic content confirming the production of IL-10 *in vivo*. Of note, these young IL-10 KO mice do not develop colitis despite the administration of DNBS, so we assess the biological efficacy of these recombinant bacteria on a well characterized low-grade intestinal inflammation model (Martín et al., 2014; Allain et al., 2016).

Several studies have reported beneficial effects of different strains of *B. bifidum* in a murine model of acute colitis (Preising et al., 2010; Alard et al., 2018). Our *B. bifidum* BS42 seems to have no beneficial impact as demonstrated on the murine model of low-grade intestinal inflammation. In our experiments, BS42:BEST$_{BL1181}$:IL-10 had a greater beneficial effect on gut inflammation with a significant

decrease in LCN-2 level than BS42. Despite not significant, BS42:BEST$_{BL1181}$:IL-10 improved systemic inflammation as observed by the decrease in IL-6 and IFN-γ cytokines. Impact of BS42:BEST$_{Exp4}$:IL-10 was less obvious confirming that IL-10 secretion is an advantage. In a previous work Martín et al. (2014) tested a recombinant strain of *L. lactis* producing murine IL-10 in the same model of low-grade intestinal inflammation and using the same dose of bacteria (*i.e.*, 1×10^9 CFU/mouse) for the same administration period (11 days). If we compare the efficacy between the two recombinant strains, the effects of our BS42:BEST$_{BL1181}$:IL-10 were more efficient regarding the weight evolution, the macroscopic and histologic scores and LCN-2 levels. In contrast, IL-10-producing *L. lactis* had a better effect on gut permeability and in cytokines levels. Despite each strain display its intrinsic properties, in the two cases, the presence of IL-10 in the intestine has beneficial effect on the low-grade intestinal inflammation model. It will be interesting to test in a near future a combination of these two recombinant strains producing IL-10 in different murine models of either IBS or IBD. Indeed, the ecological niche of *L. lactis* is the small intestine whereas for *Bifidobacterium* it is the colon and we can thus expect a synergic beneficial effect.

CONCLUSION

In conclusion, we succeeded in the construction of a new Bifidobacteria Expression SysTem (BEST) that allowed *in vivo* IL-10 delivery by *B. bifidum*. Furthermore, the use of recombinant *B. bifidum* BS42 secreting IL-10 cytokine in a model of low-grade intestinal inflammation showed its ability to decrease local inflammation and confirmed therefore its potential for delivery of therapeutic molecules in the colon. Despite we consider our expression system is promising, more studies are needed, such as evaluate either daily or weekly bacteria administration which will allow us to determine the advantages of the higher persistency of this genus *versus* other recombinant bacteria (e.g., lactococci and lactobacilli).

AUTHOR CONTRIBUTIONS

LB-H and A-JW designed the study. AF performed the cloning. AM performed the *in vitro* characterization of IL-10 production. AM, FC, PR, and A-JW performed the animal experiments. AM performed all dosages. BR read the histological score. SG provided the analytical tools. AM, LB-H, and A-JW interpreted the data and wrote the manuscript. PL and M-JB provided the critical feedback, and helped to shape the research, analysis, and 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.2018.03075/full#supplementary-material

FIGURE S1 | Western blot analyses of IL-10 in the C and S sample following or not 42°C induction.

FIGURE S2 | *In vivo* production of IL-10. IL-10 KO-mice received daily BS42:BEST$_{Exp4}$:IL-10 or BS42:BEST$_{BL1181}$:IL-10, during 11 days by oral gavage. IL-10 were then measured in **(A)** caecal content and **(B)** colonic content by ELISA.

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REVIEW
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Engineering Components of the *Lactobacillus* S-Layer for Biotherapeutic Applications

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Lactic acid bacteria (LAB) are frequently harnessed for the delivery of biomolecules to mucosal tissues. Several species of *Lactobacillus* are commonly employed for this task, of which a subset are known to possess surface-layers (S-layers). S-layers are two-dimensional crystalline arrays of repeating proteinaceous subunits that form the outermost coating of many prokaryotic cell envelopes. Their periodicity and abundance have made them a target for numerous biotechnological applications. In the following review, we examine the multi-faceted S-layer protein (Slp), and its use in both heterologous protein expression systems and mucosal vaccine delivery frameworks, through its diverse genetic components: the strong native promoter, capable of synthesizing as many as 500 Slp subunits per second; the signal peptide that stimulates robust secretion of recombinant proteins; and the structural domains, which can be harnessed for both cell surface display of foreign peptides or adhesion enhancement of a host bacterium. Although numerous studies have established vaccine platforms based on one or more components of the *Lactobacillus* S-layer, this area of research still remains largely in its infancy, thus this review is meant to not only highlight past works, but also advocate for the future usage of Slps in biotherapeutic research.

Keywords: *Lactobacillus*, probiotic, S-layer, biotherapeutic, mucosal vaccine, CRISPR

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INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, anaerobic or microaerophilic, non-sporulating microorganisms that inhabit diverse environments including milk and plant surfaces, as well as the mouth, gastrointestinal tract, and vaginal tract of humans and animals (Liu et al., 2014). Traditionally, they have been employed for food and dairy fermentations, but have more recently garnered attention for their health-promoting properties with many species used widely as probiotics (Klein et al., 1998; Klaenhammer et al., 2005). Recombinant LABs are frequently harnessed for mucosal delivery of biomolecules such as therapeutic proteins or vaccine antigens (Berlec et al., 2012). In comparison to traditional intravenous or intramuscular vaccine administration, the mucosal route enables immunizations to be performed orally, reducing potential side effects while increasing specificity for chronic illnesses and infections associated with mucosal tissues (Bermudez-Humaran et al., 2011;

Wyszynska et al., 2015). Furthermore, many LABs are bile and acid tolerant, act as natural adjuvants, and interact with cells of the immune system (Wells and Mercenier, 2008), making them ideal candidates for antigen carriage.

The LABs most frequently chosen for vaccine delivery are *Lactococcus lactis* and select species of the *Lactobacillus* genus (Wells and Mercenier, 2008; Bermudez-Humaran et al., 2011, 2013; Wyszynska et al., 2015). However, unlike *L. lactis*, several species *Lactobacillus* have been shown to possess surface-layers (S-layers) (Hynonen and Palva, 2013). S-layers have been detected on both Gram-positive and Gram-negative bacteria and are nearly ubiquitous in archaea (Fagan and Fairweather, 2014). They are defined as two-dimensional crystalline arrays composed of repeating proteinaceous subunits that constitute the outermost layer of a cell envelope (Fagan and Fairweather, 2014). The S-layer proteins (Slps) attach to the underlying peptidoglycan via electrostatic interactions and possess inherent, entropy-driven affinities to self-assemble with each other (Hynonen and Palva, 2013). Thus far, S-layers have been characterized for their role in maintaining cell shape; acting as molecular sieves; serving as binding sites for large molecules, ions, or phages; and mediating surface adhesion (Sleytr et al., 2014). Additionally, Slps are some of the most abundant proteins synthesized by the cell, making them metabolically expensive but also underscoring their importance to the organism (Sara and Sleytr, 2000; Fagan and Fairweather, 2014). Their high expression, periodicity, and self-assembling properties have made them a target for numerous applications in biotechnology and nanotechnology (Avall-Jaaskelainen and Palva, 2005; Hynonen and Palva, 2013; Sleytr et al., 2014).

In the following review, we examine Slp applications in recombinant protein expression and biotherapeutic delivery via their distinct genetic building blocks: the strong native promoter, which can synthesize as many as 500 Slp subunits per second; the signal peptide, that can trigger robust secretion of target molecules; and the structural domains, which can be harnessed for both cell surface display of heterologous proteins or enhancement of host adhesion (**Figure 1A**). Despite the existence of several recombinant protein expression systems based on one or more components of the *Lactobacillus* S-layer, this area of research still remains largely underexploited. Thus, the purpose of this review is to not only shed light on past S-layer studies, but also to advocate for future utilization of Slps in mucosal vaccine and biotherapeutic delivery research.

HARNESSING THE STRONG NATIVE PROMOTER

The bacterial S-layer array is composed of an estimated 5×10^5 subunits, representing 10–15% of the total protein content of the bacterium (Sleytr and Messner, 1983; Avall-Jaaskelainen and Palva, 2005). During exponential growth phase, approximately 500 subunits per second must be synthesized, translocated to the cell surface, and incorporated into the existing lattice structure (Sleytr et al., 2014). In order to obtain these high levels of transcription, S-layer genes possess strong, efficient promoters, which can be harnessed for protein production systems (**Figure 1B**). Within the *Lactobacillus* genus, this research has predominately been limited to *slp* promoters of *Lactobacillus acidophilus* and *Lactobacillus brevis*.

Several studies have investigated the versatility of *Lactobacillus* S-layer promoters for driving heterologous protein expression in various LAB hosts. Lactobacilli regularly possess multiple *slp* genes within the same strain that are not all concurrently active (Hynonen and Palva, 2013). Although SlpA is the major constituent of the *L. acidophilus* S-layer, it can be moved to an inactive position triggering expression of the once silent SlpB (Boot et al., 1996a; Konstantinov et al., 2008). When the *L. acidophilus ATCC 4356 slpA* and *slpB* promoters were evaluated in *Lactobacillus casei* ATCC 393, only *slpA* remained active under all tested growth phases (Boot et al., 1996b). However, the same *slpA* promoter, although highly efficient in *L. lactis*, was nearly inactive in isolates of *Lactobacillus reuteri* (Lizier et al., 2010). The *L. acidophilus* NCFM *slpA* promoter was used to drive expression of a green fluorescent protein (GFP) in shuttle vectors based on *oriV1* and *oriV2* replicons (Chen et al., 2014). Similarly, the plasmids exhibited distinct properties based on which strain they were ported into as well as growth-phase-dependent effects. Both plasmids were capable of replicating in strains of *L. casei* and *Lactobacillus delbrueckii*, but only the *oriV1* plasmid, pEL5.6, could replicate in *Lactobacillus paracasei* (Chen et al., 2014). The functionality of the *L. brevis* ATCC 8287 *slpA* promoter was evaluated in three LAB hosts: *L. lactis* MG1614, *Lactobacillus plantarum* NCDO1193 and *Lactobacillus gasseri* NCK334, via the expression of various reporter genes (Kahala and Palva, 1999). The S-layer promoter was recognized in each strain, but was particularly active in *L. lactis* and *L. plantarum*. In fact, aminopeptidase N (PepN) reporter activity within *L. plantarum* was 30-fold higher compared to the *Lactobacillus helveticus* PepN native host and composed a staggering 28% of the total cellular protein during late exponential growth phase. In summary, *Lactobacillus slp* promoters are effective tools for driving recombinant protein expression, but optimization based on host and growth conditions is essential.

The S-layer promoter has also been harnessed for reporter expression *in situ*. Plasmid instability and antibiotic markers can complicate the use of these constructs when moving into human clinical trials or to market, thus chromosomal insertions have gained immense popularity. For *L. acidophilus* NCFM, a pORI-based *upp* counterselective gene replacement system (Goh et al., 2009) has considerably aided this effort (see "Engineering Platforms"). Originally intended for knockout characterizations, it was first employed for a knock-in by cloning a β-glucuronidase (*gusA*) reporter downstream of the *slpA* gene (Douglas and Klaenhammer, 2011). The resulting mutant exhibited a three log increase in GusA activity in comparison to the *gusA*-negative parent, and the study established a framework for the exploitation of highly expressed genomic regions for heterologous protein production (Douglas and Klaenhammer, 2011). Although this particular system was never evaluated within the context of

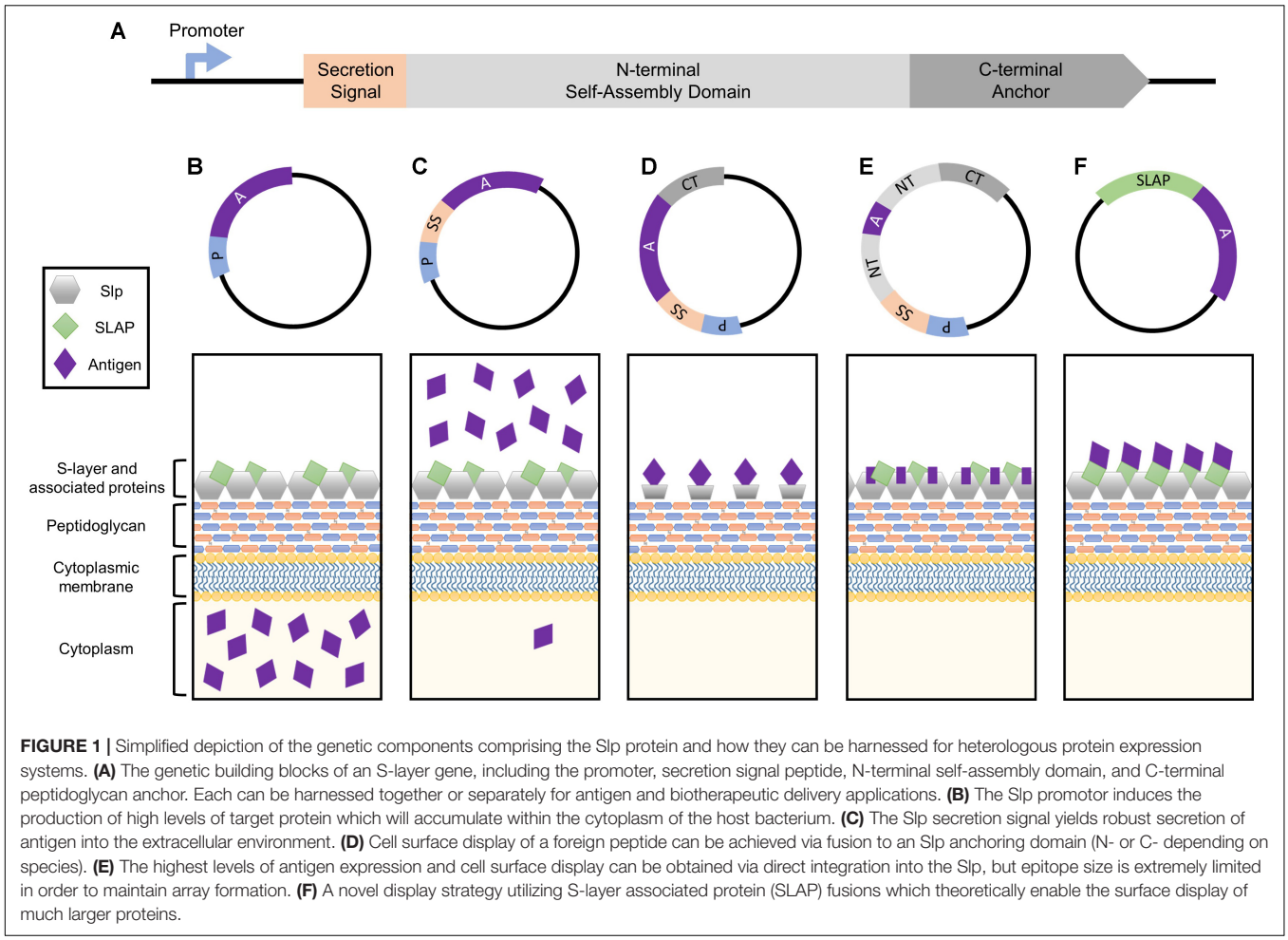

FIGURE 1 | Simplified depiction of the genetic components comprising the Slp protein and how they can be harnessed for heterologous protein expression systems. **(A)** The genetic building blocks of an S-layer gene, including the promoter, secretion signal peptide, N-terminal self-assembly domain, and C-terminal peptidoglycan anchor. Each can be harnessed together or separately for antigen and biotherapeutic delivery applications. **(B)** The Slp promotor induces the production of high levels of target protein which will accumulate within the cytoplasm of the host bacterium. **(C)** The Slp secretion signal yields robust secretion of antigen into the extracellular environment. **(D)** Cell surface display of a foreign peptide can be achieved via fusion to an Slp anchoring domain (N- or C- depending on species). **(E)** The highest levels of antigen expression and cell surface display can be obtained via direct integration into the Slp, but epitope size is extremely limited in order to maintain array formation. **(F)** A novel display strategy utilizing S-layer associated protein (SLAP) fusions which theoretically enable the surface display of much larger proteins.

vaccine delivery, the technique was shown effective for expressing antigens using an alternative highly expressed region within the *L. acidophilus* genome (O'Flaherty and Klaenhammer, 2016).

Moving beyond reporter genes and targeting more specific therapeutics, the *L. brevis* JCM 1559 *slpA* promoter was evaluated in an *L. casei* IGM393 host via incorporation into a mouse interleukin 10 (IL-10) secretion system (Kajikawa et al., 2010). Administration of IL-10 was previously shown to be an effective treatment of murine colitis when delivered via recombinant *L. lactis* (Steidler et al., 2000). The authors sought to improve upon this design by substituting in a strain hypothesized to be better adapted to mammalian body temperature. The delivery system was successful in that it yielded high levels of IL-10 secretion when cloned into *L. casei*, but accumulation of the protein varied widely based on pH (Kajikawa et al., 2010). Maximum efficiency occurred at pH 8.0 and dropped drastically as the pH became more acidic. Although the authors attributed low levels of the protein to its physical characteristics (Kajikawa et al., 2010), the selection of a promoter from a free-living species (e.g., *L. brevis*) over a low pH tolerating, vertebrate-adapted organism (e.g., *L. acidophilus*), may have also contributed (Duar et al., 2017). Nonetheless, the S-layer promoter has repeatedly demonstrated its utility for driving protein production systems, and thus merits future research with a focus on therapeutic molecule delivery.

EXPLOITATION OF THE SECRETION SIGNAL PEPTIDE

All *Lactobacillus* S-layer proteins characterized thus far are preceded by a 25–32 amino acid signal peptide sequence indicative of secretion through the general secretory pathway (Hynonen and Palva, 2013). By cloning this short sequence upstream of an antigen or biotherapeutic molecule, one can obtain robust secretion of a target protein (**Figure 1C**), thus this peptide is frequently harnessed for biotechnological applications.

The *L. brevis* ATCC 8287 *slpA* signal sequence, along with the promoter and transcription terminator, were used to drive expression and secretion of the *Escherichia coli* β-lactamase (*bla*) reporter gene in *L. lactis*, *L. brevis*, *L. plantarum*, *L. gasseri,* and *L. casei,* using a low-copy-number plasmid derived from pGK12 (Savijoki et al., 1997). In all hosts

tested, Bla was expressed and released into the culture medium, though highest yields were obtained by recombinant *L. lactis* and the strain of *L. brevis* from which the SlpA components were derived. Production of Bla was mainly restricted to exponential growth phase (Savijoki et al., 1997). Since the system was under control of an *slpA* promoter, it was unsurprising that efficiency was host-specific and growth-phase-dependent (see "Harnessing the strong native promoter").

The Slp secretion signal has frequently been harnessed for targeted therapeutic applications. In a study seeking to develop a recombinant *L. lactis* mucosal vaccine against porcine post-weaning diarrhea and edema disease, signal peptides from the *L. lactis* major extracellular protein, Usp45, and *L. brevis* SlpA, were used to stimulate the secretion of an *E. coli* F18 fimbrial adhesion protein (FedF) fused to a proteinase PrtP cell wall anchor (Lindholm et al., 2004). Both expression systems induced secretion of all tested FedF-PrtP fusions, however, the quantity of fusion proteins found in the culture medium was four to sixfold higher in those containing the SlpA signal peptide. This was a compelling find considering that the Usp45 signal sequence, previously shown to be one of the most effective *L. lactis* secretion signals (Nouaille et al., 2003), was outperformed by the heterologous SlpA signal sequence. A similar result was obtained with the human interferon alpha 2b (hIFNα-2b) gene (Zhang et al., 2010), used worldwide for the treatment of diseases such as hepatitis B and C (Chelbi-Alix and Wietzerbin, 2007). The addition of the *L. brevis* SlpA signal peptide increased the secretion efficiency threefold in comparison to the lactococcal Usp45 signal sequence (Zhang et al., 2010).

The SlpA signal sequence has also been employed for secretion of chromosomally inserted heterologous proteins. The *Bacillus anthracis* protective antigen (PA), fused to a dendritic cell (DC)-targeting peptide, was previously shown to induce protective immunity when delivered on a plasmid via *L. acidophilus* NCFM (Mohamadzadeh et al., 2009) and *L. gasseri* (Mohamadzadeh et al., 2010). Since a major advantage of using recombinant microbes for vaccine delivery is their ability to express multiple antigens (Wells and Mercenier, 2008), the *B. anthracis* PA was co-expressed with the *Clostridium botulinum* Serotype A neurotoxin heavy-chain antigen (O'Flaherty and Klaenhammer, 2016). The *C. botulinum* vaccine cassette, which utilizes an *L. acidophilus* SlpA secretion signal, was chromosomally inserted downstream of the highly expressed enolase gene. Western blot analysis and RNA sequencing confirmed expression of the two antigens (O'Flaherty and Klaenhammer, 2016). Although this strain was never evaluated *in vivo*, a similarly, constructed strain, also utilizing the SlpA secretion signal, but carrying only the *C. botulinum* antigen (O'Flaherty and Klaenhammer, 2016), was used to vaccinate mice but unable to confer complete protection (Sahay et al., 2018). Rather, vaccine efficacy was enhanced by intradermal injection of the purified immunogenic subunit, which elicited robust memory B cell responses and rendered mice resistant to lethal doses of botulinum neurotoxin serotype A (Sahay et al., 2018).

The Slp signal sequence is capable of stimulating ample secretion of target molecules, prompting its continued use in vaccine research platforms. Although antigen secretion by recombinant LABs has been shown effective for treating disease (Mohamadzadeh et al., 2005, 2009, 2010), exposure to proteolytic enzymes, low pH, and bile salts may encourage protein degradation and therefore decreased functionality (Wells and Mercenier, 2008); consequently cell wall anchoring has become a popular alternative. However, surface display is a balancing act. High exposure of a protein implies optimal host interaction, but also increased susceptibly to degradation. Alternatively, low exposure, via compact protein folding or embedment within the cell wall, confers protection in exchange for diminished efficacy (Michon et al., 2016). Several S-layer-mediated surface display approaches have attempted to resolve this relationship.

THE SELF-ASSEMBLING AND ANCHORING DOMAINS OF THE Slp

Cell wall anchoring via fusion to Slp structural domains (**Figure 1D**) enables direct interaction of target peptides with host mucosal tissues while simultaneously protecting them from degradation (Bermudez-Humaran et al., 2011; Wang et al., 2016). The first S-layer-mediated display of a foreign epitope was generated by fusing the S-layer homology (SLH) domain of *B. anthracis* to the normally secreted levansucrase of *Bacillus subtilis* (Mesnage et al., 1999). The surface-exposed levansucrase retained its enzymatic and antigenic properties, prompting a new area of research which exploits the anchoring abilities of the Slp for cell surface presentation. *Lactobacillus* Slps do not possess SLH domains and are instead composed of two structural domains: a variable terminal for monomer self-assembly (N- or C- depending on the species), and a highly conserved peptidoglycan anchor (Smit et al., 2001; Hynonen and Palva, 2013).

Several studies have used S-layer-mediated anchoring for recombinant protein display on S-layer-deficient bacteria. The cell wall binding domain of the *L. crispatus* F5.7 Slp (LbsA) in conjunction with the Slp promoter and secretion signal, were used for surface display of a GFP on several *Lactobacillus* chicken isolates (Mota et al., 2006). The intention was to generate a vaccine delivery framework for the immunization of broilers against infectious diseases, but never evolved past proof of concept. Vaccination via recombinant bacteria is a particularly attractive option for livestock and poultry operations since the lyophilized microorganisms can be blended into feed; a process that is easily scaled up (Wang et al., 2016). For human applications, the complete SlpA from *L. acidophilus* ATCC 4356 was fused to a GFP reporter for external presentation on a plasmid-cured, lactose-deficient derivative of *L. casei* ATCC 334 (Qin et al., 2014). The authors were able to develop a food-grade cell surface display vector by substituting lactose metabolism genes in place of antibiotic selection markers and verified gastrointestinal stability via *in vitro* modeling (Qin et al., 2014). The *L. crispatus* K2-4-3 SlpB C-terminal domain,

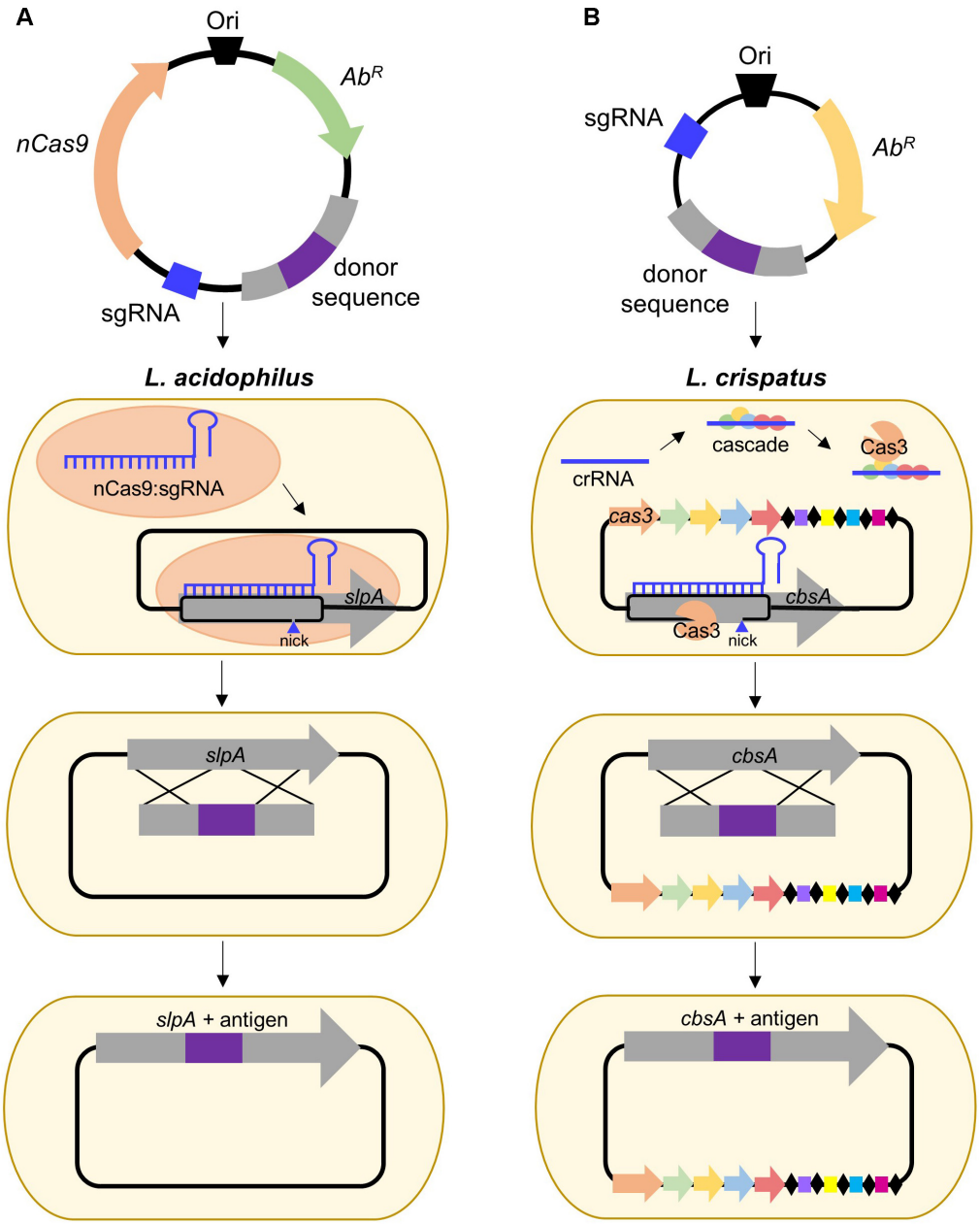

FIGURE 2 | Proposed strategies for CRISPR-based genome editing in relevant S-layer-forming lactobacilli. **(A)** Antigen integration into *L. acidophilus* SlpA via an exogenous Type II system employing a Cas9 nickase variant which introduces a guide RNA-targeted single-stranded break. Unlike wild-type Cas9 which generates blunt double-stranded breaks (DSB), nickases cut only one strand of the DNA, permitting genome editing in bacteria deficient in DSB repair (Chiang et al., 2016; Song et al., 2017). **(B)** Antigen integration into *L. crispatus* CbsA using the endogenous Type I system which consists of the CRISPR-associated complex for antiviral defense (Cascade) and the signature Cas3 nuclease (Barrangou, 2015).

LcsB, was employed for the display of not only a GFP reporter (Hu et al., 2011), but also a carcinoembryonic antigen (CEA) (Zhang et al., 2016). Previous studies have shown that CEA is capable of eliciting strong T-cell and humoral immune responses, which can hinder tumor growth (Greiner et al., 2002). Oral administration of recombinant CEA-presenting *L. lactis* to mice yielded significantly higher levels of CEA-specific secretory IgA and a higher spleen index in comparison to CEA antigen alone or the negative control, demonstrating the potential of *L. lactis* CEA as a cancer vaccine (Greiner et al., 2002).

Since the S-layer of *L. crispatus* is capable of binding intestinal extracellular matrices such as collagen and laminin, heterologous expression of Slps has also been used to improve the adhesive capacity of host organisms. For example, the *L. crispatus* JCM5810 collagen-binding S-layer protein, CbsA (Martinez et al., 2000), as well as its individual domains (Antikainen et al., 2002),

were expressed on the surface of *L. casei* enabling recombinant organisms to bind various extracellular matrices. Similarly, a surface display cassette consisting of the *L. brevis* ATCC 8287 SlpA receptor-binding domain fused to a PrtP spacer enhanced *L. lactis* adherence to Intestine 407 cells (Avall-Jaaskelainen et al., 2003). Both approaches were able to significantly increase the adhesive capacity of engineered organisms, but yet to be evaluated is the probable synergistic effect of combining improved adhesion with an S-layer anchored antigen in what could potentially be a potent vaccine design platform.

Unlike much of the work presented in this review, a handful of studies achieved Slp-mediated display of foreign proteins without DNA manipulation in the host by exploiting the inherent ability of Slps to anchor and self-assemble. For instance, the *L. acidophilus* C-terminal anchor (SAC) was attached to a GFP reporter, then produced and purified in *E. coli*. The SAC-GFP fusion protein was capable of binding lithium chloride-pretreated surfaces of wild-type *L. acidophilus*, *L. helveticus*, and *L. crispatus* (Smit et al., 2001). Similarly, *L. crispatus* K2-4-3 LcsB-GFP fusions were able to associate with SDS-pretreated surfaces of various S-layer-forming LABs including *L. brevis*, *L. helveticus*, *L. crispatus*, and *Lactobacillus salivarius* as well as several non-S-layer-formers, including *L. lactis, L. delbrueckii*, *Lactobacillus johnsonii*, and *Streptococcus thermophilus* (Hu et al., 2011). Interestingly, neither the SAC-GFP fusion nor LcsB-GFP were able to bind the surface of *L. casei* (Smit et al., 2001; Hu et al., 2011). This approach is unique in that it offers an alternative way to deliver foreign proteins while also circumventing the GMO (genetically modified organism) label, but is limited by its inability to generate additional heterologous protein *in vivo* and susceptibility to replacement by wild type Slps. However, in general, studies using heterologously expressed Slps and Slp anchors are hindered by inadequate secretion across the cell wall (Hu et al., 2011) or inability to form an array due to irregular folding and/or lack of cell surface exposure (Martinez et al., 2000). Consequently, rather than tease apart the efficient Slp secretion and display system, there is now interest in harnessing it as a whole through the direct insertion of foreign peptides within the context of the protein.

DELIVERY VIA DIRECT INTEGRATION WITHIN THE Slp

The extraordinarily high, stable abundance of the Slp, makes it an enticing target for antigen display and delivery via direct integration into its genome sequence (**Figure 1E**). The presentation of an exogenous protein within the context of the Slp was first achieved in *Caulobacter crescentus* through the random introduction of a pilin peptide from *Pseudomonas aeruginosa* strain K. Eleven potential sites of successful insertion were identified, demonstrating for the first time the capacity of the Slp to act as a carrier for foreign epitopes (Bingle et al., 1997).

Many subsequent studies have focused on mapping the S-layer to gain insight into ideal positioning for novel insertions, including in *Lactobacillus*. In *L. acidophilus* ATCC 4356, peptides ranging from 7 to 13 amino acids were randomly introduced into the Slp (Smit et al., 2002). Within the variable N-terminal (SAN), five of these positions maintained paracrystalline structure formation *in vitro*, while four others resulted in the complete abolishment of any array-forming capacity. Unsurprisingly, an insertion into the cell wall-binding domain had no effect on assembly (Smit et al., 2002). Similarly, the *L. brevis* SlpA was mapped via cysteine-scanning mutagenesis combined with sulfhydryl modification to identify locations of high surface accessibility and verify that the mutations did not alter self-assembly properties (Vilen et al., 2009). Combined, these works established several stable, surface-accessible insertion sites within the *Lactobacillus* Slp, yet few researchers have capitalized on this knowledge.

Currently, only two studies have successfully integrated antigens within the context of the *Lactobacillus* Slp. Through an inducible expression system, the poliovirus VP1 epitope was evaluated in four potential *L. brevis* ATCC 8287 *slpA* insertion sites (Avall-Jaaskelainen et al., 2002). The location that demonstrated the best surface expression was then targeted for chromosomal insertion of the c-Myc epitope via direct double-crossover integration (Avall-Jaaskelainen et al., 2002). A uniformly chimeric S-layer was obtained without any effect on array formation. More recently, the membrane proximal external region (MPER) epitope from human immunodeficiency virus type 1 (HIV-1) was inserted into *L. acidophilus* NCFM SlpA (Kajikawa et al., 2015). *L. acidophilus* NCFM is regularly employed for mucosal vaccine delivery due in part to its direct interactions with the dendritic cell-specific antigen DC-SIGN (Konstantinov et al., 2008) and adaptation to the harsh conditions associated with gastric transit (Sanders and Klaenhammer, 2001). Vaccination via the recombinant organism, in conjunction with an IL-1β adjuvant, successfully stimulated MPER-specific antibody production in both the serum and mucosal secretions of mice (Kajikawa et al., 2015). This study marks the first and only instance of an Slp-integrated antigen being evaluated *in vivo*.

The establishment of a uniformly chimeric S-layer translates to approximately 10^5 instances of epitope display on the surface of a single bacterium (Sleytr and Messner, 1983). Despite these considerable numbers, insert size is exceptionally limited in order to preserve S-layer array formation (Smit et al., 2002). Currently, peptides longer than 19 amino acids are unable to be inserted into SlpA without disrupting the lattice structure (Kajikawa et al., 2015). Therefore, alternative methods exploiting auxiliary proteins associated with the S-layer are now being investigated for the display and delivery of larger antigens, as seen in Slp-mediated anchoring studies (see "The self-assembling and anchoring domains of the Slp"), but at frequencies more akin to direct integration.

S-LAYER ASSOCIATED PROTEINS

The *Lactobacillus* S-layer, once thought to be solely composed of repeating monomeric Slp subunits, is actually far more

complex (Johnson et al., 2013, 2015). It is now widely accepted that S-layers can act as scaffolds for the external display of numerous auxiliary proteins, termed S-layer associated proteins (SLAPs), which can confer additional physiological functionalities (Johnson et al., 2013, 2015, 2017; Hymes et al., 2016; Johnson and Klaenhammer, 2016; Celebioglu and Svensson, 2017). Recently, the SLAP profile of *L. acidophilus* NCFM was quantified via multiplexing mass spectrometry (Klotz et al., 2017). Although results revealed significant growth stage-dependent alterations, they also highlighted several proteins with consistent high expression in both logarithmic and stationary growth phases (Klotz et al., 2017). The surface location and abundance of these proteins make them excellent targets for biotherapeutic delivery. Unlike Slp integrants, SLAP fusions are theoretically less limited in epitope size as they are not prone to S-layer array disruption (**Figure 1F**). Both the native SLAP promoter and secretion signal can be harnessed for this process with the intent to maintain high expression, secretion, and surface localization coupled with the display and delivery of a significantly larger and therefore more potent epitope.

TABLE 1 | S-layer protein applications in recombinant protein expression systems and biotherapeutic delivery platforms.

S-layer-forming lactobacilli	Slp	Slp Component	Host	Antigen/Reporter	Results	Reference
L. acidophilus	SlpA; SlpB	Promoter	*L. casei*	CAT	Evaluated *slpA* and *slpB* promoters; only *slpA* remained active under all tested growth conditions	Boot et al., 1996b
	SlpA	Promoter	*L. lactis; L. reuteri* chicken crop isolates	eGFP	The *slpA* promoter was highly efficient in *L. lactis* but nearly inactive in *L. reuteri* isolates	Lizier et al., 2010
	SlpA	Promoter	*L. casei; L. paracasei; L. plantarum; L. lactic; L. helveticus; L. acidophilus; L. lactis; E. coli*	GFP	Plasmids encoding *slpA* promoter exhibited distinct properties based on host and growth phase	Chen et al., 2014
	SlpA	Promoter	*L. acidophilus*	GusA	The *slpA*-driven GusA activity increased three logs in comparison to the *gusA*-negative parent	Douglas and Klaenhammer, 2011
	SlpA	Secretion	*L. acidophilus*	*B. anthracis* protective antigen (PA); *C. botulinum* Serotype A neurotoxin heavy-chain antigen	The SlpA signal sequence generated stable and robust secretion of the *C. botulinum* antigen	O'Flaherty and Klaenhammer, 2016
	SlpA	Secretion	*L. acidophilus*	*C. botulinum* Serotype A neurotoxin heavy-chain antigen	Recombinant organism was unable to confer complete protection against an experimental botulism challenge	Sahay et al., 2018
	SlpA	Integration	*L. casei*	GFP	Generated a food-grade SlpA-based cell surface display vector and verified gastrointestinal stability *in vitro*	Qin et al., 2014
	SlpA	Integration	*L. acidophilus*	HIV-1 membrane proximal external region (MPER)	Delivery of MPER peptide via direct integration into SlpA stimulated antigen-specific antibody production in both serum and mucosal secretions of vaccinated mice	Kajikawa et al., 2015
L. brevis	SlpA	Promoter	*L. lactis; L. plantarum; L. gasseri*	GusA; Luc; PepN	The *slpA* promoter was recognized in all strains but especially *L. lactis* and *L. plantarum*	Kahala and Palva, 1999
	SlpA	Promoter	*L. casei*	Mouse IL-10	The *slpA* promoter yielded high levels of IL-10 but was sensitive to low pH	Kajikawa et al., 2010

(Continued)

TABLE 1 | Continued

S-layer-forming lactobacilli	Slp	Slp Component	Host	Antigen/Reporter	Results	Reference
	SlpA	Promoter; secretion	*L. lactis; L. brevis; L. plantarum; L. gasseri; L. casei*	*E. coli* β-lactamase (Bla)	Bla was expressed in all hosts, but most efficiently in *L. lactis* and *L. brevis*; production was restricted to exponential growth phase	Savijoki et al., 1997
	SlpA	Secretion	*L. lactis*	*E. coli* F18 fimbrial adhesion protein (FedF)	The SlpA signal sequence increased FedF secretion efficiency four to sixfold in comparison to the lactococcal Usp45 signal sequence	Lindholm et al., 2004
	SlpA	Secretion	*L. lactis*	Human interferon alpha 2b (hIFNα-2b)	SlpA signal sequence increased hIFNα-2b secretion efficiency threefold in comparison to the lactococcal Usp45 signal sequence	Zhang et al., 2010
	SlpA	Secretion; structural domain	*L. lactis*	None	Surface expression of SlpA receptor-binding domain increased adherence to Intestine 407 cells	Avall-Jaaskelainen et al., 2003
	SlpA	Integration	*L. brevis*	Poliovirus VP1 epitope; c-Myc epitope	Directly inserted epitopes into SlpA without disrupting array formation	Avall-Jaaskelainen et al., 2002
L. crispatus	LbsA	Promoter; secretion, structural domain	*Lactobacillus* chicken isolates	GFP	Achieved expression, secretion and surface presentation of GFP	Mota et al., 2006
	LcsB	Structural domain	*L. lactis*	GFP	Achieved surface presentation of GFP	Hu et al., 2011
	LcsB	Structural domain	*L. lactis*	Carcinoembryonic antigen (CEA)	LcsB-mediated display of CEA stimulated higher levels of antigen-specific secretory IgA and a higher spleen index when fed to mice	Zhang et al., 2016
	CbsA	Structural domain	*L. casei*	None	Recombinant *L. casei* expressing CbsA was able to bind immobilized collagens	Martinez et al., 2000
	CbsA	Structural domain	*L. casei*	None	Recombinant expression of CbsA domains enabled adhesion to laminin and collagen	Antikainen et al., 2002

Slp ENGINEERING PLATFORMS

Presently, the two most popular techniques for engineering LABs are the NICE (NIsin Controlled gene Expression) system in *L. lactis* and the pORI-based *upp* counterselective gene replacement system in *L. acidophilus*. There is also growing interest surrounding the use of CRISPR-Cas technology, though its application in bacterial genome editing remains relatively underrepresented (Selle and Barrangou, 2015; Hidalgo-Cantabrana et al., 2017).

The NICE System

The NICE system uses nisin to drive heterologous protein expression in *L. lactis* (Kuipers et al., 1995). Through the insertion of signal transduction genes from a nisin gene cluster into a nisin-negative *L. lactis* strain, NZ9000 was created (Kuipers et al., 1998). Subsequently, when a gene of interest is inserted downstream of the inducible *nisA* promoter, expression of that gene can be obtained by the addition of nisin to the culture medium (Mierau and Kleerebezem, 2005). Since its conception, NICE has become one of the most successful and widely used expression systems in Gram-positive bacteria (Mierau and Kleerebezem, 2005). Indeed, the NICE system was even employed for the production a number of the S-layer fusion proteins mentioned above (Hu et al., 2011; Zhang et al., 2016) and to render the non-adhesive *L. lactis* NZ9000 adhesive via the addition of an *L. brevis* SlpA receptor-binding domain (Avall-Jaaskelainen et al., 2003). The availability of an easily engineered, non-S-layer-forming organism, has greatly accelerated not only our understanding of the biological role of an S-layer but also how we can exploit it.

The pORI-Based *upp* System

The establishment of the pORI-based *upp* counterselective gene replacement system in S-layer-former *L. acidophilus* NCFM, first employed for the functional characterization of SlpX (Goh et al., 2009), has since become an invaluable tool for S-layer component engineering. The system uses a *upp*-encoded uracil phosphoribosyltransferase (UPRTase) as a counterselection marker to positively select for double crossover homologous recombination events. The method has been adapted for numerous Slp studies ranging from reporter integration (Douglas and Klaenhammer, 2011), anchoring/adjuvant assessment (Kajikawa et al., 2011), and targeted antigen delivery systems for disease protection (Kajikawa et al., 2015; O'Flaherty and Klaenhammer, 2016). Similar counterselective systems have also been developed in non-S-layer-formers including *L. gasseri* ATCC 33323 (Selle et al., 2014) and *L. casei* ATCC 393 (Song et al., 2014), but have yet to be harnessed for Slp analyses. In general, the technique remains a superior approach for characterizing the functional genetics of lactobacilli without the additional pressures required for plasmid maintenance.

CRISPR

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) together with CRISPR-associated (Cas) proteins, form the prokaryotic adaptive immune system which provides DNA-encoded, RNA-guided, sequence-specific protection against viral invaders (Barrangou and Doudna, 2016). The current classification system uses two broad classes consisting of six major types (I–VI) which can be allocated into approximately 30 subtypes (Klompe and Sternberg, 2018). The simplest of these is the Type II system which relies on the activity of a singular Cas9 endonuclease and has gained immense popularity for its ability to be repurposed for genome editing (Chylinski et al., 2014; Barrangou and Doudna, 2016; Klompe and Sternberg, 2018). Depending on the organism, this can be done via harnessing of the endogenous system or supplying an exogenous Cas9:single guide RNA (sgRNA) complex. To date, no CRISPR-based genome editing has been performed in S-layer-forming lactobacilli, but it has been conducted in *L. casei* (Song et al., 2017) and *L. lactis* (Berlec et al., 2018), frequent hosts for recombinant Slp investigative research. Nevertheless, the development and delivery of a functional CRISPR-Cas9 plasmid remains a critical need, particularly in organisms such as *L. acidophilus* NCFM (depicted in **Figure 2A**), which does not possess an active CRISPR system (Crawley et al., 2018), but has proven to be hugely impactful in both probiotic and Slp research.

Despite the popularity of Type II engineering, Type I systems have been harnessed for genome editing in *Clostridium* and archaea (Pyne et al., 2016; Cheng et al., 2017) and transcriptional regulation in *E. coli* (Luo et al., 2015). S-layer formers, *L. crispatus,* and *L. helveticus*, frequently possess Type I systems (Crawley et al., 2018) and are also of interest for vaccine delivery. *L. crispatus* is a natural inhabitant of the human vaginal tract (Lepargneur, 2016), rendering it suitable for delivery of antigens targeting sexually transmitted diseases, such as HIV, while *L. helveticus* is predominately associated with dairy (Taverniti and Guglielmetti, 2012), which could be advantageous when considering modes of delivery and stability of the vaccine. A proposed strategy for Type I genome editing in *L. crispatus* is illustrated in **Figure 2B**. Harnessing of the endogenous system or delivery of a functional exogenous system both possess the potential to be powerful tools for advancing Slp-mediated biotherapeutic research.

The NICE and pORI-based *upp* systems have both proven effective for S-layer-mediated biotherapeutic delivery; however, next-generation genome editing tools, such as CRISPR-Cas, hold tremendous potential for bacterial engineering overall (Selle and Barrangou, 2015; Hidalgo-Cantabrana et al., 2017). Many S-layer-forming lactobacilli possess endogenous CRISPR systems, making them promising candidates for future S-layer engineering studies. Alternatively, delivery of a functional CRISPR to strains devoid of a system, will also greatly accelerate the pace at which recombinant organisms can be generated. Ironically, despite CRISPR originating as the bacterial adaptive immune system, CRISPR-based bacterial genome editing still remains relatively underexploited, though recent studies have provided valuable insights for its widespread future implementation (Selle and Barrangou, 2015).

FUTURE DIRECTIONS

The Slp is a multi-faceted engineering target with both biotherapeutic and biotechnological applications (Sleytr et al., 2014). However, within the *Lactobacillus* genus, harnessing of this protein remains early in its development (Avall-Jaaskelainen and Palva, 2005; Hynonen and Palva, 2013); nonetheless, the research highlighted above (summarized in **Table 1**) advocates for its continued pursuit. The *slp* promoter and signal peptide are undoubtedly adept at driving robust expression and secretion of target proteins, while the structural domains have successfully displayed foreign epitopes and improved the adhesive capacity of host cells. More complex and novel display strategies, such as direct integration into the Slp or SLAP fusions, are innovative approaches for cell surface presentation that also exploit the inherent properties of S-layer-forming lactobacilli. In general, eliciting consistent immune responses via the mucosal route of administration is hindered by rapid elimination or inability to make contact with M cells and other mucosal tissues involved in antigen uptake and processing (Ogra et al., 2001). Thus the ability of recombinant LABs, and the S-layer in particular, to promote antigen uptake and stimulate the adaptive immune response is highly desirable (Konstantinov et al., 2008).

Numerous studies have established vaccine platforms based on one or more components of the *Lactobacillus* Slp, however, there remains a disconnect between delivery and efficacy. Despite extensive reviews touting the effectiveness of the LAB-based vaccines *in vivo* (Wells and Mercenier, 2008; LeCureux and Dean, 2018), few S-layer-based delivery frameworks have moved into animal models. Although successful secretion and/or surface display of the reporter/antigen is regularly achieved, only three of the recombinant organisms presented above were tested in mice

(Kajikawa et al., 2015; Zhang et al., 2016; Sahay et al., 2018), whereas the remainder were more focused on establishing that antigen production/display was even possible. Thus, an important step moving forward will be connecting the delivery of these antigens with an actual vaccination event, therefore surpassing proof of concept studies and ultimately demonstrating disease protection.

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CK and RB wrote and edited the manuscript.

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Discovery of Bacterial Deaminases That Convert 5-Fluoroisocytosine Into 5-Fluorouracil

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Cytosine is one of the four letters of a standard genetic code, found both in DNA and in RNA. This heterocyclic base can be converted into uracil upon the action of the well-known cytosine deaminase. Isocytosine (2-aminouracil) is an isomer of cytosine, yet the enzymes that could convert it into uracil were previously mainly overlooked. In order to search for the isocytosine deaminases we used a selection strategy that is based on uracil auxotrophy and the metagenomic libraries, which provide a random pool of genes from uncultivated soil bacteria. Several genes that encode isocytosine deaminases were found and two respective recombinant proteins were purified. It was established that both novel deaminases do not use cytosine as a substrate. Instead, these enzymes are able to convert not only isocytosine into uracil, but also 5-fluoroisocytosine into 5-fluorouracil. Our findings suggest that novel isocytosine deaminases have a potential to be efficiently used in targeted cancer therapy instead of the classical cytosine deaminases. Use of isocytosine instead of cytosine would produce fewer side effects since deaminases produced by the commensal *E. coli* gut flora are ten times less efficient in degrading isocytosine than cytosine. In addition, there are no known homologs of isocytosine deaminases in human cells that would induce the toxicity when 5-fluoroisocytosine would be used as a prodrug.

Keywords: metagenomics, deaminase, isocytosine, 5-fluorouracil, cancer therapy

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INTRODUCTION

Cytosine is a common cellular pyrimidine. It is a part of the cytidine triphosphate (CTP), which can serve as a high-energy molecule and a co-factor in metabolic processes. Cytosine is also one of the nitrogenous bases both in DNA and in RNA, which makes it an important molecule with many functions in the cell. It is a genomic "wild card" that is a key to generating genomic flexibility (Nabel et al., 2012). The cytosine modifications in messenger RNA precursors have significant impact on the functions of the proteins they encode (Gerber and Keller, 2001). Due to cytosine deamination, the AID/APOBEC family of cytidine deaminases have the unique potential to influence both epigenetic and genetic forms of heritable information, making these enzymes the link between both forms of inheritance (Chahwan et al., 2010). It is obvious that cytosine has objectives in the cell that are beyond the storage of information in the genome and most of these functions rely on the enzymes that are capable of modifying it.

Cytosine deaminase (CD) from *E. coli* is a member of the amidohydrolase superfamily that catalyzes the hydrolytic deamination of cytosine, forming uracil, and ammonia (Hall et al., 2011).

Members of amidohydrolase superfamily are predominantly found in bacteria and also in certain fungi, but not in mammalian cells (Finn et al., 2016). Due to their ability to convert the non-toxic prodrug, 5-fluorocytosine (5-FC) into a well-known anticancer drug 5-fluorouracil (5-FU) (Raza et al., 2015) cytosine deaminases are among the key enzymes used in the prodrug-mediated control of variety of cancer types. Bacterial and yeast CDs have been investigated in the context of cancer therapy in detail by many research groups (Malekshah et al., 2016). After activation by CD, 5-FU can be further changed into potent pyrimidine antimetabolites by other cellular enzymes. It was shown that the 5-FU produced upon the action of this enzyme mediates the growth inhibition and apoptosis-mediated cell death of a variety of tumor types both *in vitro* and *in vivo* (Karjoo et al., 2016; Gaded and Anand, 2018). In this study, we applied our genetic screening system in order to search for new enzymes that could be beneficial for this type of cancer therapy. We chose isocytosine as the substrate for these putative enzymes.

Isocytosine (2-aminouracil) is an isomer of cytosine. Together with isoguanine it was one of the first non-natural nucleobases used in experiments of unnatural base pair studies. These studies seek to understand how the complementarity originally appeared in the forms of the A-T and G-C pairs and ultimately – to reveal the origin of the natural nucleic acids (Hirao et al., 2012). Isocytosine pairs in a normal manner with non-natural isoguanine and also in a "reversed Watson-Crick" manner with natural guanine. Therefore, it is used for structural studies of nucleic acids (Yang et al., 1998) as well as in physical-chemical studies involving metal complex binding (Gupta et al., 2004), hydrogen-bonding (Camacho-García et al., 2015), tautomerism and proton transfer (Delchev and Mikosch, 2007) in nucleobases. Moreover, following the logic of cytosine deaminase–5-fluorocytosine enzyme–prodrug pair, isocytosine could provide a base for a putative new prodrug 5-fluoroisocytosine, if it was used together with a putative isocytosine deaminase (**Figure 1**).

Metagenomes are an appealing pool of otherwise inaccessible genes that could reveal new enzymatic activities and even biochemical pathways (Popovic et al., 2015). In this study, we used the metagenomic libraries and an *E. coli* uracil auxotroph-based selection strategy in order to search for putative isocytosine deaminases. Since the expected enzymatic activity of the isocytosine deaminase is the conversion of isocytosine into uracil, we used an *E. coli* strain lacking several *pyr* genes that are responsible for the pyrimidine *de novo* biosynthesis (Aučynaitė et al., 2018). This strain is unable to grow in the defined synthetic medium without a source of uracil. Therefore, it could be used as a host strain for functional screening of the metagenomic libraries for the uracil generating enzymes. In this study isocytosine was used as a substrate supposing that the clones encoding the appropriate deaminases would convert isocytosine to uracil hence allowing the growth on the mineral medium.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Primers, Media and Reagents

Escherichia coli DH5α (Thermo Fisher Scientific) was used for routine DNA manipulations. *E. coli* DH10B (Thermo Fisher Scientific) was used for disruption of *pyr* genes to obtain the DH10BΔ*pyr* strain (Aučynaitė et al., 2018). *E. coli* BL21(DE-3; Novagen) was used to produce the recombinant deaminase proteins.

High copy number cloning vector pUC19 (Thermo Fisher Scientific) was used for the preparation of metagenomic libraries (Aučynaitė et al., 2018). Vectors for inducible expression of C-terminally 6xHis-tagged proteins pET21b(+), pET28a(+; Novagen) and pQE-70 (Qiagen) were used for cloning of the URA3, Vcz, and *E. coli* CD CodA genes, respectively.

Standard techniques were used for DNA manipulations (Sambrook and Russell, 2001). The URA3 deaminase gene was amplified by PCR using primers *URA3FW* 5′-ATATACATATGGCCAAAACACTCTTGG-3′ and *URA3RV* 5′-TATATCTCGAGTTCACCCATGACC-3′, digested with *Nde*I and *Xho*I and cloned into the corresponding site of pET21b(+) vector. The Vcz deaminase gene was amplified by PCR using primers *VczFW* 5′-ATATACCATGGACAAAAGAACGCTGC-3′ and *VczRV* 5′-TATATCTCGAGCAGCCGATGCCGGTT-3′, digested with *Nco*I and *Xho*I and cloned into the corresponding site of pET28a(+) vector. The *E. coli* CD CodA gene was amplified by PCR using primers *CodAFW*: 5′-ATTAAGCATGCCGAATAACGCTTTA-3′ and *CodARV*: 5′-TAATTAAGCTTTCAACGTTTGTAATCGAT-3′, digested with *Sph*I and *Hind*III and cloned into the corresponding site of pQE-70 vector. All of the DNA primers were synthesized at Metabion International AG. All of the restriction endonucleases were purchased from Thermo Fisher Scientific.

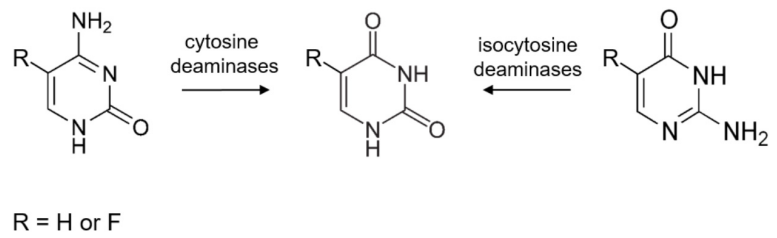

FIGURE 1 | A schematic representation of the classical cytosine deaminase (CD) and the predicted isocytosine deaminase substrates and products.

Escherichia coli strains transformed with recombinant plasmids were grown in nutrient broth (NB; Oxoid, Thermo Fisher Scientific) medium supplemented with either 100 mg/L ampicillin or 50 mg/L kanamycin, as required, at 37°C with aeration (unless noted otherwise). *E. coli* DH10BΔ*pyr* cells transformed with metagenomic libraries were grown in M9 minimal medium with casamino acids (Ausubel et al., 2003) supplemented with 100 mg/L ampicillin, 15 mg/L kanamycin, 20 mg/L cytosine, isocytosine or uracil, as required, at 37°C with aeration. A final concentration of 0.1 mM IPTG was added to the M9 minimal medium for induction of *E. coli* CD CodA gene expression.

Uracil and 5-fluorouracil were purchased from Sigma-Aldrich Co., Isocytosine was purchased from Combi-Blocks Inc. Cytosine was purchased from Alfa Aesar. 5-fluoroisocytosine was synthesized using the established protocols, with slight modifications (Chen et al., 2009 WO/158011 A1).

Over-Expression and Purification of the Recombinant 6xhis Tagged Isocytosine Deaminase Proteins

Vcz and URA3 deaminase genes were cloned into pET28a(+) or pET21b(+) vectors, respectively, and transformed in to the BL21(DE-3) cells. The resulting bacteria were grown in LB medium containing 50 mg/L kanamycin (for pET28a(+)) or 100 mg/L ampicillin (for pET21b(+)). The culture was grown at 37°C until OD_{600} reached 0.5–0.6. It was then cooled on ice and the inducer isopropyl-1-thio-β-D-galactopyranoside (IPTG, Thermo Fisher Scientific) was added to a final concentration of 0.5 mM. The induced cells then were incubated at 20°C overnight. The cells were then collected by centrifugation, resuspended in 50 mM TRIS–HCl, pH 8, and disrupted by sonication at 750 W for 1 min using a VC-750 ultrasound processor (Sonics 0026 Materials, Inc.). Cell debris was removed by centrifugation at 16000 × *g* for 10 min.

Cell extracts were loaded onto a Ni-NTA column (GE Healthcare) previously equilibrated with 50 mM TRIS–HCl, pH 8. The adsorbed proteins were eluted with 50 mM TRIS–HCl, pH 8 using linear gradient of 0–500 mM imidazole. The fractions containing the proteins were pooled and desalted by dialysis against 50 mM TRIS–HCl, pH 8. The purity of recombinant proteins was confirmed by electrophoresis on a 12% SDS–PAGE gel visualized by Coomassie Brilliant Blue staining. The concentration of recombinant proteins was measured using Lowry method (Lowry et al., 1951) with bovine serum albumin as the standard.

Substrate Specificity Assays

The enzymatic reactions were carried out at 37°C in 50 mM TRIS–HCl, pH 8.0 buffer and incubated for 1 h. The 20 μL final volume of the reaction mixture contained 1 μg of purified recombinant 6xHis tagged Vcz or URA3 deaminase protein and 20 mM of substrate (cytosine, isocytosine, or 5-fluoroisocytosine).

After the incubation, 1 μL of the reaction mixture was used for the thin layer chromatography (TLC) analysis. TLC was performed on aluminum sheets coated with silica gel 60 F254 using the methanol:chloroform (1:9) mixture as a mobile phase. The spots were detected and visualized under the 254 nm UV light.

HPLC-MS analyses were performed using a high performance liquid chromatography system, equipped with a photo diode array detector (SPD-M20A) and a mass spectrometer (LCMS-2020), equipped with an electrospray ionization (ESI) source. The chromatographic separation was conducted using a YMC Pack Pro column, 3 mm × 150 mm at 40°C and a mobile phase that consisted of 0.1% formic acid water solution (solvent A) and acetonitrile (solvent B). Mass spectrometry data was acquired in both positive and negative ionization mode and analyzed using the LabSolutions LCMS software.

Enzyme Activity Measurements

The activity of isocytosine deaminases were assayed spectrophotometrically at 21°C by monitoring the decrease in absorbance at 285 nm (ε_{285} = 3760 M^{-1} cm^{-1}) or 260 nm (ε_{260} = 2715 M^{-1} cm^{-1}) resulting from the deamination of isocytosine or 5-fluorisocytosine to uracil or 5-fluorisocytosine, respectively, by using a Helios gamma UV-visible spectrophotometer (Thermo Fisher Scientific). The reaction was initiated by the addition of the appropriate amount of the enzyme to 0.05 M TRIS–HC1, pH 8.0, buffer supplemented with isocytosine (5–700 μM) and the initial reaction rates were recorded. The kinetic parameters (k_{cat} and K_m) of the purified Vcz and URA3 deaminases were determined by fitting the experimental data from three independent experiments using a Michaelis-Menten equation (SigmaPlot 10).

Synthesis of 5-Fluoroisocytosine

Ethylfluoroacetate (1.93 mL, 20 mmol) and ethylformate (1.74 mL, 20 mmol) were added to the cooled suspension of sodium hydride (960 mg, 20 mmol) in 30 mL of anhydrous ether. The reaction mixture was stirred for 20 h at room temperature. The solvent was evaporated and the residue was cooled in an ice bath. Guanidine hydrochloride (1.38 g, 60 mmol) was neutralized by addition to a solution of sodium ethylate (2.5 M in ethanol, 24 mL) cooled in an ice bath. The solution was stirred for 30 min, the formed sodium chloride was filtered and the filtrate was added to the cooled reaction mixture. The mixture was refluxed for 17 h. After the reaction was completed (as monitored by TLC), the formed brownish precipitate was filtered. The filtrate was concentrated under reduced pressure and acidified with 1 N HCl solution to pH 4.0. The crude reaction mixture was twice purified by reverse phase column chromatography (C-18 cartridges, water/methanol mixture, 10:0→10:2). The solvents were removed under reduced pressure to afford 780 mg (30% yield) of 5-fluoroisocytosine. MS (ESI^+), m/z 128.05 $(M-H)^-$, 130.05 $(M + H)^+$. UV λ_{max} 263 nm. ^1H NMR (DMSO–d_6, 400 MHz): δ = 6.50 (s, 2H, NH_2); 7.64 (d, 1 H, J = 4.3 Hz, CH); 11.42 (s, 1H, NH). ^{13}C NMR (DMSO–d_6, 100 MHz): δ = 142.43; 144.77; 153.25; 156.15.

RESULTS

Screening for Isocytosine Deaminases

The *E. coli* DH10BΔ*pyr* strain is unable to grow in the defined synthetic medium without a source of uracil, because its pyrimidine *de novo* biosynthesis pathway has been disrupted. Therefore, these cells can be used as a host while searching for isocytosine deaminases in the metagenomic libraries, if the M9 minimal medium is supplemented with isocytosine as a sole source of uracil. Of the several positive hits, three transformants exhibited strong growth on isocytosine, but not cytosine (**Figure 2**, Vcz, URA3, and URA4); one, which could use isocytosine as well as cytosine, was also selected as control (**Figure 2**, KANOS), as it was reminiscent of classical CD (**Figure 2**, CodA). It is shown in **Figure 2** that proteins encoded all four of the selected DNA fragments are functional *in vivo* and are able to convert isocytosine into uracil thus restoring the growth phenotype of *E. coli* DH10BΔ*pyr* cells.

Plasmid DNA was isolated from these transformants and sequenced using primer-walking method. The presence of a single ORF encoding a deaminase was revealed in each of the DNA fragments. The DNA fragments, ORFs and corresponding

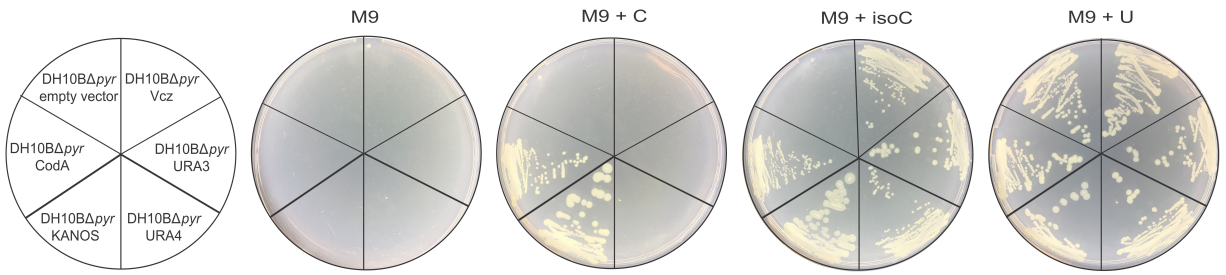

FIGURE 2 | *In vivo* functionality of *E. coli* CD (CodA, in pQE70 expression vector) and the DNA fragments that were selected from four metagenomic libraries: KANOS, URA4, URA3, and Vcz (in pUC19 vector). Empty pQE70 vector was used as a negative control. Minimal medium (M9) was supplemented 100 mg/L ampicillin, 15 mg/L kanamycin, 0.1 mM IPTG, and either with 20 mg/L cytosine (M9 + C), isocytosine (M9 + isoC) or uracil (M9 + U, positive control).

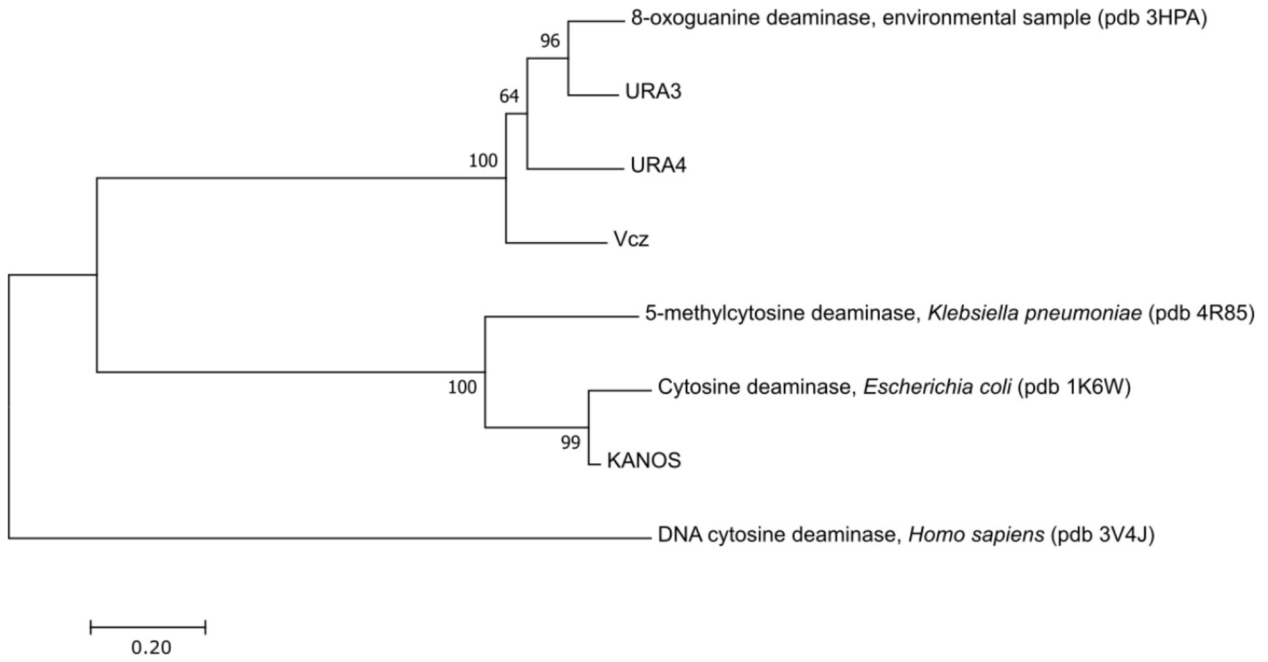

FIGURE 3 | Evolutionary relationships of deaminases. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 3.9 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 8 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 207 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). This analysis demonstrates that the enzymes found in metagenomic libraries belong to two groups as depicted in the tree: the classical cytosine deaminases (KANOS) and the 8-oxoguanine deaminases (URA3, URA4, and Vcz).

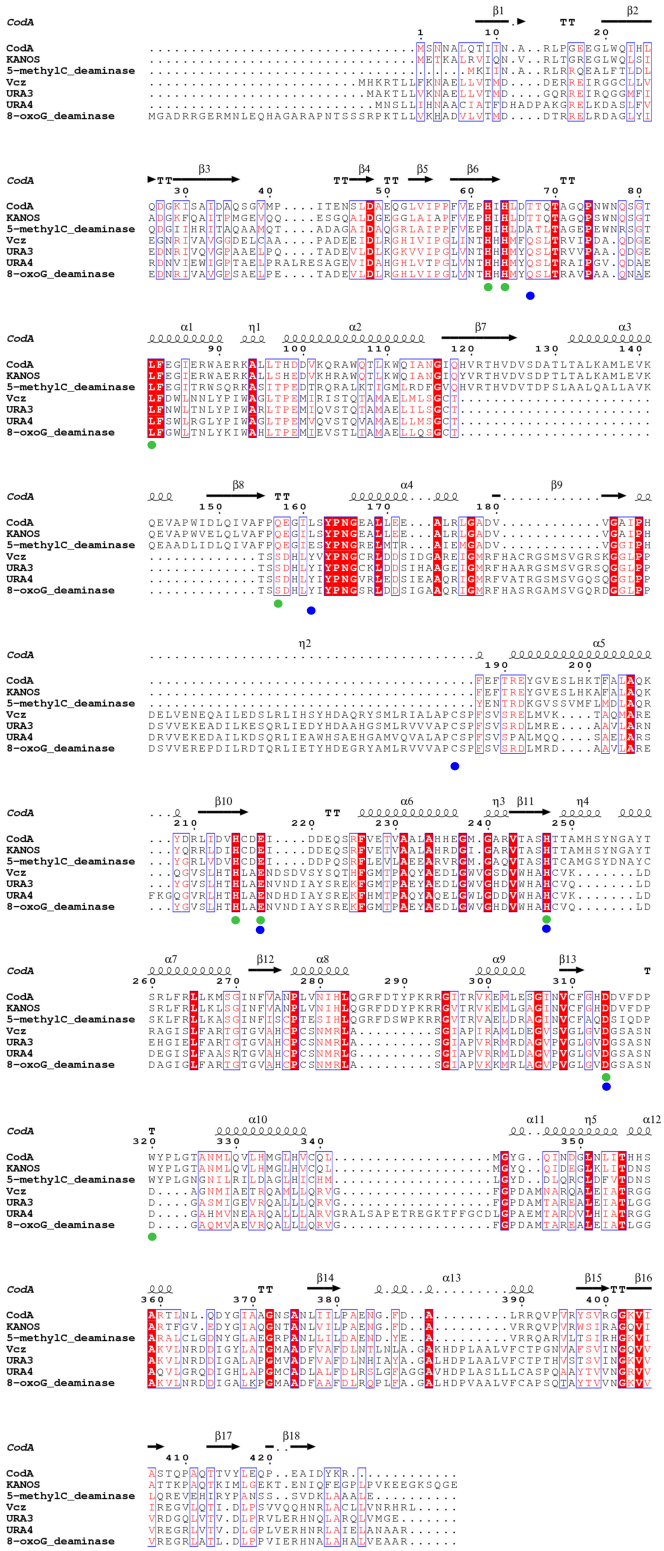

FIGURE 4 | Multiple amino acid sequence alignment of deaminases from the metagenomic libraries (KANOS, Vcz, URA3, and URA4) and those with confirmed functions and tertiary structures. CodA: *E. coli* cytosine deaminase, amino acid residues forming its active site are indicated in green circles (Ireton et al., 2002); 5-methylC_deaminase: *Klebsiella pneumoniae* 5-methylcytosine deaminase (Hitchcock et al., 2014); 8-oxoG_deaminase: 8-oxoguanine deaminase, amino acid residues forming its active site are indicated in blue circles (Hall et al., 2010). Highly similar residues are in red and framed in blue, strictly identical residues are in white on a red background. The alignment was performed using Clustal Omega (Sievers et al., 2011) and ESPript (Robert and Gouet, 2014).

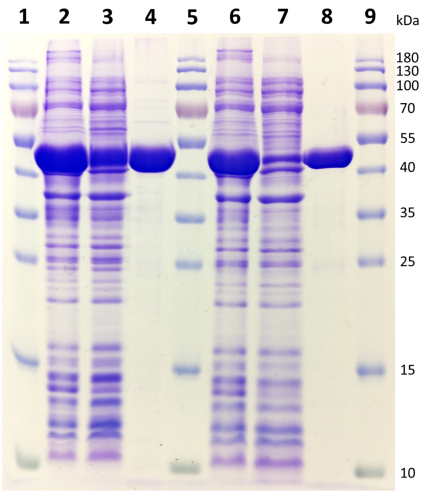

FIGURE 5 | 12% SDS–PAGE gel representing the purification of recombinant Vcz and URA3 deaminases. 1, 5, and 9: PageRuler^{TM} Prestained Protein Ladder (Thermo Fisher Scientific); 2: total proteins obtained from induced *E. coli* BL21(DE-3) bacteria transformed with pET28a-Vcz; 3: soluble protein fraction of *E. coli* BL21(DE-3) bacteria transformed with pET28a-Vcz; 4: ∼40 μg of recombinant 6xHis-tagged Vcz deaminase; 6: total proteins obtained from induced *E. coli* BL21(DE-3) bacteria transformed with pET21b-URA3; 7: soluble protein fraction of *E. coli* BL21(DE-3) bacteria transformed with pET21b-URA3; 4: ∼30 μg of recombinant 6xHis-tagged URA3 deaminase.

proteins were named after the metagenomic libraries they were found in: Vcz, URA3, URA4, and KANOS [accession numbers MH015236, MH015234, MH015235, and MH015237 in GenBank (Benson et al., 2012), respectively].

Ensuing phylogenetic analysis using MEGA7 (Kumar et al., 2016) demonstrated that these proteins belong to two phylogenetic groups as depicted in **Figure 3** (human single-strand DNA CD APOBEC3G (Li et al., 2012) was used as an outgroup). The KANOS protein is closely related to the classical cytosine deaminases (represented by CodA of *E. coli* (Ireton et al., 2002), see also **Figure 4**), therefore it was not chosen for further experiments. The other three proteins are closely related to 8-oxoguanine deaminases represented by the 8-oxoguanine deaminase from an environmental sample of Sargasso sea (Hall et al., 2010), but are different from a bacterial 5-methylcytosine deaminase (Hitchcock et al., 2014), which is related to the cytosine deaminases.

Enzymatic Activity of the Recombinant Deaminase Proteins

Two of the deaminase proteins (URA3 and Vcz) were overproduced in the *E. coli* BL21(DE-3) cells and purified using the Ni-ion chromatography (the purification steps are shown in **Figure 5**). The molecular weights of URA3 and Vcz (approx. 50 kDa) corresponded to the theoretical ones (48.8 and 49.5 kDa, respectively) (**Figure 5**). Third putative deaminase URA4 was not purified since the attempts to clone it into the expression vectors have failed. The recombinant URA3 and Vcz deaminases were tested for the enzymatic activity *in vitro* by incubating them with isocytosine and other substrates. As depicted in **Figure 6**, neither of these deaminases use cytosine as a substrate (**Figures 6A–D**), but they do convert isocytosine into uracil (**Figures 6E–H**) as well as 5-fluoroisocytosine into 5-fluorouracil (**Figures 6I–L**). The Vcz and URA3 enzymes efficiently catalyzed the deamination of isocytosine but were not active toward cytosine. For Vcz, the values of k_{cat}, K_m, and k_{cat}/K_m for isocytosine were 1.64 ± 0.06 s^{-1}, 1860 ± 45 μM, and $8.8 \pm 0.3 \times 10^2$ M^{-1} s^{-1}, respectively. For URA3, the values of k_{cat}, K_m, and k_{cat}/K_m for isocytosine were 2.00 ± 0.15 s^{-1}, 1085 ± 120 μM, and $1.8 \pm 0.1 \times 10^3$ M^{-1} s^{-1}, respectively. For Vcz, the values of k_{cat}, K_m, and k_{cat}/K_m for 5-fluorisocytosine were 0.1 ± 0.007 s^{-1}, 1270 ± 128 μM, and $7.9 \pm 0.08 \times 10^1$ M^{-1} s^{-1}, respectively. For URA3, the values of k_{cat}, K_m, and k_{cat}/K_m for isocytosine were 0.02 ± 0.003 s^{-1}, 1330 ± 305 μM, and $1.5 \pm 0.3 \times 10^1$ M^{-1} s^{-1}, respectively.

DISCUSSION

The isocytosine deaminases that were discovered in this study are all members of the amidohydrolase superfamily of enzymes that catalyze the hydrolysis of a wide range of substrates and contains most of the enzymes known to catalyze the deamination of nucleobases. In all known cases, the nucleophilic water molecule is activated through complexation with a mononuclear or binuclear metal center (Zn^{2+} in the case of 8-oxoguanine deaminases) that is perched at the C-terminal end of the β-barrel core within a $(\beta\alpha)_8$ structural domain (Seibert and Raushel, 2005). The phylogenetic analysis that includes deaminases of amidohydrolase superfamily with known enzymatic activities and tertiary structure solved, demonstrated that the deaminases we have discovered fall into 2 distinct groups. The first group includes the URA4, URA3, and Vcz, which are closely related to 8-oxoguanine deaminases – these enzymes have previously been shown to act on isocytosine (Hall et al., 2010). The second group includes the classical CD (CodA from *E. coli*), 5-methylcytosine deaminase Kpn00632 from *Klebsiella pneumoniae* (Hitchcock et al., 2014) and KANOS (**Figure 3**). Based on the similarity to 8-oxoguanine deaminase discovered by Hall et al. (2010), we tested the enzymatic activity of both URA3 and Vcz enzymes and discovered that they are capable of converting both guanine into xanthine and 8-oxoguanine into uric acid. The limited solubility of guanine and 8-oxoguanine prevented accurate detection via TLC. Nonetheless, according to the HPLC-MS results that were obtained, URA3 and Vcz deaminases convert guanine into xanthine and 8-oxoguanine into uric acid, although the detection of UV spectra and molecular ions is complicated (data not shown). The sequence comparison of newly discovered isocytosine deaminases with the ones that act on either cytosine, 5-methylcytosine or 8-oxoguanine (**Figure 4**) allowed us to make predictions regarding the requirements for the enzymatic activity and substrate specificity. The tertiary structures and the enzymatic activities of the above mentioned deaminases were studied in detail (Ireton et al., 2002; Hall et al., 2010, 2011; Hitchcock et al., 2014). Sequence alignment (**Figure 4**) demonstrates that all these deaminases are metal

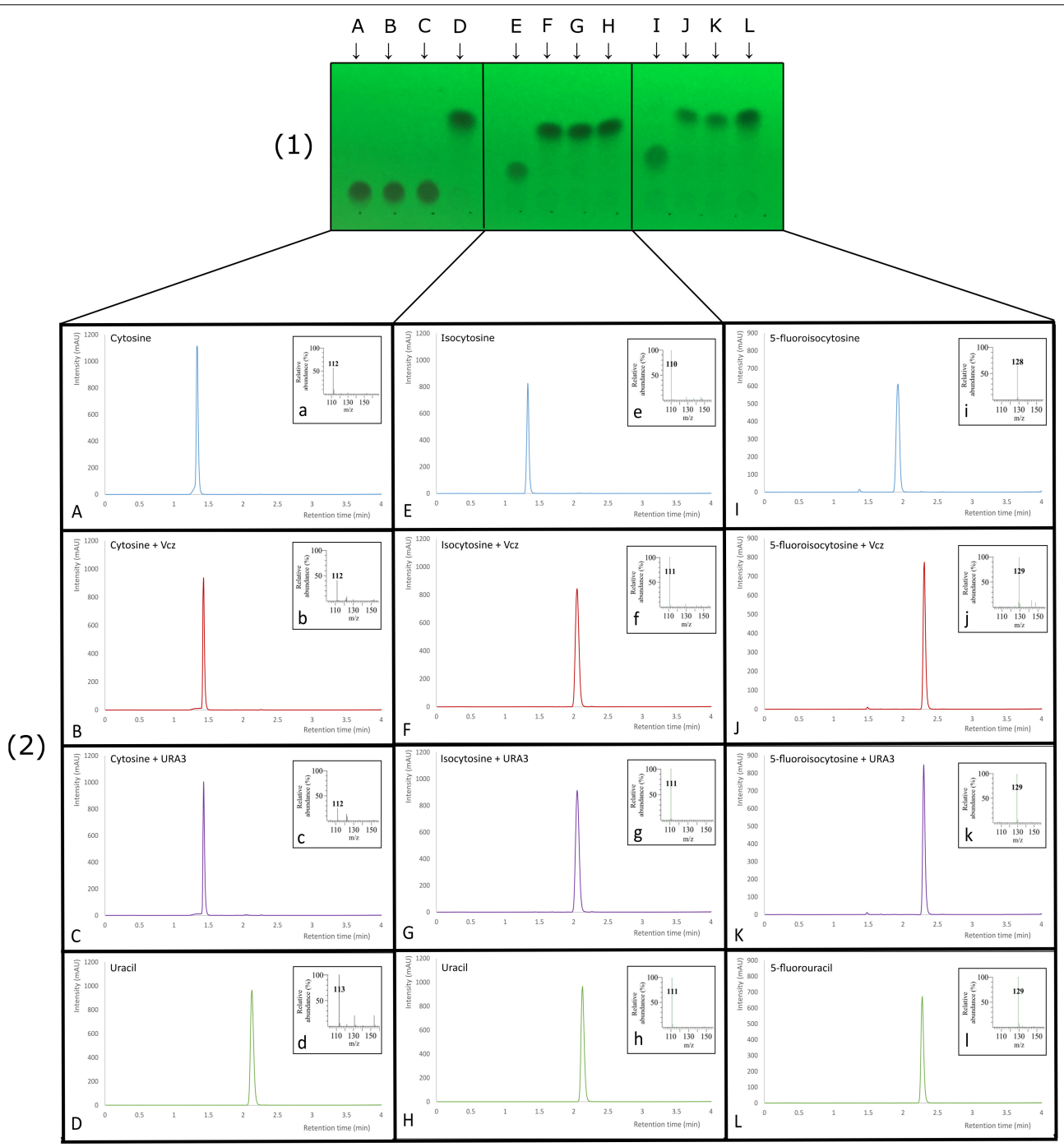

FIGURE 6 | Thin layer chromatography plate **(1)** and corresponding LC-MS analyses **(2)** of substrates **(A,E,I)**, enzymatic isocytosine deaminase Vcz **(B,F,J)** and URA3 **(C,G,K)** reactions and products **(D,H,L)**. **(A)** TLC and UV chromatogram of cytosine standard, **(a)** MS-spectra (*m/z* of 112 consistent with $[M + H]^+$ of cytosine), **(B)** TLC and UV chromatogram of the reaction mixture using cytosine and Vcz deaminase, **(b)** MS-spectra (*m/z* of 112 consistent with $[M + H]^+$ of cytosine), **(C)** TLC and UV chromatogram of the reaction mixture using cytosine and URA3 deaminase, **(c)** MS-spectra (*m/z* of 112 consistent with $[M + H]^+$ of cytosine), **(D)** TLC and UV chromatogram of uracil standard, **(d)** MS-spectra (*m/z* of 113 consistent with $[M + H]^+$ of uracil), **(E)** TLC and UV chromatogram of isocytosine standard, **(e)** MS-spectra (*m/z* of 110 consistent with $[M-H]^-$ of isocytosine), **(F)** TLC and UV chromatogram of the reaction mixture using isocytosine and Vcz deaminase, **(f)** MS-spectra (*m/z* of 111 consistent with $[M-H]^-$ of uracil), **(G)** TLC and UV chromatogram of the reaction mixture using isocytosine and URA3 deaminase, **(g)** MS-spectra (*m/z* of 111 consistent with $[M-H]^-$ of uracil), **(H)** TLC and UV chromatogram of uracil standard, **(h)** MS-spectra (*m/z* of 111 consistent with $[M-H]^-$ of uracil), **(I)** TLC and UV chromatogram of 5-fluoroisocytosine standard, **(i)** MS-spectra (*m/z* of 128 consistent with $[M-H]^-$ of 5-fluoroisocytosine), **(J)** TLC and UV chromatogram of the reaction mixture using 5-fluoroisocytosine and Vcz deaminase, **(j)** MS-spectra (*m/z* of 129 consistent with $[M-H]^-$ of 5-fluorouracil), **(K)** TLC and UV chromatogram of the reaction mixture using 5-fluoroisocytosine and URA3 deaminase, **(k)** MS-spectra (*m/z* of 129 consistent with $[M-H]^-$ of 5- fluorouracil), **(L)** TLC and UV chromatogram of 5-fluorouracil standard, **(l)** MS-spectra (*m/z* of 129 consistent with $[M-H]^-$ of 5- fluorouracil).

proteins with binuclear metal centers that are defined by the conservative His62, His64, His215, and Asp314 residues (numbering according to CodA in **Figure 4**, green dots). These residues were previously reported as involved in metal coordination and substrate binding in the active site of *E. coli* CD (Ireton et al., 2002). The residues that form the active center of 8-oxoguanine deaminases (Hall et al., 2010) are conserved in the Vcz, URA3 and URA4 deaminases (**Figure 4**, blue dots). Three of these residues are conservative in CodA (and in 5-methylcytosine deaminase as well) and are involved in its active site formation (**Figure 4**, CodA numbering: Glu218, His247, and Asp314, green and blue dots). However, the Asp315, which is present in CodA and KANOS (CodA numbering in **Figure 4**), is replaced by glycine in URA3, URA4, Vcz, and 8-oxoguanine deaminase (or by serine in 5-methylcytosine deaminase). This residue is critical for the substrate specificity of deaminases: CodA is essentially unable to catalyze the deamination of 5-methylcytosine, but when the Asp315 residue was replaced by alanine, the mutant CodA enzyme displayed activity toward 5-methylcytosine (Hitchcock et al., 2014). Comparison of 3D structures of CodA and the 8-oxoguanine deaminase demonstrates that β7/α3/β8 helixes are missing in the latter as well as in the Vcz, URA3, and URA4 deaminases, but an $\alpha3_{10}$ (η2) helix is present instead, in which a cysteine residue that is involved in active site formation is present (**Figure 4**, η2 helix, blue dot). In contrast to all known isocytosine/cytosine deaminases, URA4 protein contains an additional loop between helixes α10 and α11. The functional role of this structural element is not clear. It was demonstrated that 8-oxoguanine deaminase from *Pseudomonas aeruginosa* PA01 is capable of isocytosine deamination, albeit with 10-fold lower efficiency than that of 8-oxoguanine. Moreover, cytosine was not a substrate of this protein (Hall et al., 2010). The URA3 and Vcz deaminase values of k_{cat}/K_m for isocytosine were similar to those obtained by Hall et al. (2010), using the 8-oxoguanine deaminase from *Pseudomonas* sp. However, their homolog from Sargasso sea metagenome is a tenfold more efficient in deamination of isocytosine, as well as is the *E. coli* CD (Hall et al., 2011).

Although the isocytosine deaminases, as predicted, do convert isocytosine into uracil, another substrate – 5-fluoroisocytosine – is a gateway to a potential future application of newly discovered enzymes, namely, the enzyme-prodrug therapy of cancer. The purpose of cancer therapy is to kill cancer cells selectively without harming the non-cancer cells. The classical bacterial or yeast CD and its prodrug 5-fluorocytosine (5-FC) have been one of the most investigated enzyme-prodrug pairs for many years (Malekshah et al., 2016). This type of cancer therapy relies on the non-toxic prodrug 5-FC, which is converted to its active form, 5-fluorouracil (5-FU), by CD activity (Mullen et al., 1992; Huber et al., 1993).

The 5-FU, which is a common chemotherapy drug, is further converted into potent pyrimidine antimetabolites by other cellular enzymes and can inhibit thymidylate synthase which leads to the cell cycle arrest and apoptosis (Yata et al., 2012). 5-FU is widely used in the treatment of a range of cancers, including that of the aero-digestive tract, breast, head, and neck, but the greatest response rates were observed in the case of colorectal cancer. However, the administration of 5-FU is toxic to the patients – up to 80% of administered 5-FU is broken down in the liver (Longley et al., 2003). Hence, the efficient tumor-localized expression of CD enzymes remains one of the main problems that the enzyme-prodrug therapy has to face.

The use of the non-toxic prodrug and localized expression of CD enables the reduction of the systemic side effects of 5-FU. Still, it remains one of the problems with CD/5-FC enzyme/prodrug therapy, mainly because of the presence of CD in normal gut flora, which is also able to convert 5-FC to 5-FU (Harris et al., 1986; Malet-Martino et al., 1991). We propose that 5-fluoroisocytosine, together with the new URA3 or Vcz isocytosine deaminases, could be used as a novel enzyme-prodrug pair. We believe that the gut flora would not metabolize 5-fluoroisocytosine as efficiently as 5-fluorocytosine. Although it was shown that the CD from *E. coli* is able to catalyze the deamination of isocytosine, the kinetic parameters (k_{cat}/K_m) differ by a 10-fold in favor of cytosine (Hall et al., 2011). Moreover, there are no known homologs of Vcz and URA3 isocytosine deaminases in the human cells, as revealed by BLAST (Altschul et al., 1990) analysis (data not shown). The new isocytosine deaminase/5-fluoroisocytosine enzyme/prodrug pair would alleviate the toxic side effects of cytosine deaminase/5-fluorocytosine pair in cancer therapy. More biochemical experiments may be required in order to determine the exact kinetic parameters of these enzymes with different substrates and also to pinpoint the exact amino acids involved in the catalysis. The alanine scanning and/or random mutagenesis experiments similar to those performed for the 5-FC deaminase (Mahan et al., 2004a,b) would allow the selection of the most efficient variants of URA3 and Vcz enzymes. In order to test the applicability of isocytosine deaminase/5-fluoroisocytosine enzyme/prodrug pair for cancer gene therapy, several experiments are under way. These additional studies are aimed at production of cell lines/bacterial strains that express the isocytosine deaminases and also at the action of this novel enzyme/prodrug pair on cancer cells *in vitro* and in animals.

CONCLUSION

Three isocytosine/8-oxoguanine deaminases that belong to the amidohydrolase superfamily were discovered using soil-based metagenomic libraries, a selection method employing *E. coli* uracil auxotrophy and isocytosine as a substrate. It was demonstrated *in vitro* that two of these deaminases are capable of converting isocytosine, but not cytosine, into uracil as well as 5-fluoroisocytosine into 5-fluorouracil. The latter finding suggests that these enzymes, together with 5-fluoroisocytosine, could be used for cancer therapy as a novel enzyme/prodrug pair, which might alleviate problems associated with the classical cytosine deaminase/5-fluorocytosine enzyme/prodrug pair.

AUTHOR CONTRIBUTIONS

AA performed the genetic screens, cloning, enzymatic activity measurements, and wrote the manuscript. RR purified the recombinant proteins and reviewed the manuscript. DT synthesized 5-fluoroisocytosine, performed the HPLC-MS analyses, and reviewed the manuscript. RM planned the experiments, analyzed the results, provided the metagenomic libraries, and reviewed the manuscript. JU planned the experiments, analyzed the results, and wrote the manuscript.

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Whey Protein Isolate-Supplemented Beverage, Fermented by *Lactobacillus casei* BL23 and *Propionibacterium freudenreichii* 138, in the Prevention of Mucositis in Mice

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Mucositis is a clinically important gastrointestinal inflammatory infirmity, generated by antineoplastic drugs cytotoxic effects. The inflammatory process caused by this disease frequently leads to derangements in the alimentary tract and great malaise for the patient. Novel strategies are necessary for its prevention or treatment, as currently available treatments of mucositis have several limitations in relieving its symptoms. In this context, several research groups have investigated the use of probiotic bacteria, and in particular dairy bacterial strains. Compelling evidences reveal that milk fermented by certain probiotic bacteria has the capacity to ameliorate intestinal inflammatory disorders. In addition, innovative probiotic delivery strategies, based on probiotics incorporation into protective matrices, such as whey proteins, were able to increase the therapeutic effect of probiotic strains by providing extra protection for bacteria against environmental stresses. Therefore, in this study, we evaluated the role of the whey protein isolate (WPI), when added to skim milk fermented by *Lactobacillus casei* BL23 (*L. casei* BL23) or by *Propionibacterium freudenreichii* CIRM-BIA138 (*P. freudenreichii* 138), as a protective matrix against *in vitro* stress challenges. In addition, we investigated the therapeutic effect of these fermented beverages in a murine model of mucositis induced by 5-Fluorouracil (5-FU). Our results demonstrated that milk supplementation with 30% (w/v) of WPI increases the survival rate of both strains when challenged with acid, bile salts, high temperature and cold storage stresses, compared to fermented skim milk without the addition of WPI. Moreover, treatment with the probiotic beverages prevented weight loss and intestinal damages in mice receiving 5-FU. We conclude that the presence of WPI maximizes the anti-inflammatory effects of *L. casei* BL23, but not for *P. freudenreichii* 138, suggesting that whey protein enhancement of probiotic activity might be strain-dependent.

Keywords: *Lactobacillus*, mucositis, probiotics, *Propionibacterium*, stress tolerance, whey protein isolate

INTRODUCTION

Mucositis is a severe inflammation that affects the entire extension of the Alimentary Tract (AT) of individuals undergoing malignancy treatment based on chemotherapy or radiotherapy (Sonis, 2004). One of the main drugs associated with this condition is 5-Fluorouracil (5-FU). This is a antimetabolic drug commonly prescribed for the treatment of head, neck and gastrointestinal cancer (Longley et al., 2003). 5-FU unfortunately presents non-specific cytotoxicity toward cells, inhibiting the proliferation of both cancer cells and normal cells with high replication rates, such as the enterocytes of the gastrointestinal tract (GIT) (Carvalho et al., 2017a). A series of clinical symptoms, such as nausea, weight loss, vomiting, severe abdominal pain and diarrhea are commonly reported in patients receiving 5-FU during cancer treatment (Bastos et al., 2016). Moreover, mucositis frequently increases predisposition to local and systemic secondary infections, thus generating additional costs and extending the patient's hospitalization time (Carvalho et al., 2017a). Mucositis is characterized by pathological changes in the small bowel. These changes include the presence of degenerate enterocytes (Ciorba et al., 2016), submucosal vessel damage, leukocyte infiltrate in the *lamina propria*, with accumulation of neutrophils and eosinophils (Antunes et al., 2016), increased mucin production and degeneration of goblet cells (Stringer, 2013), atrophy of villi, hypoplasia and apoptosis of intestinal crypts (Chang et al., 2012). Currently, there is no treatment that is completely successful in the prevention and treatment of mucositis. However, there has been a growing interest in the use of probiotics as promising candidates for the treatment of this disease (Carvalho et al., 2017a).

Probiotics are included in a variety of products, including fermented foods, dietary supplements, formulas for newborns and infants, as well as various pharmaceutical formulations (Cousin et al., 2011). Currently, fermented beverages by one or more bacteria, have gained the functional food status, which makes them an important part of our diet as well as our main daily source of beneficial microbes (Leroy and De Vuyst, 2014; Carmo et al., 2017).

Selected strains of lactic acid bacteria (LAB) were reported as probiotic with beneficial effects provided by different mechanisms of action and can be used in functional foods withal (Carvalho et al., 2017b; Eales et al., 2017; Tang et al., 2017). Some studies have shown that the administration of lactobacilli strains can reduced some parameters of mucositis in mice model induced by 5-FU, such as prevent weight loss, attenuate the diarrhea and intestinal damage (Justino et al., 2015). *L. casei* BL23 has also been considered as a good probiotic strain, according to results obtained in others inflammatory models. Some studies demonstrated that *L. casei* BL23 was able to alleviates colitis symptoms in a dextran sulfate sodium (DSS) model (Foligne et al., 2007; Rochat et al., 2007) and the ability of these strain to attenuate intestinal inflammation can be enhanced using a protection matrix (Lee et al., 2015b). Other important group of bacteria widely used in the food industry, particularly in Swiss-type cheese manufacture, is propionic acid bacteria (PAB). *Propionibacterium freudenreichii* represents the main species thereof and is listed in the Qualified Presumption of Safety list (QPS) by the European food safety authority and it has recently been considered a promising probiotic (Rabah et al., 2017). *P. freudenreichii* produces metabolites are considered as prebiotics, such as 1,4-dihydroxy-2-naphtoic acid (DHNA) and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ), both associated to bifidogenic effects. Furthermore, *P. freudenreichii* is the only GRAS bacterial species producing food-grade vitamin B12 at the industrial scale (Rabah et al., 2017). Selected *P. freudenreichii* strains have been associated with therapeutical effects based on *in vitro* and *in vivo* properties to attenuate colitis model induced by trinitrobenzene sulfonic acid (TNBS) (Cousin et al., 2012a, 2016; Plé et al., 2015). A dairy propionibacteria, *P. freudenreichii* 138, demonstrated a pro-apoptosis capacity, in HTG-1 human gastric cancer cells without toxicity effects in healthy human cells (Cousin et al., 2012a, 2016). Moreover, Cousin et al. (2012b) shown the persistence of *P. freudenreichii* 138 in piglets colon, withal metabolic activity and producing propionate, which is a SCFA with probiotic properties (Cousin et al., 2012b).

An extremely important factor for the therapeutic effects of probiotics is the ability of the bacteria to survive during transit through the GIT or during industrial processes (Cousin et al., 2012b; Rabah et al., 2017). Digestion indeed imposes harsh conditions including gastric acid and presence of bile salts, which may severely affect bacterial viability (Leroy and De Vuyst, 2014; Huang et al., 2016a). A probiotic microorganism must, however, tolerate these stresses for a long persistence in the host and for an enhanced beneficial effect (Carmo et al., 2017). In the aim to maximize the tolerance of bacteria to stressful environments and thus to increase their probiotic ability, the food matrix used in the manufacture of fermented products plays a key role as a protective medium (Gagnaire et al., 2015; Lee et al., 2015a). As an example, milk proteins, as well as whey protein isolates, constitute very promising protective matrices for probiotics, besides being an efficient delivery vehicles to target protective molecules and microorganisms to digestive epithelium (Livney, 2010; Cousin et al., 2012a; Vargas et al., 2015; Huang et al., 2016a). Whey proteins have been recognized for their various functional and nutritional properties. The functional properties are mainly due to their physical, chemical and structural characteristics and the nutritional value is directly linked to the concentration of essential amino acids (Yadav et al., 2015). Some studies have also demonstrated the potential of whey proteins to enhance the survival and viability of probiotic bacteria during production and storage (Marshall, 2004; Madureira et al., 2007; Almeida et al., 2009; Huang et al., 2016b; Baruzzi et al., 2017; Dąbrowska et al., 2017).

The aims of this work were (i) to evaluate whether whey protein isolate is a good protective matrix for *L. casei* BL23 and *P. freudenreichii* CIRM-BIA 138, against adverse environmental conditions and (ii) to investigate the therapeutic effect of administration of whey protein isolate-supplemented beverage, fermented by both strains, in the prevention of mucositis induced by 5-FU.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactobacillus casei BL23 strain was kindly supplied by the UMR1219 Micalis Institute (INRA-AgroParisTech, Jouy-En-Josas, France) and the *P. freudenreichii* CIRM-BIA138 (alias ITG P9) strain by the Biological Resource Center (International Center of Microbial Resources, INRA, Rennes, France). *L. casei* BL23 was grown in MRS broth at 37°C for 24 h, without shaking. *P. freudenreichii* 138 was grown in YEL culture medium at 30°C for 72 h, without agitation (Malik et al., 1968).

Dairy Beverage Formulation and Supplementation With Whey Protein Isolate

The fermented beverage was prepared using skimmed milk (SM) powder 12% w/v (Itambé, Brazil). For cultivation of *L. casei* BL23 (SMLC), the SM was supplemented with yeast extract (Kasvi Curitiba, Brazil) and glucose (Merck, Germany) (Tharmaraj and Shah, 2003). For cultivation of *P. freudenreichii* 138 (SMPF), the milk was supplemented with casein peptone (5g/L) (KASVI, Curitiba, Brazil) and sodium lactate (50 m/M) (Sigma, St. Louis, MO, United States) (Cousin et al., 2012b). Both milk were autoclaved at 110°C for 15 min. The SMs was supplemented with whey protein isolate (WPI), natural flavor, 90% protein (Vulgo Supplements, Brazil) at concentrations of 5, 15, and 30% w/v, and strains cultivated in skimmed milk without WPI was used as control. The same growth conditions used to cultivate the strains in MRS or YEL were applied for growth in fermented beverages.

Stress Challenges

Samples of 10 ml from the stationary-phase of *L. casei* BL23 and *P. freudenreichii* 138 in culture media or in skim milk, supplemented or not with WPI were subjected to acid, bile salts, and heat challenges (Huang et al., 2016b). For acid stress, the samples were incubated in MRS broth or YEL broth, previously adjusted to pH 2.0 using HCl, at 37°C for 60 min. Briefly, for bile salts stress, the samples were incubated in MRS broth or YEL broth containing 1.0 g/L of bile salts (an equimolar mixture of cholate and deoxycholate, Sigma Chemical, St. Louis, MO, United States) and then, incubated at 37°C for 60 min. Finally, to simulate the pasteurization temperature established by the International Dairy Foods Association (IDFA), the samples were incubated in their specific culture media pre-heated to 63°C for 30 min. After stresses challenges, aliquots of each sample were subjected to 1:10 serial dilutions using peptone water (9 g/L peptone, 5 g/L NaCl) and plated on MRS agar or YEL agar medium. Plates of *L. casei* BL23 were incubated for 48 h at 37°C. Plates of *P. freudenreichii* 138 were incubated for 144 h (6 days) at 30°C in jars containing anaerobiosis generator (Anaerocult A®, Merck Millipore). The number of viable bacteria was determined by counting of colony forming unit (CFU) after incubation. The bacteria survival rate (%) through each stress condition was calculated through the following equation (Ferreira et al., 2017):

$$\text{Survival Rate (\%)} : \frac{\log\ N}{(\log\ N_0\ \times\ 100)} \quad (1)$$

Where N refers to the number of bacteria population (CFU mL^{-1}) in culture medium after stress challenges, and N_0 refers to the number of initial population (CFU mL^{-1}) before the stress challenges.

Bacterial Survival During Storage at 4°C

The long-term survival of *L. casei* BL23 and *P. freudenreichii* 138 in dairy beverage supplemented with 30% of WPI was assessed during the storage process at 4°C for 90 days kept away from light (Huang et al., 2016b). For the evaluation of bacterial survival during cold storage, plate seeding was performed on days 0 (pre-storage time), 7, 14, 21, 30, 60, and 90 after storage. The *L. casei* BL23 and *P. freudenreichii* 138 plates were incubated according to their specific conditions on agar media (see above). The number of viable bacteria during storage was determined by counting CFU in the culture after incubation. The evolution of acidification of stored samples was also screened in the same days. To evaluate if both fermented beverages could survive GIT stress conditions after being stored at 4°C, we performed acid stress and biliary stress *in vitro* challenges for the dairy beverage supplemented with 30% of WPI. Acid and bile salts stresses were performed on days 30, 60, and 90 after the storage start.

Evaluation of Therapeutic Effects of Beverages Fermented by *L. casei* BL23 or *P. freudenreichii* 138 in a Mice Model of Mucositis

Animals

Conventional female BALB/c mice between 6 and 8 weeks of age were obtained at Federal University of Minas Gerais (UFMG–Belo Horizonte, Brazil). Mice were kept in a temperature-controlled room with *ad libitum* access to water and standard chow diet. The study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (CEUA-UFMG, Brazil, protocol 366).

Probiotic Treatment, Mucositis Induction, and Experimental Groups

For probiotic treatment, mice received 0.5 ml of fermented beverages supplemented or not with 30% of WPI via gastric gavage, during 13 days. In order to induce mucositis, mice received a single intraperitoneal injection of 5-FU (Fauldfluor – Libbs) (300 mg/kg) on day 11, and were euthanized 72 h after induction of mucositis, in 14th of experimental day (Carvalho et al., 2017a). An injection of saline (NaCl 0.9%) was used in control groups. After euthanasia, a longitudinal abdominal incision was performed to remove the intestine for further analyses. Body weight of mice was determined throughout the experiment. For *in vivo* experimentation, BALB/c mice were divided into sixteen groups according to **Table 1**. All experiments

TABLE 1 | Experimental groups and the respective treatments.

Non-inflamed groups Injection of 300 mg/kg of saline (0.9% NaCl)		Inflamed groups Injection of 300 mg/kg of 5-FU	
Group	**Treatment**	**Group**	**Treatment**
Water	H_2O	Water	H_2O
SMLC + WPI	Skim milk specific for *L. casei* BL23 + WPI	SMLC + WPI	Skim milk specific for *L. casei* BL23 + WPI
SMPF + WPI	Skim milk specific for *P. freudenreichii* 138 + WPI	SMPF + WPI	Skim milk specific for *P. freudenreichii* 138 + WPI
SMLC + BL23	Skim milk specific for *L. casei* BL23 fermented by *L. casei* BL23	SMLC + BL23	Skim milk specific for *L. casei* BL23 fermented by *L. casei* BL23
SMLC + WPI + BL23	Skim milk specific for *L. casei* BL23 fermented by *L. casei* BL23 + WPI	SMLC + WPI + BL23	Skim milk specific for *L. casei* BL23 fermented by *L. casei* BL23 + WPI
SMPF + 138	Skim milk specific for *P. freudenreichii* 138 fermented by *P. freudenreichii* 138	SMPF + 138	Skim milk specific for *P. freudenreichii* 138 fermented by *P. freudenreichii* 138
SMPF + WPI + 138	Skim milk specific for *P. freudenreichii* 138 fermented by *P. freudenreichii* 138 + WPI	SMPF + WPI + 138	Skim milk specific for *P. freudenreichii* 138 fermented by *P. freudenreichii* 138 + WPI
Association (Assoc)	Equal mixture of SMLC + WPI + BL23 and SMPF + WPI + 138	Association (Assoc)	Equal mixture of SMLC + WPI + BL23 and SMPF + WPI + 138

All groups were gavaged daily, with 0.5 ml of the appropriate treatments, for 13 days.

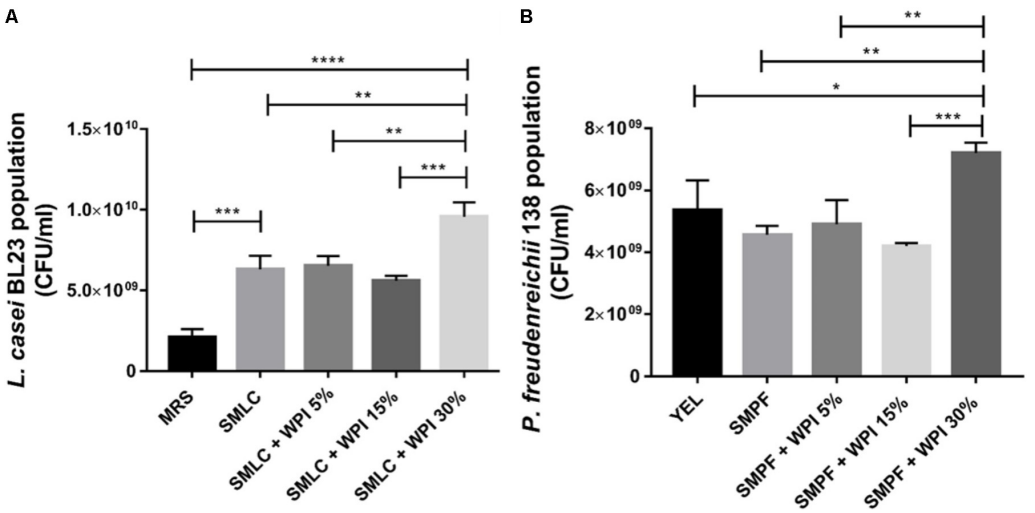

FIGURE 1 | Different concentrations of WPI in skim milk impacted on *Lactobacillus casei* BL23 and *Propionibacterium freudenreichii* 138 growth. **(A)** Colony-forming unit (CFU) counting of *L. casei* BL23, after 24 h of incubation at 37°C and **(B)** Population of *P. freudenreichii* 138, after 78 h of anaerobic incubation at 30°C were assessed by CFU counts. These assays were performed in triplicate technique and biological triplicate. Asterisks represent statistically significant differences and were indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

were performed simultaneously therefore, the same control groups were used for all experimental probiotic assays. Each group containing 6–9 animals. All beverages contained 10^9 CFU mL^{-1} bacteria.

Histological Analysis

The distal portion of the small bowel (ileum) from the mice was collected and washed with PBS. Afterwards, rolls were prepared for histomorphological analysis. Histological materials were immersed in 4% buffered formaldehyde solution for tissue fixation. Then, the material was embedded in paraffin, and a 4 μm section of each sample was placed on a glass slide and stained with Hematoxylin-Eosin (HE). The histological score was determined using a score that measures the intensity of the infiltrate of mononuclear and polymorphonuclear cells in the *lamina propria* of the ileum, the presence of ulceration and erosion and changes in mucosal architecture (Soares et al., 2008). For each parameter a classification was given according to the

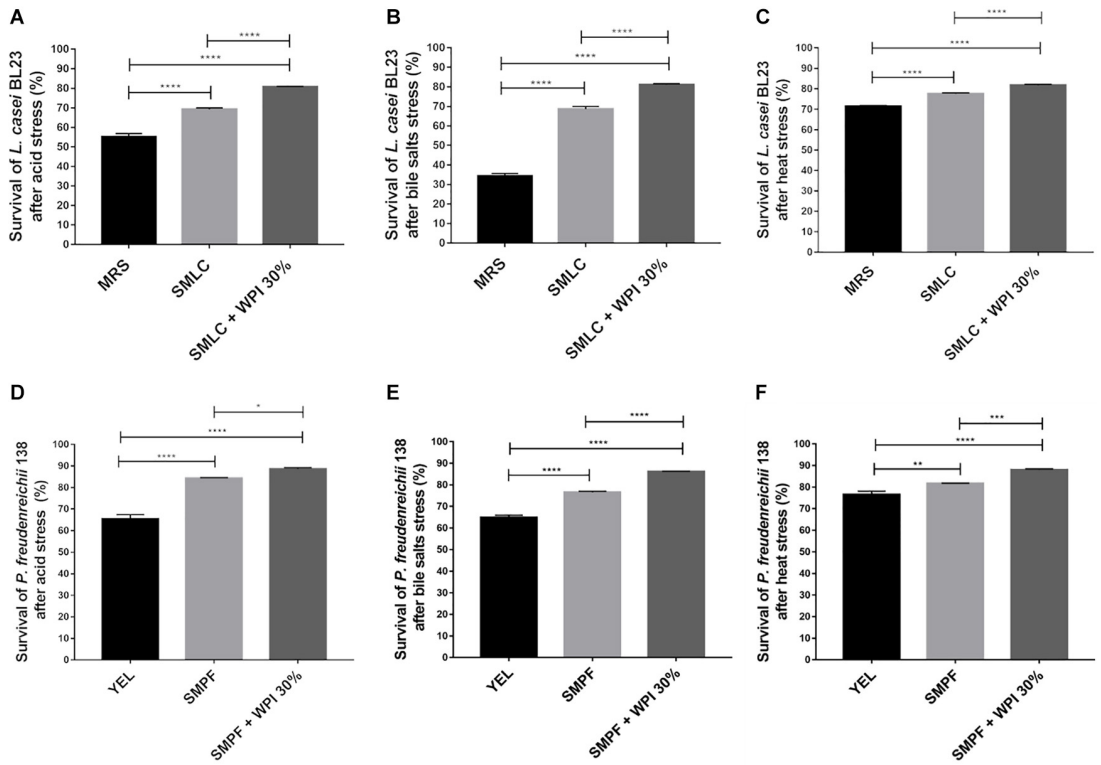

FIGURE 2 | Whey protein isolate (WPI) confers stress tolerance on *L. casei BL23* and *P. freudenreichii* 138. *L. casei* BL23 was cultured for 24 h in the indicated growth media until stationary phase and then subjected to **(A)** acid stress (pH 2 for 60 min at 37°C); **(B)** bile salts stress (1 g/liter for 60 min at 37°C) or **(C)** heat stress (63°C for 30 min). *P. freudenreichii* 138 was cultured for 72 h in each culture media until stationary phase, and then subjected to **(D)** acid stress, **(E)** bile salts stress, or **(F)** heat stress. Viable bacteria were enumerated by counting colonies in the challenged and control cultures and then, expressed as percent survival (means ± standard deviations). These assays were performed in triplicate technique and biological triplicate. Asterisks represent statistically significant differences and were indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

severity of the lesion in the tissues: absent (0), mild (1), moderate (2) and severe (3). For morphometric analysis, 10 images of the ileum of each animal were randomly captured and analyzed using ImageJ software (version 1.8.0). Villi height and the crypt depth were measured vertically from the tip of villi to the base of the adjacent crypt. Additional cuts in the paraffinized samples from the ileum were stained by the Periodic Acid-Schiff (PAS), technique to determine the number of goblet cells in the tissues (Prisciandaro et al., 2011). Ten random field images of each sample were made using the 40× objective and the intact goblet cells were counted using ImageJ software (version 1.8.0) and expressed as the number of cells per high-power field (hpf) (40×, 108.2 μm^2).

Measurement of Secretory IgA

Levels of secretory IgA (sIgA) were determined by enzyme-linked immunosorbent assay (ELISA) in small bowel intestinal fluids (Carvalho et al., 2017a). Microtiter plates (Nunc-Immuno Plates, MaxiSorp) were coated with anti-IgA antibodies (Southern Biotechnology, Birmingham, AL, United States) for 18 h at 4°C. The plates were washed with saline (NaCl 0.9%) added with Tween 20 (0.05%) and blocked with 200 µl PBS-casein (0.05%) for 1 h at room temperature. Intestinal fluid samples were diluted in PBS-casein (0.25%) and then added to the plate. After incubation for 1 h at room temperature, the wells were washed and biotin-conjugated anti-mouse IgA antibody (Southern Biotechnology) diluted in PBS-casein (0.25%) (1: 10,000). The plates were incubated for 1 h at 37°C and anti-IgA conjugated to streptavidin peroxidases (1:10,000) were added (Southern Biotechnology). After 1 h of incubation, 100 µl of orthophenylenediamine (OPD) (Sigma, St. Louis, MO, United States) and H_2O_2 (0.04%) were added to each well. Plates were kept away from light until the coloration developed. The reaction was stopped by addition of 2 N H_2SO_4. Reading was performed on a plate reader (Bio-Rad Model 450 Microplate Reader) at 492 nm absorbance. The results were measured in concentration of sIgA (µg) per ml of intestinal fluid, according to the standard curve.

Statistical Analyses

The results were reported as the mean ± standard deviation and analyzed using Student's *t*-test, Holm–Sidak *t*-test, One-Way ANOVA or Two-Way ANOVA followed by the Tukey or Sidak post-test. Non-parametric data's were analyzed using Kruskal-Wallis data followed by the Dunns post-test. Graphs and statistical analyzes were performed in GraphPad Prism

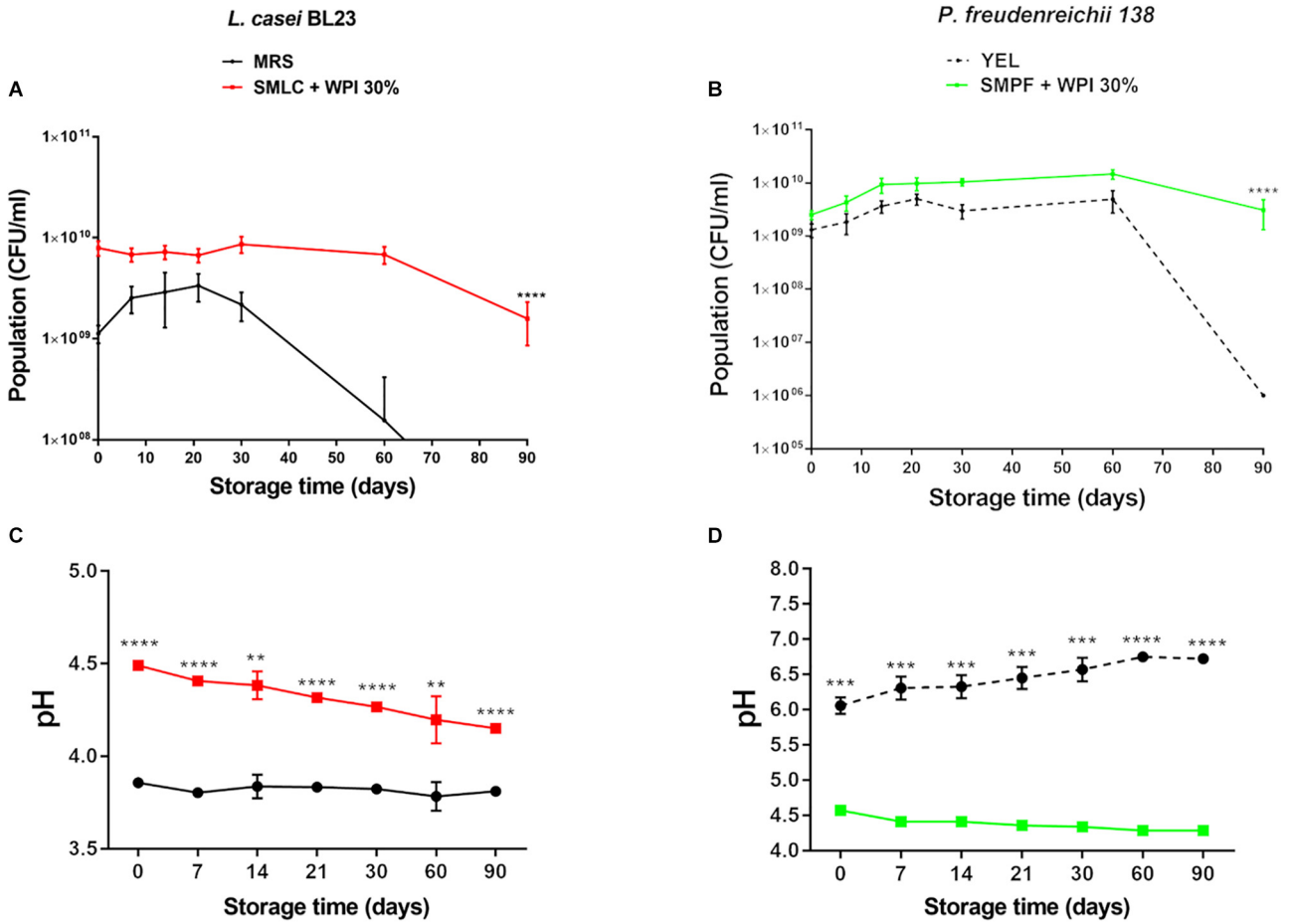

FIGURE 3 | Viability of *L. casei BL23* and *P. freudenreichii* 138 are maintained during 90 days of cold storage when are cultured in skim milk supplemented with WPI. Final population (CFU ml^{-1}) of **(A)** *L. casei* BL23, and **(B)** *P. freudenreichii* 138 in the indicated culture media. Bacteria were culture until the stationary phase and then storage at 4°C for 90 days. Viable bacteria were enumerated by counting the colonies on days 0, 7, 14, 21, 30, 60, and 90 after storage. **(C,D)** Variation of pH values throughout storage days. Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

version 7.00 for Windows (GraphPad Software, San Diego, CA, United States). *P*-values under 0.05 were considered significant.

RESULTS

Different Concentrations of Whey Protein Isolate Alter the Growth of Bacteria

The final population of *L. casei* BL23 and of *P. freudenreichii* 138 was monitored after growth in skim milk supplemented with different concentrations of WPI (**Figure 1**). We observed a significant increase in the CFU counting of *L. casei* BL23 when cultivated in SMLC after 24 h (6.3×10^9 CFU mL^{-1}), in comparison with MRS medium (2.1×10^9 CFU mL^{-1}). Moreover, the largest final population of *L. casei* BL23 was found in skim milk supplemented with 30% WPI (SMLC + WPI 30%) (9.5×10^9 CFU mL^{-1}). The final population of *P. freudenreichii* 138 also showed a similar result (**Figure 1B**). In this case, the highest population (7.2×10^9 CFU mL^{-1}) was obtained in skim milk supplemented with 30% of WPI (SMPF + WPI 30%). This condition allowed the highest growth of the propionibacteria, when compared with all other culture media used.

Whey Protein Isolate Improves the Tolerance of Bacteria Toward Environmental Stresses

The survival rate of *L. casei* BL23 and *P. freudenreichii* 138 was evaluated after acid stress, bile salts stress and high-temperature stress (**Figure 2**). *L. casei* BL23 showed enhanced survival rate after acid stress, when cultured in SMLC medium (69.2%), compared to the MRS control (55.1%) (**Figure 2A**). The survival was further increased when SMLC was supplemented with 30% WPI, leading to the highest survival rate of *L. casei* BL23 (80.6%). This value was significantly higher than skim milk supplemented with 5% and 15% of WPI (data not shown). The population after acid stress in SMLC + WPI 30% was 8.5×10^7 CFU mL^{-1}. A similar result was observed for *P. freudenreichii* 138 (**Figure 2D**). After acid stress, the highest

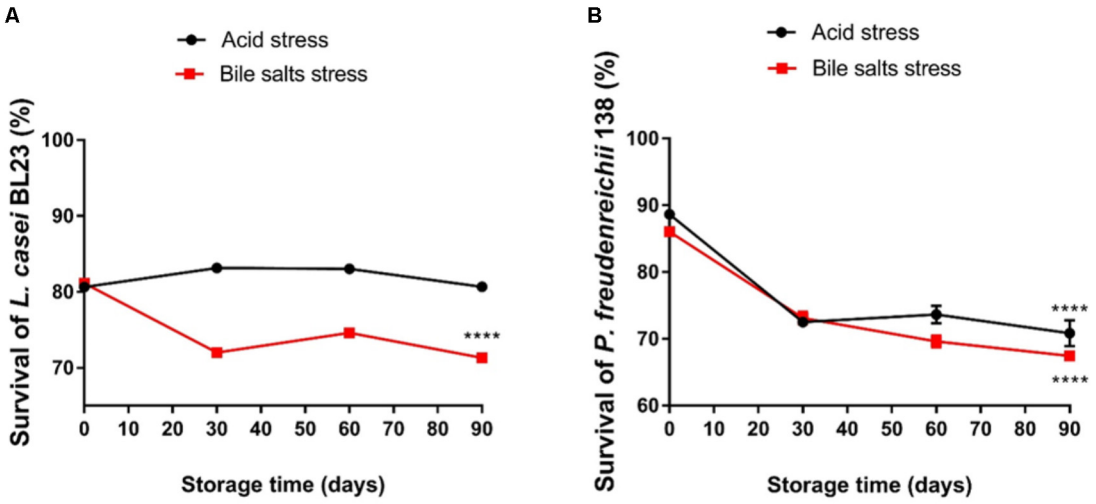

FIGURE 4 | *Lactobacillus casei* BL23 **(A)** and *P. freudenreichii* 138 **(B)** maintain the ability to tolerate acid stress and bile salts stress even during storage when cultured in skim milk supplemented with WPI. Viable bacteria were enumerated by counting colonies on cultures after acid and bile salt stress on days 0, 30, 60, and 90 post-storage and then expressed as percent survival (means ± standard deviation). The assays were performed in triplicate technique and biological triplicate. Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

tolerance was observed when *P. freudenreichii* 138 was grown in skim milk supplemented with 30% WPI (SMPF + WPI 30%) (88.6%), compared with skim milk without supplementation (SMPF) (84.3%) or YEL medium (65.3%). In addition, the survival rate of *P. freudenreichii* 138 grown in milk added with 5% of WPI or 15% are lower than supplementation with 30% (data not shown). The population of *P. freudenreichii* 138 after acid stress in SMPF + WPI 30% was 7.6×10^8 CFU mL^{-1}. Likewise, our results show enhanced tolerance toward bile salts stress when *L. casei* BL23 and *P. freudenreichii* 138 were cultured in beverage containing 30% of WPI (**Figures 2B,E**). After temperature stress, we observed that both strains presented a high tolerance to 63°C, independent of culture medium. However, the highest survival rate was obtained when bacteria were cultured in milk added with 30% of WPI (**Figures 2C,F**).

Bacteria Remained Viable in the Fermented Milk Supplemented With WPI During Storage at 4°C

Changes in bacterial population in fermented skim milk supplemented with whey protein were monitored during storage at 4°C for 90 days (**Figures 3A,B**). The viability of both strains, in skim milk contained 30% of WPI, remained practically unchanged, with a small CFU reduction after 90 days. After cold storage, the *L. casei* BL23 population in the skim milk culture media was maintained at 1.5×10^9 CFU mL^{-1}, while the *P. freudenreichii* 138 population at 7.4×10^9 CFU mL^{-1}. In contrast, viable *L. casei* BL23 and *P. freudenreichii* 138 was significantly decreased when grown in culture media (MRS and YEL, respectively) over the entire storage time, presenting a final population below 2×10^2 CFU mL^{-1}, for *L. casei* BL23 and 1.0×10^6 CFU mL^{-1} for *P. freudenreichii* 138. **Figures 3C,D** represents the evolution of the pH values in the culture of both strains during the storage. We observed a decrease in pH values in beverages containing WPI. In the order hand, in controls the pH values did not decline over the storage time, remaining constant for *L. casei* BL23 while a slight increase in the pH value was detected for *P. freudenreichii* 138. **Figures 4A,B** represent the survival rates of *L. casei* BL23 and *P. freudenreichii* 138, upon acid and bile salt stresses, following storage at 4°C. The survival rate of *L. casei* remained almost constant between days 0 and 90 of storage (**Figure 4A**). For *P. freudenreichii* 138, the acid and bile salts tolerance slightly decreased, between days 0 and 30 (**Figure 4B**). However, propionibacteria survival remained around 70% after 90 days of storage.

Probiotic Beverage Fermented by *L. casei* BL23 or *P. freudenreichii* 138 Reduces the Weight Loss in Mice With Mucositis

Time-course of the weight of mice during the 14 experimental days is shown in **Figure 5**. None of the treatments with probiotic beverages was able to significantly alter the weight of mice during the first 11 days of gavage preceding mucositis induction. As expected, mice receiving 5-FU began to lose weight soon after the drug injection on day 11 (**Figures 5A,C,E**). Interestingly, we observed that the treatment with probiotic beverage fermented by *L. casei* BL23 (**Figure 5B**) or fermented by *P. freudenreichii* 138 (**Figure 5D**) was able to reduce the weight loss of inflamed mice, in the presence or absence of WPI in the medium. However, the association of both bacterial strains were not efficient to further limit the weight loss, compared to mice that received no probiotic treatment (**Figure 5F**).

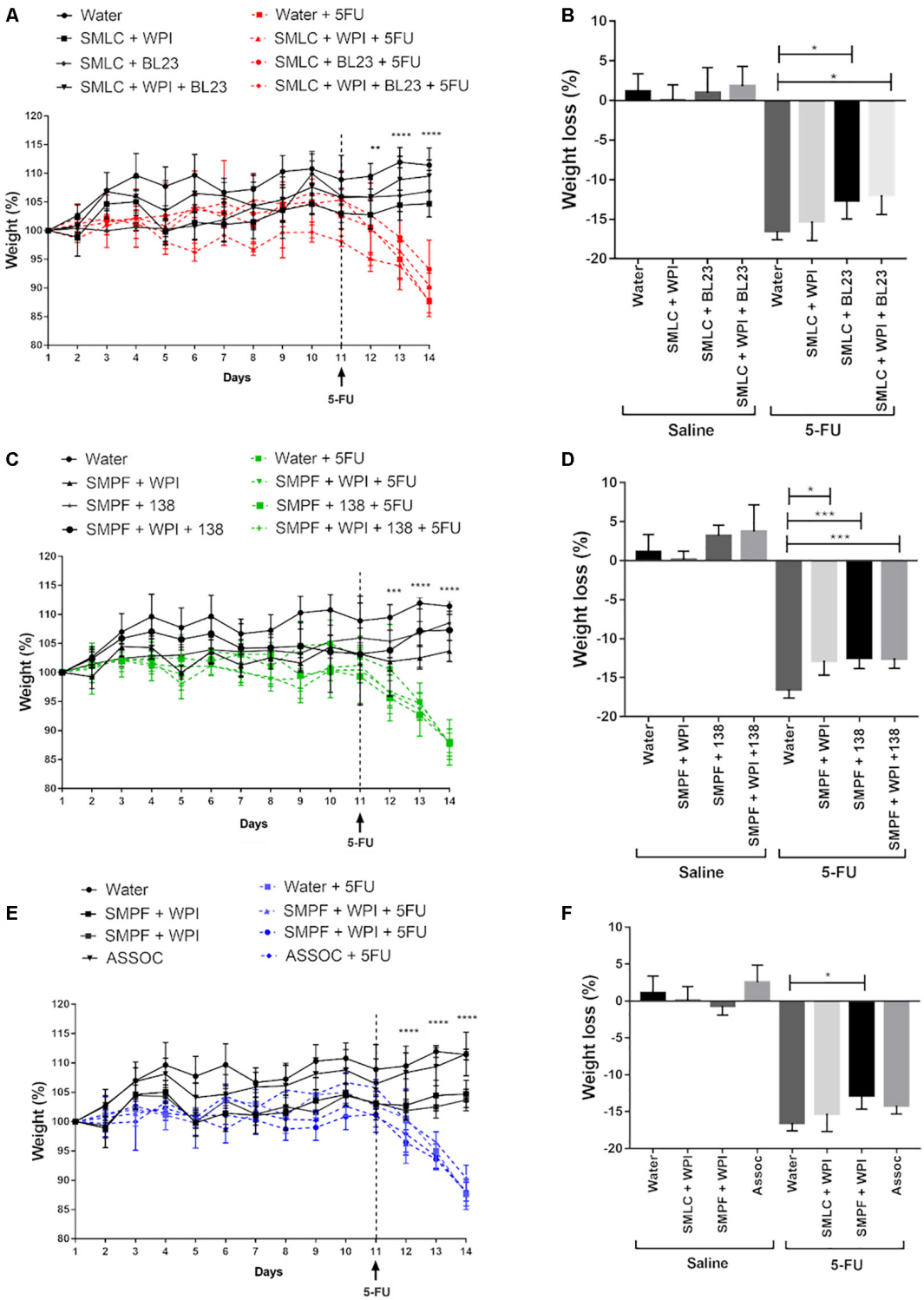

FIGURE 5 | Time-course of body weight for mice treated with **(A)** probiotic beverage fermented by *L. casei* BL23; **(C)** probiotic beverage fermented by *P. freudenreichii* 138 and **(E)** probiotic beverage fermented by association with *L. casei* BL23 and *P. freudenreichii* 138. **(B,D,F)** Weight loss observed after 5-FU injection and differences across groups. $N = 6–9$. Mice were weighted daily during 14 days. Asterisks represent statistically significant differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

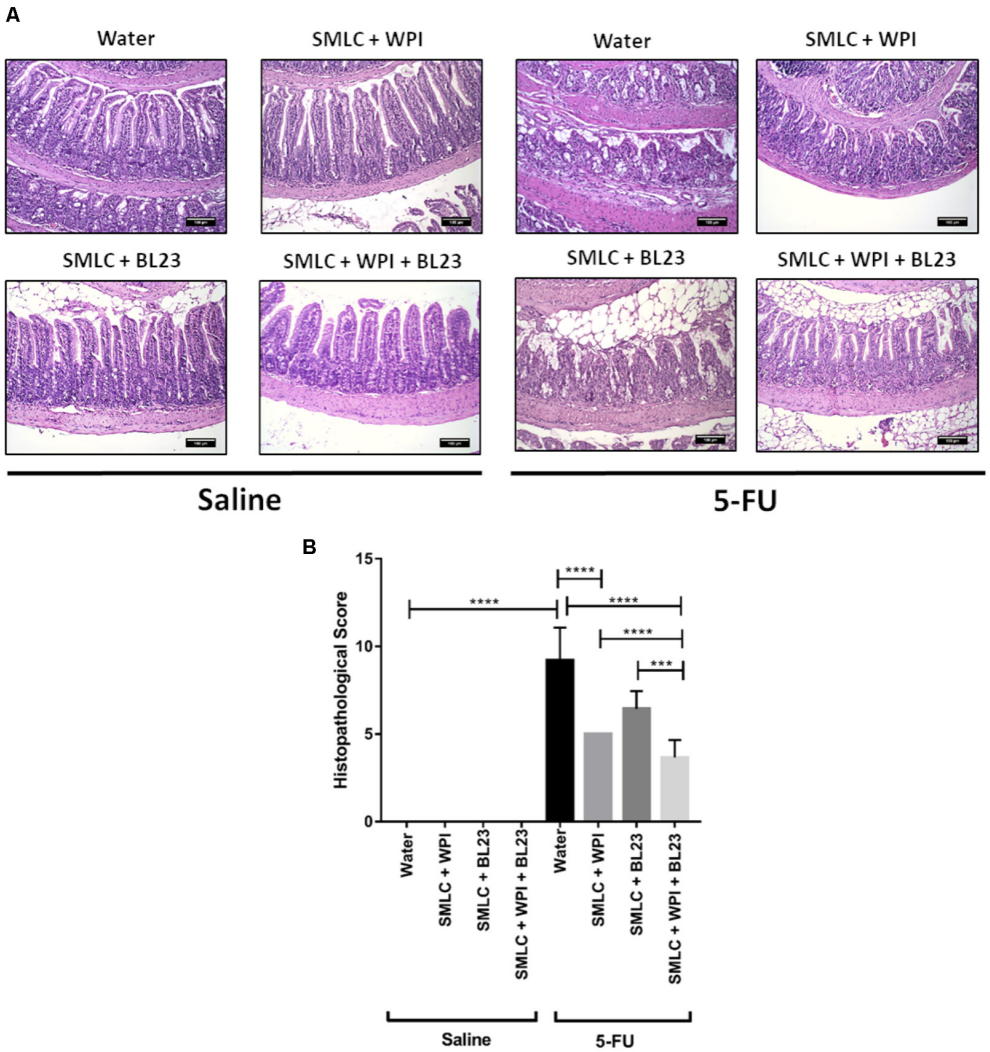

FIGURE 6 | Administration of skim milk supplemented with WPI fermented by *L. casei* BL23 prevents mucosal damage in mice. **(A)** Representative H&E-stained images from mucosal histopathology and **(B)** histopathological score obtained in mice treated. The image acquisition was done with a 20× magnification objective. Scale bar = 100 μm. Same control groups were used for all experimental probiotic assays. Asterisks represent statistically significant differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Beverage Supplemented With Whey Protein Isolate Improves Mucosal Preservation in the Inflamed Mice

Histological analysis revealed a mucosal pattern within normal limits in all groups injected with saline (0.9% NaCl), showing that the probiotics beverages did not alter gut mucosal morphology (**Figures 6–8**). On the other hand, mice submitted to mucositis demonstrated alterations in the morphological structure of the ileum, which was evidenced by an increase in the histopathological parameters. This reflected mainly inflammatory cell infiltration in the *lamina propria*, submucosa and muscular layer, and a prominent alteration in villus structure. However, mice treated with probiotic beverages showed decreased mucosal damage, compared to inflamed mice that did not receive any probiotic treatment. Moreover, supplementation with WPI was able to improve the anti-inflammatory effects of *L. casei* BL23 beverage (**Figure 6**) but not for *P. freudenreichii* (**Figure 7**). Mice treated with the association of *L. casei* BL23 and *P. freudenreichii* 138 in skim milk, presented a reduced histopathological score compared to mice receiving only water, however, the histopathological scores obtained are no better than the treatment using the individually fermented milks by *L. casei* BL23 or *P. freudenreichii* 138 (**Figure 8**).

Treatment With Probiotic Beverages Prevented Villus Shortening and Degeneration of Goblet Cells

Morphometric analysis was carried out to evaluate epithelial integrity. A decrease in villus height and in crypt depth was

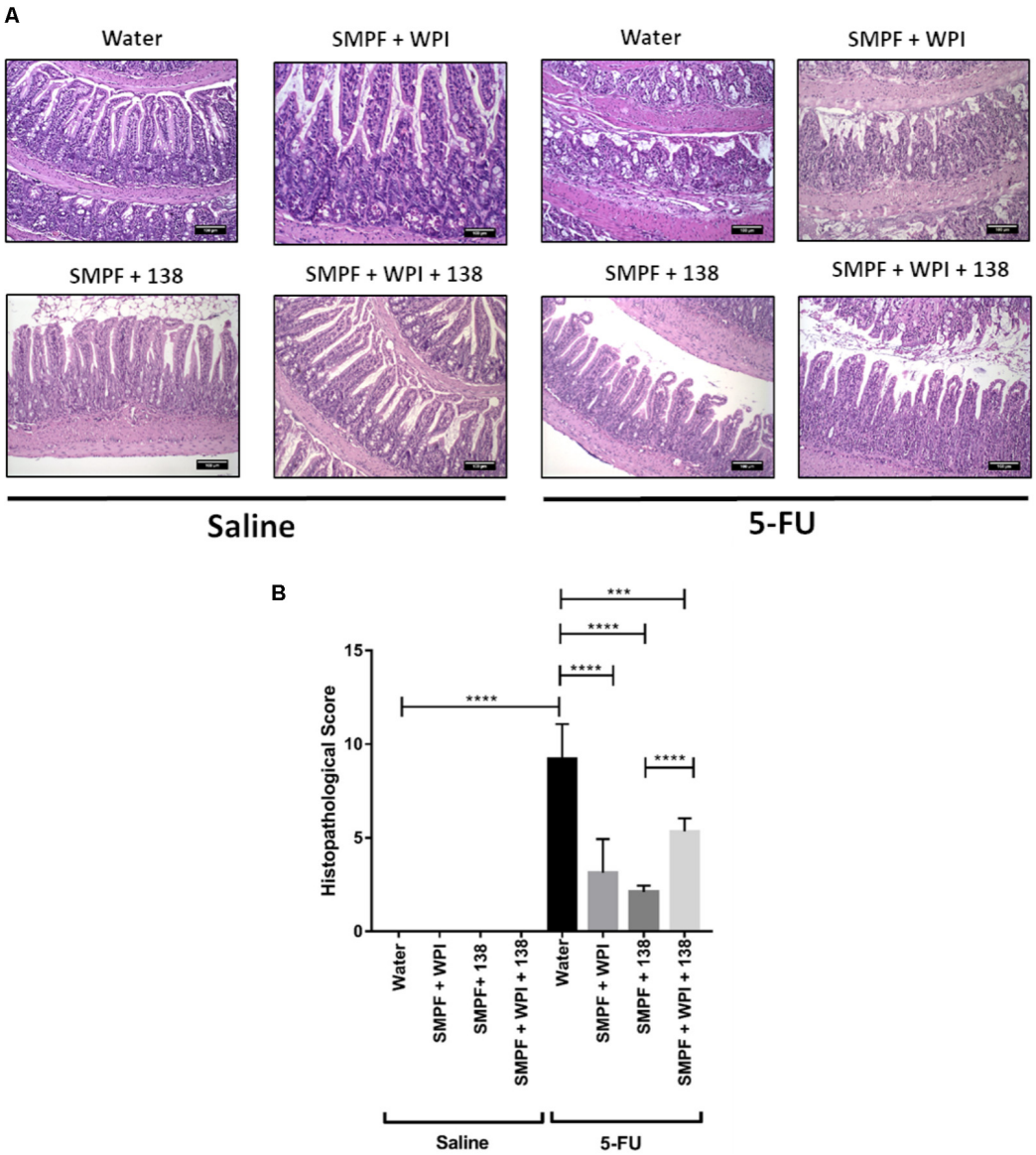

FIGURE 7 | Administration of skim milk without supplemented with whey protein isolate and fermented by *P. freudenreichii* 138 prevents mucosal damage in mice. **(A)** Representative H&E-stained images from mucosal histopathology and **(B)** histopathological score obtained in mice. The image acquisition phase was done with a 20× magnification objective. Scale bar = 100 μm. Same control groups were used for all experimental probiotic assays. Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

observed in mice after 5-FU injection (66 μm) (**Figure 9**). Treatment with probiotic beverages showed increased villus height, especially in groups treated with SMLC + WPI + BL23 (129.2 μm) (**Figure 9A**) and SMPF + 138 (176.04 μm) (**Figure 9C**). No difference was found in crypt depths either in treated or untreated mice. As expected, the mucositis induction resulted in substantial decrease in goblet cells number (9.08 goblet cell/hpf) (**Figure 10**) when compared to the groups injected with 0.9% saline (51.4 goblet cell/hpf). In the other hand, administration of probiotics beverages prevented the degeneration of goblet cells in the mice ileum. The highest goblet cell count was found in mice treated with SMLC + WPI + BL23 (34.7 goblet cell/hpf) (**Figure 10A**) and SMPF + 138 (27.9 goblet cell/hpf) (**Figure 10B**).

Administration of Probiotic Beverages Did Not Change the Secretory IgA Production

Figure 11 indicates the concentration of IgA secreted in the small intestine of healthy animals or after induction of 5-FU mucositis, treated or not with *L. casei* BL23, with *P. freudenreichii* 138, or with the association of the two strains. Our results showed that

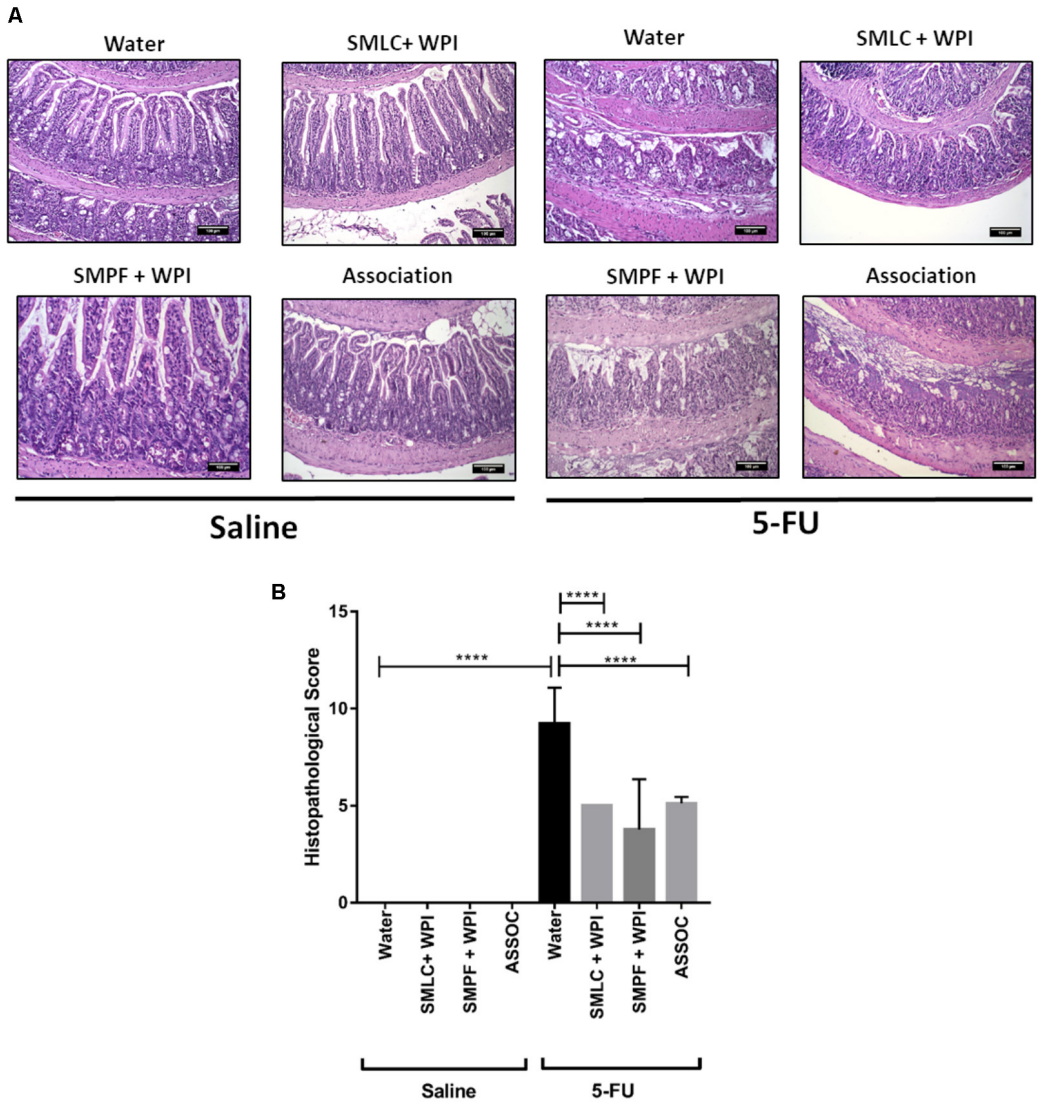

FIGURE 8 | Association of skim milk fermented by *L. casei* BL23 and skim milk fermented by *P. freudenreichii* 138 does not provide additional effect in prevents mucosal damage. **(A)** Representative H&E-stained images from mucosal histopathology and **(B)** histopathological score obtained in the animals treated with different beverages. The image acquisition phase was done with a 20× magnification objective. Scale bar = 100 μm. Same control groups were used for all experimental probiotic assays. Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

there was no significant difference across the groups evaluated in this study.

DISCUSSION

Mucositis is a gastrointestinal inflammation that affects the quality of life of patients undergoing malignancy treatments (Sonis, 2004). Currently, classical therapies available for the prevention and treatment of the disease are not very effective and therefore new options have been suggested, such as the use of probiotic bacteria (Carvalho et al., 2017a). Several of these probiotics required the addition of protective matrices, in order to confer a protection via an efficient delivery of probiotic bacteria to the GIT, and enhance the therapeutics effects in the disease context (Carmo et al., 2017). The present study investigated the effects of whey protein isolate as a protective matrix for two bacterial strains and study the probiotic potential of these fermented beverages in a mucositis mice model induced by 5-FU.

Our data show that the addition of whey protein isolate boosted the growth of *L. casei* BL23 and of *P. freudenreichii* 138. This result may be due to a larger amount of nutrients provided by milk constituents and by WPI in the beverages, including carbohydrate and nitrogen sources, as they are essential for the energy metabolism of both *L. casei* BL23 and *P. freudenreichii* 138 (Cousin et al., 2011; Pessione, 2012). This result corroborates previous studies showing that other bacteria experienced enhanced growth as a result of whey proteins

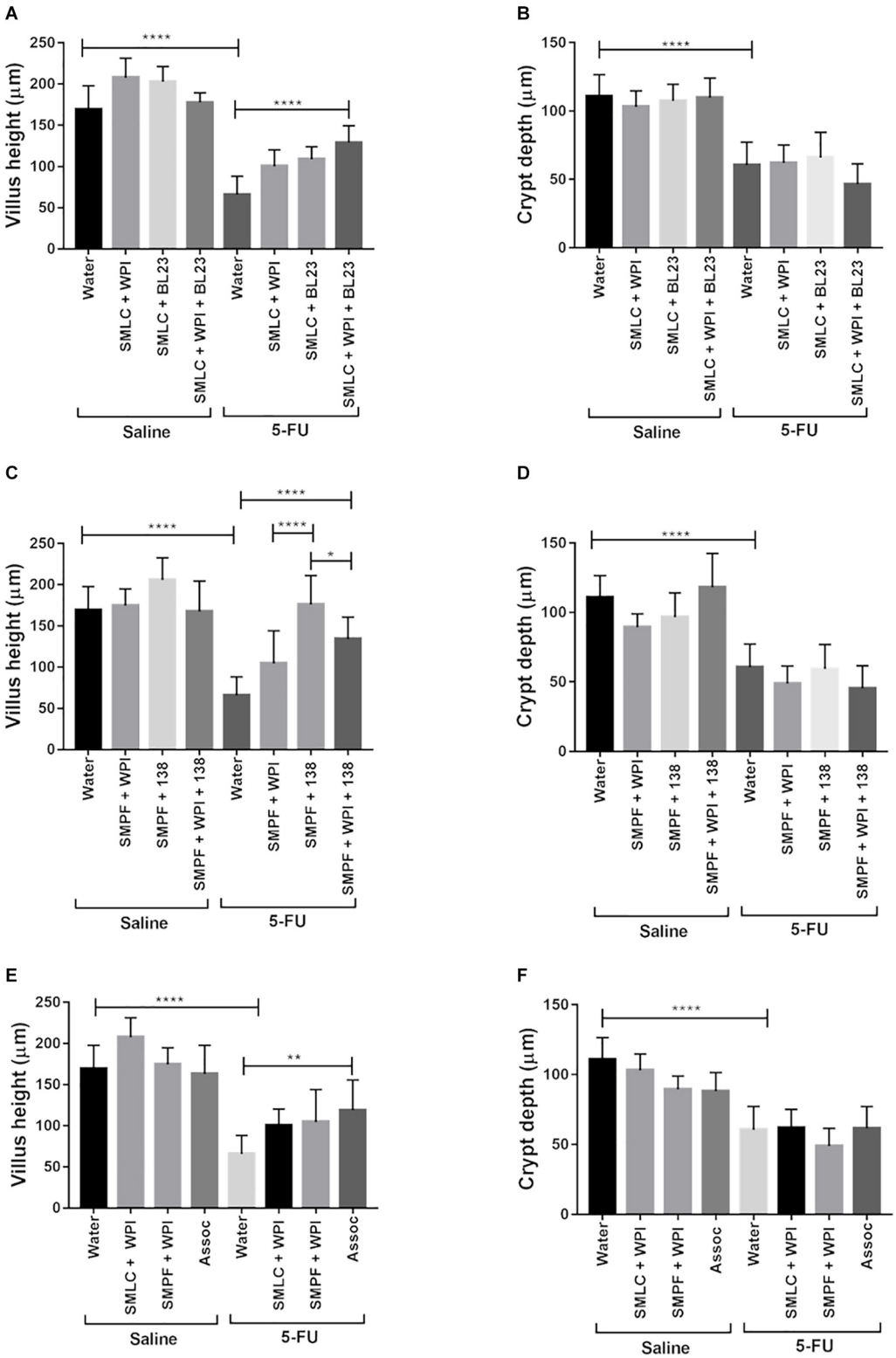

FIGURE 9 | Administration of probiotic beverages improves villus architecture. Morphometric analysis of villus height and crypt depth of animals treated with **(A,B)** beverages fermented by *L. casei* BL23; **(C,D)** beverages fermented by *P. freudenreichii* 138 or **(E,F)** beverages fermented by the association of both bacteria following 5-FU or saline administration. Values were obtained by measuring ten random images of the ileum of mice. $N = 6, 9$. Asterisks represent statistically significant differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

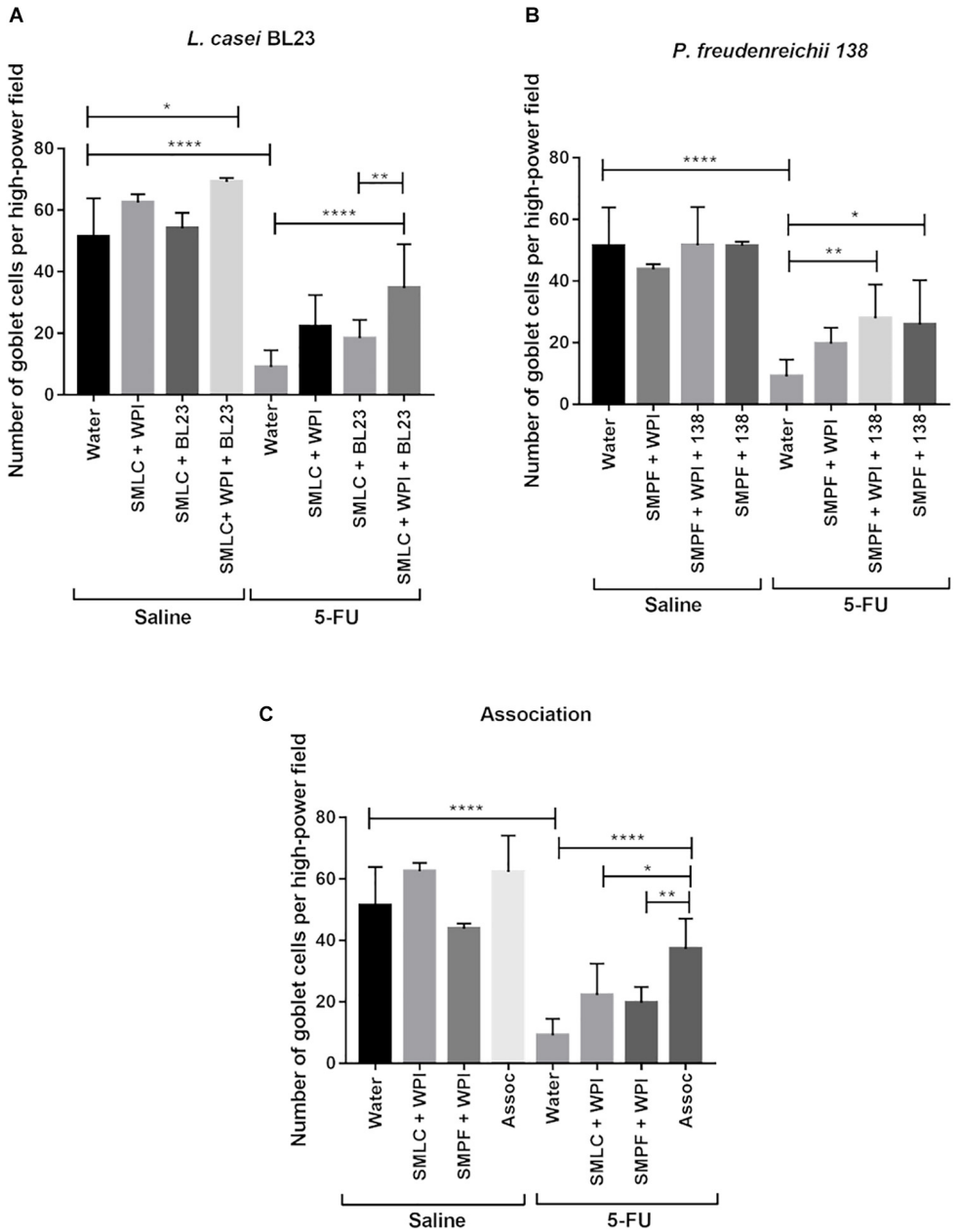

FIGURE 10 | Administration of probiotic beverages prevented the marked degeneration of goblet cells in the mice ileum. Quantification of intact goblet cells in the animals ileum treated with **(A)** beverages fermented by *L. casei* BL23; **(B)** beverages fermented by *P. freudenreichii* 138 or **(C)** beverages fermented by the association of both bacteria following 5-FU or saline administration. Values were obtained by counting intact cells in ten random field images of mice. Results were expressed as means ± standard deviation. $N = 6–9$. Asterisks represent statistically significant differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

addition (Almeida et al., 2009; Skryplonek and Jasińska, 2015; Huang et al., 2016b; Baruzzi et al., 2017).

Due the fact that the products to be considered as a probiotic must be in sufficient quantities of viable bacteria in your local of action (Cousin et al., 2012a; Rabah et al., 2017), we decided to investigate the efficiency of WPI as a protective matrix for bacteria. For this, we evaluated the survival rate of *L. casei* BL23 and of *P. freudenreichii* 138 strains in environments that simulated stomach pH (pH 2), in the presence of bile salts in GIT and at high temperature. Our results demonstrated that the tolerance of *L. casei* BL23 and of *P. freudenreichii* 138 to these stressing conditions was significantly higher in the presence of WPI. Therefore, beverages containing WPI also showed a high viable cell counts after these stresses. Thus, skim milk supplemented with WPI is an effective matrix for the two strains used in this work, considering acid, bile salts

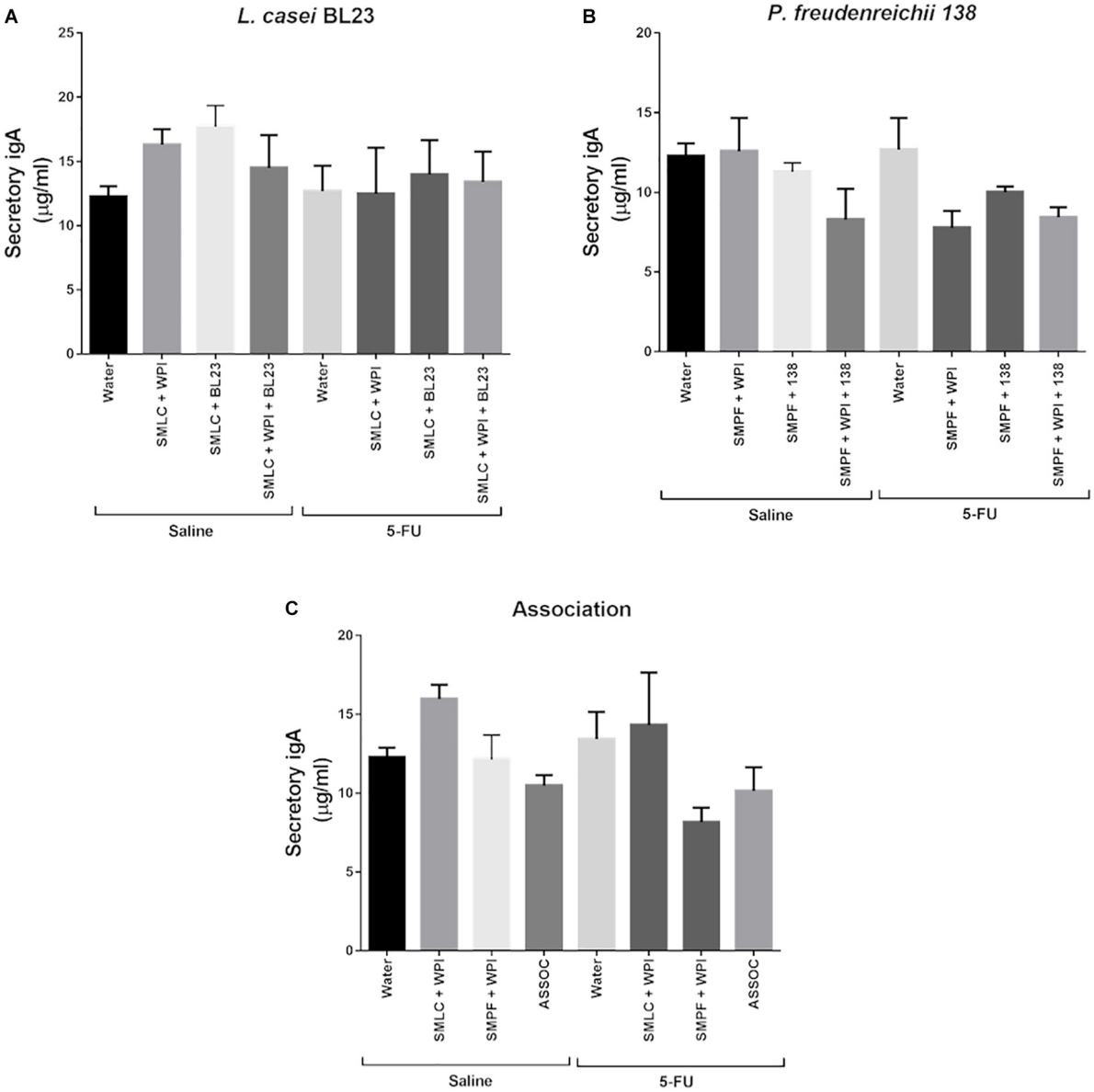

FIGURE 11 | Administration of probiotic beverages did not alters the secretory IgA levels. Quantification of immunoglobulin A secretion (sIgA) in the small intestine of healthy or inflammation mice, treated for 14 days with **(A)** *L. casei* BL23 **(B)** *P. freudenreichii* 138, or **(C)** association of *L. casei* BL23 and *P. freudenreichii* 138. Results were expressed as means ± standard deviation. *N* = 6–9.

and high-temperature stresses. Similar results were described by Vargas et al. (2015), who demonstrated that the probiotic strains *Streptococcus thermophilus* and *L. bulgaricus* presented a higher tolerance to the same stress conditions in the presence of WPI in the culture medium (Vargas et al., 2015). Cousin et al. (2012b) have also shown that *Propionibacterium* strains survived better in acid and biliary stresses when included in a dairy matrix (Cousin et al., 2012b). In a study by Huang et al. (2016b) was also demonstrated that the *P. freudenreichii* CIRM-BIA 129 tolerates simulated GIT stresses when included in hyperconcentrated sweet whey (Huang et al., 2016b). Studies suggest that dairy proteins protect probiotic bacteria via a process called coacervation. In this process, the proteins form microspheres that pack the microorganism inside, thus forming a kind of barrier that protects them from adverse environmental conditions (Silva et al., 2015; Coghetto et al., 2016).

The current definition of probiotic stipulates that microorganisms should be consumed alive (WHO, 2002; Hill et al., 2014). Thus, the survival of bacteria at low temperatures is an important parameter for the development of an effective probiotic product and fermented dairy products are generally stored at 4°C (Cousin et al., 2012b). Both bacteria tested in this work remained viable during at least 90 days at 4°C when cultured in skim milk supplemented with WPI, reaching up

to 10^9 CFU mL^{-1}, after 90 days. Furthermore, it is possible to suggest that these bacteria were also metabolically active as a pH decrease was observed, due to production of lactic acid and propionic acid (main fermentation products of *L. casei* and *P. freudenreichii,* respectively) during cold storage. In addition, stress tolerance of *L. casei* BL23 and of *P. freudenreichii* 138 was maintained upon cold storage in skim milk plus WPI. Therefore, the amount of nutrients provided by milk plus WPI was sufficient to sustain survival of the bacteria over 90 days of cold storage, in accordance with previous reports (Cousin et al., 2012b; Vargas et al., 2015; Moslemi et al., 2016; Shori, 2016; Baruzzi et al., 2017).

In our probiotic beverages, WPI supplementation increases the bacteria's survival rate to environmental stresses, which is an essential parameter for therapeutic effects (Rabah et al., 2017). Since, *L. casei* BL23 is able to reduce inflammation parameters in colitis model (Rochat et al., 2007; Watterlot et al., 2010), and *P. freudenreichii* 138 has been shown some probiotic effects *in vitro* and *ex vivo* model (Cousin et al., 2012a,b), which is interesting to check their potentials in other disease models. In this context, we tested whether the probiotic beverages were able to exert beneficial effects in mice submitted to experimental mucositis and whether the addition of WPI would enhance these probiotic effects. 5-FU treatment caused weight loss, shortening of intestinal villi and an inflammation of mucosa in mice, in accordance with the literature (Carvalho et al., 2017a). Moreover, probiotic beverages fermented by *L. casei* BL23 and *P. freudenreichii* 138 were able to decrease 5-FU-induced intestinal inflammation in BALB/c mice, with preservation of the mucosal integrity and reduced weight loss. Same results were observed by oral administration of Simbioflora®, that containing *L. paracasei*, *L. rhamnosus*, *L. acidophilus* and *Bifidobacterium lactis* plus fructooligosaccharide in a 5-FU-mucositis mice model and in a treatment using a probiotic mixture, named VSL#3, in mucositis model induced by Irinotecan in rats (Bowen et al., 2007; Trindade et al., 2018). Our study also shows that addition of WPI improved *L. casei* BL23 beneficial effects in the ileum, but not for *P. freudenreichii* 138.

Another important feature evaluated in this study was the number of goblet cells throughout the tissue. Goblet cells are responsible for producing a layer of mucus that covers the entire surface of the intestinal epithelium and is mainly composed of high molecular weight glycoproteins known as mucins (Johansson et al., 2013). This mucus prevents the direct adhesion of microorganisms to the epithelium and their translocation to the internal layers of the intestine, besides being important for the lubrication of the intestinal walls and for the protection of the epithelium against digestive acidic fluids and toxins (Kim and Khan, 2013). Previous studies have described that the intestine cells need a series of amino acids, mainly threonine, cysteine, and serine, for the synthesis of this mucus in healthy conditions (Faure et al., 2006). However, during inflammatory processes such as mucositis, a superactivation of the goblet cells occurs, aiming to increase the protection of the epithelium damaged by the inflammatory process (Stringer et al., 2007, 2009a). Consequently, the requirement for amino acids by the cells is increased. However, these amino acids are usually insufficient during the inflammation, compromising adequate mucus barrier functioning (Stringer et al., 2009b). The demand for threonine, cysteine, and serine can be adequately supplied by the diet in order to increase the availability of these amino acids (Faure et al., 2006). WPI used in this study is rich in these three amino acids. Our probiotic beverages prevented the degeneration of goblet cells, suggesting that the presence of milk and WPI may have increased the availability of these amino acids, increasing the production of mucus and consequently improving the framework of protection and tissue repair observed in the histological analyses. Similar results were shown in a probiotic treatment with *Saccharomyces cerevisiae* UFMG A-905 in a murine model of irinotecan-induced mucositis (Bastos et al., 2016), as well as in a mice treated with a mixture of *L. acidophilus* and *Bifidobacterium bifidum* in a 5-FU-induced intestinal mucositis model (Yeung et al., 2015). Prisciandaro et al. (2011) also shown that a *Escherichia coli* Nissle 1917 (EcN) probiotic derived supernatants was able to partially maintained acidic-mucin producing goblet cells in the jejunum and neutral mucin producing goblet cells in the ileum, in 5-FU mucositis model in mice (Prisciandaro et al., 2011). Furthermore, due to the capacity to preserve globet cells and consequently to maintenance of mucin production, this is possible that adhesion of *L. casei* BL23 and *P. freudenreichii* 138 strains to the intestinal epithelial cells can be enhanced in mouse GIT, leading to an increase the probiotic therapeutic effect (Ouwehand and Salminen, 2003).

In summary, our results indicate that the developed probiotic beverages have anti-inflammatory effects in mucositis. Thus, we sought to investigate the role of IgA in the regulation of inflammatory conditions in these mice. IgA is the main antibody type found in mucosal secretions (Pabst et al., 2016) and has many important functions such as modulation of intestinal microbiota and mucosal protection against invading pathogens (Lycke and Bemark, 2017). These functions are naturally important in intestinal mucositis because this disease is associated with dysfunctions related to imbalances in the intestinal microbial community (Clemente et al., 2012). Destruction of the physical barrier that covers the GIT facilitates the invasion of pathogens from the lumen (Bischoff et al., 2014). However, none of the probiotic beverages used in this study was able to alter IgA production, ruling out a probiotic effect via stimulation of sIgA. Accordingly, administration of another probiotic species, *Lactococcus lactis* NCDO, in a DSS-induced colitis model, does not enhance the levels of sIgA (Luerce et al., 2014).

CONCLUSION

We have demonstrated that the supplementation of skim milk with 30% of whey protein isolate is a good matrix to provide protection for the *L. casei* BL23 and for *P. freudenreichii* 138 against environmental stresses. Furthermore, both probiotic beverages developed here were efficient in preventing mucositis induced by 5-Fluorouracil in BALB/c mice. *L. casei* BL23 protective effect was further enhanced by the addition of WPI. The benefits of adding WPI for the prevention of mucositis thus depends on the bacterial strain used.

AUTHOR CONTRIBUTIONS

BC performed the *in vitro* analysis, animal experimentation regarding mucositis pre-treatment with the probiotic strains, interpreted the data regarding the immunological parameters that were assessed, and was a major contributor in the writing of the manuscript. EO and BS were major contributors in the animal experimentation. EF and SS performed, analyzed, and interpreted the histological analysis from ileum slides. JA analyzed and interpreted the morphometric analysis. LA performed the *in vitro* analysis and data interpretation. LL and HA performed, analyzed, and interpreted the secretory IgA quantification assay. AF, AV, LG, and VA contributed to the data interpretation and were major contributors in the writing of the manuscript. GJ and YLL were responsible for ceding the strains, contributed to the data interpretation, and were major contributors in the writing of the manuscript. RC and FC have contributed equally in the supervision, performing experiments, analysis and interpretation of the immunological data, and were major contributors equally in the writing of the manuscript.

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Microencapsulation of Lactic Acid Bacteria Improves the Gastrointestinal Delivery and *in situ* Expression of Recombinant Fluorescent Protein

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The microencapsulation process of bacteria has been used for many years, mainly in the food industry and, among the different matrixes used, sodium alginate stands out. This matrix forms a protective wall around the encapsulated bacterial culture, increasing its viability and protecting against environmental adversities, such as low pH, for example. The aim of the present study was to evaluate both *in vitro* and *in vivo*, the capacity of the encapsulation process to maintain viable lactic acid bacteria (LAB) strains for a longer period of time and to verify if they are able to reach further regions of mouse intestine. For this purpose, a recombinant strain of LAB (*L. lactis* ssp. *cremoris* MG1363) carrying the pExu vector encoding the fluorescence protein mCherry [*L. lactis* MG1363 (pExu:*mCherry*)] was constructed. The pExu was designed by our group and acts as a vector for DNA vaccines, enabling the host cell to produce the protein of interest. The functionality of the pExu:*mCherry* vector, was demonstrated *in vitro* by fluorescence microscopy and flow cytometry after transfection of eukaryotic cells. After this confirmation, the recombinant strain was submitted to encapsulation protocol with sodium alginate (1%). Non-encapsulated, as well as encapsulated strains were orally administered to C57BL/6 mice and the expression of mCherry protein was evaluated at different times (0–168 h) in different bowel portions. Confocal microscopy showed that the expression of mCherry was higher in animals who received the encapsulated strain in all portions of intestine analyzed. These results were confirmed by qRT-PCR assay. Therefore, this is the first study comparing encapsulated and non-encapsulated *L. lactis* bacteria for mucosal DNA delivery applications. Our results showed that the microencapsulation process is an effective method to improve DNA delivery, ensuring a greater number of viable bacteria are able to reach different sections of the bowel.

Keywords: recombinant *Lactococcus lactis*, sodium alginate encapsulation, mCherry reporter protein, pExu vector, DNA delivery

INTRODUCTION

The intestinal microbiota is composed of a huge diversity of ecological niches, encompassing more than 50 genera of bacteria (Holdeman et al., 1976) with different characteristics: harmful, beneficial, or both. Among the bacteria with beneficial activity, the LAB can be highlighted. These are Gram-positive, saccharolytic, rod shaped, non-sporulating bacteria with low-GC content genomes, that could reside in the large intestine (Salminen et al., 1998). The majority of LAB have a 'Generally Recognized as Safe' (GRAS) status according to the United Sates Food and Drug Administration (US FDA) and fulfill of the Qualified Presumption of Safety (QPS) notion criteria developed by the European Food Safety Authority (EFSA).

Some strains are classified as probiotic, promoting them to be beneficial for consumer health (Sanders et al., 2013). This intrinsic advantage of some LAB, as well as the easiness to design them in recombinant lactic acid bacteria (rLAB) makes them a good alternative to systemic delivery systems compared with other mucosal delivery vehicles such as liposomes and attenuated pathogens. For these reasons, over the past two decades, LAB has been intensively studied as potential carriers of compounds with therapeutic or prophylactic effects at mucosal surface (Wells and Mercenier, 2008; Bermúdez-Humarán, 2009; Zurita-Turk et al., 2014; Souza et al., 2016; Mancha-Agresti et al., 2017). In this context, plasmid-cured strains of *L. lactis* ssp. *lactis* IL1403 and *L. lactis* ssp. *cremoris* MG1363, whose genomes were sequenced in 1999/2001 and 2007, respectively, are the strains most commonly used (Bolotin et al., 2001; Wegmann et al., 2007; Linares et al., 2010) for production of recombinant molecules in mucosal vaccination (Wells and Mercenier, 2008). In addition, interest in genus *Lactobacillus* has also increased (Li et al., 2007; Hongying et al., 2014; Allain et al., 2016). In DNA delivery system the eukaryotic host cells express antigens encoded by the vaccine. Epitopes exposed by the recombinant protein (antigen) are very similar to those in their native form (Běláková et al., 2007), consequently they are presented to the immune system in an analogous form found in nature.

Oral administration of rLAB producing/coding therapeutics proteins have the benefit of direct proteins delivery *in situ*, avoiding degradation either by enzymatic action or adverse conditions encountered in the stomach such as severe acid challenges. Also, rLAB could be protect against stress elicited by the exposure to high bile and salt conditions of the intestine faced during transit through the gastrointestinal tract (Drouault et al., 1999). On the other hand, studies have shown that the percentage of viable bacteria that reaches the gut may be considerably lower compared to the number of administered bacteria (Charteris et al., 1998; Cook et al., 2012), due to above mentioned stress conditions.

To circumvent this drawback, bacterial microencapsulation technique using polymer matrix offers adequate conditions to reduce bacterial loss, in the gastrointestinal tract, as well as in food products (Fávaro-Trindade and Grosso, 2002). The most widely used matrix for microencapsulation is sodium alginate, a non-toxic and biocompatible polymer which is able to establish a versatile matrix which protects active components, as well as probiotic microorganisms sensible to pH, heat, oxygen, and other factors (Goh et al., 2012). Some studies report an increase of up to 80–95% survival of encapsulated probiotics with alginate beads (Sheu and Marshall, 1993; Jankowski et al., 1997; Krasaekoopt et al., 2003). Lower bacteria population reduction during exposure to simulated gastric environment and bile solution (Picot and Lacroix, 2004) are some features improved by encapsulation of probiotics in alginate. Encapsulation in alginate of *Bifidobacterium longum* as well as, *Lactobacillus rhamnosus* improved survival at acid pH (pH 2.0) up to 48 h and also, death rate decreased proportionately with increased alginate concentrations (2–4%) and bead size (Lee and Heo, 2000; Goderska et al., 2003).

In previous work, our group developed a new vector (pExu – Extra Chromosomal Unit) to be used as a DNA delivered by *Lactococcus lactis* and *Lactobacillus* strains. We showed eGFP expression (Enhanced Green Fluorescent Protein), by enterocytes in the duodenal region between 12 up to 72 h after oral administration of the recombinant strain *L. lactis* (pExu:*egfp*) (Mancha-Agresti et al., 2016). Nevertheless, tissue auto fluorescence is in the same GFP protein wavelength, hindering tissue analysis and eGFP expression detection in the lower bowel region. Nowadays, there are many fluorescent proteins available (Shaner et al., 2005; Ai et al., 2008; Day and Davidson, 2009). Among the best red members in the fruit series, the RFP (Red Fluorescent Protein) mCherry, can be highlighted. This protein is obtained from successive modifications of the *Discosoma* ssp. fluorescent protein DsRed, and it has longest excitation and emission wavelengths (587/610 nm, respectively) with shortest maturation time (Patterson et al., 2001; Shaner et al., 2004; Jach et al., 2006) in comparison with other RFPs. Proteins that emit fluorescence in red zone are more desirable for cellular studies than those which emit in green or blue zones because red light presents less dispersion allowing deep tissue penetration (Nienhaus and Wiedenmann, 2009), therefore increasing sensibility (Deliolanis et al., 2008).

The aim of this study was to encapsulate the *L. lactis* ssp. *cremoris* MG1363 strain carrying the pExu vector encoding RFP *mCherry* gene in alginate matrix and evaluate potential delivery across gut portions after oral administration by comparing non-encapsulated and encapsulated bacteria.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are listed in **Table 1**. *E. coli* Top10 strain was grown in Luria-Bertani (LB) medium (Acumedia, Lansing, MI, United States) at 37°C with vigorous shaking. *L. lactis* ssp. *cremoris* MG1363 was grown statically at 30°C in M17 medium (Sigma-Aldrich, St. Louis, MO, United States) supplemented with 0.5% glucose (w/v) (Labsynth, São Paulo, Brazil) (G-M17). The Erythromycin antibiotic (Sigma-Aldrich) was added at the indicated concentration as necessary; 500 μg/mL for *E. coli* and 125 μg/mL for *L. lactis*. Pure cultures of bacteria were kept as stock cultures in 40% glycerol (v/v)

TABLE 1 | Bacterial strains and plasmids used in this work.

Bacterial strain	Characteristics	Source
Escherichia coli TOP10	*E. coli* K-12-derived strain; F-mcrA 1 (mrr-hsdRMS-mcrBC) 8 80lacZ1M15 1lacX74 nupG recA1 araD139 1 (ara-leu)7697 galE15 galK16 rpsL (StrR) endA1 λ.	Invitrogen
Escherichia coli TOP10 (*pTP:mCherry*)	*E. coli* TOP10 carrying the pTP:*mCherry* plasmid; Km^r	This work
Escherichia coli TOP10 (*pExu:mCherry*)	*E. coli* TOP 10 carrying the pExu:*mCherry* plasmid; Ery^r	This work
Lactococcus lactis MG1363	*L. lactis* ssp. *cremoris*	Gasson, 1983
Lactococcus lactis MG1363 (pExu:*mCherry*)	*L. lactis* MG1363 carrying the pExu:*mCherry* plasmid; Ery^r	This work
Plasmids	***Characteristics***	**Source**
Zero Blunt® TOPO®	Cloning vector (KmR, ccdB gene fused to the C-terminus of the LacZα fragment)	Invitrogen
pXJM19:*mCherry*	ori colE1, oricg, ptac, *mCherry,* Cm^r	Ott et al., 2012
pTP:*mCherry*	TOPO® vector with the *mCherry* ORF; Km^r	This work
pExu:*mCherry*	pCMV/Ery^r/RepA/ RepC/mCherry	This work

Plac: promoter of operon lac; Km^r: gene that confers resistance to kanamycin; pUc ori: origin of replication; ccdB: lethal gene; ZeoR: gene resistance to zeomycin, ori ColE1: origin of replication for E. coli; Amp: gene that confers ampicillin resistance; pCMV: cytomegalovirus promoter; Ery: gene that confers erythromycin resistance; RepD and RepE: origins of prokaryotic replication for L. lactis and Lactobacillus; egfp: coding sequence of enhancer fluorescent green protein; Cm: gene that confers resistance to chloramphenicol; mCherry: coding sequence of red fluorescent monomeric protein.

(Sigma-Aldrich) for *E. coli* and 25% glycerol (v/v) for *L. lactis* at −80°C.

DNA Manipulations

The protocols of DNA manipulation were performed following Sambrook protocols (Green and Sambrook, 2012) with some modifications. For plasmid DNA extraction from *L. lactis*, to prepare the protoplasts, TES buffer (25% sucrose, 1 mM EDTA, and 50 mM Tris-HCl pH 8) containing lysozyme (Sigma- Aldrich) (10 mg/mL) was added for 1 h at 37°C. Restriction enzymes were used as recommended by suppliers. Transformation of *L. lactis* was performed by electroporation (2,400 V, 25 μF capacitance and 200 Ω of resistance). *L. lactis* transformants were plated on G-M17 agar plates containing erythromycin and were counted after 48 h incubation at 30°C, whereas *E. coli* transformants were plated onto LB agar plates containing the required antibiotic for 24 h at 37°C.

pExu:*mCherry* and *L. lactis* ssp. *cremoris* MG1363 (pExu:*mCherry*)

The ORF of reporter gene *mCherry* was amplified by PCR technique using Hot Start® high-fidelity DNA Polymerase (Qiagen, Hilden, Germany). Oligonucleotides primers used for mCherry were: 5′ ggcGCGGCCGCAATGGTGAGCAAGGGCGAGG 3′ (forward) and 5′ ggcCTCGAGTTACTTGTACAGCTCGTCCATGC 3′ (reverse). Artificial restriction sites of *Not*I and *Xho*I enzyme and also, customized Kozak sequence were added in the forward oligonucleotides. Purified PCR product and pExu plasmid (Mancha-Agresti et al., 2016) were digested with the same restriction enzymes, as recommended by suppliers. The digested vector and insert were purified by Illustra™ GFX™ PCR DNA kit and then were ligated with T4 DNA ligase (Invitrogen, Carlsbad, CA, United States) and transformed into *E. coli* Top10, creating the *E. coli* Top10 (pExu:*mCherry*) strain. Sequence analysis was done to confirm the insert integrity using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, United States) and ABI3130 sequencing equipment. Lastly, pExu:*mCherry* was transformed into competent cells of *L. lactis* ssp. *cremoris* MG1363 strain generating *L. lactis* MG1363 (pExu:*mCherry*) strain, used in this study.

mCherry Production by Eukaryotic Cells *in vitro*

Chinese hamster ovarian cell line [Flp-In™-CHO (Invitrogen)] (CRL 12023)-ATCC was used to evaluate the mCherry expression. For this intent, CHO cells were cultured in complete Nutrient Mixture F12 Ham media (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Gibco-Thermo Scientific, Waltham, MA, United States), 1% L-glutamine (Sigma-Aldrich), and 1% HEPES (Sigma-Aldrich). Cells were seeded at 1×10^6 in a 6-well plate and at 90–95% of confluence, CHO cells were transfected with 4 μg of pExu:*mCherry* vector or no plasmid (negative control) using Lipofectamine™ 2000 (Invitrogen), according to supplier's recommendation. Eukaryotic cells protein expression was checked by epifluorescent microscope (Zeiss Axiovert 200, filter 585/42 nm, Oberkochen, Germany) and by flow cytometry (BD FACSCanto™, BD Bioscience, Franklin Lakes, NJ, United States). Duplicate transfection assays were performed.

Bacterial Doses and Microencapsulation

To prepare the doses of *L. lactis* (pExu:*mCherry*), on the first day, the culture was grown at 30°C in M17 medium (Sigma-Aldrich) supplemented with 0.5% glucose (Labsynth) (GM17) and 125 μg/mL of erythromycin (Sigma-Aldrich). On the second day, a 1/20 dilution was performed until the culture reaches $OD_{600\ nm}$ = 1.0. Then 2 mL, which corresponds to one dose, centrifuged for 10 min at 4°C at 4,000 rpm. Supernatant was discarded, and the pellet was washed twice with PBS (0.01 M) and then it was resuspended in 30 μL of sterile PBS, immediately frozen in ultra-freezer −80°C until use. At the moment of gavage, sterile PBS (0.01 M) was added (∼70 μL) to the prepared

doses to reach a final volume of 100 μL. The final quantities of administrated bacteria were 10^{14} CFU/dose. This concentration was calculated after adequate dilution (10^{-14}), where colonies were counted by pour plate method (G-M17 agar-medium with erythromycin).

For encapsulated doses, after washing the pellet with PBS (0.01 M), bacteria culture [10^{14} colony forming unit (CFU)] was mixed with 50 μL of sterile PBS (0.01 M)and 50 μL of 1% of Sodium Alginate Solution matrix as described by Krasaekoopt et al. (2003). For this procedure, alginate solution was prepared and sterilized by autoclaving (121°C for 15 min). The mixture was homogenized carefully and this homogeneous solution (Bacteria+PBS+1% of sodium alginate solution) was extruded through a 21-gauge nozzle into a sterile 3% $CaCl_2$ solution magnetically stirring (a cross-linking solution), forming beads (encapsulated bacteria) by contact of both solutions. Afterward, beads were harvested by filtering using Cell Strainer 40 μm Nylon (Corning, NY, United States) and $CaCl_2$ remnants were removed by pipetting. Then, they were transferred to a sterile micro tube and stored in ultra-freezer −80°C until use. Before gavage, ∼100 μL of sterile PBS was added to frozen encapsulated doses.

Encapsulation Efficiency and Viability After Freezing

To determine the encapsulation efficiency (EE), the beads were mixed with 100 μL of 2% (w/v) sterile sodium citrate buffer (pH = 8.36) during 10 min to dissociate the capsules. After this time, the mixture was vigorously homogenized and subsequently the entrapped viable bacteria, after adequate dilution (10^{-10}), were counted by pour plate method (G-M17 agar-medium). Non-encapsulated bacteria were also submitted to the same treatment to guarantee the same experimental condition. This process was performed during 5 days after doses production to see viability of beads after freezing. Encapsulation efficiency after freezing was calculated by the following equation:

$$EE\% = \frac{\text{CFU encapsulated cells}}{\text{CFU non} - \text{encapsulated cells}} \times 100$$

* CFU is a number of colony forming unit on the agar plate.

Efficiency was verified for freshly encapsulated and non-encapsulated bacteria (day 0) and for doses conditioned at −80°C (days 1 to 5). For the last condition, single doses were unfrozen and plated each day.

Viability of Encapsulated Bacteria to Artificial Juice

To verify the viability of entrapped bacteria, stomach environment was simulated. In a 20 mL solution containing sodium chloride 0.9% (w/v) and 0.3% of pepsin (w/v) (Sigma-Aldrich) (pH = 2), 10^{14} CFU (one dose) of encapsulated and non-encapsulated bacteria was added and incubated for 2 h at 37°C, with shaking at 50 rpm (Chávarri et al., 2010). Afterward, both suspensions were centrifuged and the supernatant was discarded (the encapsulated solution was filtered using Cell Strainer 40 μm Nylon-corning to avoid capsule loss during discard of the supernatant). Then, the bacterial content was washed twice with PBS (0.01 M) and centrifuged in order to remove the remaining acid solution. Finally, pellets were ressuspended with 100 μL of sodium citrate buffer (pH = 8.36) and after that, 100 μL of this mixture was assayed to pour plate method. This experiment was performed in duplicate. The survival (%) of free and encapsulated bacteria was calculated by spread plate count on G-M17 agar after incubation at 30°C for 48 h as shown in the following equation:

$$\%\text{Survival} = \frac{\log(\text{CFU beads after 2 hours exposure to acid condition})}{\log(\text{CFU initial beads count})} \times 100 \quad (1)$$

Mice

Conventional 4 to 6-week-old (25–30 g) male and female C57BL/6 mice, were obtained from Centro de Bioterismo (CEBIO) of Universidade Federal de Minas Gerais (UFMG, Brazil). Procedures and manipulation of animals followed the rules of Ethical Principles in Animal Experimentation, accepted by the Ethics Committee on Animal Experiments (Protocol # 114/2010, CETEA/UFMG/Brazil). All animals were maintained in collective cages in an environmentally controlled room with a 12-h light/dark cycle and given free access to water and food *ad libitum*.

Mice Handling: Gavage of Recombinant *L. lactis* MG1363 Strain (Encapsulated and Non-encapsulated) Into C57BL/6 Mice

Mice were split into three groups as follows: (i) PBS group, (ii) non-encapsulated *L. lactis* ssp. *cremoris* MG1363 (pExu:*mCherry*), and (iii) encapsulated *L. lactis* MG1363 (pExu:*mCherry*). Two independent experiments were performed with 24 mice in each group (72 mice/experiment). The animals were separated according to each evaluated time (three animals for each time). The recombinant strains (10^{14} CFU bacterial suspensions in a final volume of 100 μL of PBS) were orally administered by gavage. The administration was performed in one go (at zero time) and, at the following intervals: 12, 24, 48, 72, 96, 120, 144, and 168 h (post-gavage), animals were euthanized by cervical dislocation and intestinal sections of duodenum, jejunum, ileum and colon were analyzed by confocal microscopy and qRT-PCR. Images were captured using Zeiss LSM 540 META inverted confocal laser-scanning microscope and analyzed by Zeiss LSM Image Browser software.

mRNA Extraction and Real-Time PCR (qRT-PCR)

Samples of intestinal sections (duodenum, jejunum, ileum, and colon) at 24 h post-gavage, were collected to perform qRT-PCR assay to confirm mCherry expression in enterocytes after oral gavage. We even collected intestinal samples (duodenum,

jejunum, ileum, and colon) at different times post-gavage (12, 48, 72, 96, 120, 144, and 168 h). Samples from each gut portion were collected after mice euthanasia and stored at −20°C in RNA later (Invitrogen) until RNA extraction. Total RNA was obtained using TRIzol reagent (Invitrogen), in compliance with manufacturer instructions. The quality and quantity of RNA samples were evaluated in agarose gel electrophoresis, and also through spectrophotometer analysis on NanoDrop® 2000 spectrophotometer (Thermo Scientific) taking into account absorbance ratios of 280/260 and 260/230 nm. Extracted RNA samples were treated with DNAse I (Invitrogen) which was subsequently deactivated. The complementary deoxyribonucleic acid (cDNA) synthesis was performed using 1 μg of RNA and ImProm-II reverse transcriptase (Promega, Madison, WI, United States) in compliance with its manual.

Quantitative reverse transcription PCR (qRT-PCR) was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States) and gene specific-primers for *mCherry* open reading frame (ORF). Housekeeping gene *Actb* was used as reference for normalization (Giulietti et al., 2001). Experimental approach was optimized by adjusting concentrations of primers for optimal specificity and efficiency (**Table 2**). Amplification reactions were performed in final volume of 10 μL, using 5 μL of SYBR green supermix and 10 ng of cDNA (5 ng/μL). The PCR cycle parameters were as follows: initial denaturation at 95°C for 30 s, annealing/extension at 60°C for 60 s, 40 cycles of 95°C for 15 s followed by a dissociation stage for recording the melting curve. Expression levels in control group were used as calibration data (i.e., animals which received PBS). Results were shown graphically as fold changes in gene expression, using the means and standard deviations of target gene expression amount in accordance with Hellemans et al. (2007). Data were analyzed according to the relative expression using $2^{-\Delta\Delta Ct}$ method (Hellemans et al., 2007).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Statistical significance between the groups was calculated using a Student's *t*-test. A 95% confidence limit was significant at a value of $P < 0.05$.

RESULTS

Recombinant Strain of *L. lactis* – *L. lactis* MG1363 (pExu:*mCherry*)

The mCherry ORF (711bp) (GenBank No. AST15061.1) was successfully cloned into the pExu vector (Mancha-Agresti et al., 2016). This vector has an eukaryotic unit harboring the cytomegalovirus mammalian promoter (pCMV), the polyadenylation signal of the bovine growth hormone (BGH), and a prokaryotic region containing RepD/RepE replication origins for *L. lactis* and also OriColE1 replication origin for *E. coli*. The resistance marker Erythromycin (Ery), was used to select recombinant strain. pExu:*mCherry* vector construction was successfully confirmed by molecular techniques: PCR, enzymatic digestion (*Not*I and *Xho*I), and also DNA sequencing. pExu:*mCherry* was transformed into *L. lactis* ssp. *cremoris* MG1363 wild type strain generating *L. lactis* MG1363 (pExu:*mCherry*) recombinant strain.

Eukaryotic Cells Are Able to Express the mCherry Protein

Functionality of pExu:*mCherry* was established after transfection of CHO cells in two independent assays. In the first one, fluorescence microscopy analysis showed that transfected eukaryotic cells were able to express the reporter protein. In contrast, non- transfected cells showed no fluorescence, as expected. In **Figure 1**, the pictures A, B, and C show the eukaryotic cell expressing or not the fluorescent protein. Images were captured by fluorescence microscopy (**Figures 1A–C**).

In the second one, the percentage of expressing cells was evaluated by flow cytometry. After 48 h, 19% of transfected cells with pExu:*mCherry* showed ability to express the reporter protein. No expression was observed in the non-transfected cells, and also in cells transfected with pExu:*empty* (data not shown). In **Figure 1** pictures D and E show the dot plot of non-transfected and transfected cells respectively. These results confirmed functionality of pExu:*mCherry* plasmid in eukaryotic cells.

Encapsulation Efficiency in 1% (w/v) Alginate and Loss of Viability After Encapsulation and Freezing of *L. lactis* MG1363 (pExu:*mCherry*)

At first, alginate concentration for encapsulation was standardized by testing three different concentrations (0.5, 1, and 2% w/v) with 3% $CaCl_2$ (w/v). Results obtained at this stage showed that the uniformity and spherical beads at 0.5% (w/v) of alginate concentration were not satisfactory due to low viscosity and consequently, less number of binding sites for Ca^{2+} ions (cross-linkage) (Chandramouli et al., 2004; Lotfipour et al., 2012). With 2% (w/v) alginate concentration, beads were too viscous to be extruded from the 21G needle. Thus, the 1%

TABLE 2 | Targets and primers concentration of qRT-PCR performed in this study.

RNA target	Primer sequence (5′–3′)		Amplicon size (pb)	Primer (μmol l^{-1})
	Forward	Reverse		
mCherry	CACTACGACGCTGAGGTCAA	GTGGGAGGTGATGTCCAACT	97	0.25
β-Actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT	138	0.25

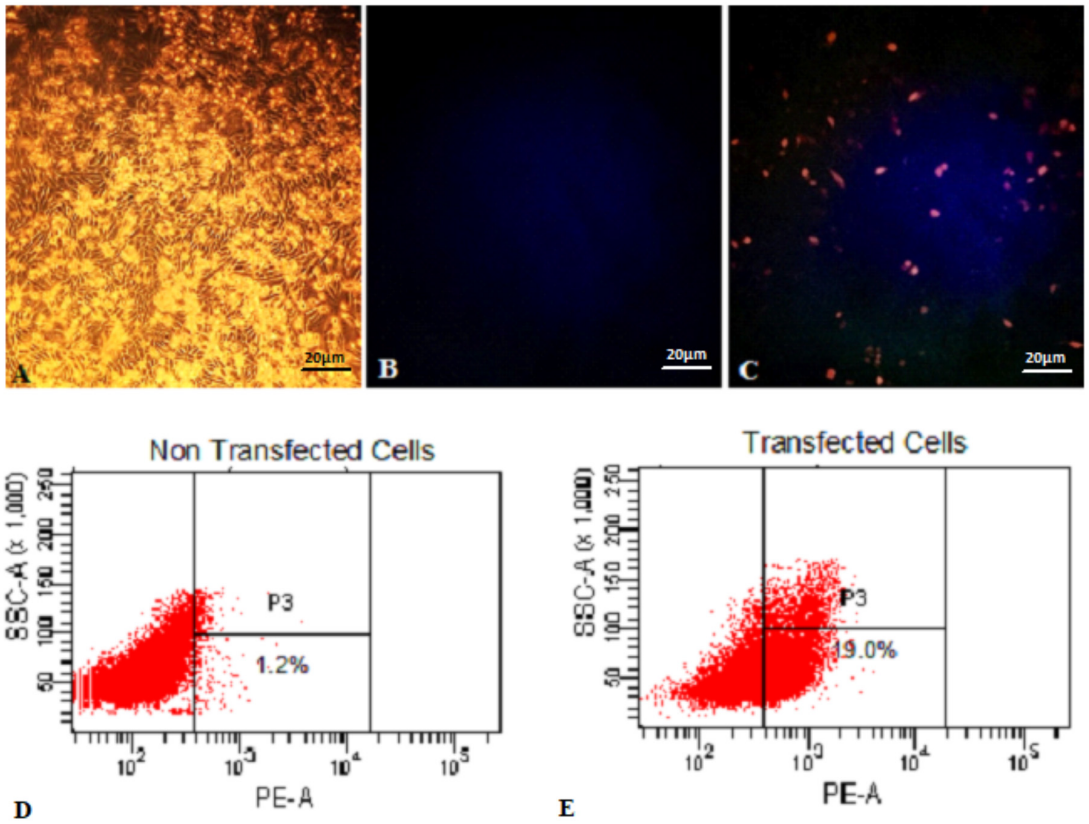

FIGURE 1 | Expression of mCherry protein by Eukaryotic cells transfected with pExu:*mCherry* plasmid evaluated by Fluorescence Microscopy and Flow Cytometry. **(A)** Light field capture of non-transfected CHO cells, **(B)** non-transfected CHO cells, **(C)** transfected CHO cells with pExu:*mCherry* (20X), **(D)** Dot plot of non-transfected cells, **(E)** Dot plot transfected cells with pExu:*mCherry*. The fluorescence images were obtained using the epifluorescent microscope (Zeiss Axiovert 200, filter 585/42 nm). The dot plot graph shows the cell count on the y axis and the PE-A filter on the x axis examined through BD FACSDiva™ Software-BD-biosciences.

(w/v) alginate concentration presented the best conditions for encapsulation.

Regarding efficiency of encapsulation, our results showed that at the time of doses preparation it had 2.8×10^{14} CFU. After the encapsulation process (represented as 0 time), we observed a decrease in the number of CFU (1.6×10^{14}), being about 60% efficiency, in comparison with non-encapsulated ones. It was possible to observe that both strains (encapsulated and non-encapsulated) lost viability during freezing days, in equivalent form, as demonstrated in **Figure 2**. Therefore, in this study, only recent confectioned doses were used in oral administration to mice.

In artificial gastric juice, our results showed that the number of cultivable cells observed corresponds to 4.5×10^4 CFU and 4.7×10^4 CFU for non-encapsulated and encapsulated, respectively. Regarding initial doses in non-encapsulated bacteria (2.8×10^{14} CFU) and encapsulated (1.6×10^{14} CFU), we can observe that the percentage survival of non-encapsulated bacteria was 25.3%, while the survival of encapsulated strain was 32.9%. The bacterial survival showed around an 8% increase in encapsulated ones. These results lead to the conclusion that encapsulated bacteria has slightly more resistance to survive in artificial gastric juice, demonstrating that encapsulate process can be successfully used in oral administration strategies.

mRNA and Protein Expression in Mice Bowel

In order to investigate the expression of mCherry protein by eukaryotic cells *in vivo*, we evaluated mRNA expression of mCherry in different parts of the bowel, after gavage. Our results showed that animals which received encapsulated bacteria were able to express 4.34x fold increase in duodenum and 3.03x fold in jejunum portion when compared with non-encapsulated bacteria. However, no significant differences were seen in ileum and colon portions as shown in **Figure 3**. When the kinetics qRT-PCR experiments were performed, it was observed the same behavior as in confocal analysis. Animals which received the encapsulated doses have higher relative expression in duodenum and in jejunum sections while in ileum and colon the relative expression was lower. Nevertheless, in the colon no statistical differences were observed. The same behavior was observed in the relative expression of non-encapsulated bacteria, where the levels of expression in the duodenum and in the jejunum sections

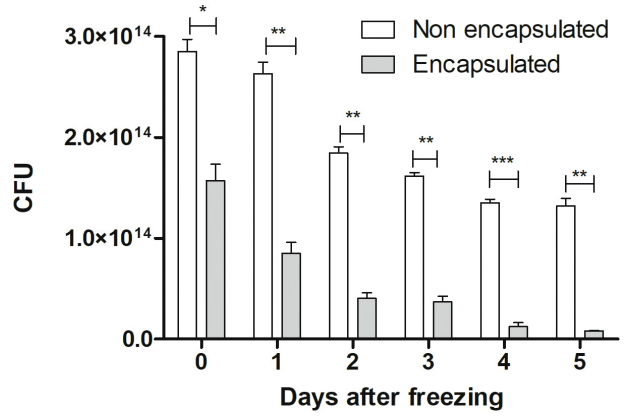

FIGURE 2 | Cellular viability: viability of non-encapsulated and encapsulated cells of *L. lactis* MG1363 (pExu:*mCherry*) from 0 to 5 days after freezing (–80°C). The 0 represents the number of CFU by plate count immediately after the encapsulation process. $*P < 0.05$, $**P < 0.001$, and $***P < 0.0001$.

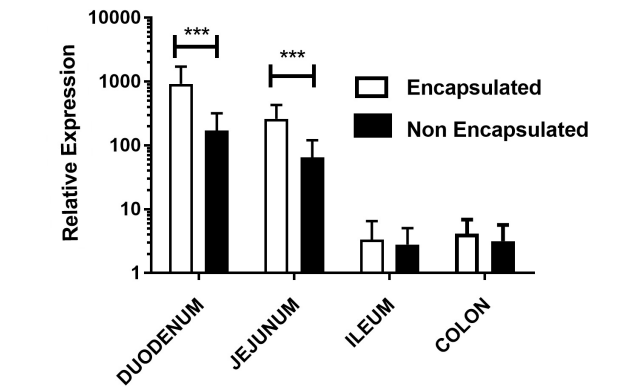

FIGURE 3 | Relative expression of mCherry protein in mouse intestinal cells: evaluation of mCherry protein expression by epithelial cells from different sections of the gut of mice treated orally with, non-encapsulated or encapsulated, *L. lactis* MG1363 (pExu:*mCherry*), by the qRT-PCR technique. Axis y shows the relative expression, and axis x shows the gut sections. $***P < 0.0001$.

were higher compared to ileum and colon ones (**Supplementary Figures 1**, **2**).

Intestinal Cells of Mice Orally Administered With Encapsulated *L. lactis* MG1363 (pExu:*mCherry*) Showed mCherry Expression in Distant Regions of the Bowel

After oral administration of encapsulated and non-encapsulated *L. lactis* MG1363 (pExu:*mCherry*) to C57BL/6 mice at different times (12, 24, 48, 72, 96, 120, 144, and 168 h), the expression of mCherry protein by eukaryotic cells was investigated. The eukaryotic cells of the duodenum and jejunum were able to express mCherry protein until 3 days post-gavage with non-encapsulated bacteria as was shown in previous report with eGFP protein (Mancha-Agresti et al., 2016), while encapsulated bacteria was able to keep the protein expression for 7 and 4 days in duodenum and jejunum, respectively. When we investigated the ileum and colon sections, there was no expression found after gavage with non-encapsulated bacteria. Nevertheless, ileum of animals which received the encapsulated *L. lactis* expressed the recombinant protein between 2 and 4 days after gavage, as shown in **Figure 4**. In the colon section no expression was found (data not shown). The confocal microscopy showed two important aspects. First, there was more expression of mCherry protein by eukaryotic cells when encapsulated *L. lactis* was administered, as were shown in qRT-PCR results. The other aspect is the encapsulated strain is able to reach distant parts of the bowel, as ileum, for example (**Figure 4**).

DISCUSSION

Lactococcus lactis is commonly studied as a host for heterologous protein production and as a delivery platform for therapeutic molecules (Morello et al., 2008; Bahey-El-Din et al., 2010). Different strategies have been proposed in order to improve the delivery in the gut, including the membrane fragilization (Tao et al., 2011) or the expression of invasin proteins in the bacteria membrane (Innocentin et al., 2009). Even though these strategies have drawbacks, many reports showed delivery efficiency when using the DNA vaccine platform, by both oral (Zurita-Turk et al., 2014; Pereira et al., 2015; Souza et al., 2016) or nasal administration (Mancha-Agresti et al., 2017).

The eGFP expression by enterocytes, after mice oral administration of non- encapsulated *L. lactis* MG1363 (pExu:*egfp*), was successfully reported by our group (Mancha-Agresti et al., 2016). It was also reported that delivery by this recombinant strain only occurred in the duodenum section between 12 and 72 h after gavage. The eGFP protein is the marker of choice for many biological studies. However, there are limitations to its use, both *in vivo* and *in vitro* experiments. For example, GFP may lose its direct fluorescence during tissue fixation, as well as for subsequent processing which leads to commercial use of antibodies for immunostaining for GFP detection. Also, GFP expression shows substantial biological variability, even among similar cell types in a single animal (Anderson et al., 2005; Brazelton and Blau, 2005). In addition, GFP protein could have diminished brightness, variability, or loss of fluorescence due to auto-fluorescence of the cells in green wavelengths, and also a delay between protein synthesis and fluorescence development (Cubitt et al., 1995; Aspiras et al., 2000; Shaner et al., 2005). Even more, conventional fluorescent proteins, such as GFP, emit in visible spectrum, where light absorption by tissue is strong (Deliolanis et al., 2008), therefore, its sensibility is low.

In order to improve our previous results and circumvent the drawbacks of *egpf* or *gfp* reporter gene, we decided to evaluate another reporter, the *mCherry*, using the same system, the pExu vector delivered by *L. lactis* ssp. *cremoris* MG1363. The mCherry is a monomeric, non-toxic protein and doesn't need any stress induction to host organisms expressed at high levels (Carroll

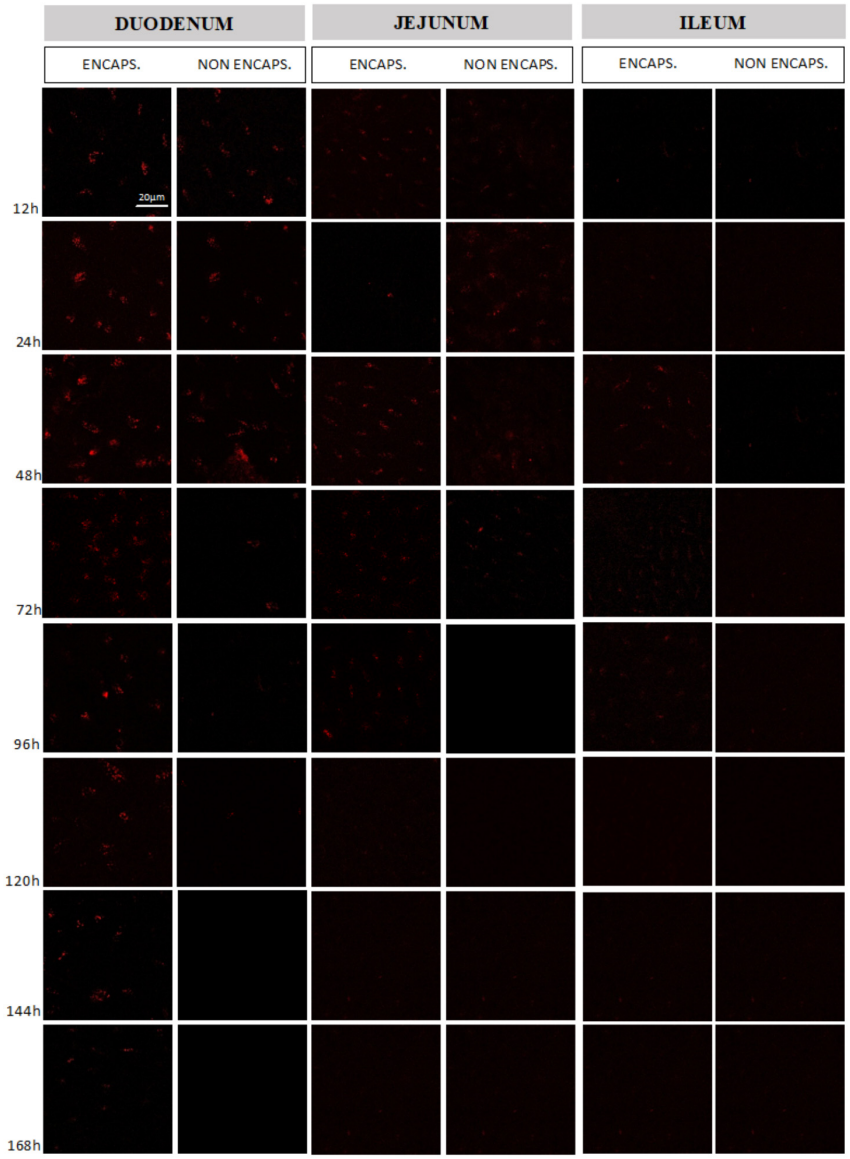

FIGURE 4 | Evaluation of mCherry protein expression by mouse intestinal cells. Comparison between the expression obtained in the different intestinal sections (duodenum, jejunum, and ileum) from the delivery of the vector pExu:*mCherry* by non-encapsulated and encapsulated bacteria. The expression was evaluated between 12 and 168 h after mice gavage. The images were obtained using Zeiss LSM 510 META inverted confocal laser-scanning microscope and the images were analyzed by Zeiss LSM Image Browser software.

et al., 2010). It is highly resistant to photobleaching, consequently losing less fluorescence overtime (Viegas et al., 2007). An important issue to highlight about mCherry in comparison with other red-shifted fluorescent proteins, is their fluorescence emission beyond the 600 nm spectra, where light absorption by tissues is substantially reduced, this showcases enhanced detection sensitivity in whole body imaging applications by at least two orders of magnitude over GFP (Deliolanis et al., 2008).

A previous study using mCherry reporter gene was conducted by van Zyl et al. (2015) to analyze, after mice gavage, the migration and colonization in the gastrointestinal tract (GIT) of two recombinant LAB: *Enterococcus mundtii ST4SA* and *Lactobacillus plantarum 423*. This study reports mCherry as a promising reporter system for living cell imaging studies *in vitro*, *in vivo*, and *ex vivo*. They showed that both bacteria predominantly colonized the cecum and colon, and also persisted in the gut for at least 24 h (van Zyl et al., 2015). Another study used *mCherry* ORF cloned in prCR12 vector (heterologous expression of mCherry protein), transfected in three strains of *Lactobacillus* (*L. plantarum* Lp90, *L. plantarum* B2, and *L. fermentum* Pbcc11.5) showed great strategy in estimating gut colonization in zebrafish model using probiotics. Another interesting point highlighted by these authors is to avoidance of animal sacrifice (Russo et al., 2015).

Acid gastric secretion, a major defense mechanism against the majority of ingested microorganisms, is the first stress confronted by orally administered bacteria (Marteau et al., 1997). Once *L. lactis* has overcome the gastric barrier, they need to envisage the enteric secretions encountered in the duodenum region such as mucus, lysozyme, and phospholipase A2 (Peeters and Vantrappen, 1975; Harwig et al., 1995), defensins secreted by Paneth cells (Mallow et al., 1996) and also bile salt and pancreatic juices. Due to these adversities, high cell mortality is detected in duodenum (Drouault et al., 1999). Divergence may occur once distinct bacterial species demonstrate different reductions in viability, as shown in a study comparing survival of *Bifidobacterium bifidum, Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus* in the gastric compartment (Marteau et al., 1997).

To prevent this situation, and consequently increase *L. lactis* viability in duodenum region and also reach further regions from the intestine, we investigated the encapsulation technique, that has been extensively studied and standardized in *in vitro* experiments (Sheu and Marshall, 1993; Rowley et al., 1999; Krasaekoopt et al., 2003; Desai and Jin Park, 2005; Urbanska et al., 2007; Nedovic et al., 2011).

Once the focus of our group is the use of LAB as DNA vaccine platform in the treatment of different gut disease, like inflammatory bowel disease (IBD), an important issue concern how the encapsulation doses could be affected by the freezing. Demonstrating that bacteria remains viable after freezing could help the development of new therapeutical strategies, for example, preparation of encapsulate doses in one moment and administrate them in another one. Even more, in developing countries this application could help to solve logistic issues, for example frozen doses could be transport further distances without big damage. In order to provide support for *in vivo* studies, we initially performed *in vitro* assays to verify the efficiency of encapsulation process with alginate matrix and the viability of bacteria doses for 5 days after freezing.

In contrast with Kailasapathy (2006) studies, in which EE of *Lactobacillus acidophilus* and *Bifidobacterium lactis* was tested after storage, we unexpectedly verified a decrease in the CFU of *L. lactis* ssp. *cremoris* MG1363 doses soon after the encapsulation process when compared with non-encapsulated. However, when these bacteria were submitted to low pH conditions, simulating gastric environment, the viability of encapsulated bacteria increased around 8% when compared with non-encapsulated. The important issue of this result is to highlight that encapsulation matrix was able to protect the bacteria against these astringent conditions.

Our findings are in accordance with Mortazavian et al. (2007) who tested three different pH (extreme, intermediate, and normal), in extreme and normal conditions they showed that encapsulated *L. acidophilus*, with 2% sodium alginate, had slightly higher percentages of survival than non-encapsulated, being 17.4 and 16%, respectively (Mortazavian et al., 2007). Also, an increase of 9.52 and 7.52% was described in the survival of encapsulated bacteria (*L. acidophilus)* with again 2% of sodium alginate at pH = 3.0 for 3 h in 2 and 1% of bile salt respectively (Chandorkar et al., 2018). These authors highlighted that microencapsulation is a competent technique able to protect probiotics against gastrointestinal environment. All this data supports the idea that encapsulated techniques improve the survival of probiotic bacteria against unfavorable conditions.

This finding was corroborated by our *in vivo* results where the expression of mCherry protein in the duodenum was higher for encapsulated bacteria and was also detected in ileum. The qRT-PCR provided quantitative results that were in line with those obtained in confocal microscopy, specially, in duodenum and jejunum portions, evidencing higher expression of mCherry protein in animals which received encapsulated doses. Although fluorescence had been observed by confocal microscopy at ileum portion, no significant differences were observed in qRT-PCR assay for both, ileum and colon.

Microencapsulation process shows to protect bacteria doses against environment through the GIT. Our hypothesis is that the alginate matrix protects the bacteria against the adversities (stomach and duodenum), allowing their movement across the bowel, being the bacteria able to deliver the plasmid in further sections, as jejunum, ileum, and also slightly in colon.

To our knowledge, this is the first study describing the comparison of encapsulated vs. non-encapsulated *L. lactis* using DNA delivery strategy, *in vivo*. Compared with our previous results (Mancha-Agresti et al., 2016) microencapsulation technique enables higher and longer recombinant protein expression in the gut. Furthermore, this process increases bacteria survival allowing further segments of the gut such as ileum to be reached. The methodology of encapsulation adopted in this work is able to enhance the potential for mucosal delivery by LAB. These findings are uplifting for mucosal therapy for different kinds of bowel diseases.

AUTHOR CONTRIBUTIONS

NC-R, MD, CC, LJ, and PM-A conceived the project. NC-R, MD, PM-A, and SCS wrote the original draft of the manuscript. NC-R, MD, PM-A, SL, AN, and VA wrote, reviewed and edited the manuscript. VA obtained the funding and supervised the project.

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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.2018.02398/full#supplementary-material

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Mutation of the Surface Layer Protein SlpB Has Pleiotropic Effects in the Probiotic *Propionibacterium freudenreichii* CIRM-BIA 129

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Propionibacterium freudenreichii is a beneficial Gram-positive bacterium, traditionally used as a cheese-ripening starter, and currently considered as an emerging probiotic. As an example, the *P. freudenreichii* CIRM-BIA 129 strain recently revealed promising immunomodulatory properties. Its consumption accordingly exerts healing effects in different animal models of colitis, suggesting a potent role in the context of inflammatory bowel diseases. This anti-inflammatory effect depends on surface layer proteins (SLPs). SLPs may be involved in key functions in probiotics, such as persistence within the gut, adhesion to host cells and mucus, or immunomodulation. Several SLPs coexist in *P. freudenreichii* CIRM-BIA 129 and mediate immunomodulation and adhesion. A mutant *P. freudenreichii* CIRM-BIA 129Δ*slpB* (CB129Δ*slpB*) strain was shown to exhibit decreased adhesion to intestinal epithelial cells. In the present study, we thoroughly analyzed the impact of this mutation on cellular properties. Firstly, we investigated alterations of surface properties in CB129Δ*slpB*. Surface extractable proteins, surface charges (ζ-potential) and surface hydrophobicity were affected by the mutation. Whole-cell proteomics, using high definition mass spectrometry, identified 1,288 quantifiable proteins in the wild-type strain, i.e., 53% of the theoretical proteome predicted according to *P. freudenreichii* CIRM-BIA 129 genome sequence. In the mutant strain, we detected 1,252 proteins, including 1,227 proteins in common with the wild-type strain. Comparative quantitative analysis revealed 97 proteins with significant differences between wild-type and mutant strains. These proteins are involved in various cellular process like signaling, metabolism, and DNA repair and replication. Finally, *in silico* analysis predicted that *slpB* gene is not part of an operon, thus not affecting the downstream genes after gene knockout. This study, in accordance with the various roles

attributed in the literature to SLPs, revealed a pleiotropic effect of a single *slpB* mutation, in the probiotic *P. freudenreichii*. This suggests that SlpB may be at a central node of cellular processes and confirms that both nature and amount of SLPs, which are highly variable within the *P. freudenreichii* species, determine the probiotic abilities of strains.

Keywords: bacteria genomic, bacteria proteomic, surface layer protein, HDMSE, shotgun proteomic

INTRODUCTION

Probiotic bacteria 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). This term was further used by International Scientific Association for Probiotics and Prebiotics (ISAP) (Hill et al., 2014). Clinical proofs of efficiency were indeed obtained, in the context of antibiotic- and *Clostridium difficile*-associated diarrhea (Rondanelli et al., 2017), lactose intolerance (Oak and Jha, 2018), irritable bowel syndrome (IBS) (Ford et al., 2014), and ulcerative colitis, one of the disorders that constitute Inflammatory bowel disease (IBD) (Plaza-Díaz et al., 2017). The mechanisms underpinning these effects mainly belong to three categories: (i) metabolic effects, (ii) modulation of the gut microbiota, and (iii) probiotic/host molecular interactions. Although lactobacilli and bifidobacteria were mainly considered for probiotic usage, promising effects were also reported for dairy propionibacteria (Rabah et al., 2017).

The probiotic properties of dairy propionibacteria are strain-dependent and include microbiota modulation, apoptosis modulation in colonic cells and immunomodulation. Some of these probiotic abilities were validated at the clinical level. Microbiota modulation by dairy propionibacteria result in a bifidogenic effect (Roland et al., 1998; Seki et al., 2004; Suzuki et al., 2006). The corresponding molecular mechanisms were elucidated, and two molecules are shown to be involved in bifidogenic effect: 1,4-dihydroxy-2-naphtoic acid (DHNA) and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) (Isawa et al., 2002; Furuichi et al., 2006). The pro-apoptotic effect of dairy propionibacteria was evidenced using *in vitro* cellular models (Jan et al., 2002) and animals models (Lan et al., 2008). This effect is mainly due to the production of the short chain fatty acids (SCFA) acetate and propionate by dairy propionibacteria (Lan et al., 2007; Cousin et al., 2016). The anti-inflammatory effect was suggested in IBD patients (Mitsuyama et al., 2007) and confirmed in animal colitis models (Foligné et al., 2010; Plé et al., 2015, 2016). Immunomodulatory properties are due to several metabolites as SCFAs and to cells wall component (Rabah et al., 2017). Indeed, surface proteins considered as microorganism-associated molecular patterns (MAMP) play a pivotal role in interaction with host's immune system (Deutsch et al., 2012; Le Maréchal et al., 2015). This includes SlpB and SlpE, surface proteins anchored to the cell wall via surface-layer homology (SLH) domains (Deutsch et al., 2017; do Carmo et al., 2018). Indeed, mutation of *slpB* and *slpE* genes clearly affected the immunomodulatory properties of *P. freudenreichii* (Deutsch et al., 2017). We have recently shown that SlpB is involved both in immunomodulation and in adhesion to cultured human intestinal epithelial cells (do Carmo et al., 2017).

In probiotic bacteria, extractable surface proteins play several role in bacterium/host interaction, protection against environmental stresses, inhibition of pathogens, survival within the host digestive tract, and determination or maintenance of cell shape (Hynönen and Palva, 2013; do Carmo et al., 2018). In this study, we investigated the impact of *slpB* gene mutation on the physiology of *P. freudenreichii* CIRM-BIA 129 using a proteomic approach. In this purpose, we investigated alterations in extractable surface proteins and in the whole-cell proteome. We compared wild-type CIRM-BIA 129 with mutant CB129Δ*slpB*. We report pleiotropic effects of this single mutation on physicochemical properties of this propionibacteria.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The wild-type *P. freudenreichii* CIRM-BIA 129 (WT) strain and genetically modified *P. freudenreichii* CIRM-BIA 129Δ*slpB* strain (CB129Δ*slpB*) (do Carmo et al., 2017) were grown at 30°C in Yeast Extract Lactate (YEL) broth (Malik et al., 1968). For the CB129Δ*slpB*, YEL 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 ($OD_{650\,nm}$), as well as by counting colony-forming units (CFUs) in YEL medium containing 1.5% agar. *P. freudenreichii* strains were harvested in a stationary phase (76 h, 2×10^9 $CFU.mL^{-1}$, determined by plate counts) by centrifugation (8,000 × g, 10 min, 4°C).

Inventory of Extractable Surface Proteins Using Guanidine Hydrochloride and MS/MS

Proteins were guanidine-extracted, trypsinolysed and subjected to mass spectrometry (Le Maréchal et al., 2015). Peptides were separated by Nano-LC-MS/MS using a Dionex U3000-RSLC nano-LC system fitted to a Qexactive mass spetrometer (Thermo Scientific, San Jose, CA, USA) equipped with a nano-electrospray ion source (ESI) (Proxeon Biosystems A/S, Odense, Denmark). Peptides were identified from MS/MS spectra using the X!Tandem pipeline 3.4.3 software (Langella et al., 2017) for search into two concatenated databases: (i) a homemade database containing all the predicted proteins of the *P. freudenreichii* CIRM-BIA 129 used in this study and (ii) a portion of the UniProtKB database corresponding to taxonomy 754252: *P. freudenreichii* subsp. *shermanii* (strain ATCC 9614/CIP 103027/CIRM-BIA1).

Zeta Potential Analysis

Electrophoretic mobility (zeta potential) was determined according to the well-described protocol of Schär-Zammaretti and Ubbink (2003). Bacteria were harvested from a 5 mL stationary phase culture by centrifugation (8.000 × g, 10 min, room temperature) and washed twice with a PBS buffer pH 7.0. Cell count of the final suspensions was approximately 10^8 CFU/ml. The pellet was resuspended in a 10 mM KH_2PO_4 solution (pH 7.0). The electrophoretic mobility was measured by using a ZetaSizer nanoZS (Malvern Instruments, Malvern, United Kingdom) and a glass capillary Zetasizer Nanoseries DTS 1061 (Malvern Instruments, Malvern, United Kingdom) as the electrophoretic cell. Electrophoretic mobilities were converted to the ζ-potential using the Helmholtz-Smoluchowski equation (Schär-Zammaretti and Ubbink, 2003). All experiments were done in biological and technical triplicates.

Cell Surface Hydrophobicity Analysis

The Microbial Adhesion To Hydrocarbons (MATH) assay was performed as described by Kos et al. (2003). The optical density of the stationary phase bacteria was adjusted to an $OD_{650\,nm} = 1$. The samples were centrifuged for 5 min, 10,000 × g at room temperature and the pellets washed twice with the same volume of PBS pH 7.0 prior to resuspension in 15 mL of 0.1M KNO_3, pH 6.2. An aliquot of each bacterial suspension (4 ml) was mixed with 1 mL of the solvent (Xylene, chloroform and ethyl acetate), incubated for 5 min at room temperature and mixed by vortex during 120 s. Subsequently, samples were incubated during 60 min to allow phases separation, the aqueous phase was carefully removed and absorbance ($OD_{600\,nm}$) was determined as above. Cell surface hydrophobicity in terms of per cent (H %) was calculated using the following formula: H % = (1–A1/A0) × 100. All experiments were done in biological and technical triplicates.

Transmission Electron Microscopy Assay

Cultures were grown on YEL medium to an $OD_{650\,nm}$ of 1. Transmission electron microscopy was executed after bacteria were washed with PBS and fixed overnight at 4°C in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde. Fixed bacteria were rinsed and stored at 4°C in cacodylate buffer containing 0.2 M sucrose. They were then postfixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate and 2% uranyl acetate in water before gradual dehydration in ethanol (30% to 100%) and embedding in Epon. Thin sections (70 nm) were collected on 200-mesh cooper grids and counterstained with lead citrate before examination. The thickness of the cell wall was determined using the imageJ software in both strains analyzed by Transmission Electron Microscopy (TEM) as described (Foligné et al., 2010; Deutsch et al., 2012).

Stress Conditions Challenge

P. freudenreichii strains in stationary phase were subjected to lethal doses of different stresses. The acid challenge was carried out at pH 2.0 for 1 h as described (Jan et al., 2000). The bile salts stress was induced by adding 1.0 g/l of bile salts for 60 s as described (Leverrier et al., 2003). For the thermic stress, bacteria were heated for 30 min at 63°C. Viable cells were determined by serial dilutions of samples made up in peptone water (0.1% bacteriological peptone, Kasvi, Brazil), adjusted to pH 7.0 and containing 0.9% NaCl, into YEL medium containing 1.5% agar. CFU were counted after 6 days of anoxic incubation at 30°C (Anaerocult® A - Merck Millipore). All experiments were done in biological and technical triplicates.

Whole-Cell Protein Extraction and Preparation of Total Bacterial Lysates

The optical density of the stationary phase bacteria was adjusted to an $OD_{650\,nm} = 1$. The cultures were centrifuged for 5 min, 10,000 × g at room temperature and the bacterial pellets from biological triplicates were resuspended in 1 mL of lysis buffer containing 42% urea, 15% thiourea, 4% SDC (sodium deoxycholate), 12.5 mM Tris-HCl pH 7.5 and 1.5% dithiothreitol (DTT) with 10 µL of protease inhibitor (GE HealthCare, Pittsburgh, USA). Next, whole-cell proteins were extracted as described (Silva et al., 2014) and quantified by Qubit 2.0 fluorometer (Invitrogen, Carlsbad, USA). 100 µg of each protein extract were denatured with 0.2% of RapiGest SF solution (Waters, Milford, USA) at 80°C for 15 min, reduced with 100 mM DTT at 60°C for 30 min, and alkylated with 300 mM iodoacetamide at room temperature in a dark room for 30 min (Leibowitz et al., 2017). Subsequently, proteins were enzymatically digested with 10 µl of trypsin at 0.5 $\mu g.\mu L^{-1}$ (Promega, Madison, USA), and the digestion stopped with the addition of 10 µL of 5% trifluoroacetic acid (TFA) (Sigma Aldrich, Saint Louis, USA) (Silva et al., 2017). Tryptic peptides were subjected to SDC removal (Lin et al., 2010), desalted using C18 MacroSpin Columns (Harvard Apparatus, Holliston, USA), according to the manufacturer's instructions, and dried under vacuum in the Eppendorf™ Vacufuge™ Concentrator (Eppendorf, Hamburg, Germany) (Wong et al., 2013). Prior to injection, the peptides were resuspended in 20 mM ammonium formate (Sigma Aldrich) and transferred to Waters Total Recovery vials (Waters).

LC-HDMS^E Analysis and Data Processing

Quantitative proteomics analyses were conducted with Bidimensional Nano Ultra-Performance Liquid Chromatography (nanoUPLC) tandem Nano Electrospray High Definition Mass Spectrometry (nanoESI-HDMS^E) both using a 1-h reverse-phase (RP) gradient from 7 to 40% (v/v) acetonitrile (0.1% v/v formic acid) and a 500 $nL.min^{-1}$ nanoACQUITY UPLC 2D Technology system (Waters) (Gilar et al., 2005). A nanoACQUITY UPLC High Strength Silica (HSS) T3 1.8 µm, 75 µm × 150 mm column (pH 3) was used in conjunction with a RP Acquity UPLC Nano Ease XBridge BEH130 C18 5 µm, 300 µm × 50 mm nanoflow column (pH 10) (Silva et al., 2017). Typical on-column sample loads were 500 ng of total protein digests for each sample of the 5 fractions (500 ng per fraction/load).

The measurements for all samples by mass spectrometer was operated in resolution mode with a typical m/z resolving power of at least 25,000 Full Width at Half Maximum (FWHM) and an ion mobility cell that was filled with helium gas and a cross-section resolving power at least 40 Ω/Δ Ω. The effective

resolution with the conjoined ion mobility was 25,000 FWHM. Analyses were performed using nano-electrospray ionization in positive ion mode nanoESI (+) and a NanoLock-Spray (Waters) ionization source. The lock mass channel was sampled every 30 s. The mass spectrometer was calibrated with an MS/MS spectrum of [Glu1]-Fibrinopeptide B human (Glu-Fib) solution (100 fmol.μL^{-1}) that was delivered through the reference sprayer of the NanoLock-Spray source. The double-charged ion ($[M + 2H]^{2+} = 785.8426$) was used for initial single-point calibration, and MS/MS fragment ions of Glu-Fib were used to obtain the final instrument calibration.

The multiplexed data-independent acquisition (DIA) scanning with added specificity and selectivity of a non-linear "T-wave" ion mobility ($HDMS^E$) device was performed with a Synapt G2-Si HDMS mass spectrometer (Waters) (Giles et al., 2011). Synapt G2-Si HDMS was automatically planned to switch between standard MS (3 eV) and elevated collision energies $HDMS^E$ (19–45 eV) applied to the transfer "T-wave" collision-induced dissociation cell with nitrogen gas. The trap collision cell was adjusted to 1 eV, using a millisecond scan time that was previously adjusted based on the linear velocity of the chromatographic peak that was delivered through nanoACQUITY UPLC (Waters). A minimum of 20 scan points was generated for each single peak, both in low-energy and high-energy transmission at an orthogonal acceleration time-of-flight (oa-TOF) and a mass range from m/z 50 to 2,000.

Mass spectrometric analysis of tryptic peptides was performed using a mass spectrometer equipped with a T-Wave-IMS device (Waters) in MS^E mode following the method previously described (Distler et al., 2014). Stoichiometric measurements based on scouting runs of the integrated total ion account prior to analysis were performed to ensure standardized molar values across all samples. Therefore, the tryptic peptides of each strain were injected with the same amount on the column. The radio frequency (RF) offset (MS profile) was adjusted such that the nanoESI-$HDMS^E$ data were effectively acquired from m/z 400 to 2000, which ensured that any masses less than m/z 400 that were observed in the high energy spectra with arose from dissociations in the collision cell (Silva et al., 2017).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD009804.

Proteins Identification and Quantification

$HDMS^E$ raw data were processed using Progenesis QI for Proteomics (QIP) v.2.0 (Nonlinear Dynamics, Newcastle, UK) as described by Kuharev et al. (2015). For proteins identification, the peptides were searching against a *P. freudenreichii* strain CIRM-BIA 129 database as described above. The reversed sequences were joined together to the original sequences using ProteinLynx Global Server (PLGS) v 3.0.2 (Waters) database management tool. The reversed sequences were used to calculate the false positive rate during identification process. Next, the following parameters were used for peptide identification: digest reagent = trypsin; maximum missed cleavage = one; maximum protein mass = 600 kDa; modifications: carbamidomethyl of cysteine (fixed), acetyl N-terminal (variable), phosphoryl (variable), oxidation of methionine (variable); search tolerance parameters: peptide tolerance = 10 ppm, fragment tolerance = 20 ppm, maximum false discovery rate (FDR) = 1%.

The protein-level quantitation was performed with Relative Quantitation using Hi-N algorithm. Proteins identified with at least two peptides and presents in at least two of the three biological replicates were considered (Silva et al., 2014). The proteins list was exported by the function "export protein measurements" and was used to subsequent bioinformatics analysis. Proteins were considered to be differentially expressed between mutant and wild type if there were a significant ($p < 0,05$, ANOVA) change in expression $\geq$ 2-fold (log2 ratio ≥ 1.0). A volcano plot was generated to visualize the differentially expressed proteins across these strains.

Extraction of Genomic DNA of the CB129Δ*slpB* Strain

Genomic DNA was extracted from CB129Δ*slpB* culture grown in YEL medium supplemented with chloramphenicol (10 μg ml^{-1}), during the phase (76 h at 30°C). Samples was centrifuged at 4°C and 8,000 × g for 10 min. Bacterial pellets were resuspended in 1 ml Tris/EDTA/RNase [10 mM Tris/HCl (pH 7.0), 10 mM EDTA (pH 8.0), 300 mM NaCl, 50 μg RNase A ml^{-1}] with 50 mg of Glass beads VK01 and cell lysis occurred in Precellys®24 by 2 cylces of 15 s at 6,500 rpm. DNA was purified using phenol/chloroform/isoamyl alcohol and precipitated with ethanol according with Sambrook and Russell (2001). DNA concentrations were determined spectrophotometrically in Thermo Scientific NanoDrop 1000.

Genome Sequencing, Assembly and Annotation of the CB129Δ*slpB* Strain

CB129Δ*slpB* strain sequencing libraries were constructed using 100 ng of genomic DNA. The gDNA was sheared with the Ion Shear™ Plus Reagents Kit and barcoded using the Ion Xpress Fragment Library kit and Ion Xpress™ Barcode Adapters (Life Technologies, USA), according to the manufacturer's protocol. Size selection of ∼400 bp was performed with 2% E-Gel® SizeSelect™ Agarose Gels (Invitrogen, USA) and quantified with the Ion Library Quantitation Kit. The libraries were amplified with the OneTouch Template 400 kit on the Ion One Touch™ 2 (Life Technologies) and enriched on the Ion OneTouch™ ES (Life Technologies). Genomic libraries were enriched using Ion PI™ Hi-Q™ Sequencing Polymerase in the Ion 318™ v2 Chip, according to the manufacturer's protocols, and they were sequenced using Ion Torrent Personal Genome Machine (PGM). The amplification processes were performed using Ion PGM™ Hi-Q™ Sequencing 400 Polymerase with required 1,100 flows. Finally, signal processing was performed using Torrent Suite 4.2.1 to conclude the sequencing process.

De novo assembly was conducted using the software Newbler v 2.9 (Roche 454, USA). The assembled contigs were oriented to generate a scaffold using CONTIGuator v 2.7 (Galardini et al., 2011) and the strains *P. freudenreichii* CIRM-BIA 1 (FN806773.1) and *P. freudenreichii* JS17 (LT618789) as reference.

The *P. freudenreichii* CIRM-BIA 1 strain (without the *slpB* gene) was used for comparative analysis as it is a reference from INRA strain collection strain and *P. freudenreichii* JS17 strain was used due to the presence of the s-layer gene *slpB*. CLC Genomics Workbench 7.0 (Qiagen, USA) was used to map the raw reads against the reference genome and to generate the consensus sequence used to the gap filling. The plasmid that integrated within and disrupted the *slpB* gene was not found in the scaffold, but its sequence was found within the contigs that were excluded during the scaffold generation. It was manually inserted to the scaffold by mapping its ends on the *slpB* gene and using the overlap sequences as coordinates for the insertion. The insertion was validated by mapping the reads on the assembly and checking for mismatches on the regions flanking the plasmid. The genome of CB129Δ*slpB* strain was annotated automatically using RAST pipeline (Aziz et al., 2008; Brettin et al., 2015).

Bioinformatics Analyses

The predicted proteins of CB129Δ*slpB* and WT strain were analyzed using the SurfG+ v1.0 tool (Barinov et al., 2009) to predict sub-cellular localization. It enabled the classification of proteins within the following categories: cytoplasmic (CYT), membrane (MEM), potentially surface-exposed (PSE) and secreted (SEC). The prediction of orthologous groups by functional category the sequences was performed using Cluster of Orthologous Genes (COG) database version 2014db (Galperin et al., 2015). The COG database search was performed using an *in-house* script (available at https://github.com/aquacen/blast_cog). The number of predicted proteins in relation to subcellular localization and functional category were visualized in plots generated using TIBCO SpotFire software 7.0 (TIBCO, Boston, USA) from the protein list exported of QIP. The Interactivenn web-based tool (Heberle et al., 2015) was used to evaluate the shared proteins among strains through Venn diagram.

Protein-protein interaction (PPI) network was constructed using interolog mapping methodology and metrics according to Folador et al. (2014). To generate a preview of the interaction network was generated using Cytoscape version 2.8.3 (Shannon et al., 2003) with a spring-embedded layout. To indicate the reliability of our predicted PPIs in the database STRING, the network was selected using the score 500 (0.5). In the PPI network, the interactions with score close to 500 are with red or yellow lines and, above 700 in dark green lines. The score indicating how much the pair of proteins in the interaction is similar (homologous) to the interaction according to the database. In the PPI, they interact with at least 65% identity with at least 65% coverage.

A circular map comparing the chromosome of CB129Δ*slpB* with *P. freudenreichii* CIRM-BIA 1 and JS17 strains was generated using BLAST Ring Image Generator(BRIG) software v0.95 (Alikhan et al., 2011). Operon prediction in CB129Δ*slpB* strain was performed using FGENESB (http://www.softberry.com).

Statistical Analyses

Growth curve, MATH assay, Zeta potential measure, and stress challenges were performed with three technical replicates and three biological replicates. The results were expressed as means ± standard deviations. Statistical analyses were performed in GraphPad Prism Software version 7 (GraphPad Software) using Student's *t*-test, one-way or two-way ANOVA with SIDAK's or Tukey *post-hoc* analyses for multiple comparisons. Asterisks represent statistically significant differences and were indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS

Impact of *slpB* Mutation on *P. freudenreichii* Extractable Surface Proteins

SLPs play a key role in probiotic/host interactions and we have shown that such interactions are impaired in an *slpB* mutant. Electrophoretic analysis of guanidine extracts confirmed the disappearance of the corresponding SlpB protein (do Carmo et al., 2017). In the present study, we further investigated these extractable fractions in order to decipher the impact of such a single mutation on the inventory of SLPs, and more widely, of extractable surface proteins, including surface layer associated proteins (SLAPs). Using nanoLC-MS/MS, we identified 40 surface extractable proteins in CB129Δ*slpB* strain, yet 33 in the parental wild-type CIRM BIA 129 one (**Table 1**). The core of extractable proteins, non-covalently bound to the cell wall, common to mutant and parental strains, was composed of 23 proteins, including solute-binding protein of the ABC transport system (BopA), internalin A (InlA), surface protein with SLH Domain E (SlpE), and surface-Layer Protein A (SlpA). Moreover, it comprised a series of cytoplasmic proteins involved in different biological processes like Heat shock 70 kDa protein 1 (HSP70 1), Clp chaperone, GroL1 and GroL2, Elongation factor Tu, and subunits of Methylmalonyl-CoA mutase and subunits of Methylmalonyl-CoA carboxytransferase. Among extractable proteins specific of the CB129Δ*slpB*, we identified proteins involved in metabolic processes like Coenzyme A transferase involved in acetyl-CoA metabolic process and Pyruvate phosphate dikinase Pyruvate synthase involved in pyruvate metabolic process. Furthermore, this specific subset also comprised another protein involved in stress response (HSP70 2). As expected, the SlpB protein was found only in the parental wild type CIRM BIA 129, yet not in the CB129Δ*slpB* mutant.

Impact of *slpB* Mutation on *P. freudenreichii* ζ-Potential and Cell Surface Hydrophobicity

Propionibacterial SLPs, with a low isoelectric point, confer negative charges to the cell surface. In order to identify if the net surface charge was altered in the mutant strain, we conducted ζ-potential and cell surface hydrophobicity assays in both strains. As shown in the **Figure 1A**, the WT strain exhibited a zeta potential of −21.73 ± 1.63 mV, reflecting a negative net charge, in accordance with the low isoelectric point of *P. freudenreichii* SlpB protein. By contrast, mutation of *slpB* gene significantly affected the zeta potential of the CB129Δ*slpB* strain, which

TABLE 1 | Proteins identified in the extraction of surface proteins non-covalently bound to the cell wall using guanidine hydrochloride of CB 129 wild-type and CB129Δ*slpB* strains[1].

Strain						Wild-type							CB129Δ*slpB*			
Group ID[a]	Sub-group ID[b]	locus_tag	Protein description[c]	SurfG+[d]	COG letter[e]	MW[f]	log(*e*-value)[g]	Coverage[h]	Uniques[i]	Specific uniques[j]	emPAI[k]	log(*e*-value)[g]	Coverage[h]	Uniques[i]	Specific uniques[j]	emPAI[k]
a1	a1.a1	PFCIRM129_05460	Surface protein with SLH domain (S-layer protein E)	SEC	O	59.2	−125.6	50	19	18	47.3	−138.8	55	18	17	41.8
a1	a1.a2	PFCIRM129_00700	Surface layer protein B (S-layer protein B)	SEC	O	56.8	−263.2	74	34	33	37274.9	–	–	–	–	–
a1	a1.a3	PFCIRM129_09350	Surface layer protein A (S-layer protein A)	SEC	O	58.3	−174.3	75	24	23	16.0	−143.5	68	22	21	9.0
a2	a2.a1	PFCIRM129_12235	Internaline A	SEC	S	145.5	−464.8	67	53	–	89.1	−426.3	65	49	–	42.3
a3	a3.a1	PFCIRM129_03680 & PFCIRM129_03685	MERGED=TRUE	–	–	95.9	−186.7	43	18	–	283.8	−196.6	47	20	–	431.9
a4	a4.a1	PFCIRM129_10100	60 kDa chaperonin 2 (Protein Cpn60 2) (groEL protein 2) (Heat shock protein 60 2)	CYT	O	56.4	−82.5	38	13	–	5.3	−112.6	49	18	–	14.8
a5	a5.a1	PFCIRM129_07835	60 kDa chaperonin 1 (Protein Cpn60 1) (groEL protein 1) (Heat shock protein 60 1)	CYT	O	56	−89.2	38	12	–	2.2	−134.7	60	21	–	7.5
a6	a6.a1	PFCIRM129_06355	Chaperone clpB 2 (ATP-dependent Clp protease B2) (Clp chaperone)	CYT	O	94.2	−39.6	19	10	9	1.2	−103.0	29	18	–	3.0
a7	a7.a1	PFCIRM129_06315	Chaperone protein dnaK 1 (Heat shock protein 70 1) (Heat shock 70 kDa protein 1) (HSP70 1)	CYT	O	65.3	−24.0	13	5		0.6	−61.2	34	15	12	3.8
a7	a7.a2	PFCIRM129_08775	Chaperone protein dnaK 2 (Heat shock protein 70 2) (Heat shock 70 kDa protein 2) (HSP70 2)	CYT	O	67.1	–	–	–	–	–	−43.7	23	10	7	2.0
a9	a9.a1	PFCIRM129_08275	Elongation factor Tu	CYT	J	43.6	−43.7	33	7	–	2.0	−32.4	28	7	–	3.4
b11	b11.a1	PFCIRM129_11405	30S ribosomal protein S1	CYT	J	53.5	−5.4	7	2	–	0.3	−58.9	27	8	–	1.6
b12	b12.a1	PFREUD_01840	Pyruvate synthase/Pyruvate-flavodoxin oxidoreductase	CYT	C	136.4	–	–	–	–	–	−67.5	16	14	–	1.2
b13	b13.a1	PFCIRM129_10305	Methylmalonyl-CoA carboxytransferase 5S subunit. (transcarboxylase 5S) 505 bp	CYT	C	55.5	−23.3	16	5	–	0.7	−37.1	23	9	–	1.8
b14	b14.a1	PFCIRM129_06950	Trigger factor (TF)	CYT	O	57.3	−8.3	6	2	–	0.3	−36.7	20	6	–	2.0

(Continued)

TABLE 1 | Continued

Strain						Wild-type							CB129Δ*slpB*			
Group ID[a]	Sub-group ID[b]	locus_tag	Protein description[c]	SurfG+[d]	COG letter[e]	MW[f]	log(*e*-value)[g]	Coverage[h]	Uniques[i]	Specific uniques[j]	emPAI[k]	log(*e*-value)[g]	Coverage[h]	Uniques[i]	Specific uniques[j]	emPAI[k]
b15	b15.a1	PFCIRM129_07240	Methylmalonyl-CoA mutase large subunit (Methylmalonyl-CoA mutase alpha subunit) (MCM-alpha) (MUTB-(R)-2-Methyl-3-oxopropanoyl-CoA CoA-carbonylmutase)	CYT	I	80.1	−15.6	7	4	–	0.4	−38.3	15	8	–	1.0
b16	b16.a1	PFCIRM129_06070	Enolase 1	CYT	G	45.9	−26.5	20	5	–	1.1	−41.5	25	7	–	1.7
b17	b17.a1	PFCIRM129_07235	Methylmalonyl-CoA mutase small subunit (Methylmalonyl-CoA mutase beta subunit) (MCB-beta)	CYT	I	69.5	−16.2	9	4	–	0.4	−59.4	26	9	–	1.2
b19	b19.a1	PFCIRM129_10180	Iron-sulfur protein	CYT	C	57.2	−26.1	18	6	–	1.1	−16.7	8	3	–	0.4
b20	b20.a1	PFCIRM129_08670	Cell-wall peptidases, NlpC/P60 family SEC protein	SEC	M	58.7	−51.6	22	8	–	1.7	−9.5	6	2	–	0.4
b21	b21.a1	PFCIRM129_09300	FAD-dependent pyridine nucleotide-disulphide oxidoreductase:4Fe-4S ferredoxin, iron-sulfur binding:Aromatic-ring hydroxylase	CYT	C	59.7	–	–	–	–	–	−42.1	20	8	–	1.2
b22	b22.a1	PFCIRM129_00205	Succinate dehydrogenase flavoprotein subunit	CYT	C	74.7	−17.1	5	3	–	0.3	−20.8	6	4	–	0.5
b23	b23.a1	PFCIRM129_08495	NADH-quinone oxidoreductase chain G (NADH dehydrogenase I, chain G)	CYT	C	84.8	−22.3	6	3	–	0.2	−28.3	9	5	–	0.4
b24	b24.a1	PFCIRM129_09980	Peptidyl-prolyl cis-trans isomerase	SEC	O	35.9	−23.0	22	4	–	5.8	−11.5	7	2	–	1.2
b25	b25.a1	PFCIRM129_10295	Methylmalonyl-CoA carboxytransferase 12S subunit (EC2.1.3.1) (Transcarboxylase 12S subunit). 610 bp	CYT	I	56.3	−31.2	11	5	–	0.7	−15.2	7	3	–	0.4
b26	b26.a1	PFCIRM129_11300	Glyceraldehyde-3-phosphate dehydrogenase / erythrose 4 phosphate dehydrogenase	CYT	G	37.7	−48.7	39	9	–	2.9	–	–	–	–	–

(Continued)

TABLE 1 | Continued

Strain						Wild-type						CB129Δ*slpB*			
Group ID[a]	Sub-group ID[b]	locus_tag	Protein description[c]	SurfG+[d]	COG letter[e]	MW[f]	log(*e*-value)[g]	Coverage[h]	Uniques[i]	Specific uniques[j]	emPAI[k]	log(*e*-value)[g]	Coverage[h]	Uniques[i]	Specific uniques[j]	emPAI[k]
b27	b27.a1	PFCIRM129_05155	ATP synthase subunit alpha (ATPase subunit alpha) (ATP synthase F1 sector subunit alpha)	CYT	C	58.8	−7.9	5	2	–	0.2	−17.9	11	5	–	0.6
b30	b30.a1	PFREUD_10490	ATP synthase subunit beta (ATPase subunit beta) (ATP synthase F1 sector subunit beta)	CYT	C	52.4	−12.0	9	3	–	0.4	−15.0	12	4	–	0.7
b31	b31.a1	PFCIRM129_11080 & PFCIRM129_11085	MERGED=TRUE	–	–	35.4	–	–	–	–	–	−17.1	20	4	–	0.9
b32	b32.a1	PFCIRM129_10995	Glycerol kinase (ATP:glycerol 3-phosphotransferase) (Glycerokinase) (GK)	CYT	C	55.6	–	–	–	–	–	−17.7	11	5	–	1.3
b33	b33.a1	PFCIRM129_01440	Coenzyme A transferase (Putative succinyl-CoA or butyryl-CoA:coenzyme A transferase)	CYT	C	55.6	–	–	–	–	–	−14.5	7	3	–	0.5
b34	b34.a1	PFCIRM129_11710 & PFCIRM129_11715	MERGED=TRUE	–	–	58.8	−12.1	4	2	–	0.4	−30.6	13	5	–	1.2
b35	b35.a1	PFCIRM129_05730	D-lactate dehydrogenase	CYT	C	63.6	−9.5	9	3	–	0.3	−14.4	11	4	–	0.4
b36	b36.a1	PFCIRM129_00390	Cysteine synthase 2	CYT	E	33.5	−40.2	38	6	–	1.7	–	–	–	–	–
b37	b37.a1	PFCIRM129_08120	Solute binding protein of the ABC transport system	SEC	E	61.4	−7.3	7	3	–	0.4	−11.9	4	2	–	0.3
b38	b38.a1	PFCIRM129_05105	Hypothetical protein	CYT	–	64	–	–	–	–	–	−17.0	10	5	–	0.6
b40	b40.a1	PFCIRM129_01500	Pyruvate phosphate dikinase	CYT	G	95.7	–	–	—-	–	–	−11.2	3	2	–	0.1
b41	b41.a1	PFCIRM129_03550	Alanine dehydrogenase	CYT	E	39.3	–	–	–	–	–	−5.8	6	2	–	0.4
b43	b43.a1	PFCIRM129_10420	iolA (Myo-inositol catabolism IolA protein) (Methylmalonic acid semialdehyde dehydrogenase)	CYT	C	52.7	–	–	–	–	–	−9.1	6	2	–	0.3
b44	b44.a1	PFCIRM129_08025	Resuscitation-promoting factor	SEC	L	37.7	−15.9	11	2	–	0.9	–	–	–	–	–
b45	b45.a1	PFREUD_14570	Polyribonucleotide nucleotidyltransferase (Polynucleotide phosphorylase) (PNPase) (Guanosine pentaphosphate synthetase)	CYT	J	79.3	–	–	–	–	–	−9.5	3	2	–	0.2
b46	b46.a1	PFCIRM129_08280	Elongation factor G (EF-G)	CYT	J	76.5	–	–	–	–	–	−5.4	2	2	–	0.2

(Continued)

TABLE 1 | Continued

Strain						Wild-type						CB129Δ*slpB*				
Group ID[a]	Sub-group ID[b]	locus_tag	Protein description[c]	SurfG+[d]	COG letter[e]	MW[f]	log(*e*-value)[g]	Coverage[h]	Uniques[i]	Specific uniques[j]	emPAI[k]	log(*e*-value)[g]	Coverage[h]	Uniques[i]	Specific uniques[j]	emPAI[k]
b48	b48.a1	PFCIRM129_08935	FAD linked oxidase domain protein	CYT	C	100.4	–	–	–	–	–	–18.2	5	3	–	0.2
b49	b49.a1	PFCIRM129_08300	DNA-directed RNA polymerase beta chain (RNAP beta subunit) (Transcriptase beta chain) (RNA polymerase subunit beta)	CYT	K	128.5	–	–	–	–	–	–8.8	3	2	–	0.1
b50	b50.a1	PFCIRM129_00200	Succinate dehydrogenase	CYT	C	27	–8.4	10	2	–	0.6	–	–	–	–	–
b51	b51.a1	PFCIRM129_10175	Hypothetical protein	CYT	S	23.1	–11.5	16	2	–	0.7	–	–	–	–	–

[a] *The Group to which the protein belongs. All the proteins in a group have at least one peptide in common.*

[b] *The Sub-Group to which the protein belongs. All the proteins in a sub-group are identified with the same valid peptides.*

[c] *Protein description as it appears in the header of the fasta file.*

[d] *SurfG+ localization prediction.*

[e] *Cluster of Orthologous Group category – A, RNA processing and modification; B, Chromatin Structure and dynamics; C, Energy production and conversion; D, Cell cycle control and mitosis; E, Amino Acid metabolis and transport; F, Nucleotide metabolism and transport; G, Carbohydrate metabolism and transport; H, Coenzyme metabolis; I, Lipid metabolism; J, Translation; K, Transcription; L, Replication and repair; M, Cell wall/membrane/envelope biogenesis; N, Cell motility; O, Post-translational modification; P, Inorganic ion transport and metabolism; Q, Secondary Structure; T, Signal Transduction; U, Intracellular trafficking and secretion; Y, Nuclear structure; Z, Cytoskeleton; R, General Functional Prediction only; S, Function Unknown.*

[f] *Molecular weight of the protein expressed in KDa.*

[g] *Protein e-value expressed in log. Statistical value representing the number of times this protein would be identified randomly. Calculated as the product of unique peptide e-values in the sample.*

[h] *Percentage of protein sequence covered by identified peptides.*

[i] *The number of unique peptide sequence assigned to this protein.*

[j] *The number of unique peptide sequence specific to this subgroup of proteins. It is only available if there are more than one subgroup within a group.*

[k] *The Exponentially Modified Protein Abundance Index (emPAI) computation (Ishihama et al., 2005).*

[1] *Part of these results were previously published in do Carmo et al. (2017).*

was −6.75 ± 0.55 mV, showing a reduced electronegativity, in accordance with a disorganization of the S-layer at the bacterial cell surface. As shown in **Figure 1B**, the wild type strain also showed a high affinity to the hydrocarbons tested, whereas the CB129Δ*slpB* mutant showed a decreased adhesion, whatever the hydrocarbon used in the assay. Adhesion, respectively to mutant and WT strains, were as follow: to Xylol, 0.33 ± 0.52% and 43.67 ± 6.31%, to Chloroform 16.5 ± 10.7% and 75 ± 5.88, and to Ethyl Acetate 5.33 ± 7.17% and 43.83 ± 5.74%. Cell surface properties being drastically affected, we then sought morphological changes caused by the mutation (**Figure 2**). Both strains exhibited a similar cell wall thickness, 24.33 ± 0.4154 nm and 24.90 ± 0.4154 nm, respectively. No significant difference in term of bacteria morphology, cell wall thickness and shape was observed between the two strains using transmission electron microscopy.

Impact of *slpB* Mutation on *P. freudenreichii* Growth and Stress Tolerance

A single mutation, inactivating a key gene, may affect bacterial fitness and thus probiotic efficacy. We therefore monitored *P. freudenreichii* growth and tolerance toward acid, bile salts and heat challenges, in the wild type and in the mutant. The growth curves showed a similar pattern for both strains (**Figure 3A**). The bacterial count at the stationary phase end was also equivalent for both strains, with a viable population count of 1.63×10^9 CFU.mL^{-1} and 1.75×10^9 CFU.mL^{-1} for the wild type and the mutant strains, respectively. Tolerance toward stress challenges is reported in **Figure 3B**. In the case of acid stress, we observed a significant decrease in viability for the CB129Δ*slpB* strain 0.71 ± 0.13% (7.3×10^6 CFU.mL^{-1}) compared to the WT strain 5.76 ± 1.48% (5.76×10^7 CFU.mL^{-1}). During the bile salts stress, we observed the same trend in the tolerance. Indeed, the survival rate for the CB129Δ*slpB* strain was significantly decreased 0.37 ± 0.24% (3.71×10^6 CFU.mL^{-1}), compared to the WT strain 2.19 ± 1.01% (2.19×10^7 CFU.mL^{-1}). The same stands for heat challenge, with a reduced survival in CB129Δ*slpB* 0.71 ± 0.16% (9.01×10^6 CFU.mL^{-1}) compared to WT strain 5.76 ± 1.35% (5.86×10^7 CFU.mL^{-1}).

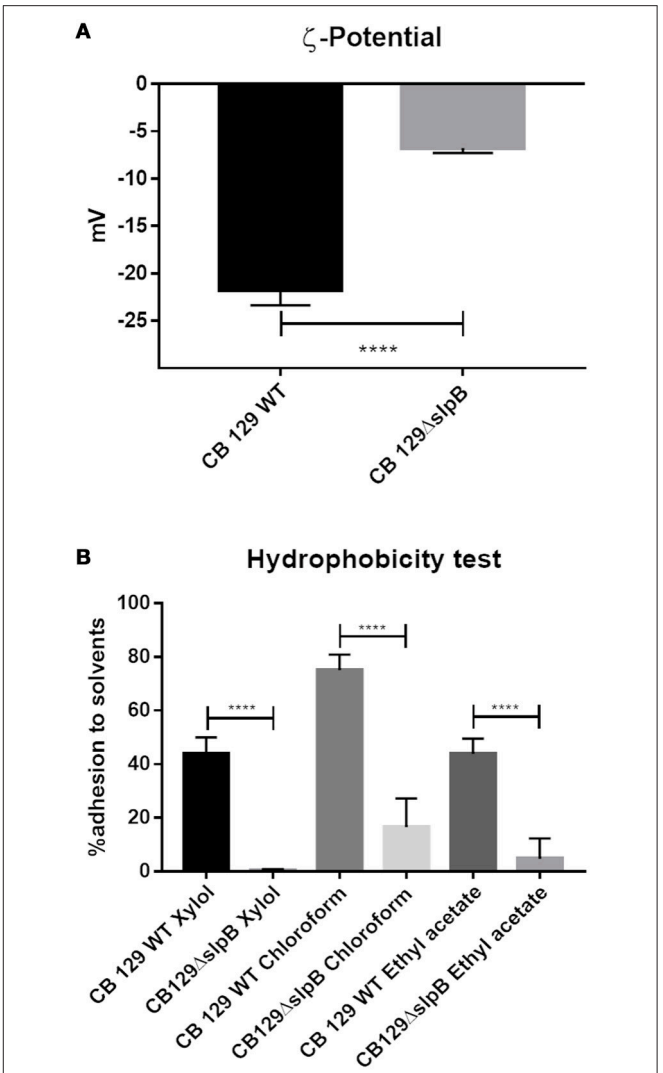

FIGURE 1 | Mutation of *slp*B gene drastically affects surface proprieties in *P. freudenreichii* CIRM-BIA 129. **(A)** The surface net charge was determined by measuring the ζ-potential. **(B)** The surface hydrophobicity was determined by quantifying adhesion to solvents (Xylol, Chloroform, and Ethyl Acetate). Wild-type (WT) and mutant CB129Δ*slpB* strains were compared. Bar represents the mean SD of three biological replicates and three technical replicates. The asterisks (****) denotes the statistical significance of the represented value between CB 129 WT and CB 129Δ*slpB* ($p > 0.0001$).

Impact of *slpB* Mutation on *P. freudenreichii* Qualitative and Quantitative Proteome

Considering the major alterations in surface extractable proteins, bacteria cell surface physicochemical properties, and stress tolerance, a qualitative and quantitative analysis of the total proteome was performed to elucidate the impact of the *slpB* gene knockout in the mutant strain. A total of 1,288 quantifiable proteins (53.26% of predicted proteome) wherein 1,253 proteins (reported in **Figure 4A**) were identified (**Table S1**). In the WT strain 1,227 proteins were found, whereas in the CB129Δ*slpB* strain, we detected 1,252 proteins. Comparative analysis revealed a core-proteome, composed by 1,226 proteins, shared by both strains (**Figure 4A**). Differences in protein abundance were observed by proteomic quantitative analysis (**Figure 4B**). A total of 97 proteins (4.2% of the predicted proteome) of these common proteins showed differences in the level of expression among strains, including 36 up-regulated and 61 down-regulated proteins in CB129Δ*slpB* in comparison with the WT strain (**Table 2**).

According to the predicted subcellular localization of the 1,253 proteins identified, 1,081 proteins are CYT (61% of predicted proteome), 71 are MEM (18% of predicted proteome), 77 are PSE (41% of predicted proteome) and 24 are SEC (38% of predicted proteome). In the analysis of non-differentially expressed proteins, we classified 1,001 as CYT proteins, 67 as MEM proteins, 70 as PSE proteins and 22 as SEC proteins

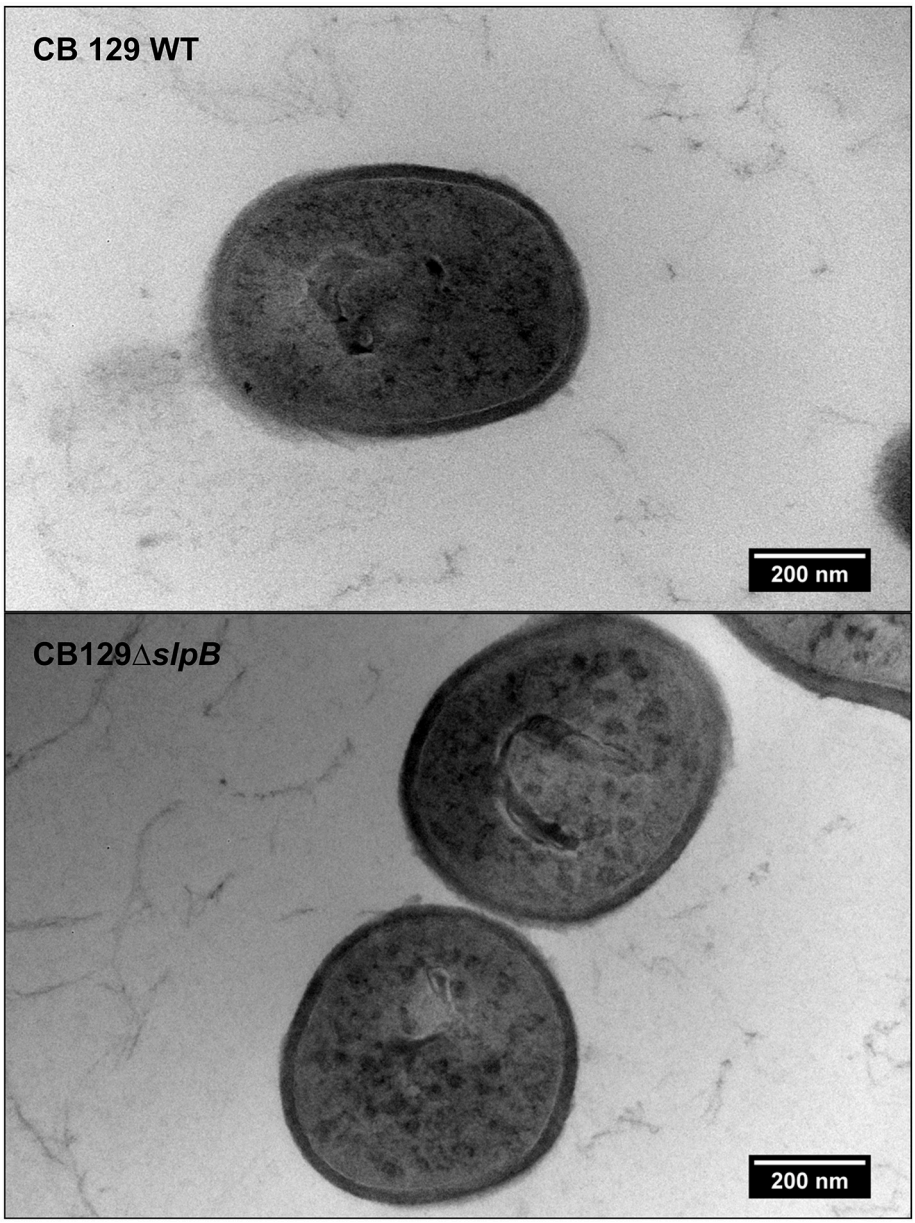

FIGURE 2 | Mutation of *slp*B gene does not affect envelope thickness in *P. freudenreichii* CIRM-BIA 129. Wild-type (WT) and mutant CB129Δ*slpB* strains were analyzed by transmission electron microscopy (TEM). No difference in morphology and cell wall tickeness was found.

(**Figure 4C**). Meanwhile, between the *P. freudenreichii* WT and the CB129Δ*slpB* strains, from 97 proteins differentially expressed, the subcellular localization were predicted as follow: 81 CYT, 2 MEM, 7 PSE, and 7 SEC proteins (**Figure 4C**).

According to COG functional classifications, the differentially expressed proteins were classified into 20 biological processes (**Figure 5A**). A general category of differentially regulated proteins in CB129Δ*slpB* strain core proteome showed 27 proteins involved in information storage and processing, 25 associated to metabolism and, 18 proteins related to cellular processes and signaling (**Figure 5A**). Proteins that mediate different biological process were dysregulated in the mutant strain. As seen in **Figure 5B**, 11 proteins were classified as having general functions, 10 proteins related to process of replication, recombination and repair, other 10 proteins linked to posttranslational modification, chaperones, protein turnover, and 9 proteins involved in the transcription process. The differentially expressed proteins between wild-type and mutant strains detected in each functional category are shown in **Table 2**. In addition, we detected proteins exclusive to the proteome of each strain. WT strain exhibits a unique exclusive protein, the Putative carboxylic ester hydrolase, which is involved in

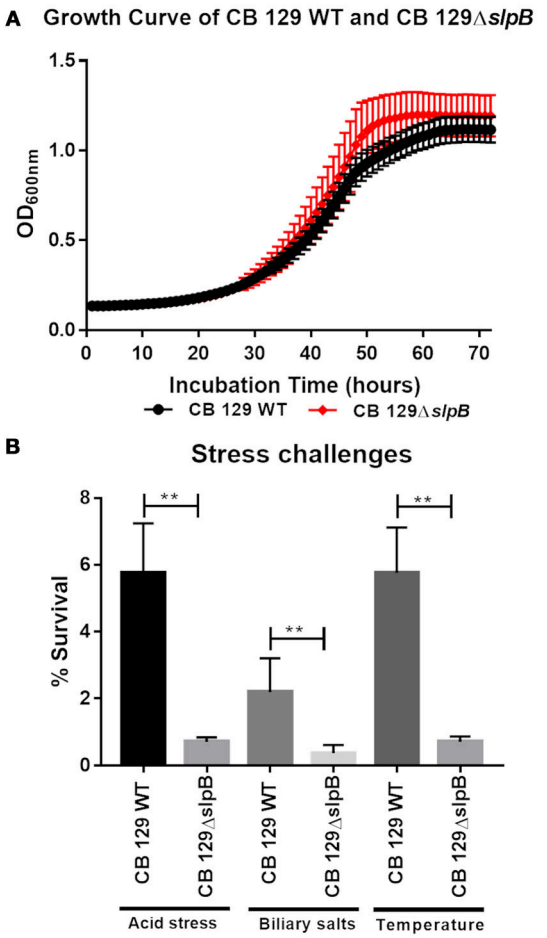

FIGURE 3 | Mutation of *Slp*B drastically affects stress tolerance in *P. freudenreichii* CIRM-BIA 129. **(A)** The growth curve of Wild-type (WT) and mutant CB129Δ*slpB* strains was determined at 30°C in YEL broth until stationary phase (72 h). Growth was monitored by $OD_{650\,nm}$ as a function of time. No statistically significant difference was found in growth curve between strains. **(B)** Wild-type (WT) and mutant 129Δ*slpB* strains were subjected to acid, bile salts and thermal challenges. Viable propionibacteria were enumerated by plate counting before and after each challenge. Asterix represent statistically significant differences between strains and were indicated as follows: $^{**}p < 0.01$.

metabolism, especially in hydrolase activity. Interestingly, 27 proteins were found exclusively in the mutant strain, they are involved in several processes like metabolism and replication, recombination and repair (**Table S1**).

slpB Gene Mutagenesis and Whole-Genome Co-localization

Complete genome of CB129Δ*slpB* (BioProject - PRJNA476583, Accession - CP030279) strain was sequenced and assembled in a circular chromosome, which exhibits a length of 2.6815.18 bp, with a G+C content of 67.28%, and a total of 2,479 CDSs, 6 rRNA genes (5S, 16S, and 23S), and 45 tRNA genes. The circular map showed a high similarity when comparing CB129Δ*slpB* with the CIRM-BIA 1 and the JS17 reference strains (**Figure 6A**). **Figure 6B** shows the localization of the plasmid inserted within the *slpB* gene during its knockout and **Figures S1**, **S2** shows the read mapping before and after the insertion. Genomic analyses of genetic context, i.e., the sequences upstream and downstream the *slpB* gene, confirmed that this locus is not part of an operon and thus should not affect the expression of downstream genes or upstream genes. Complete genome sequence of CB129Δ*slpB* strain further ruled out any homologous recombination (HR) in other genome sites.

Protein-Protein Interaction (PPI)

We performed a PPI network to evaluate the interactions among the proteins differentially regulated in WT and CB129Δ*slpB* strains (**Figure 7**). The interactome analysis revealed 118 interactions between identified proteins. In PPI network, we observed that upregulated proteins, such as DNA-directed RNA polymerase alpha chain (PFCIRM129_08045), and 50S ribosomal protein L2 (PFCIRM129_08225), which exhibit high interaction, are involved in Transcription and Translation, respectively. Moreover, downregulated proteins such as GTP binding signal recognition particle protein (PFCIRM129_00245), DNA polymerase III alpha subunit (PFCIRM129_04260) and Enolase 2 (PFCIRM129_06035) showing high interaction, are involved in metabolism, DNA repair and main glycolytic pathway, respectively.

DISCUSSION

Propionibacterium freudenreichii CIRM-BIA 129 has emerged as a probiotic strain with a great immunomodulatory potential in the context of inflammatory bowel disease, according to promising results obtained in animal models (Plé et al., 2015, 2016). Recently, our group has studied the role of the surface SlpB protein of *P. freudenreichii* CIRM-BIA 129 in adhesion to the intestinal epithelial cells, a probiotic property linked to beneficial effects. Knocking-out of the *slpB* gene evidenced a direct involvement of this protein in the adhesion to HT-29 cells. Electrophoretic analysis of guanidine extracts confirmed the disappearance of the corresponding SlpB protein (do Carmo et al., 2017). Surface layer proteins are associated to several functions (do Carmo et al., 2018). Therefore, in order to better understand the impact of this mutation, we performed a more thorough proteomic analysis by applying nanoLC-MS/MS to these extracts. Differences were found between the parental wild type CIRM BIA 129 and the isogenic CB129Δ*slpB* mutant strains of *P. freudenreichii*, in terms of surface extractable proteins. As shown in **Table 1**, proteins previously identified in CB 129 WT strain guanidine-extracted proteins (Le Maréchal et al., 2015) were detected in both strains, including in particular, surface proteins anchored in the peptidoglycan cell wall via surface layer homology (SLH) domains, such as SlpA, SlpB, SlpE, and InlA like as previously reported by Carmo and collaborators (do Carmo et al., 2017). However, this set of SLH domain-containing proteins was reduced in the mutant strain guanidine-extracted proteins, with the expected absence of SlpB protein, thus validating the directed mutagenesis. Analysis

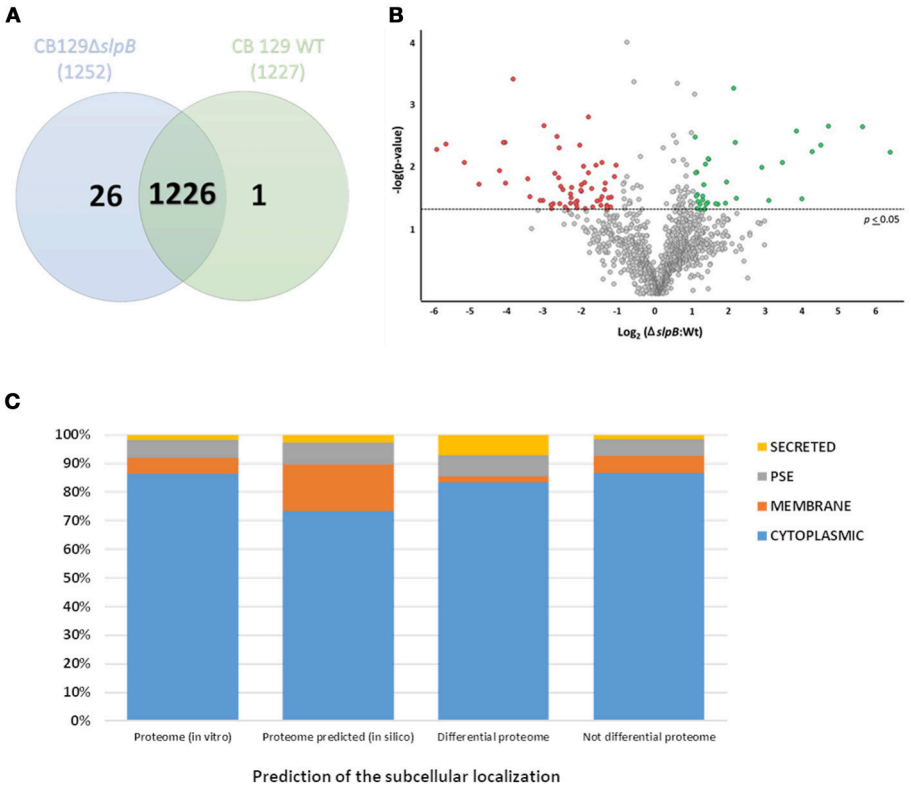

FIGURE 4 | Label-free quantification of proteins from *P. freudenreichii* CIRM-BIA 129 and CB 129Δ*slpB* strain. **(A)** Distribution of the proteins identified in the proteome of WT and CB129Δ*slpB* strains, represented by a Venn diagram. **(B)** Volcano Plot showing Log(2) Fold Change of the differentially expressed proteins in CB129Δ*slpB* strain in relation to WT strain. Green (up-regulated proteins) and red circles (down-regulated proteins) represent proteins statistically different ($p \leq 0.05$, ANOVA) in abundance between strains by 2-fold or more. **(C)** Prediction of the subcellular localization of the proteins identified by LC/MS and organized as cytoplasmic (CYT), membrane (MEM), potentially surface-exposed (PSE) or secreted proteins (SEC).

of CB129Δ*slpB* strain guanidine-extracted proteins, identified several proteins, including chaperones, such as ClpB, DnaK, and GroEL, and Enolase (carbohydrate metabolism) involved in stress tolerance, as previously reported for *Propionibacterium* ssp. strains by enzymatic shaving of the surface proteins using trypsin (Jan et al., 2000; Gagnaire et al., 2015; Huang et al., 2016). Another noticeable difference was the higher number of guanidine-extracted proteins, in the mutant strain, compared to the wild type strain. This included proteins usually described as cytoplasmic: enzymes of the central carbon metabolic pathways, such as pyruvate synthase, or the two subunits of the methylmalonyl-CoA mutase, a recognized cytoplasmic marker, previously described as an extracellular marker of autolysis (Valence et al., 2000). Interestingly, the HSP 70 cytoplasmic stress-related protein present at the surface of the mutant strain could be responsible for preventing protein denaturation. It is as such considered a factor of virulence and pathogenesis in some specific pathogens (Ghazaei, 2017), in *Neisseri meingitidis* (Knaust et al., 2007) and in *Mycobacterium* spp. (Das Gupta et al., 2008). This appeals further investigation, as it suggests a profound modification of the envelope structure and cell surface properties of the mutant strain.

SLAPs are known to determine key parameters of the surface layer of bacteria, in terms of charge and hydrophobicity (Wilson et al., 2001). Not only amino acid residues, but also covalent modification may endow the S-layer lattice with a strong negative charge. Thus, we determined the surface charge in both *P. freudenreichii* WT and CB129Δ*slpB* strains by measuring the zeta potential, which reflects the mobility rate of cells within an electric field. A lower negative value is reportedly linked with higher hydrophobicity, consequently improving adhesion (de Wouters et al., 2015). Likewise, considering the presence of surface proteins and their role in zeta potential, van der Mei *et al.* have shown that some wild type strains, like the *L. acidophilus* ATCC4356, with SLPs, are more negatively charged at pH 7 than strains without SLPs, such as *L. johnsonii* LMG9436 and *L. gasseri* LMG9203 (van der Mei et al., 2003). We thus further investigated the hydrophobicity of the cell surface, a parameter thought to be correlated with *in vitro* adhesion of bacteria to mucin, collagen, fibronectin, and to human epithelial cells (Duary et al., 2011). The cell surface hydrophobic and hydrophilic properties have been studied in lactic acid bacteria (Sandes et al., 2017) and can be correlated to the adhesion process to intestinal epithelial cells of apolar surface proteins (Guo et al., 2010). Using the MATH assay, we showed that the CB129Δ*slpB* strain has a strongly decreased ability to adhere to xylol, as well as to chloroform and to ethyl acetate solvents, indicating a change in the global properties of the cell surface, affecting adhesion to surfaces.

TABLE 2 | Differentially regulated proteins at CB129Δ*slpB* in relation to CB 129 wild-type.

Accession	Score	Description	LOG(2) ratio fold-change	Anova (p)	COG biological process
UP-REGULATED PROTEINS					
PFCIRM129_09610	41.9018	Protein of unknown function	6.16	0.006	Coenzyme transport and metabolism and Signal transduction mechanisms
PFCIRM129_09540	37.0751	Protein of unknown function	5.43	0.003	Transcription
PFCIRM129_09590	102.7882	Protein of unknown function	4.53	0.002	Cell wall/membrane/envelope biogenesis
PFCIRM129_09465	51.2086	Protein of unknown function	4.33	0.005	–
PFCIRM129_09585	90.2656	Protein of unknown function	4.10	0.006	General function prediction only
PFCIRM129_04060	38.8837	Guanylate kinase, Guanosine monophosphate kinase (GMP kinase)	3.83	0.033	Nucleotide transport and metabolism
PFCIRM129_09570	44.4682	Protein of unknown function	3.69	0.003	Cell motility
PFCIRM129_07005	243.5985	DNA ligase (NAD+)	3.32	0.009	Replication, recombination and repair
PFCIRM129_01620	59.7705	Stomatin/prohibitin	2.96	0.036	Posttranslational modification, protein turnover, chaperones
PFCIRM129_10485	35.443	Spermidine synthase	2.76	0.011	Amino acid transport and metabolism
PFCIRM129_10870	30.3436	Protein of unknown function	2.09	0.033	General function prediction only
PFCIRM129_09930	56.3355	Hypothetical protein	2.06	0.004	Posttranslational modification, protein turnover, chaperones
PFCIRM129_09935	87.1427	Aldo/keto reductase	2.02	0.001	Secondary metabolites biosynthesis, transport and catabolism
PFCIRM129_08225	203.0233	50S ribosomal protein L2	1.84	0.018	Translation, ribosomal structure and biogenesis
PFCIRM129_05110	60.8023	Nuclease of the RecB family	1.80	0.039	Replication, recombination and repair
PFCIRM129_02560	52.268	Transcriptional regulator	1.61	0.041	Coenzyme transport and metabolism
PFCIRM129_08430	75.8136	Pyruvate flavodoxin/ferredoxin oxidoreductase	1.55	0.040	Energy production and conversion
PFCIRM129_09920	380.2718	Hypothetical secreted protein	1.37	0.008	Translation, ribosomal structure and biogenesis
PFCIRM129_04715	57.0906	Hypothetical protein	1.36	0.008	Signal transduction mechanisms
PFCIRM129_09175	100.5874	NAD-dependent epimerase/dehydratase	1.33	0.038	General function prediction only
PFCIRM129_12405	136.3375	UDP-glucose 4-epimerase	1.29	0.041	Cell wall/membrane/envelope biogenesis
PFCIRM129_01790	34.8378	3-dehydroquinate dehydratase	1.27	0.010	Amino acid transport and metabolism
PFCIRM129_07890	128.6333	Putative O-sialoglycoprotein endopeptidase	1.26	0.048	Translation, ribosomal structure and biogenesis
PFCIRM129_00585	212.5378	Polyphosphate glucokinase	1.24	0.048	Transcription and Carbohydrate transport and metabolism
PFCIRM129_07790	140.7785	Cysteine synthase 1	1.23	0.020	Amino acid transport and metabolism
PFCIRM129_09600	51.9916	Protein of unknown function	1.21	0.034	Replication, recombination and repair
PFCIRM129_11300	522.5826	Glyceraldehyde-3-phosphate dehydrogenase/erythrose 4 phosphate dehydrogenase	1.21	0.030	Carbohydrate transport and metabolism
PFCIRM129_00690	23.8969	Protein of unknown function	1.14	0.049	Function unknown
PFCIRM129_01510	22.4775	Carbohydrate or pyrimidine kinases PfkB family	1.14	0.040	Carbohydrate transport and metabolism
PFCIRM129_03870	27.4108	Glutamine-dependent NAD(+) synthetase	1.08	0.036	General function prediction only
PFCIRM129_00225	85.9906	16S rRNA processing protein	1.06	0.028	Translation, ribosomal structure and biogenesis
PFCIRM129_11255	221.963	Pyridoxal biosynthesis lyase pdxS	1.06	0.047	Coenzyme transport and metabolism

(Continued)

TABLE 2 | Continued

Accession	Score	Description	LOG(2) ratio fold-change	Anova (p)	COG biological process
PFCIRM129_03920	293.1815	Pyridine nucleotide-disulphide oxidoreductase	1.05	0.013	Energy production and conversion
PFCIRM129_07930	409.5736	Glucosamine–fructose-6-phosphate aminotransferase (Hexosephosphate aminotransferase, D-fructose-6-phosphate amidotransferase)	1.04	0.031	Cell wall/membrane/envelope biogenesis
PFCIRM129_11805	158.7382	Magnesium (Mg2+) transporter	1.03	0.013	Inorganic ion transport and metabolism
PFCIRM129_08045	417.9752	DNA-directed RNA polymerase alpha chain (RNAP alpha subunit) (Transcriptase alpha chain) (RNA polymerase subunit alpha)	1.01	0.004	Transcription
DOWN-REGULATED PROTEINS					
PFCIRM129_06035	66.5616	Enolase 2	−1.09	0.010	Carbohydrate transport and metabolism
PFCIRM129_06325	41.3227	Trypsin-like serine protease	−1.13	0.015	Posttranslational modification, protein turnover, chaperones
PFCIRM129_00315	221.1159	Beta-lactamase-like:RNA-metabolizing metallo-beta-lactamase	−1.21	0.031	Translation, ribosomal structure and biogenesis
PFCIRM129_04530	19.2011	Hypothetical protein	−1.23	0.045	Function unknown
PFCIRM129_06605	17.9867	Metal-dependent hydrolase	−1.29	0.032	General function prediction only
PFCIRM129_10030	162.9893	DNA repair protein	−1.32	0.042	Replication, recombination and repair
PFCIRM129_06500	87.9342	Hypothetical protein	−1.33	0.048	Nucleotide transport and metabolism
PFCIRM129_10650	33.3856	Hypothetical protein	−1.33	0.045	Cell wall/membrane/envelope biogenesis
PFCIRM129_03835	79.2532	Pyrazinamidase/nicotinamidase	−1.37	0.019	Coenzyme transport and metabolism and Signal transduction mechanisms
PFCIRM129_10070	83.9023	Hypothetical protein	−1.39	0.024	General function prediction only
PFCIRM129_00245	381.851	GTP binding signal recognition particle protein	−1.45	0.031	Intracellular trafficking, secretion, and vesicular transport
PFCIRM129_05955	85.1759	Peptide-methionine (S)-S-oxide reductase	−1.46	0.009	Posttranslational modification, protein turnover, chaperones
PFCIRM129_09830	327.7897	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B (Asp/Glu-ADT subunit B)	−1.49	0.042	Translation, ribosomal structure and biogenesis
PFCIRM129_09395	77.9918	Protein of unknown function	−1.50	0.035	Replication, recombination and repair
PFCIRM129_07355	38.1315	Hypothetical protein	−1.59	0.036	Amino acid transport and metabolism
PFCIRM129_02750	19.5802	Anti-sigma factor	−1.61	0.010	Transcription
PFCIRM129_09840	37.8042	Glutamyl-tRNA(Gln) amidotransferase subunit C (Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C)	−1.71	0.045	Translation, ribosomal structure and biogenesis
PFCIRM129_12290	113.782	Hypothetical protein	−1.73	0.023	Translation, ribosomal structure and biogenesis
PFCIRM129_02880	157.3643	Zn dependant peptidase	−1.82	0.002	General function prediction only
PFCIRM129_01675	171.214	Flavin-containing amine oxidase	−1.82	0.013	Amino acid transport and metabolism
PFCIRM129_00465	78.5201	Thiamine biosynthesis protein	−1.90	0.047	Coenzyme transport and metabolism
PFCIRM129_09980	56.8929	Peptidyl-prolyl cis-trans isomerase	−1.91	0.019	Posttranslational modification, protein turnover, chaperones
PFCIRM129_02370	174.428	L-aspartate oxidase (LASPO) (Quinolinate synthetase B)	−1.94	0.010	Coenzyme transport and metabolism

(Continued)

TABLE 2 | Continued

Accession	Score	Description	LOG(2) ratio fold-change	Anova (p)	COG biological process
PFCIRM129_05120	33.1399	Putative carboxylic ester hydrolase	−1.99	0.020	Lipid transport and metabolism
PFCIRM129_04475	54.968	Transporter	−2.01	0.026	Function unknown
PFCIRM129_12425	80.5954	Protein of unknown function FUZZYLOCATION=TRUE	−2.02	0.025	Transcription
PFCIRM129_04980	227.0852	D-alanine–D-alanine ligase (D-alanylalanine synthetase)	−2.05	0.005	Cell wall/membrane/envelope biogenesis and General function prediction only
PFCIRM129_11215	88.6965	Dioxygenase	−2.12	0.047	Inorganic ion transport and metabolism and Secondary metabolites biosynthesis, transport and catabolism
PFCIRM129_10195	96.8755	Transcriptional regulator	−2.12	0.036	Transcription
PFCIRM129_08985	30.4822	Hypothetical protein	−2.13	0.042	General function prediction only
PFCIRM129_04260	287.3443	DNA polymerase III alpha subunit	−2.15	0.038	Replication, recombination and repair
PFCIRM129_02065	15.789	Ferrous iron uptake protein A 9.a.8.1.x	−2.25	0.022	Inorganic ion transport and metabolism
PFCIRM129_04725	106.5589	Hypothetical protein	−2.27	0.032	Cell wall/membrane/envelope biogenesis
PFCIRM129_05460	489.2107	Surface protein with SLH domain	−2.29	0.039	Posttranslational modification, protein turnover, chaperones
PFCIRM129_04925	12.884	Hypothetical protein	−2.29	0.028	Carbohydrate transport and metabolism
PFCIRM129_10690	9.166	Protein of unknown function	−2.37	0.049	Function unknown
PFCIRM129_05620	65.4691	MscS transporter, small conductance mechanosensitive ion channel	−2.43	0.044	Cell wall/membrane/envelope biogenesis
PFCIRM129_06895	73.9719	Thiredoxine like membrane protein	−2.49	0.024	Posttranslational modification, protein turnover, chaperones
PFCIRM129_10610	181.5134	Phosphocarrier, HPr family	−2.54	0.021	Signal transduction mechanisms and Carbohydrate transport and metabolism
PFCIRM129_02565	36.2455	Hypothetical protein	−2.57	0.039	Defense mechanisms
PFCIRM129_00850	58.5524	Cation-transporting ATPase	−2.59	0.005	Inorganic ion transport and metabolism
PFCIRM129_02970	142.4983	Hypothetical protein	−2.60	0.016	Energy production and conversion
PFCIRM129_00010	145.5914	Argininosuccinate lyase (Arginosuccinase)	−2.65	0.004	Amino acid transport and metabolism
PFCIRM129_02590	36.8971	Hypothetical transmembrane protein	−2.71	0.013	Inorganic ion transport and metabolism
PFCIRM129_02910	44.2268	Hypothetical protein	−2.74	0.039	Replication, recombination and repair
PFCIRM129_10040	39.9232	Hypothetical protein	−2.78	0.048	Carbohydrate transport and metabolism
PFCIRM129_12235	1098.1026	Internaline A	−2.80	0.041	Posttranslational modification, protein turnover, chaperones
PFCIRM129_00040	20.5108	N-acetyl-gamma-glutamyl-phosphate reductase (AGPR) (N-acetyl-glutamate semialdehyde dehydrogenase) (NAGSA dehydrogenase)	−2.99	0.002	Amino acid transport and metabolism
PFCIRM129_03005	41.8204	Hypothetical protein	−3.01	0.035	Secondary metabolites biosynthesis, transport and catabolism
PFCIRM129_05445	69.2875	Transcriptional Regulator, TetR family	−3.09	0.036	Transcription
PFCIRM129_02960	83.486	Cold shock-like protein CspA	−3.36	0.031	Transcription

(Continued)

TABLE 2 | Continued

Accession	Score	Description	LOG(2) ratio fold-change	Anova (p)	COG biological process
PFCIRM129_00705	46.3689	Surface protein of unknown function	−3.42	0.016	–
PFCIRM129_08670	192.0452	Cell-wall peptidases, NlpC/P60 family secreted protein	−3.80	0.000	General function prediction only
PFCIRM129_03390	45.4963	Superfamily II RNA helicase	−4.01	0.019	Replication, recombination and repair
PFCIRM129_06155	35.4	Hypothetical protein	−4.03	0.004	Carbohydrate transport and metabolism
PFCIRM129_06085	371.7356	Transcription-repair coupling factor	−4.07	0.004	Replication, recombination and repair and Transcription
PFCIRM129_01360	47.9803	NUDIX hydrolase	−4.17	0.012	Nucleotide transport and metabolism
PFCIRM129_11775	48.0011	Surface protein D with SLH domain	−4.70	0.020	Posttranslational modification, protein turnover, chaperones
PFCIRM129_00700	461.2371	Surface layer protein B (S-layer protein B)	−5.10	0.009	Posttranslational modification, protein turnover, chaperones
PFCIRM129_11140	154.2908	Type I restriction-modification system DNA methylase	−5.58	0.005	Defense mechanisms
PFCIRM129_04135	15.2209	Uncharacterized ATPase related to the helicase subunit of the holliday junction resolvase	−5.82	0.006	Replication, recombination and repair

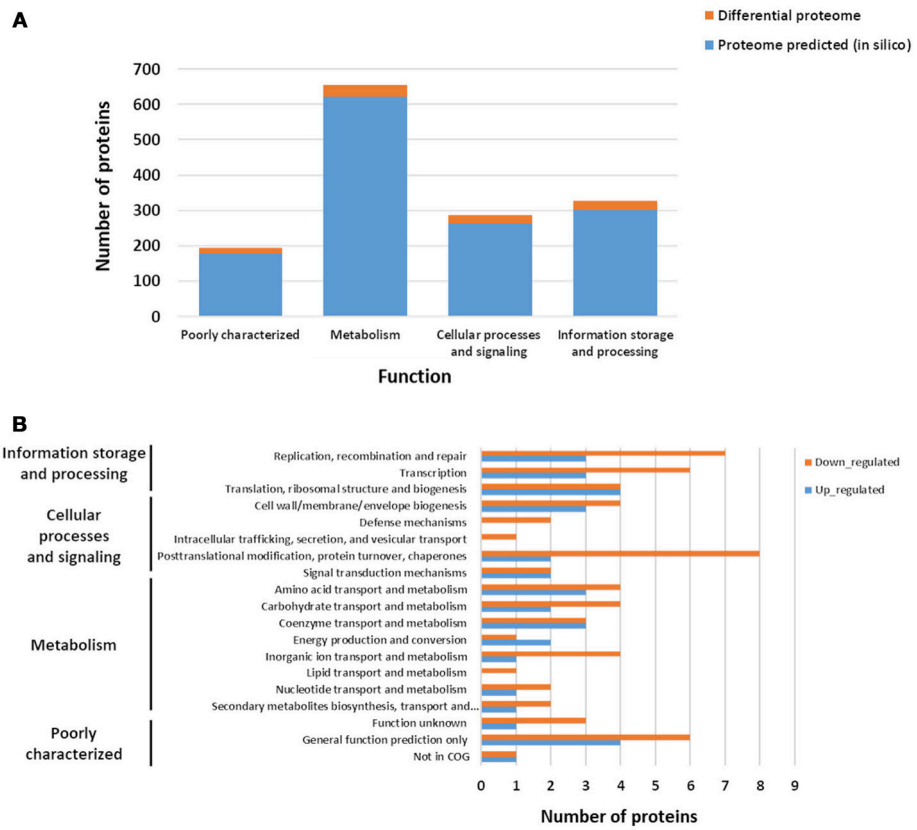

FIGURE 5 | Repartition of differential proteins in biological processes. **(A)** Functional distribution of the predicted theoretical proteome and of the experimental differential proteins when comparing Wild-type (WT) and mutant CB129Δ*slpB* strains. **(B)** Repartition of differential proteins in Biological processes. Functional distribution and Biological process were predicted based on the functional classifications of COG database.

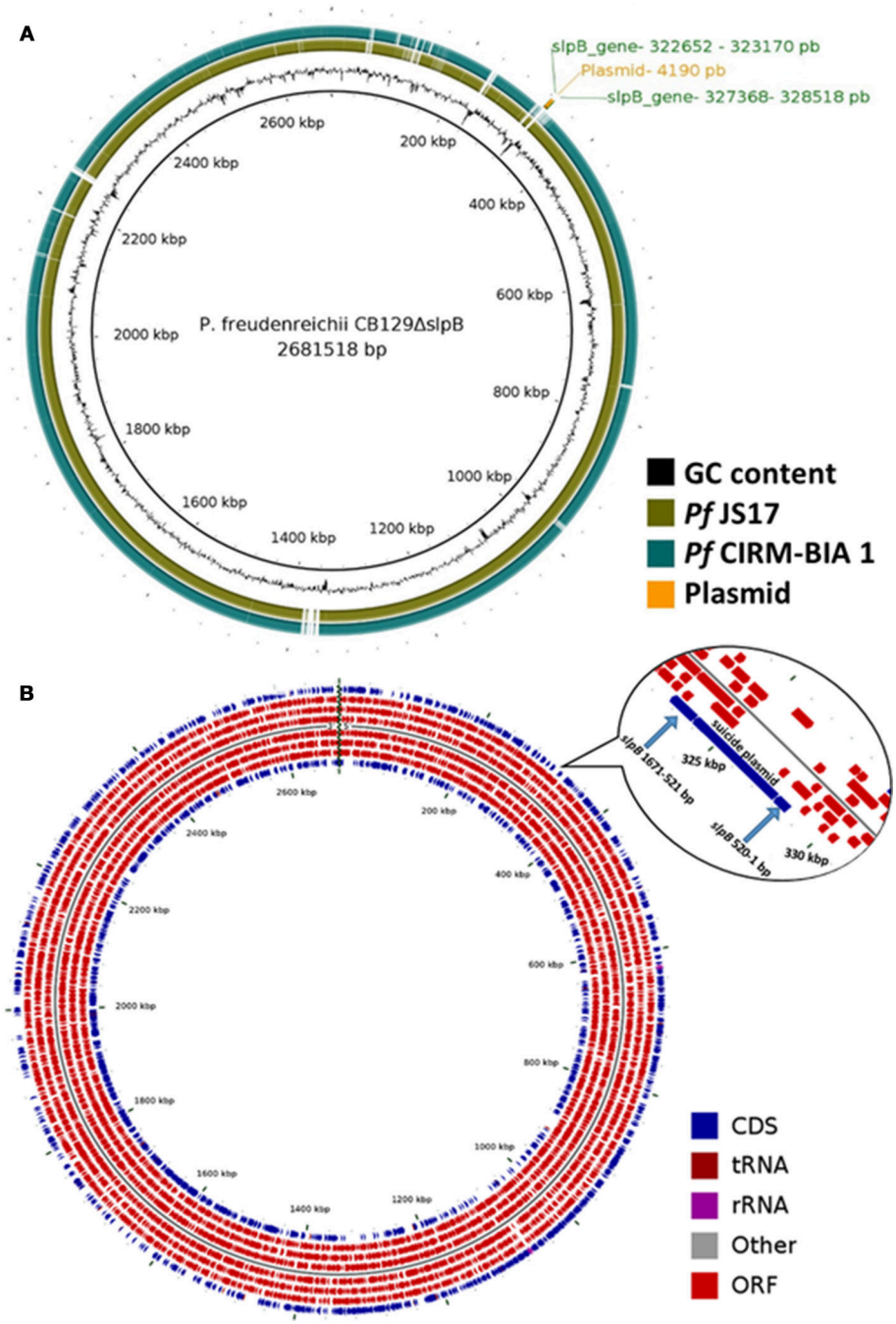

FIGURE 6 | Comparative genomic map generated with BRIG and Map of Circular genome generated with CGview. **(A)** *P. freudenreichii* CIRM-BIA 1 and *P. freudenreichii* JS17 were aligned using CB129Δ*slpB* strain as a reference. **(B)** In the outermost ring the genes localization in genome, followed by CDS, tRNAs, rRNAs, other RNAs, and CDSs. The insertion site of the plasmid for the *slpB* gene mutation is visualized in the zoom image.

These results corroborate with the previous study showing a decreased adhesion to HT-29 human intestinal epithelial cells (do Carmo et al., 2017). Hydrophobicity and ζ-potential are factors correlated with bacterial adhesion to the epithelial cells, which are guided by charge and hydrophobicity of the bacterial surface.

The presence of surface layers being reportedly linked to tolerance toward stresses (do Carmo et al., 2018), we decided to investigate the impact of such a mutation on the CB129Δ*slpB* strain tolerance toward stress challenges that are relevant for the selection of new probiotics. The ability to survive acid stress in the stomach and bile salts stress in the duodenum during the passage

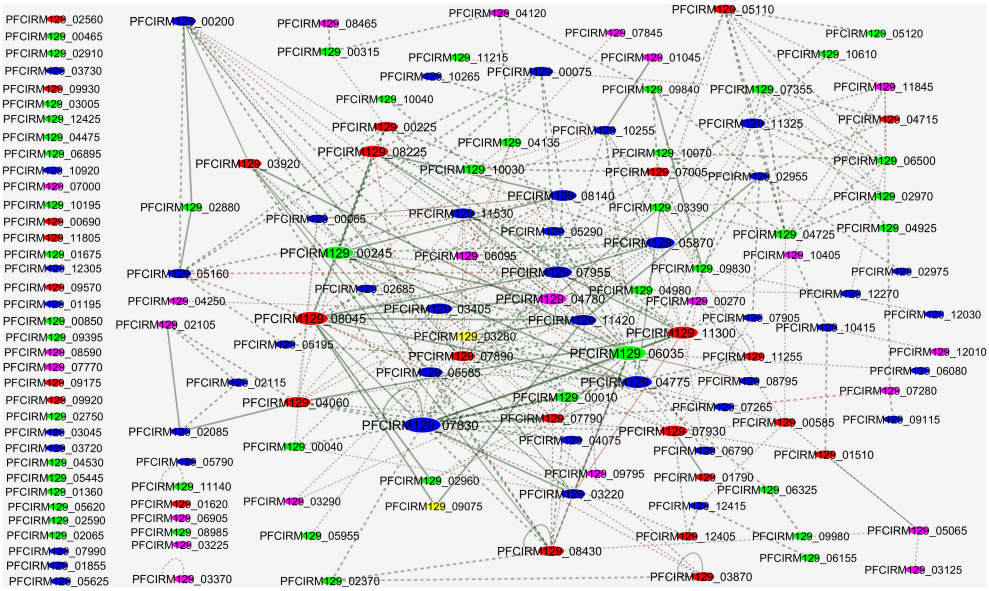

FIGURE 7 | Protein-protein interactions of the proteins identified as differentially expressed in CB 129Δ*slpB*. The sizes of the nodes represent the degree of interaction for each gene/protein; the major nodes demonstrate greater interactions. Red, up-regulated; Blue, unchanged; Green, down-regulated; Yellow, Exclusive identified at WT strain; Purple, Exclusive identified at CB129Δ*slpB* strain.

through the digestive tract, is important for probiotic interaction with the host (Rabah et al., 2017). Accordingly, *in vitro* assays can be used to simulate digestive stresses, mimicking the exposure to acidic conditions (pH 2.0) or to biliary salts (1 g.L^{-1}) (Jan et al., 2000). For *P. freudenreichii*, commonly used as a cheese starter, the heat stress tolerance constitutes a relevant technological ability of this strain (Rosa do Carmo et al., 2017). Overall, we observed a large decrease in tolerance to the environmental stresses, confirming a role of SlpB in toughness. In the guanidine-extracted proteins of the mutant strain, the chaperones and heat shock proteins, DnaK1, DnaK2, ClpB 2, GroE1, and GroE2 were found. Inside the cell, they are responsible for protein folding and are correlated to acid and bile adaptation (Leverrier et al., 2005; Gagnaire et al., 2015). Here, they were found at the surface of the CB129Δ*slpB* mutant, which was more susceptible to extreme acid stress and temperature, compared to wild type strain. Previous work showed that *L. acidophilus* ATCC 4356 adapts to harsh environments by increasing the expression of the s-layer SlpA protein upon bile, acidic pH and heat stress exposition (Khaleghi et al., 2010; Khaleghi and Kasra, 2012). Moreover, changes in the cell surface properties could alter the transmembrane protein complex responsible for the extrusion of protons from the cytoplasm, which are responsible for surviving environmental stresses (Ruiz et al., 2013; Rosa do Carmo et al., 2017).

Profound modifications of *P. freudenreichii* physiology and surface properties suggested that modifications, wider than the disappearance of a single protein, occurred as a result of *slpB* gene inactivation. To understand this impact of the mutation, a comparative proteomic analysis was performed to identify significant alterations in the whole proteome profile of the mutant strain, using label-free quantitative proteomic analysis. Prediction of sub cellular localization using the SurfG+ tool (Barinov et al., 2009) evidenced changes in all the categories (CYT, MEM, PSE and SEC) in the differential proteome of CB129Δ*slpB*. In addition, differential proteome was functionally classified using COG, showing a functional implication of differential proteins in cellular processes such as signaling, information storage, processing, and metabolism. Specifically, this study showed that the moonlighting enolase and NlpC/P60 are both exported (Frohnmeyer et al., 2018), as it was recently observed in the cutaneous *Propionibacterium acnes* strain (Jeon et al., 2017). These moonlighting proteins were downregulated in CB129Δ*slpB*. Interestingly, in the *Bifidobacterium* and *Lactobacillus* genera, moonlighting proteins, such as enolase, also play a role in immunomodulation and adhesion (Sánchez et al., 2010; Kainulainen and Korhonen, 2014; Vastano et al., 2016). Furthermore, in the PPI network we observed high interactions between the downregulated Enolase (PFCIRM129_06035), reportedly involved in human gut colonization and stress adaptation (Ruiz et al., 2009), with other proteins involved in several other processes, including metabolism and DNA repair. Moreover, all surface layer-associated proteins SlpA, SlpD, SlpE, and InlA were downregulated in CB129Δ*slpB*. These proteins form a protective layer on the surface of the bacteria, and have been associated with environmental stress tolerance (Fagan and Fairweather, 2014). As seen previously, a decreased amount of these proteins could be directly associated with stress susceptibility and with altered hydrophobicity. SLAPs can directly influence these properties (Pum et al., 2013), and consequently alter adhesion to epithelial cells (do Carmo et al., 2017).

We performed the complete genome DNA sequencing of the CB129Δ*slpB,* which, in turn, allowed us to evaluate whether the *slpB* gene disruption had major consequences on the mutant strain genome. The *slpB* gene is not part of an operon, which suggests that homologous recombination using the suicide plasmid pUC:Δ*slpB*:*Cm*R (do Carmo et al., 2017) did not affect the expression of upstream and downstream genes. Analysis of the genetic context, upstream and downstream, revealed that the homologous recombination process was site-specific, and not affecting other genes in the genome of the mutant strain CB129Δ*slpB*. However, we were unable to evaluate possible rearrangements in the genome of CB129Δ*slpB*, which could have affected the transcription of other genes. Therefore, more studies are necessary to explore whether any probiotic potential was lost after the single mutation of the *slpB* gene in *Propionibacterium freudenreichii* CIRM-BIA 129 strain.

CONCLUSION

This study evidenced the pleiotropic impact of the surface layer protein *slpB* mutation in the probiotic strain *Propionibacterium freudenreichii* CIRM-BIA 129 in relation to its physicochemical proprieties, stress challenges, surfaceome and whole cell quantitative proteome. It confirmed the key role of SLPs and strongly suggests that expression of specific ones, such as *P. freudenreichii* SlpB, should be used as criteria for selecting strains with probiotic potential.

AUTHOR CONTRIBUTIONS

FC performed *in vitro* assays, microscopy, proteomic assays and data interpretation. WS, FP, GT, and ROC performed proteomic assays, data interpretation and bioinformatics analyses. BC, EO, and SS performed *in vitro* assays. II and HR data interpretation. EF performed PPI network. CC performed microscopy. MC, AC, and RS performed genomics and data interpretation. VA, GJ, HF, and YL contributed to the supervision, analysis, and interpretation of data and were major contributors to revising the manuscript. All authors contributed in writing 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.2018.01807/full#supplementary-material

Figure S1 | Verification of assembly error by read mapping. The plasmid pUC:Δ*slpB*:*Cm*R was not inserted in the *slpB* gene during de novo genome assembly. The read mapping on the *slpB* gene shows misalignments upstream and downstream insertion site, confirming the assembly error. The read mapping was performed using in CLC Genomics Workbench 7.0.

Figure S2 | Assembly curation and validation by read mapping. The manual insertion of plasmid pUC:Δ*slpB*:*Cm*R in the *slpB* gene was validated by read mapping. The correct read alignments upstream **(A)** and downstream **(B)** the plasmid validate the manual insertion. The read mapping was performed using in CLC Genomics Workbench 7.0.

Table S1 | Total list of proteins identified in the core-proteome of CB 129 wild-type and CB129Δ*slpB*.

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Characterization of a Prophage-Free Derivative Strain of *Lactococcus lactis* ssp. *lactis* IL1403 Reveals the Importance of Prophages for Phenotypic Plasticity of the Host

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Lactococcus lactis is a lactic acid bacterium of major importance for the dairy industry and for human health. Recent sequencing surveys of this species have provided evidence that all lactococcal genomes contain prophages and prophage-like elements. The prophage-related sequences encompass up to 10% of the bacterial chromosomes and thus contribute significantly to the genetic diversity of lactococci. However, the impact of these resident prophages on the physiology of *L. lactis* is presently unknown. The genome of the first sequenced prototype strain, *L. lactis* ssp. *lactis* IL1403, contains six prophage-like elements which together represent 6.7% of the IL1403 chromosome. Diverse prophage genes other than those encoding phage repressors have been shown to be expressed in lysogenic conditions, suggesting that prophage genes are indeed able to modulate the physiology of their host. To elucidate the effect of resident prophages on the behavior of *L. lactis* in different growth conditions, we constructed and characterized, for the first time, a derivative strain of IL1403 that is prophage-free. This strain provides unique experimental opportunities for the study of different aspects of lactococcal physiology using the well-defined genetic background of IL1403. Here, we show that resident prophages modify the growth and survival of the host strain to a considerable extent in different conditions, including in the gastrointestinal environment. They also may affect cellular autolytic properties and the host cells' susceptibility to virulent bacteriophages and antimicrobial agents. It thus appears that prophages contribute significantly to lactococcal cell physiology and might play an important role in the adaptation of *L. lactis* to cultivation and environmental conditions.

Keywords: ***Lactococcus lactis*** **IL1403, prophages, prophage impact, prophage-cured strain, physiology of** ***Lactococcus lactis***

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INTRODUCTION

Lactococci (*Lactococcus lactis* species) are widely used in the industrial manufacturing of fermented dairy products (Leroy and De Vuyst, 2004; Smit et al., 2005). *L. lactis* is also of particular interest for its ever-growing therapeutic and epidemiological applications in the domains of human and animal health (Bermudez-Humaran et al., 2013). For many years, lactococci were an object of intensive fundamental and applied studies, which now have progressed to the fields of "omics-" and

systems biology (de Vos, 2011; Kok et al., 2017). Currently, 34 complete and 97 partially assembled lactococcal genomes are available in NCBI's GenBank[1]. In each one, the genomes of temperate bacteriophages (complete prophages) and prophage remnants have been identified (Chopin et al., 2001; Ventura et al., 2007; Kelleher et al., 2018). Prophage-related sequences encompass from 3 to 10% of the total genome, and represent a significant part of the observed genomic differences among *L. lactis* strains (Kelleher et al., 2018). Genome sequence analyses of lactococcal prophages indicated that they are affiliated with the P335 group of phages, one of the three dominant phage species commonly found in dairy factories (Deveau et al., 2006). The two other species (c2- and 936-like) are composed of phages that are exclusively virulent (Deveau et al., 2006).

The impact of prophages on cell physiology and bacterial ecology has mainly been studied in *Escherichia coli* (Wang et al., 2010) and some bacterial pathogens (reviewed in Fortier and Sekulovic, 2013). Although resident prophages are able to provoke the destruction of a cell, there is a growing appreciation for the beneficial effects they can have for a host. Specifically, there is evidence that prophages can increase host fitness under specific environmental conditions, improve its resistance against infecting phages, or modify cellular metabolism by introducing novel functions or altering pre-existing ones (Wang et al., 2010; Fortier and Sekulovic, 2013; DeBardeleben et al., 2014; Howard-Varona et al., 2017). However, questions about the overall impact of prophages on different aspects of *L. lactis* physiology have not yet been completely resolved. The main reason for this is the lack of an experimental model appropriate for this type of study, namely, a prophage-free strain of *L. lactis* in which all phage-related chromosomal elements have been removed. Progress has been made toward this goal, but the strains generated to date have several drawbacks. For example, a putatively prophage-cured derivative of *L. lactis* ssp. *cremoris* UC509 (UC509.9) still carries a remnant prophage (Ainsworth et al., 2013; Kelleher et al., 2018). Likewise, the derivative strains have been produced of *L. lactis* IL1403, but these still contain three prophage remnants (Visweswaran et al., 2017). Finally, a prophage-free derivative of *L. lactis* ssp. *cremoris* NZ9000 was generated, but in the process several non-essential host genes were also deleted (Zhu et al., 2017).

Lactococcus lactis subsp. *lactis* IL1403 strain is the first completely sequenced lactococcal strain (Bolotin et al., 2001). It has been widely used for both fundamental and applied research, including diverse studies of lactococcal genetics and physiology, as well as lactococcal phage biology (Chopin et al., 2005; Douillard and De Vos, 2014; Kok et al., 2017). The genome of IL1403 contains six prophage-like elements, which represent in total 6.7% of the IL1403 chromosome (Chopin et al., 2001). Transcriptional analyses of IL1403 and other lactococcal strains have revealed that, in addition to the genes encoding repressor proteins, other phage-specific genes are expressed in the lysogenic state under different growth conditions (Boyce et al., 1995; Blatny et al., 2003; Seegers et al., 2004; Xie et al., 2004; Cretenet et al., 2011; Dijkstra et al., 2016; Visweswaran et al., 2017). As yet, though, it is unknown if or how these prophage-encoded proteins affect the physiology of the lactococcal cell.

Here, we describe the construction and initial characterization of a derivative of *L. lactis* IL1403 strain in which we deleted all prophage-like elements. We then examined the impact of IL1403 prophages on aspects of cell physiology that are of particular relevance for the industrial and health applications of *L. lactis*. Our results reinforce previous findings of the complex effect of prophages on the behavior of the bacterial cell, and demonstrate the significant contributions of prophages to the adaptive phenotypic plasticity and fitness of *L. lactis*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Phages, and Media

Lactococcus lactis ssp. *lactis* IL1403, IL1946 (Chopin et al., 1989; Bolotin et al., 2001), and derivative strains were grown at one of two defined temperatures (30°C or 37°C, specified for each experiment) in M17 medium supplemented with 0.5% glucose (M17glu). *E. coli* strain TG1 was grown at 37°C in LB medium. When needed, ampicillin (Ap; 100 μg/ml for *E. coli*), tetracycline (Tc; 5 μg/ml for *L. lactis* and 10 μg/ml for *E. coli*), chloramphenicol (Cm; 10 μg/ml), and erythromycin (5 μg/ml for *L. lactis*) were added to the culture medium. Heme (Sigma) stock solution (0.5 mg/ml) was freshly prepared in alkaline water (0.05 N NaOH). Plasmid pIL1237 is a derivative of pBluscript II SK+ that carries the Tc^R gene of pAT182 (Rasmussen et al., 1994), while plasmid pIL253 and pIL253 derivative, which carry Cm^R gene, were from our laboratory collection. Reference prolate phages (c2, bIL67), small isometric-headed phages (sk1, bIL66, bIL170, bIL41), and phages isolated from industrial samples (bIL8, bIL10, bIL13, bIL14, bIL19, bIL20, bIL32, and bIL39) were also from our laboratory collection. Phages were sequentially propagated in suitable *L. lactis* hosts and, when required, in *L. lactis* IL1403 or *L. lactis* IL6288 strains using M17glu medium supplemented with 10 mM $CaCl_2$. Phages were enumerated as described in (Anba et al., 1995).

Measurement of Growth Rate and Cell Survival

For growth-rate experiments, *L. lactis* strains were incubated overnight in M17glu medium at 30°C in static (non-aerated) conditions, then diluted to OD_{600} 0.05 (measured with a Novaspec II Visible Spectrophotometer, Pharmacia Biotech) in fresh M17glu medium that contained, when required, different concentrations of heme (Sigma), from 0 to 100 μg/ml. We then transferred 200 μl of each sample to a 96-well plate. All plates (with the lids on to avoid evaporation) were incubated at the temperature indicated for each experiment, with or without constant shaking, in a multimode microplate reader (Synergy 2, BioTek Instruments, Inc) for the time indicated. The OD_{600} was measured at 15-min intervals; the static culture was gently agitated before each measurement. Growth experiments were repeated at least four times, and each experiment included four

[1]http://www.ncbi.nlm.nih.gov/genome/genomes/156

independent cultures of each strain. For the survival experiments, time and temperature conditions are specified in the Results and Discussion section, but the general procedure was as follows. Bacterial cultures were grown overnight in M17glu medium, then diluted in fresh medium. Static cultures were grown without agitation (culture volume 20 ml in 50-ml bottle) at 30°C or 37°C. To determine cell viability, aliquots were taken at different time points, serially diluted in Ringer's saline solution (Merck, Germany), and plated on M17glu agar. Colony-forming units (CFUs) were counted after 36 h of incubation at 30°C. Each data point (CFU/ml) was the mean of the counts of at least three independent cultures.

Lysozyme and Nisin Susceptibility Assays

The susceptibility of *L. lactis* to antimicrobials was determined using M17 agar plates. Overnight cultures of IL1403 and its derivative strains were diluted 10-fold in Ringer's saline solution (Merck). Drops (5 μl) of the dilutions were spotted on M17glu agar that contained different concentrations of inhibitors: from 0 to 0.5 mg/ml of lysozyme (Fluka) and from 0 to 300 ng/ml of nisin (Sigma). Plate counts were performed after 36 h of incubation at 30°C. The experiment was reproduced three times.

Plate Test of Autolytic Activity

Lytic activity of growing *L. lactis* cells was examined by plating the diluted overnight cultures on M17 agar plates that contained 0.2% (wt/vol) autoclaved, lyophilized cells of *Micrococcus luteus* ATCC 4698 (Sigma). Plates were incubated at the indicated temperatures for 48 h and then examined for the appearance of halos around the lactococcal colonies. The experiment was performed three times. Images were acquired with a ChemiDoc MP system (Bio-Rad).

Autolysis in Buffer Solution

Lactococcus lactis strains were grown in M17 medium to an OD_{600} of 0.6. Cells were harvested by centrifugation at 5000 *g* for 15 min at 4°C, washed once with sterile 50 mM potassium phosphate (PBS) buffer (pH 7.0), and resuspended in the same buffer to OD_{600} 0.7; supplemented when required with 0.05% Triton X-100 (Bio-Rad). Cell suspensions were transferred into 100-well sterile microplates and incubated at 30°C or 37°C. Autolysis was monitored by measuring the OD_{600} of the cell suspensions at 15-min intervals with an automated multi-mode plate reader (Synergy 2, BioTek Instruments, Inc). This experiment was performed twice. Each experiment included four independent cultures of each strain. The extent of autolysis was expressed as the percentage decrease in OD_{600}.

Heme-Induced Toxicity Assay

Autoclaved heme (Fluka) was added to exponentially growing cultures of *L. lactis* IL1403 and IL6288 (OD_{600} of 0.6) at concentrations ranging from 0 to 50 μg/ml. Cultures were incubated at 30°C under non-aerated conditions. After 1 h, the number of viable cells was determined by diluting the cultures in Ringer's saline solution and spotting 3 μl onto M17glu agar. Images were acquired with a ChemiDoc MP system after 36 h of incubation at 30°C. This experiment was performed twice.

Molecular Cloning and DNA Sequence Analysis

The procedures used here for DNA manipulation, cloning, and transformation of *E. coli* and *L. lactis* were carried out, for the most part, as described in (Sambrook et al., 1989; Anba et al., 1995). Polymerase chain reactions (PCRs) were performed using the Gene AMP PCR System 9700 thermal cycler (Applied Biosystems) and ExTaq (Takara Biomedicals) essentially as recommended by the supplier. Nucleotide sequencing was performed on PCR products using appropriate primers (**Supplementary Table S1**), Taq polymerase (Applied Biosystems), and fluorescent dideoxyribonucleotides on a 377A DNA Sequencer (Applied Biosystems).

Curing of Resident Prophages From IL1403 Chromosome

First, we constructed plasmids that carried DNA fragments corresponding to the early genome regions of different IL1403 prophages; this was performed by cloning *SmaI*-digested phage-specific DNA into the *Eco*RV site of the pIL1237 plasmid vector. DNA fragments used for cloning were amplified using the IL1403 chromosome as a template and specific oligonucleotides (AA07/AA08 for bIL309; AA30/AA31 for bIL286; AA32/AA33 for bIL310; AA34/AA35 for bIL312) that contained *SmaI* sites (see **Supplementary Table S1** for oligonucleotides used in this study). Cloning was performed in *E. coli* strain TG1. The resulting plasmids were designated pIL1237::bIL309, pIL1237::bIL286, pIL1237::bIL310, and pIL1237::bIL312.

To delete bIL309 prophage, we started with the previously constructed bIL285-free strain IL1946 (Chopin et al., 1989) and transformed it with the pIL1237::bIL309 plasmid. Five Tc^R colonies were tested by PCR to verify the correct integration of pIL1237 at the bIL309, using oligonucleotide pairs AA26/MCC31, AA27/MCC32, and AA26/AA27. One PCR-positive clone was selected for induction experiments. To induce prophage excision, cells were grown to OD_{600} 0.6, serial dilutions were spotted on M17 agar plates, and plates were irradiated with UV light (at 254 nm) with an energy of 40 J/m^2 for 2–4 s with a Stratalinker 2400 UV source (Stratagene). Next, 24 Tc^S clones selected after irradiation were tested by PCR with the oligonucleotide pair AA26/AA27 to detect the excision of bIL309; 4 PCR-positive clones were subsequently verified by sequencing. The resulting *L. lactis* strain (bIL285- and bIL309-free) was named IL6248 (**Table 1**).

Next, to delete bIL286 prophage from this strain, IL6248 cells were transformed with the pIL1237::bIL286 plasmid. Seven IL6248 Tc^R colonies were tested by PCR for chromosomal integration using the oligonucleotide pairs AA28/AA41, AA30/AA29, AA39/AA40, AA37/AA40, AA36/AA37, and AA36/AA39. One PCR-positive clone was used for induction experiments. Induction was performed by irradiation with UV light (254 nm) with an energy of 60 J/m^2 for 2–4 s. Verification of the prophage-free derivative strain was performed by PCR using

TABLE 1 | Prophage-free derivative strains of *L. lactis* IL1403.

Strain	Parental strain	Resident prophages					
IL1403		bIL285	bIL286	bIL309	bIL310	bIL311	bIL312
IL1946	IL1403		bIL286	bIL309	bIL310	bIL311	bIL312
IL6248	IL1946		bIL286		bIL310	bIL311	bIL312
IL6250	IL6248				bIL310	bIL311	bIL312
IL6254	IL6250					bIL311	bIL312
IL6260	IL6250					bIL311	bIL312
IL6277	IL6260						bIL312
IL6288	IL6277						
IL6345	IL1403	bIL285		bIL309	bIL310	bIL311	bIL312
IL6353	IL6345	bIL285			bIL310	bIL311	bIL312
IL6351	IL1946			bIL309	bIL310	bIL311	bIL312
IL6328	IL6260					bIL311	
IL6354	IL6288		bIL286				

the oligonucleotide pair AA28/AA29. The resulting *L. lactis* strain (bIL285-, bIL309-, and bIL286-free) was named IL6250 (**Table 1**).

To delete bIL310 prophage, strain IL6250 was transformed with the pIL1237::bIL310 plasmid. Seven IL6250 Tc^R colonies were tested by PCR for chromosomal integration at the bIL310 using the oligonucleotide pairs MCC35/AA41, MCC44/AA42, and AA41/AA42. One PCR-positive clone was used for induction experiments. Induction was performed by irradiation with UV light (254 nm) with an energy of 60 J/m^2 for 2–4 s. Verification of the prophage-free derivative strain was performed as described above. Two clones of the resulting *L. lactis* strain (bIL285-, bIL309-, bIL286-, and bIL310-free) were named IL6254 and IL6260 (**Table 1**). To delete bIL312 prophage, strain IL6254 was transformed with the pIL1237::bIL312 plasmid, but all efforts to obtain Tc^R integrants were unsuccessful. We then checked for the presence of bIL312 in strains IL6254 and IL6260 (both bIL285-, bIL309-, bIL286-, bIL310-free) using the oligonucleotide pairs AA43/AA44, AA43/AA56, AA34/AA35, AA44/AA37, and AA56/AA57; we wanted to identify clones in which bIL312 had been spontaneously lost as the result of the previous treatment with UV light. Among approximately 1000 clones, three clones derived from IL6260 did not carry bIL312. The verification of the selected clones was performed as described above. The resulting *L. lactis* strain (bIL285-, bIL309-, bIL286-, bIL301-, and bIL312-free) was named IL6328 (**Table 1**).

A bIL311-free strain was obtained with the use of the pGhost9 plasmid integration mutagenesis system (Maguin et al., 1996). DNA fragments that corresponded to the early region of the bIL311 genome were amplified using the oligonucleotide pairs AA53/AA60 and AA47/AA58. They were digested with *SmaI* and *Kpn*I and cloned into pGhost9 in *E. coli* TG1 cells. Next, the resulting plasmid was introduced into IL6260 strain. Selection of integrants was performed after shifting the temperature to 37.5°C and cells plating on M17glu agar supplemented with Em. The integration was confirmed by PCR amplification. Excision of the integrated pGhost construct, which led to the deletion of bIL311, was performed by shifting the temperature to 30°C and selecting for Em^S clones. The resulting *L. lactis* strain (bIL285-, bIL309-, bIL286, bIL310, and bIL311-free) was named IL6277 (**Table 1**). Deletion of bIL312 prophage from IL6277 was performed via transformation with the pIL1237::bIL312 plasmid, the selection of four Tc^R integrants, and consequent induction of the prophage by irradiation with UV light (254 nm) with an energy of 60 J/m^2 for 2–4 s. The resulting, completely prophage-free, strain IL1403 (bIL285-, bIL309-, bIL286-, bIL301-, bIL312-, and bIL311-free) was verified by sequencing and named IL6288 (**Table 1**). Strain IL6351 (bIL285-, bIL286-free) was constructed from IL1946 using the pIL1237::bIL286 plasmid essentially as described above (**Table 1**). In addition, bIL286 was deleted from IL1403 strain as described above, the resulting strain was named IL6345 (**Table 1**). Next, IL6353 (bIL286, bIL309-free) stain was constructed from IL6345 by the constitutive insertion and induction of pIL1237::bIL309 plasmid.

Finally, we constructed the lysogenic strain IL6354 by re-introducing bIL286 into the prophage-free strain IL6288; this was performed using the superinfection plate assay with total phage lysate as described in (Reyrolle et al., 1982). Briefly, phage lysate was prepared from 10 ml of exponentially growing IL1403 culture treated with mitomycin C (final concentration of 0.5 μg/ml/) at 30°C. Lysis was detected by measuring the OD_{600} for 4 h at 30-min intervals following the addition of mitomycin C. Cellular debris was removed by centrifugation at 8,000 rpm for 10 min at 4°C. Between 10 and 20 μl of the lysate were immediately spotted on M17glu agar plates in which the top agar had been mixed with IL6288 cells. A non-treated culture served as the control. Plates were incubated at 30°C for 36 h. The individual colonies that grew within the lysis zones were examined for the presence of the prophage using PCR with the oligonucleotide pairs mentioned above and subsequent sequencing. One clone carried the bIL286 prophage at its initial location; it was retained and named IL6354 (**Table 1**).

Mouse Experiment

Germ-free mice (8-weeks-old, C3H) were provided by ANAXEM, the germ-free rodent breeding facility of the MICALIS institute (INRA, Jouy-en-Josas, France). All animals were housed in flexible-film isolators (Getinge-La Calhène,

Vendôme, France). Three isolators were used; each contained one cage of four mice. Mice were provided, *ad libitum*, sterile tap water and a gamma-irradiated standard diet (R03-40, S.A.F.E., Augy, France). Their bedding was composed of wood shavings and they were also given cellulose sheets as enrichment. The light/dark cycle was 12 h/12 h, the temperature was maintained between 20 and 22°C, and humidity was between 45 to 55%. All procedures related to the use of germ-free mice were approved by the Local Ethics Committee (Comethea: reference number 45) and by the French Ministry of Research, and were recorded under the project number 3441-2016010614307552. Once a day for 3 consecutive days, mice were orally gavaged with 16% glycerol solution in PBS buffer that contained either 10^9 CFUs of strain IL1403, or 10^9 CFUs of strain IL6288, or the mixture of IL1403 (pIl253:Em^R) and IL6288 (pIL253: Cm^R) strains (10^9 CFUs of each strain). Feces were collected and weighed at the indicated day after gavage. Then, feces were resuspended, vortexed and diluted in PBS buffer. The survival and stability of *L. lactis* was checked by plating fecal material on M17glu agar either antibiotic-free or supplemented with Em or Cm for the detection of IL1403 or IL6288, respectively, in the pure or the mixed cultures. CFUs were counted after 36 h of anaerobic cultivation at 30°C.

RESULTS AND DISCUSSION

Construction of Prophage-Free Derivatives of *L. lactis* IL1403

As was described previously (Bolotin et al., 2001; Chopin et al., 2001), the genome of *L. lactis* strain IL1403 contains six randomly distributed prophage-like elements: bIL285 (35538 bp), bIL286 (41834 bp), bIL309 (36949 bp), bIL310 (14957 bp), bIL311 (14510 bp), and bIL312 (15179 bp) (**Figure 1A**). The bIL309 is integrated into a $tRNA^{arg}$ gene. Five other prophages are integrated into non-coding regions of the IL1403 chromosome. The bIL312 is integrated between the $tRNA^{met}$ gene and the *hslA* gene, which codes for the bacterial histone-like protein HU and is transcribed in the opposite direction of the phage integrase gene. The bIL285 is integrated between the *ykhD* gene (codes for redox-sensing transcriptional regulator) and the *radC* gene (codes for DNA repair protein), which are oriented in the opposite direction to the prophage lysin and integrase genes, respectively. The bIL286 is integrated between the $tRNA^{ser}$ gene and the *yofM* gene. This latter gene is transcribed in the opposite direction of the phage integrase gene and it appears to be similar to the *ylxM* gene of *Bacillus subtilis*, which encodes a component of a putative signal recognition particle. Prophage bIL310 is integrated between the genes *yofE* and *mtlD*, which encode, respectively, a protein of unknown function and mannitol-1-phosphate 5-dehydrogenase. Finally, prophage bIL311, which carries two IS983 elements within its early-genes region, is located between the *yucF* and *pdc* genes, which code for a protein of unknown function and phenolic acid decarboxylase, respectively.

Prophage-like elements can be divided into two groups: the first comprising the three prophages with large genomes (33–42 kb; bIL285, bIL286, bIL309) and the second composed of the three prophages with small genomes (14–15 kb; bIL310, bIL311, and bIL312) (**Figure 1B**). The three smaller IL1403 prophages appear to lack the genes required for phage morphogenesis and lysis of the host cell; they have thus been considered to be prophage remnant elements. It was reported previously that the repression system that maintains lactococcal prophages in the lysogenic state in different lactococcal strains is quite tight: prophages do not appear to be activated by individual stressors such as low or high temperatures, high osmotic pressure, hydrochloric or lactic acids, hydrogen peroxide, or the antibiotic bacitracin, which targets peptidoglycan synthesis (Xie et al., 2004; Ho et al., 2016). However, treatment with ultraviolet (UV) light does cause the induction of all IL1403 prophages with the exception of bIL311 (Chopin et al., 2001), a finding that was previously employed for the deletion of the bIL285 prophage from IL1403 strain (Chopin et al., 1989). We used this existing bIL285-free strain (IL1946) and eliminated four of the remaining prophages (bIL286, bIL309, bIL310, and bIL312) via the construction of single-copy integrants using the non-replicative pIL1237 plasmid that carried the Tc^R gene. Prophage genomes that were marked with Tc^R gene were induced by UV light to enable the selection of Tc^S prophage-free clones (see the Section "Materials and Methods"). However, this method was not successful in deleting the bIL311 prophage, which was subsequently accomplished using the pGhost9 plasmid integration mutagenesis system (Maguin et al., 1996) (see the Section "Materials and Methods"). The final prophage-free derivative of strain IL1403, cured of all six prophages, was designated IL6288. In addition, we constructed several intermediate IL1403 derivatives that contained different combinations of prophages. The IL6345 and IL6353 strains were constructed by the successive deletions of bIL286 and bIL309 prophages from the parental IL1403 strain. We also evaluated the ability of the prophage-free strain, IL6288, to serve as a potential host in re-lysogenization experiments that used total phage lysate obtained after the induction of lysogenic strain IL1403 (see the Section "Materials and Methods"). Using this approach, we found that only phage bIL286 was able to re-lysogenize the prophage-free derivative strain; this was consistent with a previous suggestion that all the other prophages are defective (Chopin et al., 2001). The relevant genotypes of all constructed strains are listed in **Table 1**.

Resident Prophages Modify the Susceptibility of Host Cells to Virulent Phages

The presence of prophages in a bacterial chromosome alters the sensitivity of the host strain to infecting phages by different mechanisms. Constitutive expression of prophage-encoded repressor proteins ensures immunity of the host to infection with related temperate phages. Two other mechanisms, the abortive infection (Abi) and superinfection exclusion (Sie) resistance systems protect bacteria from virulent phages (Chopin et al., 2005; Labrie et al., 2010). Strain IL1403 does not possess any Abi system, but four prophage-encoded *sie* genes have been formally identified in its genome: *orf2* (*sie_{285}*) in bIL285, *orf2*

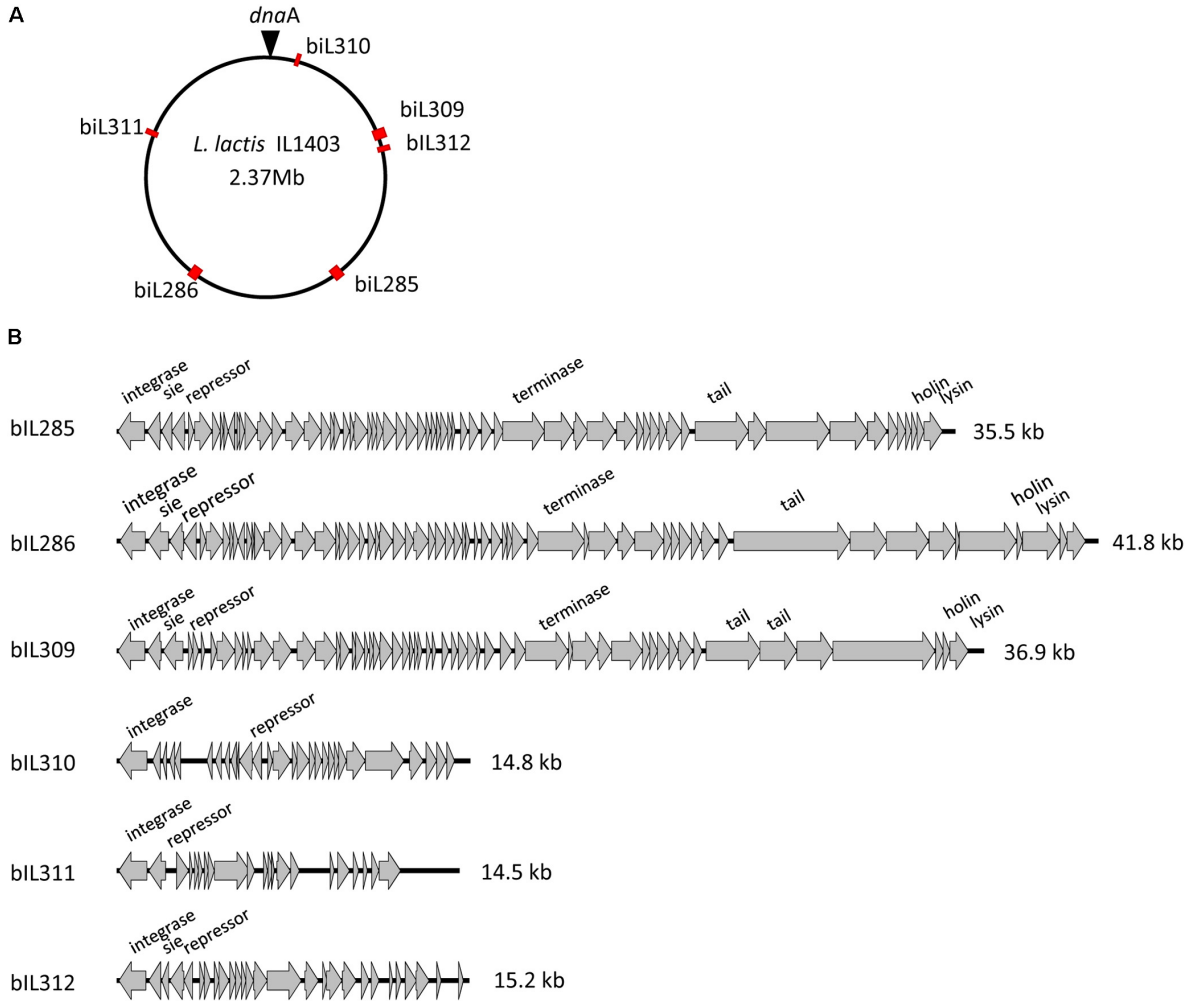

FIGURE 1 | *L. lactis* IL1403 prophages. **(A)** Location of prophages on the chromosome of *L. lactis* IL1403. The prophage positions are marked by red rectangles; the position of *dnaA* within the *oriC* region is designated by a triangle. **(B)** Genome organization of the *L. lactis* IL1403 prophages. Genome boundaries correspond to *attB* sites. Genes are represented by arrows oriented in the direction of transcription. Functions of selected early- and late-gene products are indicated.

($orf2_{286}$), in bIL286, *orf2* (sie_{309}) in bIL309, and *orf2* (sie_{312}) in bIL312 (McGrath et al., 2002; Mahony et al., 2008). These *sie* genes are located within the phage lysogeny modules; they are expressed in the lysogenic state and encode proteins with membrane-spanning regions and/or a hydrophobic N-terminus (McGrath et al., 2002; Mahony et al., 2008). When produced using the nisin-inducible expression system in *L. lactis* ssp. *cremoris* strain MG1363, sie_{309} and sie_{312} provided resistance against several phages from the 936 phage group by blocking phage DNA injection. Sie proteins have been detected in different IL1403 proteomes, indicating that these proteins are produced in substantial amounts (Drews et al., 2004; Beganovic et al., 2010).

To examine the impact of resident prophages on a cell's sensitivity to infection with virulent phages, *L. lactis* IL1403 and its derivative strains were assayed against a range of 936- and c2-like phages (see the Section "Materials and Methods"). For 12 of the 14 virulent phages from our laboratory collection, phage titers and plaque morphology were identical for all tested strains (**Table 2**, data shown for bIL170 and bIL67). However, the presence or absence of resident prophages had a detectable effect on the activity of phages bIL14 and c2; these two virulent phages had different titers and showed variable plaque morphology when propagated on the parent strain IL1403 or its prophage-free derivative IL6288 (**Table 2**). Both properties were unaffected by the identity of the host strain (IL1403 or IL6288) that had been used for initial phage propagation (data shown for phage stocks prepared in IL1403 strain).

The development of the 936-like phage bIL14 was positively affected by the deletion of all six prophages: the titer of bIL14 obtained by plating on IL6288 was more than ten times higher than on the parental strain (**Table 2**). Additionally, no turbid plaques were detected on strain IL6288 (**Figure 2A**). Instead, bIL14 appeared to have varying effects on the intermediate strains. The development of bIL14 in strains IL1946, IL6353, IL6260, IL6328, and IL6277 was similar to that in IL1403, while the development of bIL14 in IL6250, IL6345, and IL6354 was

TABLE 2 | Resident prophages of *L. lactis* IL1403 interfere with infection by virulent phages.

Strain	Relevant genotype						Phage titer[a] *(PFU/ml)*[b]			
							936 Phage species		c2 Phage species	
							bIL170	bIL14	bIL67	c2
IL1403	bIL285	bIL286	bIL309	bIL310	bIL311	bIL312	1×10^8 c[c]	2.7×10^6 c; 1.2×10^6 t[d]	1×10^8 c	1.1×10^5 c, s[e]; 3.3×10^6 t
IL1946		bIL286	bIL309	bIL310	bIL311	bIL312	1×10^8 c	8.4×10^5 c; 8×10^5	1×10^8 c	2.5×10^4 c, s; 5.1×10^4 t
IL6345	bIL285		bIL309	bIL310	bIL311	bIL312	1×10^8 c	4.4×10^7 c	1×10^8 c	2.5×10^5 c, s; 2.6×16^5 t
IL6351			bIL309	bIL310	bIL311	bIL312	1×10^8 c	2×10^5 c	1×10^8 c	1.8×10^3 c, s; 9.1×10^4
IL6248		bIL286		bIL310	bIL311	bIL312	1×10^8 c	4.1×10^5 c	1×10^8 c	1×10^5 c, s; 2.2×10^5 t
IL6353	bIL285			bIL310	bIL311	bIL312	1×10^8 c	1×10^6 c; 6×10^5 t	1×10^8 c	2.3×10^5 c, s; 1.5×10^6 t
IL6250				bIL310	bIL311	bIL312	1×10^8 c	4.5×10^7 c	1×10^8 c	8.9×10^4 c, s; 7.2×10^4 t
IL6260					bIL311	bIL312	1×10^8 c	1.5×10^6 c; 5×10^6 t	1×10^8 c	3×10^4 c, s; 3×10^5 t
IL6328					bIL311		1×10^8 c	2.1×10^6 c; 3.8×10^6 t	1×10^8 c	4×10^5c; 2×10^5t
IL6277						bIL312	1×10^8 c	1.8×10^6 c; 5.1×10^6 t	1×10^8 c	1×10^6 c; 6×10^6 t
IL6354		bIL286					1×10^8 c	4.5×10^7 c	1×10^8 c	9.5×10^6 c; 1×10^6 t
IL6288	Prophage-free						1×10^8 c	4.9×10^7 c	1×10^8 c	8.8×10^6 c; 1.5×10^7 t

a, average of three independent experiments; b, PFU: plaque forming units; c, c: clear plaques; d, t: turbid plaques; e, s: small plaques.

similar to the prophage-free strain (**Figure 2A** and **Table 2**). Thus, it appeared that bIL14 had the maximal plating efficiency on strains, which were deleted for bIL286 either singly (as in strain IL6345) or together with other large prophages (IL6250). This could be evidence that the bIL286-encoded $orf2_{286}$ *sie* gene plays an important role in bIL14 infection. However, the stimulatory effect of bIL286 deletion on the efficiency of bIL14 infection disappeared with the single deletion of either bIL285 or bIL309 (IL6351 and IL6353, respectively, **Table 2**), which suggests that the relationship between the three prophages is complex. Furthermore, the simultaneous deletion of the three small prophages (bIL310, bIL311, and bIL312) increased bIL14 plating efficiency to the level found in the prophage-free strain, even in the presence of bIL286 (IL6354). Instead, the intermediate strains IL6248 and IL6351 showed lower sensitivity to bIL14 than the parental strain did (**Figure 2A**). These strains carry all three small prophages (bIL310, bIL311, bIL312) and either bIL286 or bIL309, but lack bIL285. At present, the molecular basis for the behavior of the bIL14 phage cannot be explained because its genome sequence is unknown. However, these results are consistent with previously reported data that indicated that prophage-encoded Sie defense systems were effective only against a limited group of the virulent 936-like phages, based on a highly specific direct interaction between the Sie protein and a structural element of the adsorbed phage (Mahony et al., 2008).

For the prolate-headed phage c2, the highest titers and clear plaques were likewise found in the prophage-deleted strain, IL6288, while the lowest titers and the mixture of small clear and turbid plaques were found in IL1403 and any strain carrying the two small prophages bIL311 and bIL312 (i.e., strains IL6250, IL1946, IL6248, IL6345, IL6351, IL6353, and IL6360) (**Figure 2B** and **Table 2**). The presence of phage bIL311 played a major role in the inhibition of c2 infection (compare strains IL6328 and IL6277, **Table 2**), but this inhibitory effect was further intensified by the presence of bIL312 (compare strains IL6328 and IL6260, **Figure 2B** and **Table 2**). Previous investigations

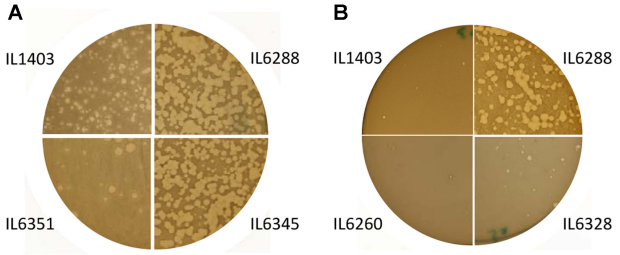

FIGURE 2 | Morphology of the lysis plaques formed by **(A)** the 936-like small isometric-headed phage bIL14 and **(B)** the prolate-headed phage c2 in the parental strain IL1403 and its derivative strains: IL6351 (bIL285-, bIL286-free), IL6345 (bIL286-free), IL6260 (bIL285-, bIL286-, bIL309-, bIL310-free), IL6328 (bIL285-, bIL286-, bIL309-, bIL310-, bIL312-free), and IL6288 (prophage-free). The experiment was reproduced at least three times. The results shown are from a representative experiment.

of the bIL312 phage found that its *orf2* (sie_{312}) gene provided resistance against 936-like phages, but not c2 phages; instead, no putative resistance genes have yet been identified in the genome of bIL311 (McGrath et al., 2002; Mahony et al., 2008). This suggests that the effects observed here were caused by as-yet-unidentified bIL311- and bIL312-encoded resistance function(s). It has been shown recently that two late bIL311 genes, *orf17* and *orf18*, and one bIL312 gene, *orf21*, are expressed in the host IL1403 strain (Dijkstra et al., 2016). The predicted Orf17 protein shows homology to proteins from the GNAT family of acetyltransferases. The predicted Orf18 protein contains a helix-turn-helix DNA-binding motif. No homology to proteins of known function can be detected for bIL312 *orf21*.

It should be noted that the genomes of two prolate-headed phages tested here, bIL67 and c2, are highly similar at the overall nucleotide level, but there is a region that is much less conserved (51.9% of identity) which contains three late-expressed genes involved in host-range determination (Lubbers et al., 1995;

Millen and Romero, 2016). To infect a host cell, all c2-like phages first recognize carbohydrate receptors on the cell surface in order to ensure the reversible binding of the phage to the host cell. Next, c2 phage interacts with the cell-membrane protein receptor Pip (phage infection protein), which results in irreversible binding and is essential for subsequent injection of phage DNA (reviewed in Mahony et al., 2016). However, bIL67 requires a different cell-membrane-associated protein receptor, YjaE. The factors that determine the utilization of either Pip or YjaE are encoded by genes from the non-conserved genomic regions of c2 and bIL67, respectively (Millen and Romero, 2016). This may explain the different behavior observed here between c2 and bIL67 phages during infection of the wild-type and prophage-free strains: it could be that the prophage-encoded proteins modify the arrangement of the specific cellular receptors required for infection by c2, but not by bIL67.

Altogether, our results indicate that prophage genes may contribute to the resistance of their host to virulent phage infection. In addition to the *sie* genes previously identified in the IL1403 genome, other as-yet-unidentified prophage-encoded functions (for example, those encoded by bIL311) are probably involved in phage resistance and await further investigation.

Prophage-Free Strain Shows Increased Resistance to Cell-Wall-Specific Antimicrobials

To compare the susceptibility of IL1403 and its prophage-free derivative to antimicrobial agents, we selected two commonly used cell-wall inhibitors, lysozyme, and nisin. Lysozyme, which is an important element of the innate immune system in animals, acts through hydrolysis of peptidoglycan (PG) sugar chains. In addition to this enzymatic activity, lysozyme, like nisin, also has non-enzymatic inhibitory effects based on cationic antimicrobial peptide (CAMP) activity; this kills bacteria through the formation of pores and destabilization of the cytoplasmic membrane via mechanisms involving interactions with phospholipids (Herbert et al., 2007; Roy et al., 2009). Bacterial resistance to CAMP activity occurs through modification of the cellular membrane or the interception of CAMPs by cell envelope-associated or secreted proteases (Roy et al., 2009; Koprivnjak and Peschel, 2011).

To test the impact of the resident prophages on their host's sensitivity to antimicrobial agents, we examined the growth of IL1403 and its derivatives in the presence of lysozyme or nisin (see the Section "Materials and Methods"). As shown in **Figures 3A,B**, parental strain IL1403 was highly sensitive to both lysozyme and nisin, while its prophage-free derivative, IL6288, was much more resistant to both antimicrobials. The IL6353 strain, which carried the bIL285 prophage, showed the same pattern of growth inhibition as IL1403, while the bIL285-free derivatives tested (IL1946, IL6351, IL6248) were similar to IL6288 in showing increased resistance to both antimicrobials (data shown for IL6248).

Our results demonstrated that the resident prophages, especially bIL285, increase the sensitivity of IL1403 to antimicrobials. We suggest that the cause of this may lie in modifications made by prophage-encoded proteins to the bacterial membrane and/or PG that increase host sensitivity to lysozyme and nisin. For example, the *orf2* (sie_{285}) protein, encoded by the constitutively expressed lysogeny module, could serve as a mediator of the CAMP-membrane interaction. *orf2* (sie_{285}) was detected in the proteome of *L. lactis* (Drews et al., 2004) and later recognized as a membrane protein that contains a bacterial Pleckstrin-homology (PH*b*) domain (Xu et al., 2010). It has been shown that membrane-associated PH*b*-containing proteins can interact with phosphatidylinositol lipids (Xu et al., 2010). Thus, it may be that the *orf2* (sie_{285}) protein modifies the phospholipid organization of the membrane and thereby increases host sensitivity to antimicrobials. In addition, the cell-wall hydrolase activity encoded by the bIL285 and/or bIL286 prophages (Visweswaran et al., 2017) may also contribute to fragility of the cell wall and increase the sensitivity of the parent strain to cell-wall-specific antimicrobials. Similarly, an increased sensitivity to lysozyme was recently demonstrated for lysogenic *Streptococcus suis* strain SS2-4 compared to its prophage-free derivative, but no molecular mechanism was proposed to explain this observation (Tang et al., 2013).

The experimental validation of the involvement of phage-encoded lysins and/or *orf2* (sie_{285}) in the sensitivity of lactococci to antimicrobials requires further investigation.

Resident Prophage Modifies the Autolytic Properties of *L. lactis* IL1403

The autolytic properties of *L. lactis* are crucial for its use in dairy fermentation and are also considerated in health applications (Lortal and Chapot-Chartier, 2005; Bermudez-Humaran et al., 2013). Bacterial autolysis is a complex phenomenon determined by multiple factors: the composition and structure of cellular PG, the expression and activity of peptidoglycan hydrolases (PGHs) and proteolytic enzymes, and the activity of prophage-encoded lytic enzymes (Lepeuple et al., 1998; O'Sullivan et al., 2000; Pillidge et al., 2002; Lortal and Chapot-Chartier, 2005; Visweswaran et al., 2017). Altogether, these factors determine important variations in autolysis levels that are found among lactococcal strains, and make the selection of an appropriate autolytic strain a difficult task. Previous studies of the autolysis of lactococcal cells have investigated the role of the main cellular autolytic enzyme, *N*-acetyl muramidase (AcmA), and the influence of the prophages bIL285, bIL286, and bIL309, which possess two-component lysis cassettes that encode lysin-holin complexes (Pillidge et al., 2002; Labrie et al., 2004; Visweswaran et al., 2017). Expression of the corresponding prophage genes was not detected by global transcriptome analyses, but was instead demonstrated by qRT-PCR (Xie et al., 2004; Cretenet et al., 2011; Visweswaran et al., 2017). Phage-specific endolysin activity was detected in the cell-free extracts only after UV induction of prophages (Visweswaran et al., 2017).

To examine the impact of the resident prophages on the autolysis of growing lactococcal cells, we first applied the standard plate assay for the detection of PGH activity, using lyophilized *M. luteus* cells at both 30°C (to simulate fermentation conditions) and at 37°C (to simulate the gastrointestinal environment) (see the Section "Materials and Methods").

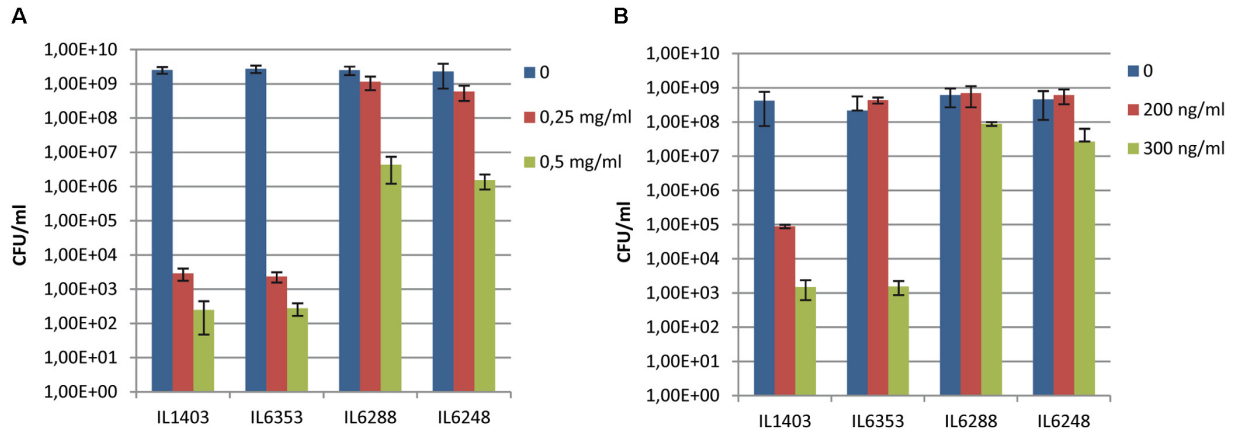

FIGURE 3 | Sensitivity of *L. lactis* IL1403 and its derivative strains IL1946 (bIL285-free), IL6353 (bIL286-, bIL309-free), and IL6288 to **(A)** lysozyme and **(B)** nisin. Serial dilutions of overnight cultures (from 10^{-3} to 10^{-5}) were spotted on M17glu agar containing either lysozyme (from 0 to 0.5 mg/ml; **A**) or nisin (from 0 to 300 ng/ml; **B**). Colonies were counted after 36 h of incubation at 30°C. The bars represent average values from three independent experiments, including two biological replicas for each strain.

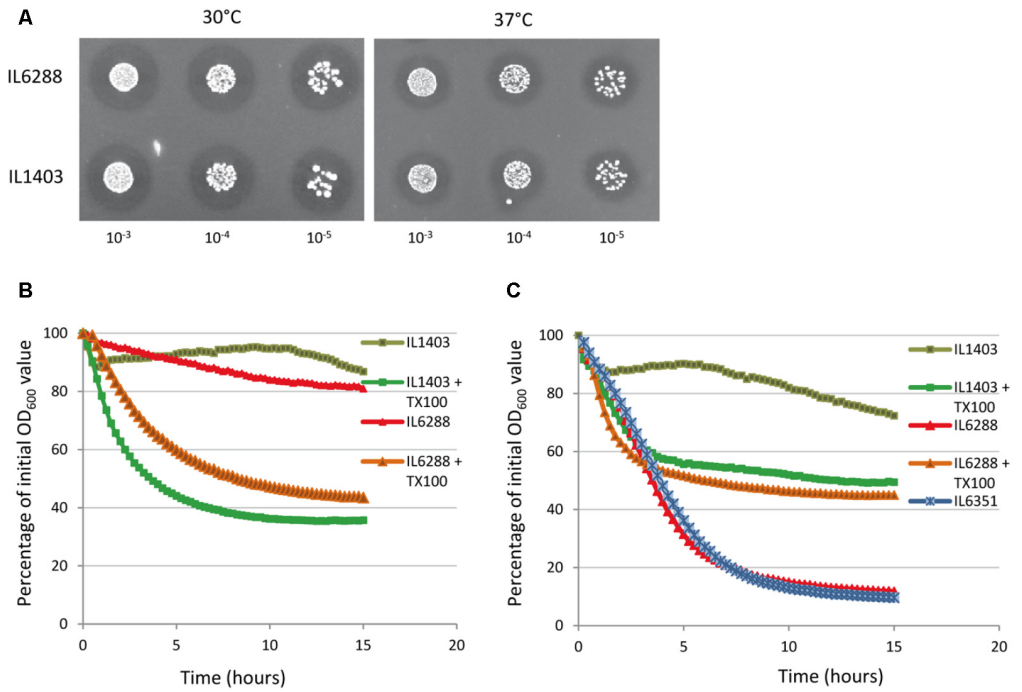

FIGURE 4 | Autolytic properties of IL1403 and its prophage-free derivative strain IL6288. **(A)** Lytic activity of growing *L. lactis* IL1403 and IL6288 cells was examined by plating serial dilutions of overnight cultures on M17glu agar plates that contained 0.2% (wt/vol) autoclaved, lyophilized cells of *M. luteus* ATCC 4698. Images were acquired with a ChemiDoc MP System after 48 h of incubation at 30°C or 37°C. Autolysis of *L. lactis* IL1403 and its derivative strains IL6351 (bIL285-, bIL286-free) and IL6288 (prophage-free) at 30°C **(B)** and 37°C **(C)**. Exponentially grown cells were washed and re-suspended in 50 mM potassium phosphate buffer in the absence or presence of 0.05% Triton X-100 (+TX100). Lysis was monitored by measuring the OD_{600} of the cell suspensions at 15-min intervals with an automated multimode plate reader (Synergy 2, BioTek Instruments, Inc). For each strain, the mean values of three independent suspensions, analyzed simultaneously, are plotted and expressed as the percentage decrease in OD_{600}, the SDs were $\leq$ 10%. Similar autolytic profiles were obtained in three independent experiments. The results presented are of a representative experiment.

We observed no difference between the halos formed by the parent and the derivative strains during growth at either 30°C or 37°C (**Figure 4A**, data shown for IL1403 and IL6288). This suggests that the main cellular autolysin, AcmA, exhibits the same level of activity in all tested strains and that the contribution of the prophage-encoded lytic enzymes to cellular autolytic activity is insignificant in the tested growth conditions. Next, to determine the impact of the resident prophages

on autolysis of non-growing cells, we performed a standard turbidimetric assay using autolysis buffer that contained the non-ionic detergent Triton X-100 (see the Section "Materials and Methods"); Triton X-100 permeabilizes bacterial cells via solubilization of integral membrane proteins and does not affect PG composition or the activity of PGHs (Schnaitman, 1971). As shown in **Figures 4B,C**, the prophage-free strain exhibited an autolysis rate similar to that of the parental strain at both 30°C and 37°C. In order to examine the putative roles of the prophage-encoded integral membrane proteins in IL1403, we repeated the autolysis test without the use of Triton X-100. At 30°C, both strains exhibited weak autolytic activity, which is characteristic for non-induced autolysis. Furthermore, IL1403 showed similar autolysis profiles at 37°C in the presence or absence of Triton X-100. Instead, in the absence of the detergent the autolysis rate of IL6288 at 37°C increased significantly compared to that of IL1403 (**Figure 4C**). To identify the prophage(s) primarily responsible for the observed phenotype, we assessed the autolytic properties at 37°C of *L. lactis* IL1403 derivatives that contained different prophages. In the presence of Triton X-100, all showed autolytic profiles identical to the parental strain. However, in the absence of Triton X-100, profiles differed among strains. Strains IL6351 and IL6248 (both lacking bIL285) showed autolytic profiles similar to that of prophage-free IL6288 (**Figure 4C**, data shown for IL6351). The unusual autolytic profiles of the non-permeabilized bIL285-free *L. lactis* cells suggest that bIL285 encodes protein(s) that protect *L. lactis* IL1403 cells from autolysis under the non-growth conditions, In this, *L. lactis* may be similar to *Streptococcus pneumonia*, in which it was recently shown that the presence of a phage-like element correlated with a delay in autolysis (DeBardeleben et al., 2014). This inhibition of autolysis was not attributed to alterations in the amount or activity of the major autolysin LytA, but was instead explained by increased cross-linking within the cell wall.

These results suggest that bIL285-encoded protein(s) may change the autolytic properties of the host by modifying the cellular membrane rather than through interfering with the activity of autolysins. The influence of temperature on this phenomenon is of special interest and may have implications for the industrial and therapeutic applications.

Prophages Interfere With the Growth Rate and Survival of *L. lactis* Cells

The impact of prophages on bacterial fitness is not obvious. Some prophage-free derivative strains exhibit improved fitness compared to parental strains under conditions that trigger prophage induction, but in other cases prophages can be beneficial for their hosts (Wang et al., 2010; Verghese et al., 2011; Baumgart et al., 2013; Wiles et al., 2013; Martínez-García et al., 2014). Most research conducted to date has focused on the roles of resident prophages and prophage-like elements in *E. coli* and different bacterial pathogens, but less information is available for lysogenic bacteria from other ecological systems (Wang et al., 2010; Casas and Maloy, 2011; Verghese et al., 2011; Fortier and Sekulovic, 2013; Tang et al., 2013).

To address the overall effect of the resident prophages on the fitness of *L. lactis* IL1403, we first investigated the growth rate of lysogenic and prophage-free strains at 30°C and at 37°C in rich M17 medium (see the Section "Materials and Methods"). At 30°C, no significant difference was observed between the growth rates of the parental and the prophage-free derivative strains within the time of our experiment (∼15 h) (**Figure 5A**). At 37°C, instead, both strains grew at a uniform rate, but the prophage-free IL6288 strain reached and maintained a plateau of stationary growth at a higher OD than that of the parental strain (**Figure 5A**). These results indicate that the prophages present in *L. lactis* IL1403 do not play an important role during exponential growth under the standard laboratory conditions, but the prophage-free derivative has a growth advantage at the increased incubation temperature. The decrease in growth observed for the parental strain was not caused by prophage induction, as high temperature (37°C) does not provoke the excision of phage DNA (Chopin et al., 2001; Ho et al., 2016).

Next, we examined the effect of resident prophages on the survival of host cells during stationary-phase growth (**Figures 5B,C**). Long-term viability of lactococcal strains is a crucial characteristic for their use in fermentation and storage of dairy foods (Stanton et al., 2005; Bron and Kleerebezem, 2011). Both strains IL1403 and IL6288 showed a rapid decrease in viability during growth at 37°C (**Figure 5C**). In contrast, the survival of IL1403 after 75 h of growth at 30°C was higher than that of its prophage-free derivative (**Figure 5B**). Both strains entered into the stationary phase at the same time, but IL1403 retained at least 90% of its viability for approximately 50 h after reaching the stationary phase, while the viability of the prophage-free strain decreased over that time. To examine the contribution of individual prophages to the survival of the host cells, we then estimated the viability of the intermediate prophage-free derivative strains. The viability of the IL6250 strain carrying only small prophages during the stationary-phase growth at 30°C was intermediate between the parental strain and its prophage-free derivative (**Figure 5B**).

Our results demonstrate that prophage genes improve the stationary-phase survival of lysogenic *L. lactis* strains at a temperature appropriate for growth. Indeed, this type of conditional selection was suggested to contribute to the stabilization of the domesticated prophage genomes (Bobay et al., 2014). Thus, prophage genes could represent an important selective advantage for *L. lactis* strains during the fermentation process.

Prophages Adversely Affect Aerobic and Heme-Activated Respiration Growth of *L. lactis*

Survival of *L. lactis* in aerobic environments is generally poor, as oxygen induces DNA, protein and membrane damage (Duwat et al., 2001; Rezaïki et al., 2004). However, *L. lactis* growth can be greatly improved under these conditions if there is a supply of heme, an iron−containing porphyrin essential for activation of the respiration electron transfer chain. Compared with aerobic growth, respiratory growth increases lactococcal biomass and

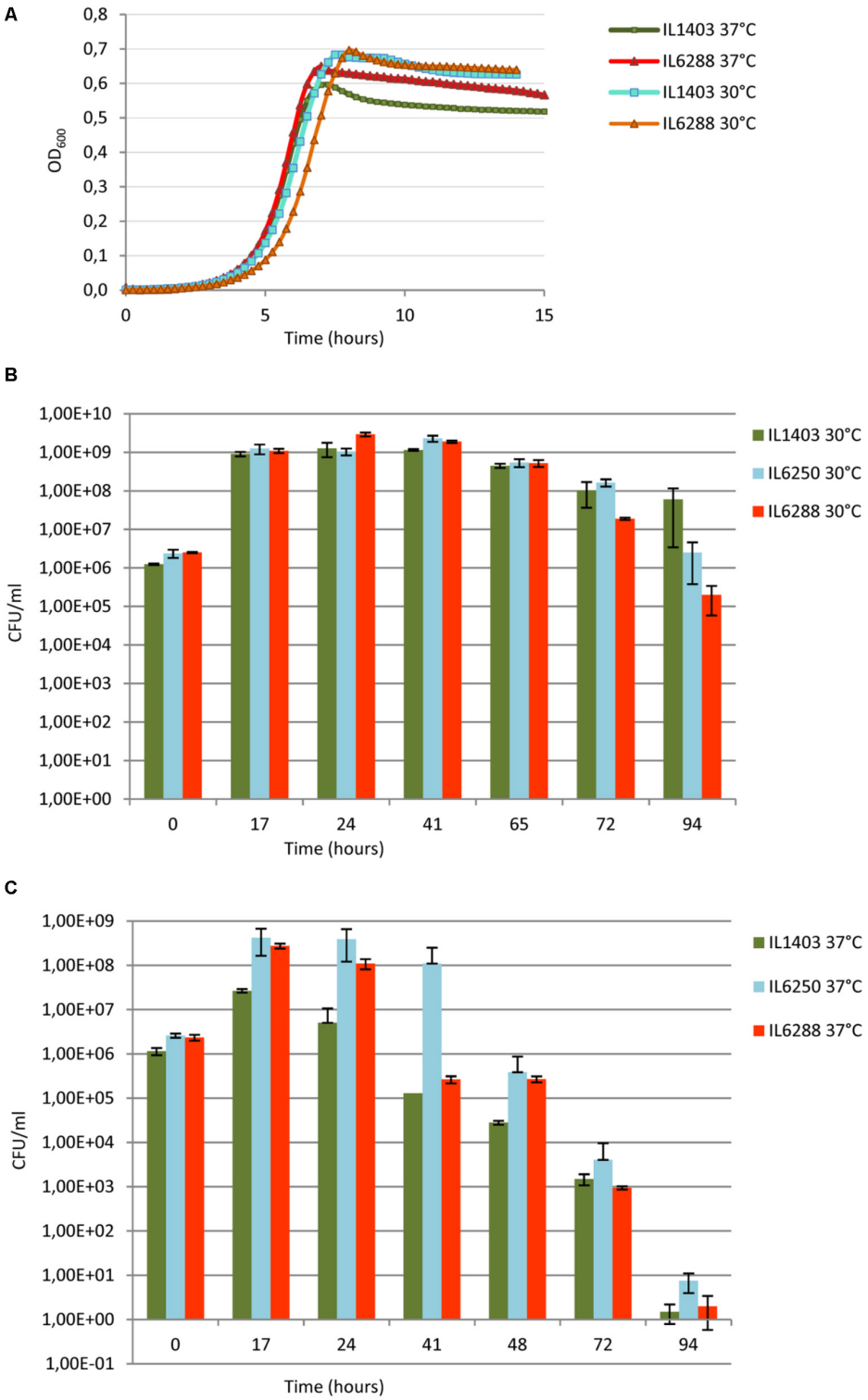

FIGURE 5 | Growth and survival of *L. lactis* IL1403 and its derivative strains at 30°C and 37°C. **(A)** For growth experiments, non-aerated cultures of strains IL1403 and IL6288 were incubated in M17glu medium at the given temperature in a multimode microplate reader (Synergy 2, BioTek Instruments, Inc). OD_{600} was measured at 15-min intervals for the indicated time; cultures were gently agitated prior to sampling. Each experiment included four independent cultures of each strain. For each strain, the mean values of three independent cultures, analyzed simultaneously, are plotted, and the SDs were $\leq$ 10%. Growth curves of each strain were established at least four times. Survival experiments were performed in static growth conditions at 30°C **(B)** and 37°C **(C)**. Cultures of IL1403, IL6250 (bIL285-, bIL286-, bIL309-free), and prophage-free IL6288 were incubated in M17glu medium at the indicated temperature. Cell survival was monitored by counting CFUs after plating bacterial cultures on M17glu agar. Colonies were counted after 36 h of incubation at 30°C. Each data point (CFUs/ml) is the mean of three counts.

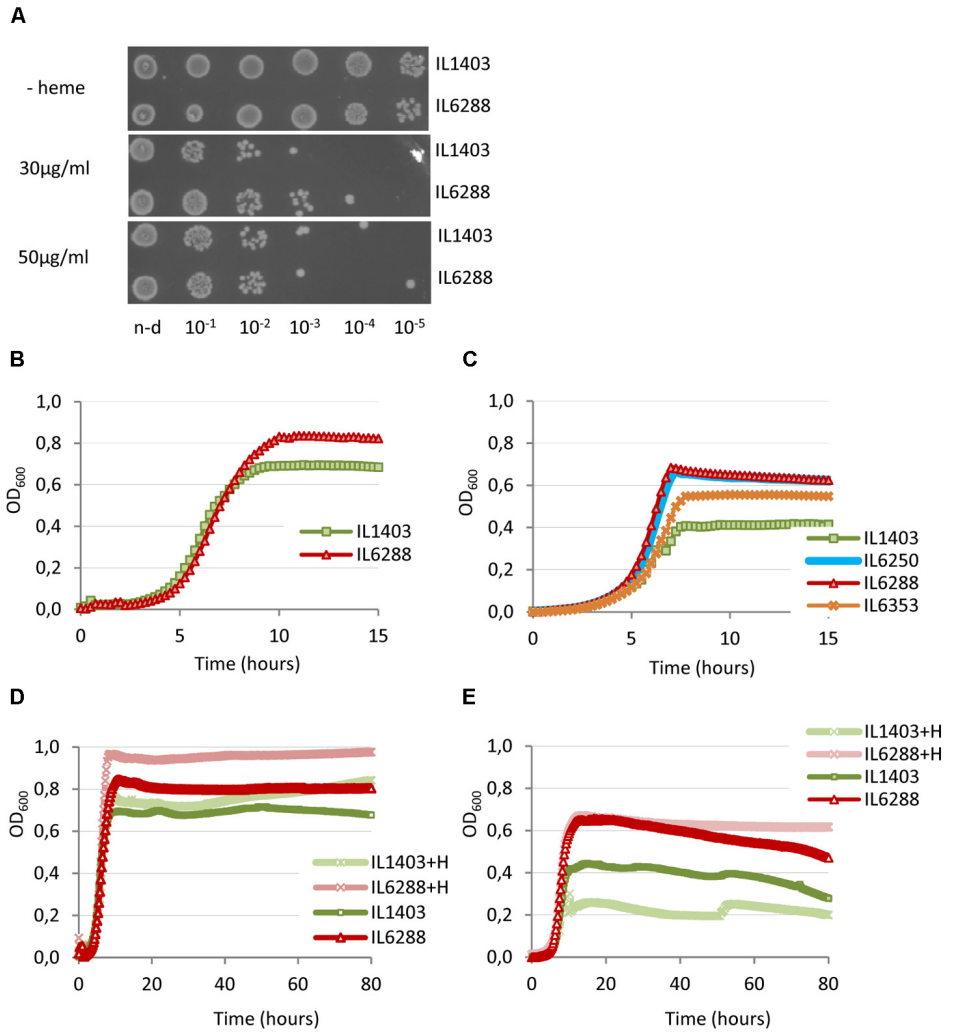

FIGURE 6 | Effect of heme on the growth of *L. lactis* IL1403 and its prophage-free derivative strain IL6288. For the assay of heme-induced toxicity **(A)**, autoclaved heme was added to static cultures of *L. lactis* IL1403 and IL6288 at an OD_{600} of 0.6 at the indicated concentrations. Cultures were incubated at 30°C under non-aerated conditions. After 1 h, viable cell numbers were determined by spotting indicated serial dilutions of the bacterial cultures onto M17glu agar. Images were acquired with a ChemiDoc MP system after 36 h of incubation at 30°C. This experiment was performed twice; the results of a representative experiment are shown. For the characterization of growth under aerated conditions **(B–E)**, cultures of IL1403 and its derivative strains were incubated in M17glu medium with (+H) or without heme in a multimode microplate reader (Synergy 2, BioTek Instruments, Inc) with constant shaking at 30°C **(B,D)** or 37°C **(C,E)**. Each experiment included four independent cultures of each strain. For each strain, the mean values of three independent cultures, analyzed simultaneously, are plotted. Growth curves of each strain were established three times. The results presented are of a representative experiment.

long-term survival (Rezaïki et al., 2004). Because of this, heme-activated respiration conditions have been already used for dairy starter production and may open new avenues for the use of *L. lactis* in health-related contexts (Teusink and Smid, 2006; Pedersen et al., 2012). However, free heme causes toxicity at high concentrations due to its ability to generate reactive oxygen species (Lechardeur et al., 2011).

Considering the importance of this aspect of lactococcal physiology, we wanted to investigate how prophage content influences the resistance of *L. lactis* IL1403 to heme and its survival under aeration and/or respiration conditions. First, we determined whether resident prophages influence the tolerance of the bacterial host to heme (Materials and Methods). As shown in **Figure 6A**, there were only slight differences between strains IL1403 and IL6288 in sensitivity to treatment with heme, which was performed under static conditions of growth at 30°C. This suggests that resident prophages have little or no impact on the regulation of heme uptake and degradation, which are known to determine heme tolerance in bacteria (Lechardeur et al., 2011).

We next compared the growth of the parental strain and its derivatives under aerobic conditions at 30°C and 37°C (see the Section "Materials and Methods"). For all strains, the negative effects of oxygen on cell growth were more apparent at 37°C than at 30°C. As shown in **Figures 6B,C**, the growth period of prophage-free strain IL6288 was extended compared to that of the parental strain, so that IL6288 cultures entered into

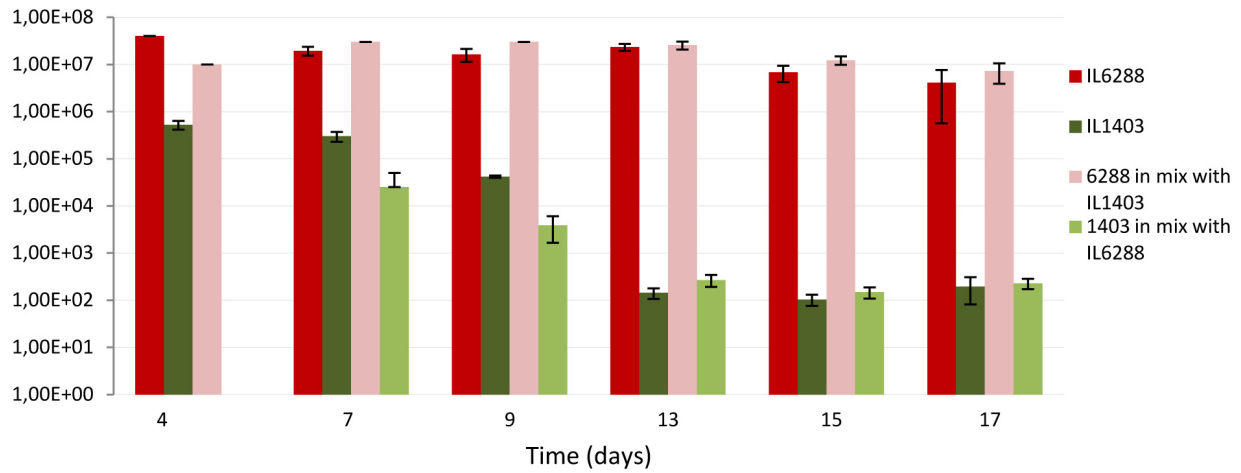

FIGURE 7 | Survival of *L. lactis* IL1403 and its prophage-free derivative strain, IL6288 after passage through the gastrointestinal tract of germ-free mice. Over 3 consecutive days, mice were orally gavaged with 16% glycerol solution in PBS buffer containing either 10^9 CFUs of strain IL1403, or 10^9 CFUs of strain IL6288, or the mixture of 10^9 CFUs of strain IL1403:Em^R plus 10^9 CFU of strain IL6288:Cm^R. Each strain was recovered from mouse feces at the indicated time points; cell survival was determined by counting CFUs on M17glu agar plates after 36 h of incubation at 30°C. Each data point (CFUs/ml) is the mean of at least three measurements.

the stationary phase at a higher optical density than those of IL1403. In general, the growth kinetics of the prophage-free strain were similar to those of intermediate strains that carried only the three small prophages (IL6250) or strains with the small prophages and either bIL309 or bIL286 (**Figure 6C**; data shown for IL6250 grown at 37°C). Instead, the strains that carried the three small prophages together with bIL285 (strain IL6353) or with bIL309 and bIL286 (strain IL1946) showed intermediate growth profiles (**Figure 6C**; data shown for IL6353 grown at 37°C).

It has been shown that the cleavage of the lactococcal phage repressors by RecA results in prophage induction (Chopin et al., 2001). In *L. lactis*, oxygen provokes DNA-damage and an increase of RecA activity (Rezaïki et al., 2004). Thus, the oxidative stress may stimulate induction of the resident prophages, which in turn may negatively affect bacterial growth. In addition, negative effect of the increased temperature in the conditions stimulating DNA damage might be due to the thermosensitive nature of the double-strand break repair enzyme, RexAB, responsible for the main recombination-mediated DNA repair pathway in lactococci (El Karoui et al., 1998).

Because DNA damage of *L. lactis* cells under respiration conditions is reduced by the presence of heme (Rezaïki et al., 2004), we next examined the effect of heme addition on the growth of two *L. lactis* strains in aerobic conditions (see the Section "Materials and Methods"). As shown in **Figures 6D,E**, the growth of the prophage-free strain was improved by the addition of heme at both 30°C and at 37°C. Conversely, the growth of IL1403 was improved at 30°C but was weakened at 37°C. This suggests that heme is more toxic to cells grown under aeration conditions at increased temperature. Moreover, this effect correlates with the presence of prophages.

These data demonstrate that deletion of the resident prophages from strain IL1403 positively affects the aerobic growth of lactococcal cells and significantly increases the positive effect of heme-activated respiration.

Prophage-Free Strain Has Improved Survival in the Digestive Tract of Germfree Mice

The majority of *L. lactis* strains do not survive the passage through the digestive tract. However, this bacterium's high potential for therapeutic use has stimulated the search for factors that increase its ability to persist in the gastrointestinal environment (Vesa et al., 2000; Kimoto et al., 2003). Under these conditions, bacterial physiology may be affected in a number of ways. For example, the different physicochemical stressors in the digestive tract (e.g., nitric oxide or bile salts) might activate the bacterial SOS response and subsequently, the induction of prophages. Even prior to induction, though, prophage-encoded functions might modify the host's PG and/or cell membrane, weaken the cell wall, and increase the sensitivity of lysogenic bacteria to lysozyme and other host defense factors (see above).

To evaluate the impact of the resident prophages on lactococcal survival and/or colonization in the digestive tract, we used a germfree mouse model. Specifically, we determined the viability of *L. lactis* IL1403 and IL6288 cells after oral gavages of either pure (IL1403, IL6288) or mixed (50:50 IL1403:IL6288) bacterial cultures (see the Section "Materials and Methods"). After passage through the stressful gastrointestinal conditions, strain IL6288 demonstrated significantly enhanced survival compared to the parent strain. As shown in **Figure 7**, the population of IL1403 cells decreased considerably during the experimental period (from approximately 10^5 CFUs/g feces at the 4th day after administration to 10^2 CFU/g feces at the 17th day, either in pure IL1403 culture or in mixed culture with IL6288). This is consistent with previously published results

demonstrating the poor survival of lactococcal strains in the digestive tract (Drouault et al., 1999; Vesa et al., 2000; Kimoto et al., 2003). In contrast, the population of IL6288 cells was relatively stable, remaining at approximately 10^7 to 10^6 CFUs/g feces both in the pure and the mixed culture during the experimental period (17 days).

We suggest that, the increased resistance to lysozyme (see above) can be an important factor determining the survival of IL6288 cells through the digestive tract of mice. On the other hand, the repression system(s) of IL1403 prophages may be rather sensitive to some specific gastrointestinal stresses. Thus, prophage induction may also contribute negatively to the survival of the lysogenic strain.

These data indicate that the deletion of the resident prophages was a decisive factor in the ability of *L. lactis* cells to survive within the gastrointestinal environment. This finding may have major implications for the use of *L. lactis* strains as biotherapeutic agents, because prophage-free derivatives may be able to function more effectively as vaccines and/or probiotic strains.

CONCLUSION

Our results reveal the significant contribution that resident prophages make to diverse aspects of *L. lactis* cell physiology, many of which are relevant to the use of *L. lactis* for industrial and health applications. They also indicate that a resident prophage, alone or in combination with others, shapes the cellular phenotype and influences its plasticity in response to the environment. Under experimental conditions that were comparable to those of industrial fermentation, the lactococcal prophages were mostly beneficial for host survival. However, the presence of prophages had clear adverse effects when *L. lactis* was exposed to the conditions of the gastrointestinal environment. Our construction of a prophage-free strain of *L. lactis* opens new avenues for both fundamental and applied studies of different aspects of lactoccoccal cell behavior, including the response to environmental stresses and bacteria-phage and bacteria-host interactions in a "pure" bacterial genetic background. As *L. lactis* continues to grow in economic importance, this work can assist in the rational selection of strains for both the industrial manufacturing of fermented dairy products and therapeutic applications for human and animal health.

AUTHOR CONTRIBUTIONS

EB, FC, and PL designed the experiments. AA and EB performed all the major experiments. AA and FC carried out the animal experiment. EB wrote the manuscript. All the authors approved the manuscript for publication.

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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.2018.02032/full#supplementary-material

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Standardized Preparation for Fecal Microbiota Transplantation in Pigs

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The intestine of pigs harbors a mass of microorganisms which are essential for intestinal homeostasis and host health. Intestinal microbial disorders induce enteric inflammation and metabolic dysfunction, thereby causing adverse effects on the growth and health of pigs. In the human medicine, fecal microbiota transplantation (FMT), which engrafts the fecal microbiota from a healthy donor into a patient recipient, has shown efficacy in intestinal microbiota restoration. In addition, it has been used widely in therapy for human gastrointestinal diseases, including *Clostridium difficile* infection, inflammatory bowel diseases, and irritable bowel syndrome. Given that pigs share many similarities with humans, in terms of anatomy, nutritional physiology, and intestinal microbial compositions, FMT may also be used to restore the normal intestinal microbiota of pigs. However, feasible procedures for performing FMT in pigs remains unclear. Here, we summarize a standardized preparation for FMT in pigs by combining the standard methodology for human FMT with pig production. The key issues include the donor selection, fecal material preparation, fecal material transfer, stool bank establishment, and the safety for porcine FMT. Optimal donors should be selected to ensure the efficacy of porcine FMT and reduce the risks of transmitting infectious diseases to recipients during FMT. Preparing for fresh fecal material is highly recommended. Alternatively, frozen fecal suspension can also be prepared as an optimal choice because it is convenient and has similar efficacy. Oral administration of fecal suspension could be an optimal method for porcine fecal material transfer. Furthermore, the dilution ratio of fecal materials and the frequency of fecal material transfer could be adjusted according to practical situations in the pig industry. To meet the potential large-scale requirement in the pig industry, it is important to establish a stool bank to make porcine FMT readily available. Future studies should also focus on providing more robust safety data on FMT to improve the safety and tolerability of the recipient pigs. This standardized preparation for porcine FMT can facilitate the development of microbial targeted therapies and improve the intestinal health of pigs.

Keywords: fecal microbiota transplantation, pigs, standard, stool bank, intestinal microbiota

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INTRODUCTION

The mammalian intestine harbors trillions of microbes (including bacteria, fungi, and viruses). These microbes play vital roles in the maintenance of gut homeostasis and host health (Sommer and Backhed, 2013). Currently, gut microbes are regarded as "microbial organs" functioning in nutrient absorption and metabolism (Backhed et al., 2007), host immune system development (Ivanov et al., 2009), the intestinal epithelium differentiation (Sommer and Backhed, 2013), and intestinal mucosal barrier maintenance (Garrett et al., 2010) in mammals. However, several factors, including host genetic characteristics, diet, environment, and antibiotic use, may affect the intestinal microbial diversity and function (Willing et al., 2011a; Schroeder and Backhed, 2016). Intestinal microbiota disorders can cause host gastrointestinal or non-gastrointestinal diseases (Brandt, 2013) such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and metabolic syndrome (Borody and Khoruts, 2011). Currently, antibiotics play important roles in intestinal disease prevention. However, antibiotics-induced resistance and spread of antibiotic-resistant pathogens have emerged as serious problems worldwide (Allen et al., 2014). Antibiotic therapy may also alter the intestinal microbial community and lead to intestinal microbial dysbiosis. Although probiotics (benign microbes) have shown efficacy in improving host intestinal health, their efficacy may be weak. This is because probiotic microbial composition is simple, and exogenous microbes may not colonize persistently to adapt to the dynamic intestinal homeostatic environment (Tannock et al., 2000; Sartor, 2004). Focus on the novel fecal microbiota transplantation (FMT) for the prevention and treatment of intestinal disorders has been increasing in the human medicine (Smits et al., 2013). More and more clinical applications of FMT have provided convincing proofs that modification of the intestinal microbiota is an effective therapy for intestinal dysbiosis-related diseases (Sekirov and Finlay, 2009; Smits et al., 2013). The urgent need for alternative therapies to antibiotics and the therapeutic potential of intestinal microbial manipulation promoted the development of FMT (Hamilton et al., 2012).

Fecal microbiota transplantation refers to the engraftment of fecal suspension from a healthy donor into the recipient's intestinal tract to restore the community and function of intestinal microbiota (Khoruts and Sadowsky, 2016). The first use of donor feces as a therapeutic agent for food poisoning and diarrhea was recorded in the *Handbook of Emergency Medicine* by a Chinese, Hong Ge, in the 4th century (Drew, 2016). During the 16th century, Shizhen Li described the effective treatment of many intestinal diseases with fecal material in the *Compendium of Materia Medica* and the fecal suspension was called "Huanglong Tang" (Zhang et al., 2012). FMT has been applied in veterinary medicine to treat intestinal disorders of ruminants and equines since the 17th century (Borody et al., 2004). In human medicine, the FMT was firstly used to treat pseudomembranous enterocolitis performed by Eiseman et al. (1958). Presently, FMT is highly recognized as an effective treatment option for recurrent *Clostridium difficile* infection (CDI) in human. It is gradually being used as a therapy for some diseases including IBD, IBS, intractable constipation, and intestinal immunodeficiency in human (van Nood et al., 2013; Borody et al., 2015). The representative cases for FMT in mammals are shown in **Table 1** (Anderson et al., 2012; Ridaura et al., 2013; van Nood et al., 2013; Sivan et al., 2015; Diao et al., 2016; Xiao et al., 2017). Growing evidences have revealed the similarity between intestinal microbiota of recipients and donors as well as the normalization of gut microbial compositions and functions in recipients after FMT therapy in human (Khoruts et al., 2010; Li et al., 2016). Rather than continuing to disturb the composition of normal intestinal microbiota, FMT efficiently restores gut microbiota of the recipients (Kelly et al., 2014). Currently, pigs encounter multiple stressors and overuse of antibiotics (Campbell et al., 2013; Barton, 2014), which destroy the normal community structure of intestinal microbiota and lead to the emergence of multidrug-resistant microorganisms in the intestine (Laxminarayan et al., 2013; Xu et al., 2015). The use of antibiotics in livestock farming was gradually banned (Casewell et al., 2003) due to that antibiotics-induced resistance, spread of antibiotic-resistant pathogens, and antibiotic residues in foods have emerged as serious problems worldwide (Allen et al., 2014). Thus, finding alternatives to antibiotics is important to livestock farming and food safety. Because of the similarities between human beings and pigs in terms of intestinal microbiota and nutritional physiology (Garthoff et al., 2002; Heinritz et al., 2013), FMT may be a promising method for intestinal microbiota reconstitution and health improvement in pigs. However, feasible procedures for performing FMT in pigs remain unclear. In this study, we summarize a standardized preparation for porcine FMT, which is used in the pig industry to prevent and treat intestinal disorders.

DONOR SELECTION

Optimal donors should be selected to ensure the efficacy of porcine FMT and reduce the risks of transmitting infectious diseases during the transfer of fecal suspension. Selection of donors which are not fit may have adverse effects on the stability and tolerance of the intestinal microbiota, thereby causing intestinal rejection. Importantly, porcine FMT may lead to pathogen transmission because donor feces may carry pathogenic and conditional pathogenic microbes. Thus, potential donors should be selected using strict exclusion criteria, including the genetic backgrounds, phenotypic characteristics, infectious diseases, common pathogens, and other indicators. We proposed the standard for donor screening in porcine FMT based on studies related to human donors screening and pig production.

Studies have revealed that maternal-line first-degree relatives or intimate contacts (e.g., mating, common-bond) can share environmental risk factors (Owens et al., 2013). Immediate family members may contain a mass of the same microbial species in their gastrointestinal tract. As a result, recipients are more tolerant to gut microbiota from donors who are immediate family members (Kelly et al., 2015). Intestinal microbial community has been recognized to be potentially associated with the pathogenesis of diseases and intestinal disorders

TABLE 1 | Characteristics of donors and recipients, transplantation method, and effect of FMT on recipients.

Donors	Recipients	Transplantation method	Effect on recipients
Healthy human (Anderson et al., 2012)	Patients with IBD	Colonoscopy/enema or enteral tube	Prevent infectious diarrhea in patients with IBD
Healthy human (van Nood et al., 2013)	Patient with recurrent CDI	Colonoscopy orduodenal Infusion	Normalize bowel functioning and treat CDI
Jackson Laboratory (JAX) mice (Sivan et al., 2015)	Taconic Farms (TAC) mice	Oral gavage	Facilitate antitumor immunity
Obese twin and lean twin (Ridaura et al., 2013)	Germ-free mice	Oral gavage	Transfer the characteristics of donor obesity from human to mice
Yorkshire pigs, Tibetan pigs, and Rongchang pigs (Diao et al., 2016)	Germ-free mice	Oral gavage	Transfer the gut characteristics from pigs to mice
Yorkshire and Tibetan pigs (Xiao et al., 2017)	Commercial hybrid newborn piglets	Oral gavage	Improve the intestinal anti-inflammatory function

(Bakken et al., 2011). Phenotypic features and behaviors are the most intuitive reflections of health status in pigs. In addition, it is important to verify whether there is a history of genetic disease based on genetic spectrum analysis of ancestors of potential donors. Moreover, the potential risks of transmitting infectious diseases should be assessed. Importantly, the donor pig candidates should be isolated from other pigs to avoid the transmission of pathogens among individuals. Below are the exclusion criteria in detail (**Box 1**).

To ensure that donors are of safety for porcine FMT, serological testing and stool testing should be performed to monitor infectious pathogens and other risk factors (**Table 2**). Common infectious diseases-related pathogens in the pig industry are as follows (Meurens et al., 2012; Denner and Mueller, 2015; Lee, 2015; Renukaradhya et al., 2015; Hu et al., 2017).

- Porcine infectious diseases-associated viruses include hog cholera virus, porcine pseudorabies virus, porcine parvovirus, porcine influenza virus, porcine encephalitis virus, porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, porcine epidemic diarrhea virus, transmissible gastroenteritis virus, rotavirus, bocavirus, corona virus, cytomegalovirus, and porcine enterovirus.
- Porcine infectious diseases-associated bacteria include pathogenic *Escherichia coli*, *Salmonella*, *Haemophilus parasuis*, *Bordetella bronchiseptica*, *Toxigenic pasteurellamultocida, Actinobacillus pleuropneumoniae*, pathogenic *Streptococcus, Brachyspira hyodysenteriae, Lawsonia intracellularis*, *Clostridium perfringens*, and *Shigella*.
- Porcine infectious diseases-associated parasites include Toxoplasma gondii, porcine whipworm, porcine ascarid, Clonorchis sinensis, Coccidia, and Cysticercus cellulosae.

Serological tests are widely used to detect infectious diseases-associated pathogens based on the antigen–antibody binding reactions *in vitro*. We can use the antigen–antibody binding reactions to monitor the invasive pathogens which could stimulate host to generate the corresponding antibodies in serum. Blood samples are obtained from the porcine blood vessel and coagulated at 4°C. The serum is finally collected from the supernatant after the coagulated blood samples are centrifuged. Serological tests mainly include the serum neutralization test, hemagglutination inhibition test, enzyme-linked immunosorbent assay (ELISA), agar diffusion test, and complement fixation test. Specifically, ELISA tests have been a powerful approach to detect the infectious diseases-associated pathogens in serological tests because its several advantages, including good sensitivity and specificity (Sattler et al., 2014; Shin et al., 2015). Given that feces may carry some infectious pathogens, stool testing for donor pigs is crucial to reduce the infectious risk of porcine FMT directly. We should extract the fecal DNA from donor pigs and then perform PCR amplification reaction to

BOX 1 | Key issues of criteria for donor screening in porcine FMT.

Selection of phenotypic characteristics:

► Age <5 months preferably.
► Normal body temperature of 38∼39.5°C (rectal temperature).
► *Ad libitum* access to feed and water.
► Normal behavioral characteristics (including breathing status, feeding behavior, excreting behavior, social behavior, and reproductive behavior).
► No hemorrhagic spot and wound in body skin.
► No other abnormal behaviors.

Risk of infectious agents for donor screening:

► Recent (<2 weeks) vaccination with live attenuated virus.
► Recent (<2 weeks) copulation (or artificial insemination).
► Contact with other pigs with a history of infectious diseases in the past.
► Appearance of diarrhea, constipation or hematochezia.
► History of exposure to other endemic diarrhea areas.
► History of using antibiotics or other drugs.

TABLE 2 | General serological testing and stool testing to monitor potentially infectiouspathogens.

General serological testing	Stool testing
Hog cholera virus	Porcine epidemic diarrhea virus
Pseudorabies virus	Transmissible gastroenteritis virus
Porcine parvovirus	Rotavirus
Porcine influenza virus	Bocavirus
Porcine encephalitis virus	Corona virus
Porcine reproductive and respiratory syndrome virus	pathogenic *Escherichia coli*
Porcine circovirus type 2	*Salmonella*
Cytomegalovirus	Porcine Whipworm
Haemophilus parasuis	Porcine ascarid
Bordetella bronchiseptica	Clonorchis sinensis
Toxigenic pasteurellamultocida	Coccidia
Actinobacillus pleuropneumoniae	Cysticercus cellulosae
Pathogenic *Streptococcus*	*Brachyspira hyodysenteriae*
Toxoplasma gondii	*Lawsonia intracellularis*
Porcine enterovirus	*Clostridium perfringens*
	Shigella

confirm whether corresponding pathogens are present in the feces (Borewicz et al., 2015). RNA-virus pathogens could be detected using a combined method of the fecal RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Specific primers should be designed according to the gene sequences of the standard pathogens. Whether the potential pathogens are present in feces could be judged through the corresponding amplified products. Moreover, ELISA test could be also used to detect pathogens-associated antigens in feces directly to confirm whether corresponding pathogens (such as porcine epidemic diarrhea virus) are present in the feces (Opriessnig et al., 2014).

Recent studies have used FMT to restore the phenotypes of donors in recipients, suggesting the key roles of intestinal microbiota in mammalian host health such as obesity (Ridaura et al., 2013; Goodrich et al., 2014), colon cancer (Wong et al., 2017), pathogens resistance (Lawley et al., 2012), and anti-tumor immunity (Sivan et al., 2015). Thus, it is crucial to select optimal pig donors because the fecal microbiota compositions of donors may have critical effects on the efficacy of the porcine FMT. Growing evidences have suggested that intestinal microbiota-mediated colonization resistance against intestinal pathogens (Buffie and Pamer, 2013). Several studies have revealed that probiotics (include *Lactobacillus*, *Bifidobacterium*, and *Bacillus* spp.) contribute to decrease the level of colonization with enterotoxigenic *E. coli* (ETEC) and maintain intestinal microbial balance (Konstantinov et al., 2008; Chiang et al., 2015). A recent study showed that *Lactobacillus johnsonii* may have the potential efficacy to reduce *Salmonella* invasion of intestinal epithelium in pigs (Casey et al., 2004). Thus, we should select optimal donor pigs which have high-abundance "functional microbes" according to the results of fecal microbial compositions and functions analyzed by 16S rDNA sequencing and metagenomics in porcine FMT.

PREPARATION OF FECAL MATERIAL

Previous studies have suggested that at least 30 g of fecal material should be used for the FMT in human (Mattila et al., 2012; Satokari et al., 2015). However, varieties of stool diluents, such as sterile saline (0.9%, NaCl) and phosphate buffer solution (PBS) (Gough et al., 2011; Brandt and Aroniadis, 2013) can be used as alternatives. The stool material should be diluted 3–5 times with large volumes of the solvent and buffer solution (Cammarota et al., 2017). Considering the heterogeneity in the fecal microbes between different individuals or donors (Smits et al., 2013), we suggested that the dilution ratio of the fecal materials could be adjusted in porcine FMT. Importantly, all equipment used in the fecal suspension preparation should be strictly sterile.

Preparation of Fresh Fecal Material

Fresh feces used for the porcine FMT should be transported on ice to a specialized laboratory within 2 h after defecation (Lee et al., 2016). Approximately, 30 g fecal samples are diluted with 150 ml sterile saline and homogenized in a standard blender. The slurry is then filtered three times through gauze (Mattila et al., 2012), strainer, or 0.25 mm stainless steel sieves to eliminate the undigested and small particulate matter in the fecal suspension (Owens et al., 2013). We suggest that the fecal suspension could be centrifuged at 6,000 × *g* for 15 min (Hamilton et al., 2012). The precipitate, without the supernatant, is re-suspended in fresh sterile saline, and then, the resulting suspension should be transferred to the recipients directly (Hamilton et al., 2012). Because the fecal microbes are predominantly anaerobes, reducing the time of oxygen exposure in fecal material preparation is crucial to ensuring fecal microbial viability. All fecal material preparation processes should be carried out at a room temperature of 20–30°C; preferably in an anaerobic incubator (Rossen et al., 2015) (**Figure 1**).

Preparation of Frozen Fecal Material

Preparation of frozen fecal suspension is an optimal choice to ensure sample availability, whenever there is the need for porcine FMT (Cammarota et al., 2017). Comparative studies have demonstrated that frozen fecal material does not only simplify the practical steps of clinical human FMT, but also has the similar efficacy to fresh fecal material (Satokari et al., 2015). To improve the fecal microbial survival rates during the cryopreservation, fresh stool samples should be diluted with sterile saline homogenized and filtered using the protocol used in the preparation of the fresh fecal material in porcine FMT. Subsequently, the resulting suspension should be added to glycerol to get a final concentration of 10% (Lee et al., 2016). Finally, the fecal suspensions are labeled accurately and then stored at −80°C (Satokari et al., 2015). Importantly, frozen fecal material should be stored at a low temperature as soon as possible (−80°C refrigerator or liquid nitrogen) to ensure the fecal microbial survival. When there is the need for porcine FMT, the frozen fecal suspension should be thawed at 37°C (water bath) (**Figure 1**). Upon frozen fecal suspension

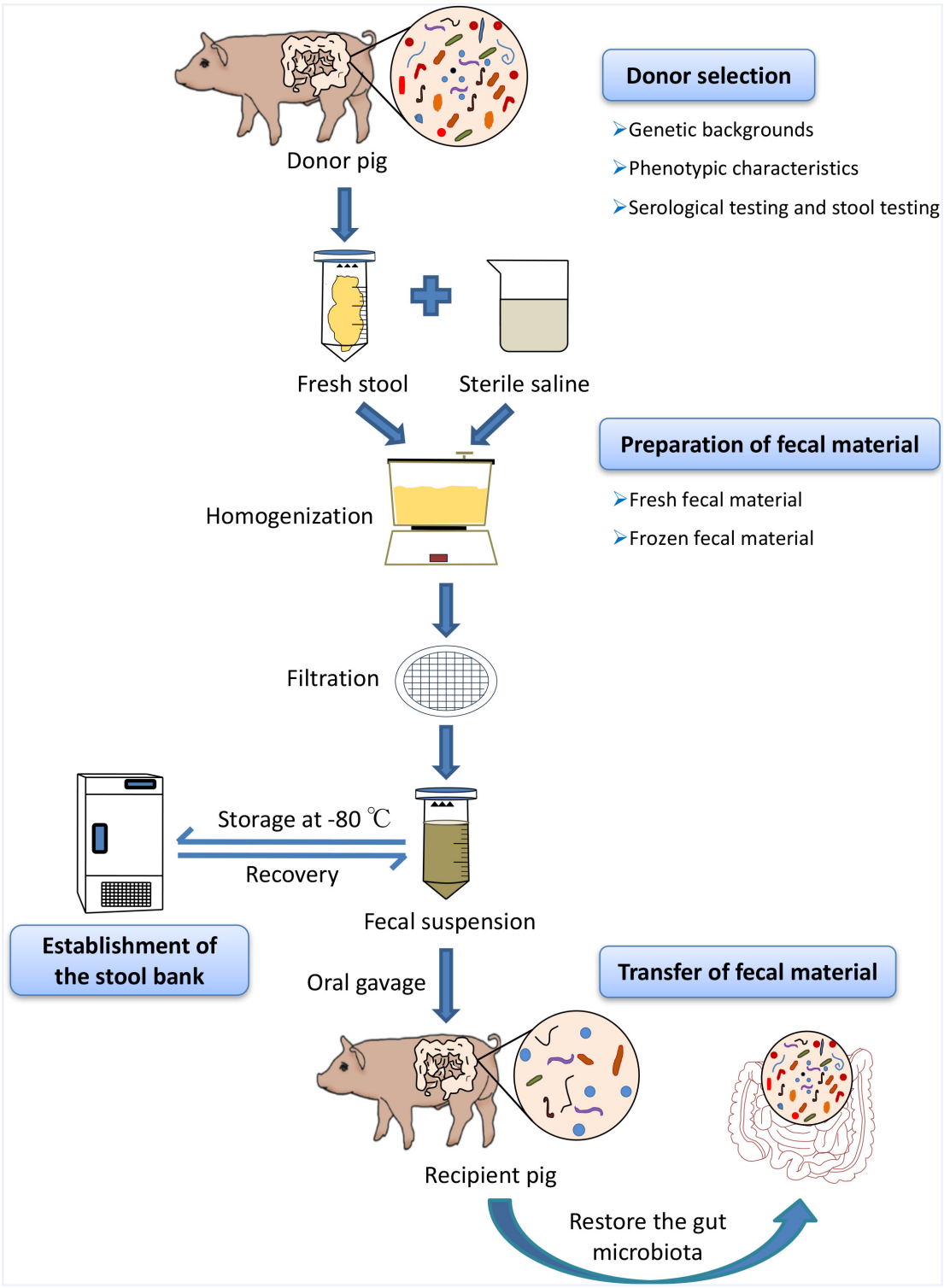

FIGURE 1 | Schematic workflow of fecal microbiota transplantation (FMT) in pigs. At first, optimal donors should be selected to ensure the efficacy of porcine FMT and reduce the risks of transmitting infectious diseases during the transfer of fecal suspension via strict genetic backgrounds investigation, phenotypic characteristics, and serological test and stool testing. Fresh feces from healthy donor pigs were homogenized with sterile saline (0.9% NaCl) in blender and the stool materials should be diluted 3–5 times with large volumes of the buffer solution. The slurry was then filtered through sterile sieves and the suspension is either transferred to the recipients or mixed with 10% sterile glycerol to store at –80°C immediately. When there is the need for porcine FMT, the frozen fecal suspension should be thawed at 37°C (water bath).

thawing, sterile saline solution can be added to obtain a required concentration and the infusion of fecal suspension should be implemented as soon as possible at room temperature (Satokari et al., 2015).

TRANSFER OF FECAL MATERIAL

In the human medicine, different routes for fecal material transfer have been reported, including the upper gastrointestinal tract (oral administration); middle gastrointestinal tract (endoscopy, nasogastric tube, nasal jejunum, and nasal duodenum); lower gastrointestinal tract (colonoscopy and enema) (Drekonja et al., 2015). In mice models, oral gavage and cohousing are used to transfer the fecal microbiota from donor mice to recipient mice (Willing et al., 2011b). Cohousing takes advantage of the natural tendency of mice to feed on the feces of littermates (Endt et al., 2010). Recent studies have suggested that porcine FMT via oral gavage using fecal suspension from donor pigs can improve growth performance, intestinal barrier, and innate immune function in recipient pigs (Hu et al., 2017; Xiao et al., 2017). However, a recent study has shown that porcine FMT via oral gavage have a negative effect on the growth performance of pigs (McCormack et al., 2018). Considering the practicality of fecal material transfer in pig production, we proposed that oral administration of fecal suspension could be an optimal method for fecal material transfer in porcine FMT (**Figure 1**). Moreover, the prepared fecal materials could be mixed with diet for direct feeding or formulated into multi-layered capsules to be administrated with diet or oral gavage directly (Hirsch et al., 2015; Youngster et al., 2016). The frequency of fecal material transfer could also be adjusted according to practical situations in pig industry. The transfer of fecal material should be performed as soon as possible because the microbial cells are fragile and sensitive.

In the human medicine, patients with CDI should be treated with vancomycin at least for 3 days and then discontinue antibiotic for 12–48 h before FMT in order to inhibit the abundance of *C. difficile* in the intestine and reduce the load of intestinal microbes (Hamilton et al., 2012; Cammarota et al., 2017). However, recent studies on FMT in animal models have shown that antibiotic pretreatment could reduce the diversity of native microbiota and may be not beneficial to the establishment of exogenous microbiota (Manichanh et al., 2010). Thus, we suggest that all recipient pigs don't receive the antibiotics over 2 weeks before FMT and are free to water and diet.

ESTABLISHMENT OF THE STOOL BANK

To meet the potential large-scale requirement in the pig industry, it is important to establish a stool bank to make porcine FMT readily available. Fecal donors need to be recruited beforehand and rigorously screened systematically in porcine FMT. Several key issues should be considered. First, strict screening of donors including stool and serology testing is essential to prevent the transfer of infectious pathogens and reduce the risk of susceptibility in recipient (Smith et al., 2014). In addition, the supply of donor feces must meet the growing demand. Thus, we suggested that donor pigs should be segregated from other pigs since the stool and serological tests are conducted. After a series of stool and serological tests, feces will be continuously collected from eligible donor pigs (Kazerouni et al., 2015). The fecal material prepared for transplantation should be mixed with 10% sterile glycerol and stored at −80°C within 6 months, without diminishing the therapeutic efficacy (Costello et al., 2015). Establishment of stool bank not only saves the time for FMT, but also reduces cost since a single excellent donor can serve for multiple recipients (Hamilton et al., 2012). Furthermore, stool banks retain information about donors, thereby ensuring traceability during the FMT therapeutic process (Terveer et al., 2017). Procedures for the stool bank must comply with basic safety rules. Thus, the establishment of stool bank may optimize the practical procedures of porcine FMT and facilitate the development of this therapeutic method.

SAFETY OF FMT

Currently, most clinical experiences that focused on the use of FMT in humans have shown that FMT is safe in humans. Patients treated with FMT did not experience any serious adverse events (Borody and Khoruts, 2011), except minor symptoms such as slight diarrhea, constipation, vomiting, and abdominal discomfort (Kump et al., 2013; Rossen et al., 2015). It has been reported that the most common adverse events after FMT treatment of CDI and IBD include diarrhea, abdominal distention, abdominal cramps, constipation, and fever in human medicine (Cui et al., 2015; Agrawal et al., 2016). Some patients who received FMT treatment may suffer diarrhea on the day of transplantation, but the diarrhea generally disappears in a short term. In human medicine, adverse events are often associated with methods used to deliver fecal material, underlying diseases, and physical conditions of patients (Sokol et al., 2016). However, the evidence on the safety of FMT in pigs is relatively limited because porcine FMT has been applied before large and long-term comparable trials were conducted to assess the safety. Although recent studies have reported that the fecal microbes from donors extensively colonized in the recipients and coexisted with intestinal microbes of recipients over 3 months in human medicine (Li et al., 2016). The effects of fecal metabolites and heterogeneous substances on the intestinal microbiota of recipients are still unclear. Considering that pork is the main meat food for human, we should carefully reflect on the potential effects of porcine FMT on pork food safety. Firstly, strict donor screening is essential for reducing the risks of pathogen transmission during porcine FMT. Importantly, the potential antibiotics and drugs residues in pork of recipient pigs should be avoided via strict donor selection in which donor pigs have the history of using antibiotics or other drugs in diets or injection should not be used. Moreover, the potential

effects of fecal metabolites and heterogeneous substances on pork safety should also be further investigated in porcine FMT.

PERSPECTIVES

Based on the FMT procedures in human medicine and the pig industry, we proposed the standardized preparation (including donor selection, fecal material preparation, and fecal materials transfer) for the porcine FMT used in pig production. This standardized preparation for porcine FMT can increase the feasibility in the clinical operation for FMT and improve the intestinal health of pigs. Considering that the intestinal microbiota of piglets may be immature and sensitive to intestinal microenvironment, we conclude that porcine FMT on piglet production stage may be most effective. It is crucial to select optimal pig donors because the fecal microbes from donors may confer efficacy on the porcine FMT and the transfer of fecal materials may increase the risk of infectious pathogens transmission. Growing evidences have linked long-term diet habits to the composition of fecal microbiota (Matijasic et al., 2014). To ensure the fecal microbes from donor pigs can adapt to the intestinal microenvironment of recipient pigs, we suggest that the diets formulated according to NRC requirements for donor pigs and recipient pigs should be same if donor pigs and recipient pigs are same breed. It is known to us that there is a difference between pig breeds in diets because of the difference in nutrients requirements. Considering that there is no diet requirement for donors and recipients in human FMT, we suggest that the diets should be formulated according the nutrients requirements for different pig breeds, respectively. Although no relevant study has evaluated the survival rate of fecal microbes (including facultative anaerobic microbes, strict anaerobic microbes, and aerobic microbes) exposed to the atmosphere conditions (Yamashiro, 2017), the process of fecal material preparation will directly affect the efficacy of porcine FMT. Thus, it is important to shorten the time for fecal material preparation and transfer as soon as possible during porcine FMT. Considering that the effects of fecal metabolites and heterogeneous substances on the intestinal microbiota in recipients is still unclear, we should further improve the efficiency of fecal materials purification (besides the methods of filtration and centrifugation) to maximize the potential of porcine FMT. The method of fecal material transfer may also affect the efficacy of porcine FMT. Some microbes belonging to the phylum Firmicutes can form spores, which require growth factors in the upper digestive tract to survive (Burns et al., 2010). In addition, some microbes belonging to phylum Bacteroidetes may be denatured in the acidic environment of the stomach during the transfer (Damman et al., 2012). Therefore, it is crucial to identify the functional microbiota and choose an optimal method for delivery. A recent report has shown that the fecal microbial compositions in recipients are highly similar to that in donors by 14 days post-transplantation in human FMT (Khoruts et al., 2010). It is still difficult to conclude when the effect of FMT will be visible and how long the effect of FMT will be last because the purposes and experimental conditions for porcine FMT may be different in different assay. Thus, more studies are needed to identify the intestinal microbial dynamics induced by porcine FMT and when the effect of porcine FMT should become visible.

Fecal microbiota transplantation has been widely used in human therapy for gastrointestinal diseases, including CDI, IBD, and IBS. Interestingly, some recent studies have used FMT to restore the phenotypes of donors in recipients and suggested the key roles of intestinal microbiota in mammalian host health such as obesity (Ridaura et al., 2013; Goodrich et al., 2014), colon cancer (Wong et al., 2017), pathogens resistance (Lawley et al., 2012), and anti-tumor immunity (Sivan et al., 2015). Thus, characterization of porcine intestinal microbial functions via FMT is of great significance and requires further investigation. The underlying mechanism of FMT and the gut microbes conferring efficacy on FMT are still unclear. Thus, precise manipulation of gut microbiota through probiotics (benign microbes) has currently emerged as a promising therapeutic strategy for gastrointestinal disorders (Foxx-Orenstein and Chey, 2012; Lawley et al., 2012; Buffie et al., 2015; Schieber et al., 2015). Recently, developed high-throughput approaches (including metagenomics, metatranscriptomics, and metabolomics) have been applied to identify the association between host health and the composition and functionality of gut microbiota (Kootte et al., 2012; Costea et al., 2017). Further studies should identify specific intestinal microbial candidates that are specific to disease pathogenesis and provide novel therapeutic strategies to take advantage of such beneficial microbes (Everard et al., 2013).

AUTHOR CONTRIBUTIONS

JH and LC wrote the paper with the help of all authors. JH, LC, YT, CX, BX, MS, WZ, SZ, XW, LL, YY, TY, YN, QH, and XX prepared the materials for this manuscript. XY revised this manuscript. All authors read and approved the final version of the manuscript.

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Antimicrobial Susceptibility and Virulence Surveillance of *Campylobacter* spp. Isolated From Patients in Two Tertiary Medical Centers in Taiwan

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Campylobacter spp. may cause fever, vomiting, and diarrhea in humans. Antibiotic treatment is suggested for patients with severe campylobacteriosis. However, the interpretative criteria for antibiotic susceptibility are inconsistent between Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing guidelines. The aim of the study is to investigate the antibiotic susceptibility and prevalence of cytolethal distending toxin genes and to evaluate antibiotic susceptibility testing methods in the clinical laboratories of two tertiary medical centers in Taiwan. In total, 236 bacterial isolates were collected between 2001 and 2014. The disk diffusion and *E*-test methods were used to evaluate the antibiotic susceptibility, and broth dilution results were used as a reference. The virulence genes *cdtA*, *cdtB*, *cdtC*, and *ceuE* were detected through polymerase chain reaction. The antimicrobial sensitivity rates for erythromycin, ciprofloxacin, and tetracycline using the broth dilution assay were 80.4, 5.4, and 3.4%, respectively. No significant differences were observed in the antibiotic susceptibility of the isolates obtained from southern and northern Taiwan. However, some differences were observed between species. The susceptibility test for erythromycin (disk diffusion) showed that the isolates with small inhibition zone diameters were all resistant, and five isolates (4.0%) with large IZDs were non-sensitive. The error rate of the disk diffusion method according to the CLSI M45-A3 guideline was 5.4% (8/148). The incompatibility rates between the *E*-test and broth dilution methods for erythromycin, ciprofloxacin, and tetracycline were 8.0, 5.3, and 1.3%, respectively. The positive rates of the genes *cdtA* and *cdtC* were considerably higher in *Campylobacter jejuni* than in *C. coli*. Erythromycin is recommended as the first choice of treatment for campylobacteriosis. The disk diffusion method is suitable for prescreening *Campylobacter* susceptibility by using the CLSI M45-A2 and EUCAST criteria

(low error rate of 3.4%). If antibiotic treatment fails or IZDs are between 6 and 20 mm, minimum inhibitory concentration testing by using the *E*-test method is highly recommended because the results of the *E*-test and broth dilution methods exhibit high agreement. The error rate of disk diffusion method using CLSI M45-A3 criteria is only slightly higher than B, which is also a suitable criteria.

Keywords: *Campylobacter*, antibiotic susceptibility, virulence factor, disk diffusion, *E*-test

INTRODUCTION

Campylobacter spp. are zoonotic bacteria. First named in 1963, the *Campylobacter* genus currently comprises more than 20 species (Sebald and Veron, 1963). The main causes of human infection are *Campylobacter jejuni* and *Campylobacter coli* (van der Beek et al., 2010). There are a few reports of infections in other species (Man, 2011). People who are in close contact with animals, consume raw meat, are employed in animal husbandry, or own pets are at a high risk of campylobacteriosis (Dasti et al., 2010). *Campylobacter* infections may cause watery diarrhea, abdominal cramps or fever (Kaakoush et al., 2015). Severe campylobacteriosis has also been reported (Ge et al., 2013). Antibiotic treatment is suggested for patients with severe campylobacteriosis (Kaakoush et al., 2015). There are several common antimicrobial agents for erythromycin, ciprofloxacin, tetracycline, and doxycycline in campylobacteriosis therapy (Ge et al., 2013). The Clinical and Laboratory Standards Institute (CLSI) M45 guidelines recommend the susceptibility test by using the disk diffusion and broth dilution minimum inhibitory concentration (MIC) methods (Clinical and Laboratory Standards Institute, 2016). According to the M45-A2 (2010 version) guidelines, *Campylobacter* can be reported as being resistant to erythromycin and ciprofloxacin if no inhibition zone is observed in disk diffusion screening. If the inhibition zone diameter (IZD) is >6 mm, the susceptibility can be confirmed using the MIC method according to the interpretive criteria. The sensitivity of *Campylobacter* to tetracycline and doxycycline can only be confirmed using the broth micro-dilution method. In the M45-A3 (2016 version) guidelines, the IZD interpretive criteria were newly added, but the MIC interpretive criteria were the same as those in the previous version. However, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines include another criterion for susceptibility, which is different from the CLSI criterion for susceptibility in *C. jejuni* and *C. coli* (EUCAST, 2018). Whether the use of the 2016 version criterion is feasible and whether the IZDs are consistent with the MIC results remain unclear in Taiwan. In addition, whether the results of the *E*-test method for MIC determination are consistent with those of the broth micro-dilution method, which is more complex and expensive than the *E*-test, requires investigation.

Studies have shown that the resistance of *Campylobacter* is increasing, particularly its resistance to commonly prescribed ciprofloxacin and tetracycline (Luangtongkum et al., 2009). Because most hospitals in Taiwan do not report the antimicrobial susceptibility of *Campylobacter* spp., the resistance of *Campylobacter* isolates is unknown in Taiwan. In this study, we intended to examine the antimicrobial susceptibility of *Campylobacter* to provide reliable data for clinical use and for reducing the dependence on empirical medication and literature recommendations when treating campylobacteriosis. In addition, susceptibility data from different years were analyzed to identify drug resistance trends. We analyzed susceptibility data from different areas in Taiwan to determine whether regional differences exist in antibiotic resistance.

Campylobacter produces several cytotoxins, including the extensively studied cytolethal distending toxin (CDT), which is also produced by many gram-negative bacteria (Bolton, 2015). *Campylobacter* species that produce toxins are more likely to cause serious illnesses than species that do not (Fais et al., 2016). Analysis of virulence factors is helpful to provide evidence for active treatment.

MATERIALS AND METHODS

Analysis of Case Numbers of Campylobacteriosis

Analysis of the number of cases of campylobacteriosis in Chang Gung Memorial Hospital, Linkou, from 2005 to 2014 showed that the average number of cases of intestinal infection per year was 181. The average number of cases of parenteral infection per year was 9 in the same period; however, the number of cases was slightly higher in 2008–2011 probably because of the influence in the area (**Supplementary Material A**). The positive rate of virulence was higher in children than in adults, and the rate has not changed significantly from 2010 to 2014 (**Supplementary Material B**). No seasonal differences in the case numbers were observed, but the positive rate was higher in winter because fewer samples were collected in winter (**Supplementary Material C**). Intestinal infection was predominantly observed in children aged 1–6 years, accounting for 34.4% of the total case number; however, parenteral infection was predominantly observed in adults aged > 45 years, accounting for 68.6% of the total case number (**Supplementary Material D**).

Bacterial Isolates and Growth Conditions

In total, 236 bacterial isolates were analyzed. The isolates consisted of 165 isolates from patients in northern Taiwan (2001–2012: 59 isolates of *C. jejuni* and 29 isolates of *C. coli*; 2013–2014: 45 isolates of *C. jejuni*, 31 isolates of *C. coli*, and 1 isolate of *C. fetus*) and 71 isolates from patients in southern Taiwan (2013–2014: 57 isolates of *C. jejuni* and 14 isolates of *C. coli*). Stool (193), blood (41), and dialysate (2) samples of the patients were collected. The clinical isolates obtained from

northern and southern Taiwan were collected consecutively in different periods from two tertiary medical centers, namely Linkou Chang Gung Memorial Hospital and Kaohsiung Chang Gung Memorial Hospital, respectively. All samples were plated on Columbia blood agar plates containing 5% sheep blood agar and were incubated at 42°C in a microaerophilic atmosphere(CampyPak; BBL; Becton Dickinson, Rutherford, NJ, United States) for 24–48 h. Species identification was performed through matrix-assisted laser desorption ionization time of flight mass spectrometry and multiplex polymerase chain reaction (PCR) assay (Wang et al., 2002; Schulthess et al., 2013; Randall et al., 2015). The accuracy of MALDI-TOF mass identification in *Campylobacter* is close to 100% (Randall et al., 2015). The mass identifications and data analyses were performed using the Bruker LT microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). A direct smear method with a 70% formic acid overlay was used. The gene of PCR is *hipO* from *C. jejuni*; glyA from *C. coli*, *C. lari*, and *C. upsaliensis*; *sapB2* from C. fetus subsp. fetus; and the internal control 23S rRNA.

Broth Micro-Dilution Method

Minimum inhibitory concentration were determined for the 236 *Campylobacter* isolates using a commercial susceptibility plates (Sensititre, Trek Diagnostic Systems) containing serial double fold dilutions of nine antimicrobial agents, namely erythromycin, azithromycin, telithromycin, clindamycin, tetracycline, nalidixic acid, ciprofloxacin, florfenicol, and gentamicin. The broth dilution protocol was based on that provided in the CLSI guidelines (Clinical and Laboratory Standards Institute, 2016). The results of susceptibility to erythromycin, ciprofloxacin, and tetracycline were interpreted using epidemiological cutoff values based on MICs reported in the CLSI guidelines. The CLSI interpretive criteria for the other six antibiotics are not available. Quality control is the operation of *C. jejuni* ATCC 33560 specified by the manufacturer.

Disk Diffusion Method

Susceptibility testing of erythromycin, tetracycline and ciprofloxacin was performed for 148 isolates of *Campylobacter* spp. obtained from patients in northern (n = 77) and southern (n = 71) Taiwan from 2013 to 2014 by using the disk diffusion method. The disk diffusion protocol, quality control and the interpretative criteria used were based on the CLSI guidelines (Clinical and Laboratory Standards Institute, 2016). Culture condition is Mueller Hintion agar with 5% sheep blood, 42°C for 48 h in a microaerophilic atmosphere(CampyPak; BBL; Becton Dickinson, Rutherford, NJ, United States).

E-Test Method

The *E*-test was performed for 75 isolates (40 from northern and 35 from southern Taiwan) selected randomly from the 148 isolates obtained during 2013–2014; erythromycin, ciprofloxacin, and tetracycline were used for this test. The MICs of erythromycin, ciprofloxacin, and tetracycline for the *Campylobacter* isolates were determined using the standard *E*-test method. After inoculation with McFarland 0.5 turbidity standard *Campylobacter* culture, 90-mm plates containing *E*-test strips (AB BIODISK, Solna, Sweden) were incubated at 42°C for 48 h in a microaerobic environment (CampyPak; BBL; Becton Dickinson, Rutherford, NJ, United States). Quality control is the operation of *C. jejuni* ATCC 33560.

Virulence Factor Analysis

The genes encoding CDT, namely *cdtA*, *cdtB*, *cdtC*, and *ceuE*, were detected in the 148 isolates obtained from 2013 to 2014 through PCR, as previously described (Bang et al., 2003). The primers sequence is *cdtA* (GNW: 5′-GGAAATTGGATTTGGGGCTATACT-3′; IVH: 5′-ATCACAAGGATAATGGACAAT-3′; Amplicon: 165 bp), *cdtB* (VAT2: 5′-GTTAAAATCCCCTGCTATCAACCA-3′; WMI-R 5′-GTTGGCACTTGGAATTTGCAAGGC-3′; Amplicon: 495 bp), *cdtC* (WMI-F: 5′-TGGATGATAGCAGGGGATTTTAAC-3′; LPF-X: 5′-TTGCACATAACCAAAAGGAAG-3′; Amplicon: 555 bp), *ceuE* for *C. jejuni* (*ceuEJ*) (JEJ1: 5′-CCTGCTCGGTGAAAGTTTTG-3′; JEJ2: 5′-GATCTTTTTGTTTTGTGCTGC-3′; Amplicon: 794 bp), *ceuE* for *C. coli* (*ceuEC*) (COL1: 5′-ATGAAAAAATATTTAGTTTTTGCA-3′; COL2: 5′-ATTTTATTATTTGTAGCAGCG-3′;Amplicon: 894 bp). The conditions of PCR are 94°C1 min/42°C2 min/72°C3 min/(30 cycles) at *cdtA/cdtB/cdtC* and 95°C3 min/57°C30 s/72°C1 min/30 cycles at *ceuEJ* and 95°C3 0 s/57°C30 s /72°C1 min/30 cycles at *ceuEC*.

Statistical Analysis

The Student t-test was used to determine the significance of differences. A difference was considered statistically significant if $p < 0.05$.

Ethics Approval and Consent to Participate

The isolate was not for the study, but for the treatment of infectious diseases in routine hospital laboratory procedure. We only used the bacterial isolate retained in the bacterium library, and the patient data are kept anonymous. Since this study only focuses on a bacterial isolate rather than patients, ethical approval was not necessary for the study according to the Swedish act concerning the ethical review of research involving humans, Etikprövningslagen (2003:460).

RESULTS

Susceptibility Analysis

Antibiotic sensitivity was analyzed using the MIC and disk diffusion methods. Clinical isolates either from northern or southern Taiwan were used for analysis. The sensitivity rate for erythromycin detected using the MIC assay was 119/148 (80.4%) (**Table 1A**). Low drug resistance indicated that erythromycin is a suitable choice for empirical therapy. In the disk diffusion method, 24 (30.0%) isolates with small IZDs (≤6 mm) were all resistant, and 5 (4.0%) isolates with large IZDs (>6 mm) were resistant. Eight isolates exhibited different results from those reported in CLSI M45-A3 guidelines. Three isolates which diameter less than 13 mm were sensitive and five isolates larger than 16 mm were resistant. The high agreement between the

TABLE 1A | Compliance analysis between the inhibition zone diameters and MICs by using broth dilution method for erythromycin (A), ciprofloxacin (B), and tetracycline (C).

			Disk diffusion (mm)						
Interpretation			**≤6**	**7–12**	**13–15**	**16–19**	**20–23**	**≥24**	
CLSI guideline M45-A3 2016			**R**		**I**	**S**			**Total (*n*)**
EUCAST disk diffusion guideline in *C. jejuni*			**R**				**S**		
MIC (μg/ml)		<1						30	30	119
	S†	1–8		3*#		1#	3	82	89	
	I†	16						1*#	1	1
		32–64							0	28
	R†	>64	24				1*#	3*#@	28	
	Total (n)		24	3		1		120	148	

**Error (5.4%, 8/148) of disk diffusion based on CLSI M45-A3 guidelines 2016. #Error (6.1%, 9/148) of disk diffusion based on EUCAST disk diffusion guidelines for C. jejuni. @Including two isolates with inhibition zone diameter > 40 mm. †S, susceptible; I, intermediate; R, resistant.*

TABLE 1B |

			Disk diffusion (mm)			
			≤ 6	**7–23**	**≥24**	**Total (*n*)**
MIC (μg/ml)		<1	3		4	7
	S†	1			1	1
	I	2		1	1	2
		4–64	107	6	3@	116
	R	>64	22			22
	Total (n)		132	7	9	148

†S, susceptible; I, intermediate; R, resistant. @Including one isolate with inhibition zone diameter > 40 mm.

TABLE 1C |

			Disk diffusion (mm)			
			≤6	**7–25**	**≥26**	**Total (*n*)**
MIC (μg/ml)		<1			2	2
	S†	1–4			3	3
	I	8				0
		16–64	7	4		11
	R	>64	121	9	2@	132
	Total (*n*)		128	13	7	148

†S, susceptible; I, intermediate; R, resistant. @Inhibition zone of two isolates is >40 mm.

two methods indicated that the disk diffusion method with erythromycin is useful for susceptibility screening. The resistant rates for ciprofloxacin and tetracycline determined through MIC assays were 138/148 (93.2%) and 143/148 (96.6%) (**Tables 1B,C**), respectively. High antimicrobial resistance showed that both these antibiotics are not suitable choices for empirical therapy. The isolates with small IZDs (≤6 mm) were all resistant to tetracycline, but not all isolates (129/132, 97.7%) were resistant to ciprofloxacin. The isolates with large IZDs (>6 mm) that had sensitivity rates of 11/16 and 15/20 were non-sensitive to ciprofloxacin and tetracycline, respectively. Unexpectedly, some of the isolates with very large IZDs (>40 mm) were found to be resistant in the MIC assay (**Table 1**). This disagreement between the results of the two methods indicates that the disk diffusion method with ciprofloxacin and tetracycline is not useful for susceptibility screening.

Differences in Antibiotic Susceptibility Between the Isolates From Northern and Southern Taiwan

Several differences were observed in susceptibility to the nine antibiotics, namely erythromycin, azithromycin, telithromycin, clindamycin, tetracycline, nalidixic acid, ciprofloxacin, florfenicol, and gentamicin, between *Campylobacter* isolates from northern and southern Taiwan (**Table 2**). In *C. coli,* azithromycin

TABLE 2 | Minimum inhibitory concentration operated by broth dilution of *C. jejuni* and *C. coli* isolated from patients in two tertiary medical centers in northern and southern Taiwan.

Antibiotics	Species	Area	Cumulative isolates (%) inhibited, by MIC(mg/L)							
			0.12	0.25	0.5	1	2	4	8	>8
Erythromycin	*C. coli*	North			2(6.5)	8(25.8) #	14(45.2) #	17(54.8) #		31(100.0)
		South				1(7.1) #	4(28.6) #	6(42.9) #		14(100.0)
	C. jejuni	North	1(2.2)	4(8.9)	12(24.4)	32(71.1) #	37(82.2)#	40(88.9) #	42(93.3)	45(100.0)
		South		6(10.5)	17(29.8)	39(68.4) #	51(89.5) #	53(93.0) #		57(100.0)
Azithromycin	*C. coli*	North	11(35.5) *#	18(58.1)						31(100.0)
		South	1(7.1) *#	5(35.7)						14(100.0)
	C. jejuni	North	29(64.4) #	34(75.5)	35(77.8)	36(80.0)	38(84.4)	39(86.7)		45(100.0)
		South	43(75.4) #	51(89.5)	53(93.0)		54(94.7)			57(100.0)
Telithromycin	*C. coli*	North			1(3.2)	3(9.7)	10(32.3) #	14(45.2) #	17(54.8) #	31(100.0)
		South	1(7.1)				2(14.3) #	4(28.6)#	7(50.0)#	14(100.0)
	C. jejuni	North	1(2.2)	3(6.7)	9(20.0)	14(31.1)				45(100.0)
		South		1(1.8)	9(15.8)	16(28.1)				57(100.0)
Clindamycin	*C. coli*	North		6(19.4)	7(22.6)#	10(32.3) #	16(51.6)#	19(61.3)#	20(64.5)#	31(100.0)
		South			1(7.1) #	4(28.6) #	6(42.9)#		7(50.0)	14(100.0)
	C. jejuni	North	7(15.6)	15(33.3)	31(68.9) #	36(80.0)#	39(86.7)#	40(88.9)#	42(93.3) #	45(100.0)
		South	9(15.8)	23(40.4)	36(63.2) #	46(80.7) #	52(91.2) #			57(100.0)
Tetracycline	*C. coli*	North			1(3.2)					31(100.0)
		South								14(100.0)
	C. jejuni	North			1(2.2)					45(100.0)
		South					2(3.5)			57(100.0)
Nalidixic Acid	*C. coli*	North								31(100.0)
		South								14(100.0)
	C. jejuni	North						1(2.2)	2(4.4)	45(100.0)
		South							2(3.5)	57(100.0)
Ciprofloxacin	*C. coli*	North			1(3.2)				5(16.1)	31(100.0)
		South					1(7.1)		2(14.3)	14(100.0)
	C. jejuni	North	2(4.4)	3(6.7)				6(13.3)	9(20.0)	45(100.0)
		South		1(1.8)	3(5.3)		4(7.0)	6(10.5)	9(15.8)	57(100.0)
Florfenicol	*C. coli*	North				2(6.5)	16(51.6)	24(77.4)	29(93.5)	31(100.0)
		South				3(21.4)	10(71.4)#	11(78.6)	12(85.7)	14(100.0)
	C. jejuni	North			3(6.7)	5(11.1)	16(35.6)	26(57.8)	40(88.9)	45(100.0)
		South				7(12.3)	18(31.6)#	36(63.2)	57(100.0)	57(100.0)
Gentamicin	*C. coli*	North			1(3.2)	13(41.9)	20(64.5)	22(71.0)		31(100.0)
		South			1(7.1)	4(28.6)	5(35.7)			14(100.0)
	C. jejuni	North	1(2.2)		17(37.8) *	36(80.0)	39(86.7)		40(88.9)	45(100.0)
		South	1(1.8)		35(61.4)*	51(89.5)	55(96.5)	56(98.2)		57(100.0)

**Significant difference in area ($p < 0.05$). #Significant difference in species ($p < 0.05$). MIC of C. fetus is 1, 0.25, 4, 2, 2, >64, 1, 8, and 0.5 from top as in the aforementioned order.*

■ *Non-susceptible in CLSI guideline M45-A3 2016 (erythromycin ≥ 16 μg/mL; tetracycline ≥ 8 μg/mL; ciprofloxacin ≥ 2 μg/mL).*

■ *Susceptible in CLSI guideline M45-A3 2016 (erythromycin ≤ 8 μg/mL; tetracycline ≤ 4 μg/mL; ciprofloxacin ≤ 1 μg/mL).*

■ *No MIC interpretive criteria in CLSI guideline M45-A3 2016.*

resistance was higher in the isolates obtained from southern Taiwan than in those from northern Taiwan, but in *C. jejuni*, gentamycin resistance was higher in the isolates obtained from northern Taiwan than in those from southern Taiwan. Other antibiotic resistance rates (erythromycin, telithromycin, clindamycin, tetracycline, nalidixic acid, ciprofloxacin and florfenicol) were not significantly different between the isolates obtained from the two areas. Significant differences were observed in the antibiotic susceptibilities of *C. coli* and *C. jejuni* to erythromycin, azithromycin, telithromycin, clindamycin, and gentamicin in each area; however, no significant difference was observed between the isolates obtained from the two areas.

Verification Analysis by Using the *E*-Test and Microdilution MIC Methods

The MICs of three antibiotics, namely erythromycin, ciprofloxacin, and tetracycline, in 75 *Campylobacter* isolates were further verified using the *E*-test and microdilution methods.

TABLE 3 | Comparison between the *E*-test and broth-dilution methods for erythromycin **(A)**, ciprofloxacin **(B)**, and tetracycline **(C)**.

Antibiotics			*E*-test		
			Susceptible	**Intermediate**	**Resistant**
Broth-dilution	**(A) Erythromycin**	Susceptible	59	1*	3*#
		Intermediate	0	0	0
		Resistant	2*#	0	10
	(B) Ciprofloxacin	Susceptible	3	0	1*#
		Intermediate	0	0	0
		Resistant	3*#	0	68
	(C) Tetracycline	Susceptible	3	0	0
		Intermediate	0	0	0
		Resistant	0	1*	71

**Inconsistency between different methods. #Major error. Interpretive criteria is based on the CLSI M45-A3 guidelines, 2016. Erythromycin: ≤8, 16, and ≥32 μg/mL; ciprofloxacin: ≤1, 2, and ≥4 μg/mL; tetracycline: ≤4, 8, or ≥16 μg/mL as susceptible, intermediate, or resistant.*

TABLE 4 | Positive rate of the presence of each *cdt* gene in clinical isolates.

***Campylobacter* isolate (n)**	**Specimen (n)**	***cdtA***	***cdtB***	***cdtC***	***ceuEJ***	***ceuEC***
C. coli (45)	Other (6)*	0.0%	100.0%	0.0%	N/A	100.0%
	Stool (39)	21.9%	100.0%	15.4%	N/A	100.0%
C. fetus (1)	Other (1)*	0.0%	100.0%	100.0%	N/A	N/A
C. jejuni (102)	Other (6)*	83.3%	100.0%	83.3%	100.0%	N/A
	Stool (96)	97.9%	100.0%	62.7%	99.0%	N/A

**The other specimens are blood (n = 11) and dialysate (n = 2). N/A, not applicable.*

TABLE 5 | Antibiotic resistance rates of *Campylobacter* from 2001 to 2014 in northern Taiwan.

	2001–2006			**2007–2012**			**2013–2014**		
	Stool(16) %	**Blood(8) %**	**Total(24) %**	**Stool(42) %**	**Blood(22) %**	**Total(64) %**	**Stool(67) %**	**Blood(10) %**	**Total(77) %**
Ciprofloxacin	75.0	100.0	83.3	76.2	86.4	79.7	76.1	80.0	76.6
Erythromycin	18.8	25.0	20.8	14.3	18.2	15.6	19.4	40.0	22.1
Tetracycline	81.3	100.0	87.5	69.0	77.3	71.9	97.0	90.0	96.6

As shown in **Table 3**, several disagreements were observed between the results of the two methods. The incompatibility rates for erythromycin, ciprofloxacin, and tetracycline were 8.0% (6/75), 5.3% (4/75), and 1.3% (1/75), respectively. Furthermore, the major error rates of erythromycin and ciprofloxacin are 6.7% (5/75) and 5.3% (4/75), and tetracycline has no major errors.

Presence of Bacterial Virulence Genes

The genes encoding CDT, namely *cdtA*, *cdtB*, *cdtC*, *ceuEJ*, and *ceuEC*, were analyzed in the related *Campylobacter* species *C. coli* and *C. jejuni* (**Table 4**). The positive rates of *C. jejuni* for *cdtA* and *cdtC* in stool are 97.9% and 62.7%, respectively, and those for *C. coli* is 21.9% and 15.4% respectively. The positive rates for *cdtA* and *cdtC* were considerably higher in *C. jejuni* than in *C. coli* ($p < 0.05$). However, the positive rates of the other three virulence factors were nearly 100%, and no differences were observed between *C. coli* and *C. jejuni*.

Antibiotic Susceptibility Analysis of *Campylobacter* Species Isolated From Patients in Different Periods

We further analyzed antibiotic resistance rates in three periods, namely 2001–2006 ($n = 24$), 2007–2012 ($n = 64$), and 2013–2014 ($n = 77$) in northern Taiwan (**Table 5**). The resistance rates for all antibiotics, except tetracycline, were high in *Campylobacter* isolated from blood than those from stool samples. Although the resistance rate for tetracycline in 2007–2012 was slightly low and the resistance rate for erythromycin in

blood isolates was slightly high; the difference was not statistically significant.

DISCUSSION

The antibiotic resistance interpretive criteria in the CLSI M45-A2 guidelines suggest that erythromycin is resistant when an IZD (≤6 mm) is not observed in a 15-μg paper disk, and uncertain if the IZD exceeds 6 mm. When the resistance results of the disk diffusion methods are uncertain, the MIC method can be used to determine drug resistance; accordingly, an MIC of ≥32 μg/mL represents that the isolate is resistant to the tested drug, whereas MICs of ≤8 and 16 μg/mL represent that the isolate is sensitive and intermediate, respectively. In addition to CLSI, other reference standards, such as EUCAST, are used in Europe. EUCAST also recommends the use of a 15-μg paper disk of erythromycin to test the susceptibility of *C. jejuni*. According to the criteria for *C. jejuni*, IZD < 20 mm indicates resistance, whereas IZD ≥ 20 mm indicates sensitivity; however, for *C. coli*, IZD < 24 mm indicates resistance, whereas IZD ≥ 24 mm indicates sensitivity. In addition, according to the criteria of the MIC method in EUCAST, an MIC of >4 μg/mL indicates resistance, whereas an MIC of ≤4 μg/mL indicates sensitivity, without an intermediate category. In the CLSI M45-A3 guidelines, disk diffusion criteria were added (IZD ≤ 12 mm indicates resistance, an IZD of 13–15 mm indicates the intermediate category, and IZD ≥ 16 mm indicates sensitivity).

Currently, antibiotic susceptibility testing for *Campylobacter* is not widely implemented in Taiwan. However, based on the needs of clinical treatment, susceptibility testing may be introduced in the future (Ge et al., 2013). Our hospital, similar to other hospitals in Taiwan, uses the antibiotic resistance interpretive criteria of the CLSI guidelines. However, we wish to identify the difference between these two criteria and between different antibiotic susceptibility testing methods.

In this study, all the 24 isolates with IZDs ≤ 6 mm for erythromycin showed MIC > 16 μg/mL (CLSI criteria). However, 4 of the 28 isolates considered resistant according to the EUCAST criteria (IZD < 20 mm) were sensitive according to the MIC method and CLSI criteria (MIC ≤ 8 μg/mL). The isolates with IZD ≥ 20 mm were considered sensitive according to the EUCAST criteria. Most of the isolates were sensitive, with the exception of five isolates with MIC > 8 μg/mL, which denotes the resistance criterion of CLSI. The discrepancies between disk diffusion and IZD < 20 mm (sensitive) and IZD ≥ 20 mm (resistant) are 14.2% (4/28) and 4.1% (5/120), respectively. We believe that the criteria of the disk diffusion method in EUCAST or CLSI for determining erythromycin susceptibility are feasible, despite a few errors, because the requirements of labor, material, resources, and turn-around-time for the disk diffusion method are less than those for the MIC method. Al-Natour's study on *Campylobacter* isolated from animals showed a high degree of agreement between the two methods although the criteria used were different from those used in the present study (Al-Natour et al., 2016). However, our study showed that the disk method was only suitable for erythromycin and not for ciprofloxacin and tetracycline. The difference in suitability may be due to differences in drug resistance in isolates from different regions.

Although the erythromycin-resistant isolates identified using the M45-A2 criteria (IZD < 6 mm represents resistance) of CLSI were entirely correct, the use of these criteria is complicated for the isolates for which the resistance cannot be determined because of the discrepancy of intermediate isolates in the M45-A3 criteria and the complexity of the broth dilution MIC method. The combination of the CLSI and EUCAST criteria might provide additional information, such as ≤6 mm represents resistance, where >20 mm represents sensitivity (low error rate of 3.5%, 5/144). Because of a high error rate (75%, 3/4), if the IZD is between 6 and 20 mm or if antibiotic treatment failed, the MIC method is recommended.

Among the isolates, resistance to ciprofloxacin and tetracycline, regardless of the CLSI or EUCAST criteria, was high (>90%), thus indicating that these two antibiotics are not suitable for empirical treatment. Even if the IZD exceeded the "sensitive" criteria of EUCAST (≥26 mm for ciprofloxacin and ≥30 mm for tetracycline), a large proportion of the isolates were shown to be resistant according to the results of the MIC method. For ciprofloxacin, the rate of non-compliance was 33.3% (3/9) based on the CLSI MIC criteria and >56% based on the EUCAST MIC criteria. For tetracycline, the rate of non-compliance was 50% on average. The sensitivity to these two antibiotics could not be determined using the disk diffusion method; hence, the MIC method was used. Two methods of MIC determination are routinely used, broth dilution and *E*-test. The broth dilution method requires the preparation of antibiotic solutions or commercial kits that are expensive (US$25/isolate and nine antibiotics) and require more complicated operations. Commercial kits have a short shelf life, and are associated with difficulty of interpretation; however, the *E*-test is considerably simpler and have a long shelf life. Although the price of *E*-test for nine antibiotics is similar to that of broth dilution, the price of *E*-test are more flexible in price because we can choose a few antibiotics we need. We compared the rates of compliance of the two MIC methods to determine whether we can use the *E*-test result instead of the broth dilution method to obtain MIC data. The results showed that *E*-test method results exhibit high agreement with those of the broth dilution method according to the CLSI criteria, 94.6% (71/75), 94.6% (71/75), and 98.6% (74/75) for erythromycin, ciprofloxacin, and tetracycline, respectively.

Although previous studies have indicated an increase in *Campylobacter* resistance in recent years (Luangtongkum et al., 2009), no significant difference was obtained among the data of different years in our analysis, thus suggesting that the increase in ciprofloxacin and tetracycline resistance may have occurred before 2001. According to a study in

Taiwan duck farms from 2013 to 2014, antimicrobial resistance differs between northern and southern Taiwan (Department of Veterinary Medicine, National Taiwan University, unpublished data). This discrepancy may be due to differences in the use of antibiotics in the livestock sector or in the natural distribution of such zoonotic bacteria. Because human infection partly results from meat consumption, we analyzed the regional difference of antibiotic susceptibility in patients with campylobacteriosis. We analyzed the differences in resistance to nine antibiotics between two tertiary medical centers, Linkou and Kaohsiung Chang Gung Memorial Hospitals, with a distance of 250 km between them, which represent northern and southern Taiwan, respectively. The results showed that the effects of regional differences on human infection were not significant because we observed differences in only two analysis groups, namely gentamycin for *C. jejuni* and azithromycin for *C. coli*, with higher and lower resistance, respectively, in the northern than in the southern area.

The number of *Campylobacter* bacteremia cases was highest in the middle-aged population in our study; the result is similar to that of a recent study in Israel in 2016 (Hussein et al., 2016). In our study, however, we did not find *Campylobacter* species other than C. *jejuni*, *C. coli*, and *C. fetus*, although the increasing prevalence of uncommon *Campylobacter* has been reported in the literature. Differences between species were observed in four antibiotics, namely azithromycin, erythromycin, telithromycin and clindamycin; *C. coli* showed higher resistance to all the drugs than *C. jejuni* did. Pigs are the main natural reservoirs of *C. coli*. We do not know whether the discrepancy is due to differences in the feeding process of pigs between regions or livestock or other differences such as the nature of species. According to a report by Riley in Canada, *C. coli* is more resistant to ciprofloxacin and erythromycin than *C. jejuni* (Riley et al., 2015). Moreover, our study reported that *C. coli* was less resistant to tetracycline than other species. Similar studies have provided different results on drug resistance (El-Adawy et al., 2015; Cha et al., 2016; Kim et al., 2016). Therefore, the establishment of a bacterial local susceptibility database is required because of the regional difference.

Campylobacter is more toxic to hosts when it contains virulence factors (Bolton, 2015; Lai et al., 2016). We analyzed the differences between four virulence factor genes, namely *cdtA, cdtB, cdtC,* and *ceuE,* between *C. jejuni* and *C. coli*. The results showed that the positive rate of *cdtA* and *cdtC* in *C. jejuni* (*cdtA*: 97.9% in stool and 83.3% in the other; *cdtC*: 62.7% in stool and 83.3% in the other) was significantly higher than that in *C. coli* (*cdtA*: 21.9% in stool and 0.0% in the other; *cdtC*: 15.4% in stool and 0.0% in the other). However, the difference of positive rate in *cdtB* and *ceuE* between *C. jejuni* and *C. coli* is not significant due to their positive rate nearly 100%. According to a study of *Campylobacter* in storks in Europe (Szczepanska et al., 2015), the positive rate of *cdtB* in *Campylobacter* was 58–88%, which is less than that observed in our study. The positive rate of *C. coli* in the above study was higher than that of *C. jejuni*, which was also different from our human clinical case study. We believe that this difference is attributable not only to differences in regions but also differences in species. However, the number of *Campylobacter* studies on the positive rate of *cdt* genes in human clinical infection cases has not increased in recent years. Although the positive rate of the virulence factor in *C. jejuni* is higher than in *C. coli*, the number of parenteral infection cases (*C. jejuni*: *C. coli* = 1:1) caused by *C. coli* is higher than that of intestinal infection cases (*C. jejuni*: *C. coli* = 1:0.4). The use of antibiotics and other invasive factors may contribute to these differences. Additional studies are needed to explain these differences.

CONCLUSION

Cases of severe or invasive campylobacteriosis require the use of antibiotics; erythromycin is suitable as the first choice, and the ciprofloxacin and tetracycline are not suitable because of their high drug resistance (observed in more than 95% of cases in Taiwan). The disk diffusion method with erythromycin can be used as a susceptibility screening method, as determined using the CLSI ($\leq$6 mm represents resistance) and EUCAST ($\geq$20 mm represents sensitivity) criteria. The error rate of the combination method was only 3.5% (5/144). If antibiotic treatment fails or when the IZD is between 6 and 20 mm, the MIC method is recommended. The error rate of disk diffusion method using CLSI M45-A3 criteria is only slightly higher than B, which is also a suitable criteria. The *E*-test is an alternative approach for the broth dilution method because of the high agreement of its results with broth dilution results.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

M-CG, S-FK, and J-JL designed the experiments. C-CC, and H-LY integrated the experiments for bacterial isolates and control of antibiotic susceptibility test. M-CG and S-FK participated in other experiments. S-CC performed the statistical analyses. M-CG and J-JL wrote the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

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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.2018.03186/full#supplementary-material

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