INSIGHTS INTO MICROBE-MICROBE INTERACTIONS IN HUMAN MICROBIAL ECOSYSTEMS: STRATEGIES TO BE COMPETITIVE

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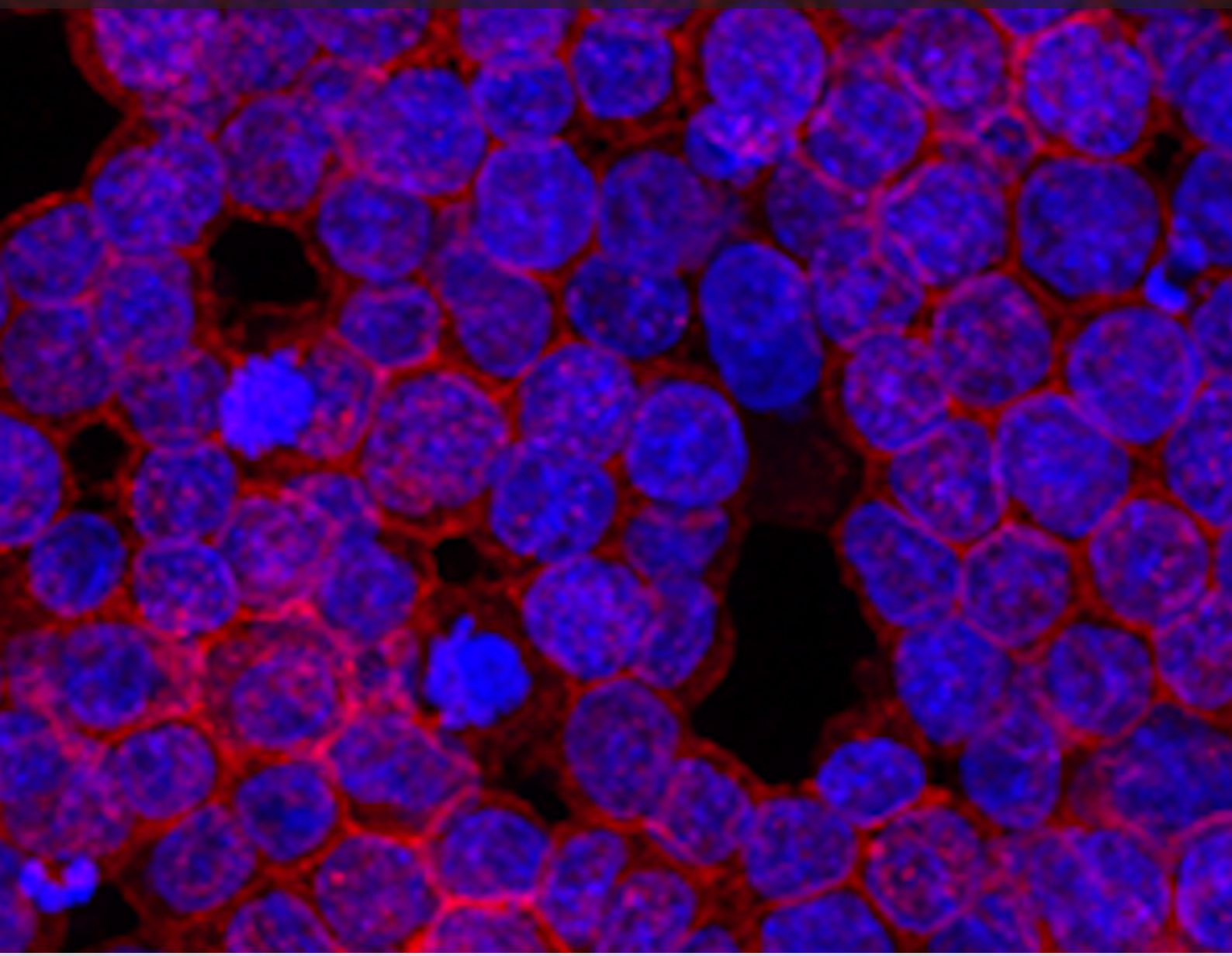

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INSIGHTS INTO MICROBE-MICROBE INTERACTIONS IN HUMAN MICROBIAL ECOSYSTEMS: STRATEGIES TO BE COMPETITIVE

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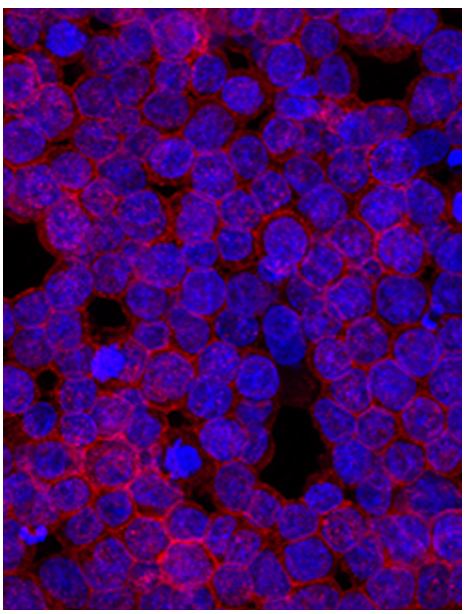

Immunofluorescence image obtained by confocal scanning laser microscopy of cell line HT29 after 20 h of incubation with the toxigenic *Clostridium difficile* supernatant Tox-S by the combination of DAPI-stained nucleus (blue) and F-actin stained with Phalloidin-Alexa-Fluor-568 probe (red).
Image owned by P. Ruas-Madiedo, M. Gueimonde and L. Valdés-Varela

All parts of our body having communication with the external environment such as the skin, vagina, the respiratory tract or the gastrointestinal tract are colonized by a specific microbial community. The colon is by far the most densely populated organ in the human body. The pool of microbes inhabiting our body is known as "microbiota" and their collective genomes as "microbiome". These microbial ecosystems regulate important functions of the host, and their functionality and the balance among the diverse microbial populations is essential for the maintenance of a "healthy status". The impressive development in recent years of next generation

sequencing (NGS) methods have made possible to determine the gut microbiome composition. This, together with the application of other high throughput omic techniques and the use of gnotobiotic animals has greatly improved our knowledge of the microbiota acting as a whole.

In spite of this, most members of the human microbiota are largely unknown and remain still uncultured. The final functionality of the microbiota is depending not only on nutrient availability and environmental conditions, but also on the interrelationships that the microorganisms inhabiting the same ecological niche are able to establish with their partners, or with their potential competitors. Therefore, in such a competitive environment microorganisms have had to develop strategies allowing them to cope, adapt, or cooperate with their neighbors, which may imply notable changes at metabolic, physiological and genetic level.

The main aim of this Research Topic was to contribute to better understanding complex interactions among microorganisms residing in human microbial habitats.

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EDITORIAL
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Editorial: Insights into Microbe–Microbe Interactions in Human Microbial Ecosystems: Strategies to Be Competitive

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Keywords: human microbiota, quorum sensing, gnotobiotic mice, biofilm, *Bifidobacterium*, bacterial-pathogen infection, short chain fatty acids, breast milk

The Editorial on the Research Topic

Insights into Microbe–Microbe Interactions in Human Microbial Ecosystems: Strategies to Be Competitive

The human body is colonized by trillions of commensal microorganisms (bacteria, archaea, viruses, and microscopic eukaryotes) that are collectively referred to as the human microbiota. The microbiota colonizes the skin and mucosal body surfaces of humans and animals, where they are engaged in a constant crosstalk with the host immune system and metabolism. This human microbiota displays a vast genetic catalog, the so called microbiome, contributing functions that are not encoded by our own human genome (Li et al., 2014). The classical tools to analyze its taxonomy and diversity, such as microscopy and cultivation, have been gradually replaced by culture-independent approaches. Initially the study of the human microbiota focused on taxonomy but interests have shifted to understanding the functional role of these human microbial ecosystems and their implications for the host (Salazar et al., 2014). It is also well established that the composition and functionality of this microbiome is essential for maintaining a "healthy status." The microbes living on and within the human body inhabit competitive and complex environments, and deploy different ecological strategies for survival, which may imply notable changes on these microorganisms at metabolic, physiological and genetic level.

The current Research Topic covers a collection of reviews, mini-reviews and original research articles that discuss how bacteria adapt to the specific human niches by competing, or otherwise co-existing with other bacteria and host cells. Recent development of high-throughput analytical tools and "meta-omics" technologies has allowed us to obtain complete overviews of community composition and diversity as well as inferred functionality of genes and metabolic pathways in a wide range of body habitats.

The mini-review by Palau-Rodríguez et al. discusses the use of the metabolomic approach as a powerful tool for exploring the crosstalk between microbial and host metabolism in order to identify human gut microbial-host co-metabolites in the context of metabolic diseases such as obesity and type 2 diabetes.

The laboratory mice are useful experimental models for the study of microbial communities in a mammalian host. The review by Martín et al. provides a comprehensive overview of the application of gnotobiotic animals as tools to decipher the mechanisms underlying microbe–microbe and microbe–host interactions. In addition, the combination of gnotobiotic techniques with new approaches ("omics" and genetic engineering) has revealed causative associations between alterations in the commensal microbiota and several diseases.

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Cell-cell communication in Firmicutes populations by quorum sensing is mostly mediated by peptides that are released to the extracellular environment. The molecular mechanisms underlying bacterial cell-cell communication are not completely understood in spite of their importance for elucidating the microbial contribution to human health and disease. The mini review of Pérez-Pascual et al. presents the current state of research on the biological relevance in Gram positive bacteria of RRNPP, a family of cytoplasmic transcriptional peptide-associated regulators that modulate the expression of target genes involved to host-microbe interactions and with key roles in the context of commensalism or pathogenesis of certain bacteria in human microbial ecosystems.

It is widely recognized that many bacteria form structured multicellular communities, also known as biofilms. Cell-to-cell interactions lead to the establishment of complex and highly structured communities that are responsible for 75% of human microbial infections. The mini-review of Miquel et al. summarizes strategies for prevention of biofilm growth and biofilm control and focuses on catheter-related infections. The review of García et al. goes deeper into the interaction of pathogenic bacteria with host cells and describes the proteoglycans (PG) family which are complex and ubiquitous host molecules which have a different distribution and composition depending on the tissue, and act as key mediators of bacterial infections. The characterization of PG-pathogen interactions can lead to more effective control of infections, and help to overcome antimicrobial resistance, a world health issue of increasing importance.

The original research of Kaur et al. describes how the bacterium *Pseudomonas aeruginosa* inhibits the growth of *Scedosporium aurantiacum*, an opportunistic fungal pathogen in cystic fibrosis, using a combination of solid plate assays and liquid cultures. The results of this study highlight the importance of biofilm formation by *P. aeruginosa* for inhibiting the growth of *S. aurantiacum* in a mimicked lung environment.

Clostridium difficile is an opportunistic pathogen inhabiting the human gut, and is the most frequent aetiological agent of nosocomial diarrhea. Valdés-Varela et al. explore the anti-toxin activity of some *Lactobacillus* and *Bifidobacterium* strains upon the human intestinal epithelial cell line HT29. These two genera are common habitants of the human gastrointestinal tract and some of their members are considered as probiotics.

The human gut microbiome also participates in the biosynthesis and transformation of compounds that are important for both microbial and host physiology. *Bacteroides* are able to use dietary or host-derived glycans as energy sources. In the original research of Rios-Covián et al., the authors have studied the metabolism of the species *Bacteroides fragilis* in the presence of different carbohydrates, including exopolysaccharides synthetized by bifidobacteria. The results show the versatility of *B. fragilis* for adapting to complex carbohydrates and amino acids present in the intestinal environment.

The mini-review by Rios-Covián et al. summarizes the current knowledge on the intestinal microbiota metabolic pathways leading to the production of short chain fatty acids from undigested complex dietary substrates in the gut. Bacterial cross-feeding interactions are involved in the production of a substantial part of these bacterial metabolites, with a huge impact on human health.

The establishment of the gut microbiota is a crucial process influenced by perinatal factors, including the type of infant feeding. Breast milk is considered the optimum food for newborns and health benefits associated with breast-feeding have been reported (Le Huerou-Luron et al., 2010). The research article of Boix-Amorós et al. presents a combined approach to identify the relationships between milk microbiota composition, bacterial load, macronutrients and human cells during lactation using molecular techniques and flow cytometry.

In summary, together the articles of this research topic make a substantial contribution toward understanding the complex interaction among microorganisms residing in human microbial habitats.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication. The editorial was written jointly by the editors of the topic.

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This topic was organized to update the current status of research in the field of human microbiota and the complex interaction between the different microorganisms present in the human ecosystems and with the host. We would like to specially thank all the researchers who contributed their valuable work to this topic, by enriching it. Our deep gratitude to the reviewers and Associate Editors who contributed with their respected criticism for improving this work.

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REVIEW
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Metabolomic insights into the intricate gut microbial–host interaction in the development of obesity and type 2 diabetes

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Gut microbiota has recently been proposed as a crucial environmental factor in the development of metabolic diseases such as obesity and type 2 diabetes, mainly due to its contribution in the modulation of several processes including host energy metabolism, gut epithelial permeability, gut peptide hormone secretion, and host inflammatory state. Since the symbiotic interaction between the gut microbiota and the host is essentially reflected in specific metabolic signatures, much expectation is placed on the application of metabolomic approaches to unveil the key mechanisms linking the gut microbiota composition and activity with disease development. The present review aims to summarize the gut microbial–host co-metabolites identified so far by targeted and untargeted metabolomic studies in humans, in association with impaired glucose homeostasis and/or obesity. An alteration of the co-metabolism of bile acids, branched fatty acids, choline, vitamins (i.e., niacin), purines, and phenolic compounds has been associated so far with the obese or diabese phenotype, in respect to healthy controls. Furthermore, anti-diabetic treatments such as metformin and sulfonylurea have been observed to modulate the gut microbiota or at least their metabolic profiles, thereby potentially affecting insulin resistance through indirect mechanisms still unknown. Despite the scarcity of the metabolomic studies currently available on the microbial–host crosstalk, the data-driven results largely confirmed findings independently obtained from *in vitro* and animal model studies, putting forward the mechanisms underlying the implication of a dysfunctional gut microbiota in the development of metabolic disorders.

Keywords: metabolomics, gut microbiota, obesity, type 2 diabetes, co-metabolism

Abbreviations: ^{1}H-NMR, proton nuclear magnetic resonance; BA, bile acids; IGT, impaired glucose tolerance; FMO3, flavin monooxygenase 3; FXR, Farnesoid X Receptor; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; NAFLD, non-alcoholic fatty liver disease; OGTT, oral glucose tolerance test; T2D, type 2 diabetes; TGR-5, *G*-protein coupled receptor; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide.

GUT MICROBIOTA AND *DIABESITY*: ROLE IN ENERGY HARVEST, GUT BARRIER INTEGRITY, ENDOCRINE MODULATION, AND METABOLIC INFLAMMATION

Obesity is a complex,multifactorial disease characterized by an excessive accumulation of fat due to an imbalance between energy intake and expenditure. The linear rise in the prevalence of T2D throughout the normal, overweight and obese ranges is so high that the relative risks of diabetes are 40 times higher when BMI increases above 35 kg/m^2 (Hu et al., 2001; Mokdad et al., 2003; Poirier et al., 2006; World Health Organization [WHO], 2013). The public concern over the obesity epidemic mostly lies in the intimate connection between obesity and T2D (so-called *diabesity*; Astrup and Finer, 2000) and makes the elucidation of mechanisms underlying the co-occurrence of the two diseases a central focus of current biomedical research.

Recently, consideration has started to be given to the gastrointestinal tract as a key point in the development and progression of complex metabolic diseases, since it represents the milieu where interactions between exogenous (i.e., diet, microbiome) and endogenous (i.e., genetic) factors predisposed to disease and the body's defenses (physical barrier, immune system response) actually take place. Increasing evidence indicates in particular the impact of changes in the composition of the human gut microbiota on host metabolism and a variety of diseases (Bäckhed et al., 2005; Moreno-Indias et al., 2014; Shoaie et al., 2015).

Firmicutes (Gram-positive), Bacteroidetes (Gram-negative) and Actinobacteria (Gram-positive) represent over 90% of the phyla and dominate the gut microbiota (DiBaise et al., 2008), but a relevant change in their relative proportion has been described in obesity and T2D. A favorable prevalence of Firmicutes bacteria toward healthy subjects has been observed in both animal models of obesity (Ley et al., 2005) and human obesity (Ley et al., 2006; Turnbaugh and Gordon, 2009), also reviewed in (Turnbaugh and Gordon, 2009; Sanz et al., 2013; Moreno-Indias et al., 2014), although with some discrepancies among data (Schwiertz et al., 2010). Although the potential impact of specific species on host metabolism has already been elucidated, most of the data so far available have reported observed changes at the phylum level. Furthermore, the physiological contribution of Firmicutes in the development of the obese phenotype is still being debated. In turn, some studies have observed a positive correlation between ratios of Bacteroidetes to Firmicutes and plasma glucose concentration, but not with BMI, although this was expected (Larsen et al., 2010).

Different mechanisms have been proposed in the attempt to understand the impact of microbiota both in maintaining metabolic health and in the development of obesity and T2D. Essentially, the intestinal microbial variability has been hypothesized as an important factor in four different processes, namely: (i) the modulation of energy homeostasis by regulating the energy harvest from diet, fat storage, lipogenesis, and fatty acid oxidation (host energy metabolism; Tilg et al., 2009; Musso et al., 2010); (ii) the modulation of the gut barrier integrity by regulating the epithelial permeability, the intestinal motility and the transport of digestion products such as short-chain fatty acids, which are an energy source for colonocytes (Samuel et al., 2008); (iii) the regulation of gastrointestinal peptide hormone secretion, by suppressing the secretion of the lipoprotein lipase inhibitor (fasting-induced adipose factor), determining the release of fatty acids from circulating triglycerides and lipoproteins in muscle and adipose tissue and promoting fat mass accumulation (Bäckhed et al., 2007); and (iv) the modulation of the host inflammatory state by contributing to the systemic increase of lipopolysaccharide, which impairs insulin sensitivity (metabolic endotoxemia; reviewed in Bäckhed et al., 2007; Cani et al., 2007, 2012; Sun et al., 2010; Vrieze et al., 2010; Shen et al., 2013). Evidence of the role of gut microbiota in the preservation of metabolic health also comes from the effect of prebiotics, such as non-digestible carbohydrates, namely non-digestible ingredients that are fermented by specific beneficial bacterial strains, selectively promote the growth and/or activity (release of end-products of bacterial fermentation) of the gastrointestinal microbiota, affecting favorably the host health (Gibson et al., 2010). The intake of prebiotics has in fact been described to act on host endocrine secretion, improve gut barrier integrity by increasing the release of glucagon-like peptide-2 (Cani et al., 2012; Dewulf et al., 2013), stimulate postprandial release of peptides involved in energy homeostasis and/or pancreatic functions such as the anorexigenic glucagon-like peptide-1 and peptide YY, and the decrease of orexigenic peptides such as ghrelin in plasma which in turn modulates food intake (regulators of appetite) and energy expenditure across the entire gastrointestinal tract (Piche et al., 2003; Delzenne and Cani, 2011; reviewed in Vrieze et al., 2010). Furthermore, evidence suggests that the modulation of the host metabolic health by prebiotics intake can be mediated to specific fermentation products (i.e., short-chain fatty acids, predominantly acetate, propionate and butyrate) produced by cross-feeding between *Eubacterium rectale* and *Bifidobacterium thetaiotaomicron* (Venema, 2010); *Propionibacterium* sp. and *Bacteroides* sp. (Hosseini et al., 2011); *Faecalibacterium prausnitzii* and *Roseburia intestinalis/Eubacterium rectale* (Duncan et al., 2004; Venema, 2010) respectively.

THE METABOLOMIC APPROACH

Due to the species specificity of several enzymatic machineries, the gut microbial composition and activity are likely to be characterized by the profile of small metabolites produced in the intestinal lumen, eventually absorbed through the intestinal barrier and further biotransformed by the host. Consequently, the complexity of microbial–host exchanges may be reflected in the specific chemical signature of host circulating biofluids (Nicholson et al., 2012). Metabolomics has recently attracted attention as the most suitable *-omics* technology for investigating complex, polygenic and multifactorial diseases with a strong metabolic etiology, such as obesity and T2D as well as the crosstalk of distinct predisposing factors in disease development

and progression (Faber et al., 2007; Llorach et al., 2012; Du et al., 2013; Kurland et al., 2013). Aimed at the comprehensive analysis of the low- molecular- weight compounds contained in a biological system –by definition, metabolites comprise a plethora of primary or secondary derivatives of the intermediate metabolism (molecular weight below 900 and 2000 Dalton, depending on sources; Beckonert et al., 2010; Psychogios et al., 2011; Hadacek, 2015) metabolomics represents a powerful tool for exploring the crosstalk between the microbial and host metabolism in a more exhaustive fashion.

The workflow applied in metabolomic studies is broadly categorized into five main steps: (1) sample collection, (2) sample preparation, (3) data acquisition, (4) data analysis, and (5) biological interpretation of the results obtained (Llorach et al., 2012). The analytical techniques most commonly used for the characterization of the metabolome of a biological sample are MS and ^{1}H-NMR. Both technologies have their advantages and disadvantages. ^{1}H-NMR implies a non-destructive, non-selective, cost-effective, and relatively sensitive analysis while, compared to ^{1}H-NMR, MS mainly offers potential advantages in terms of sensitivity and, if coupled to different separation techniques such as LC or GC, it provides a means of detecting a broader and complementary range of biomarkers (Faber et al., 2007). LC coupled to electrospray ionization MS is becoming the method of choice for the acquisition of profiling metabolites in complex biological samples (Scalbert et al., 2009) through both targeted (i.e., triple quadrupole-driven) and non-targeted (e.g., quadrupole time-of-flight-, linear trap quadrupole orbitrap-driven) approaches.

The present review aims to summarize the gut microbial–host cometabolites identified so far in humans in relation to obesity and/or T2D by targeted and untargeted metabolomic studies. Since the potential impact of some specific species in host metabolism has already been elucidated, an attempt to associate bacterial producers of the co-metabolites with the metabolic alterations related to the obese, diabetic, or diabese phenotype was also made. A critical view of the current limitations and future directions of metabolomics will accompany the discussion.

MATERIALS AND METHODS

Search Strategy

The following keywords were searched for in the PubMed and Web of Science electronic databases: (Metabolom* [TW] or co-metabol* [TW] or host-gut metabo* [TW] or nuclear magnetic resonance [TW] or MS [TW] or magnetic resonance spectroscopy [TW]) AND (OBES* [TW] OR DIABET* [TW] OR DIABES* [TW]) AND (gut micro* [TW]). Species (human), language (English), and publication date restrictions (2000 to date, last search on November 27th, 2014) were imposed, but there were none for gender, age or ethnicity. Relevant references cited in the selected articles were additionally reviewed. Targeted and untargeted metabolomic approaches driven by ^{1}H-NMR or MS techniques were both included in the selection. Low-molecular-weight (<1000 Da) metabolites significantly up- or downregulated in overweight and obese subjects with/without impaired glycemic control, with respect to controls (i.e., lean, healthy subjects), were the primary outcomes of interest of the review.

RESULTS AND DISCUSSION

Characteristics of the Studies and Metabolic Variations

Only eight human studies successfully met the eligibility criteria for inclusion in the review (details in the Supplementary Material File). As summarized in **Table 1**, seven observational and one interventional study have so far applied a metabolomic approach and specifically identified changes in products of the gut microbial–host co-metabolism in overweight to obese individuals (BMI > 25 kg/m^2) and/or several degrees of impaired glycemic control (ranging from IGT up to T2D) compared to control individuals. Other comorbidities were not described (i.e., hypertension, renal or liver dysfunction).

Overall, the study subjects, designs and objectives were quite heterogeneous despite the small number of retrieved studies (Supplementary Material File), thereby complicating an otherwise integrated and consistent picture of the metabolomic changes observed.

Urine (Salek et al., 2007; Calvani et al., 2010; Zhao et al., 2010; Huo et al., 2015), fasting serum (Huo et al., 2009; Zhang et al., 2009; Suhre et al., 2010) and plasma (Zhao et al., 2010; Campbell et al., 2014) were the biological samples used in these studies. A data-driven untargeted approach was chosen in the majority of the studies (Salek et al., 2007; Huo et al., 2009; Zhang et al., 2009; Calvani et al., 2010; Zhao et al., 2010; Campbell et al., 2014) while two of them provided quantitative information about known targeted metabolites (Suhre et al., 2010; Huo et al., 2015). The metabolic changes observed in these studies and the related interpretations are summarized in **Table 2**.

Co-metabolism of Bile Acids

Two of the metabolomic studies described in this review highlighted a change in the circulating pool of BA in obese patients with insulin resistance or T2D, compared with BMI-matched healthy individuals (Suhre et al., 2010; Zhao et al., 2010). Alterations involved both human-derived (hepatic) structures (primary BA) and gut microbial-produced derivatives (secondary BA).

To the best of our knowledge, it is currently accepted that the bacterial enzymes involved in the biotransformation from primary to secondary BA are not shared across the whole microbial community, although they have been described so far in genera belonging to the four major phyla Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria (Labbé et al., 2014). Furthermore, according to Jones et al. (2014) Actinobacteria and Firmicutes clones would be the only ones able to degrade all conjugated BA, with Bacteroidetes species being limited to tauro- conjugation activities.

After their production in the liver and the eventual glyco- and tauro-conjugation (*N*-acyl amidation with glycine or taurine

TABLE 1 | Human metabolomic studies showing gut microbial–host co-metabolites significantly altered in obese and/or T2D diagnosed patients, respect to controls.

Observational Studies

Disease	Participants[1]	Medication[2]	Approach (analytical technique)	Specimen	Changes respect to the CT group	Reference
Obesity + pre-T2D	Group 1 = 15 (0F) morbidly OB with IR	No	Non-targeted	Spot urine, fasting	↓Hippuric acid, *N*- methylnicotinate ↑ 2-hydroxyisobutyrate	Calvani et al., 2010
	Group 2 (CT) = 10 (0F) healthy NW (with NGT)	No	(^{1}H-NMR)			
Obesity + T2D (treated vs. not)	Group 1 = 15 (8F) OW with treated T2D	Metformin (15)	Non-targeted	Serum, fasting	↑Trimethylamine-*N*-oxide	
	Group 2 (CT) = 20 (10F) OB with untreated T2D	No	(^{1}H-NMR)			
Obesity + T2D (treated vs. not)	Group 1 = 20 (11F) OB with treated T2D	Glyburide (10), glimepiride (6), Gliclazide (4)	Targeted	Spot urine, fasting	↓Hippuric acid (untreated T2D) ↑hippuric acid (with anti-T2D drugs)	Huo et al., 2015
	Group 2 = 20 (11F) OB with untreated T2D	No	(UPLC-MS)			
	Group 3 (CT) = 20 (10F) healthy OB (with NGT)	No				
Obesity + T2D	Group 1 = 30 (13F) OW to OB with untreated T2D	No	Non-targeted	Spot urine, fasting	↓Hippuric acid, *N*- methylnicotinate, ↓*N,N*-dimethylglycine, *N,N* dimethylamine	Salek et al., 2007
	Group 2 (CT) = 12 (4F) healthy NW to OW (with NGT)	No	(^{1}H-NMR)			
Obesity + T2D	Group 1 = 40 (0F) OB with T2D	No (7), antidiabetic medication	Targeted	Serum, fasting	↓Cholate ↑deoxicholate ↓Gamma murichолate	Suhre et al., 2010
	Group 2 (CT) = 60 (0F) healthy OW (with NGT)		(UPLC-MS/MS)			
(pre-)T2D	Group 1 = 74 (42F) NW with T2D	No (48), metformin, acarbose, glipizide or repaglinide as a monotherapy (26)	Non-targeted	Serum, fasting	↓Choline (vs. NGT and vs. IGT)	Zhang et al., 2009
	Group 2 = 77 (44F) NW with IGT	No	(^{1}H-NMR)			
	Group 3 (CT) = 80 (46F) healthy NW (with NGT)	No				
Obesity + pre-T2D	Group 1 = 12 (?F) OB with IGT	No	Non-targeted	Plasma, fasting	↑Glycochenodeoxycholic acid	Zhao et al., 2010
	Group 2 (CT) = 39 (?F) healthy OB (with NGT)		(UPLC-qToF-MS)	Spot urine, fasting	↓Hippuric acid, 3-hydroxyhippuric acid, methyluric acid, methylxanthine	

Single-arm intervention study (weight-loss plan with calorie restriction and exercise)

Disease	Participants	Duration and associated clinical outcomes	Approach (analytical technique)	Specimen	Changes from baseline	Reference
Obesity + pre-T2D	Group = 15 (15F) OB with IR (only 12 up to the end)	0, 14–17 weeks ↓BMI, body fat, V02, fasting	Non-targeted (GC-ToF-MS)	Plasma, fasting and 30, 60, 90, 120 min after OGTT	↑Tricarballylic acid (fasting and after OGTT)	Campbell et al., 2014

[1] *'healthy' stands for NGT subjects. Abbreviations: F, number of females in studies; CT, control; BMI, Body Mass Index; OW, overweight; OB, obese; NW, normal weight; NGT, normal glucose tolerance; IGT, impaired glucose tolerance; IR, insulin resistance; T2D, type 2 diabetes; pre-T2D, prediabetes;*[1]*H NMR, proton nuclear magnetic resonance; UPLC-qTOF-MS, reversed-phase ultra performance liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; GC-TOF-MS, gas chromatography/time-of-flight mass spectrometry.*

[2] *Medication during the study.*

TABLE 2 | Summary of the most significant gut microbial and host co-metabolites identified in the selected studies.

Class	Metabolite	Disease	Change[1]	Anti-T2D drugs effect	Sample	Interpretation	Reference	*Related bacteria (phyla)*
Bile acids (primary)	γ-murichолate (hyocholate)	Obesity + T2D	↓		Blood fluids	Bile acids are proposed as new metabolic integrators of whole body energy homeostasis that influence glucose and lipid metabolism. Subjects with diabetes exhibit alterations in the composition of the bile acid pool and their related biosynthetic pathway. A higher rate of conversion of primary to secondary bile acids by the gut microbiota has been implicated in the observed variation.	Suhre et al., 2010	Firmicutes[3]
	cholate	Obesity + T2D	↓		Blood fluids		Suhre et al., 2010	
	glycochenodeoxycholate	Obesity + pre-T2D	↑		Blood fluids		Zhao et al., 2010	
Bile acids (secondary)	Deoxycholate	Obesity + T2D	↑		Blood fluids		Suhre et al., 2010	
Vitamin metabolites	Choline	(pre-)T2D	↓↓[2]		Blood fluids	In the absence of anti-T2D treatment, alteration of choline metabolism (increased degradation) noticed in T2D patients may result from: (a) an altered demand, possibly by altered lipoprotein turnover/biosynthesis, (b) an altered gut microbiotal activity associated with T2D development, or (c) an osmotic compensation for raised blood glucose concentrations. Low levels of choline would also associate to the prevalence of OB/T2D complications, namely nonalcoholic fatty liver. When associated with metformin, may indicate a possible two-way relationship between the anti-T2D treatment and the gut microbiota. The intestinal bacteria composition would influence glucose metabolism and the mechanisms of action of metformin, and the drug would regulate back the gut microbial function.	Zhang et al., 2009	Firmicutes, Proteobacteria and Actinobacteria[4]
	TMAO	Obesity + T2D		↑	Blood fluids		Huo et al., 2009	
	DMA, DMG	Obesity + T2D	↑		Urine		Salek et al., 2007	
	N-methylnicotinate (trigonelline)	Obesity + (pre-)T2D	↓		Urine	Gut microbial class-specific product of the metabolism of niacin, which is an essential vitamin involved in major physiological functions such as coenzyme in tissue respiration, carbohydrate and lipid metabolism. Trigonelline regenerates glutathione stores that are depleted by oxidative stress in obesity. Moreover, low levels of trigonelline could suggest perturbation in nucleotide metabolism during T2D.	Salek et al., 2007; Calvani et al., 2010	NA

(Continued)

TABLE 2 | Continued

Class	Metabolite	Disease	Change[1]	Anti-T2D drugs effect	Sample	Interpretation	Reference	*Related bacteria (phyla)*
Organic acids and derivates	2-hydroxyisobutyric acid	Obesity + pre-T2D	↑		Urine	Since their production is species specific at the colonic level, changes in their level may reflect (a) significant shifts in the subjects' gut microbe ecology or functional differences in the microbiome metabolic activity between OB with IR and healthy lean individuals and (b) changes in the host metabolism/uptake of gut-derived metabolites, possibly related to a variation in the intestinal mucosa permeability after weight-loss plan with calorie restriction and exercise.	Calvani et al., 2010	Firmicutes[5]
	tricarballylic acid	Obesity + pre-T2D	↑		Blood fluids		Campbell et al., 2014	
Phytochemical and purine metabolites	hippuric acid	Obesity + (pre-)T2D	↓↓↓↓	↑	Urine	Changes in the production of hippurate and derivatives are generally connected to diet and gut microbial activities with the human metabolic phenotype and the blood pressure of individuals. They could indicate a relevant role of the gut microbiota in the pathogenesis of the pre-T2D state and could be related to age progression and gender effects on metabolism in T2D. The reversion of these changes by sulfonylurea treatment would confirm a beneficial effect of anti-T2D drugs on gut microbiota metabolism, besides glucose homeostasis. Higher concentrations in obese humans could reflect the known role of gut microbiota in energy metabolism and immune function of the host.	Salek et al., 2007; Calvani et al., 2010; Zhao et al., 2010; Huo et al., 2015	Firmicutes[5]
	3-Hippuric acid hydroxyhippuric acid				Urine		Zhao et al., 2010	
	Methyluric acid	Obesity + pre-T2D	↓		Urine	Gut microbiota-associated metabolite biomarkers, related to IGT. Accumulating evidence indicates that the gut microbiota is instrumental in the energy metabolism and immune function of the host.	Zhao et al., 2010	NA
	Methylxanthine	Obesity + pre-T2D	↓		Urine		Zhao et al., 2010	

OB, obesity; T2D, type 2 diabetes; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; SU, sulfonylurea; M, metformin hydrochloride; TMAO, trimethylamine-N-oxide; NMN, N-methylnicotinate; DMG, N, N dimethylglycine; DMA, dimethylamine. [1]*In respect to healthy controls.* [2]*Also in T2D vs. IGT but not IGT vs. NGT.* [3]*From: Begley et al., 2005; Ridlon et al., 2006; Fukiya et al., 2009; Labbé et al., 2014.* [4]*From: Craciun and Balskus, 2012.* [5]*From: Li et al., 2008.*

substituents), primary BA are secreted in the small intestine through the bilis (Ridlon et al., 2006; Hofmann and Hagey, 2008; Swann et al., 2011), where they are first subjected to deconjugation by a bacterial bile salt hydrolase enzyme produced by species of the four phyla, such as *Clostridium, Bacteroides, Lactobacillus, Bifidobacterium,* and *Enterococcus* (Begley et al., 2005). In the ileum, more than 95% of these BA undergo enterohepatic recycling (Swann et al., 2011; Kootte et al., 2012; Nicholson et al., 2012), are absorbed from the intestine and returned to the liver (Ridlon et al., 2006). The remaining 5% escape the enterohepatic circulation and reach the large bowel where the bioconversion into secondary BA is completed, especially by Firmicutes phyla *(Eubacterium* sp. and *Clostridium* sp.; Midtvedt, 1974; Nguyen and Bouscarel, 2008; Swann et al., 2011).

A decrease of primary BA and an increase of secondary BA was observed in the fasting serum of overweight patients with T2D, compared to healthy subjects (Suhre et al., 2010). The authors hence concluded that differences in the gut microbiota of diabetic patients may lead to higher rates of conversion from primary to secondary BA. In turn, Zhao et al. (2010) only observed an increase of glycochenodeoxycholic acid (primary BA) in the plasma of prediabetic individuals, while no changes were noticed in urine. Despite the only partial overlapping of the results, both studies suggested that overweight and obesity may not be the predominant factor implied in the metabolomic changes observed, and thus in linking impaired glucose homeostasis to alterations in BA pool composition. As argued in those studies, the variation of the BA pool in biofluids may depend on different factors, namely a change in the prevalence or activation of the gut microbial species implied in BA bioconversion or an altered reabsorption of BA through the gut mucosa, in turn produced by the disease itself, by dietary or other external changes (i.e., induced by bariatric surgery), or by a combination of these. In any case, the results indicated a probable implication of the modulation of BA biosynthetic pathways in the relationship between gut microbiota and insulin resistance (**Figure 1**).

These findings are in line with independent studies that recently associated changes in BA turnover with *diabesity*. In turn, a reduction of the bacterial enzymatic activities involved in the conversion of primary into secondary BA was observed in diabetic patients compared to healthy controls, and linked to Firmicutes phyla (Labbé et al., 2014). A very similar pattern was also reported in obese patients with diagnosed metabolic syndrome, treated with antibiotic agents (vancomycin and amoxicillin) and associated with both a decreased prevalence of the Firmicutes population and a reduction of peripheral insulin sensitivity (Vrieze et al., 2014). Taken together, these data suggest a possible link between BA and metabolic health (Lefebvre et al., 2009). In line with these findings, BA have recently been proposed as metabolic integrators of whole-body energy homeostasis implicated in the regulation of various metabolic pathways, including their own synthesis and enterohepatic circulation, triglyceride, glucose, and energy homeostasis, by acting as signaling molecules through receptor-dependent and -independent pathways. The role of a dysregulation of this

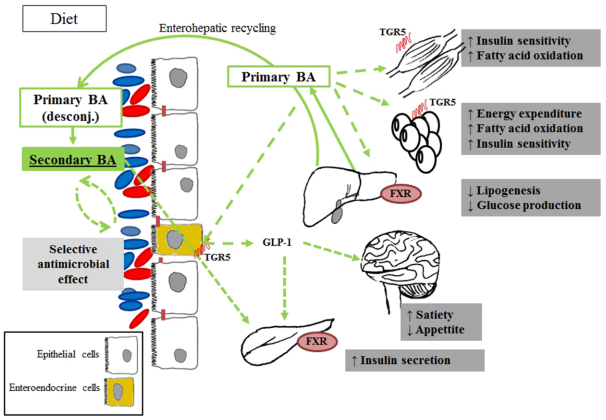

FIGURE 1 | Products of bile acids co-metabolism associated with obesity and diabetes via metabolomic studies. After the ingestion of food, conjugated BA are secreted into the intestinal lumen, are subjected to deconjugation by the intestinal bacteria, and converted to secondary BA. The main effect of secondary BA is mediated by the FXR activation, which in turn act increasing insulin secretion and sensitivity in the pancreas. Primary BA is also involved in glucose metabolism by TGR-5 activation and glucagon-like peptide-1 (GLP-1) release. Host endogenous processes are in solid lines, while microbial production and metabolite actions are in dotted lines.

BA-mediated metabolic control in the pathogenesis of T2D and co-morbidities, such as its attractiveness as a therapeutic target, is now beginning to be elucidated (reviewed in Prawitt et al., 2011). Their action on energy metabolism regulation would occur via both the activation of the nuclear receptor FXR and FXR-independent pathways, such as through the membrane receptor TGR5 expressed in several tissues including gall bladder, ileum, colon, and brown and white adipose tissue.

It is worth noting that preliminary evidence has shown that not all BA activate equally, and the microbial-derived production of secondary BA could be an important mechanism in the regulation of signaling pathways involved in the development of *diabesity* (Nguyen and Bouscarel, 2008). When gut microbial-derived secondary BA are bound to TGR5, the receptor is internalized and a series of adenylate cyclase-dependent signaling is triggered by activating distinct pathways involved in glucose and lipid energy metabolism (Kawamata et al., 2003; Thomas et al., 2008). In tissues, such as brown adipose tissue and muscle, this would lead to an increased mitochondrial activity and oxidative phosphorylation, which has been linked to an insulin sensitization both in genetic and diet-induced models of *diabesity* (Watanabe et al., 2006). In enteroendocrine L-cells (Kawamata et al., 2003), in turn, these signaling pathways would enhance glucose metabolism by stimulating the production of glucagon like peptide, thereby promoting insulin secretion. Finally, recent studies have also shown that BA secretion improves insulin secretion, insulin sensitivity and whole-body glucose homeostasis (reviewed in Thomas et al., 2008), improving liver and pancreatic function in obese mice (Thomas et al., 2009; Tremaroli and Bäckhed, 2012).

Co-metabolism of Vitamins

Products of the gut microbiota-driven metabolic pathway of vitamins such as choline and niacin have been associated with obesity and diabetes.

Choline Metabolism

Although humans may produce choline endogenously (*de novo* hepatic synthesis), dietary intake (e.g., from egg yolk, liver, muscle meats, fish, nuts, legumes) is necessary to meet the demand for body health maintenance (Blusztajn, 1998; Zeisel, 2000). Once dietary choline reaches the intestine, anaerobic intestinal microorganisms, mainly of Firmicutes and Proteobacteria phyla (Romano et al., 2015) catalyze its conversion to TMA, which may be further degraded to several methylamines by the gut microbiota (sym-xenobiotic pathway; Harris et al., 2012), or is absorbed and oxidized to TMAO by the hepatic FMO3 enzyme. Choline may also be converted into betaine and further products (e.g., dimethylglycine) by mammalian mitochondrial pathways in the liver and kidney (Lever and Slow, 2010) (**Figure 2**). The bacterial gene clusters responsible for anaerobic choline degradation started to be identified only recently. Bacterial species belonging to Firmicutes, Actinobacteria and Proteobacteria phyla have been revealed as possessing the necessary enzymatic activities, while Bacteroidetes would be apparently deprived (Craciun and Balskus, 2012). However, the complete diversity of species that contribute to TMAO production in humans still remains unknown (Romano et al., 2015).

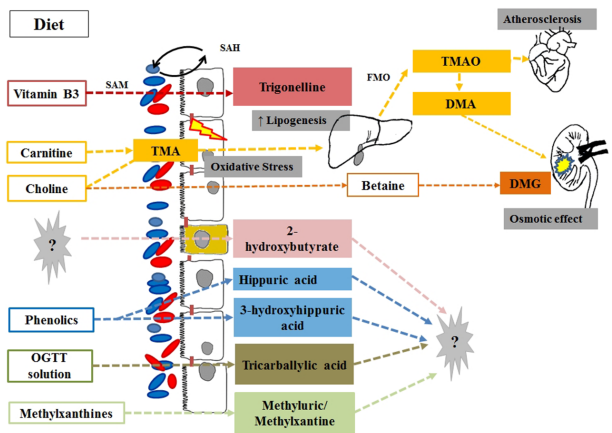

FIGURE 2 | Products of vitamin, phytochemical and purine co-metabolism associated with obesity and diabetes via metabolomics studies. Intestinal microorganisms catalyze the conversion of dietary choline and carnitine into TMA with a direct effect on the intestinal mucose (increased oxidative stress) and is the substrate for the hepatic production of TMAO (associated with cardiovascular disease risk) and TMA. Choline may escape microbial degradation and convert into betaine and further products [i.e., dimethylglycine (DMG)] by mammalian mitochondrial pathways in the liver and kidney where they have an detrimental osmotic effects. For most of the host microbial co-metabolites associated with the diabese phenotype, the eventual role in glucose and lipid metabolism remains unknown. OGTT, Oral glucose tolerance test; SAH and SAM, *S*-adenosylmethionine to *S*-adenosylhomocysteine (methionine cycle).

Recent studies have shown that circulating levels of choline and TMAO are related to cardiovascular disease risk (Dumas et al., 2006; Micha et al., 2010; Wang et al., 2011; Koeth et al., 2013; Warrier et al., 2015), and the gut microbiota-driven TMA-FMO3-TMAO pathway has been particularly recognized as a key regulator of lipid metabolism and inflammation. Increased levels of TMAO have been observed in a leptin-deficient murine model of obesity and T2D (Gipson et al., 2008; Won et al., 2013) and revealed a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice (Dumas et al., 2006). The systemic perturbations of key metabolites of choline have also been related to the progression of T2D, suggesting that in the early stages of diabetes an attenuated conversion of choline into dimethylglycine may occur, which can be observed by the increased levels of TMAO and TMA, with a reversion of this behavior at a later stage of the disease (Guan et al., 2013).

Messana et al. (1998) published the first study linking TMAO and T2D in humans. Using a ^{1}H-NMR approach, increased levels of TMAO and dimethylamine were observed in the urine of diabetic individuals compared to a group of healthy individuals, and were present in high concentrations even in diabetics with good metabolic control (i.e., absence of glycosuria and glycohemoglobin). In the last decade, little progress has been made on the mechanisms that would directly or indirectly involve TMAO in the development of diabetes. Nevertheless, the potential role of an altered composition of the microbiota and its ability to metabolize choline in glucose homeostasis and disease development has become increasingly relevant (Dumas et al., 2006).

To the best of our knowledge, three further metabolomic studies observed a change in choline metabolism, which was associated with impaired glycemic control in humans, within a wide range of BMI (Salek et al., 2007; Huo et al., 2009; Zhang et al., 2009; **Table 1**). In all of them, fasting biosamples were analyzed, thereby avoiding fluctuations in choline concentrations due to dietary intake. Salek et al. (2007) carried out a ^{1}H-NMR-driven metabolomic comparison of urinary changes linked to T2D both in animals (obese mice and rats with autosomal recessive defects in the leptin receptor gene – *db/db* and Zucker *fa/fa*, respectively), and individuals who were overweight to obsese (BMI = 25–40) compared to healthy lean controls. An increased excretion of a product of choline biotransformation, namely *N,N*-dimethylglycine and *N,N*-dimethylamine, distinguished the urinary metabolome of T2D in all species in the study (Salek et al., 2007), suggesting a possible increase in the choline turnover. The authors assumed that the *diabese* phenotype may have a major demand for choline, possibly due to an altered biosynthesis of lipoproteins, an altered metabolism of methylamines – which would play an important osmoregulatory role by degrading dietary choline – or to an altered intestinal microbiota composition (Salek et al., 2007). Although there is a scarcity of data in this regard, Firmicutes and Proteobacteria seem to be the most implicated phyla in the conversion of choline to TMAO (Romano et al., 2015). For this reason, the decline of choline circulating levels and increase of choline subproducts such as TMAO and DMA in obese subjects would be in line

with the increase of the Firmicutes phylum associated with obese phenotype (Ley et al., 2006).

The role of BMI in the observed association was partly downsized in a second study (Zhang et al., 2009). By applying a similar untargeted and ^{1}HNMR-driven approach, in fact, Zhang et al. (2009) demonstrated a decrease in the serum levels of choline in normal-weight subjects with T2D (BMI $= 25.9 \pm 9.0$) compared to non-diabetic lean individuals (normal or IGT); however, no changes in the downstream products of choline metabolism were detected. Aside from increased insulin resistance, the decrease in serum choline is linked to a specific shift in the gut microbial community in the diabetic patients (relative increase in Firmicutes) and to an increase in the prevalence of T2D complications, as NAFLD (Zhang et al., 2009). The role of the microbial community hosted by *diabese* subjects in altering choline metabolism was also tested by assessing the effects of antidiabetic medication (Huo et al., 2009). As shown in **Table 1**, Huo et al. (2015) observed increased serum levels of TMAO in overweight diabetic subjects receiving metformin treatment *versus* untreated *diabese* controls, which may indicate an intestinal bacterial regulation function of metformin. It has already been suggested that antidiabetic treatments have a beneficial effect on gut microbiota metabolism (Huo et al., 2015), although the exact mechanisms are still unclear (Moreno-Navarrete et al., 2012). The authors suggested a link between the deregulation of choline metabolism in T2D and a rupture of the intestinal barrier by oxidative stress (Wei et al., 2008). In any case, a possible two-way relationship between anti-T2D treatment and gut microbiota has been hypothesized.

Niacin Metabolism

Alterations in the niacin (vitamin B3) metabolism have also been observed in association with obesity and T2D, and due to the overlapping in the choline/niacin catabolic pathways (i.e., via betaine and glycine metabolism), may also reflect a dysregulation in choline metabolism (Huang et al., 2012). Through a LC–MS-driven metabolomic approach, an altered urinary excretion of nicotinuric acid (*N*-nicotinoyl-glycine) was recently proposed as a potential marker of metabolic syndrome diagnostic traits and of cardiometabolic risk (Huang et al., 2012). Similarly, an association between the presence of trigonelline (betaine nicotinate) and obese and diabetic phenotypes has been proposed. Despite having a possible exogenous (dietary) origin, trigonelline is mostly biosynthesized by the gut microbiota during the conversion of *S*-adenosylmethionine to *S*-adenosylhomocysteine (methionine cycle). By applying a ^{1}H-NMR-based metabolomics approach, Salek et al. (2007) found lower levels of trigonelline in the urine of diabetic (*db/db*) mice and humans, associated with a change in energy and tryptophan metabolism. Further animal (Salek et al., 2007; Won et al., 2013) and human studies (Calvani et al., 2010) confirmed a decline of trigonelline in obesity and diabetes, some authors suggesting that oxidative stress possibly has a role (i.e., via glutathione store depletion) in the observed relationship (Calvani et al., 2010). Trigonelline is known to be involved in major physiological functions including lipid and carbohydrate metabolism.

Co-metabolism of Organic Acids and Derivates

Calvani et al. (2010) identified high levels of 2-hydroxyisobutyrate in the urine of obese people, and the change was associated with a reduced bacterial diversity in 'obese' gut microbiota possibly involved in nutrient and energy harvest (**Tables 1** and **2**). In particular, 2-hydroxyisobutyrate is a product of the microbial degradation of dietary proteins that escape digestion in the upper gastrointestinal tract, and its production has been associated with the presence of specific microbial members such as *Faecalibacterium prausnitzii* (Li et al., 2008), butyrate-producer species with anti-inflammatory effect and to be in low levels in obese and *diabese* individuals compared to healthy subjects (Qin et al., 2012). In addition, Campbell et al. (2014) observed that obese subjects involved in a dietary weight loss program had higher levels of tricarballylic acid after an OGTT compared with the fasting concentrations. Interestingly, tricarballylic acid is a product of gut microbial metabolism of food-derived trans-aconitate, described as an additive contained in the OGTT solution. Once again, the authors suggested a two-way relationship between the obese and gut microbial phenotype (tricarballylic acid production would in turn increase the metabolic activity of specific gut microbes associated with its production).

Co-metabolism of Phytochemicals and Purines

Hippuric acid and 3-hydroxyhippuric acid are two normal urinary components mainly derived from the degradation of plant (poly)phenols and aromatic amino acids (i.e., phenylalanine and tryptophan) by a range of gut microbes, recently found to belong to *Clostridium* sp. (Li et al., 2008). The resulting benzoic acid is then absorbed, subjected to glycine conjugation reaction (by mitochondrial glycine *N*-acyltransferase) and finally excreted in urine (Huo et al., 2015). Decreased levels of hippuric acid (Salek et al., 2007; Calvani et al., 2010; Zhao et al., 2010) and 3-hydroxyhippuric acid in urine have been related to IGT and obesity (Zhao et al., 2010) in both animal and human studies. In turn, the downregulation was reduced in T2D patients after the treatment with sulfonylurea, suggesting the drug potentially has a protector effect on gut microbiota metabolism (Huo et al., 2015). However, a strict dietary assessment is mandatory to dismiss any diet-dependent variation among groups, due to the wide range of phenolic compounds leading to these last-step metabolites following microbial and human biotransformations (lack of specificity; Salek et al., 2007). Moreover, the reasons for their putative associations with obesity and T2D are unknown. Salek et al. (2007) suggested that hippurate could be related to age progression and gender effects on metabolism in T2D, but these suppositions need to be further investigated.

Zhao et al. (2010) observed that subjects with IGT had a reduced excretion of methyluric acid and methylxanthine, which are products of the microbial metabolism of methylxanthines contained for instance in coffee and tea. The authors tentatively interpreted the observed changes as the result of an altered gut

microbiota in the presence of insulin resistance, although their putative role in glucose metabolism is still unknown.

CONCLUSION

Current public health burdens such as obesity and T2D are complex, polygenic, multifactorial diseases with a strong metabolic etiology. Gut microbiota have recently been proposed as a crucial environmental factor in their development, but the metabolic complexity of the symbiotic interaction between the host individual and its microbial community, as well as the impact of this crosstalk between body weight changes and glucose homeostasis, are still unclear.

However, our review highlighted how few metabolomic studies have been specifically conducted so far to identify the role of the gut microbiota in the development and progression of obesity and T2D, at least in humans.

Despite the scarcity, heterogeneity and intrinsic limitations of the metabolomic studies conducted so far aimed at identifying the role of the gut microbiota in the development and progression of obesity and T2D (i.e., wide range of BMI and/or glycemic status evaluated, important sources of variability not considered including ethnic, gender effects, and dietary assessments), the results obtained by these data-driven metabolomic approaches are in line with findings independently obtained from *in vitro* or animal model studies. Products of the microbial/host metabolism of BA, vitamins (choline, niacin), branched fatty acids, purines and phenolic compounds have been described as being altered in (pre-)diabetic subjects, with or without increased BMI, compared with healthy controls. Moreover, few articles show a clear relation between metabolites and their bacterial producers in terms of the complexity of the gut microbiota and the cross-feeding mechanisms that would have combined bacterial effects in the colon ecosystem.

More efforts should be directed in the future toward expanding our knowledge of the metabolic interactions of the host and the gut microbiota, particularly through a strict evaluation of the lifestyle factors (i.e., diet) strongly involved in the modulation of this crosstalk.

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SUPPLEMENTARY MATERIAL

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Gnotobiotic Rodents: An *In Vivo* Model for the Study of Microbe–Microbe Interactions

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Germ-free rodents have no microorganisms living in or on them, allowing researchers to specifically control an animal's microbiota through the direct inoculation of bacteria of interest. This strategy has been widely used to decipher host–microbe interactions as well as the role of microorganisms in both (i) the development and function of the gut barrier (mainly the intestinal epithelium) and (ii) homeostasis and its effects on human health and disease. However, this *in vivo* model also offers a more realistic environment than an assay tube in which to study microbe–microbe interactions, without most of the confounding interactions present in the intestinal microbiota of conventionally raised mice. This review highlights the usefulness of controlled-microbiota mice in studying microbe–microbe interactions. To this end, we summarize current knowledge on germ-free animals as an experimental model for the study of the ecology and metabolism of intestinal bacteria as well as of microbe–microbe interactions.

Keywords: germ-free, gnotobiology, bacterial interactions, intestinal bacteria, microbiota

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INTRODUCTION

Like human beings, conventional rodents harbor trillions of bacteria and viruses (Williams, 2014). Germ-free (GF) animals, instead, are completely free of these microbes. In the field of gnotobiology [from the Greek "*gnōtos*" (known) and "*biotic*" (life)] (Gustafsson, 1959), such animals are used to study the effects of inoculation with specific, known microbes. To raise GF mice, researchers must separate pups from their mother's wombs surgically, by aseptic cesarean or hysterectomy, thus avoiding contact with microorganisms present in the mother's vagina and skin. Alternatively, GF pups can also be generated via the process of embryo transfer, which occurs in an isolator environment and thus enables the implantation of cleansed embryos into GF recipients under well-controlled conditions. A recipient female gives birth normally and cares for the offspring as if they were her own pups, thus enhancing the pups' survival rate. GF pups are kept in a sterile environment their whole lives. In order to guarantee GF status, recurrent contamination tests are performed on their feces. Once GF animals have been produced, it is possible to expand the colony by crossing GF individuals. The new members of the colony have a natural birth and continue their life under the same sterile conditions as their progenitors.

The accuracy of GF technology lies in the ability to control the composition of the environment in which an organism develops and functions (Falk et al., 1998). To this end, gnotobiotic facilities eliminate microbes present in food, water, and bedding through specific sterilization protocols. Typically, these materials are heated to temperatures above 100°C in order to kill

bacteria and viruses. Possible contamination is prevented through a fail-safe double-door system, in which autoclaved supply cylinders are docked to a double-door port built into the isolator wall. These security measures enable inoculation by a single microorganism of interest, as well as the consequent reproduction of mono-associated animals, known as monoxenics. Animals inoculated with two microorganisms are known as dixenics, those with three are trixenics, and so on (**Figure 1A**) In the 1960s, Schaedler (Rockefeller University) investigated the effects of different mixtures of bacteria in GF mice. He was the first to study how a specific bacterium colonizes the gut of initially GF mice (Schaedler et al., 1965a,b), and his descriptions of what subsequently became known as the "Schaedler flora" have served as important tools in standardizing the microbiota of laboratory populations of rodents around the world (Schaedler et al., 1965a,b). This microbiota has recently been updated to include extremely oxygen-sensitive (EOS) bacteria, which were not included in the first survey due to the technical difficulties inherent in their isolation (Wymore Brand et al., 2015). Nowadays, this altered Schaedler microbiota (ASF) is used in several laboratories around the world for the study of bacteria-host dynamics (Wymore Brand et al., 2015). In addition, the ability to control the microbiota also enables the transfer of a simplified or complete human microbiota to the GF animals in order to create human-like conditions (Goodman et al., 2011) (**Figure 1B**). However, if the transferred microbiota is not completely defined, the resulting animals are not considered gnotobiotic animals.

Typically, gnotobiotic animals are used to study the effects of different members of the microbiota on the host. As is the case with most biological research (Williams, 2014), the field of gnotobiotics began with studies in which the system of interest—in this case, the microbiota of various animals such as mice, rats, guinea pigs, and chicks—was removed in order to examine its role in different processes (Reyniers, 1959).

Studies with these animals have demonstrated that the crosstalk between microbes and their host is essential for the well-being of the host, and have indicated that the physiology of healthy host individuals is dependent on the composition of the gut microbiota. Indeed, changes in the gut microbiota affect processes as varied as epithelial cell renewal, differentiation, and architecture (Cherbuy et al., 2010); intestinal motility (Falk et al., 1998); and host glycosylation patterns and gene expression (Hooper et al., 2001). The study of gnotobiotic rodents has also allowed researchers to identify and characterize key microbes responsible for the development of the intestinal immune system (Umesaki and Setoyama, 2000; Umesaki, 2014). Thanks to gnotobiology, it is now widely recognized that the microbiome serves its hosts in capacities that go far beyond its role in food digestion. GF animals have dramatic alterations in practically every phenotype that has been studied, including the immune system, brain development, metabolism, behavior, and the function of the heart, lungs, and lymph nodes (Williams, 2014). In addition, the combination of gnotobiotic techniques with other new approaches has revealed causative associations between alterations in the commensal microbiota and diseases such as inflammatory bowel disease (IBD), obesity, and multiple sclerosis, among others (Balish and Warner, 2002; Ley et al., 2006; Turnbaugh et al., 2006; Berer et al., 2011).

WHY USE GNOTOBIOTIC RODENTS IN STUDIES OF MICROBE–MICROBE INTERACTIONS?

In the mammalian gut, microbes exist in a complex network of cooperation and competition. Simplified experimental culture conditions, in many cases, do not adequately represent the *in vivo* activities and connections among different members of the network, which prevents accurate evaluations of their individual contributions to overall microbiota functionality (Stecher et al., 2013a). Instead, gnotobiology offers a model in which microbe–microbe interactions can be assessed directly in a more complex environment. Furthermore, the use of initially GF animals enables complete knowledge of the microbial composition of a host, with consequent advantages for the interpretation of results compared to studies of conventional mice. For all these reasons, gnotobiotic studies offer a wide range of advantages compared to both other animal models and *in vitro* conditions. Gnotobiotics represents the optimal compromise between the realistic complexity of conventional rodents and the controlled nature of *in vitro* tests, without the incomplete knowledge and control of the environment inherent in the former and the oversimplifications of the latter.

Gnotobiotics as a Model of Synergistic Microbial Interactions (Cooperative Network Analysis)

The diverse intestinal microbiota is characterized by extensive synergistic ecological interactions (Stecher et al., 2013a). Gnotobiotic animals have been employed for deeper analyses of these important microbial interactions, in particular (i) bacterial succession, (ii) cross-feeding, and (iii) phage regulation of bacterial populations.

Most studies of bacterial succession have focused on the initial gut colonization process (primo-colonization; Fanaro et al., 2003). In these studies, different bacteria are introduced (sequentially or not) into GF animals in order to mimic the progressive colonization of the sterile intestine that begins immediately after birth. For instance, Tomas et al. (2013) compared the transfer of two different microbiota to GF rats: one from suckling rats, rich in early colonizing bacteria, and the other obtained from adult rats, representative of a mature microbiota. In rats with the early colonizing microbiota, strictly anaerobic bacteria succeeded aero-tolerant bacteria. Because of this coordinated process of bacterial succession, at the end of the experiment the microbiotas of both groups had converged on the same species profile, that of the mature inoculum. Similar results have been described for the process of microbiota acquisition in conventional mice (Gillilland et al., 2012). However, the mechanisms behind this succession remain poorly described, as this study focused only on host responses and not the evolution in microbe–microbe interactions (Tomas et al., 2013).

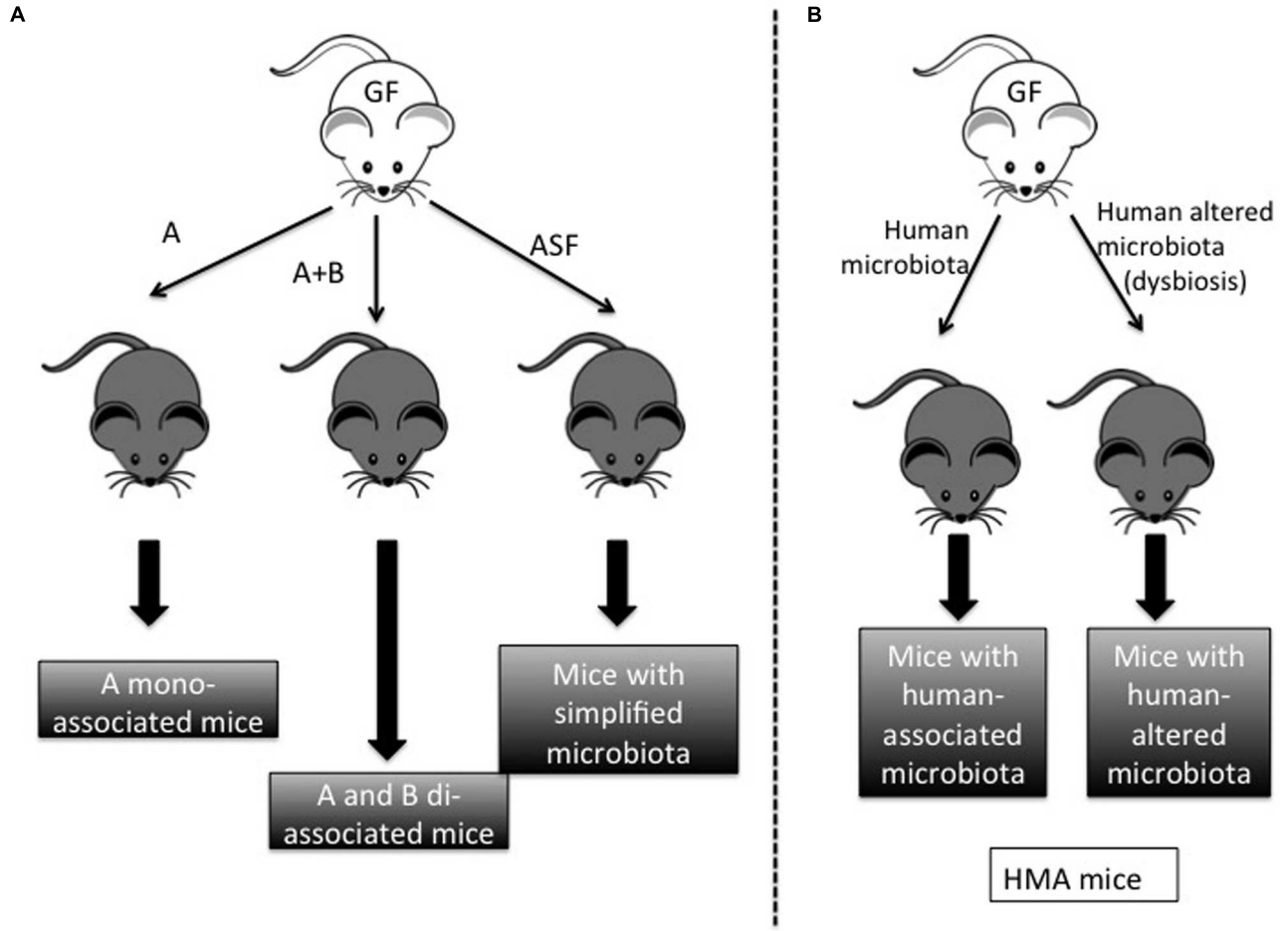

FIGURE 1 | Different models derived from germ-free (GF) mice. (A) Gnotobiotic models: GF mice can be colonized by one bacterium (microorganism A) in order to obtain A-mono-associated mice. Similarly, GF mice can be colonized by microorganisms A and B to obtain A + B di-associated mice. When mice are colonized by a basic microbiota such as the altered Schaedler Flora (ASF), we obtain mice with a simplified microbiota. **(B)** Non-gnotobiotic models: when mice are colonized by human microbiota from healthy or unhealthy individuals (normally with dysbiosis), we obtain mice with human-associated microbiota (HMA).

Aero-tolerant bacteria are thought to "prepare" the colonic environment for the growth of the strictly anaerobic bacteria that will later predominate. Researchers have taken advantage of this phenomenon to introduce certain strictly anaerobic bacteria into mice with a controlled microbiota. For example, the bacterium *Faecalibacterium prausnitzii* has well-known anti-inflammatory properties that contribute to its role as a sensor and promoter of intestinal health (Miquel et al., 2013, 2014). However, because it is EOS, it is quite difficult to obtain monoxenic *F. prausnitzii*-associated rodents. By preparing the environment with *Escherichia coli*, though, Miquel et al. (2015) were able to generate gnotobiotic mice that harbored both *F. prausnitzii* and *E. coli*. Similarly, dixenic *F. prausnitzii*-associated rats were also obtained by first inoculating the rats with *Bacteroides thetaiotaomicron* (Wrzosek et al., 2013) (**Figure 2**). *B. thetaiotaomicron* is an anaerobic bacterium; rather, it decreases the oxido-reductive potential of the gastrointestinal tract (GIT), thus enabling colonization by *F. prausnitzii*. In addition, *B. thetaiotaomicron* and *F. prausnitzii* complement each other metabolically: the former is acetate-producing whereas the latter is acetate-consuming and butyrate-producing (Duncan et al., 2002; Mahowald et al., 2009). In di-associated rats, *B. thetaiotaomicron* produces acetate and *F. prausnitzii* transforms this into butyrate (Wrzosek et al., 2013). These short-chain fatty acids (SCFAs) are also able to stimulate the host response. For instance, SCFA trigger pleiotropic signals in the host, including signals regulating mucin synthesis and secretion (Gaudier et al., 2004; Burger-van Paassen et al., 2009). Both of these dixenic models illustrate the importance of bacterial interactions during the colonization of GF rodents by microorganisms. Since strictly anaerobic bacteria are major components of the intestinal microbiota, these mutualistic relationships are likely of vital importance in microbiota acquisition and maintenance.

As mentioned above, the study of bacterial cross-feeding phenomena is one area in which the tools of gnotobiology will continue to be indispensable. Special models of human microbiota have been developed for this purpose, as only 15% of

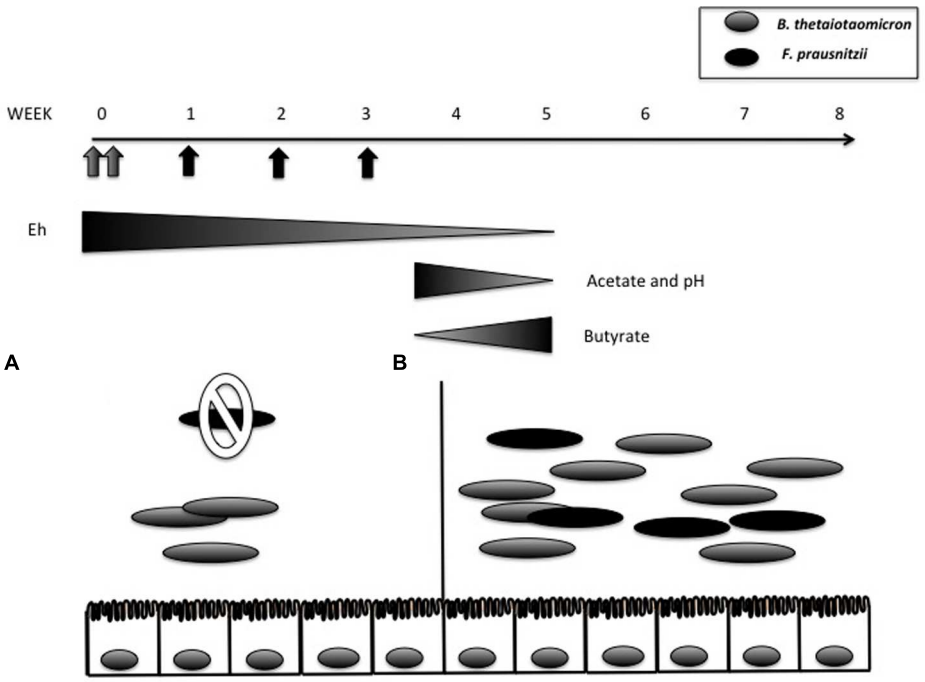

FIGURE 2 | *Bacteroides thetaiotaomicron* (gray oval) and *Faecalibacterium prausnitzii* (black oval) di-associated mice production process. (A) When *B. thetaiotaomicron* and *F. prausnitzii* were inoculated individually (gray arrows), only *B. thetaiotaomicron* colonized. **(B)** *F. prausnitzii* was successfully implanted after week 4 following three weekly inoculations (black arrows). The oxido-reductive potential (measured by Eh value) in the cecum progressively decreased in the presence of *B. thetaiotaomicron* (from 178 mV in GF rats to 141.1 mV at day 2) and decreased further in the presence of *F. praustnizii* (−274 mV at day 30). Comparison of *B. thetaiotaomicron* mono-associated rats with dixenic rats revealed that in the presence of *F. praustnizii* there was a decrease in pH and acetate and an increase in butyrate. Adapted from Wrzosek et al. (2013).

the bacterial species found in mice are also present in humans (Tomas et al., 2012). Human microbiota-associated (HMA) mice have also been employed as models in the study of the ecology and metabolism of the human intestinal microbiota (Hirayama and Itoh, 2005). For instance, studies of GF mice colonized by human microbiota have clarified the cross-feeding activities of *Akkermansia muciniphila* (Van den Abbeele et al., 2011), a highly specialized bacterium capable of utilizing mucus as a sole carbon and nitrogen source (Derrien et al., 2004; Everard et al., 2013). The mucus-degrading ability of *A. muciniphila* and its localization within the mucus layer have revealed its specific niche and function within the gut (Swidsinski et al., 2009; Png et al., 2010). By degrading mucus, *A. muciniphila* has the ability to produce SCFAs (acetate and propionate) (Derrien et al., 2004), which can stimulate microbiota interactions and a host response. Specifically, oligosaccharides and acetate stimulate growth and metabolic activity in commensal bacteria growing close to the mucus layer, which may in turn discourage pathogenic bacteria from crossing the mucus layer to reach the intestinal cells (Derrien et al., 2004, 2010, 2011). Furthermore, an increase in acetate and propionate due to the degradation of mucus by *A. muciniphila* could stimulate other bacterial groups with well-known butyrate-producing capacities as it has been proved *in vitro* with other acetate producers (Rios-Covian et al., 2015).

Other scientific research on the human microbiome has stimulated interest in probiotics, health-promoting agents that are able to modulate the intestinal microbiota. In a recent experiment, HFA mice were used to study the ability of *Bifidobacterium longum* strain BB536 to modulate the gut environment (Sugahara et al., 2015). HFA mice that were supplemented with strain BB536 showed increased fecal levels of butyrate and pimelate, a precursor of biotin. In addition, the BB536-supplemented mice also had metabolic alterations related to biotin synthesis, specifically involving *Bacteroides caccae* and the increased prevalence of *Eubacterium rectale*, a butyrate producer (Sugahara et al., 2015). All these possible interactions of *B. longum* BB536 with the microbial community might explain, at least in part, various reports of its beneficial physiological effects on the host, including anti-allergy effects, competition against harmful bacteria, and improvement in defecation frequency and stool characteristics (Odamaki et al., 2007, 2012; Xiao et al., 2007; Namba et al., 2010).

Gnotobiotics also offers clear advantages for the study of phage-bacteria dynamics in the human gut. Bacterial viruses (phages) are the most abundant biological group on earth (Rodriguez-Valera et al., 2009). In some ecosystems, they are capable of maintaining high levels of diversity of bacterial strains through lysis of their hosts, in a process described by the constant-diversity dynamics model of bacterial diversity; this model is based on the fact that many of the genes that differ between strains affect regions that are potential phage recognition targets (Rodriguez-Valera et al., 2009). Recently,

Reyes and coworkers introduced sequenced human gut bacteria into GF mice and followed this with a controlled phage attack using virus-like particles (VLPs) purified from the fecal microbiota (Rodriguez-Valera et al., 2009).The authors observed transient changes in bacterial community structure and bacterial acquisition of resistance to phage attack through both ecological and epigenetic mechanisms. These results illustrate the utility of gnotobiotic mice in characterizing ecological relationships among all components in the gut, viruses as well as bacteria.

Gnotobiotic Animals as Models of Antagonistic Microbial Interactions (Competitive Network Analysis)

Microbe–microbe interactions are not always cooperative. Antagonistic cross-talk is a fundamental link in microbial ecology and vital for the maintenance of host health. Gnotobiotic animals have been employed to obtain deeper insight into the ability of the microbiota to protect against infections, a process termed colonization resistance (Stecher et al., 2013b).

Alterations in microbiota homeostasis generally result in dysbiosis, an imbalance among bacterial species that often occurs in the digestive tract. This condition, also known as dysbacteriosis, is associated with illnesses such as IBD, chronic obesity, cancer, and bacterial vaginosis (Martin et al., 2013, 2014a,b). Efforts to link these diseases to specific changes in the microbiota have been hindered, though, by a lack of knowledge of healthy bacterial communities. Indeed, it is not well understood if dysbiosis itself is a consequence or a cause of an imbalance in bacteria–bacteria relationships in the gastrointestinal tract.

When colonization resistance is disrupted through antibiotic-mediated perturbations in the microbiota or other similar phenomena, infections by nosocomial pathogens tend to increase (Ubeda and Pamer, 2012; Stecher et al., 2013b). This observation could be explained by the 1983 proposal of Freter et al. (1983a,b) that populations of most intestinal bacteria are held in check by competition for limited substrates. GF animals provide an ideal testing ground in which to explore this hypothesis, as the level of competition within a particular host can be manipulated through the introduction of varying numbers of bacterial strains. Studies using this approach have found that, because they lack competing bacterial species, GF animals are more susceptible than conventional ones to infections by *E. coli, Clostridium difficile, Vibrio cholerae*, or *Citrobacter rodentium* (Collins and Carter, 1978; Butterton et al., 1996; Stecher et al., 2005; Kamada et al., 2012; Reeves et al., 2012). This hypothesis was further supported by experiments in which the transplantation of healthy fecal microbiota increased host resistance (Barman et al., 2008; Endt et al., 2010; Lawley et al., 2012). Thus, Freter's theory provides a straightforward explanation of why gnotobiotic mice are more sensitive to nosocomial infections: the lack of substrate competition reduces barriers to colonization. However, this conclusion is probably over-simplistic. Nowadays, studies from various fields, including gnotobiology, have concluded that colonization resistance is the result of not only a highly complex interplay among members of the commensal microbiota, but also of interactions among the microbiome [the entire community of microbes and their genetic material; (Martin et al., 2014a), the intestinal mucosa, and the immune system (Stecher et al., 2013b)]. For example, GF mice have been used to examine how individual bacteria species or bacterial communities influence colonization resistance to *C. difficile*. In particular, GF mice that had been inoculated only with a murine isolate from the family *Lachnospiraceae* demonstrated partially restored colonization resistance to *C. difficile* (Reeves et al., 2012), confirming that the indigenous microbial community of the GIT and the inter-relationships among its members determine susceptibility to colonization and growth of *C. difficile* (Reeves et al., 2011, 2012). These results highlight the potential of gnotobiotic mice as a controlled environment in which to study antagonist microbial interactions.

Colonization resistance is not the only example of antagonistic interactions in the microbiota. For example, gnotobiotic models have also been used to analyze growth competition between the well-known yoghurt bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. The growth and lactate production of these two bacteria were monitored in different media and in the GIT of GF rats. When GF rats were not supplemented with lactose, they were colonized only by *S. thermophilus,* not by *L. bulgaricus*. However, when the rats' drinking water was supplemented with lactose, both bacteria were able to colonize. Interestingly, when lactose-supplemented GF rats were inoculated with a mix of both bacteria, *S. thermophilus* colonized the GIT faster and more extensively than *L. bulgaricus* did. This work showed that *S. thermophilus* has a competitive and growth advantage over *L. bulgaricus in vitro* as well as *in vivo* in the GIT of GF rats (Ben-Yahia et al., 2012).

FUTURE TRENDS IN GNOTOBIOLOGY

At this point, gnotobiology is a mature science with applications that go far beyond conventional ones. The classical experiments and protocols used routinely in the study of microbes' interactions with their hosts can easily be modified in order to investigate interactions between and among multiple members of the microbiota. As an example, a study aiming to understand the effect of a microbe of interest on a particular disease would benefit greatly from the use of mice with a controlled, described microbiota. Such mice would then also be the perfect experimental system in which to analyze the relationship of the same microbe with the rest of the microbiota. Furthermore, this analysis could be performed in both healthy and diseased hosts. Similarly, our understanding of a pathogen's activity in a host could be enhanced through the use of various monoxenic mice: such mice could be infected with a pathogen and monitored in order to directly assess the effect of individual resident microorganisms on the disease caused by the pathogen. This model presents unique opportunities to analyze the possible antagonistic interactions between resident and invading microorganisms.

In brief, the options with gnotobiology are almost unlimited. Furthermore, the combination of gnotobiotics with new-*omics* approaches (e.g., transcriptomics, metabolomics, and

genomics) and molecular genetics (genetically modified mice and bacteria) could provide important insights into the roles of microbe–microbe interactions. This knowledge could improve our understanding of how commensal bacteria change into opportunistic pathogens, how pathogens are able to proliferate, and how probiotic bacteria could help to resolve dysbiosis.

CONCLUDING REMARKS

Here, we have summarized the most important outcomes of gnotobiological research in the field of microbe–microbe interactions as well as other concerns with relevance to the field. The most significant questions in microbiota analysis, including microbial interaction, rely on gnotobiotic animals for further advancements in the field. GF animals will be indispensable in future investigations of how certain microorganisms are able to colonize and survive in the host, while others, e.g., pathogens, are not. Studies of interaction and communication within the microbiota will also depend heavily on gnotobiotic approaches.

AUTHOR CONTRIBUTIONS

RM, LB-H, and PL designed the mini-review. RM wrote the manuscript. LB-H and PL corrected the manuscript. All the authors approved the final version of the manuscript.

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

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Relationship between Milk Microbiota, Bacterial Load, Macronutrients, and Human Cells during Lactation

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Human breast milk is considered the optimal nutrition for infants, providing essential nutrients and a broad range of bioactive compounds, as well as its own microbiota. However, the interaction among those components and the biological role of milk microorganisms is still uncovered. Thus, our aim was to identify the relationships between milk microbiota composition, bacterial load, macronutrients, and human cells during lactation. Bacterial load was estimated in milk samples from a total of 21 healthy mothers through lactation time by bacteria-specific qPCR targeted to the single-copy gene fusA. Milk microbiome composition and diversity was estimated by 16S-pyrosequencing and the structure of these bacteria in the fluid was studied by flow cytometry, qPCR, and microscopy. Fat, protein, lactose, and dry extract of milk as well as the number of somatic cells were also analyzed. We observed that milk bacterial communities were generally complex, and showed individual-specific profiles. Milk microbiota was dominated by *Staphylococcus*, *Pseudomonas*, *Streptococcus,* and *Acinetobacter. Staphylococcus aureus* was not detected in any of these samples from healthy mothers. There was high variability in composition and number of bacteria per milliliter among mothers and in some cases even within mothers at different time points. The median bacterial load was 10^6 bacterial cells/ml through time, higher than those numbers reported by 16S gene PCR and culture methods. Furthermore, milk bacteria were present in a free-living, "planktonic" state, but also in equal proportion associated to human immune cells. There was no correlation between bacterial load and the amount of immune cells in milk, strengthening the idea that milk bacteria are not sensed as an infection by the immune system.

Keywords: human microbiome, breast milk, lactation, qPCR, flow cytometry, somatic cells, 16S rRNA, bacterial load

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INTRODUCTION

Human milk is a complex fluid adapted to satisfy the nutritional requirements of the infant, and also protective compounds which help to create the right microenvironment for gut development and maturation of the immune system (Petherick, 2010; Walker, 2010). More recently, milk has been recognized to host commensal and potential probiotic bacteria, which together with milk's growth

factors and other components may have health implications. For example, they could be involved in the digestion of nutrients, facilitating the digestion process, although the most likely role for these microorganisms is immune modulation (Fernández et al., 2013) Culture-dependent methods have long confirmed the presence of viable bacteria in aseptically collected samples (Heikkilä and Saris, 2003). However, an important part of the species has not yet been cultured under laboratory conditions, and subsequently the diversity of human milk could be underestimated by classical approaches. Although partial contamination from skin microbes occurs, the presence of strictly anaerobic species such as *Bifidobacterium*, *Clostridium*, and some *Bacteroides* spp., which are absent in the skin microbiota, supports that breast milk hosts a unique microbiome (Hunt et al., 2011; Cabrera-Rubio et al., 2012a; Jost et al., 2013). Accumulating evidence suggeststhat milk microbiota is influenced by perinatal factors such as mode of delivery, lactation time, gestational age, maternal health, or geographical locations (Khodayar-Pardo et al., 2014; Cabrera-Rubio et al., 2016).

It has been estimated that an infant consumes ∼800 mL/day, ingesting between 1×10^4 and 1×10^6 bacteria daily (Heikkilä and Saris, 2003) but those data were based on culture techniques and may have underestimated the total load of microorganisms. Other non culturable-dependent methods, such as molecular techniques or cytometry should be implemented in order to make more accurate estimates of milk's bacterial densities (Collado et al., 2009). Knowing total bacterial numbers in milk will be useful to understand bacterial behavior and also, to estimate the bacterial load under infectious situations. This would open new possibilities to develop potential tools to detect problems in the nursing mother. Furthermore, it's known that milk contains a wide range of nutrients, such as lactose, fat or proteins, which can be used as bacterial food source (Petherick, 2010). Milk also contains a variable number of human cells, including epithelial and immune cells, and the number of the latter has been related to lactational mastitis problems (Hassiotou et al., 2013). Thus, the relationship between bacterial load and other factors such as milk developmental stage, nutrient composition, number of somatic immune cells, or bacterial diversity have not been studied in depth.

Therefore, the purpose of the present study was to develop and establish a protocol using molecular techniques and flow cytometry to calculate the exact number of bacteria present in milk at three lactation stages from different mothers, and correlate this bacterial load to the abovementioned factors that could influence it.

MATERIALS AND METHODS

Subjects and Sampling

A total of 21 healthy Spanish mothers with exclusive breast feeding practices participated in the study and provided samples of breast milk (BM) within 1 month after delivery. Breast-milk samples were collected within 5 days after mothers gave birth (colostrum), between 6 and 15 days (transition) and after 15 days (mature). However, only 57 samples were analyzed, as not all of mothers provided a sample at the three time points. Details of delivery and gestational age were collected after birth. Written informed consent was obtained from the participants and the study protocol was approved by the Ethics Committee of the CSIC (Spanish National Research Council).

Before sample collection, the mothers were given oral and written instructions for standardized collection of samples. Previously, nipples and mammary areola were cleaned with soap and sterile water and soaked in chlorhexidine to reduce bacteria residing on the skin. The milk samples were collected in a sterile tube manually, discarding the first drops, with a sterile milk collection unit. All samples were kept frozen at −20°C until delivery to the laboratory.

DNA Isolation

Milk samples (5–10 mL) were thawed and centrifuged at 4000 × g for 20 min to separate fat and cells from whey. Thereafter, total DNA was isolated from the pellets by using the MasterPure Complete DNA and RNA Purification Kit (Epicenter) according to the manufacturer's instructions with some modifications (Simón-Soro et al., 2015). Two hundred and fifty microliters of saline solution and 250 μl of lysis buffer were added to the pellets, together with Pathogen Lysis Tubes (QIAGEN) glass beads. Both chemical and physical cells disruption was performed after mixing vigorously the samples in a TissueLyser II (QIAGEN) during 5 min at 30 Hz, incubating in dry ice 3 and 5 min at 65°C in a thermoblock, repeating the process 2 times. Fifty microliters of lysozyme (20 mg/ml) and 5 μl of lysostaphin (20 μg/ ml) were added to the tubes, and the samples were incubated for 1 h at 37°C. Two microliters of proteinase K were added and samples were incubated for 15 m at 65°C. The reaction was ended putting tubes on ice, and proteins were precipitated using 350 μl of the protein precipitation agent, discarding the pellets. DNA was precipitated using isopropanol, washed with 70% Ethanol and resuspended with 30 μl TE buffer. The total DNA isolated was quantified with a NanoDrop ND-1000 (ThermoScientific) spectrophotometer.

Quantitative Real-Time Polymerase Chain Reaction Analysis and Bacterial Load

qPCR amplification and detection were performed with primers targeted to the fusA gene, a bacterial gene which is present in a single and unique copy per bacterial cell (Santos and Ochman, 2004), making it a more accurate target for bacterial load estimations compared to the 16S rRNA gene, which is present in variable copy numbers among different bacterial species. The use of a single-copy gene in qPCR analysis implies that the number of gene copies equals the number of bacterial cells, improving measures of bacterial densities. In this work, we used modified fusA gene primers from Santos and Ochman (2004), based on multiple alignments with all sequences of this gene in the Ribosomal Database Project Functional Gene Repository (Fish et al., 2013) as available on January 2015, using an annealing temperature of 62°C in a Light Cycler 480 Real-Time PCR System (Roche Technologies). The primer sequences were as follows: 138F- GCTGCAACCATGGACTGGAT, and 293R- TCRATGGTGAAGTCAACGTG. Each reaction mixture of 20 μl was composed of KAPA Sybr Fast qPCR Kit (KAPA

Biosistems), 0.4 µl of each primer (10 µM concentration) and 1 µl of template DNA using an annealing temperature of 62°C in a Light Cycler 480 Real-Time PCR System (Roche Technologies). All amplifications were performed in duplicates. The bacterial concentration in each sample was calculated by comparison with the Ct values obtained from standard curves. These were generated using serial 10-fold dilutions of DNA extracted from 10 million bacteria quantified and sorted from a pool of four milk samples from different mothers using a MoFlo XDP cytometer, after mild sonication to separate aggregated cells (Simón-Soro et al., 2015).

PCR Amplification and Pyrosequencing

Partial 16S rRNA genes were amplified by PCR with the universal bacterial primers 8F and 785R (Simón-Soro et al., 2014) by the use of high-fidelity AB-Gene DNA polymerase (Thermo Scientific) with an annealing temperature of 52°C and 20 cycles. A secondary amplification was performed by using the purified PCR product as a template, in which the universal primers were modified to contain the pyrosequencing adaptors A and B and an 8-bp "barcode" specific to each sample, following the method used in Benítez-Páez et al. (2013). The final DNA per sample was purified by using an Ultrapure PCR purification kit (Roche), and its concentration was measured by PicoGreen fluorescence in a Modulus 9200 fluorimeter from Turner Biosystems. PCR products were pyrosequenced from the forward primer end using a 454 Life Sciences system, in a GS-FLX sequencer with Titanium chemistry (Roche) at the Foundation for the Promotion of Health and Biomedical Research (FISABIO) in Valencia, Spain. Sequences were deposited in the MG-RAST public repository under the project name "Relationship between milk microbiota, bacterial load, macronutrients, and human cells during lactation" with Accession Numbers 4689674.3-4689703.3.

Sequence Analysis

Sequences with an average quality value <20 and/or with >4 ambiguities in homopolymeric regions in the first 360 flows were excluded from the analysis. Obtained 16S rRNA reads were end-trimmed in 10 pb sliding windows with average quality value >20, then length (200 bp) and quality filtered (average $Q > 20$). Only sequences longer than 400 bp were considered and chimeric reads were eliminated using UCHIME (Edgar et al., 2011). Sequences were assigned to each sample by the 8-bp barcode and phylum-, family-, and genus-level taxonomic assignment of sequences that passed quality control were made using the Ribosomal Database Project classifier software (Wang et al., 2007) within an 80% confidence threshold. Sequences >97% identical were considered to correspond to the same operational taxonomical unit (OTU), representing a group of sequences that presumably correspond to the same species (Yarza et al., 2008). Sequences were clustered at 97% nucleotide identity over 90% sequence alignment length using the CD-hit software (Li and Godzik, 2006). Rarefaction curves were calculated with the RDP pyrosequencing pipeline (Cole et al., 2009) using the same number of randomly selected sequences per group and Chao1 and Shannon estimations (representing species richness and diversity, respectively) were obtained. For those genera found at higher than 1% frequency, a BLASTn (Altschul et al., 1997) was performed against the RDP database, selecting those hits with nucleotide identity values >97% and alignment lengths >400 bp, following (Cabrera-Rubio et al., 2012b).

Milk Composition Analysis

We analyzed 38 milk samples from 17 mothers with known bacterial load to elucidate their fat, protein, and lactose composition (% w/w) by spectrophotometry using a MilkoScan FT 6000 (FOSS), and the number of somatic cells (cells/ml) using an Integrated Milk Testing Fossomatic FC(FOSS) cytometer, in LICOVAL, Valencia, Spain.

Bacterial Fractions in Milk

Bacterial distribution in human milk was determined after analyzing 10 ml of colostrum ($n = 9$) and mature milk ($n = 9$) samples, using a MoFloXDP cytometer with sorter. Transition samples were not analyzed due to lack of volume availability. Light was produced by an argon laser of 400 nm (blue light) and 200 Mw. First, the machine was calibrated using electromagnetic beads (Fluorospheres, Beckman Coulter Inc.) with known size (1,

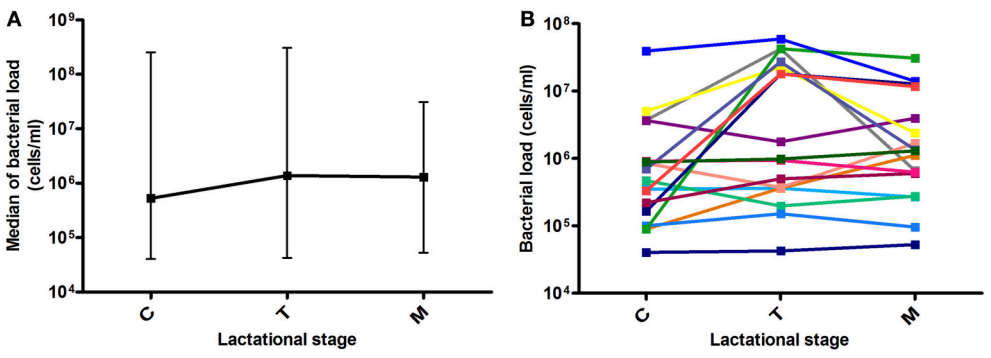

FIGURE 1 | Bacterial load over lactational stages. (A) Data show the median with ranges (maximum and minimum values for each group) of bacterial load at the three time points. C, colostrum samples ($n = 19$); T, transition milk samples ($n = 20$); M, mature milk samples ($n = 17$). **(B)** Lines show individual bacterial load for each mother at the three time points ($n = 17$).

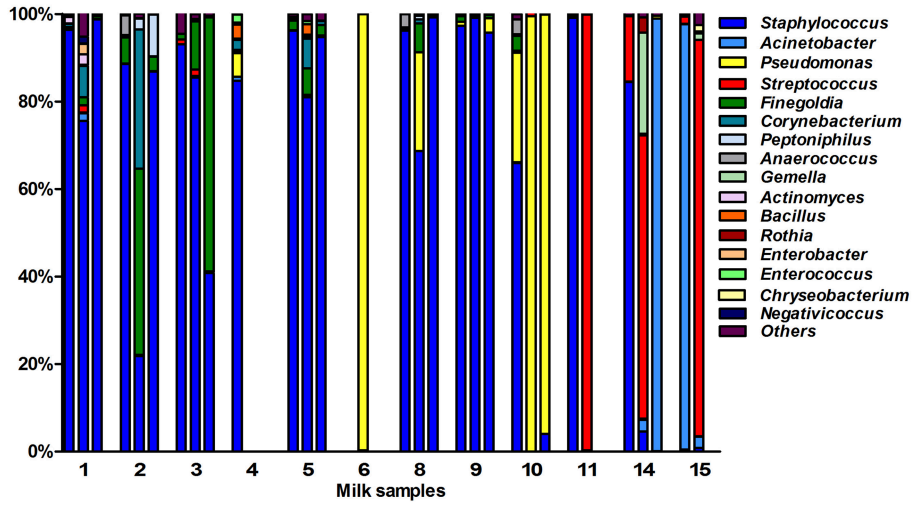

FIGURE 2 | Bacterial taxonomic composition of human breast milk. The bars show the proportion of bacterial genera as inferred by PCR amplification and pyrosequencing of the 16S rRNA gene in healthy mothers ($n = 12$). Each number in the X axis represent a donor, with first column representing the colostrum sample, second the transition milk and third the mature milk samples. In some cases, data from the three breastfeeding stages could not be obtained due to sample unavailability or sequencing failure. Bacterial genera that were under 1% were grouped in the "Others" category.

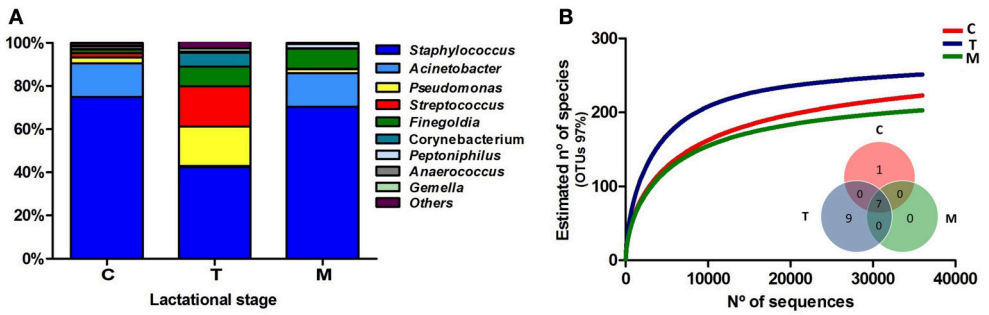

FIGURE 3 | Bacterial diversity of human breast milk. (A) Shows the proportion of each bacterial genera in the three lactational-stages, as inferred by PCR amplification and pyrosequencing of the 16S rRNA gene. **(B)** Shows the rarefaction curves of the three groups, relating the sequencing effort with an estimate of the number of bacterial species, as inferred by the number of OTUs. An OTU is a cluster of 16SrRNA sequences that were >95% identical, a conservative estimate for the boundary between species, established at 97% for full-length 16S rRNA sequences. The inlet Venn's diagram shows the number of bacterial genera shared between and unique to the three sample types, excluding bacterial genera present at <1% proportion. Seven genera are shared at the three breastfeeding stages: *Finegoldia, Streptococcus, Corynebacterium, Staphylococcus, Acinetobacter, Peptoniphilus,* and *Pseudomonas*. C, colostrum samples ($n = 11$); T, transition samples ($n = 11$); M, mature samples ($n = 8$).

3, and 10 μm). Then, events under 3 μm (containing planktonic bacteria) and those over 3 μm (containing human cells) were counted and sorted in two different tubes. DNA was extracted from both fractions for each sample, and qPCR was performed to determine the number of bacteria present in each of them, corresponding to free-living and human cells-associated bacteria. Fluorescence microscopy was performed on a selected number of samples after marking with DAPI dye, and visualized on a Nikon Eclipse E800 microscope. For Scanning Electron Microscopy, samples were kept on Karnovsky solution and further fixed with 1% OsO_4 in PBS buffer. Samples were then dehydrated with ethanol and critical-point drying, attached to a stub and coated with gold. Images were obtained in a Hitachi S-4800 Scanning electron Microscope with default settings at University of Valencia.

RESULTS AND DISCUSSION

Bacterial Load in Milk

Bacterial load values at each milk maturation stage are shown in **Figure 1**. After analyzing 56 milk samples by qPCR, results showed large individual differences in bacterial load over time between samples from the different mothers and in some cases even within individuals at different time points, indicating that human milk samples are highly variable in microbial content. Median values for colostrum, transition and mature milk were

TABLE 1 | Prevalence of bacterial genera and species in breast milk samples.

Genera	Prevalence[a]	Species	Prevalence[b]
Staphylococcus	24/30	*Staphylococcus epidermidis*	22/24
		Staphylococcus lugdunensis	5/24
		Staphylococcus hominis	5/24
		Staphylococcus microti	3/24
		Staphylococcus warneri	1/24
		Staphylococcus equorum	1/24
Streptococcus	13/30	*Streptococcus mitis*	7/13
		Streptococcus infantis	6/13
		Streptococcus cristatus	5/13
		Streptococcus salivarius	4/13
		Streptococcus mutans	3/13
		Streptococcus sanguinis	3/13
		Streptococcus gordonii	1/13
		Streptococcus sanguinosus	1/13
Finegoldia	9/30	*Finegoldia magna*	9/9
Pseudomonas	8/30	*Pseudomonas deceptionensis*	3/7
		Pseudomonas fragi	3/7
		Pseudomonas meridiana	3/7
		Pseudomonas gessardii	2/7
		Pseudomonas moorei	1/7
		Pseudomonas japonica	1/7
		Pseudomonas sasplenii	1/7
Acinetobacter	7/30	*Acinetobacter haemolyticus*	4/7
		Acinetobacter junii	2/7
		Acinetobacter ursingii	2/7
		Acinetobacter lwoffii	2/7
		Acinetobacter parvus	1/7
		Acinetobacter guillouiae	1/7
		Acinetobacter pittii	1/7
		Pseudomonas alcaliphila	1/7
Anaerococcus	5/30	*Anaerococcus octavius*	5/5
		Anaerococcus murdochii	1/5
		Anaerococcus prevotii	1/5
Actinomyces	4/30	*Actinomyces radingae*	3/4
		Actinomyces neuii	2/4
Enterobacter	4/30	*Enterobacter cancerogenus*	2/3
		Enterobacter aerogenes	1/3
		Enterobacter hormaechei	1/3
		Enterobacter asburiae	1/3
		Enterobacter kobei	1/3
Peptoniphilus	3/30	*Peptoniphilus lacrimalis*	1/3
		Peptoniphilus gorbachii	1/3
		Peptoniphilus harei	1/3
Gemella	3/30	*Gemella haemolysans*	3/3
Rothia	3/30	*Rothia mucilaginosa*	3/3
Corynebacterium	2/30	*Corynebacterium simulans*	1/2
		Corynebacterium xerosis	1/2
		Corynebacterium amycolatum	1/2
Bacillus	2/30	*Bacillus thuringiensis*	1/2
		Bacillus circulans	1/2
		Bacillus megaterium	1/2
Chryseobacterium	1/30	*Chryseobacterium daeguense*	1/1

Assignment to the species taxonomic level was performed by BLASTn selecting only alignments >300 bp and sequence identity >97%.

[a] *Data indicate the number of samples containing the indicated genus.*

[b] *Data indicate the number of samples containing the indicated species referred to the number of samples containing the corresponding genus.*

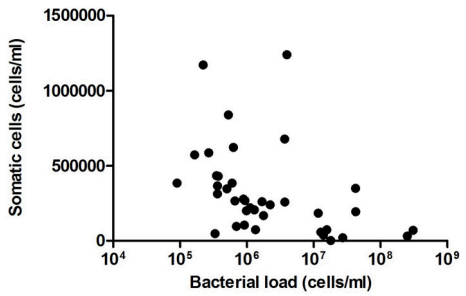

FIGURE 4 | Relationship between number of somatic cells and bacterial load in milk samples. The graph shows a comparison between the number of bacterial cells per milliliter (estimated by qPCR) and the number of somatic cells per milliliter, estimated with an Integrated Milk Testing Fossomatic 5000 (FOSS) cytometer. ($n = 38$, $R^2 = 0.0066$). C, colostrum samples ($n = 12$); T, transition samples ($n = 15$); M, mature samples ($n = 11$).

around 10^6 bacterial cells per ml, with no significant differences between the three time points. Data from other researchers had indicated bacterial densities of 10^3–10^4 per ml of breast milk, but they were based on laboratory culture (Heikkilä and Saris, 2003), or on qPCR methods calibrated by culture (Khodayar-Pardo et al., 2014), which account for a limited fraction of total bacteria in human samples. In addition, a significant fraction of microorganisms were found to be adhered to the extracellular matrix of human cells (see Section Bacterial Distribution in Milk Below), which could further prevent the growth on culture media. The molecular approach used in the current manuscript expands these pioneering estimates, allowing now the study of potential relationships between bacterial load and other parameters. Although our molecular-based methods suggest bacterial loads between two and three orders of magnitude higher than those estimated by culture, it has to be taken into account that DNA from non-viable bacteria and extracellular DNA would also be amplified by qPCR, and therefore the real number of viable bacteria would probably be lower.

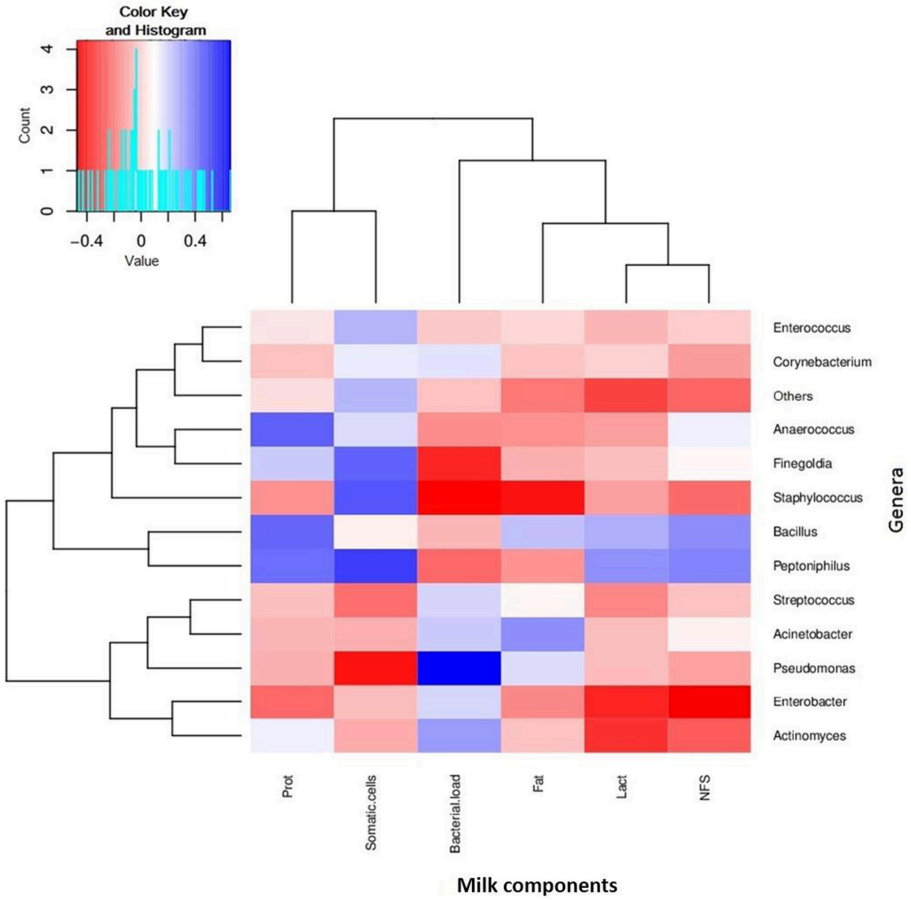

FIGURE 5 | Relationships between bacterial composition and nutritional or cellular content of human breastmilk. The figure shows a heatmap where samples have been clustered according to its compositional profile. Bacterial genera appear color-coded according to their under- (red) or over-representation (blue) in the samples, and its proportion is correlated to the amount of protein content (indicated as "prot" in the figure), fat content (Fat), lactose content (Lact), and non-fatty solid content (NFS), as well as the density of bacterial and human somatic cells. $n = 30$.

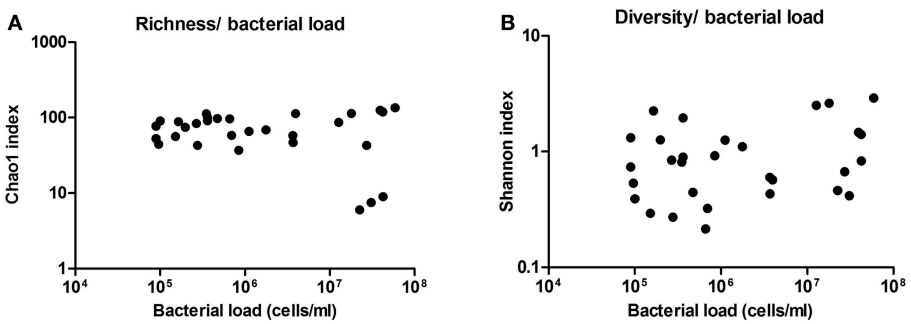

FIGURE 6 | Richness and diversity of milk samples. (A) Shows the richness in the samples as inferred by computation of Chao1 index, compared with bacterial load in cells per ml, as estimated by qPCR. **(B)** Shows the diversity in the samples as inferred by Shannon index, compared with bacterial load. ($n = 30$ in both cases).

Milk Bacterial Composition across Lactation Time

After quality filtering and length trimming, 174,886 16S rRNA sequences were analyzed, with an average number of taxonomically assigned, high-quality sequences of 4353 reads per sample. The taxonomic assignment of the sequences showed that human breast milk composition is dominated by *Staphylococcaceae,* which account for >62% of the total

number of sequences obtained (**Figure 2**). At the three lactation times, the most common genera was *Staphylococcus,* followed by *Acinetobacter* in colostrum*, Pseudomonas* and *Streptococcus* in transition milk and also *Acinetobacter* in mature milk samples (**Figure 3A**). Milk from the three lactation points showed different patterns of bacterial diversity, but no statistically significant differences were found between timepoints for any bacterial genus. Rarefaction curves after analyzing 35,000 reads per lactation time point indicated 223 OTUs in colostrum samples, 251 in transition and 203 in mature samples when sequences were clustered at 97% sequence identity (the consensus value for determining species boundaries; **Figure 3B**). The number of OTUs obtained suggests values of several hundred species in human breast milk, with transition samples having higher diversity than colostrum and mature milk, containing up to nine genera that were only found at that stage (**Figure 3B**). Similar estimates of several hundred bacterial species were also obtained by other studies (Hunt et al., 2011, Cabrera-Rubio et al., 2012a), confirming that human breast milk is highly diverse. However, most diversity in the samples corresponded to a few bacterial genera, which appeared to be dominant. Among them, we found a core of seven genera that were present at the three time points: *Finegoldia, Streptococcus, Corynebacterium, Staphylococcus, Acinetobacter, Peptoniphilus,* and *Pseudomonas.* Although determining the bacterial species composition with partial 16S rRNA sequences has to be taken with care, the relatively long sequences obtained by pyrosequencing (average read length 718 bp) allowed us to assign reads to the species taxonomic level with some degree of reliability. This analysis revealed that the most common species within Staphylococci was *S. epidermidis*, and *S. aureus* was not detected in these healthy mothers (a full list of species composition can be found in **Table 1**). It must be underlined that although some bacteria typically associated to human breast milk like *Bifidobacterium* spp (Collado et al., 2009) were detected at low proportions in our samples, this could be due to low amplification efficiency of "universal" primers in these high G+C content taxa (Sim et al., 2012).

It is interesting to note that the bacterial genera found in our samples, which were obtained from Spanish mothers, was different to those found in other high-throughput sequencing studies from American or Finnish milk samples (Hunt et al., 2011, Cabrera-Rubio et al., 2012a), suggesting that geographic, genetic, and dietary factors could be influencing microbial diversity in breast milk.

Relationship between Bacterial Load and Milk's Composition and Diversity

After comparing the number of somatic cells and bacterial load in the same samples, no significant correlation was found (**Figure 4**). Given that the number of somatic cells in milk is considered the gold standard for detecting infection (e.g., lactational mastitis) in farm animals (Olechnowicz and Jaśkowski, 2012), the absence of a somatic cell increase in our samples suggests a lack of significant immune response. Thus, the data presented in the current work suggest that high counts

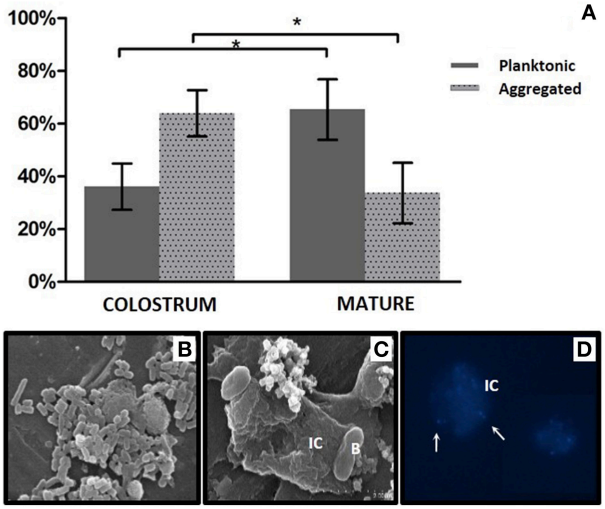

FIGURE 7 | Bacterial fractions in human breast milk. (A) Proportion of bacteria present in a free-living, "planktonic" state and aggregated to human immune cells in colostrum and mature milk samples. Bacteria from 10 ml of milk were counted and sorted by size and complexity using a Moflo cytometer. *indicates a $p < 0.05$, Mann–Whitney test. **(B)** Planktonic bacteria in milk observed by SEM microscopy. **(C)** Bacteria associated to human immune cells, observed with SEM microscopy. **(D)** Bacteria associated to human immune cells, observed with fluorescence microscopy. DNA was stained with DAPI fluorophore. Bacteria are indicated with arrows. IC, human immune cell; B, bacteria.

of bacteria in milk are not associated with infection in these healthy mothers without lactation problems. However, a positive correlation was found between the proportion of the common mastitis pathogen *Staphylococcus* and the number of somatic cells (Pearson correlation coefficient: 0.48, $p = 0.0457$). Given that a negative relationship was found between the proportion of *Staphylococcus* and the total bacterial load (correlation coefficient: -0.456, $p = 0.056$), the data suggest that it is not the number of bacteria but the specific composition of the milk microbiota that could be inducing an immune response in the mammary gland, although the major mastitis pathogen *S. aureus* was not detected in our samples (**Table 1**). Other bacteria appeared to show a positive relationship with the number of somatic cells were *Peptoniphilus* and *Finegoldia* (**Figure 5**), although the correlations were not statistically significant in these cases. It has to be kept in mind that breast milk contains several anti-inflammatory (He et al., 2016) that could partly reduce somatic cells counts.

Additionally, we analyzed the diversity and richness of the bacteria present in these samples by the statistic indexes "Shannon" and "Chao1," respectively. Neither diversity nor richness increased or decreased significantly with bacterial load (**Figure 6**). This also supports a lack of subclinical or sub-acute mastitis, as an increase of a few dominant bacteria would be expected in case of infection, and suggests that milk microbiota is not activating an immune response in the host, although inflammatory markers have not been measured.

We also analyzed fat, protein, lactose and non-fatty solid fractions in milk, and compared them with the

bacterial load, in order to find any possible correlations (**Supplementary Figure 1**). No significant correlations were found with the number of bacteria per ml. However, some positive and negative relationships were found between some nutrients and specific bacterial genera (**Figure 5**). For instance, the amount of proteins were positively correlated with the proportion of *Bacillus*, *Peptoniphilus,* and *Anaerococcus* in the samples, whereas lactose levels were negatively correlated with *Enterobacter* and *Actinomyces*, indicating potential prebiotic and antagonistic effects for bacterial growth, which should be evaluated in bigger sample sizes. In the case of fat, whose content in milk is known to increase through breastfeeding, it was negatively correlated with the proportion of *Staphylococcus* (Pearson correlation coefficient: -0.425, $p = 0.0443$), and therefore if this negative relationship is confirmed in larger cohorts, high fat content in milk could potentially be protective of mastitis risk.

Bacterial Distribution in Milk

Bacterial loads in planktonic and human cell-associated fractions of nine samples of colostrum and nine samples of mature milk were calculated, showing that the microorganisms were present in both fractions, although aggregated bacteria appeared to be more abundant in colostrum (65.75%), and planktonic bacteria were found to be more abundant in mature samples (63.92%; **Figure 7A**). Mann-Whitney statistical tests showed significant differences ($p < 0.05$) between the two time points (but not within the same time point) for both free and human cell-associated bacteria. The high proportion of bacteria associated with human immune cells was confirmed by fluorescence and Scanning Electron microscopy (**Figures 7B–D**). Bacteria in the aggregated fraction seemed to be adhered to the membrane of human cells (identified as immune cells according to their shape and size) but not intracellular. We confirmed the presence of live bacteria moving inside the extracellular matrix of immune cells (**Supplementary Video**). Bacterial cells in this extracellular matrix have also been observed in blood samples from pregnant mothers by other researchers (Donnet-Hughes et al., 2010).An "entero-mammary pathway" has been proposed to explain the translocation of bacteria to the mammary gland through blood and/or lymph stream through its association to human immune cells (Martín et al., 2004). If this translocation process is confirmed, the milk cell-bacterial association described here could be a consequence of such a relationship. An alternative explanation would be that bacteria originated from skin and the oral cavity of the lactating child invades the mammary gland and binds to immune cells without eliciting a response (Hagi et al., 2013). Future studies should determine the kind of immune cells involved in the observed bacterial adhesion and the nature of the bacteria-human recognition (Langa, 2006; Perez et al., 2007), including the identification of which microorganisms are free and which ones are human cell-associated.

CONCLUSION

Our estimates of bacterial load provided by molecular methods indicate that a lactating infant feeding 800 ml of breastmilk per day could ingest 10^7–10^8 bacterial cells daily, about 100 times higher than previous estimates based on laboratory culture methodologies. Our data show that samples with higher bacterial load in healthy mothers do not suffer from lower diversity, as it would be expected from microbial infections. In addition, no correlation between human and bacterial cells was found in milk, suggesting that milk microbiota is not seen as an infection by the mother's immune system, and that the immune response is directed toward specific microorganisms such as *Staphylococcus*. Furthermore, specific relationships between macronutrients and specific bacteria have been described. However, more studies with higher number of samples are needed to confirm and identify key interactions between bacteria and nutrients and their potential impact in infant health. Thus, the biological function of these potentially symbiotic bacteria for infant health could be relevant, including a role in the development of their immune system, and should be elucidated.

AUTHOR CONTRIBUTIONS

AM and MC conceived the study project. AB, MC, and AM designed experiments. AB performed experiments and analyzed the data. All authors contributed to interpretation, drafted, and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00492

Supplementary Video | Time-lapse photography of a human milk immune cell containing a live bacterial cell embedded in its surface. Apart from being free-living, between 40 and 60% of milk bacteria were found attached to human cells, as estimated by qPCR of plancktonic and aggregated bacteria.

Supplementary Figure 1 | Relationship between bacterial load and macronutrients in human milk. The graphs show the comparison between bacterial load and: **(A)** protein, **(B)** lactose, **(C)** fat, and **(D)** non fatty solids in the samples ($n = 38$).

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frontiers in Microbiology

MINI REVIEW
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Bacterial Cell–Cell Communication in the Host via RRNPP Peptide-Binding Regulators

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Human microbiomes are composed of complex and dense bacterial consortia. In these environments, bacteria are able to react quickly to change by coordinating their gene expression at the population level via small signaling molecules. In Gram-positive bacteria, cell–cell communication is mostly mediated by peptides that are released into the extracellular environment. Cell–cell communication based on these peptides is especially widespread in the group Firmicutes, in which they regulate a wide array of biological processes, including functions related to host–microbe interactions. Among the different agents of communication, the RRNPP family of cytoplasmic transcriptional regulators, together with their cognate re-internalized signaling peptides, represents a group of emerging importance. RRNPP members that have been studied so far are found mainly in species of bacilli, streptococci, and enterococci. These bacteria are characterized as both human commensal and pathogenic, and share different niches in the human body with other microorganisms. The goal of this mini-review is to present the current state of research on the biological relevance of RRNPP mechanisms in the context of the host, highlighting their specific roles in commensalism or virulence.

Keywords: quorum sensing, firmicutes, virulence, commensalism, cell–cell communication

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INTRODUCTION

Throughout the human body, all surfaces with portals to the exterior are covered in microbes (Foxman and Martin, 2015), mostly bacteria, which adapt to the specific environmental conditions of each niche by fighting, competing, or co-habiting with other bacteria and host cells.

In these kinds of complex consortia, bacteria have developed sophisticated communication mechanisms wherein signaling molecules are exported into the extracellular environment, accumulated, and then detected by a sensor protein. This sensing leads bacteria to modulate the expression of target genes, thus enabling them to escape from immune defense or attack the host (in the case of virulence) or compete with other bacteria (in commensalism).

In Firmicutes, cell–cell communication, or quorum sensing, is mostly mediated by peptides. Most of these peptides are encoded by short open reading frames but a few of them are produced by the proteolytic degradation of signal peptides of lipoproteins. All of them are then released into the extracellular environment (Antunes and Ferreira, 2009; Atkinson and Williams, 2009). Some of these peptides can be detected in the extracellular medium by two-component systems, while others are actively imported back into bacteria by the oligopeptide transporter Opp, at which point they interact with sensors in the cytoplasm. This interaction modulates the activity of the sensors and thus the expression of target genes (Rocha-Estrada et al., 2010; Cook and Federle, 2014; Monnet and Gardan, 2015). These sensors belong to the RRNPP

family of cytoplasmic regulatory receptors (name given from the different sensors described: Rgg, Rap, NprR, PlcR, and PrgX). They are characterized at the structural level by tetratricopeptide repeats, which are involved in the regulator/peptide interaction (Declerck et al., 2007; Parashar et al., 2015). Rgg, NprR, PlcR, and PrgX are transcriptional regulators *sensu stricto* and will be examined in this review (**Table 1**), whereas the Rap phosphatases found in bacilli will not. RAP proteins have a phosphatase activity and regardless of this catalytic activity, they can also inhibit the transcriptional activity of response regulators of two component systems. They have been mainly studied in *Bacillus subtilis* and are involved in the control of sporulation, competence, and production of degradative enzymes and antibiotics (for review see Pottathil and Lazazzera, 2003).

Different RRNPP mechanisms have been deciphered in detail (**Figure 1**) and described in several recent reviews (Cook et al., 2013; Slamti et al., 2014; Fontaine et al., 2015). However, these mechanisms have been validated mostly *in vitro*, and very few studies have explored how they function in complex environments. The RRNPP members that have been studied so far, are found in bacilli, streptococci, or enterococci. Some of the species of interest are human opportunistic pathogens and share different niches in the human body with other bacteria. This mini-review will focus on the data available concerning the functioning of these mechanisms in the context of the host. In addition, we will examine whether these systems are linked to virulence or commensalism.

PrgX/TraA REPRESSORS AND VIRULENCE IN ENTEROCOCCI

Conjugation is a horizontal gene transfer mechanism that controls the transfer of genes from donor cells to recipient cells after contact. Conjugation of sex pheromone plasmids in *Enterococcus faecalis* is controlled by a transcriptional repressor in the PrgX/TraA family that is able to bind either an activating or a repressing peptide. The conjugation of the pCF10 plasmid has been studied in detail. Very briefly, when bound to the activating peptide cCF10, PrgX adopts a dimeric form, which allows the expression of *prgQ* and the conjugation genes. In this way, conjugation can occur between pCF10 of the donor and the recipient cell. Instead, when bound to the inhibitory peptide iCF10, the PrgX repressor adopts a tetrameric conformation; this represses the transcription of *prgQ*, which encodes iCF10, as well as that of downstream conjugation genes. Therefore, the conjugation cannot occur (**Figure 1**) (Clewell et al., 2014). This mechanism and additional regulatory pathways at the transcriptional and post-transcriptional levels have been thoroughly described in multiple recent reviews (Dunny, 2007, 2013; Dunny and Johnson, 2011).

E. faecalis is a commensal bacterium that colonizes the intestinal tract of humans as well as the vagina and oral cavity, but it is also an opportunistic pathogen which causes significant nosocomial infections. Over 20 plasmids belonging to the sex pheromone family have been found to be more prevalent in clinical enterococcal strains (Coque and Murray, 1995). *In vivo* conjugation has been reported in human plasma (Hirt et al., 2002) as well as in different animal models such as the gastrointestinal tract of hamsters, rabbit plasma, and the endocarditis or subdermal chamber in rabbits (Huycke et al., 1992; Hirt et al., 2002).

Part of the regulon of the PrgX system is the aggregation substance (AS), a surface protein that mediates donor/recipient attachment and which is encoded by the *prgB* gene. Interestingly, synthesis of AS is induced, through the PrgX mechanism, in donor cells in contact with human plasma, in the absence of recipient cells (Hirt et al., 2002). This observation led to the proposal of a model in which a plasma component, probably an albumin/lipid complex, inactivates the inhibitory peptide iCF10, thus allowing the expression of the conjugation genes, including *prgB* (Chandler et al., 2005). Beyond its role in conjugation, AS has been shown to be a virulence factor in different rabbit models of endocarditis; in some of these models it produces cardiac vegetations and higher mortality (Chow et al., 1993; Schlievert et al., 1998; Hirt et al., 2002). AS also accelerates early biofilm development in an *ex vivo* porcine heart valve model (Chuang-Smith et al., 2010). It promotes adhesion to cultured renal tubular cells from pork (Kreft et al., 1992) and adhesion to and invasion of different types of cultured human enterocytes (Olmsted et al., 1994; Sartingen et al., 2000; Wells et al., 2000; Waters et al., 2003, 2004). Finally, AS also protects *E. faecalis* against attack by human neutrophils despite phagocytosis and neutrophil activation (Rakita et al., 2000) and by human macrophages (Sussmuth et al., 2000).

TABLE 1 | RRNPP family of transcriptional regulators with their associated peptides and their biological roles.

Name of regulator	Peptide	Group	Role of mechanism
Rgg	SHP, ComS (XIP)	Streptococci	Commensalism/Virulence
NprR	NprX	Bacilli	Necrotrophism
PlcR	PapR	Bacilli	Virulence
PrgX, TraA	cCF10 iCF10	Enterococci	Virulence
TprA	PhrA	Streptococci	Commensalism

PlcR/PapR AND VIRULENCE IN THE *Bacillus cereus* GROUP

The *B. cereus* group comprises multiple species (Bottone, 2010), including the insect pathogen *Bacillus thuringiensis*, the etiological agent of anthrax *Bacillus anthracis*, (Kolsto et al., 2009), and *B. cereus sensu stricto*, an opportunistic pathogen causing food-borne gastroenteritis and systemic infections (Stenfors Arnesen et al., 2008). In *B. cereus* and *B. thuringiensis*, PlcR is a pleiotropic transcriptional activator that induces the expression of 80% of the genes coding for extracellular factors, including some virulence factors such as degradation enzymes and cytotoxic and cell surface proteins (Agaisse et al., 1999; Gohar et al., 2002, 2008). Interaction of the peptide PapR

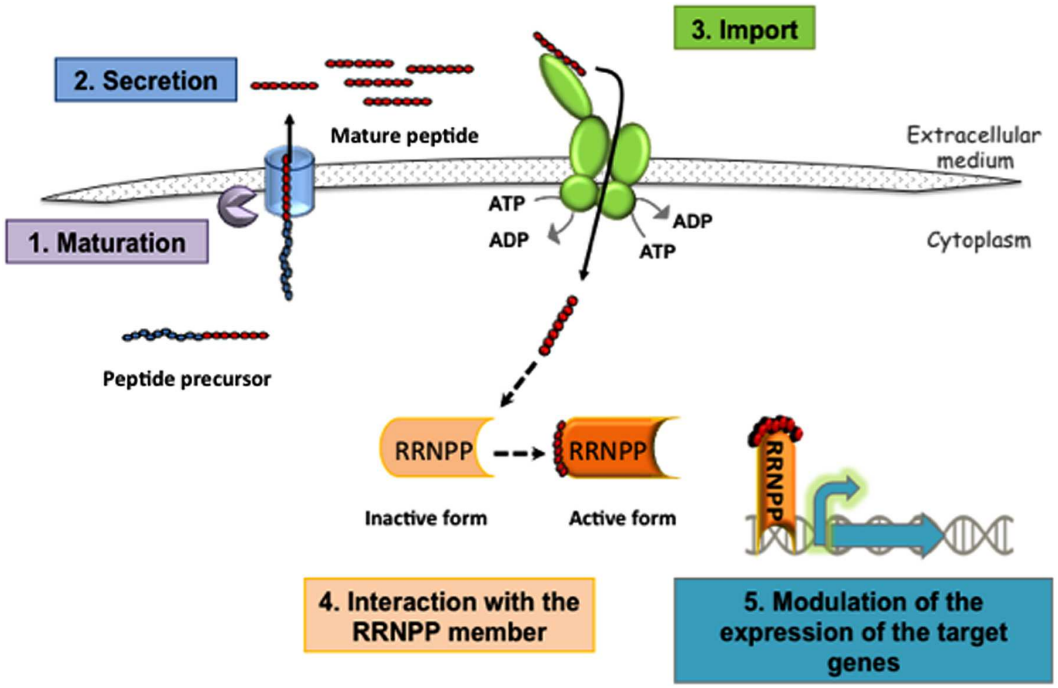

FIGURE 1 | Schematic representation of the RRNPP mechanism involving transcriptional regulators. (1) Maturation of a peptide precursor (or protein in *E. faecalis*) and (2) secretion of the mature peptide releases a signaling peptide outside the bacterium. These two processes involve various exporters and proteases, depending on the bacteria, and some have yet to be identified. The order in which these steps occur is also largely unknown. (3) Next, the signaling peptide is imported into the cell by Opp, a transporter from the ATP-binding cassette (ABC) family. (4) Finally, the signaling peptides interact with their specific sensors (Rgg, NprR, PlcR, or PrgX), and (5) modulate the expression of their target genes. For NprR, PlcR, or the Rgg which function as activators binding to their cognate peptide allows them to positively control the expression of their target genes. For TprA or the Rgg3 that function as repressor binding to their cognate peptide alleviates the repressor effect. The repressing function of PrgX is either strengthened by the binding of an inhibitory peptide or weakened by the binding of an activating peptide.

with PlcR allows the complex to bind to DNA and enables transcription of the target genes (**Figure 1**) (Grenha et al., 2013). In *B. thuringiensis*, disruption of the *papR* gene inactivated the expression of the PlcR regulon, resulting in decreased hemolytic activity and a significant reduction in virulence in an insect infection model (Slamti and Lereclus, 2002).

Other studies have also identified a role for PlcR in the regulation of pathogenesis in *B. cereus* and *B. thuringiensis*. In both of these bacteria, PlcR inactivation provokes a significant reduction in virulence in mice (Salamitou et al., 2000) and rabbits (Callegan et al., 2003). Similarly, in *ex vivo* cell culture assays, cytotoxicity of *B. thuringiensis* strain Bt407 in Hela or Caco-2 human epithelial cells was PlcR-dependent. In addition, this toxic effect was cell-contact-independent, which supports the hypothesis that at least one of the secreted factors regulated by PlcR is responsible for this cytotoxicity (Ramarao and Lereclus, 2006). Three important enterotoxins in the *B. cereus* group – the hemolysin BL (Hbl), the cytotoxin K (CytK), and the non-hemolytic enterotoxin (Nhe) – are under control of the PlcR–PapR system (Agaisse et al., 1999). Hbl and Nhe are pore-forming toxins, which induce cell lysis in different eukaryotic cell models (Jessberger et al., 2014) and CytK is cytotoxic toward the Caco-2 intestinal cell line (Hardy et al., 2001; Fagerlund et al., 2004; Jessberger et al., 2014). More generally, it is hypothesized that these enterotoxins are responsible for the abdominal cramps and diarrhea that are symptoms of infection (Ramarao and Sanchis, 2013). However, *plcR* mutant strains are not completely avirulent (Tran et al., 2011), suggesting that other virulence factors non-regulated by PlcR play a role during *B. cereus* infections (Guillemet et al., 2010).

NprR/NprX AND NECROTROPHISM IN THE *B. cereus* GROUP

The NprR/NprX system appears to be functional in three of the species in this group: *B. anthracis*, *B. cereus*, and *B. thuringiensis* (Rice et al., 2015). The sensor NprR interacts with the signaling peptide NprX, and the complex then activates the expression of NprA metalloprotease (Perchat et al., 2011), as well as other genes that encode degradative enzymes like lipases or peptidases (**Figure 1**) (Dubois et al., 2012). Interestingly, the PlcR–PapR complex stimulates the transcription of the *nprR–nprX* operon (Dubois et al., 2013).

In *B. thuringiensis*, it has been proposed that this system participates in the necrotrophism process in an insect larval model (Dubois et al., 2012). However, little is known about its activity in the mammalian environment, with one exception: in *B. anthracis,* the *nprR* gene was highly expressed during the outgrowth of spores within murine macrophages (Bergman et al.,

2007), which suggests that this system plays a role in *B. anthracis* pathogenesis.

Rgg/SHP IN COMMENSALISM AND VIRULENCE IN STREPTOCOCCI

The Rgg transcriptional regulators are widespread among many species of the phylum Firmicutes. However, Rgg-associated peptides have only been found in Streptococcaceae thus far. Two subfamilies of Rgg are distinguished: (i) Rgg associated with the SHP (small hydrophobic peptides) family of peptides, which regulates genes of diverse functions, and (ii) the Rgg called ComR, which, in association with the ComS (XIP) family of peptides, controls the triggering of competence in multiple species (Ibrahim et al., 2007b; Fleuchot et al., 2011).

Peptide-associated Rgg mechanisms were first deciphered in *Streptococcus thermophilus* (Ibrahim et al., 2007a; Fontaine et al., 2010, 2013; Fleuchot et al., 2011) and later in three opportunistic pathogenic streptococci (**Figure 1**). The first of these was the human commensal associated with dental caries, *Streptococcus mutans*, which contains a ComS/ComR system (Mashburn-Warren et al., 2010). To our knowledge, no data are available *in vivo* for this bacterium. The second was the human-restricted pathogen *Streptococcus pyogenes* (or GAS, from Group A *Streptococcus*), which has two interrelated SHP/Rgg systems, SHP2/Rgg2 and SHP3/Rgg3, in addition to a ComS/ComR system (Chang et al., 2011). More recently, the opportunistic human and animal pathogen *Streptococcus agalactiae* (or GBS, from Group B *Streptococcus*; Cook et al., 2013; Perez-Pascual et al., 2015) was determined to have one active SHP/Rgg, called SHP/RovS.

GAS is commonly carried asymptomatically in the oropharynx or on the skin but can become invasive and cause severe life-threatening diseases. The *shp2/rgg2* and *shp3/rgg3* loci are present in all sequenced strains of GAS and the complex Rgg2/3 pathway of strain NZ131 has recently been extensively studied in chemically defined medium (Lasarre et al., 2013; Cook and Federle, 2014). In addition to the *shp2/3* genes, Rgg2/3 control the transcription of downstream genes whose function remains unclear. However, this pathway modulates the levels of biofilm produced by GAS (Chang et al., 2011). Moreover, SHP/Rgg signaling is induced considerably in the presence of mannose, one of the prominent carbohydrates present in the glycoconjugates on airway epithelia. In addition, these inducing conditions confer upon GAS resistance to lysozyme, an antimicrobial host defense mechanism present in mucosal secretions and in macrophages (Chang et al., 2015). Additional experiments also showed that the peptidase PepO, whose gene is negatively regulated by CovR, can degrade SHP2 and SHP3 and, therefore, inhibit SHP/Rgg signaling. CovR is a two-component system regulator that inhibits the expression of many genes, including those encoding potential virulence factors. Despite the lack of direct *in vivo* experimental evidence for the biological relevance of these SHP/Rgg systems in GAS, these results led the authors to hypothesize that the Rgg2/3 pathway more likely plays a role in asymptomatic carriage in the host (Wilkening et al., 2015). However, in contrast to this hypothesis, another study in strain SF370 demonstrated that Rgg2 and its associated regulon were implicated in infection development, as the inactivation of *rgg2* decreased the ability of GAS to adhere to epithelial cells and increased biofilm formation. Additionally, a Δ*rgg2* mutant strain was unable to grow in human blood and showed avirulent behavior in a murine model (Zutkis et al., 2014).

GBS is a commensal of the human intestinal and vaginal tract in 15–30% of healthy adults, but remains one of the most important invasive pathogens in newborns and the elderly (Le Doare and Heath, 2013). Nearly all sequenced GBS strains present a unique copy of the *shp*/*rovS* locus. This cell–cell communication mechanism has been recently deciphered (Cook et al., 2013; Perez-Pascual et al., 2015), demonstrating that the SHP/RovS mechanism plays an important role in bacterial pathogenesis. Mice infected by isogenic *shp* or *rovS* deletion mutants showed a significant decrease in the bacterial burden in the liver and spleen compared to mice infected by the parental strain. Further exploration revealed that disruption of *shp* and *rovS* resulted in a significant decrease in the ability of GBS to adhere to and invade human HepG2 hepatic cells (Perez-Pascual et al., 2015). In addition, in chemically defined medium, at least three genes are under the control of this mechanism: *shp* and *gbs1556* (encoding a secreted protein), which are positively and directly controlled by SHP/RovS, and the *fbsA* gene, which codes for a fibrinogen-binding protein involved in GBS pathogenesis (Schubert et al., 2004) and is repressed by SHP/RovS. In rich medium, RovS also seems to control a set of genes related to virulence, such as *sodA* and *cylE*, which encode a superoxide dismutase and a protein required for the production of hemolysin, respectively (Samen et al., 2006).

Another peptide-associated Rgg transcriptional regulator has been studied in *Streptococcus suis* (Zheng et al., 2011). In this bacterium, inactivation of Rgg attenuated pathogenicity in a piglet infection model, probably due to growth and metabolism changes in the mutant. Surprisingly, a Δ*rgg* mutant showed hyper-adhesion to epithelial cells and increased hemolytic activity. DNA microarray analysis revealed that Rgg is a global regulator that affects genes with varied functions, such as the metabolism of non-glucose carbohydrates (e.g., lactose or maltose) or defense mechanisms. The authors suggest that Rgg is a global transcriptional regulator that plays a relevant role in *S. suis* survival during pathogen–host interaction (Zheng et al., 2011).

In streptococci, the *comX* gene encodes an alternative sigma factor that controls the transcription of the genes necessary for natural transformation, and transcription of *comX* is either controlled by a two-component system or a peptide-associated Rgg-like regulator, the ComS/ComR system. The involvement of ComS/ComR in the triggering of transformation has been experimentally validated in laboratory conditions for four different species: *S. mutans*, *S. thermophilus*, *Streptococcus infantarius*, and *Streptococcus macedonicus* (Fontaine et al., 2015). Interestingly, in GAS, in spite of the fact that no natural transformation has been observed in many laboratory conditions (Mashburn-Warren et al., 2012), transformants were recovered at low levels from cells grown

in biofilm on epithelial cells or *in vivo* from mice colonized intranasally with biofilm bacteria (Marks et al., 2014).

TprA/PhrA AND COMMENSALISM IN *Streptococcus pneumoniae*

Recently, new families of peptide-associated regulators have been identified in *S. pneumoniae,* which is commensal in the human nasopharynx, but also the etiological agent of serious diseases such as pneumonia, bacteremia, or meningitis (Shak et al., 2013). These new families comprise a transcriptional regulator, Tpr, and its allied cognate peptide Phr (Hoover et al., 2015). Blast analysis revealed that this system appears to be orthologous to the PlcR/PapR system in bacilli. One such system, TprA/PhrA, regulates the expression of a putative lantibiotic biosynthesis operon. TprA represses the expression of its own encoding gene, as well as that of the *phrA* gene and the lantibiotic genes. However, the PhrA peptide antagonizes TprA activity. A study of this regulation following the expression of the *phrA* gene revealed that, whereas glucose can inhibit *phrA* expression, galactose, the main sugar of the human nasopharynx, can induce it (Hoover et al., 2015). Tn-seq analysis (sequencing of insertion transposon sites by high throughput sequencing methods) highlighted that disruptions of *tprA* or some of the lantibiotic genes reduced the fitness of *S. pneumoniae* for nasopharynx colonization in a murine model (van Opijnen and Camilli, 2012). These results led Hoover et al. (2015) to hypothesize that expression of the lantibiotic genes through the TprA/PhrA system may be important during niche colonization, where *S. pneumoniae* competes with other bacteria for space and resources. This was supported by the observation that the TprA/PhrA system and its regulated lantibiotic gene cluster are not needed for invasive disease development in a murine model (van Opijnen and Camilli, 2012; Hoover et al., 2015).

CONCLUSIONS AND PERSPECTIVES

In Firmicutes, peptides are the best-known signaling molecules. The peptides that interact with RRNPP regulators are short ($<$ 10 amino acids), without post-translational modifications, are secreted and then reimported into bacteria through an oligopeptide transporter. The RRNPP transcriptional regulators are activated by these peptides via tetratricopeptide domains involved in peptide-protein interactions. RRNPP regulators and reimported short peptides act together as a communication system, which clearly differs from a second group of communication systems. In this second group, which includes the well-documented Agr system from *Staphylococcus aureus*, peptides are post-translationally modified and activate two components systems at the outside bacterial surface.

The number of putative members of the RRNPP family has increased dramatically; for example, in streptococci, the Tpr/Phr and Rgg/SHP families are predicted to have 53 and 68 members, respectively, (Fleuchot et al., 2011; Hoover et al., 2015) and it is highly probable that others remain to be discovered. In order to better understand these regulators, much work remains, especially focusing on their role and *in vivo* function in opportunistic pathogens. Research conducted thus far indicates that RRNPP members regulate a variety of biological functions. For example, PlcR/PapR as well as PrgX/TraA have a direct impact on virulence, through controlling the expression of target genes that encode virulence factors. The TprA/PhrA system has been implicated in the commensal behavior of *S. pneumoniae* and seems to be involved in the expression of a bacteriocin-encoding operon. The role of Rgg/SHP mechanisms is more complex. Whereas the RovS/SHP system in GBS seems to participate in disease development, the orthologous mechanism in GAS, Rgg2/SHP2, has been predominantly linked to commensal behavior. Moreover, this system is expressed at variable levels in different strains of GAS (Chang et al., 2015).

Existing conclusions regarding the regulatory function of RRNPP members in the host are derived from indirect results and require further validation. In particular, this field would benefit from *in vivo* imaging of the induction of these mechanisms during host colonization. Furthermore, gene expression studies at the single-cell level are rare; to our knowledge, only one such study exists, an investigation of PlcR and NprR of *B. thuringiensis* in an insect model (Verplaetse et al., 2015). These innovative approaches should greatly improve the identification of host-associated environmental conditions that induce these mechanisms.

Finally, when viewed in the context of emerging resistance to antibiotics, the RRNPP systems represent an attractive target for research into new mechanisms for bacterial control. In the future, it is possible that infections by some opportunistic pathogens could be prevented by decreasing the expression of virulence factors, without altering the composition of the global microbiota, via modulation of the activity of RRNPP regulators.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Anti-biofilm Activity as a Health Issue

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The formation and persistence of surface-attached microbial communities, known as biofilms, are responsible for 75% of human microbial infections (National Institutes of Health). Biofilm lifestyle confers several advantages to the pathogens, notably during the colonization process of medical devices and/or patients' organs. In addition, sessile bacteria have a high tolerance to exogenous stress including anti-infectious agents. Biofilms are highly competitive communities and some microorganisms exhibit anti-biofilm capacities such as bacterial growth inhibition, exclusion or competition, which enable them to acquire advantages and become dominant. The deciphering and control of anti-biofilm properties represent future challenges in human infection control. The aim of this review is to compare and discuss the mechanisms of natural bacterial anti-biofilm strategies/mechanisms recently identified in pathogenic, commensal and probiotic bacteria and the main synthetic strategies used in clinical practice, particularly for catheter-related infections.

Keywords: anti-biofilm, biofilm, probiotics, pathogens, lock solution

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INTRODUCTION

Biofilms are multimicrobial communities enclosed in self-synthetized polymeric matrices, attached to biotic or abiotic surfaces. Eighty percent of the world's microbial biomass are found in the biofilm state, and sessile cells are thus considered as the predominant mode of life for microorganisms in nature. These cells frequently express phenotypes different from their non-adherent planktonic counterparts, with a high capacity to colonize new surfaces and a high tolerance to exogenous stress (Donlan and Costerton, 2002; Macfarlane and Dillon, 2007). Depending on the microbial species and their localization (environmental/biomedical/industrial), biofilms can be either beneficial or detrimental for humans. According to the National Institutes of Health, more than 75% of microbial infections that occur in the human body are promoted by the formation and persistence of biofilms. Some bacterial biofilms, such as the intestinal microbiota, also play protective and functional roles. Intestinal commensal and beneficial bacteria–bacteria interactions are directly involved in host homeostasis (Wrzosek et al., 2013). In human health, an imbalance of microbiota, called dysbiosis, is associated with several diseases (Martín et al., 2014a). This correlation is in part due to bacterial interplay between members of bacterial communities such as group effect, cooperation, kin competition, genetic expression profiles, and phenotypic diversification (Rendueles and Ghigo, 2015) that could be encompassed by the adjective "anti-biofilm". Interference interactions have already inspired the design of alternatives to antibiotics in the fight against pathogenic microorganisms (Rasko and Sperandio, 2010). Recently, major challenges and opportunities in this field were addressed during the workshop "Biofilms, Medical Devices, and Anti-Biofilm Technology" (Phillips et al., 2015). Many medical device-associated and

persistent infections can be attributed to biofilm-associated microbes. To tackle the overarching public health issue of the contribution of biofilms to health care-associated infections it was suggested that clinicians and health care workers should be more closely involved in their detection and treatment. It was also suggested that the applied science of biofilm formation and prevention would provide greater knowledge of the contamination of medical devices. Some answers are to be found in the development of the anti-biofilm activities of beneficial microbes and/or the understanding and diversion of the anti-biofilm capacities of pathogenic bacteria. In this review, after establishing a definition of the term anti-biofilm, we will focus on bacterial anti-biofilm activities with examples of probiotic and pathogenic bacteria. With reference to clinical examples, we will then discuss the use, challenges and limitations of anti-biofilm strategies.

ANTI-BIOFILM ACTIVITY: WHAT DOES IT MEAN?

Biofilms were initially defined as structured communities of bacterial cells enclosed in self-produced polymeric matrices and adherent to inert or living surfaces (Costerton et al., 1999). Later, it became obvious that biofilms exhibit altered phenotypes compared with corresponding planktonic cells, especially with regard to gene transcription (Lindsay and von Holy, 2006). Biofilms are ubiquitous and nearly all species of microorganisms, bacteria, fungi, yeasts, algae, protozoa, and viruses are able to adhere to surfaces and/or to each other to form biofilms (Wingender and Flemming, 2011). Biofilms formed by pathogenic bacteria are the most extensively documented, such as *Klebsiella pneumoniae* biofilms seen in **Figure 1** on abiotic (1A) and biotic surfaces (epithelial cell monolayer; 1B). Biofilms are increasingly recognized by the public health community as an important source of pathogens (Donlan and Costerton, 2002; Wingender and Flemming, 2011). They are involved in specific infectious diseases such as osteomyelitis, otitis media, peridontitis, and dental caries (Costerton et al., 1999) and in chronic diseases such as pulmonary infections of cystic fibrosis patients. They are also involved in nosocomial infections due to opportunistic pathogens, especially urinary tract, lower respiratory tract, and surgical site infections and bacteremia, and mostly when invasive medical device are being used. In 2012, a prevalence survey of 1,938 healthcare facilities and 300,330 patients carried out by the French association RAISIN "Réseau d'alerte, d'investigation et de surveillance des infections nosocomiales" showed that the most frequent microorganisms associated with nosocomial infections (RAISIN, 2015) were *Escherichia coli*, *Staphylococcus aureus* (38.1% resistant to methicillin, MRSA), *Pseudomonas aeruginosa,* and *K. pneumoniae*, all of which are high biofilm producers (**Figure 1C**).

Biofilms pose significant clinical problems because sessile bacterial cells are inherently recalcitrant to antimicrobial agents such as antibiotics (for review, Lebeaux et al., 2014). Several factors are responsible for the biofilm-associated resistance, including the density and the physiological state of the cells, but also the physical structure of the biofilm. Exopolysaccharides and extracellular DNA (eDNA) of the biofilm matrix can act as a barrier to diffusion and thus reduce penetration of antibiotics into biofilms. The effectiveness of this barrier varies between antibiotics; tetracyclines, rifamycins, fluoroquinolones, and daptomycin penetrate better than beta-lactams, aminoglycosides, and glycopeptides (Stewart et al., 2009; Singh et al., 2010; Cha et al., 2011; Doroshenko et al., 2014). The effects of antibiotics can also be affected by the particular microenvironment of biofilm, such as acidic pH and low level of oxygen encountered in the deep layers of the aggregates (Siala et al., 2014). The cells within biofilm are generally less metabolically active than planktonic cells and therefore significantly less sensitive to mechanism of action by many antimicrobials targeting synthesis of macromolecules or metabolic pathways such beta-lactams and quinolones (Xie et al., 2005; Mascio et al., 2007). In addition, a percentage of cells within biofilm may be persister cells, which are transiently antibiotic tolerant without harboring genetic changes seen in antimicrobial resistance. The presence of antibiotics can *per se* induce persistence (Dörr et al., 2010; Kwan et al., 2013) and/or enhance biofilm formation, particularly at sub-Minimal

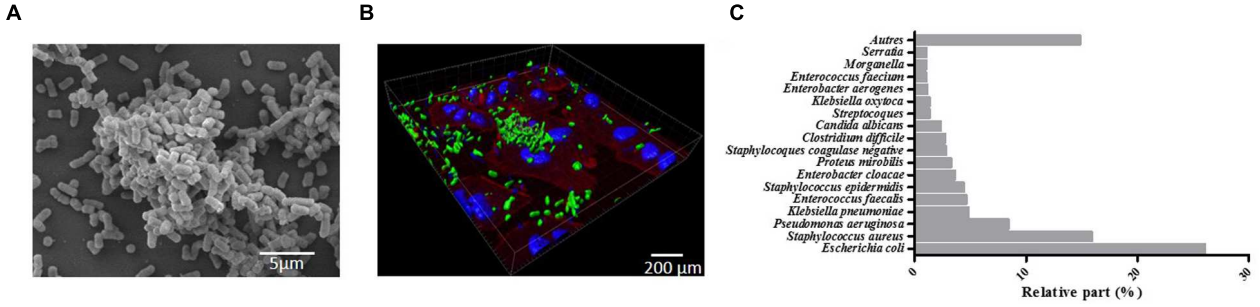

FIGURE 1 | Four-hour-old biofilm of *Klebsiella pneumoniae*. (A) scanning electron microscopy (SEM) observation of the biofilm formed on Thermanox slide performed as previously described (Goncalves et al., 2014). **(B)** Confocal microscopic observations of the biofilm (green) formed onto A549 pulmonary cell monolayer stained with Phalloidin (red) and DAPI (blue). Observations were performed as previously described (Marquès et al., 2015). **(C)** Relative percentage of the most frequent micro-organisms associated with nosocomial infections in France (adapted from RAISIN, 2015).

Inhibitory concentrations (MIC) (Wang et al., 2010b; Kaplan et al., 2012; Ng et al., 2014; Lázaro-Díez et al., 2016) and thus lead to treatment failure. The *in vitro* determination of MICs is restricted to planktonic cells growing exponentially under conditions that are optimal for action of the drug but unlikely to be met in biofilm populations. *In vitro* and *in vivo* experiments demonstrated that the MIC and the minimum bactericidal concentration (MBC) for biofilm bacterial cells are usually much higher (approximately 10–10,000 times) than their counterpart planktonic cells (Hengzhuang et al., 2012; Marquès et al., 2015). The effective antibiotic MBC *in vivo* for biofilm eradication are therefore impossible to reach by conventional antibiotic administrations due to the toxicities and the side effects of antibiotics and the limitation of renal and hepatic functions. Combination of antibiotics with different killing mechanisms leading to synergism remains nowadays the best solution for the treatment of biofilm infections. Rifampicin and fosfomycin-based combinations have shown *in vitro* enhanced activities against biofilm embedded *Staphylococcus aureus* isolates (Tang et al., 2012, 2013) but their *in vivo* efficiencies still remain to be determined (**Table 1**).

In addition, biofilm phenotype provides resistance to host defenses, in particular, leukocyte phagocytosis. There are various possible mechanisms of action by which bacteria escape from the immune system including inhibition of inclusion of biofilm cells by phagocytes (Günther et al., 2009) and low immunogenicity of the biofilm matrix (Thurlow et al., 2011). These resistance properties and the genetic and phenotypic versatilities of cells within biofilm prompted workers to look for biofilm-specific therapies to eradicate this common cause of persistent infections. The term "anti-biofilm" appeared in the literature during the 1990s and is now widely used without, however, being fully defined. On the basis of current knowledge, this review proposes the use of the term anti-biofilm as "a natural or induced process, leading to reduction of bacterial biomass through the alteration of biofilm formation, integrity and/or quality".

Studies have shown that two different anti-biofilm mechanisms are able to modulate biofilm formation: inhibition of bacterial surface attachment and destabilization/disruption of mature biofilms irreversibly attached. Many of the existing anti-biofilm agents are non-biocidal, but some bactericidal molecules could be considered as anti-biofilm agents as they are still active against mature biofilms protected by their architecture. Bactericidal anti-biofilm agents should be very specifically targeted otherwise their use could impair the composition of established ecosystems and damage beneficial microbiota. Nevertheless, anti-biofilm strategies represent interesting approaches for medical biotechnology as attested by the large number of recent publications (Rumbaugh and Ahmad, 2014). Anti-biofilm agents fall into two large groups, synthetic and natural (**Table 2**). Synthetic biofilm inhibitors, in part listed below, are mostly derived from bactericidal technologies.

Non-thermal Plasma (NTP) Technology

Plasma is a unique state of matter that results from a rapid ionization of the gas obtained through subjecting gas to extremely high temperatures or passing gas through high-voltage electricity (Scholtz et al., 2015). The unspecific character of

TABLE 2 | The different classes of anti-biofilm agents.

Synthetic	Natural product
Non-thermal plasma	Antibiotics
Photodynamic substances	Protozoan grazing
Nanoparticles	Plant products
Surface topographic modifications	Bacteriophages
Other peptides and molecules	Microbial agents

TABLE 1 | Effects of different antibiotics family against *Staphylococcus* biofilms.

Antibiotic		Species	Assay	Effect on biofilm	Reference
Beta-lactams	Penicillins and most cephalosporins	*Staphylococcus aureus*	*in vitro*	Induction of biofilm formation at Sub-MICs	Ng et al., 2014 Kaplan et al., 2012 Lázaro-Díez et al., 2016
	Ceftaroline			Bactericidal anti-biofilm activity after prolonged exposure	Landini et al., 2015
Rifampicin		*S. aureus*	*in vitro*	Anti-biofilm activity, synergistic with fusidic acid and tigecycline	Tang et al., 2013
		S. epidermidis		High anti-biofilm activity alone or in combination with vancomycin or daptomicin	Olson et al., 2010
Vancomycin		*S. aureus*	*in vitro*	Promotion of biofilm formation through an autolysis-dependent mechanism	Hsu et al., 2011
		S. epidermidis		Induction of eDNA release at sub-MICs leading to increased biofilm formation	Doroshenko et al., 2014
Daptomycin		*S. aureus*	*in vitro*	Induction of viable but non-cultivable cells in biofilm at low concentrations	Pasquaroli et al., 2014
				Anti-biofilm effect in monotherapy	LaPlante and Woodmansee, 2009
			in vivo	Prevention of the emergence of rifampin resistance mutants	Cirioni et al., 2010
Fosfomycin		*S. aureus*	*in vitro*	Anti-biofilm activity synergistic with linezolid or minocycline or vancomycin	Tang et al., 2012

eDNA, extracellular DNA; MIC, minimal inhibitory concentrations.

their anti-microbial activity, low toxicity for human tissues and absence of long-living toxic compounds make non-thermal plasmas (NTPs) a very promising tool for biofilm prevention and control in the decontamination of foods and biological materials (Ermolaeva et al., 2015).

Photodynamic Substances

To produce an antimicrobial photodynamic therapy (PDT), three major components are needed, light, oxygen, and a photosensitizer. The excitation of the photosensitizer by light generates reactive oxygen species (ROS), which leads to the oxidation of biomolecules of microorganisms and results in cell damage and death (Hamblin and Hasan, 2004). It was recently shown that the photosensitizer 5-aminolevulinic acid (5-ALA), once absorbed by proliferating bacteria, is converted into the natural photosensitizer Protoporphyrin IX (PpIX), which has synergic effects with the antibiotic gentamicin against the biofilm of several Gram-positive bacteria (Barra et al., 2015). For example, photoactive TiO_2 antibacterial coating was proposed to reduce pin tract infections and proved to have antibacterial effect against *Staphylococcus* strains (Villatte et al., 2015).

Nanotechnology

Two kinds of nanotechnologies can affect biofilm formation on a surface. First, nano-modifications of surface topography (roughness and nanostructure) limit primary bacterial adhesion without the use of any biocide molecules (Desrousseaux et al., 2013). However, results on their efficacy have been conflicting and inhibition of primary adhesion seems to be dependent mostly on the spatial organization of the nano features. Second, surfaces can be chemically modified by addition of nanoparticles made of iron, silver, zinc, or titanium (Neethirajan et al., 2014). Most of these nanoparticles exert antibacterial activity by interacting electrostatically with the bacterial membrane, which leads to membrane disruption (Beyth et al., 2015). In addition, the bioavailability of these nanoparticles due to their high surface-to-volume ratios allows them to penetrate a mature biofilm and thus to target bacterial cells not only at the surface but also within the deep layers of biofilm (Bakkiyaraj and Pandian, 2014).

Other molecules could be added to this list, in particular detergents and antiseptics and synthetic peptides (de la Fuente-Núñez et al., 2014). However, the increasing interest in promoting health by natural means has concentrated research trends on natural biofilm inhibitor products with less biocidal activity.

Protozoan Grazing

Protozoan grazing is believed to be the major trophic pathway whereby the biomass produced by bacteria, cyanobacteria and algae re-enters the food web. However, this type of microorganism's biomass control is hard to adapt to human health. *In vitro*, the ciliate *Colpoda maupasi* has been shown to reduce the thickness of mature biofilms of opportunistic pathogens formed by *Klebsiella pneumoniae*, *Pseudomonas fluorescens,* and *Staphylococcus epidermidis* (Huws et al., 2005). In addition, the presence of protozoa in drinking water distribution systems can regulate the autochthonous and allochthonous bacterial populations (Sibille et al., 1998), which suggests that this process could be used to decrease or limit nosocomial infections caused by environmental contamination.

Plant Products

Plants represent a huge resource of bioactive molecules. A recent study showed that some of them contain anti-biofilm compounds that inhibit growth, interrupt quorum sensing (QS) and/or prevent bacterial adhesion (Husain et al., 2015). Garlic acts as a QS-interfering compound in the treatment of bacterial infections, owing to the production of ajoene, a sulfur-rich molecule (Jakobsen et al., 2012). Cranberry is also an anti-adhesion agent (Allison et al., 2000; Cai et al., 2014) able to prevent urinary infections (Rafsanjany et al., 2015), dental caries (Girardot et al., 2014), and skin infections (Morán et al., 2014).

Bacteriophages

The most abundant category of microorganisms on earth, are viruses whose interactions with biofilm members are ecologically important in horizontal gene transfer between bacteria (transduction). Bacteriophages play other important roles in microbial communities such as the modulation of bacterial populations. They also produce a number of enzymes able to disrupt the protection afforded by the biofilm matrix, thereby modifying biofilm architecture and increasing its susceptibility to antibiotics (Abedon, 2015). However, there are several drawbacks to the use of phages: (i) phage lytic activity releases Gram-negative bacterial-membrane-endotoxins, (ii) phage-resistant bacteria can arise rapidly (Örmälä and Jalasvuori, 2013) and (iii) phages can spread bacterial virulence genes (Rossmann et al., 2015).

Bioinspired anti-biofilm molecules can be isolated from eukaryotes, such as the lactoferrin (Ammons and Copié, 2013), but most are derived from microbial phenomena occurring within the biofilms themselves. In fact, bacterial fitness within biofilm relies on the ability of a given strain not only to adhere, settle, and develop as a biofilm, but also to inhibit others from doing so (Rendueles and Ghigo, 2015).

BACTERIAL-DERIVED BIOFILM INHIBITORS

Intra- and interspecies interactions and competition between microorganisms within biofilm are governed by ecological and evolutionary parameters (Rendueles and Ghigo, 2015). Bacterial interferences are present at different levels of biofilm development; they can affect primary adhesion and/or maturation via exclusion/competition phenomena, modify matrix composition or enhance dispersal. Bacterial anti-biofilm activities govern microbe–surface interactions and microbe–microbe interactions and they are shared by commensal, pathogen, and probiotic bacteria (**Figure 2**). The increasing interest in promoting a natural approach to health has intensified research in the field of probiotics worldwide over the last two decades. Probiotics, recently redefined by an expert panel of the International Scientific Association for Probiotics and Prebiotics

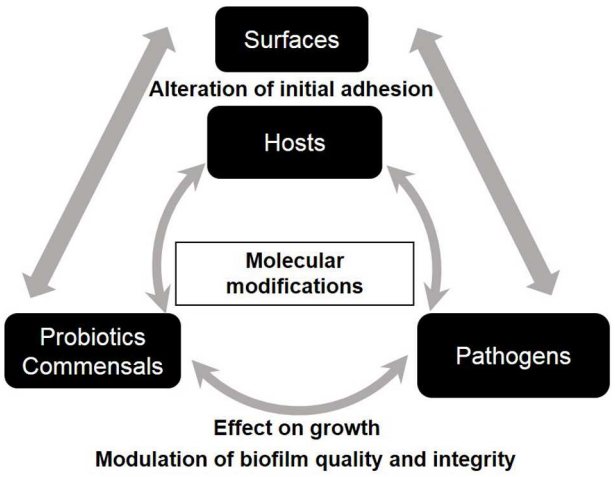

FIGURE 2 | Schematic representation of the impact of bacterial anti-biofilm activities on microbe-surface interactions and on microbe-microbe interactions.

(ISAPP) as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014), have gained increasing medical attention because of their antagonist effects against numerous pathogens. Probiotics with anti-biofilm properties, especially *Lactobacilli*, seem promising in the treatment of oral, wound and vaginal infections in both clinical trials and *in vitro* studies (Vuotto et al., 2014). For some probiotics, this beneficial activity is boosted when grown as biofilm (Rieu et al., 2014). Pathogens also exhibit anti-biofilm properties when competing with other bacteria to reach new ecological niches.

Effect on Growth

Most beneficial and pathogen bacteria are able to secrete antibacterial substances such as antimicrobial peptides (AMPs), lantibiotics, bacteriocins, microcins, lactic acid, and hydrogen peroxide. Using different mechanisms of action, such as membrane permeabilization and interference with essential enzymes, these molecules inhibit bacterial growth or cause bacterial death. Some of them are highly effective against sessile cells such as the bacteriocins, nisin A, lacticin Q, sonorensin, and colicin R (Okuda et al., 2013; Rendueles et al., 2014; Chopra et al., 2015). Interestingly, colicin R produced by the commensal *Escherichia coli* ROAR029 strain, preferentially targets sessile rather than planktonic bacteria (Rendueles et al., 2014). The enhanced sensitivity of sessile cells to colicin R has been attributed to a reduced growth rate caused by diminished turnover of outer membrane components or increased stress within the biofilm. Several bacteriocin-producing strains have filed patent application for food and biomedical applications (Benmechernene et al., 2013). Interestingly, the probiotic lactobacilli strains *Lactobacillus casei* Shirota and *L. rhamnosus* HN001 inhibited growth and biofilm formation of the pathogen *Streptococcus mutans* by producing an acid environment and bacteriocin-like polypeptides, suggesting the synergic properties of these two mechanisms of action (Lin et al., 2015). Anti-biofilm agents impairing bacterial growth usually display narrow spectra. For instance, AMPs have shown a considerably stronger antimicrobial activity against biofilm formed by Gram-positive bacteria than by Gram-negative strains, justifying their potential use in the specific treatment of ocular infections, which are mostly due to Gram-positive bacteria (Dawgul et al., 2014).

Alteration of Initial Adhesion by Surface Modification

The most effective strategy to antagonize the first step of biofilm formation is the use of biosurfactants and bioemulsifiers able to modify the physicochemical cell surface properties and thus to impair microbial adhesion. This is illustrated notably by the production of *Pseudomonas aeruginosa* rhamnolipids, which are able to disrupt the cohesiveness of biofilm formed by *Bordetella bronchiseptica* (Irie et al., 2005), *Bacillus subtilis*, *S. aureus,* and *Micrococcus luteus* (Quinn et al., 2013). Another anti-biofilm biosurfactant is the surfactin produced by *Bacillus subtilis*, which is able to reduce colonization of surfaces by the food pathogenic bacteria *Listeria monocytogenes*, *Enterobacter sakazakii,* and *Salmonella enteritidis* (Nitschke et al., 2009). Other pathogenic bacteria share such mechanisms: *K. pneumoniae* and the uropathogenic strain *E. coli* CFT073 exhibit a broad-spectrum anti-biofilm non-bactericidal activity by secreting extracellular polysaccharide (EPS) with anti-adhesion properties (Valle et al., 2006; Goncalves et al., 2014). EPS are the essential building blocks for the biofilm matrix of most microorganisms but they can also inhibit their neighbors' biofilm structuration by interfering with initial adhesion, dispersion, cell-to-cell communication, and/or matrix formation (Rendueles and Ghigo, 2012). One example of this type of anti-biofilm mechanism of action is the interference of the capsular polysaccharides of *Actinobacillus pleuropneumoniae* serotype 5 with cell-to-cell and cell-to-surface interactions of other bacteria, which prevents them from forming or maintaining biofilms (Karwacki et al., 2013). More examples are given in the review of Rendueles and Ghigo (2012), in which the authors advise that targeting surface colonization rather than overall bacterial fitness is a more promising approach.

Homeostatic relations between the hosts and their microbiota considered as biofilms at the surface of epithelial host cells are abundant. At the intestinal level, commensal and probiotic bacteria strengthen intestinal barrier function by enhancing mucin production and tight junction integrity, and by modulating the activity of the immune system. These properties are assimilated to biotic surface modifications involved in the reduction of pathogenic-associated biofilm and thus the protection of the host from infections. For instance, the probiotic mixture VSL#3 is able *in vivo* to induce mucin gene expression (Caballero-Franco et al., 2007) and commensal bacteria such as bifidobacteria or *Bacteroides thetaiotaomicron* promote defense functions of the host epithelial cells via the production of acetate (Fukuda et al., 2012; Wrzosek et al., 2013). In contrast to these adaptations of host capacities, bacteria can specifically target the degradation of host receptors and then inhibit the adhesion process. For instance, some *S. epidermidis* strains secrete a serine

protease named Esp, which degrades human receptor proteins (e.g., fibronectin, fibrinogen, and vitronectin) recognized by *S. aureus* and involved in host-pathogen interaction and tissue colonization (Sugimoto et al., 2013).

Modulation of Biofilm Quality and Integrity

Evidence has shown that anti-biofilm bacterial agents not only modify biotic and abiotic surfaces but also alter the physical properties of bacterial surfaces involved in cell-to-cell aggregation and surface attachment processes. For instance, EPS released by *L. acidophilus* A4 exert anti-biofilm activity against a wide range of Gram-positive and Gram-negative bacteria by affecting the expression of genes involved in curli production and chemotaxis, and thus modifying cell-to-cell (autoaggregation) and cell-to-host cell (adhesion) adherence (Kim et al., 2009). More recently, inhibition of *S. aureus* was shown to be due to the physicochemical properties of the *Lactobacillus* cells surface such as hydrophobicity, autoaggregation, and coaggregation abilities (Ren et al., 2012). These kinds of inhibition processes are likely induced by alteration of the expression of key surface structures that are required for surface colonization and govern the complex interactions between pathogenic and/or common environmental bacteria. For instance, *Streptococcus intermedius* down-regulates the expression of both short (mfa1) and long (fimA) fimbriae required for attachment and biofilm development by *Porphyromonas gingivalis* (Christopher et al., 2010). The anti-biofilm activity of the oral strain of *Streptococcus* does not affect growth rate and is mediated by the surface arginine deiminase ArcA. More recently, it was shown that *Lactobacilli* strains impair fungal biofilm formation structure by down-regulating the expression of *Candida glabrata EPA6* and *YAK1* genes, encoding, respectively, an adhesin involved in the yeast biofilm development and its transcriptional regulator (Chew et al., 2015).

Anti-biofilm activities are characterized by inter-species communications not only between different genera of prokaryotes but also between prokaryotic and eukaryotic cells. Bacterial anti-biofilm activities are therefore likely to naturally regulate bacterial populations in an ecological niche. For example, the Esp protease secreted by a subset of commensal *S. epidermidis* in the nasal microbiota inhibit biofilm formation by pathogenic *S. aureus* (Iwase et al., 2010). Ecological homeostasis of polymicrobial biofilms involves exclusion, competition and displacement phenomena between pathogenic, commensal and/or probiotic bacteria for adhesion/attachment sites and/or nutriment access. A new mechanism of invasion resistance deployed by oral-derived microbial community (O-mix) to defend their domains was recently reported. The O-mix is able to restrict the colonization of exogenous *E. coli* strains by sensing the *E. coli* lipopolysaccharides (LPS) and subsequently killing them with oxygen free radicals (He et al., 2010). The underlying molecular mechanisms were recently discovered and involve the coordinated role of three commensal bacterial species (*Staphylococcus saprophyticus*, *Streptococcus infantis,* and *Streptococcus sanguinis)* acting as 'Sensor,' 'Mediator,' and 'Killer,' respectively (He et al., 2014). Numerous *in vitro* studies have shown that *Lactobacilli* can exert competitive exclusion of different pathogenic bacteria such as *S. aureus, Salmonella enterica, Shigella sonnei, E. coli,* and *Gardnerella vaginalis* by interfering with their binding sites on the epithelial cell surface (Jankowska et al., 2008; Prince et al., 2012; Zhang et al., 2012; Abedi et al., 2013; Castro et al., 2013).

Anti-biofilm activity can modulate biofilm bacterial diversity via interferences between species or between bacteria and the host surface. Different mechanisms of action can explain the chain reaction leading to the anti-biofilm process, the first of which is modification of cell-to-cell communication. The search for anti-biofilm compounds acting on QS and/or on signal molecule of targeted bacteria has already been undertaken (Leoni and Landini, 2014). For a recent review on QS inhibitors, see Brackman and Coenye (2015). It must, however, be noted that jeopardy of bacterial communication can lead to dispersion of a wide range of bacterial biofilms, and induction of biofilm dispersal by fatty acid signals may be a commonly used mechanism. For instance, the *cis*-2-decenoic acid produced by *P. aeruginosa* induces dispersion of *P. aeruginosa* PAO1 biofilm but also of those formed by a variety of Gram-negative and Gram-positive bacteria *(E. coli, K. pneumoniae, Proteus mirabilis, Streptococcus pyogenes, Bacillus subtilis, S. aureus,* and *Candida albicans*; Davies and Marques, 2009). Many bacterial enzymes involved in active biofilm dispersal have also been identified, in particular those involved in matrix degradation such as the serine protease Esp and the deoxyribonuclease I, DNase I. However, the most studied biofilm-matrix-degrading enzyme is dispersin B (Kaplan, 2009; Iwase et al., 2010; Brown et al., 2015). This glycoside hydrolase produced by the periodontopathogen *A. actinomycetemcomitans* completely inhibits biofilm formation and disperses preformed biofilm of several bacterial species: *E. coli, S. epidermidis, S. aureus, P. fluorescens, and Yersinia pestis* (Kaplan et al., 2004). As a consequence of the destruction of the physical integrity of the highly protective matrix barrier, sessile microbial cells are suddenly exposed to the external offensive of both antibiotics and innate host immune defenses (Kaplan, 2009). To avoid potential adverse effects due to the release of live bacteria from biofilms it seems essential to combine molecules with biofilm dispersing activity and anti-bacterial activity: *cis*-2-decenoic with antibiotics or disinfectants for eradication of catheter-associated biofilms (Rahmani-Badi et al., 2014; Sepehr et al., 2014) and dispersin B with KSL-W antimicrobial peptide for treatment of chronic wound infections (Gawande et al., 2014).

SUCCESSFUL USE OF ANTI-BIOFILM METHODS IN HUMAN HEALTH

Clinical trials performed with beneficial bacteria and particularly probiotics make use of exclusion and/or inhibition of growth of pathogens to protect the mucosa from the colonization of these undesirable microorganisms (Martín et al., 2014b). These strategies can be considered as anti-biofilm strategies, since aggregates formed on biotic surfaces, such as epithelia, have

molecular properties similar to those of biofilms formed on abiotic surfaces and are actually considered as such (Stacy et al., 2016). However, most molecular knowledge of the biofilm mode of life derives from studies performed on aggregates formed on abiotic surfaces, and a lot remains to be discovered about the specific biofilm host-immune response. Most anti-biofilm based randomized clinical trials have focused on infections associated with biofilm formation on abiotic surfaces: medical devices (especially ventilator-associated pneumonia and catheter-related infections) or dental surfaces. In these randomized clinical trials, the main strategy used to successfully control biofilm formation was the use of surface-coated (Berra et al., 2008; Kollef et al., 2008) or surface-treated catheters (Scannapieco et al., 2009; Quintas et al., 2015) and/or changes in surface composition of the device (Ghorbanzadeh et al., 2015). The last paragraph of this review will focus on these different strategies, with special emphasis on their advantages and limitations.

THE CASE OF CATHETER-RELATED INFECTIONS

Central venous catheters are essential in the management of patients and they are commonly used for the intravenous administration of fluids, blood products, complex drug treatments and total parenteral nutrition, for monitoring hemodynamics and for hemodialysis provision. The major concern with their use is colonization by microorganisms, which subsequently leads to infection, mostly catheter-related bloodstream infections (CRBSIs). CRBSIs are potentially devastating, entailing substantial morbidity, mortality and additional healthcare costs. It is estimated that a total of 250,000 cases of CRBSI occur yearly in the USA (Maki et al., 2006).

Catheter luminal colonization is the first step in catheter infection. In catheters with placement shorter than 21 days (short-term catheters), colonization originates mainly from the skin microbiota, with the microorganisms migrating distally along the external surface into the subcutaneous catheter tract. The microorganisms can also seed the intraluminal catheter surface from contaminated hubs, connectors and infusates. The intraluminal source of infection may be especially important in patients with long-term hemodialysis catheterization. Microbes colonizing catheter lumens constitute with host proteins a biofilm in which they proliferate and escape systemic antibiotics and immune host defenses. Ultimately, microorganisms can detach from biofilm and invade the bloodstream causing CRBSI and metastatic infections.

Multifaceted infection control interventions including maximal barrier precautions, line care bundle, development of educational programs, outcome surveillance and performance feedback of infection control practices have been implemented and succeeded in decreasing CRBSI rates. Several other preventive measures undertaken to further reduce the risk of CRBSI have been developed including coating catheter surfaces with antimicrobial agents and locking catheter lumens with antimicrobial solutions.

(1) Coating catheter with anti-infective agents (antiseptics or antibiotics) aims at inhibiting bacterial adhesion to the catheter lumen surface and preventing biofilm growth and subsequent infection. Catheters can be bonded to the inner and/or outer surface or impregnated within the material itself. The most commonly used antimicrobial agents are chlorhexidine-silver sulphadiazine (CHSS) and minocycline–rifampicin (MR).

First-generation antiseptic catheters whose outer surface was impregnated with CHSS had a lower rate of CRBSI when inserted for a short duration (<8 days) in units with a high incidence of CRBSI (>3 per 1,000 catheter-days; Walder et al., 2002). Second-generation CHSS catheters were then developed, with a long half-life of impregnation at the internal and external surfaces. Their use decreased the rate of catheter colonization but failed to diminish the incidence of CRBSI in units with acceptable rates of CRBSIs (Timsit et al., 2011). The development of bacterial resistance in relation to the use of CHSS catheters has never been observed in a clinical setting, but resistance to chlorhexidine has been detected in experimental studies (Tattawasart et al., 1999). Physicians must be aware that in rare cases hypersensitivity reactions have been reported in patients who were inserted with these catheters (Trautner and Darouiche, 2004).

Catheters impregnated intraluminally and extraluminally with MR have been widely developed. MR concentrations on the surface of these catheters decrease with placement duration but still exert antimicrobial activity through 60 days of catheterization (Darouiche et al., 2005). Their use is associated with a decrease in the rates of colonization and infection compared with standard catheters and first-generation CHSS impregnated catheters (Hockenhull et al., 2009; Wang et al., 2010a). No prospective trial has compared second-generation CHSS and MR-impregnated catheters. In a recent published retrospective study, second-generation CHSS and MR-impregnated catheters, in comparison with standard catheters, decreased the risk of CRBSI to a similar extent (Lorente et al., 2015).

There are concerns regarding the potential of MR-impregnated catheters for altering the microbiologic profile of catheter colonization/infection and promoting bacterial resistances. Data indicate that they can enhance the risk of fungal catheter colonization (León et al., 2004; Picioreanu et al., 2004; Lardon et al., 2011). However, bacterial resistance induced by the prolonged use of MR-impregnated catheters has not been demonstrated in clinical studies (Ramos et al., 2011; Bonne et al., 2015).

Other catheter materials have been tested including oligon, silver, carbon, platinum, and antimetabolite. Several prospective, randomized studies comparing these catheters with un-coated catheters in the prevention of catheter colonization and infection have yielded conflicting results (Kalfon et al., 2007; Walz et al., 2010; Lai et al., 2013).

Current evidence suggests the magnitude of the effect of antimicrobial-impregnated catheters differs according to the type of patient population. For instance, their beneficial benefit was mainly observed in critically ill patients and only inconsistently in

cancer patients and in patients receiving total parenteral nutrition (Lai et al., 2013). The decision to use these catheters should be based on a risk benefit analysis taking into account the incidence of CRBSI observed in the institution for at-risk populations and balancing the attributable costs of CRBSI, the price over cost of impregnated catheters and concerns for the emergence of bacterial resistance.

Antimicrobial-impregnated catheters should be reserved for patients whose catheter placement is expected to be longer than 5 days, in units with unacceptable rates of infections (more than three CRBSIs per 1,000 catheter-days), despite adherence to a comprehensive preventive strategy (Bach et al., 1996).

(2) The antimicrobial lock (AML) strategy is designed to prevent or to treat endoluminal catheter infections. It is intended for catheters that are not used continuously and consists of instilling a selected AML solution into the catheter lumen while the catheter is idle. The AML solution is allowed to dwell or is "locked" for a certain period of time in the catheter lumen. Thereafter, the lock solution is aspirated and discarded or flushed through the catheter into the bloodstream. This strategy can achieve local antimicrobial concentrations 100–1,000 times higher than that obtained by parenteral treatment to overcome the adaptive bacterial resistance of sessile bacteria.

An ideal AML solution should possess several important properties including widespread bactericidal activity against the microorganisms commonly involved in CRBSIs, the potential to penetrate biofilm and kill sessile cells, prolonged chemical stability that does not impair catheter integrity, a low potential for promoting antimicrobial resistance, a low risk of toxicity and adverse events, and the ability to maintain catheter patency by preventing catheter occlusion and thrombosis. Thus, most AML solutions combine anticoagulant and anti-biofilm activities.

A wide variety of antibiotics have been used alone or in combination to lock catheter lumen, including penicillins, cephalosporins, aminoglycosides, fluoroquinolones, folate antagonists, glycopeptides, lipopeptides, oxazolidinones, rifampicin, polymyxins, tetracyclines, glycylcyclines, and carbapenems. The antibiotic solution is currently mixed in unfractionated heparin (UFH) to obtain antimicrobial-anticoagulant solutions. However, there is a growing body of data supporting the use of alternative anticoagulants such as low molecular weight heparins, calcium chelators (citrate or ethylenediamine-tetra-acetic acid [EDTA]), and tissue plasminogen activator. The choice of antibiotics and their concentration is based on the expected susceptibility of biofilm to the antimicrobials and their ability to kill the biofilm cells. In addition, the decision to choose a mixture must take into account the results of studies conducted on the stability and compatibility of the solution, which depend on the type of agents combined, their respective concentration and the experimental conditions including temperature, exposure duration, and storage conditions. Experimental studies suggest that antibiotic UFH mixtures are compatible with a broad range of antibiotic concentrations when unfractionated heparin concentration is higher than 3500 U/mL, while precipitation occurs when antibiotics are diluted with unfractionated heparin at concentrations lower than 1000 U/mL (Droste et al., 2003). Numerous data are now available to guide physicians in the choice of antibiotic lock solution components and their final concentrations (Mermel et al., 2009; Justo and Bookstaver, 2014).

The widespread use of the prophylactic antibiotic lock strategy raises concerns in clinical practice because of the risk for the development of antimicrobial-resistant organisms (Landry et al., 2010; Dixon et al., 2012). Emerging gentamicin resistant bacteria have been identified as causative agents of bacteremia in chronic hemodialysis patients when tunneled catheters were prophylactically locked with a solution of gentamicin (4 mg/mL) and unfractionated UFH (5000 U/mL; Landry et al., 2010). In addition, catheter locking may induce severe adverse events when a fraction of the lock solution spills from the catheter lumen into the bloodstream during and after instillation (Agharazii et al., 2005). Detectable gentamicin serum levels have been observed in chronic dialysis patients receiving preventive gentamicin-citrate lock (Dogra et al., 2002). Severe neurological disorders have been reported after prolonged exposure to aminoglycoside-based lock solution (Dogra et al., 2002; Saxena et al., 2002). The use of prophylactic antibiotic lock solution with antibiotics routinely used to treat systemic infections remains debatable, and in our opinion should therefore be discouraged.

One way to reduce the likelihood of antibiotic resistance is the use of catheter lock solutions that do not include an antibiotic component. The most commonly used non-antibiotic lock solutions are taurolidine, a high concentration of citrate or of EDTA and ethanol.

- Taurolidine is an amino acid taurine derivative with a broad-spectrum activity against bacteria and fungi. It acts as a disinfectant by inducing irreparable microbial cell wall injury (Willatts et al., 1995). Several randomized control studies have compared taurolidine and UFH lock solutions in preventing CRBSI (Betjes and van Agteren, 2004; Bisseling et al., 2010; Solomon et al., 2010; Dümichen et al., 2012; Handrup et al., 2013) and yielded mixed results depending on the study population. In pediatric oncology patients and infants on home parenteral nutrition, taurolidine used at various concentrations (1.35 and 2%) with or without addition of 4% citrate decreased the rate of CRBSI as compared to low doses of unfractionated heparin (150 and 100 U/mL; Bisseling et al., 2010; Dümichen et al., 2012; Handrup et al., 2013). In contrast, in hemodialysis patients, the use of 1.35% taurolidine – 4% citrate solution failed to prevent CRBSI and exit site infections as compared to UFH lock solution (5,000 U/mL), but increased the need for premature catheter removal for poor flow (Liu et al., 2014; Zhao et al., 2014).
- Citrate and EDTA have anticoagulant activities similar to those of heparin by chelating ionized calcium, which results in a blockade of the coagulation pathways. In addition, they enhance the activity of antimicrobial drugs and therefore there is a growing interest in the use of cationic chelator-based lock solutions in the prevention of CRBSI. These lock solutions

have been widely studied and have been demonstrated to be highly effective in hemodialysis patients and pediatric cancer patients (Zacharioudakis et al., 2014; Zhao et al., 2014). In hemodialysis patients, the mixture of 7.0% sodium citrate, 0.15% methylene blue, 0.15% methylparaben, and 0.015% propylparaben reduced the risk of CRBSI in a randomized open label trial (Maki et al., 2011) but citrate alone was not more effective than UFH (Zhao et al., 2014). A similar result was observed with a lock solution of 4% EDTA (Kanaa et al., 2015). In addition, there have been concerns about the use of citrate at high concentrations owing to its potential toxicity, allergic reactions, arrhythmia and cardiac arrest (US Food and Drug Administration, 2000).

– Ethanol is an inexpensive antiseptic that acts by non-specific protein denaturation. It has drawn much interest as a lock solution for the prevention of CRBSI because it exerts bactericidal and fungicidal activity against a broad range of microorganisms and is unlikely to promote antimicrobial resistances. The time required by ethanol to eradicate experimental biofilm varies according to the microorganisms studied and is concentration-dependent. Ethanol has no antithrombotic properties and cannot be mixed with UFH because of potential precipitation. Randomized control studies on ethanol locks at concentrations higher than 70% v/v in the prevention of CRBSI have yielded conflicting results because of differences in study design, case mix population, and lock dwell time (Sanders et al., 2008; Slobbe et al., 2010; Broom et al., 2012; Pérez-Granda et al., 2014; Souweine et al., 2015). Either way, there are a number of concerns with the use of such high concentrations of ethanol in lock solutions for fear of catheter structural degradation, plasma protein precipitation and catheter occlusion (Mermel and Alang, 2014). However, ethanol solution at a 40% v/v concentration inhibits bacterial and fungi growth in established biofilms, does not induce catheter damage, and has satisfactory compatibility when mixed with low molecular weight heparin and heparinoids. When combined with low molecular weight heparin, 40% v/v ethanol may exert strong anticoagulant activity and has only a marginal impact on plasma protein precipitation. No clinical study has so far assessed the efficacy of such mixtures in preventing CRBSI.

FUTURE CHALLENGES AND LIMITATIONS OF ANTI-BIOFILM STRATEGIES

Conventional and current anti-biofilm therapies target one bacterial species without considering that most biofilm-related and chronic infections are due to the persistence of polymicrobial biofilms (Hall-Stoodley and Stoodley, 2009). Thus, there is no ideal solution to totally eradicate biofilm, but the key would be the simultaneous application of agents implementing mechanisms with synergic potential in order to both disturb the biofilm structure and kill bacteria (Khan et al., 2014). Multidisciplinary approaches are needed to decipher the generic networks underlying complex community interactions and to place them in their ecological and evolutionary context. The use of computational tools to comprehensively understand anti-biofilm processes seems essential. Biofilm and multispecies biofilm modeling techniques are available and take into account heterotroph parameters (Picioreanu et al., 2004; Lardon et al., 2011). Three-dimensional computer models of biofilm dynamics have been developed as tools for investigating mechanisms of protection against antimicrobial agents in biofilms (Chambless et al., 2006). They could be used for the analysis of the effects of anti-biofilm agents, in particular to assess their efficacy and to consider how they could impact the emergence of new classes of resistant microbes (Phillips et al., 2015).

The Food and Drug Administration (FDA) has received several medical device submissions that contain anti-biofilm claims (Phillips et al., 2015). However, current *in vitro* and *in vivo* assays are unable to effectively predict biofilm outcomes in humans and it is therefore important to develop reliable alternatives to clinical studies for the evaluation of anti-biofilm claims with standardized anti-biofilm procedures and validation methods that can establish correlation with clinical outcomes. That goes hand in hand with the elucidation of the mechanisms of action of the numerous anti-biofilm and bactericidal agents described so far. As already proposed in the Nutrition and Health claims domain, considering bacterial anti-biofilm agents will be useful in the future to establish a framework to help academic and industrial communities to explore their potential in accordance with health and nutrition policy (Miquel et al., 2015). In part because of the administrative complexity of these approaches, other potential applications must be envisaged such as vaccine strategy. The vaccine against the oral bacterium *Fusobacterium nucleatum* that preferentially targets FomA, an outer membrane protein involved in bacterial co-aggregation, can be considered as a pioneer anti-biofilm vaccine (Liu et al., 2010). New and specific vaccines are needed but it is necessary first to more fully investigate the interactions between biofilm and the host immune system, a domain as yet unexplored (Bryers, 2008).

Finally, modification of gene expression of pathogens within biofilm by probiotic counterparts could represent an interesting anti-biofilm approach with a dual-purpose: to limit bacterial colonization and inhibit the expression of virulence factors. For instance, some *Lactobacilli* down-regulate the expression of the virulence genes of *S. mutans, S. aureus,* and *Salmonella enterica* (Das et al., 2013; Nouaille et al., 2014; Wu et al., 2015). In the literature, the biofilm mode of life is generally opposed to the virulence capacities of bacteria (Claret et al., 2007), suggesting that biofilm ensures that bacteria stay within a specific niche and that its destabilization induces adverse effects. Further studies are required to assess the *in vivo* benefit of anti-biofilm approaches that can guarantee to have therapeutic or prophylactic benefits and to be very specific, highly effective and environmentally safe. However, due consideration should be given to the comparative risks and benefits for the patient, in particular the potential side effects on beneficial bacteria of the host microbiota and the emergence of antimicrobial resistances (Phillips et al., 2015).

CONCLUSION

The objective of the review was to give a better definition of the anti-biofilm activities involved in microbial crosstalk. The prevalence of biofilm is not only a significant problem for human health, but also in food and the food industry, and in water and sewage treatment, and warrants expenditure for the development of effective anti-biofilm strategies. Coating catheter surfaces with antimicrobial agents and locking the catheter lumens with antimicrobial solutions are two different approaches that have produced encouraging results with regard to catheter-related infections. A major obstacle will be to translate the use of original anti-biofilm agents into commercial reality, in part because of the necessity to develop specific drug delivery applications. It still remains a real challenge for scientists in this innovative cross-cutting research domain.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Surface Proteoglycans as Mediators in Bacterial Pathogens Infections

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Infectious diseases remain an important global health problem. The interaction of a wide range of pathogen bacteria with host cells from many different tissues is frequently mediated by proteoglycans. These compounds are ubiquitous complex molecules which are not only involved in adherence and colonization, but can also participate in other steps of pathogenesis. To overcome the problem of microbial resistance to antibiotics new therapeutic agents could be developed based on the characteristics of the interaction of pathogens with proteoglycans.

Keywords: infection, bacteria, host interaction, proteoglycans, glycosaminoglycans

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INTRODUCTION

According to the World Health Organization (WHO), in high-income countries people predominantly die of chronic diseases, whereas infectious diseases in low-income countries cause almost one third of all deaths (World Health Organization, 2014b). It is necessary to focus research not only on currently non-controlled infectious pathologies, but also on those pathogens which have hitherto been considered controlled in high income countries but are now re-emerging, as well as newly emerging diseases. One of the most important reasons behind the emergence or re-emergence of a disease is the fact that most such pathogens are able to evolve quickly, thus acquiring transmissible genetic modifications that give them an advantage in overcoming both the host and environmental changes, as in the case of the evolution and the transference of antibiotic resistance genes in current generation of drug-resistant strains of bacteria. The spread of these resistant strains is favored by both global trade and the mobility of individuals, resulting in infectious diseases becoming a global health problem (Morens and Fauci, 2013). The report "Antimicrobial resistance: global report on surveillance," published by the WHO in April 2014 and based on data from 114 countries worldwide, highlights the problem of the large increase in antimicrobial resistance in all regions of the world (World Health Organization, 2014a). Among other things, the WHO has drawn attention to the need to develop new diagnostic products, antibiotics and other instruments that allow health professional to combat emerging resistances.

Bacterial adherence and colonization of host cells are the crucial initial steps for infection to occur. Innovative strategies of interference in bacterial adhesion need to be considered and developed by investigating the mechanisms used by bacteria to overcome host defenses and attach to host cells. Bacteria possess diverse adherence mechanisms, including pili, fimbriae and various types of membrane proteins, all of which can exist in the same pathogen and co-operate to increase bacterial adhesion (Virji, 2009).

An interesting feature of the molecules used as receptors by pathogens is their wide distribution in tissues, while simultaneously presenting a certain degree of variability that enables the tropism known to exist in some bacterial adhesions. Proteoglycans (PGs) meet both these conditions; they

are complex ubiquitous molecules which have a different distribution and composition depending on the tissue (Esko et al., 2009). PGs consist of different types of core protein modified with chains of anionic polysaccharides called glycosaminoglycans (GAGs). GAGs are mainly made up of repeated disaccharide units, and depending on the composition of these units they can be classified as either: heparin/heparan sulfate (HP/HS), chondroitin/dermatan sulfate (CS/DS), keratan sulfate, and hyaluronic acid (HA; Esko et al., 2009; **Figure 1**). HS, composed of glucuronic acid and *N*-acetyl glucosamine, is the most widespread and physiologically relevant GAG (Sugahara and Kitagawa, 2002). It has a complex biosynthesis: after the polymerization of the sugar backbone, the chains are modified in different interdependent reactions, including *N*-deacetylation/*N*-sulfatation, epimerization, and various *O*-sulfations. This modification process is carried out by a number of correlated enzymes which act in a certain order. The first reaction, *N*-deacetylation/*N*-sulfatation, generates a domain organization of HS chains that includes *N*-acetylated (NA) and *N*-sulfated (NS) domains, which are separated by mixed NA/NS domains. Most subsequent modifications take place in NS domains, which become highly modified and hypervariable (Sugahara and Kitagawa, 2002). It has been described that NA/NS domain length and NS domain sulfation patterns are characteristic of the type of cell involved, and on its physiological state (Sugahara and Kitagawa, 2002; **Figure 2**).

Proteoglycans with HS moieties (HSPGs) can be classified according to their location: on the cell surface, where two families are found: the transmembrane syndecans and the glycosyl phosphatidylinositol-anchored glypicans; in the extracellular matrix (ECM) where there are three types of HSPGs: agrin, perlecan and type XVIII collagen; and inside intracellular vesicles, where serglycin is located (Sarrazin et al., 2011; **Table 1**). HSPGs have multiple functions, some of them dependant on the core proteins, but most related with the GAG chains because of their characteristic epimerization and sulfation pattern. This structural diversity allows HSPGs to play a key role in many processes including cell adhesion and migration, organization of ECM, regulation of proliferation, differentiation and morphogenesis, cytoskeleton organization, tissue repair and inflammation. HSPGs can bind several ligands such as cytokines, chemokines, growth factors, and morphogens, protecting them against proteolysis and controlling their gradients (Sarrazin et al., 2011). HSPGs co-operate with different molecules to define basement membrane structure and to mediate in cell-ECM attachment, cell–cell interactions, and cell motility (Sarrazin et al., 2011). Shedding of membrane-bound HSPG ectodomains can be carried out by enzymatic cleavage (Garton et al., 2006; Nam and Park, 2012; **Figure 3**), and is an important factor in host response to tissue injury and inflammation in pathophysiological processes (Garton et al., 2006).

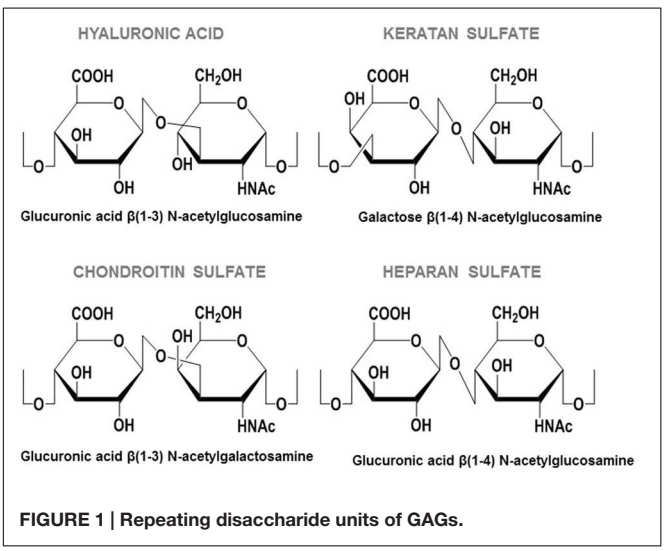

FIGURE 1 | Repeating disaccharide units of GAGs.

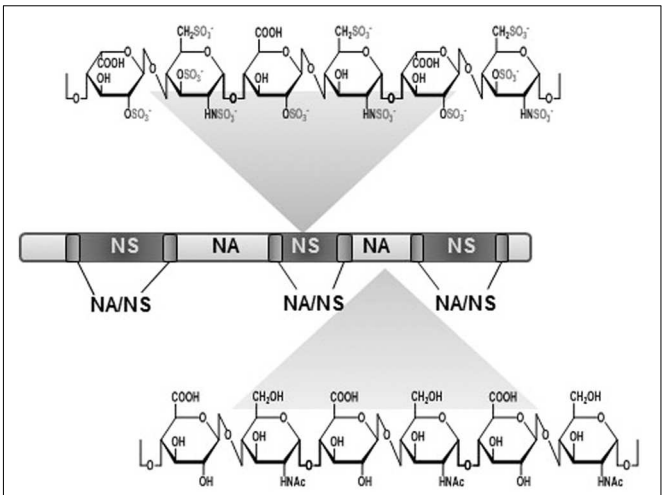

FIGURE 2 | Heparan sulfate (HS) domain architecture. The highly sulfated NS-domains are flanked on either side by less-sulfated transition zones (NA/NS). In turn, these sulphated regions are separated by non-sulfated NA-domains.

HS moieties are involved in the adherence of many microorganisms. This ranges from attaching normal human microbiota to cell surfaces and includes the genus *Lactobacillus,* which interacts primarily with HS and CS in the vaginal and intestinal epithelium (Martín et al., 2013), to various pathogenic bacteria, viruses, and parasites. Pathogens interact with HPSGs not only to achieve adherence and colonization, but also in invasion, internalization, dissemination, and toxicity (Kamhi et al., 2013; García et al., 2014). Some bacterial pathogens are even able to induce shedding of cell surface HSPGs, particularly syndecan-1. The released syndecan-1 ectodomain then binds to host defense factors, such as antimicrobial peptides and cytokines, which then become inhibited in a HS-dependent manner, resulting in deregulation of the infection response and the promotion of pathogenesis (Menozzi et al., 2002; Barlett and Park, 2010).

This review focuses on describing the role of PGs and GAGs in the principal human infectious diseases caused by bacteria (**Table 2**).

TABLE 1 | Classification of HSPGs according to their location.

Heparan sulfate proteoglycans	
Cell surface	Transmembrane syndecans
	Glycosylphosphatidylinositol-anchored glypicans
Extracelullar matrix	Agrin
	Perlecan
	Type XVIII collagen
Intracellular vesicles	Serglycin

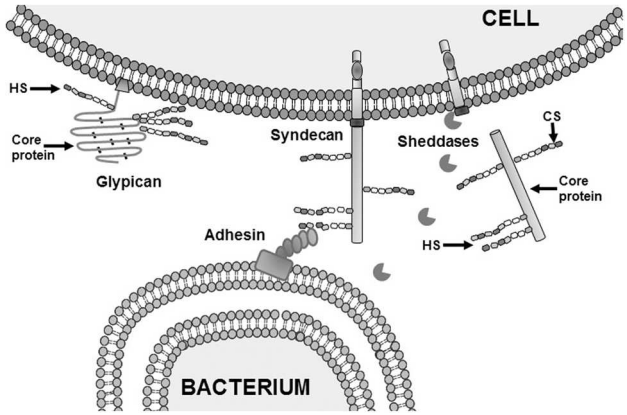

FIGURE 3 | Adhesion of bacterium to target cells mediated by HS and shedding of syndecan ectodomains. Schematic representation of the interaction between an adhesin on the outer membrane of a Gram negative bacteria and HS from syndecan on the cell surface of a eukaryotic.

INFECTIOUS RESPIRATORY DISEASES

Common upper respiratory infections include the common cold, tonsillitis, pharyngitis, epiglottitis, and laryngotracheitis. Infections of the lower respiratory tract include bronchitis, bronchiolitis, tuberculosis, and pneumonia. Flu may affect either the upper or lower respiratory tract (Dasaraju and Liu, 1996). The WHO states that the leading infectious cause of death is lower respiratory infections, which caused more than 3 million deaths worldwide in 2012 (World Health Organization, 2014b).

Pneumonia, one of the most common lower respiratory infections, is an inflammation of the lung parenchyma, commonly caused by viruses or bacteria when the functioning of the host's immune system is reduced.

The most frequent type of pneumonia is caused by streptococci, particularly by *Streptococcus pneumoniae*. This bacterium is also a significant cause of meningitis, bacteremia, and otitis media (Tuomanen et al., 1987; Hollingshead and Briles, 2001). During the colonization step, *S. pneumoniae* binds to mucosal epithelial cells using HS and CS as receptors (Tonnaer et al., 2006). Additionally, the pathogen produces extracellular glycosidases which modify various glycans and GAGs in the human airway epithelium and, as a consequence, more receptors for adherence are revealed, and the sugars released are used by the pneumococci for growth. The glycosidases secreted also negatively affect other bacteria, giving the pathogen an advantage in interspecies competition (King, 2010). Furthermore, *S. pneumoniae* has the ability to induce the ectodomain shedding of syndecan-1 from the cell surface. In fact the TIGR4 strain directly stimulates shedding through a ZmpC metalloproteinase in an intracellular signaling-independent manner in order to promote its pathogenesis. However, other strains do not express ZmpC and they shed syndecan-1 through an as yet unknown alternative mechanism (Chen et al., 2007).

Haemophilus influenzae constitutes the second most common cause of bacterial pneumonia. Strains of this bacterium are classified into typeable and non-typeable (NTHi), depending on the presence or absence, respectively, of a polysaccharide capsule (King, 2012). NTHi strains are the main cause of infections in the respiratory tract, affecting mainly non-ciliated cells or damaged mucosa (King, 2012). NTHi has several different mechanisms for adherence to mucosal surfaces, e.g., five types of pili and a large number of surface proteins, including diverse adhesins such as protein E, Hia, Hap, and protein F (Moulder, 1991). Approximately 75% of NTHi strains express the adhesins HMW1 and 2 (high molecular weight proteins 1 and 2), which bind to sulfated GAGs, particularly to HS chains (Finney et al., 2014). NTHi can undergo phase variation to promote the persistence of bacteria (Noel et al., 1994).

Another causative agent of bacterial pneumonia is the obligate intracellular human pathogen *Chlamydia pneumoniae*. This microorganism has a unique biphasic life cycle with two forms, the elementary bodies (EBs) and reticulate bodies (RBs). EBs are responsible for infecting host cells and promoting entry, after which they transform into RBs. This intracellular RB form uses the host metabolism to replicate and reorganize itself back into EBs, which are then released into the lung by cell lysis and enable the infection of new cells (Moulder, 1991). The initial attachment of EBs to the epithelium surface is mediated by HS chains, mainly through electrostatic interactions, although other co-receptors may be required for efficient attachment and entry (García et al., 2014).

Another important infection of the lower respiratory tract is tuberculosis. According to the WHO, this disease is the second leading cause of death attributable to a single infectious agent (World Health Organization, 2015). The infection commonly begins in the lungs but can spread to other tissues through the expression of a heparin-binding protein (Chen et al., 2008). *Mycobacterium tuberculosis* adheres to pulmonary epithelial cells, although the microorganism is also able to infect phagocytes. The bacteria's adherence to the epithelium is mediated predominantly by heparin-binding hemagglutinin adhesin (HBHA), which interacts with HS chains. HBHA also mediates in the triggering mechanisms for the transcytosis process, which leads to extrapulmonary dissemination of the infection (Menozzi et al., 2006; García et al., 2014).

The microorganism *Pseudomonas aeruginosa* is an opportunistic pathogen that is, among other diverse infections, the major cause of burn infections and cystic fibrosis (CF). This bacterium uses diverse strategies, which may act independently or in combination, including type IV pili and adhesins, to produce a wide range of infections (Barlett and Park, 2011). The pathogen

needs an injured respiratory epithelium in order to bind properly as such damage affects the tight junctions between epithelial cells and leaves the basolateral receptors exposed (Barlett and Park, 2011). In polarized cells, *P. aeruginosa* uses different receptors depending on the side of the cell involved, binding to complex *N*-glycans on the apical surface and to HS moieties of HSPGs on the basolateral surface (Barlett and Park, 2011). During injury and dedifferentiation of epithelium, cells are not completely polarized, and levels of HSPGs are increased on the apical surface. In these circumstances, both HSPGs and *N*-glycans act as receptors for the pathogen on the apical surface. Another strategy employed by *P. aeruginosa* to increase its pathogenicity is the virulence factor LasA, a zync metalloendopeptidase, which is able to induce syndecan-1 shedding indirectly (Barlett and Park, 2011). This shedding downregulates the host defenses, leading to increased bacterial virulence and enhances its survival (Chen et al., 2008). Circulating GAG levels in CF patients are increased, not only due to shedding, but also because of other bacterial exoenzymes which are produced to inactivate the action of molecules from the host immune system. *P. aeruginosa* produces proteinases, elastase, and alkaline proteinase all of which release DS from matrix PG decorin, which then binds to neutrophil-derived α-defensin, whose bactericidal activity is thereby neutralized (Schmidtchen et al., 2003; Barlett and Park, 2010). Released GAGs are also able to interact with LL-37 electrostatically, inhibiting its binding with bacteria and in this way disabling its bactericidal action. Unconjugated LL-37 peptide can be degradated through proteolysis by neutrophil elastase and cathepsin D, which is induced by *P. aeruginosa* (Kamhi et al., 2013).

TABLE 2 | Diverse infectious pathologies caused by bacteria and mediated by GAGs, indicating the molecular species involved.

Localization	Pathology	Bacteria	GAGs
Respiratory tract	Pneumonia	*Streptococcus pneumoniae*	HS, CS
	Pneumonia	*Haemophilus influenzae*	HS
	Pneumonia	*Chlamydia pneumoniae*	HS
	Tuberculosis	*Mycobacterium tuberculosis*	HS
	Cystic fibrosis	*Pseudomonas aeruginosa*	HS
	Pertussis	*Bordetella pertussis*	GAGs
	Pharyngitis	*Streptococcus pyogenes*	HS, DS
Systemic	Lyme disease	*Borrelia burgdorferi*	HS, DS
Central nervous system	Meningitis	*Streptococcus agalactiae*	GAGs
	Meningitis	*Streptococcus pneumoniae*	HS, CS
	Meningitis	*Neisseria meningitidis*	HS, CS
Gastrointestinal tract	Gastritis, ulcers	*Helicobacter pylori*	HS
	Inflammation	*Enterococcus faecalis*	HS
	Inflammation	*Streptococcus agalactiae*	HS
	Inflammation	*Staphylococcus aureus*	HS
	Listeriosis	*Listeria monocytogenes*	HS
Urogenital tract	Gonorrhea	*Neisseria gonorrhoeae*	HS
	Urogenital chlamidiasis	*Chlamydia trachomatis*	HS, DS
Cornea	Keratitis	*Staphylococcus aureus*	HS
	Keratitis, ulcers	*Pseudomonas aeruginosa*	GAGs

Pertussis is a highly contagious bacterial disease of the respiratory tract that mainly affects infants and young children. The WHO estimates that about 16 million cases of pertussis occurred worldwide in 2008, and it continues to be a public health concern even in countries with high vaccination coverage (World Health Organization, 2011). The infection is caused by *Bordetella pertussis,* which expresses filamentous hemagglutinin adhesin (FHA), which binds to sulfated GAGs by its C-terminal to initiate the infection of bronchial epithelial cells (Hannah et al., 1994).

The pathogen *Streptococcus pyogenes* is a member of Group A streptococci. This microorganism is able to infect different human tissues, including the respiratory tract. The bacterium interacts with different GAGs from cells surfaces, mainly with DS and HS, through different types of protein M, its major surface-expressed virulence factor (Frick et al., 2003).

SYSTEMIC INFECTION DISEASES

In some infections, the bacterial pathogens are distributed through multiple tissues. An example is Lyme disease, a chronic multisystem disorder caused by the obligate intracellular pathogen *Borrelia burgdorferi*, in which the skin, heart, joint, skin, and nervous system may be affected This bacterium has multiple surface proteins with different binding specificities to GAGs depending on the tissue affected (Leong et al., 1998). In addition, different GAGs act as receptors for *B. burgdorferi* depending on the host cells; both HP and HS are essential in adherence to primary endothelium and adult kidney Vero cells, but only DS is involved in attachment to human embryonic kidney cells, while all these GAG species mediate adherence to HeLa cells, neuronal and primary telencephalon cell lines (Leong et al., 1998; García et al., 2014).

Sepsis is the systemic host response to microbial infections, and, among other alterations, this involves an increase in the permeability of the endothelium caused by the shedding of its PGs (Henrich et al., 2010). Shedding of syndecan-1 is stimulated by proinflammatory substances and, as a consequence, levels of circulating syndecan-1 increase, thereby promoting leukocyte adherence (Nelson et al., 2008). This increase in syndecan-1 levels is correlated with the cardiovascular SOFA (Sequential Organ Failure Assessment) score. During septic shock there are high levels of some GAGs in plasma and, although there is no correlation with syndecan-1 levels, they are correlated with

mortality (García et al., 2014; Nelson et al., 2014). Specifically, the quantity of circulating HS and HA increases, while KS levels are moderately reduced, and those of CS remain unaltered (Nelson et al., 2014). These released GAGs are mainly from the altered endothelium, but they can also come from connective tissue and basement membrane (Nelson et al., 2014).

NERVOUS SYSTEM INFECTIONS

Certain microbes invade brain endothelial cells and breach the blood–brain barrier (BBB) through interactions with GAGs, thus establishing central nervous system infections (Chang et al., 2011).

Meningitis has been declared one of the top 10 causes of mortal infection worldwide, and is particularly devastating in newborns (World Health Organization, 2014b). The most common agent of neonatal bacterial meningitis is *Streptococcus agalactiae*, which uses alpha C protein (ACP) to interact electrostatically with GAGs and cross the BBB. ACP also mediates streptococci entry into epithelial cells, involving Rho GTPase-mediated actin polymerization (García et al., 2014). GAG expression patterns are important for adherence in different cells, since they determine the efficiency of bacterial dissemination during infection by *S. agalactiae* (Chang et al., 2011).

In older children and adults, meningitis is mainly caused by *S. pneumoniae* and *Neisseria meningitidis*. As described above, the binding of *S. pneumoniae* to cells happens through interactions with sulfated GAGs, mainly HS and CS4 (Tonnaer et al., 2006). The pathogen *N. meningitidis* has a large number of molecules involved in adhesion including adhesin OpC, which binds to HS to initiate epithelial cell invasion (de Vries et al., 1998).

GASTROINTESTINAL INFECTION DISEASES

More than 1200 bacterial species can inhabit the healthy human gastrointestinal tract, and there is not yet general agreement on a reliable total number of species (Rajilić-Stojanović et al., 2007).

The pathogenic bacterium *Helicobacter pylori* appears adhered to human gastric mucosa, and its presence is associated with chronic gastritis type B and ulcers. What is more, *H. pylori* has been found to be related with gastric cancer, and has been designated a class I carcinogenic agent by the International Agency for Research on Cancer (García et al., 2014). Different outer-membrane proteins of *H. pylori* bind to HS from gastric cells to bring about adhesion (Guzman-Murillo et al., 2001). The pathogen also secretes various proteins that also interact with HS chains, such as cytotoxin vacA. This toxin attaches and enters into cells inducing vacuolation, which in turn leads to host cell death (Palframan et al., 2012). The most pathogenic strains of *H. pylori* have the cag pathogenicity island, which encodes the cytotoxic protein CagA, and a type IV secretion system that injects this protein into the gastric cells (Odenbreit et al., 2000). Protein CagA affects intracellular signaling pathways, including the NF-kB pathway, which leads to upregulation of the expression of the gene encoding syndecan-4, which is commonly found overexpressed in carcinoma cell lines (Odenbreit et al., 2000).

Some of the species that reside in the human intestinal tract are typically associated with systemic infection in post-surgical, shock, and in trauma patients. This is the case of *Enterococcus faecalis*, the major cause of nosocomial infections, which affects a variety of tissues (Baldassarri et al., 2005). Enterococci use HS for initial adherence and for their internalization in professional and non-professional phagocytes, which is mediated by triggering cascades of protein kinases and the reorganization of microtubules (Kamhi et al., 2013). *E. faecalis* is able to resist specific killing mechanisms inside these phagocytes and thus continue its invasion and dissemination (Baldassarri et al., 2005). Although syndecan-1 is widely expressed in enterocytes, it has been proven not to be essentially involved in the adhesion of any enterococci (Sava et al., 2009). However, it does play a role in interactions with other enteropathogenic bacteria, such as *Listeria monocytogenes,* staphylococci and streptococci, which may use the intestinal tract as a portal of entry in special circumstances (Sava et al., 2009).

Listeriosis is a serious infection caused by eating food contaminated with the intracellular pathogen *L. monocytogenes*. The bacterium employs different membrane proteins to adhere to epithelial cells using HSPGs, particularly syndecan-1 (Henry-Stanley et al., 2003). *L. monocytogenes* has great invasive capacity, thanks to its ability to induce its own internalization in a wide range of cells (Henry-Stanley et al., 2005). In phagocytic cells, the bacterial surface protein ActA plays two important roles: extracellularly, it mediates adhesion by binding with low affinity to HSPGs; intracellularly, it directs actin assembly which is needed for the bacterium's intracellular life-cycle and motility (García et al., 2014). In non-phagocytic cells, the internalization of *L. monocytogenes* is mediated by one of two invasins, depending on the polarization of the cell. In polarized epithelial cells, invasin InlA uses molecule E-cadherin in junctional sites. However, for internalization in non-polarized cells, *L. monocytogenes* utilizes InlB to interact with GAGs through the hepatocyte growth factor receptor Met; the formation of this complex promotes the endocytosis of the receptor with the bound bacterium (Pizarro-Cerdá and Cossart, 2006).

Although *S. aureus* is a member of the normal human microbiota, it is a common cause of infections affecting different parts of the body (Barlett and Park, 2010). The adherence of the bacterium to the intestinal epithelium and its internalization involve various mechanisms. One is mediated by two bacterial fibronectin binding proteins (FnBPs) that interact with the β1 integrin (Hauck and Ohlsen, 2006; Hess et al., 2006). An alternative mechanism involves the interaction between a heparin-binding protein and HSPGs, mainly syndecan-1 (García et al., 2014), which allows adherence and internalization via non-professional phagocytes in a fibronectin independent way (Hess et al., 2006).

A group B streptococci, *S. agalactiae,* occasionally colonizes mucosal surfaces of the human gastrointestinal tract. This pathogen is potentially highly invasive and dangerous in infants, in the elderly and in diabetic patients. As in cases of its adhesion

and crossing of the BBB, the bacterium uses ACP to interact with HSPGs, mainly syndecan-1, for adherence and entrance into cells, through an actin-dependent mechanism (Kamhi et al., 2013).

SEXUAL INFECTIOUS DISEASES

Sexually transmitted infections can be caused by more than 30 different bacteria, viruses, and parasites (World Health Organization, 2013). One of the most important sexually transmitted diseases is gonorrhea, caused by *Neisseria gonorrhoeae*. The bacterium employs diverse strategies for adherence to cell surfaces, including pili and several Opa adhesins, such as OpaA, which uses syndecan-1 and -4 as receptors (García et al., 2014). Following the interaction with OpaA, the cytoplasmic domains of syndecans trigger intracellular signaling cascades, activating phosphatidylcholine-specific phospholipase C and the acid sphingomyelinase, which leads to ceramide formation. Ceramide mediates in the reorganization of actin and endocytosis of gonococci. The syndecan-Opa complexes can also induce protein kinase C activation via an integrin-mediated mechanism, through binding to the serum ECM proteins fibronectin and vitronectin (Kühlewein et al., 2006).

Another common pathogen in sexual disseases is *Chlamydia trachomatis*. The bacterium has diverse serovars, which cause different diseases: serovars A-C cause trachoma; serovars D-K are involved in sexually transmitted infections and serovars L1, L2, and L3 cause lymphogranuloma venereum. The individual serovars have different strategies for adhesion and colonization (Becker, 1996). *C. trachomatis* is able to use diverse binding mechanisms, including some that involve direct interaction with HS on cells. Depending on the serovar, the degree of HS involvement varies, it being essential for serovar L2 binding but not for serovar E (Taraktchoglou et al., 2001). Additionally, *C. trachomatis* is able to adhere to and enter cells by indirect ways due to the interaction between its EB infectious forms and fibroblast growth factor 2 (FGF2; Kim et al., 2012). The complex thus formed interacts with the FGF2 receptor, which is locally activated and mediates in EB internalization into cells. The EB-FGF2 complex may involve synergistic interactions with the EB membrane bacterial protein OmcB, which also interacts with HS from the cell surface (Fechtner et al., 2013). The internalization of bacteria increases the production of FGF2, triggering a positive feedback which upregulates the infection. FGF2 may also play additional roles in enhancing pathogenesis by potentiation of inflammatory response, inhibition of apoptosis and regulation of gene expression (Kim et al., 2012).

OCULAR INFECTIOUS DISEASES

There is a wide range of non-specific pathogens which are able to infect certain areas of the eye, mainly conjunctiva, lid, and cornea. The most common external ocular infections are caused by bacteria, which mainly interact with HS chains as receptors (Armstrong, 2000). Most keratitis are caused by *S. aureus* and *P. aeruginosa*. As described above, both interact with HSPGs from injured epithelium, a requirement for corneal invasion.

The adherence of *S. aureus* to corneal epithelium takes place via collagen-binding adhesin and FnBPs A and B, which interact with collagen and fibronectin, respectively (Armstrong, 2000). Syndecan-1 is not involved in the initial attachment, although *S. aureus* induces its shedding via α- and β-toxin (Barlett and Park, 2010; Hayashida et al., 2011). Syndecan-1 ectodomains increase pathogenicity and the potential for bacterial survival by interacting with neutrophils, whose antibacterial function is thus inhibited. The shedding of syndecan-1 also affects the inflammatory process by generating gradients of quimioatractors (Hayashida et al., 2011).

Severe keratitis and central corneal ulcers are caused by *P. aeruginosa* infections (Fleiszig and Evans, 2002). The initial attachment is mediated by surface structures such as pili and polysaccharides, which mediate biofilm formation over the epithelium and on contact lenses. Extended contact lens wearing increases bacterial adherence to corneal epithelium (Fleiszig and Evans, 2002). The bacterium is not only able to adhere to cell surfaces, but it also interacts with perlecan from ECM (Willcox, 2007). As was described earlier, the bacterium is able to produce several exoproducts that degrade PGs and produce tissue injury by degradation of basement membranes and the ECM, promoting bacterial virulence and invasion both individually and via quorum-sensing (Twining et al., 1993; O'Callaghan et al., 1996; Chen and Hazlett, 2001). The bacterium *P. aeruginosa* also induces the production of host exoproducts that contribute to corneal damage and to an excessive activation of the host defense system (Twining et al., 1993).

GLYCOSAMINOGLYCANS AS THERAPEUTIC TARGETS

The increase in the emergence of antibiotic resistant bacterial pathogens has limited the efficacy of existing treatments in infections. New therapies which can supplement or replace old treatments avoiding the selective pressures that lead to bacterial resistance should be investigated and developed urgently. GAGs have been widely explored and used as therapeutic agents in diverse biomedical treatments. This broad application of GAGs is due to their involvement in multiple physiological processes such as coagulation, thrombosis, inflammation, cancer, angiogenesis, cell differentiation, tissue repair, and microbial infections (San Antonio and Iozzo, 2001).

The therapeutic applications of certain GAGs are well-known, including the use of HA in various joint disorders and plastic surgery (Kogan et al., 2007), of HP as an anticoagulant and anti-inflammatory agent (Young, 2008), and of CS in the treatment of osteoarthritis (Clegg et al., 2006). In the last few years, GAG molecules have begun to be used as drug delivery agents in nanomedicine. GAGs interact electrostatically with different compounds or biologics used for therapeutic purposes thanks to their strong negative charge. The drugs are delivered systemically or locally to treat a variety of pathological conditions, including cancer, glaucoma, wounds, and burns (Misra et al., 2015).

The increased understanding of the structure–function relationship of GAGs has made possible the design of new compounds with a potential therapeutic role in a variety of diseases (Raman et al., 2005; Gandhi and Mancera, 2008). Some analogs and antagonists of these molecules, with suitable charge and conformation, have already been developed and used to affect some GAG functions, including anticoagulants, anti-inflammatories, antimetastatics, and also to interfere with their ability to interact with growth factors, proteases, and different host defense molecules, such as neutrophils, proinflammatory molecules, and antimicrobial peptides (Rusnati et al., 2005; Rusnati and Urbinati, 2009; Harris et al., 2014). The use of GAG based-molecules in the field of cancer is particularly interesting, several compounds having already entered the clinic, and one of them (PI-88) currently being in phase III trials (Ferro et al., 2007). Since different GAGs mediate interactions with pathogens, the characterization of the different roles played by these molecules in several infectious pathologies is essential in order to design new therapeutic molecules based on these polysaccharides which may interfere in the establishment of microorganisms.

A layer of extracellular GAGs covers the healthy urothelium; these molecules attract water, creating a chemical barrier that protects against infections. During urinary tract infections the layer of GAGs becomes damaged. The restoration of this layer with GAGs supplied exogenously has been shown to play a protective role against recurrent infections and inflammatory factors (Yazawa, 2014). Other experiments have demonstrated that intravesical therapy using some GAGs, specifically HA and CS, was more effective than antibiotic therapy in reducing recurrent episodes of infections in the urinary tract (Torella et al., 2013). Natural or synthetic GAGs can be used as prophylactic agents in treatments for recurrent urinary tract infections, and even as adjunctive therapy with classic anti-infective treatments.

Hyaluronic acid has also been useful in different therapeutic aplications, such as in the treatment of chronic bronchitis. The treatment of such patients with HA led to a reduced number of acute episodes than in patients treated with a placebo. These experiments suggest that HA reduces aggravation in patients with chronic bronchitis and may help to decrease the use of antibiotics in their therapies, diminishing the risk of generating antibiotic resistance. The investigators involved in this trial proposed that HA possibly enhances host cellular defense mechanisms (Venge et al., 1996; Souza-Fernandes et al., 2006).

Therapeutic GAG-like molecules can come from different natural sources, including mammalian tissues, non-mammalian origins such as invertebrates, and synthetic GAG mimetics. These molecules can act both as agonist or antagonist either by interfering with endogenous GAGs or by forming complexes with protein ligand and/or receptors (Rudd et al., 2012; Pomin, 2015). The possible role of GAG analogs in inhibiting pathogen interaction has been widely studied, especially in virus attachment and dissemination.

Heparin derivatives and heparin-mimicking molecules are called heparinoids, and they have been used in many therapeutic applications as inhibitors of HS-protein interactions. Fucoidans are heparinoids obtained from marine brown algae. For the past decade fucoidans isolated from different species have been extensively studied due to their wide variety of biological activities, but their potent anticoagulant action is by far the most widely investigated. These molecules also have different anti-infective activities, including antiviral activity against a wide range of viruses via receptor entry blocking or interference with replicative processes (Li et al., 2008). Various experiments have demonstrated that fucoidans also have antiparasitic activities against *Toxoplasma gondii*, *Plasmodium,* and *Leishmania*. Moreover, recent *in vitro* and *in vivo* studies have related treatment with fucoidans with prevention of infection by *H. pylori* through the inhibition of adhesion to mucosal surfaces (Fitton, 2011). All these results show the potential of these molecules to be used as prophylaxis measures.

Another heparinoid analog of HS is suramin, which also presents different therapeutic activities, mainly antiangiogenic, antiviral – through its inhibition of the reverse transcriptase of several retroviruses (Wang et al., 2014), and antiparasite (McGeary et al., 2008). The effect of suramin against pathogenic bacteria has also been analyzed in different studies and it has been found to be able to produce non-specific antibacterial resistance against *Mycobacterium bovis* and *L. monocytogenes* through macrophage activations (Brandely et al., 1986). This analog is also a potent inhibitor of RecA protein of *M. tuberculosis*, the protein that is responsible for the development of antibiotic resistance (Nautiyal et al., 2014).

Heparosan, produced and found in the capsule of some pathogenic bacteria such as *Escherichia coli* K5 and *Pasteurella multocida*, is a molecule whose structure is similar to unmodified natural HS. Heparosan can be modified by chemical or enzymatic methods, thereby creating new derivatives capable of affecting different processes, such as molecules that display antiviral activity *in vitro*, especially against the human immunodeficiency virus (HIV), herpes simplex virus, and human papilloma virus (Li et al., 2013). Both HP and heparosan are able to block the adhesion of pathogenic *E. coli, Pasteurella multocida, S. aureus* to enterocytes, while *Lactobacillus rhamnosus* adherence is not altered. In addition, treatment with probiotics supplemented with heparosan has been suggested as a preventive treatment for infections (Chen et al., 2012).

Chondroitin derivatives have also been found in nature, such as fucosylated CS (FucCS) from sea cucumber species. This molecule possesses diverse activities, such as anti-coagulant, anti-thrombotic, anti-inflammatory, and anti-cancer actions. FucCS is also able to block HIV virus entry into cells (Huang et al., 2013) and *P. falciparum* cytoadherence to endothelial cells (Bastos et al., 2014).

Other GAG analogs displaying a suitable charge and conformation can interfere with the electrostatic interaction of the GAGs themselves with host defense molecules. The bactericidal activity of some cationic antimicrobial peptides, such as LL-37, is disabled when the peptides bind to GAGs. The electrostatic interaction of LL-37 with these molecules can be disrupted by nebulized hypertonic saline treatment, included in the therapy against airway mucus of CF patients. The release of LL-37 results in a restoration of the peptide antimicrobial activity (Reeves et al., 2011).

In addition to molecules of various natural origins, knowledge of the relation between the structure and function of GAGs has allowed the design and development of various families of synthetic derivatives for different therapeutic purposes. Semi-synthetic glycosaminoglycan ethers (SAGEs) are derived from sulfation and alkylation of HA; different SAGEs can be used in the treatment and prevention of different pathologies, including cancer (Prestwich and Kennedy, 2011), or different ophthalmic therapies (Prestwich et al., 2012). SAGEs can also play a role in infection treatments; gingivitis and periodontitis are initiated by chronic bacterial infections caused among others by *Porphryomonas gingivalis*. This therapy can be a major advantage compared to treatment with antibiotics which may lead to the generation of resistant bacteria.

ReGeneraTing Agents (RGTAs) are synthetic HS mimics resistant to digestion with multiple endoglycanases such as heparanase, chondroitinase, hyaluronidase, and dextranase. They are able to interfere in the interaction of GAGs with different ligands, including various growth factors such as FGF1, FGF2, and VEGF, and can play an important role in wound regeneration. CACIPLIQ20® (OTR3, Paris, France) is an RGTA member specifically designed to restore the damaged ECM in chronic wounds. Indirectly, these molecules, based on GAGs, may be useful in preventing infections in open wounds and thus reducing the time of exposure to pathogens (Barbier-Chassefière et al., 2009; Ikeda et al., 2011).

Different monosaccharides have been chemically modified in order to interfere with GAG biosynthesis, chain elongation, or sulfation. One of them is peracetylated 6-fluoro-*N*-acetylgalactosamine [6F-GalNAc (Ac3)], which can potentially inhibit 6-*O* sulfation of GAG chains. As described above, the sulfation pattern is essential for interaction with various ligands as well as for pathogen adhesion. Another of these sugars, 6F-*N*-acetyl-D-galactosamine, is an inhibitor of GAG biosynthesis, which may affect the adhesion of many ligands, including bacteria (van Wijk et al., 2015).

Different antagonists of GAGs have also been designed for therapeutic purposes. Surfen, bis-2-methyl-4-amino-quinolyl-6-carbamide, is an antagonist of HS first described in 1938 as an excipient for the production of depot insulin. This molecule binds electrostatically to sulfated GAGs, competing in their interaction with different molecules, including FGF2, VEGF or glycoprotein D and, consequently, altering different processes, such as anticoagulant activity, cell adhesion to fibronectin, and viral infection by HIV-1 (Schuksz et al., 2008).

CONCLUSION

The report on antimicrobial resistance made by the WHO in 2014 emphasizes a previously known problem that has increased significantly in recent years: bacterial resistance to antibiotics. PGs and GAGs present unique characteristics that allow them to play essential roles in the interaction between bacterial pathogens and host cells. The characterization of GAG-pathogen interactions has allowed the development of certain therapeutic molecules which are able to fight different infections. Further studies will lead to new therapies capable of more efficiently interfering with or blocking infections, helping to overcome antimicrobial resistance.

AUTHOR CONTRIBUTIONS

BG: main author of this review, conception and design, drafting the article, final approval of version to be submitted. Experience and information about bacterial attachment to eukaryotic cells through proteoglycans and glycosaminoglycans. JM-L: design, drafting the article, final approval of version to be submitted. Experience in clinical ophthalmology and about eye infections. CM: design, drafting the article, final approval of version to be submitted. Experience and information about human normal attachment to cells through glycosaminoglycans. IA: design, drafting the article, final approval of version to be submitted. Experience in clinical ophthalmology and about eye infections. LQ: design, drafting the article, final approval of version to be submitted. Expert in proteoglycans and glycosaminoglycans. FV: correspondence author, design, drafting the article, final approval of version to be submitted. Experience in clinical microbiology and infectious diseases.

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Pseudomonas aeruginosa inhibits the growth of *Scedosporium aurantiacum*, an opportunistic fungal pathogen isolated from the lungs of cystic fibrosis patients

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The filamentous fungus *Scedosporium aurantiacum* and the bacterium *Pseudomonas aeruginosa* are opportunistic pathogens isolated from lungs of the cystic fibrosis (CF) patients. *P. aeruginosa* has been known to suppress the growth of a number of CF related fungi such as *Aspergillus fumigatus, Candida albicans,* and *Cryptococcus neoformans*. However, the interactions between *P. aeruginosa* and *S. aurantiacum* have not been investigated in depth. Hence we assessed the effect of *P. aeruginosa* reference strain PAO1 and two clinical isolates PASS1 and PASS2 on the growth of two clinical *S. aurantiacum* isolates WM 06.482 and WM 08.202 using solid plate assays and liquid cultures, in a synthetic medium mimicking the nutrient condition in the CF sputum. Solid plate assays showed a clear inhibition of growth of both *S. aurantiacum* strains when cultured with *P. aeruginosa* strains PASS1 and PAO1. The inhibitory effect was confirmed by confocal microscopy. In addition to using chemical fluorescent stains, strains tagged with yfp (*P. aeruginosa* PASS1) and mCherry (*S. aurantiacum* WM 06.482) were created to facilitate detailed microscopic observations on strain interaction. To our knowledge, this is the first study describing successful genetic transformation of *S. aurantiacum*. Inhibition of growth was observed only in co-cultures of *P. aeruginosa* and *S. aurantiacum*; the cell fractions obtained from independent bacterial monocultures failed to initiate a response against the fungus. In the liquid co-cultures, biofilm forming *P. aeruginosa* strains PASS1 and PAO1 displayed higher inhibition of fungal growth when compared to PASS2. No change was observed in the inhibition pattern when direct cell contact between the bacterial and fungal strains was prevented using a separation membrane suggesting the involvement of extracellular metabolites in the fungal inhibition. However, one of the most commonly described bacterial virulence factors, pyocyanin, had no effect against either of the *S. aurantiacum* strains. This study shows that *P. aeruginosa* has a substantial inhibitory effect on the growth of the recently described CF fungal pathogen *S. aurantiacum*. The findings also highlighted that *P. aeruginosa* biofilm formation is important but not crucial for inhibiting the growth of *S. aurantiacum* in a lung- mimicking environment.

Keywords: co-culture, *S. aurantiacum*, *P. aeruginosa*, interactions, growth inhibition, phenazines, SCFM, biofilms

Introduction

Cystic fibrosis (CF) is one of the most common, potentially lethal, genetically inherited disorders affecting mainly the European Caucasian population (O'Sullivan and Freedman, 2009). Although the disease affects a number of organs and systems in the human body, lungs remain the main site of infection in CF patients (Quinton, 1999). The inherited condition stems from the mutation of the CF transmembrane conductance regulator (CFTR) gene, which regulates the transport of chloride ions across the plasma membrane of the epithelial cells (Boucher, 2007). Impaired ion exchange reduces the mucociliary clearance, which leads to accumulation of hyper-viscous mucus in the airway surfaces, thus providing ideal conditions for the growth of microorganisms (Delhaes et al., 2012). Various molecular and microbiology based approaches have revealed the polymicrobial nature of the infections in CF with the identification of complex microbiota including bacteria, fungi, and viruses (Lynch and Bruce, 2013). Most of these microorganisms are either acquired from the environment or through contact with other infected patients (Lipuma, 2010).

Bacteria constitute the major portion of the microorganisms associated with CF. The most common bacterial inhabitants of the CF airways include *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas Aeruginosa,* and *Burkholderia cepacia* complex (BCC) (Harrison, 2007; Lipuma, 2010). Among them, *P. aeruginosa* is the most dominant bacterial species known to cause chronic respiratory infections in more than 50% of adult CF patients (Coutinho et al., 2008). *P. aeruginosa* is a ubiquitous Gram-negative bacterium possessing a wide variety of pathogenicity factors to evade the host defense system (Davies, 2002). During the early stages of infection, the bacterium attaches itself to lung epithelial cell surface receptors through specific adhesins and secretes extracellular products to prolong its survival in the CF airways (Tang et al., 1995). The extracellular products secreted by *P. aeruginosa* include enzymes such as elastase and alkaline protease, exotoxins, siderophores, and phenazines such as pyocyanin with a known role in virulence (Haas et al., 1991). Moreover, *P. aeruginosa* cells form biofilms in order to proliferate inside the lungs and protect themselves from antibiotic agents (Singh et al., 2000).

In addition to bacteria, some fungal species are also known to colonize the respiratory tracts of CF patients (Cimon et al., 2000; Pihet et al., 2009). Mycological examination of the specimens obtained from CF patients have shown that *Aspergillus fumigatus* is the most predominant fungal colonizer of the CF lungs as it has been recovered from 6 to 71% of CF patients (Bakare et al., 2003; Horre et al., 2010). However, the presence of non-*Aspergillus* fungal species often remains unnoticed owing to the lack of sensitive culture techniques to examine the sputum specimens from CF patients (Delhaes et al., 2012). Recently, a more targeted approach has been developed by combining molecular techniques with laboratory culture methods, which can now identify a wide range of fungal pathogens in the expectorated sputa (Middleton et al., 2013). Studies conducted on CF patients in Australia and certain parts of Europe have confirmed the emergence of a new fungal genus *Scedosporium* (originally called *Pseudallescheria*) that causes infections in the lungs of immunocompromised hosts (Blyth et al., 2010a; Paugam et al., 2010; Lackner et al., 2014). *Scedosporium* sp. have been isolated from the sputum specimens of 14.7–17.4% of Australian CF patients which makes it the second most common fungal respiratory pathogen associated with CF (Blyth et al., 2010a,b). *Scedosporium aurantiacum* is a recently identified, highly virulent member of the *Scedosporium* sp. complex recovered from one in six CF patients in Sydney (Heath et al., 2009; Blyth et al., 2010b; Harun et al., 2010). The clinical consequences of the *S. aurantiacum* colonization or infections in the CF patients remain to be explored (Harun et al., 2010).

According to the clinical reports, the prevalence of fungi in the respiratory tracts of CF patients is mainly affected by the bacteria present, and the interactions between the bacteria and fungi potentially impact the disease outcome (Sibley et al., 2006; Chotirmall et al., 2010; Leclair and Hogan, 2010). Several *in vitro* studies have reported an inhibitory effect of *P. aeruginosa* against the common lung co-inhabitants such as *A. fumigatus* or the yeasts *Candida albicans,* and *Cryptococcus neoformans* (Hogan and Kolter, 2002; Bandara et al., 2010; Cugini et al., 2010). Similar data for *S. aurantiacum* are lacking. Reflecting the increasing importance of *S. aurantiacum* in CF, we examined the effect of clinical *P. aeruginosa* CF isolates PASS1 and PASS2 and laboratory reference strain PAO1 on the growth of two clinical *S. aurantiacum* isolates WM 06.482 and WM 08.202 using solid plate assays and liquid co-cultures containing medium that mimics the nutritional content of human CF sputum (Palmer et al., 2007).

Materials and Methods

Growth and Maintenance of Strains

Strains used in the study are listed in **Table 1**. *P. aeruginosa* PASS1 and PASS2 were isolated from the sputum samples of CF patients (Penesyan et al., under review). A common laboratory 'reference' strain PAO1 (Lewenza et al., 2014) was also included in the study. *S. aurantiacum* strains WM 06.482 and WM 08.202 were obtained from the culture collection of the Medical Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, NSW, Australia (Kaur et al., 2015). Virulence levels of all *P. aeruginosa* strains used in this study have been tested previously using *Caenorhabditis elegans* based infection model (Lewenza et al., 2014; Penesyan et al., under review). Virulence studies of *S. aurantiacum* have been performed using *Galleria mellonella* larvae model (Kaur et al., 2015).

Pseudomonas aeruginosa strains were revived from frozen stocks stored at −80°C by streaking on LB (Luria Bertani, Sigma) plates and incubation overnight at 37°C. Bacterial colonies were inoculated into LB broth and incubated at 37°C on an orbital shaker (200 rpm) overnight. Following fractions were prepared from overnight cultures of the *P. aeruginosa* strains: (1) Heat killed cells were obtained by incubating 1 ml of an overnight cell culture at 80°C for 60 min. Absence of any viable cells was confirmed by plating on LB agar medium; (2)

TABLE 1 | *Pseudomonas aeruginosa* and *Scedosporium aurantiacum* strains used in the study.

Strain	Strain name	Source	Virulence level	Reference
PASS1	*P. aeruginosa*	Sputum sample of a cystic fibrosis (CF) patient Sydney, NSW, Australia	High	Penesyan et al. (under review)
PASS2	*P. aeruginosa*	Sputum sample of a CF patient Sydney, NSW, Australia	Low	Penesyan et al. (under review)
PAO1 (ATCC 15692)	*P. aeruginosa*	Wound exudate Melbourne, VIC, Australia	High	Holloway (1955)
WM 06.482	*S. aurantiacum*	Invasive clinical isolate from CF patient Sydney, NSW, Australia	High	Kaur et al. (2015)
WM 08.202	*S. aurantiacum*	Type strain from a wound exudate Santiago de Compostela (Spain)	Low	Kaur et al. (2015)

Cell lysates were obtained after sonicating the cells (50 ml) on ice for 10 min in an ultrasonic processor followed by collection of the supernatant after centrifugation at 10,000 × *g* for 30 min; (3) Cell culture supernatants were collected by centrifuging 50 ml of overnight cultures of *P. aeruginosa* strains at 10,000 × *g* for 30 min. Supernatants were then freeze dried and resuspended in 100 μl of 1x PBS and stored at 4°C until use.

Fungal strains were maintained on PDA (potato dextrose agar, BD, Difco™) plates at 37°C. After 5 days of growth, the conidia were scraped into sterile saline solution (0.9% w/v NaCl and 0.01% v/v Tween 80) and the suspension was filtered through a sterile cotton wool to separate the conidia from the hyphal debris. Conidia were washed with 1x PBS to remove traces of saline and the inoculum was adjusted to a McFarland standard concentration of 2.5×10^5 conidia/ml. Concentration of conidia was confirmed using Neubauer counting chamber and additional plate counting.

Construction of Strains Tagged with Fluorescent Proteins

Pseudomonas aeruginosa Strain Expressing Yellow Fluorescent Protein (YFP)

Plasmid pUCP*yfp* (Gloag et al., 2013) encoding yellow fluorescent protein (YFP) was used to transform the *P. aeruginosa* PASS1 strain. In order to make electrocompetent cells, PASS1 was cultured in 5 ml of LB broth overnight at 42°C and 200 rpm. Cells were harvested by centrifugation (14,000 *g* for 15 min at 4°C) and the ionic strength of the suspension was reduced by rigorous washing with 1x M9 minimal salts medium (Sigma) followed by two washes with ice-cold sterile milliQ water. Bacterial cells were transformed by electroporation as described by Dower et al. (1988) by adding 1 μg of the plasmid DNA to 20 μl of the washed cell aliquots. At the end of the procedure, cells were streaked on LB plates containing 8 mg/ml ampicillin and incubated for up to 48 h at 37°C to select for the transformants.

Construction of the *S. aurantiacum* Strain Expressing mCherry

The *mCherry* gene was PCR amplified from the pmcherry-c1 vector (Clontech Laboratories, USA) using *mCherry.fwd* and *mCherry.rev* primers (**Table 2**) and was expressed under the *Trichoderma reesei* pyruvate kinase (*pki*) promoter, which was amplified from the pCBH1corlin vector (Te'o et al., 2000) using *pki.fwd* and *pki.rev* primers. In addition, a DNA fragment featuring the *pki* promoter together with the hygromycin B resistance gene (*pki-hph*) was PCR amplified using primer *pkihph.fwd* and *pki-hph.rev* to allow selection of transformants. The fragments were engineered to contain restriction sites as needed (**Table 2**).

TABLE 2 | Sequence of primers used for the construction of transformation cassettes.

Primer name	Sequence (5′–3′)
mCherry. fwd	**GAA GAACCT CTT AAC CTC TAG (*pki* sequence)** ATG GTG AGC AAG GGC GAG G
mCherry. rev	CAT GCG GGT ACC (*Kpn***I**) CTA TTA CTT GTA CAG CTC GTC CAT GC
pki. fwd	TGC TGC GAT ATC (*Eco*R**V**) CTT AAG TTA G TA ACT AGT GGA TC
pki.rev	**CTC GCC CTT GCT CAC CAT (*mCherry* sequence)** CTA GAG GTT AAG AGG TTC TTC
pki-hph. fwd	TAC GCG GCG CGC C CT TAA G **(*Afl*II)** TT AG T AAC TAG TGG ATC
pki-hph.rev	CAT GCT AAG CTT **(*Hind*III)** CTA TTC CTT TGC CCG CGG AC

The primers contain engineered restriction sites shown in shading and the overlapping sequences are shown in bold.

The primers *pki.fwd* and *mCherry.rev* were used to fuse the separately amplified *pki* and *mCherry* fragments in an overlap extension PCR as described by Thornton (2015). The fragment *pki-hph* was digested with restriction enzymes *Hind*III and *Afl*II (Fermentas, Thermo Scientific, USA) and fragment *pki-mcherry* was digested with *Eco*RV and *Kpn*I. The digested products *pki-hph* and *pkimcherry* were gel purified using QIAquick gel extraction kit (Qiagen, USA) and inserted into MCS-1 (multiple cloning site) and MCS-2 of the pETDuet-1 plasmid, respectively (Supplementary Figure S1). Finally, the purified vectors and inserts were ligated using T4 ligase (Fermentas, USA) at a 1:3 molar ratio for 2 h at room temperature. The final ligated vector (pETDuet-phpm) was introduced into *Escherichia coli* DH5α competent cells as described by Inoue et al. (1990). Selection of transformants was performed on LB agar plates containing ampicillin (100 μg/ml) and incubating at 37°C. Selected transformants were grown in 3 ml of LB and plasmid DNA was isolated using QIAprep Spin Miniprep kit (Qiagen, USA). The plasmid pETDuet-phpm was sequenced by AGRF, Sydney, NSW, USA to check sequence alignment of the inserted gene cassettes.

The pETDuet-phpm DNA was introduced into highly virulent *S. aurantiacum* WM 06.482 using protoplast-mediated transformation based on the method adopted from Penttilä et al. (1987) with modifications. The young hyphae obtained from an overnight culture of WM 06.482 on PDA plates with cellophane at 28°C were digested with 10 mg/ml of lysing enzyme from *T. harzianum* (Sigma–Aldrich, Australia) to obtain protoplasts which were then filtered through a sterile sintered glass filter (porosity 1). Osmotically stabilized protoplasts were transformed with 5 μg of plasmid DNA as described by Penttilä et al. (1987). Transformed protoplasts were mixed with 10 ml of molten agar (1.5% w/v KH_2PO_4, 0.5% w/v NH_4SO_4, 2% w/v glucose, 1 M sorbitol, pH 5.5) containing hygromycin B (410 U/ml) and overlayed onto PDA plates which were incubated at 28°C for 3–5 days. Hygromycin resistant colonies were restreaked onto fresh PDA plates containing hygromycin B (410 U/ml) for a second round of selection. Transformation efficiency was calculated as number of transformants per μg of plasmid DNA. Expression of the mCherry protein in selected transformants was confirmed using Fluoview FV1000 inverted confocal microscope (Olympus) with an excitation and emission wavelength 488/633 nm (HeNe).

Growth Inhibition Assays

The effect of *P. aeruginosa* on the growth *of S. aurantiacum* was tested in different combinations on both solid and liquid growth media. Combinations of bacterial and fungal strains for the testing are presented in **Table 3**.

Cross Streak Assay using Live Cells

The effect of bacteria on fungal growth was assessed using an agar plate method described by Kerr (1999), with slight modifications adopted from Chen et al. (2013). *P. aeruginosa strains* PASS1, PASS2, and PAO1; and *S. aurantiacum* strains WM 06.482 and WM 08.202, were cultured together on a synthetic cystic fibrosis medium (SCFM) that mimics the nutritional content of human CF sputum. SCFM contains average concentrations of ions, free amino acids, glucose, and lactate present in the CF sputum samples (Palmer et al., 2007). Solid SCFM agar plates were made with an addition of 2% w/v agar to liquid SCFM medium. A sterile cotton swab was used to draw a straight vertical line of *P. aeruginosa* cells (1×10^8 CFU/ml=0.5 McFarland standard concentration) across the plate. At the same time, *S. aurantiacum* conidia (2.5×10^5 conidia/ml = 0.5 McFarland standard concentration) were inoculated with a cotton swab horizontally across the upper part of the plate preventing any direct contact between fungi and bacteria. The plates were dried at room temperature for 15 min and incubated at 37°C. Digital photography was performed after 24 h to visualize the growth of both bacterial and the fungal strains tested on the plate.

Disk Inhibition Method using Live Cells and Cell Fractions

Sterile filter paper disks (Whatman no. 1; Sigma–Aldrich), 7 mm in diameter, were impregnated with 20 μl of the *P. aeruginosa* PASS1, PASS2, and PAO1 cell fractions, i.e., cell lysates, cell culture supernatant and heat inactivated cells (see preparation in section 1.1) and placed on an SCFM plate that was freshly surface seeded with 100 μl (2.5×10^5 conidia/ml) of *S. aurantiacum* conidia (WM 06.482 or WM 08.202). A suspension of live *P. aeruginosa* cells was included for comparison. The plates were incubated at 37°C for up to 3 days and observed at regular intervals for the appearance of any clear inhibition zones around the disks. Assays were repeated in three biological replicates. A relative inhibition index was calculated for each *P. aeruginosa* isolate by dividing the area of activity (difference between the area of the inhibition zone and area of the colony) by the area of the colony.

Effect of Bacteria on the Fungal Growth in Liquid Co-cultures

Interactions between *P. aeruginosa* and *S. aurantiacum* were observed in liquid medium using both chemical fluorescent stains and genetically labeled strains of bacteria and fungi in a direct contact with each other. In case of fluorescently labeled co-cultures, 1×10^8 CFU/ml of *P. aeruginosa* PASS1, PASS2, and PAO1 and 2.5×10^5 conidia/ml of *S. aurantiacum* WM 06.482 and WM 08.202 were inoculated in 20 ml SCFM medium in 100 ml shake flasks and incubated for 24 h at 37°C on an orbital shaker at 150 rpm. Aliquots were taken on a sterile glass slide from the co-cultures after every 4 h, washed with 1x PBS and fixed using 2% v/v paraformaldehyde (Sigma–Aldrich). The co-cultures were stained with DNA specific Syto9 (0.6 μM) and mitochondria specific Mito-Tracker^R Red FM (25 nM) for 15 min in the dark as per the manufacturer's protocol (Molecular Probes, Life Technologies). Bacterial cells were expected to stain with Syto9 whereas fungal cells would stain with Mito-Tracker^R Red FM. Fixed specimens were imaged using Fluoview FV1000 inverted confocal microscope (Olympus) with an excitation and emission wavelength of 488 nm (Ar) and 633 nm (HeNe).

The genetically tagged *P. aeruginosa* PASS1yfp strain and *S. aurantiacum* WM 06.482mCherry strain were also cultured together in 20 ml of SCFM for 24 h at 37°C, shaking at 150 rpm. At the end of the incubation period, cells were washed and fixed on sterile glass slides as above. Imaging was performed with a confocal microscope using an excitation and emission

TABLE 3 | Types of cultures used to investigate the effect of different *P. aeruginosa* strains on *S. aurantiacum*.

Type of co-culture	*P. aeruginosa* strains	*S. aurantiacum* strains
Solid plate (cross streak, disk inhibition assay)	PASS1, PASS2, PAO1	WM 06.482, WM 08.202
Liquid cultures (chemical fluorescent dyes)	PASS1, PASS2, PAO1(stained with Syto9)	WM 06.482, WM 08.202 (stained with Mito tracker FR)
Liquid culture (genetically tagged strains)	PASS1 (*yfp*-labeled)	WM 06.482 *(mCherry*-labeled)
Liquid culture (addition of an antibiotic)	PASS1 (*yfp*-labeled)	WM 06.482 *(mCherry*-labeled)

wavelength of 488 nm (blue laser diode for yfp) and 561 nm (yellow–green laser for mCherry) respectively. Liquid co-cultures with genetically labeled PASS1 and WM 06.482 strains were also repeated by adding different concentrations of gentamicin (2.5–10 mg/ml), which is a commonly used antibiotic against bacteria (Doring et al., 2000; Lin et al., 2011). Image analysis for both types of co-cultures was performed using IMARIS imaging software.

Transwell Assay with Polycarbonate Membranes

In order to explore the role of secreted bacterial metabolites on the fungi, *P. aeruginosa* strains PASS1, PASS2, and PAO1 and *S. aurantiacum* strains WM 06.482 and WM 08.202 were co-cultured in SCFM in sterile six-well Transwell plates (Corning) with polycarbonate cell culture inserts (0.4 μm, Sigma–Aldrich) in order to prevent direct contact between the fungal and bacterial strains. *P. aeruginosa* (1×10^8 CFU/ml) and *S. aurantiacum* (2.5×10^5 conidia/ml) were inoculated in the bottom and top of the membrane insert, respectively. The plates were incubated at 37°C for 24 h and any inhibition of the growth of *S. aurantiacum* was measured as a difference in the dry weight of the *S. aurantiacum* cultured with or without *P. aeruginosa*. The method of calculating dry weight was adopted from Kaur et al. (2015).

Effect of Phenazines

Phenazines were extracted from all *P. aeruginosa* strains (PASS1, PASS2, and PAO1) cultured in 5 ml of LB (in three biological replicates) for 2 days at 37°C using chloroform according to a method described by Mavrodi et al. (2001). Crude phenazine extracts were dried under reduced pressure to remove the solvent, resuspended in 80% acetonitrile (ACN) and applied to the filter paper disks (Whatman paper no. 1). The presence of pyocyanin in the crude phenazine extracts was confirmed using Ultra High Performance Liquid Chromatography (UHPLC) as described by Penesyan et al. (under review). While the absolute concentration of pyocyanin in the crude phenazine extracts from the three *P. aeruginosa* strains was not known, major experimental discrepancy was minimized by using same amount (20 μl) of crude extracts for the testing. The effect of these crude extracts on the fungal growth was determined using a disk inhibition assay (as described in section Disk Inhibition Method using Live Cells and Cell Fractions) where disks containing 20 μl of phenazine extracts from different *P. aeruginosa* isolates were air-dried and placed on SCFM agar plates freshly spread with 2.5×10^5 conidia/ml of *S. aurantiacum* strains (WM 06.482 and WM 08.202). The activity of blank 80% ACN, blank LB medium extract and the solution of commercial pyocyanin (10 mM, Sigma–Aldrich) were also tested against *S. aurantiacum* for comparison. Plates were incubated for 48 h at 37°C and observed for the presence of clearing zones around the filter paper disks as an indication of inhibitory activity of the extracts on fungal growth.

Statistical Analysis

Statistical significance between the means of different experimental datasets was analyzed using two-tailed Student's *t*-test. SD with *p*-value less than 0.05 was considered significant. All experiments were performed in biological triplicates.

Results

Inhibition of *S. aurantiacum* Growth by Live *P. aeruginosa* Cells

When *S. aurantiacum* strains WM 06.482 (high virulence) and WM 08.202 (low virulence) were cross streaked against three different *P. aeruginosa* isolates PAO1, PASS1, and PASS2 on the SCFM agar medium, an area of inhibition was observed in the growth of both *S. aurantiacum* strains after 24 h (**Figures 1A–F**). It was evident from the size of the inhibition area that the bacterial strains had lesser impact on the highly virulent *S. aurantiacum* strain WM 06.482 (**Figures 1A,B**) compared to the less virulent WM 08.202 strain (**Figures 1D,E**). Out of the three bacterial strains studied, *P. aeruginosa* PASS2 had the weakest inhibitory effect on the *S. aurantiacum* strains in the plate test as seen in **Figures 1C,F**.

Effect of *P. aeruginosa* Cell Extracts on *S. aurantiacum*

The effect of different cell fractions, i.e., the culture supernatant and cell lysate, and heat inactivated cells of *P. aeruginosa* strains PASS1, PASS2, and PAO1 was further tested on the growth of *S. aurantiacum* WM 06.482 and WM 08.202 using the disk inhibition method. Following 48 h incubation, clear inhibition zones were observed on plates inoculated with living cells of *P. aeruginosa* PASS1 and the reference strain PAO1 and their respective cell lysates. The inhibitory effect of *P. aeruginosa* was expressed as a relative inhibition index (**Figure 2**).

Living cells of both PAO1 and PASS1 and their corresponding cell lysates displayed a higher inhibitory activity against the less virulent *S. aurantiacum* strain WM 08.202 compared to the high virulence strain WM 06.482. Cell supernatants and heat killed *P. aeruginosa* cells failed to elicit a response against either of the fungal strains. In a separate experiment, the effect of *S. aurantiacum* was also tested against *P. aeruginosa* by incubating filter disks impregnated with *S. aurantiacum* conidia and cell fractions on the plates freshly seeded with *P. aeruginosa* cells. As *S. aurantiacum* failed to display any inhibition against *P. aeruginosa*, these interactions were not studied further (data not shown).

Effect of *P. aeruginosa* on Fungal Physiology

Pseudomonas–Scedosporium interactions were also studied using confocal microscopy by imaging cellular aggregates from liquid co-cultures labeled with fluorescent stains. Confocal images demonstrated an inhibitory effect of the *P. aeruginosa* PASS1 (isolated from sputum of a CF patient) and the reference strain PAO1 on the growth and development of both *S. aurantiacum* strains tested (**Figures 3A–F**). In the course of 24 h, the bacteria had attached to the surface of fungal hyphae and formed biofilm-like structures containing a high density of bacterial cells but very few fungal hyphae. The tested bacterial strains had a weaker impact on the more virulent WM 06.482 compared

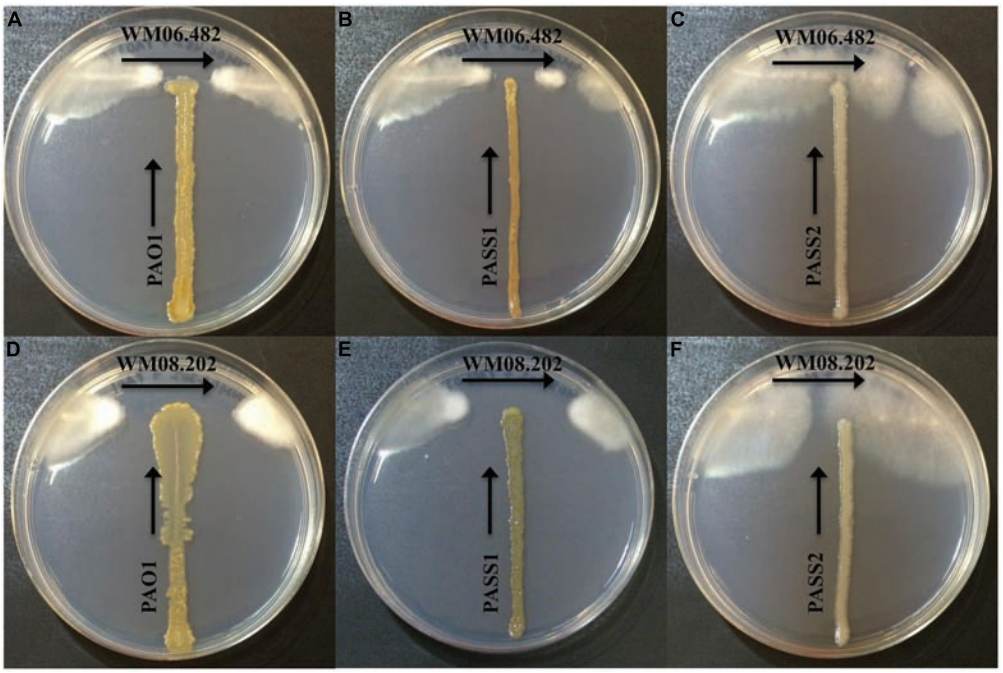

FIGURE 1 | Cross-streak plate assay between different strains of *Pseudomonas aeruginosa* and *Scedosporium aurantiacum* on synthetic cystic fibrosis medium (SCFM) agar plates. All bacterial strains were inoculated vertically whereas the fungal strains were streaked horizontally across the upper part of the SCFM agar plate. The plates were incubated at 37°C for 24–48 h. **(A–C)** Inhibition of *S. aurantiacum* strain WM 06.482 by PAO1, PASS1, and PASS2 strains of *P. aeruginosa*. **(D–F)** Inhibition of *S. aurantiacum* strain WM 08.202 by PAO1, PASS1, and PASS2 strains of *P. aeruginosa*.

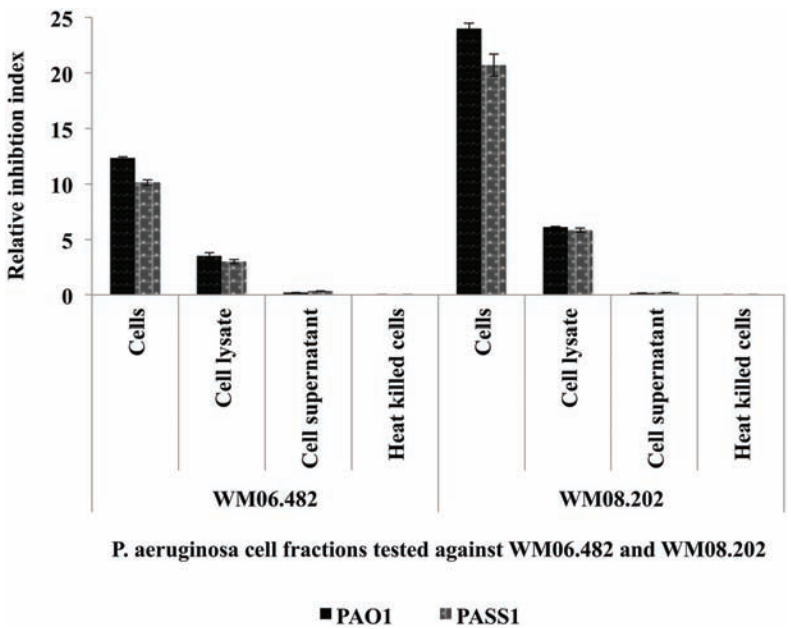

FIGURE 2 | Susceptibility of *S. aurantiacum* (WM 06.482 and WM 08.202) to *P. aeruginosa* (PAO1, PASS1, and PASS2) and their cell lysate fractions. Relative inhibition index was calculated as the average value of three replicates ($n = 3$) with a p-value <0.05 considered as significant.

to the less virulent WM 08.202, showing the resistant nature of the more virulent strain also highlighted in the plate tests. Although different fluorescent stains were used to distinguish between *P. aeruginosa* and *S. aurantiacum* in liquid cultures, it was difficult to visualize the detailed effect of bacteria on the fungal hyphae due to permeabilisation of the Syto9 dye by both

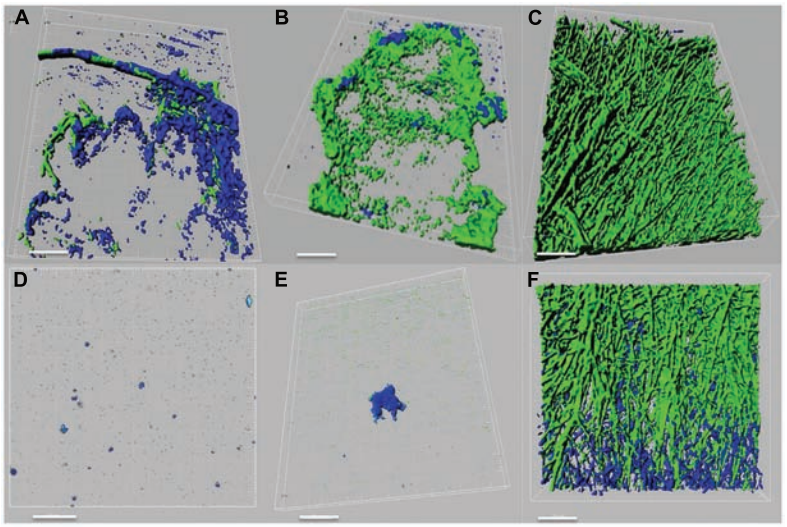

FIGURE 3 | Confocal laser scanning microscope (CLSM) images of interactions between *P. aeruginosa* (PAO1, PASS1, and PASS2) and *S. aurantiacum* (WM 06.482 and WM 08.202) as observed after co-incubating both the organisms in SCFM liquid medium at 37°C for 24 h. *P. aeruginosa* cells are stained with Syto9 (shown in green) and *S. aurantiacum* with Mito-tracker deep red FM (shown in blue). 3D re-construction of CLSM datasets was performed using IMARIS software package (Bitplane). Scale bar = 50 μm. **(A–C)** CLSM images of co-culture of WM 06.482 with PAO1, PASS1, and PASS2, respectively. **(D–F)** CLSM images of co-culture of WM 08.202 with PAO1, PASS1, and PASS2, respectively.

P. aeruginosa and *S. aurantiacum*. No growth inhibiting effect was observed when the fungal strains were co-cultured with PASS2 as indicated by dense growth of fungi in **Figures 3C,F**. These observations were also consistent with the results seen in the assays carried on plates.

Interactions between Genetically Tagged *P. aeruginosa* and *S. aurantiacum* Strains

To circumvent the difficulty in differentiating between bacteria and fungi in liquid co-cultures, genetically tagged *P. aeruginosa* strain PASS1 expressing yfp and *S. aurantiacum* strain WM 06.482 expressing mCherry were developed. With this arrangement, it was observed that the bacteria started colonizing the fungal conidia soon after incubating them together in the SCFM (**Figure 4A**). Thus, within 8 h, bacteria began aligning themselves along the length of fungal hyphae as seen in **Figures 4B,C**. After 24 h, large clumps of *P. aeruginosa* cells were observed on *S. aurantiacum* hyphal filaments (**Figure 4D**), and the amount of hyphae was also reduced in number compared to the *S. aurantiacum* control without the bacteria (**Figure 4E**).

Effect of Antibiotics used in Clinical Practice on Co-cultures

Analysis of the plate cultures and confocal images confirmed that *P. aeruginosa* had an inhibitory effect on the growth of *S. aurantiacum*. Therefore, in order to further validate this finding and to reveal the possible effect of antibiotic therapy on *S. aurantiacum* and *P. aeruginosa* mixed populations present in CF lungs, co-culturing was repeated with an addition of varying amounts of gentamicin (2.5–10 mg/ml) to selectively inhibit the growth of *P. aeruginosa*. *S. aurantiacum-P. aeruginosa* co-cultures were also maintained without the addition of gentamicin for comparison (**Figure 5A**). All bacteria were killed at a concentration of 8 mg/ml of gentamicin. As seen from **Figure 5B**, *S. aurantiacum* strain WM 06.482 was growing actively in the absence of *P. aeruginosa* strain PASS1 indicating the reversal of the inhibitory effect caused by live bacteria against the fungus.

Indirect (non-physical) Interactions between *P. aeruginosa* and *S. aurantiacum*

To investigate whether physical contact between *P. aeruginosa* and *S. aurantiacum* was important to trigger growth inhibition, co-cultures were performed in six-well plates fitted with polycarbonate membranes to prevent direct contact between *P. aeruginosa* and *S. aurantiacum* cells while allowing free exchange of nutrients and extracellular molecules between the organisms. Growth of the less virulent *S. aurantiacum* strain WM 08.202 was inhibited when co-cultured with PAO1 and PASS1, evident from the substantial decrease in the fungal biomass (**Figure 6**) when compared to the culture of WM 08.202 maintained for the same amount of time.

Pseudomonas aeruginosa isolate PASS1 and the reference strain PAO1 showed a milder inhibitory effect against the high virulence *S. aurantiacum* strain WM 06.482. The PASS2 strain had little or almost no effect on growth of either of the *S. aurantiacum* strains. The results suggested that cell–cell contact was in fact not necessary to bring about inhibition of the growth of *S. aurantiacum* by *P. aeruginosa* and that the inhibition might involve bacterial metabolites and/or extracellular signaling molecules. In addition, *S. aurantiacum* strains WM 06.482 and WM 08.202 produced a red colored pigment when co-cultured with clinical *P. aeruginosa* strain PASS1 and reference strain PAO1. No such pigment was observed in the co-cultures

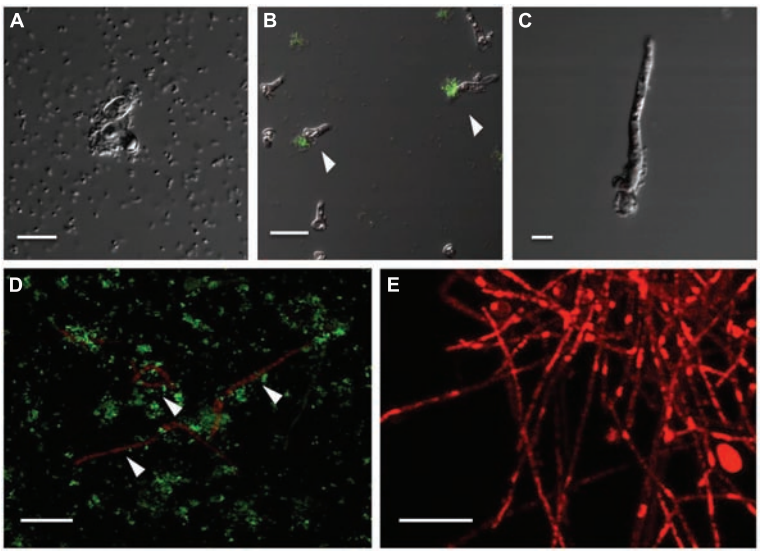

FIGURE 4 | Adhesion and colonization of mCherry-tagged *S. aurantiacum* strain WM 06.482 (shown in red) by *P. aeruginosa* strain PASS1 tagged with yfp (shown in green) during coculturing in SCFM for 24 h at 37°C. Scale bar = 20 μm. **(A)** *P. aeruginosa* cells adhered to germinating *S. aurantiacum* conidia after 2 h of incubation as viewed by DIC. **(B,C)** after 8 h, some young hyphae were surrounded by bacterial cells. **(D)** Bacteria can be seen attached to the hyphal filaments after incubation for 24 h. **(E)** Healthy growing culture of WM 06.482 expressing *mCherry* in the absence of bacteria. *White arrows indicate fungal filaments that are being colonized by the bacteria.

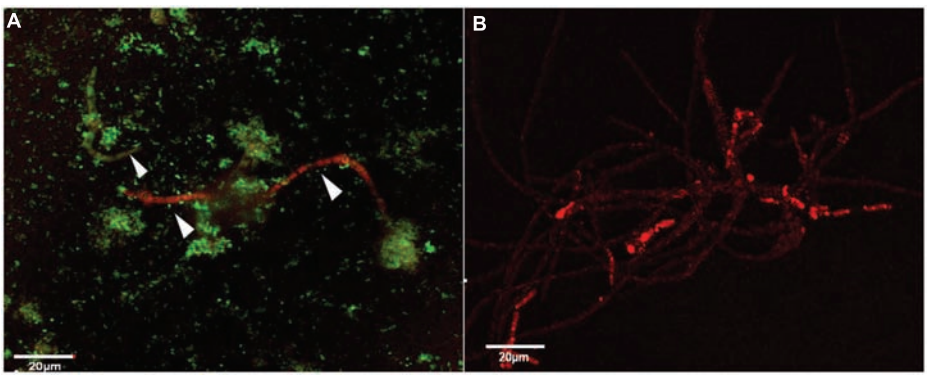

FIGURE 5 | The effect of gentamicin on *P. aeruginosa* (PASS01) and *S. aurantiacum* (WM 06.482) co-cultures growing in SCFM at 37°C for 24 h. Scale = 20 μm. **(A)** Co-culture of WM 06.482 and PASS01 without the antibiotic. **(B)** Active growth of *S. aurantiacum* in a co-culture treated with 8 mg/ml of gentamicin to eradicate the bacterial growth.

involving *S. aurantiacum* and PASS2 strain (Supplementary Figure S2).

Effect of Phenazines on the Growth of *S. aurantiacum*

To test whether known virulence factors such as phenazines secreted by *P. aeruginosa* were involved in the inhibition of *S. aurantiacum* growth, the effect of crude phenazine extracts from *P. aeruginosa* strains PAO1, PASS1, and PASS2 were tested on the two *S. aurantiacum* strains using a disk inhibition assay. No inhibition was observed with disks saturated with the crude extracts as seen in **Figure 7**. All *S. aurantiacum* strains also showed resistance to a high concentration (10 mM) of commercial phenazine pyocyanin. Phenazines are known to have an inhibitory effect against a wide range of fungal species (Kerr et al., 1999).

Discussion

Most of the studies targeting bacterial-fungal interactions *in vitro* have been performed with bacterial laboratory reference strains using either fungus-specific culture media (PDA, SABD) and/or minimal salts medium (Kerr et al., 1999; Hogan and Kolter, 2002; McAlester et al., 2008; Bandara et al., 2010; Manavathu et al., 2014). Differently to previous studies and to provide a better focus, we used a CF sputum-mimicking medium, i.e., SCFM to

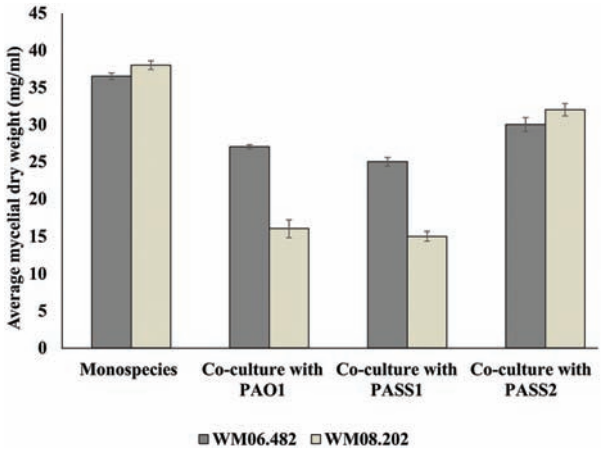

FIGURE 6 | The inhibitory effect of *P. aeruginosa* on *S. aurantiacum* during co-culture in SCFM, determined by the change in dry weight of *S. aurantiacum* hyphae. A sterile polycarbonate membrane was used to separate *P. aeruginosa* from *S. aurantiacum* in six well culture plates. Error bars on each data point represent SEM of three independent experiments. *P*-values were calculated by student's *t*-test, where $p < 0.05$ was considered significant.

explore the possible effect of *P. aeruginosa* on *S. aurantiacum* in the CF lung environment. We also used two recently isolated clinical CF strains of *P. aeruginosa*, PASS1 and PASS2, together with PAO1, a commonly used reference strain, and a clinical *S. aurantiacum* isolate (WM 06.482) with a high established virulence and a less-virulent type strain WM 08.202 to add to the clinical relevance of the findings.

Our results demonstrated that *P. aeruginosa* strains exhibit an inhibitory effect against *S. aurantiacum*. Consistent with the co-culture studies involving *P. aeruginosa* and other fungi, initial screening using plate assays suggested that presence of metabolically active (live) bacteria was necessary to inhibit the growth of the fungus as heat killed cells had no effect on *S. aurantiacum* growth (Mowat et al., 2010). Further on, extracts obtained from the bacterial monocultures failed to show any inhibitory effect. Thus it is possible that inhibition pathways might involve genes that are expressed only in bacterial-fungal co-cultures. In this respect our findings are similar to those of Rella et al. (2012) who showed that the growth of *C. neoformans* was not affected by the cell extracts obtained from *P. aeruginosa* strains PAO1 and PA14 cultured separately. The inhibition of *S. aurantiacum* by cell lysates of *P. aeruginosa* may be explained by the presence of bacterial exotoxins that are released during the cell lysis.

Confocal microscopy has been used to study interactions between chemically stained *P. aeruginosa* and major fungal lung pathogens such as *C. albicans* and *A. fumigatus* in liquid co-cultures (Bandara et al., 2010; Manavathu et al., 2014). However, the use of chemical stains was limited by the cross staining of bacteria and fungi thereby making it impossible to differentiate between them under a confocal microscope (Bandara et al., 2010). One of the key features of the current study is the use of *P. aeruginosa* and *S. aurantiacum* strains that were genetically tagged with fluorescent proteins in order to characterize the interactions in detail. To the best of our knowledge, this is the first report on successful genetic transformation of the newly described *S. aurantiacum* species. As no homologous promoters are available for this fungal species as yet, the fluorescent marker mCherry and the *E. coli hph* gene encoding hygromycin B phosphotransferase were expressed under a heterologous *pki* (pyruvate kinase) promoter derived from another ascomycetous fungus, *T. reesei* (Te'o et al., 2002; Boon et al., 2008; Klix et al., 2010). In previous studies, heterologous promoters such as *pki* and *gpdA* have been successfully used for gene expression across various phylogenetically close species (Punt et al., 1990; Jieh-Juen Yu, 1998; Ruiz-Diez and Martinez-Suarez, 1999; Almeida et al., 2007). The amount of hygromycin B required to inhibit the growth of *S. aurantiacum* was relatively high (410 U/ml) compared to some other fungi, which shows the highly resistant nature of *S. aurantiacum* also observed in antifungal susceptibility tests described in other studies (Lackner

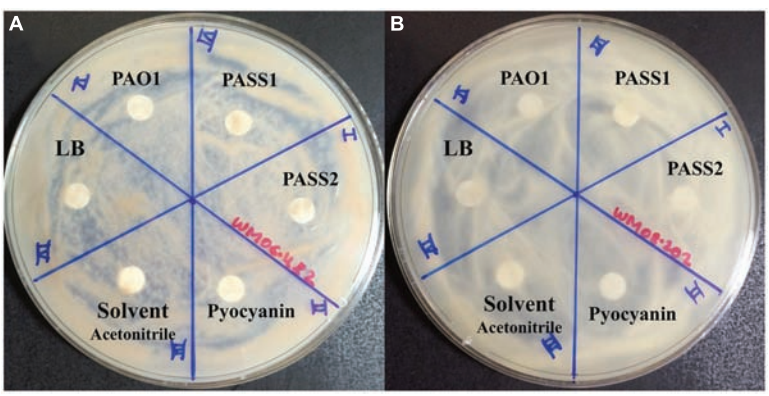

FIGURE 7 | Effect of *P. aeruginosa* phenazines on two *S. aurantiacum* isolates (A) WM 06.482 and (B) WM 08.202. Phenazines were extracted from different *P. aeruginosa* strains (PAO1, PASS1, and PASS2) and redissolved in acetonitrile (ACN). LB medium extract, ACN solvent and commercial phenazine (pyocyanin) were also tested against *S. aurantiacum*.

et al., 2012). Although the transformation efficiency was low (2.2 μg of plasmid DNA), transformant strains expressing the mCherry protein were obtained.

Confocal microscopy of the bacterial-fungal co-cultures revealed that bacteria elicit a specific inhibitory response by establishing a physical contact with the fungal hyphae. Similar types of interactions have also been observed in yeasts such as *C. albicans* and ascomycetous fungi such as *A. nidulans* and *Alternaria alternata* (Hogan and Kolter, 2002; Jarosz et al., 2011). This association might be directed toward utilization of the fungus by bacteria as an additional source of nutrients, or as an additional matrix support to form biofilms (Hibbing et al., 2010), or it may be a strategy to promote their own survival by inhibiting the fungal growth owing to nutrient limiting conditions in the medium (Brand et al., 2008).

Under nutrient limiting conditions, biofilm formation has been described as an important characteristic for *P. aeruginosa* mediated killing of other fungi such as *C. albicans* and *A. fumigatus* (Hogan and Kolter, 2002; Manavathu et al., 2014). Similarly, an inhibitory effect was also displayed by the biofilm forming strains of *P. aeruginosa* (PASS1 and PAO1) against the two *S. aurantiacum* strains in this study. PASS1 and PAO1 are high virulence strains, which share many similarities in their respective genomes. In contrast, the least virulent bacterial strain PASS2 (Penesyan et al., under review) that failed to show an effect against the fungi lacks several virulence related genes such as those encoding phenazines and the *psl* (polysaccharide synthesis locus) gene cluster which is required for biofilm formation (Ma et al., 2009; Penesyan et al., under review). The effect of bacteria on the growth of the less virulent *S. aurantiacum* strain WM 08.202 was much higher compared to the more virulent WM 06.482 both in the plate assays and in liquid co-cultures. This difference probably results from their different physiology as shown by Kaur et al. (2015) and possibly higher resistance to antifungals of the more virulent *S. aurantiacum* strain WM 06.482. These factors will be studied further when annotated *S. aurantiacum* genomes are available.

While biofilm formation and colonization of fungal hyphae in the nutrient limited SCFM liquid medium clearly contributed to the inhibition of *S. aurantiacum* by *P. aeruginosa*, it was not absolutely essential for the inhibitory effect as the cross streak assay with cultures not touching each other and disk inhibition experiments using cell lysates also resulted in inhibition of fungal growth. These indicated the possible involvement of secreted diffusible bacterial exoproducts/metabolites in fungal growth inhibition. One of these metabolites pyocyanin, a phenazine, is an extracellular redox-active virulence factor which is widely known to affect the growth of a large number of fungal species such as *A. fumigatus*, *C. albicans,* and *C. neoformans* (Kerr et al., 1999; Laursen and Nielsen, 2004; Gibson et al., 2009). Corroborating the highly resistant nature of *S. aurantiacum,* the amount of commercial pyocyanin (i.e., 10 mM) included in the test for comparison, was much higher than the MIC (minimum inhibitory concentration) of pyocyanin used for *C. albicans* and *A. fumigatus* (>0.3 mM; Kerr et al., 1999). These amounts are significantly higher than the amount of pyocyanin normally detected in the lungs of CF patients (100 μM; Wilson et al., 1988). However, neither crude phenazines nor pyocyanin showed an inhibitory effect against *S. aurantiacum* in our assays. A similar phenomenon has been observed in some ascomycetous fungi such as *A. sclerotiorum* (Hill and Johnson, 1969). Although it is not yet known if the phenazines are modified or sequestered by *S. aurantiacum*, the production of a red colored pigment in co-cultures could be due to a detoxification mechanism used by the fungus against bacterial phenazines. However, further studies into the chemical structure and UV and visible absorption spectra are required in order to ascertain if the red pigment indicates a modified phenazine.

In addition to phenazines, *P. aeruginosa* has also been reported to produce a wide variety of other exoproducts/metabolites such as proteases, elastases, haemolysin, and rhamnolipids that contribute to bacterial virulence (McAlester et al., 2008; Ben Haj Khalifa et al., 2011; Heeb et al., 2011; Rella et al., 2012; Mear et al., 2013). Their possible activity against *S. aurantiacum* will be worthy of a further study.

Most of the CF associated filamentous fungal species have been isolated from the lungs of patients with prolonged antibiotic therapies (Bakare et al., 2003). Previous clinical reports by Blyth et al. (2010b) have also showed an increased prevalence of *S. aurantiacum* in CF patients administered with antibacterial drugs indicating that the presence of bacteria has an effect on the susceptibility of the lungs to fungal infection. In support of this view, an increase in the growth of the fungus was observed upon a decline in the bacterial growth through addition of gentamicin to the co-culture medium in the present study. Therefore, it seems that the *P. aeruginosa* strains prevalent in CF patients during early stages of CF hinder fungal infection of lungs by inhibiting their growth.

Conclusion

We have assessed the effect of clinically relevant strains of *P. aeruginosa* on a newly discovered fungal lung pathogen *S. aurantiacum* in a synthetic lung-mimicking medium (SCFM) that closely resembles the chemistry of CF sputum. An inhibitory effect of *P. aeruginosa* was observed on the growth of *S. aurantiacum*, which can be mediated by the production of biologically active metabolites. Biofilm formation and colonization of fungal hyphae by bacteria were also important for *S. aurantiacum* growth inhibition. Surprisingly, the toxic *P. aeruginosa* phenazine pigments, such as pyocyanin, known to have an inhibitory effect against other fungal species including *A. fumigatus* and *C. albicans,* proved to be ineffective against *S. aurantiacum*. This suggests involvement of other virulence determinants and emphasizes the resilient nature of *S. aurantiacum* compared to other fungi present in lung infections. Further research may include transcriptomic studies of *P. aeruginosa* – *S. aurantiacum* co-cultures in order to reveal detailed molecular mechanisms underlying these interactions; these studies will be facilitated by the upcoming annotated *S. aurantiacum* genome.

Author Contributions

Conceived and designed the experiments: JK, LK, AP, AS, IP, HN. Performed the experiments: JK, SK, BP, MK. Analysed the data: JK, AP, HN. Wrote the paper: JK, HN.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00866

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Screening of Bifidobacteria and Lactobacilli Able to Antagonize the Cytotoxic Effect of *Clostridium difficile* upon Intestinal Epithelial HT29 Monolayer

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Clostridium difficile is an opportunistic pathogen inhabiting the human gut, often being the aetiological agent of infections after a microbiota dysbiosis following, for example, an antibiotic treatment. *C. difficile* infections (CDI) constitute a growing health problem with increasing rates of morbidity and mortality at groups of risk, such as elderly and hospitalized patients, but also in populations traditionally considered low-risk. This could be related to the occurrence of virulent strains which, among other factors, have high-level of resistance to fluoroquinolones, more efficient sporulation and markedly high toxin production. Several novel intervention strategies against CDI are currently under study, such as the use of probiotics to counteract the growth and/or toxigenic activity of *C. difficile*. In this work, we have analyzed the capability of twenty *Bifidobacterium* and *Lactobacillus* strains, from human intestinal origin, to counteract the toxic effect of *C. difficile* LMG21717 upon the human intestinal epithelial cell line HT29. For this purpose, we incubated the bacteria together with toxigenic supernatants obtained from *C. difficile*. After this co-incubation new supernatants were collected in order to quantify the remnant A and B toxins, as well as to determine their residual toxic effect upon HT29 monolayers. To this end, the real time cell analyser (RTCA) model, recently developed in our group to monitor *C. difficile* toxic effect, was used. Results obtained showed that strains of *Bifidobacterium longum* and *B. breve* were able to reduce the toxic effect of the pathogen upon HT29, the RTCA normalized cell-index values being inversely correlated with the amount of remnant toxin in the supernatant. The strain *B. longum* IPLA20022 showed the highest ability to counteract the cytotoxic effect of *C. difficile* acting directly against the toxin, also having the highest capability for removing the toxins from the clostridial toxigenic supernatant. Image analysis showed that this strain prevents HT29 cell rounding; this was achieved by preserving the *F*-actin microstructure and tight-junctions between adjacent cells, thus keeping the typical epithelium-like morphology. Besides, preliminary evidence showed that the viability of *B. longum* IPLA20022 is needed to exert the protective effect and that secreted factors seems to have anti-toxin activity.

Keywords: probiotics, *Clostridium difficile*, toxins, RTCA, xCelligence, *Bifidobacterium*, *Lactobacillus*, microscopy

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INTRODUCTION

Clostridium difficile is a Gram-positive, spore-forming, motile and strict anaerobe rod that can be found in the gastrointestinal tract of humans and animals (Janezic et al., 2014). The current classification of the "Bergey's Manual of Systematic Bacteriology" includes *C. difficile* in the Phylum *Firmicutes*, Class *Clostridia*, Order *Clostridiales* and Family *Peptostreptococcaceae* (Ludwig et al., 2009). A recent taxonomic study, based on 16S rRNA and ribosomal protein sequences, ascertains that *C. difficile* belongs to this family and proposes that it should be renamed as *Peptoclostridium difficile* (Yutin and Galperin, 2013); this new name appears in the taxonomic classification and nomenclature catalog of NCBI[1], but still *C. difficile* remains as the name recognized by the clinical and scientific community.

C. difficile infection (CDI) is the main cause of diarrhea associated with antibiotic use or related to health-care environments (Leffler and Lamont, 2015) and increasing incidence is reported among populations previously considered as low risk, such as pregnant women and children (Carter et al., 2012). The ubiquity of this bacterium, in combination with its capability to form spores, makes hospital environments a good source for *C. difficile* acquisition, although zoonotic (Bauer and Kuijper, 2015) and food transmissions (Troiano et al., 2015) have been proposed as well. The incidence and severity of CDI has been growing since the beginning of this century due to the global occurrence of hypervirulent strains such as BI/NAP1/027 (group BI by restriction endonuclease analysis, North American pulse-field type NAP1 by pulse-field gel electrophoresis, and ribotype 027; Rupnik et al., 2009; Yakob et al., 2015). The antibiotics metronidazole and vancomycin are the current treatments for CDI, but this does not prevent the high rates of recurrence. Thus, new emerging therapeutic options, such as fecal microbiota transplantation (FMT), new antibiotics, bacteriocins, bacteriophages, and probiotics are under evaluation for the control of CDI (Martin et al., 2013; Dunne et al., 2014; Mathur et al., 2015). Indeed probiotics, which are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO-WHO, 2001; Hill et al., 2014), have been proposed as biotherapeutic agents to help microbiota restoration after a dysbiosis caused by antibiotics or infections (Reid et al., 2011).

The information encoded on the genomes of this species, excellently reviewed by Knight et al. (2015), reveals high plasticity and very low levels of conservation among strains. This genetic diversity is reflected in its physiological adaptation to different ecosystems and in the occurrence of different phenotypes. In addition, the presence of a wide variety of transposons and phages explain the lineage evolution of clinically relevant loci, such as the antimicrobial resistance genes and the PaLoc (pathogenicity locus), among others (Knight et al., 2015). The PaLoc harbors, together with three additional genes, *tcdA* and *tcdB* coding for toxin A and toxin B, respectively, which are the major *C. difficile* virulence factors (Monot et al., 2015). The modulating environmental signals regulating the expression of PaLoc is not totally understood and a recent report shows that toxin synthesis is regulated through quorum-sensing signaling (Darkoh et al., 2015). TcdA and TcdB are large toxins whose main mechanism of action is known, although host receptors and toxin-mediated responses still remain to be fully deciphered. They act as intracellular glycosyltransferases modifying the Ras superfamily of small GTPases thus inducing intracellular changes, including *F*-actin condensation, transcriptional activation and cell apoptosis of intestinal epithelial cells. This promotes the disruption of the tight junctions and barrier integrity, leading to an increase in the gut permeability and neutrophil infiltration. Downstream effects also include modifications in the chemokine and cytokine production patterns toward an inflammatory response and fluid accumulation, ending with the clinical manifestations of leukocytosis and diarrhea (Voth and Ballard, 2005; D'Auria et al., 2013; Carter et al., 2015; Leslie et al., 2015). Therefore, anti-toxin therapies to counteract the negative effects of these potent *C. difficile* virulence factors could be valuable tools to reduce the course of CDI (Tam et al., 2015).

In a previous study we developed a biological model, using the (human) intestinal epithelial cell line HT29, to follow in real time the effect of supernatants collected from *C. difficile* cultures of a TcdA+, TcdB+ (toxinotype 0) strain. This method is based on the continuous monitoring of the impedance signal, transmitted through gold microelectrodes placed in the bottom of microtiter plates, of HT29 monolayers (Valdés et al., 2015). Our aim in the present work is to search for lactobacilli and bifidobacteria probiotic candidates with anti-toxin capability able to protect HT29 cells from the cytotoxicity caused by toxigenic *C. difficile* supernatants.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *Bifidobacterium* and *Lactobacillus* species used in this study are listed in **Table 1**. Most strains belonging to IPLA culture collection were isolated from infant feces and breast milk (Solís et al., 2010), whereas IPLA20031 and IPLA20032 were obtained after adaptation to increasing concentrations of bile salts from a parental strain isolated from a dairy product (Ruas-Madiedo et al., 2010). Strains were grown in MRSC [MRS (Biokar Diagnostics, Beauvois, France) supplemented with 0.25% L-cysteine (Sigma-Chemical Co., St. Louis, MO, USA)] at 37°C in the anaerobic chamber MG500 (Don Whitley Scientific, Yorkshire, UK) under 80% N_2, 10% CO_2 and 10% H_2 atmosphere. As standard procedure bacterial stocks, kept at −80°C in MRSC + 20% glycerol, were spread onto the surface of agar-MRSC and incubated for 3 days. A single colony was picked to inoculate MRSC broth which, after 24 h incubation, was used to inoculate (2%) 10 ml fresh MRSC broth. This culture was incubated overnight (18 h) to prepare the bacterial suspensions that will be described next.

[1]http://www.ncbi.nlm.nih.gov/taxonomy/?term=Peptoclostridium%20difficile

TABLE 1 | Strains included in this study and normalized cell index (CI) obtained at 4 and 22 h after addition of neutralized cell-free supernatants (NCFS) collected from incubations of each bifidobacteria or lactobacilli strain with 2.5% of toxigenic *Clostridium difficile* LGM21717 supernatant (Tox-S).

		Mean ± SD			
		Normalized-CI		Remnant toxin (ng/ml)	
NCFS	**Strain**	**After 4 h**	**After 22 h**	**TcdA**	**TcdB**
C. difficile Tox-S (2.5%)	LMG21717*	−0.64 ± 0.13	−0.93 ± 0.11	4.41 ± 0.01	0.48 ± 0.0
B. bifidum	LMG13195*	−0.62 ± 0.13	−0.97 ± 0.10	4.05 ± 0.33	0.15 ± 0.01
	IPLA20024	−0.43 ± 0.12	−0.92 ± 0.10	2.87 ± 0.02	0.11 ± 0.01
	IPLA20025	−0.64 ± 0.08	−1.03 ± 0.07	3.47 ± 0.09	0.14 ± 0.02
	IPLA20017	−0.71 ± 0.11	−1.04 ± 0.07	3.50 ± 0.12	0.42 ± 0.01
B. animalis	DSM15954† (Bb12)	−0.58 ± 0.09	−1.00 ± 0.13	3.71 ± 0.33	0.41 ± 0.04
	IPLA20031 (A1dOx)	−0.53 ± 0.05	−1.04 ± 0.07	4.60 ± 0.48	0.58 ± 0.26
	IPLA20032 (A1dOxR)	−0.64 ± 0.11	−1.00 ± 0.12	4.32 ± 0.3	0.42 ± 0.03
	IPLA20020	−0.62 ± 0.07	−1.05 ± 0.08	4.08 ± 0.42	0.42 ± 0.04
B. longum	IPLA20021	−0.16 ± 0.11	−0.60 ± 0.20	1.50 ± 0.14	0.29 ± 0.14
	IPLA20022	−0.06 ± 0.05	−0.06 ± 0.12	0.54 ± 0.18	0.26 ± 0.04
	IPLA20001	−0.09 ± 0.06	−0.33 ± 0.19	1.71 ± 0.05	0.26 ± 0.04
	IPLA20002	−0.07 ± 0.08	−0.57 ± 0.09	1.25 ± 0.16	0.22 ± 0.05
B. breve	IPLA20004	−0.00 ± 0.04	−0.24 ± 0.12	0.75 ± 0.09	0.21 ± 0.08
	IPLA20005	−0.03 ± 0.03	−0.25 ± 0.09	0.56 ± 0.29	0.15 ± 0.01
	IPLA20006	−0.12 ± 0.01	−0.66 ± 0.08	1.05 ± 0.37	0.12 ± 0.01
B. pseudocatenulatum	IPLA20026	−0.45 ± 0.06	−0.91 ± 0.13	3.31 ± 0.02	0.40 ± 0.12
L. crispatus	IPLA20120	−0.44 ± 0.06	−0.91 ± 0.00	4.09 ± 1.64	0.22 ± 0.09
L. gasseri	IPLA20121	−0.42 ± 0.04	−0.91 ± 0.03	3.68 ± 1.28	0.43 ± 0.16
L. paracasei	IPLA20124	−0.39 ± 0.03	−0.83 ± 0.03	4.57 ± 0.26	0.46 ± 0.03
L. rhamnosus	LMG18243* (GG)	−0.41 ± 0.03	−0.83 ± 0.03	3.35 ± 0.44	0.42 ± 0.04

**LMG: Belgian Coordinated Collections of Microorganisms" (BCCM, Gent, Belgium).*

†*DSM: German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany).*

The remnant toxins in these NCFS were quantified by ELISA tests. Data were obtained from two biological replicates each measured by duplicate.

The strain *C. difficile* LMG21717 (∼ATCC9689, Ribotype 001, genes *tcdA*+, *tcdB*+, *cdtB*-) producing both TcdA and TcdB toxins (Toxinotype 0) was purchased from the "Belgian Coordinated Collections of Microorganisms" (BCCM, Gent, Belgium). The strain was routinely grown in Reinforced Clostridium Medium (RCM, Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA) in Hungate tubes under anaerobic conditions at 37°C. Frozen stocks (−80°C in RCM + 20% glycerol) were directly activated in RCM broth incubated for 24 h and this culture was used to inoculate (2%) fresh medium that was cultivated for 13 h. This culture was used as inoculum to obtain the toxigenic supernatant.

Preparation of Toxigenic *C. difficile* Supernatant

Conditions to obtain toxigenic supernatant from *C. difficile* LMG21717 have previously been determined and published (Valdés et al., 2015). In short, 300 μl of RCM grown culture were used to inject into Hungates tubes containing 15 ml of Gifu Anaerobic Medium (GAM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). GAM cultures were incubated for 48 h and centrifuged (16,000 × *g*, 10 min) to obtain the *C. difficile*-free toxigenic supernatant (Tox-S), which was kept in several aliquots at −80°C.

Two independent ELISA tests (tgcBIOMICS GmbH, Bingen, Germany) were used to quantify the concentration of TcdA or TcdB in the toxigenic supernatant, as well as the remnant toxins in the neutralized bacterial-supernatants obtained after incubation of Tox-S with bifidobacteria and lactobacilli.

Incubation of Bifidobacteria or Lactobacilli with Toxigenic *C. difficile* Supernatant

The experimental design carried out in this study is schematized in **Supplementary Figure S1A**. Bifidobacteria and lactobacilli cultures grown for 18 h in MRSC were washed twice with PBS and resuspended at 10^9 cfu/ml in the HT29-cultivation medium (MM, described below) supplemented with 5% of Tox-S from *C. difficile* or without supplementation (controls). After incubation for 1 h under anaerobic conditions and mild stirring (∼300 rpm), the bacterial suspensions were centrifuged (16,000 × *g*, 10 min) to obtain bifidobacteria- or lactobacilli-free bacterial supernatants. Then, the pH was increased to 7.55 ± 0.05 with 1 and 0.1 N NaOH and the volume obtained was adjusted to twice the initial one with MM; this means that the maximum amount of remnant toxin that could be present was 2.5%. These neutralized cell-free supernatants (NCFS) were directly used to test their cytotoxicity upon HT29 monolayers as well as to quantify the remnant TcdA and TcdB toxins. This screening was performed with two biological replicates, each analyzed in duplicate, of each bacterial strain using HT29 monolayers of two consecutive passages (p147 and p148).

Incubation of Dead and Live *B. longum* IPLA20022 with Toxigenic *C. difficile* Supernatant

The strain *B. longum* IPLA20022 was selected in order to determine whether the capability to diminish the cytotoxic effect of *C. difficile* supernatant was dependent on bacterial viability. For that purpose UV-treated IPLA20022 suspensions were prepared from MRSC-grown cultures that were washed and resuspended in PBS at 10^9 cfu/ml. Then, the PBS suspension was poured into several petri dishes allowing a high surface spread and they were submitted to ultra violet radiation in a UV-chamber (15W, Selecta, Barcelona, Spain). Three UV cycles of 30 min were applied, homogenizing the PBS suspension in each interval, and the absence of viability was checked by plating serial dilutions of UV-treated IPLA20022 suspension in agar-MRSC (López et al., 2012). Incubation of this UV-treated suspension (dead IPLA20022) with toxigenic *C. difficile* supernatant was performed as previously described. A non UV-killed suspension (live IPLA20022) of the same culture was used as control. After incubation for 1 h, both suspensions were processed to obtain the respective NCFS (**Supplementary Figure S1A**). This experiment was carried out with three independent cultures (biological replicates) of strain IPLA20022 upon HT29 monolayers within the same passage (p149), each measured in duplicate.

Incubation of Supernatants from *B. longum* IPLA20022 with Toxigenic *C. difficile* Supernatant

To test the activity of putative secreted factors by *B. longum* IPLA20022 against *C. difficile* toxins, cell-free bifidobacterial supernatants obtained from three independent-culture replicates (each analyzed in duplicate) were incubated with 50% toxigenic (Tox-S) supernatant for 1 h under anaerobic conditions. Afterward, supernatants were neutralized (pH $\geq$ 7.5) and its cytotoxic activity tested upon HT29 monolayers (passage p149) at 2.5% in MM (**Supplementary Figure S1B**).

Intestinal Epithelial Cell Line HT29 and Culture Conditions

The intestinal cell line HT29 (ECACC 91072201), from human colon adenocarcinoma, was purchased from the "European Collection of Cell Cultures" (Salisbury, UK) and stored at IPLA under liquid N_2. McCoy's Medium (MM) supplemented with 10% foetal bovine serum (FBS), 3 mM L-glutamine and a mixture of antibiotics (50 μg/ml streptomycin-penicillin, 50 μg/ml gentamicin and 1.25 μg/ml amphotericin B) was used for HT29 cultivation. The pH value of supplemented MM was 7.48 ± 0.02. All media and reagents were purchased from Sigma–Aldrich. Maintenance of the cell line, between passages 145 to 149, was performed under standard conditions, at 37°C 5% CO_2 atmosphere, in a CO2-Series Shel-Lab incubator (Sheldon Manufacturing Inc., OR, USA).

Monitoring Behavior of HT29 in RTCA

The real time cell analyzer (RTCA-DP) xCelligence (ACEA Bioscience Inc., San Diego, CA, USA) used to monitor HT29 cells performance upon the different conditions tested, was introduced in a Heracell-240 Incubator (Thermo Electron LDD GmbH, Langenselbold, Germany) set at 37°C with 5% CO_2 atmosphere. This technology records variations in impedance due to the adhesion, growth and morphological changes of HT29 cells during interaction with gold-microelectrodes placed in the bottom of specific microtiter plates (E-plates). The impedance signal is converted in the arbitrary "cell index" (CI) unit which is recorded in the external computer allowing, as well, data analyses through the RTCA software 1.2.1 (ACEA Bioscience).

The method to monitor the damage caused by *C. difficile* toxins was previously described by Valdés et al. (2015). In short, 16-well E-plates were seeded with 2 × 10^5 HT29 cells (in 100 μl) and monitored (recording signal every 15 min) for 22 h to ensure the formation of a monolayer (confluent state). Afterward, the medium was removed and 200 μl of the different bacterial NCFS were added per well. Additionally, wells containing 200 μl of a control without bacteria but with Tox-S (added at 2.5% in MM, cytotoxic control) or 200 μl MM medium without bacteria or Tox-S added (non-cytotoxic control) were included in each experiment. The monitoring continued (every 10 min) for an additional 20–22 h under standard incubation conditions. CI values recorded were normalized by the time of the supernatant addition and by the control sample (MM) as previously described (Valdés et al., 2015). Samples of each bacterial supernatant were obtained from, at least, duplicated biological experiments (two independent Tox-S vs. bifidobacteria or lactobacilli incubations) and each NCFS was tested in duplicate (two independent wells within the same E-plate). Thus, four normalized-CI data were obtained per each bacterial strain tested.

Image Analysis of HT29 Behavior

Time-Lapsed Monitoring in Real Time

Several images were captured in real time using the compact, inverted, optical microscope (40× objective) LumaScope-600 Series (Etaluma, Carlsbad, CA, USA) which was placed inside the Heracell-240 incubator. Images were recordered in an external computer with the software LumaView600Cy 13.7.17.0 (Etaluma). To this end, 2-well μ-Slide (ibiTreat, 1.5 polymer coverslip, tissue culture treated, sterilized slides, Ibidi GmbH, Martinsried, Germany) were seeded with 2 × 10^6 HT29 cells/ml (1 ml) and placed on top of the microscope objective. Images were recorded every 15 min until the confluent state was reached (about 22 h); afterward, culture medium was removed and 1 ml of fresh medium containing 2.5% Tox-S or 1 ml of the NCFS collected after incubation of live *B. longum* IPLA20022 with Tox-S, was added in two independent μ-Slides. Image capture was performed for additional an 16 h.

End-Point CSLM Analysis

HT29 monolayers submitted to different treatments were analyzed by confocal scanning laser microscopy (CSLM) after an end-point incubation period of 20 h. For this, 8-well μ-Slide (ibiTreat, Ibidi GmbH) were seeded with 2 × 10^6 HT29 cells/ml (0.3 ml) and incubated for 22 h to reach confluent state. Afterward, supernatant was removed and wells (in duplicate) were filled with the same volume of fresh medium containing MM (control), 2.5% Tox-S, and NCFS from live or dead *B. longum* IPLA20022 incubated with Tox-S. Incubation continued for additional 20 h; then, supernatant of each well was

removed and HT29 monolayers fixed with 1 vol (0.3 ml) of cold (−20°C) acetone for 10 min. Samples were washed twice with PBS for 5 min under mild stirring and permeabilised with PBS containing 0.1% Triton 100x (Sigma) for 15 min. The nonspecific binding sites were blocked with FBS (25% in PBS) for 20 min and finally washed once with PBS. The Phalloidin-Alexa-Fluor-568 probe (Molecular Probes-Thermo Fisher, Life Technologies S.A., Madrid, Spain) toward *F*-actin was added in 0.3 ml of PBS (final concentration of 25 μl/ml) and samples were incubated overnight at 4°C in darkness. After washing twice with PBS, HT29 nucleus were stained with DAPI probe (Merck-Millipore Cor., Billerica, MA, USA) used at 1:1000 (final dilution in PBS) and incubated under the same conditions for, at least, 6 h. Finally, samples were washed and added to 0.3 ml of PBS previous visualization under microscope.

For the CSLM analysis the Leica TCS AOBS SP8 X confocal microscopy (Leica Microsystems GmbH, Heidelberg, Germany) located in the Scientific-Technical Services of Oviedo University, was used. DAPI and Alexa-Fluor-568 fluorochromes were excited at 405 nm by a blue–violet laser diode and at 578 nm by a white light laser, respectively. *Z*-stacks of HT29 samples were acquired using a 63x/1.4 oil objective applying a line average of 2 to reduce noise on the final images and a z-step of 1 micron. Details of a region were later acquired using a 2.50 optical zoom. Image-captures were recorded with the "Leica Application Suite X" software version 1.8.1.13759 (Leica).

Statistical Analysis

To assess differences in the response (normalized CI) of HT29 due to the anti-toxin activity of *B. longum* IPLA20022, one-way ANOVA followed by SNK (Student-Newman–Keuls, $p < 0.05$) mean comparison tests were performed. The statistical package IBM SPSS Statistics for Window Version 22.0 (IBM Corp., Armonk, NY, USA) was used to carry out these analyses. Legend of **Figure 4** describes the comparisons made in each type of experiment.

RESULTS

The method previously developed by our group to detect in real time the toxic effect of *C. difficile* upon intestinal cell lines was used to address the anti-toxin probiotic potential of twenty bifidobacteria and lactobacilli strains. As an initial step several parameters were optimized in order to establish conditions for the screening using as a biological model confluent-HT29 monolayers (data no shown). Finally, neutralized (pH $\geq$ 7.5) cell-free supernatants (NCFS), obtained after incubation (1 h, 37°C, anaerobiosis) of each strain (about 1×10^9 cfu/ml) with 5% *C. difficile* supernatant (Tox-S), were used for this study (**Supplementary Figure S1A**). The behavior of HT29 monolayers was monitored in real time recording the variations in the impedance signal (normalized-CI) over time due to the presence of the NCFS, the toxigenic control (2.5% Tox-S), or the culture media alone (MM; **Figure 1**). To understand the impedance graphs is worth noting that the lowest normalized-CI value indicates the highest toxigenic capability of *C. difficile* supernatant upon HT29; thus, in **Figure 1**, the red line (representing values obtained with 2.5% Tox-S) is the control for damage, whereas the pink line represents the non-toxigenic control (MM) used as a reference for normalization of all CI values being the reason to have "0 value." Regarding the effect of NCFS, those obtained after incubation of strains in MM medium without *C. difficile* toxins (dotted lines) showed normalized-CI values equal or higher to the control, therefore indicating the absence of any toxic effect induced by the putative probiotics. However, when the NCFS obtained from bacteria incubated with Tox-S were analyzed, HT29 monolayers behaved differently depending on the species considered (**Figure 1**). Graphics obtained clearly show that strains belonging to species *Bifidobacterium bifidum* and *B. animalis* subsp. *lactis* had no protective effect against *C. difficile* toxins since the normalized-CI lines obtained showed similar, or even lower, values than the toxigenic Tox-S control. By contrast, the normalized-CI obtained from the four lactobacilli tested, as well as the strains of *B. longum*, *B. breve,* and *B. pseudocatenulatum* were higher than those induced by *C. difficile* supernatant. In general, normalized-CI lines from *B. longum* and *B. breve* strains were the closest to the control, thus being the strains showing higher anti-toxin capability.

Normalized-CI obtained 4 h after NCFS addition (short term effect) or 22 h after (long term effect) were analyzed in more detail (**Table 1**). Results obtained in the short term showed that all strains belonging to *B. longum* and *B. breve*, as well as *L. gasseri* IPLA20121, *L. paracasei* IPLA20124 and *L. rhamnosus* GG, seemed to have higher values of normalized-CI than the toxigenic control. However, none of the lactobacilli were able to keep the protective effect upon HT29 for a prolonged period (22 h). The strain *C. difficile* LMG21717 used in this study produced about ten-times more TcdA than TcdB (**Table 1**) and the strains showing high protective effect were those that apparently were more effectively in reducing the concentration of TcdA, i.e., belonging to *B. longum* and *B. breve* species (**Table 1**). Indeed, the NCFS obtained from strain *B. longum* IPLA20022 that promoted the lowest damage after 22 h only had 12% of remnant TcdA. Of note is that NCFS from *B. bifidum* and *B. breve* seemed to have a good ability to reduce TcdB levels (remnant between 23 and 44%), although this fact was not correlated with higher protective effect in *B. bifidum* because this species seemed to be less effective against TcdA.

Time-lapsed microphotographs (**Figure 2**) showed that HT29 cells treated with 2.5% toxigenic *C. difficile* supernatant become spherical and the integrity of the monolayer was lost when incubation was prolonged (**Figure 2A**). However, monolayers added with NCFS from live *B. longum* IPLA20022 incubated with Tox-S remained more stable and only after a long incubation period (16 h) some cellular particles were released to the culture medium (**Figure 2B**). Furthermore, although the cytopathic mechanism of *C. difficile* toxins is well known, we performed immunohistochemistry CSLM analysis to confirm the cellular events under different treatments (**Figure 3**). Control HT29 monolayers (grown in MM for 20 h) showed a typical *F*-actin cytoskeleton in which the nucleus is imbibed, thus having an epithelial-like morphology with annexed cells well connected.

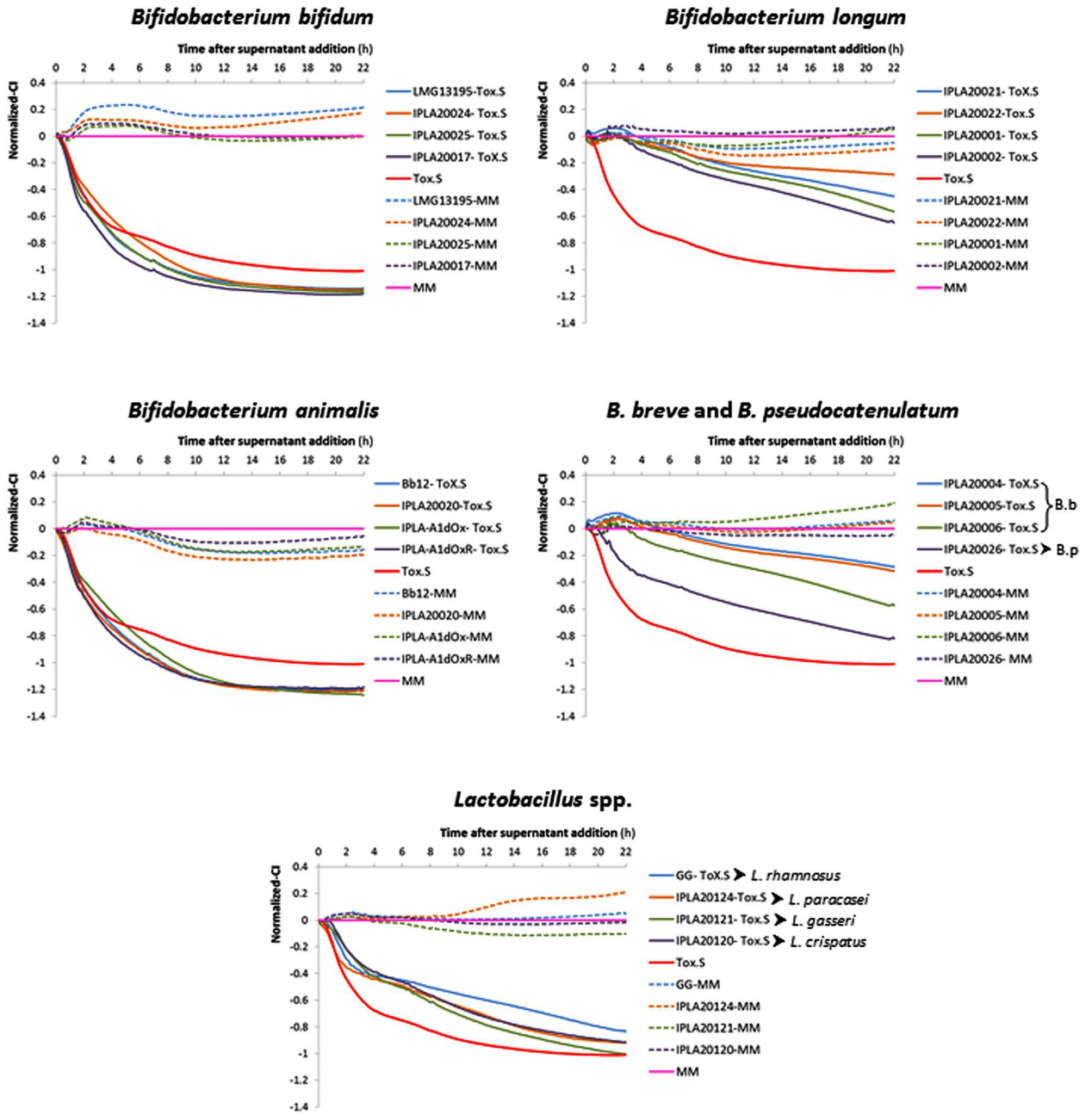

FIGURE 1 | Variation in the normalized cell index (Normalized-CI) of HT29 monolayers treated with different neutralized cell-free supernatants (NCFS; neutralized cell-free supernatants) obtained in a representative experiment after incubation of toxigenic *Clostridium difficile* supernatant (Tox-S) with different strains of *Bifidobacterium* and *Lactobacillus* species. The Tox-S was tested alone at 2.5% (red line). Normalization was performed with respect to the point of NCFS's addition and with respect to the control sample (culture medium MM without supernatant addition) which is the 0-reference control (pink line). The dotted lines represent results obtained with the NCFS obtained after incubation of the same strains in MM (without Tox-S). Representative SD values of these data are collected in **Table 1**.

However, monolayers treated for the same period with Tox-S supernatant lost the interconnection among *F*-actin filaments (**Figure 3B**) and the nucleus seems to be in the initial stages of apoptosis, i.e., the chromatin initiates the condensation showing more intense blue due to DAPI staining (**Figure 3C** and **Supplementary Figure S2**); therefore, HT29 cells become more spherical (non-epithelial morphology) and it seems that the tight junctions that maintain the monolayer integrity might have been disrupted (**Figure 3A**). The photographs obtained from HT29 monolayer treated for 20 h with the NCFS from live *B. longum* IPLA20022 were more similar to the control without toxin; the *F*-actin cytoskeleton still showed an interconnected structure and

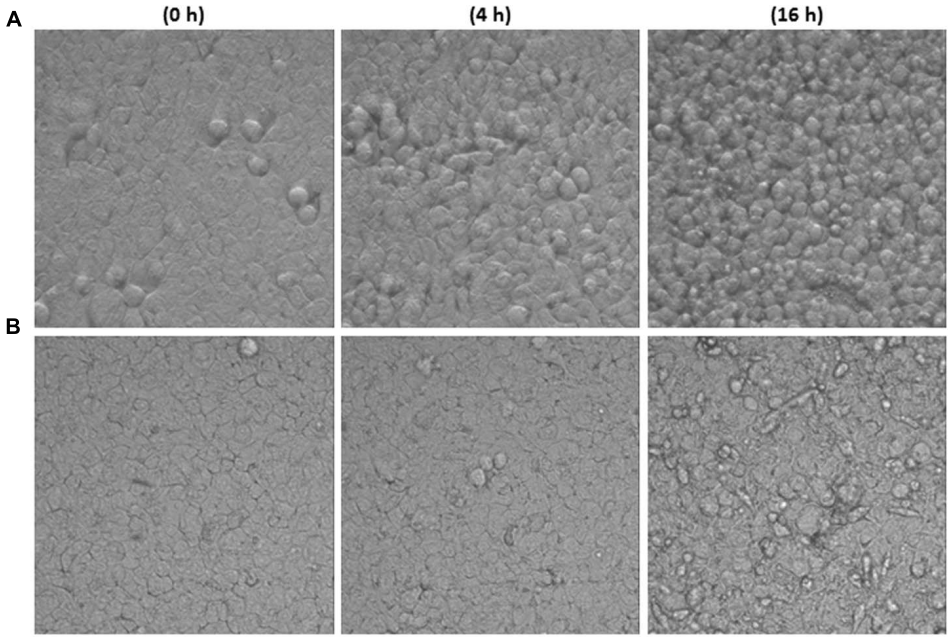

FIGURE 2 | Images of HT29 monolayers captured in real time (37°C, 5% CO_2) with the inverted optical microscope (objective 40×) at three incubation times (0, 4, and 16 h). Monolayer treated with toxigenic *C. difficile* supernatant (Tox-S, **A)** or with NCFS (neutralized cell-free supernatants) obtained after incubation of live *Bifidobacterium longum* IPLA20022 with Tox-S **(B)**.

the nucleus showed less intense DAPI staining comparable to that of the negative control than the toxigenic control. This structure, resembling that of intact epithelial monolayers, is in agreement with the presence of lower amounts of remnant toxin in the NCFS and higher normalized-CI due to the capability of this strain to counteract the effect of clostridial toxins.

In order to determine whether this strain retains its anti-toxin capability under non-viable conditions, *B. longum* IPLA20022 suspension was irradiated with UV light for 90 min. The RTCA monitoring clearly showed that this treatment modified the protective effect of the bifidobacteria upon HT29 since the normalized-CI of the dead strain followed the same tendency as the toxigenic control (**Figure 4A**). Indeed, the statistical analysis performed at 4 and 22 h after NCFS addition showed that live IPLA20022 had a significantly ($p < 0.05$) higher normalized-CI, i.e., higher protective capability, than the dead strain and the toxigenic control (**Figure 4A**). Consequently, the immunohistochemistry study confirmed that the morphology of HT29 treated for 20 h with the NCFS from dead *B. longum* IPLA20022 was more similar to that obtained with the toxigenic control (**Figure 3**). Indeed, besides the *F*-actin modification, some apoptotic bodies were evidenced in both toxigenic and dead-IPLA20022 samples (**Supplementary Figure S2**) suggesting that the UV treatment of this strain, which probably affected the structure and function of the cell envelope, abolished the anti-clostridial effect of *B. longum* IPLA20022. Finally, we have tested the activity against clostridial toxins of the supernatants obtained from overnight cultures of this bifidobacterial strain. Surprisingly, the normalized-CI values were similar to those of those obtained with (live) pellets and both of them were statistically ($p < 0.05$) higher than the toxigenic control (**Figure 4B**). This result suggests that *B. longum* IPLA20022 is able to secrete factors having activity against the toxins of *C. difficile*.

DISCUSSION

The search for novel approaches to treat or prevent CDI is a current "hot-topic" in which the scientific community is devoting much effort. Different approaches are under investigation, most of them toward restoring the dysbiotic intestinal microbiota following infection through FMT (Youngster et al., 2014; Satokari et al., 2015) or using a consortia of defined species (Lawley et al., 2012), but also toward the application of new antibiotics (Babakhani et al., 2013; Vickers et al., 2015) and drugs to treat infections (Oresic-Bender et al., 2015), as well as vaccinations with non-toxigenic *C. difficile* strains (Senoh et al., 2015) or anti-toxin antibodies (Yang et al., 2015). Probiotic bacteriotherapy is becoming an option for the prevention of *C. difficile* recurrent infection (Leffler and Lamont, 2015), and also for the attenuation of CDI symptoms. The choice of the appropriate probiotic against *C. difficile* is of pivotal relevance since, although some formulations seem to be promising (Auclair et al., 2015), not all of them are efficient (Allen et al., 2013).

Probiotic action against CDI is based on different bacterial antagonistic mechanisms, such as competition for adhesion to gut mucosa (Banerjee et al., 2009; Zivkovic et al., 2015)

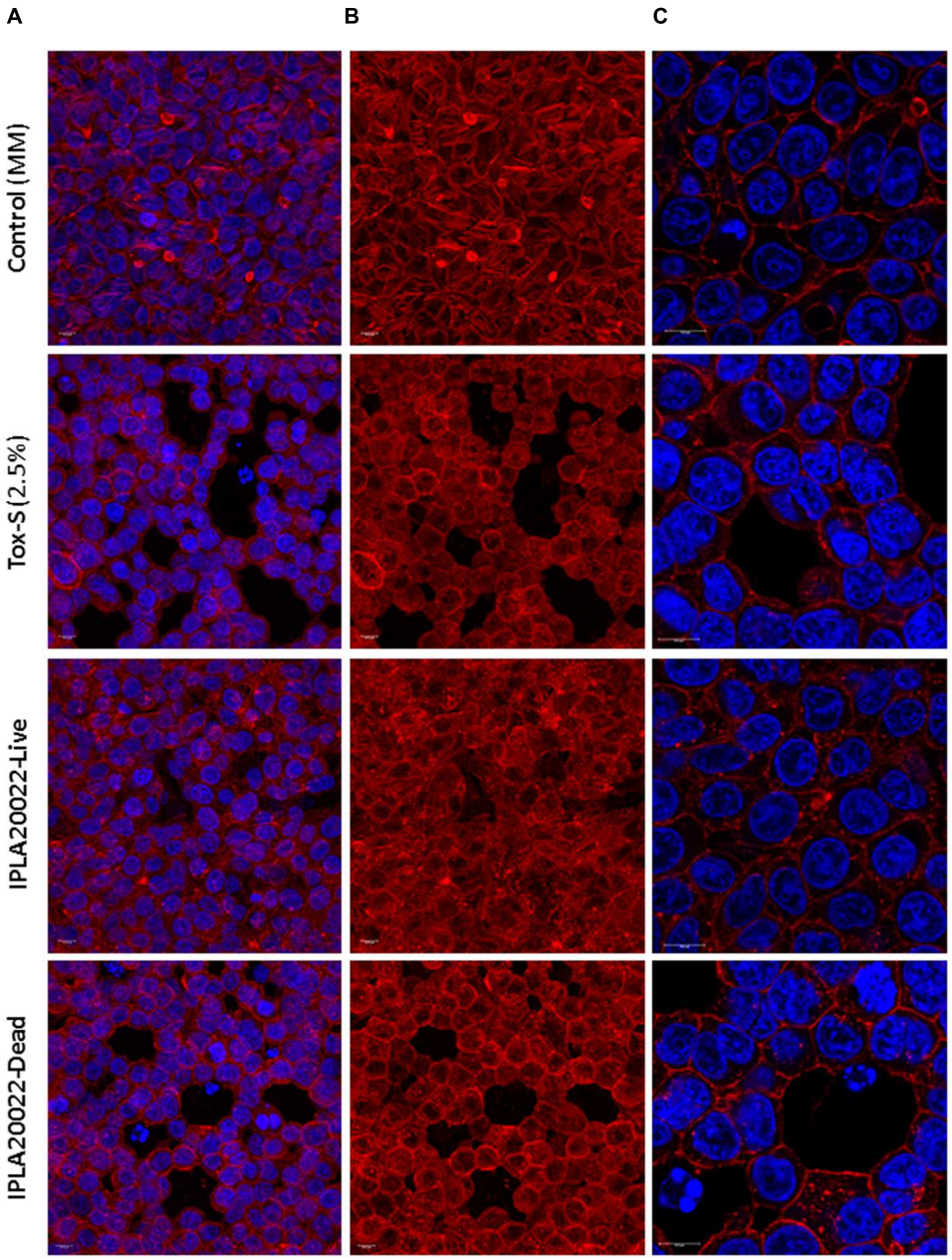

FIGURE 3 | Immunofluorescence images obtained by CSLM of HT29 after 20 h of incubation without toxigenic *C. difficile* supernatant Tox-S (control) and with Tox-S (damage control), and with NCFS (neutralized cell-free supernatants) obtained after incubation of live or dead *B. longum* IPLA20022 with Tox-S. (A) and **(B)** show a *Z*-projection (thickness about 13–15 μm) of 10 XY-slides and **(C)** shows a CSLM-zoom of a XY-region. **(A)** and **(C)** show the combination of DAPI-stained nucleus (blue, excited at 405 nm by a blue–violet laser diode) and *F*-actin stained with Phalloidin-Alexa-Fluor-568 probe (red, excited at 578 nm by a white light laser); *F*-actin is also shown as a single channel in **(B)**. Bars 10 μm.

and for colonization of the intestinal environment (Kondepudi et al., 2014), production of antimicrobial molecules (Schoster et al., 2013; Gebhart et al., 2015) or modulation of intestinal inflammation (Boonma et al., 2014). Another target for probiotic action is the reduction of toxicity caused by *C. difficile* (Trejo et al., 2013). In any case, if one of the active strains would be administered as a probiotic therapy to CDI patients, then the effect would only be present as long as the probiotic is consumed since stable colonization of probiotics in humans has not been shown yet.

In our study, we have explored the capability of twenty lactobacilli and bifidobacteria to counteract the effect of toxins (TcdA and TcdB) from *C. difficile* LMG21717 (equivalent to ATCC9689). The method used, based on impedance

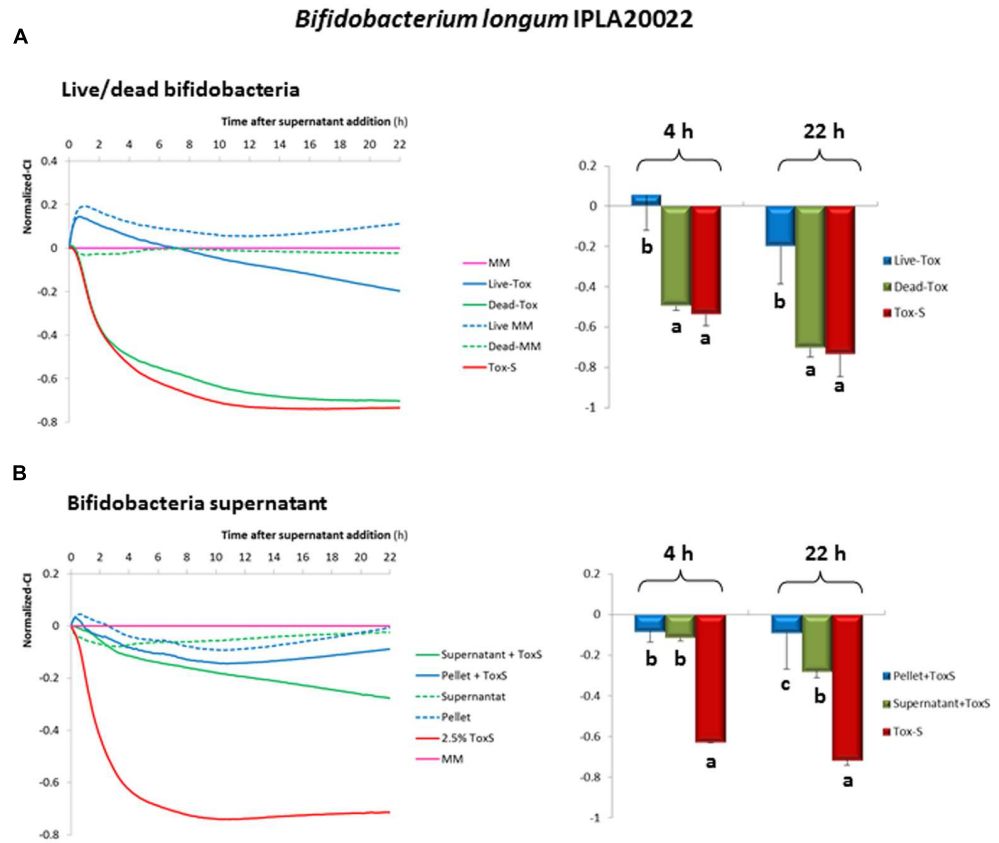

FIGURE 4 | Evolution of the normalized cell index (CI) of HT29 monolayers treated with NCFS (neutralized cell-free supernatants) obtained after incubation of toxigenic *C. difficile* supernatant (Tox-S) with live or dead *B. longum* IPLA20022 (A). Evolution of the normalized-CI of HT29 monolayers treated with 2.5% of neutralized supernatant obtained after incubation of toxigenic *C. difficile* supernatant (Tox-S) with *B. longum* IPLA20022 supernatant **(B)**. Normalization was performed with respect to the point of NCFS's addition and with respect to the control sample (culture medium MM without supernatant addition) which is the 0-reference line (pink line). The dotted lines represent results obtained with the NCFS obtained after incubation of the live or dead *B. longum* IPLA20022 or culture supernatant in MM (without Tox-S). Histograms located in the right of **(A)** and **(B)** sections, represented the mean and standard deviation of normalized-CI values obtained at 4 and 22 h from three biological replicates each measured in duplicate; within the same time, those means that do not share a common letter are statistically different ($p < 0.05$) according to one-way ANOVA and the mean comparison SNK (Student-Newman–Keuls) test.

measurement of HT29 monolayers (Valdés et al., 2015), allowed a quick search of the strains showing the highest anti-toxin ability which those were belonging to *B. longum* and *B. breve* species. This fact suggests that some species-specific characteristics could account for the observed effect, although differences were also detected among strains within the same species. As far as we know, there are few comparative studies among different probiotic species; Trejo et al. (2010) co-cultivated two *C. difficile* strains (including ATCC9689) with twenty five bifidobacteria or lactobacilli and they found that the capability to antagonize the toxic effect upon Vero line (monkey fibroblast-like kidney cells) was strain dependent, but they did not report a species-efficacy association. Nevertheless, the experimental procedure used in our screening for detecting anti-toxicity was based on the incubation of the probiotic strains with a toxigenic supernatant from *C. difficile*, previous to analyze the effect of NCFS upon the biological model HT29. Then, *a priori*, the putative mechanisms that could be behind the anti-toxin capability detected with our approach are the modification of the *C. difficile* toxin and/or its availability for acting on the epithelial cells.

Some authors have reported that probiotics are able to reduce the activity of *C. difficile* toxins. Banerjee et al. (2009) observed that *Lactobacillus delbrueckii* subsp. *bulgaricus* B-30892 releases bioactive components, of unknown nature, able to decrease the toxic effect of *C. difficile* ATCC9689 upon epithelial intestinal Caco2 cells. Similarly, *Lactococcus lactis* subsp. *lactis* CIDCA8221 secretes heat-sensitive products, higher than 10 kDa, that are not affected by treatment with proteases or protease-inhibitors, which were able to protect Vero cells from *C. difficile* toxins (Bolla et al., 2013). *Saccharomyces boulardii* releases an extracellular serin-protease that was able to breakdown the toxin A, as well as to inhibit its binding to the receptor in the brush border of ileal tissue (Castagliuolo et al., 1996). In our case, analysis of the bioactivity of the supernatant collected from strain IPLA20022 directly incubated with the toxigenic *C. difficile* supernatant showed similar effect on HT29

than that obtained with the bifidobacterial pellet. Then, it seems that this strain secreted molecules able to reduce the cytotoxic effect of clostrial toxins. As far as we could find, no exo-proteases have been described for bifidobacteria and only a few peptidases have been characterized (Janer et al., 2005; Seo et al., 2007). Additionally, other molecules inducing conformational changes in proteins that disrupt the active site of other proteins, which could putatively be involved in the inactivation of *C. difficile* toxins, have been described; these are serpins (serin protein inhibitors) found in the genome of *B. longum* (Schell et al., 2002) and *B. breve* (Turroni et al., 2010) and ion chelating agents such as the iron-chelating siderophores (Cronin et al., 2012; Vazquez-Gutierrez et al., 2015). Thus, further and extensive work will be needed in order to decipher the nature of the bifidobacterial secreted factors acting against *C. difficile* toxicity.

Regarding the adsorption as mechanism to reduce toxins activity, it has been demonstrated that the soluble S-layer protein from the surface of *L. kefir* strains diminish the damage of clostridial toxins upon Vero cells, suggesting a direct interaction between the S-layer and the toxins (Carasi et al., 2012). However, as far as we could know, this type of protein cover has not been described for bifidobacteria. Additionally, cellular extracts from *L. acidophilus* GP1B were able to interfere with quorum-sensing signals from *C. difficile* and down-regulated expression of some virulence genes; both, cellular extract and *L. acidophilus* strain, were efficient in increasing the survival rate of animals in a CDI murine model (Yun et al., 2014). The lactic acid synthesized by this lactobacilli strain also had an inhibitory effect on *C. difficile* growth. Similarly, Kolling et al. (2012) reported a bactericidal effect induced by the lactic acid synthesized by *Streptococcus thermophilus* LMD-9 and, furthermore, non-inhibitory levels (10 mM) decreased the *tcdA* expression and toxin-A release. *In vivo* (CDI mouse model) treatment with live *S. thermophilus* showed a significant inverse correlation between levels of luminal lactic acid and *C. difficile* abundance in the murine gut, thus reducing the disease activity indexes of experimentation animals (Kolling et al., 2012). In our experimental design, bifidobacteria were in contact for 1 h only with the toxigenic clostridial supernatant, but not with *C. difficile*, and the putative effect of the organic acids (lactate and/or acetate) produced in this short incubation period by lactobacilli or bifidobacteria was neutralized.

Based on the results describe in this article, the adsorption of toxins to the bifidobacterial surface as well as the presence of secreted molecules responsible for the anti-toxigenic effect observed, are both plausible mechanisms of action. Nevertheless bacterial viability, which may be also needed to keep a functional bifidobacterial envelope, is required in order to maintain the anti-clostridial activity. Finally, the highest anti-toxin capability of *B. longum* and *B. breve* strains (pointing to a species-dependent efficacy) suggests that some specific characteristics of these two phylogenetically close species (Lugli et al., 2014) could account for the anti-clostridial toxicity. Further experiments must be performed in order to understand the mechanism of action behind bifidobacterial anti-*C. difficile* toxicity. Another interesting observation that will deserve further attention is the (apparently) better capability of *B. bifidum*, and to a lower extent of *B. breve*, to specifically reduce TcdB levels.

CONCLUSION

In this work we have optimized a protocol to search for potential probiotics with anti-toxic activity against toxins synthesized by *C. difficile*. The impedance-based, RTCA xCelligence was a fast, reliable and efficient method for the screening of a large collection of bacteria allowing the selection of those strains with higher protection capability. In our case, strains from *B. breve* and *B. longum* showed the better performance, since they were able to reduce the levels of toxins from *C. difficile* supernatants. The best candidate to be used as probiotic to alleviate CDI was *B. longum* IPLA20022; this was the strain with the highest *in vitro* capability for reducing the levels of clostrial toxins, as well as for avoiding the cytopatic effect upon the intestinal epithelial cellular line HT29. Apart for elucidating the mechanism behind this anti-toxigenic capability, the next steps will be to study the efficacy of *B. longum* IPLA20022 in more complex *in vitro* and *in vivo* biological models before proposing its human application to treat CDI.

AUTHOR CONTRIBUTIONS

MG and PR-M contributed with the conception, experimental design and results interpretation of this study. LV-V carried out all experiments, OG-S advised the immunohistochemistry analysis and MA-G perform the CSLM analysis. PR-M was in charge of writing the drafted manuscript. All authors performed a critical revision of the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00577

FIGURE S1 | Final conditions used to perform the screening of the anti-*Clostridium difficile* cytotoxic activity upon HT29 monolayers of twenty bifidobacteria and lactobacilli strains (A). Scheme of the flow-work followed to test the capability of *Bifidobacterium longum* IPLA20022 culture supernatant to act against toxigenic *C. difficile* supernatant **(B)** MRSC, MRS broth supplemented with 0.25% L-cysteine; MM, McCoy's Medium added with supplements and antibiotics described in material and methods section; NCFS, neutralized cell-free supernatant.

FIGURE S2 | Immunofluorescence images obtained by CSLM of HT29 after 20 h of incubation without toxigenic *C. difficile* supernatant Tox-S (control) and with Tox-S (damage control), and with NCFS (neutralized cell-free supernatants) obtained after incubation of live or dead *B. longum* IPLA20022 with Tox-S. Images show a CSLM-2.50 optical zoom of a XY-slide. Upper-part images show the combination of DAPI-stained nucleus (blue, excite at 405 nm by a blue–violet laser diode) and *F*-actin stained with Phalloidin-Alexa -Fluor-568 probe (red, excited at 578 nm by a white light laser) **(A)**. Bottom-part images show the same magnification view at the visible (transmitted light) channel **(B)**. Arrows indicates the apoptotic bodies. Bars 10 μm.

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Different metabolic features of *Bacteroides fragilis* growing in the presence of glucose and exopolysaccharides of bifidobacteria

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Bacteroides is among the most abundant microorganism inhabiting the human intestine. They are saccharolytic bacteria able to use dietary or host-derived glycans as energy sources. Some *Bacteroides fragilis* strains contribute to the maturation of the immune system but it is also an opportunistic pathogen. The intestine is the habitat of most *Bifidobacterium* species, some of whose strains are considered probiotics. Bifidobacteria can synthesize exopolysaccharides (EPSs), which are complex carbohydrates that may be available in the intestinal environment. We studied the metabolism of *B. fragilis* when an EPS preparation from bifidobacteria was added to the growth medium compared to its behavior with added glucose. 2D-DIGE coupled with the identification by MALDI-TOF/TOF evidenced proteins that were differentially produced when EPS was added. The results were supported by RT-qPCR gene expression analysis. The intracellular and extracellular pattern of certain amino acids, the redox balance and the α-glucosidase activity were differently affected in EPS with respect to glucose. These results allowed us to hypothesize that three general main events, namely the activation of amino acids catabolism, enhancement of the transketolase reaction from the pentose-phosphate cycle, and activation of the succinate-propionate pathway, promote a shift of bacterial metabolism rendering more reducing power and optimizing the energetic yield in the form of ATP when *Bacteroides* grow with added EPSs. Our results expand the knowledge about the capacity of *B. fragilis* for adapting to complex carbohydrates and amino acids present in the intestinal environment.

Keywords: *Bacteroides fragilis*, exopolysaccharides, *Bifidobacterium*, glucose, metabolism, probiotics

Introduction

The microbes in our body reach levels of up to 100 trillion (10^{12}) cells, the majority of which reside in the colon and are anaerobes (Qin et al., 2010). The adult human distal gut microbiota is dominated by two phyla, the Firmicutes and the Bacteroidetes, the genus *Bacteroides* accounting for 20–50% in most individuals (Rigottier-Gois et al., 2003; Mahowald et al., 2009). This group of microorganisms appears during the first few days of life in the intestine of full-term neonates

(Arboleya et al., 2015) and remains at low levels (10^7 cells per gram of intestinal content) in breast-fed infants, increasing after weaning (Mackie et al., 1999). *Bacteroides* species are usually considered as symbionts or mutualists in the human intestine (Hooper and Gordon, 2001). However, in certain circumstances some species can act as opportunistic pathogens (Wexler, 2007). Indeed, the relatively large genome of *Bacteroides* enables these microorganisms to behave both as beneficial and harmful bacteria depending on the host environmental conditions. In this way, the capsular polysaccharide of certain strains of *Bacteroides fragilis* can contribute to the development and maturation of the host immune system (Mazmanian et al., 2005) but is also an important virulence determinant of this bacterium (Wexler, 2007). *Bacteroides* is an anaerobic, bile-resistant, non-spore-forming, and Gram-negative rod. It is a saccharolytic-versatile microorganism able to use dietary or host-derived glycans according to the nutrient availability (Sonnenburg et al., 2005). Members of *Bacteroides* can incorporate amino acids from the external environment (Smith and Macfarlane, 1998). Succinic, acetic, lactic, and propionic acids are produced by *Bacteroides* in variable proportions during fermentation (Rios-Covian et al., 2013). Then, these organic acids and short chain fatty acids (SCFAs) can be utilized by other intestinal microorganisms through cross-feeding mechanisms (Scott et al., 2008) or be partly reabsorbed through the large intestine, thus serving as an energy source for the host (Hooper et al., 2002).

Exopolysaccharides (EPSs) are complex carbohydrate polymers which can be produced by many microorganisms, as is the case of some *Bifidobacterium* strains (Ruas-Madiedo et al., 2007). The intestine is the normal habitat of most species of *Bifidobacterium*, some of whose strains are considered as probiotics and are being included in functional foods (Masco et al., 2005). The synthesis of EPS by bifidobacteria *in vivo* has not yet been demonstrated. However, previous evidence indicates that the presence of bile stimulates the *in vitro* production of EPS by bifidobacteria (Ruas-Madiedo et al., 2006, 2009). In addition, it has been unveiled that in the presence of EPS a relative increase of propionic acid proportions occurs as a result of the metabolic activity of colonic microbiota (Salazar et al., 2008).

The microbiota composition and its functionality in the gastrointestinal ecosystem have been intensively studied, but the dynamics of such microbiota at the metabolic level is not yet well-known. Indeed, in spite of relevant studies on the mechanisms of virulence and pathogenicity of *B. fragilis*, still little is known about the physiology of this microorganism and the adaptation of its metabolism to the gut ecosystem (Wexler, 2007). Although the effective consumption by *B. fragilis* or other intestinal microorganisms of bacterial EPSs has not been unequivocally demonstrated yet, we have recent evidence showing differential growth and metabolic patterns by *B. fragilis* depending on the carbon sources present in the external environment. Thus, a shift toward propionic acid production was found when *B. fragilis* was incubated with bifidobacterial EPS whereas in glucose, acetic acid was the most abundant metabolite formed (Rios-Covian et al., 2013). Therefore, the aim of the present work was to gain insight into *B. fragilis* metabolism grown in the presence of different carbohydrates, including EPSs produced by bifidobacteria, and to examine the results obtained in the light of the role that this bacterium plays as symbiont and opportunistic pathogen in the human gut.

Materials and Methods

Bacterial Culture Conditions

Frozen stocks of *B. fragilis* DSM2151 (DSMZ bacterial pure collection, Braunschweig, Germany) were reactivated in Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 0.25% (w/v) L-cysteine (Sigma Chemical Co., St. Louis, MO, USA) (named GAMc) and incubated at 37°C for 24 h in an anaerobic cabinet (Mac 1000; Don Whitley Scientific, West Yorkshire, UK) under a 10% H_2, 10% CO_2, and 80% N_2 atmosphere. The pH-free liquid batch cultures of *B. fragilis* were performed in 50 mL of a non-defined peptone and yeast-extract containing basal medium (BM) previously used by us in human fecal cultures (Salazar et al., 2008) and which was subsequently adapted for *Bacteroides* co-cultivation with bifidobacteria (Rios-Covian et al., 2013). The medium had the following composition: peptone (2 g/L), yeast extract (2 g/L), NaCl (100 mg/L), K_2HPO_4 (40 mg/L), KH_2PO_4 (40 mg/L), $MgSO_4$ (10 mg/L), $CaCl_2.H_2O$ (10 mg/L), $NaHCO_3$ (2 g/L), L-cysteine (2 g/L), bile salts (0.5 g/L), hemin (4 mg/L), Tween 80 (2 mL/L), $FeSO_4$ (50 μM), and $Na_3C_3H_5O(COO)_3$ (150 μM). A vitamin solution was added, resulting in final concentrations of: vitamin B_{12} (10 mg/L), vitamin K (2 mg/L), vitamin B_1 (2 mg/L), pyridoxal (1 mg/L), calcium pantothenate (2 mg/L), folic acid (1 mg/L), riboflavin (1 mg/L), biotin (1 mg/L), nicotinic acid (3 mg/L), para-aminobenzoic acid (1 mg/L). The BM was supplemented with 0.3% (w/v) glucose, or bifidobacterial EPS E44 or R1, as specified previously (Rios-Covian et al., 2013).The final pH of the medium ranged between 6.7 and 7.0. A culture of *B. fragilis* incubated in BM without external carbohydrates added was used for comparison in part of the study. The estimate of growth was obtained by measuring the optical density of cultures at 600 nm (OD_{600}). Culture media were inoculated with 1% (v/v) of an overnight culture of *B. fragilis* in BM with 1% (w/v) glucose resulting in initial population levels of around 10^6 CFU/mL.

EPS Isolation

Exopolysaccharide fractions were obtained from *Bifidobacterium animalis* subsp. *lactis* IPLA R1, a dairy origin strain (Ruas-Madiedo et al., 2006), and from *Bifidobacterium longum* IPLA E44, a fecal isolate from a healthy adult donor (Delgado et al., 2006). EPSs were isolated and purified from the cellular biomass of the producing strains harvested from agar-MRS plates supplemented with 0.25% (w/v) L-cysteine (Sigma Chemical Co.; agar-MRSc) as specified by Salazar et al. (2009b). Briefly, the biomass was collected with ultrapure water and mixed with one volume of 2 M NaOH and gently stirred overnight at room temperature; then, cells were removed by centrifugation and EPSs from supernatants were precipitated with two volumes of

absolute cold ethanol for 48 h at 4°C. After centrifugation at 10,000 × *g* for 30 min at 4°C, the EPS fraction was resuspended in ultrapure water, and dialyzed against water for 3 days at 4°C in dialysis tubes of 12- to 14-kDa molecular mass cutoff.

The isolated and purified EPS E44 and R1 fractions contained less than 2.37 and 2.03% (w/w) protein respectively, implying a final protein contribution to the culture medium of about 0.007 and 0.006% (w/v) for EPS fractions E44 and R1, respectively.

Analysis of SCFA and Organic Acids

Cell-free supernatants from cultures were filtered (0.2 μm). Quantification and identification of SCFA and branched chain fatty acids (BCFAs) was carried out by gas chromatography-mass spectrometry/flame injection detector (MS/FID) using a system composed of a 6890N GC (Agilent Technologies, Inc., Palo Alto, CA, USA) connected with a FID and a MS spectrometry 5973N detector (Agilent Technologies) as described previously (Salazar et al., 2008). Identification and quantification of organic acids was carried out on an HPLC chromatographic system composed of an Alliance 2690 module injector, a PDA 996 photodiode array detector, a 410 differential refractometer detector and Empower software (Waters, Milford, MA, USA). Chromatographic conditions were those indicated previously by Salazar et al. (2009a). Results of SCFA (acetic, propionic), BCFA (isobutyric, isovaleric), and organic acids (lactic, formic, pyruvic, succinic) concentrations were expressed in millimolar (mM). Concentrations of these compounds in media before inoculation (time 0) were subtracted from concentrations in cultures with each carbohydrate added at fixed sampling points during incubation. The sum of acetic, propionic, isobutyric, isovaleric, succinic, formic, pyruvic, and lactic acids was calculated. The molar proportion of each compound was obtained as the concentration percentage with respect to the total SCFA + BCFA + organic acids.

Amino Acids Analyses

Quantitative determination of amino acids in cell-free supernatants from cultures and in cell-free extracts (CFEs) was carried out by ultra-HPLC (UHPLC) using the method described in Redruello et al. (2013). The chromatographic system consisted of an H-Class Acquity UHPLC coupled to a PDA detector set at 280 nm (Waters). To obtain the CFEs, 10 mL of cultures were centrifuged at 6,500 × *g* for 5 min and concentrated in 1 mL of PBS. Resuspended bacterial pellets were twice broken by sonication for 30 s at 75 W and kept on ice for 1 min between sonication treatments. Cellular rests and unbroken cells were removed by centrifugation at 16,000 × *g* for 10 min at 4°C. CFE were ultra-filtered through a 3-kDa centricon following the manufacturer's instructions (Millipore, Billerica, MA, USA). One-hundred milliliters of filtered CFE or supernatants were derivatized as described in Redruello et al. (2013) and then one microliter was immediately injected into the chromatographic system. Total protein concentration of the CFE was determined using the Pierce® BCA Protein Assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA) according to the manufacturer's instructions. Results of amino acid concentrations in supernatants are expressed in millimolar (mM), whereas results of amino acid concentrations in CFEs are expressed in mmol/g of total protein. Amino acid levels of the media before inoculation (time 0) were subtracted from those in cultures containing each carbon source.

Proteomic Analyses

Differences in the proteome of *B. fragilis* growing in the presence of EPS E44 or EPS R1, with respect to glucose, were assessed separately by two-dimensional difference gel electrophoresis (2D-DIGE) as specified by Hidalgo-Cantabrana et al. (2013). In short, CFE from each culture were firstly obtained. Proteins were precipitated by the methanol-chloroform method according to Wessel and Flugge (1984), and resuspended in solubilization buffer (Destreak rehydration solution; GE Healthcare Biosciences, Uppsala, Sweden). Standard 2D gels were conducted first for each condition and were stained with Blue Silver Coomassie (Candiano et al., 2004) in order to obtain reference maps and spot-picking for mass spectrometry analyses. 2D-DIGE was then performed for each EPS and glucose cultures. The manufacturer's instruction of minimal dye protocol was adopted. Cultures of *B. fragilis* grown with glucose were labeled with Cy3 whereas cultures with each of the EPSs E44 and EPS R1 were labeled with Cy5. Gels were scanned in a Typhoon 9400 scanner (GE Healthcare) at a resolution of 100 μm and analyzed with the 2DImageMaster software (GE Healthcare). *t*-test were run between samples in each gel, and spots displaying statistical differences were excised from Coomassie stained gels and sent to the company Inbiotec (Leon, Spain) for digestion and identification by MALDI-TOF/TOF using standard protocols.

Gene Expression Analyses

Gene expression was determined by reverse transcription qPCR (RT-qPCR). Ten mL of *B. fragilis* cultures were collected at late exponential phase of growth, cells were mixed with RNA protective bacterial reagent (Qiagen GmbH, Hilden, Germany) and stored at −80°C until use. RNA extraction from cells was performed by phenol/chloroform treatment combined with physical lysis followed by the use of the RNeasy mini kit (Qiagen) as indicated by Ulve et al. (2008). The cDNA was obtained by reverse transcription of 1-μg total RNA using the kit "High Capacity cDNA Reverse Transcription" (Life Technologies, Alcobendas, Madrid). Real-time PCR was performed in an ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems) and expression levels were calculated by the ΔΔCt method as described previously (Gueimonde et al., 2007). The primers used in this study are listed in Supplementary Table S1, the 16S rRNA gene was used as endogenous control.

α-Glucosidase Activity

Two mL of culture were concentrated to 1 mL of PBS and CFE was obtained as described in the amino acid analysis section. Total protein determination was as indicated in the same section. The α-glucosidase activity was assayed both in culture supernatants and CFEs by determining the release of *p*-nitrophenol from the substrate *p*-NP α-D glucopyranoside, as described previously (Noriega et al., 2004). Specific activity in

both CFEs and supernatants was calculated relative to the amount of cell protein present in 1 mL of culture and was expressed as U/mg.

Estimation of the Redox Balance

The intracellular redox balance was determined by fluorescence spectroscopy. The fluorescence emissions of buffered cell suspensions at an OD_{600} of 0.6 in 50 mM Tris-HCl buffer pH 7.0 were monitored in an Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA, USA). The intensity values corresponding to NAD(P)H were calculated from the 413-nm emission at an excitation wavelength (λex) of 316-nm, whereas for FAD the intensity values were calculated from the 436-nm emission at a λex of 380-nm, as described by Ammor et al. (2004). The redox ratio was deduced from the NAD(P)H- and FAD-related measurements using the equation: redox ratio = FAD intensity/(FAD intensity + NAD(P)H intensity) (Kirkpatrick et al., 2005). Intensity obtained from the suspension buffer was subtracted from those obtained from the samples.

Statistical Analysis

Statistical analyses were performed using the SPSS-PC software, version 19.0 (SPSS Inc., Chicago, IL, USA). One-way analyses of variance (ANOVA) were run to compare different parameters in culture media added with bifidobacterial EPS (E44 or R1) or glucose or in media without carbohydrates added externally. When appropriate, a *post hoc* least significant difference (LSD) comparison test was applied to determine differences among the different conditions assayed. In the case of the α-glucosidase activity, *t*-tests were run between samples in the different carbohydrates. Experiments were carried out in triplicate.

Results

Growth Pattern

Growth pattern of *B. fragilis* varied depending on the culture conditions (**Table 1**). The maximum OD_{600} (OD_{600} max) was significantly higher in cultures with glucose than in the other conditions whereas the lowest values for this parameter were obtained in cultures without carbohydrates added ($P < 0.05$). Considerably lower pH values were obtained in glucose, thus reflecting more acid production and a more active metabolism and growth with this sugar. For most of the experiments performed in this work, we have collected samples when cultures reached their OD_{600} max, which corresponds to late exponential phase (**Table 1**).

TABLE 1 | Parameter values of *Bacteroides fragilis* cultures grown in the presence of glucose, EPS E44, EPS R1 or without carbohydrate source added (WCS) at OD_{600} max.

	Glucose	E44	R1	WCS
OD_{600} max	1.16 ± 0.21^{c}	0.61 ± 0.06^{b}	0.47 ± 0.07^{b}	0.23 ± 0.02^{a}
Time to reach OD_{600} max (h)	21.71 ± 0.49	23.21 ± 0.28	24.44 ± 0.24	20.6 ± 0.53
pH at OD_{600} max	5.37 ± 0.14^{a}	6.92 ± 0.12^{b}	6.92 ± 0.04^{b}	6.87 ± 0.09^{b}

Different letters indicate significant differences between cultures with different carbohydrates ($P < 0.05$).

SCFA and Organic Acids Production Profile

Molar proportions of the different SCFA and organic acids produced by *B. fragilis* during growth were clearly different in cultures with additional glucose than in cultures with EPS or without external carbohydrates added (**Figure 1**). Acetic acid was the most abundant SCFA produced in the presence of glucose (33.23%) whereas under other culture conditions the highest molar proportions corresponded to propionic acid (42.31% in cultures with EPS E44, 40.68% in cultures with EPS R1, and 44.23% in cultures without external carbohydrates added). A clear decrease of succinic and formic acids production occurred in cultures with EPS (7.88 and 9.39% for succinic acid and 7.48 and 9.85% for formic acid in EPS E44, and EPS R1, respectively) and without external carbohydrates added (12.19 and 5.22% for succinic and formic acids, respectively) as compared to glucose (18.62% for succinic acid and 20.19% for formic acid, respectively; $P < 0.05$); this happens at the expense of an increase in molar proportions of propionic acid (from 23.13% in glucose to > 40% in the other conditions) and BSCFA (0.22% in glucose in contrast with >5.5% in EPS and without carbohydrates added; $P < 0.05$). Lactic acid appeared in cultures with additional glucose but it was not detected under other conditions. A considerably higher propionic to succinic acid ratio was obtained in cultures with EPS or without carbohydrates (5.68 ± 1.28, 4.43 ± 0.73, and 3.62 ± 0.56 for EPS E44 and R1 and for cultures without carbohydrates added vs. 1.33 ± 046 in glucose; $P < 0.05$). In contrast, *B. fragilis* growing in glucose presented higher acetic to propionic acids ratio (1.44 ± 0.17 in glucose vs. 0.87 ± 0.02 and 0.83 ± 0.08 for EPS E44 and R1, and 0.63 ± 0.04 for cultures without carbohydrates added; $P < 0.05$; **Figure 1**). The production of total SCFA plus organic acids was twice as high in cultures with glucose (19.7 ± 1.06 mM) than in cultures with EPS (8.12 ± 1.85 and 7.96 ± 0.95 mM for EPS E44 and R1, respectively) and was around twofold higher in the presence of these polymers than in media without the addition of carbohydrates (4.65 ± 0.39 mM).

α-Glucosidase Activity

Cell-free extract of *B. fragilis* incubated in the presence of EPS E44 and R1 displayed higher α-glucosidase activity than in the presence of glucose, where the amount of this enzymatic activity was negligible ($P < 0.05$; **Figure 2**). No remarkable α-glucosidase activity was found in the supernatants of cultures, neither in supernatants nor CFE of cultures incubated in the absence of carbohydrates added. This indicated that the α-glucosidase activity was mainly intracellular and its production was dependent on the presence of EPS in the culture medium.

Amino Acids and Ammonia Profiles

In culture supernatants of *B. fragilis* grown in the presence of EPS or in medium without additional carbohydrates, the levels of total free amino acids hardly increased, or even decreased

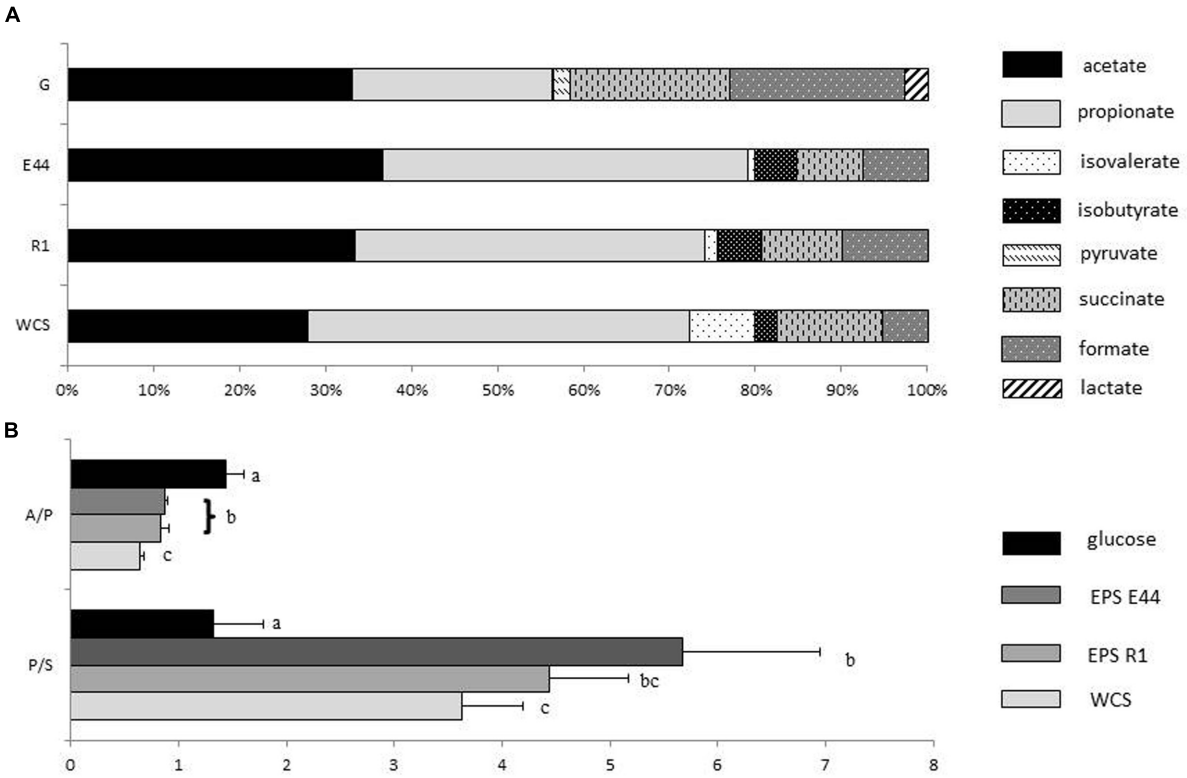

FIGURE 1 | (A) Molar proportions of SCFA, and organic acids produced by *Bacteroides fragilis* grown in glucose (G), EPS E44, and EPS R1 and without carbohydrates added (WCS). Acetate ■; propionate ■; isovalerate ■; isobutyrate ■; pyruvate ■; succinate ■; formate ■; lactate ■. **(B)** Acetate to propionate ratio (A/P) along with propionate to succinate ratio (P/S) of *Bacteroides fragilis* grown in glucose, EPS E44, EPS R1, and WCS. Glucose ■, E44 ■, R1 ■, WCS ■. Letters indicate significant differences among cultures with the different carbohydrates ($P < 0.05$). Error bars represent standard deviations. Total metabolite production in glucose: 19.7 ± 1.06, EPS E44: 8.12 ± 1.85, EPS R1: 7.96 ± 0.95, and WCS:4.65 ± 0.39 mM.

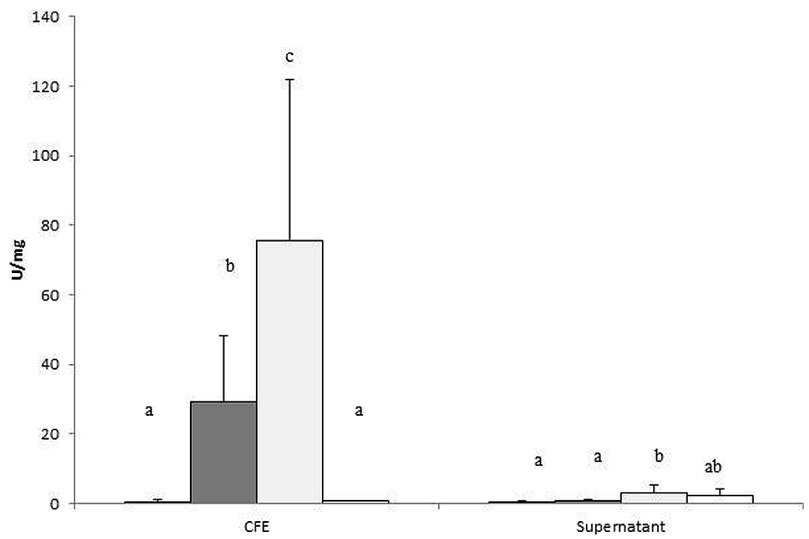

FIGURE 2 | α-glucosidase activity was determined in cell-free extract (CFE) and supernatants of *B. fragilis* grown in glucose (black bars), EPS E44 (dark gray bars), EPS R1 (light gray bars) and without carbon source added (WCS; white bars), and was referred to the amount of protein present in 1 mL of cell culture and expressed as U/mg protein. Different letters indicate significant differences among cultures with the different carbohydrates ($P < 0.05$). Error bars represent standard deviation.

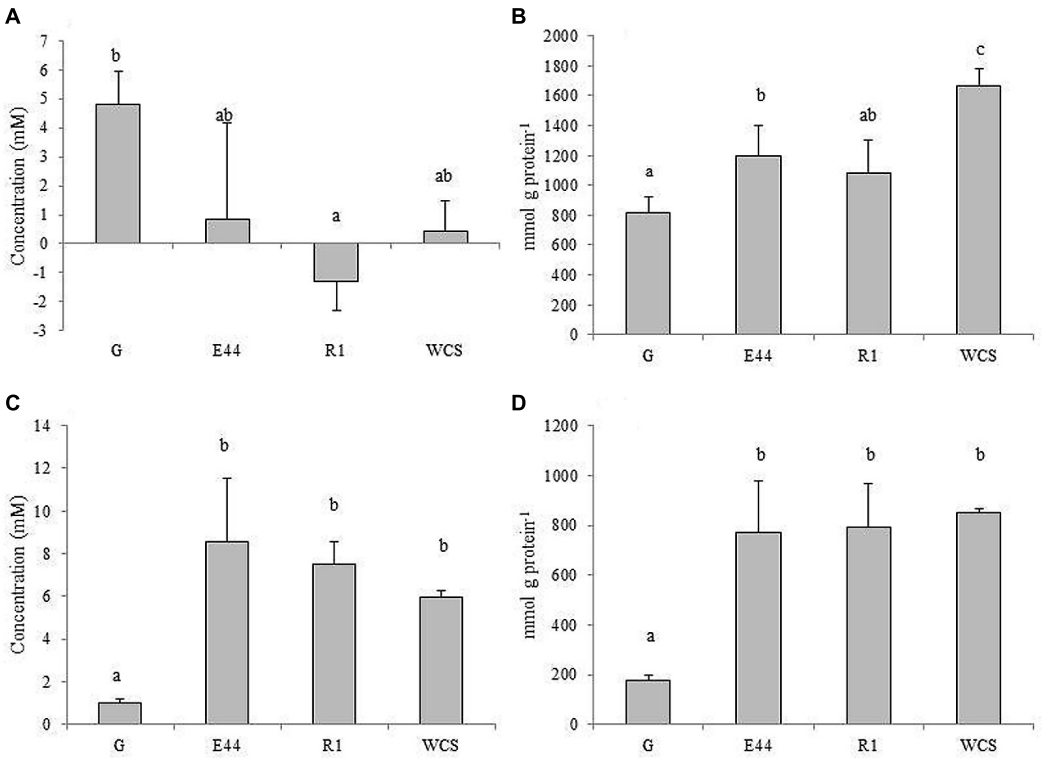

FIGURE 3 | Total amino acid concentrations in culture supernatants (A), and CFEs (B) along with ammonia levels in supernatants (C) and CFEs (D) of *B. fragilis* DSMZ2151 grown in glucose (G), EPS E44, EPS R1 and in medium without carbohydrate source (WCS). Different letters indicate significant differences among cultures with the different carbohydrates ($P < 0.05$). Error bars represent standard deviation.

after incubation, whereas with glucose the concentration of total amino acids clearly rose for the same conditions ($P < 0.05$; **Figure 3A**). In CFE, although levels of free amino acids per mg of cellular protein augmented in all cases, the most pronounced increases occurred in the absence of exogenous carbohydrates. Cultures in the presence of additional EPS displayed intermediate concentrations with respect to the other two conditions (**Figure 3B**). Levels of ammonia increased more in supernatants of cultures with EPS or without carbohydrates added than in cultures with glucose ($P < 0.05$; **Figure 3C**). Intracellular content of ammonia followed the same trend as the supernatants, with a higher accumulation in CFE of *B. fragilis* grown in the absence of carbohydrates and in media amended with EPS, than with glucose ($P < 0.05$; **Figure 3D**). All these factors taken together indicated that, proportionally, more amino acids are taken up by cells, and a concomitant release of ammonia took place when *B. fragilis* was grown in medium supplemented with EPS or in the absence of carbohydrates, as compared with glucose. Intracellular accumulation of amino acids seems to occur to a higher extent in media with EPS or without carbohydrates than in the medium containing glucose ($P < 0.05$).

In order to better understand these phenomena, the profile of amino acid concentrations was analyzed by UHPLC (Supplementary Table S2). Regardless of the culture conditions, specific amino acids were removed to variable extents, from the external medium, whereas others seemed to accumulate. The intracellular level of the different amino acids also differed. Notably, the carbohydrates present in the culture medium affected the profile of some amino acids differently. Thus, glutamic acid, glycine, threonine, GABA, tyrosine, and ornithine accumulated in the supernatants of cultures in the presence of glucose, whereas they were removed or accumulated at significantly lower levels in the presence of one or the two EPSs or in the absence of additional carbohydrates ($P < 0.05$). Conversely, with EPS or without exogenous carbohydrates, lysine and histidine, along with other amino acids, were found in supernatants at significantly higher concentrations than in cultures with glucose ($P < 0.05$). In addition, the intracellular content of six amino acids (aspartic acid, glutamic acid, tyrosine, valine, isoleucine, and lysine) appeared significantly augmented in the absence of carbohydrates added compared to cultures supplemented with glucose or EPS ($P < 0.05$). This indicated a shift in the amino acid metabolism by *B. fragilis* influenced by the type of carbohydrates available in the culture medium.

Further experimentation focused on *B. fragilis* growing in media in the presence of EPS or glucose as we aimed to ascertain the metabolic differences under these two conditions.

Proteomic Analysis of *B. fragilis* Growing in the Presence of Different Carbohydrates

The protein pool of *B. fragilis* was analyzed by means of 2D-DIGE and the proteome of the microorganism growing in the

TABLE 2 | Identification of *B. fragilis* DSMZ 2151 proteins affected by carbohydrate sources.

COG orthology	Spot no.[a]	Putative function[b]	GI number[c]	Mass[d]	pI[d]	MASCOT score	No. of peptides matched[e]	Cov.	Change fold[f]	
									E44	R1
Carbohydrate transport and metabolism	78	Transketolase	gi| 265763039	72.4	5.5	594	26	52	ND	2.48
	79	Transketolase	gi| 265763039	72.4	5.5	401	21	42	2.32	4.04
	81	Alpha-glucosidase	gi| 265762646	82.1	5.7	743	30	54	7.08	2.97
	87	Pyruvate phosphate dikinase	gi| 53713829	100.6	5.4	564	31	39	3.46	2.37
	89	Pyruvate phosphate dikinase	gi| 53713829	100.6	5.4	410	37	39	3.19	4.39
	90	Pyruvate phosphate dikinase	gi| 53713829	100.6	5.4	629	36	52	3.04	3.86
	105	Pyruvate phosphate dikinase	gi| 60682047	100.6	5.5	840	35	49	3.26	2.67
	101	Galactokinase	gi| 53712943	43.5	5.2	728	22	52	ND	2.29
Lipid transport and metabolism	82	Methylmalonyl-CoA mutase, large subunit	gi| 53715084	79.2	5.6	458	23	43	4.56	2.51
Amino acid transport and metabolism	3	Oxidoreductase (diaminopimelate dehydrogenase)	gi| 60682941	32.5	6.2	938	25	90	−2.06	−2.84
	9	NAD(P)H-utilizing glutamate dehydrogenase	gi| 60682871	49.1	6.1	526	19	53	−6.2	−7.37
	10	NAD(P)H-utilizing glutamate dehydrogenase	gi| 60682871	49.1	6.1	461	16	42	−6.17	−7.60
	11	NAD(P)H-utilizing glutamate dehydrogenase	gi| 60682871	49.1	6.1	430	17	44	−5.50	−7.19
	28	Ketol-acid reductoisomerase	gi| 53715042	38.2	5.2	1100	14	58	ND	−2.48
	62	Acetolactate synthase, large subunit, biosynthetic type	gi| 392698324	61.7	5.1	209	12	25	−2.45	−2.15
Post-translational modification, protein turnover, chaperones	12	Serine protease	gi| 53714036	54.7	6.5	296	14	30	−2.12	−2.30
Translation, ribosomal structure and biogenesis	36	Translation elongation factor 1A (EF-1A/EF-Tu)	gi| 319900910	43.7	5.3	260	8	23	ND	−4.76
	37	Elongation factor Tu	gi| 53715484	43.8	5.2	354	11	32	−2.96	−6.87
	83	Elongation factor G	gi| 265766981	80.4	5.7	489	24	38	2.59	2.83
	84	Elongation factor G	gi| 53715151	80.4	5.6	775	37	51	2.55	2.82
	103	Elongation factor G	gi| 53715468	77.9	5.1	712	23	44	ND	−2.87
	95	50S ribosomal protein L14	gi| 301161079	13.2	9.9	160	6	58	-	−2.03
	99	Translation initiation inhibitor	gi| 53714265	13.1	5.0	88	3	25	-	3.60
Cell wall, membrane, envelope biogenesis	110	Major outer membrane protein OmpA	gi| 53715321	43.3	8.7	361	15	37	ND	4.77
Inorganic ion transport and metabolism	108	Ferritin	gi| 53714336	18.1	5.0	692	12	71	2.35	2.00
Function unknown	39	Hypothetical protein BF2494	gi| 60681974	45.8	5.2	545	18	43	2.30	3.28
	41	Hypothetical protein BF2494	gi| 60681974	45.8	5.2	590	18	42	2.87	4.04
	43	Hypothetical protein BF2494	gi| 60681974	45.8	5.2	574	18	40	2.90	3.75
	92	Hypothetical protein BSHG_4061	gi| 383118833	16.3	5.1	163	8	60	−3.74	ND
	107	Hypothetical protein BF2537	gi| 53713828	20.1	6.3	533	13	79	ND	2.24

[a] *Spot numbers refer to the proteins labeled in 2D-DIGE gels.*
[b] *Putative functions were assigned from the NCBI gene database.*
[c] *GI number in the NCBI database for B. fragilis DSMZ 2151.*
[d] *As given by the NCBInr database for B. fragilis DSMZ2151. Molecular masses are expressed in kilodaltons.*
[e] *Number of tryptic peptides observed contributing to the percentage of amino acid coverage.*
[f] *Normalized change fold for each protein production derived from cells grown in EPS E44 or R1 with respect to the protein derived from cells grown in glucose. ND, ratio below 2; -, spot not detected.*

presence of each of the two EPS was compared with the proteome of the microorganism in glucose (**Table 2**). We focused only on spots corresponding to proteins displaying statistical differences ($P < 0.05$) and production ratios in EPS greater or lower than twofold compared to glucose alone, in at least one of the two polymers. According to this criterion, the production of 10 proteins was found downregulated in cultures with one or both EPSs whereas 13 proteins were upregulated. Cultures of *B. fragilis* with additional EPS R1 presented more variation in the protein-production levels with respect to glucose than cultures with EPS E44. Thus, three proteins were underproduced in the presence of the EPS R1, but not with EPS E44, whereas a unique protein

was downregulated with EPS E44 but not with EPS R1; four proteins were found upregulated only in the presence of EPS R1, but not with EPS E44 whilst the contrary was never observed. The presence of some protein spots with the same GI number and molecular mass, but differing in their experimental isoelectric points, suggested the presence of isoforms. Proteins and enzymes whose production was affected by the carbohydrates available in the culture medium belong to several functional COG categories (**Table 2**).

Figure 4 depicts a schematic representation of the main affected metabolic pathways of *B. fragilis* and the proteins involved. As shown in **Table 2**, several enzymes related to the metabolism of carbohydrates were upregulated in the presence of one or two EPS: alpha-glucosidase and galactokinase-enzymes related to the release of 1→4 α-D-glucose from complex carbohydrates and with the phosphorylation of D-galactose facilitating its entry into several metabolic routes, respectively; transketolase-enzyme from the pentose phosphate pathway that catalyzes the transfer of aldehyde or ketonic groups between monosaccharides of different carbon residues; and the pyruvate phosphate dikinase-catalyzing the interconversion of phosphoenolpyruvate (PEP) and pyruvate. The overproduction of α-glucosidase was consistent with the higher enzymatic activity found in CFE of *B. fragilis* grown with EPS added (**Figure 2**).

Several proteins participating in the metabolism of different amino acids, some of which catalyze redox reactions, were underproduced in cultures of *B. fragilis* when incubated with EPS fractions: diaminopimelate dehydrogenase–oxidoreductase participating in one of the two redundant pathways leading to the formation of lysine and diaminopimelic acid; NAD(P)H-dependent glutamate dehydrogenase–oxidoreductase participating in the assimilation of ammonia to form glutamate; ketol-acid reductoisomerase and acetolactate synthase-two enzymes, oxidoreductase and transferase respectively, involved in the biosynthesis of isoleucine, valine, and leucine. Concomitantly, several proteins related to the translation and elongation steps of protein synthesis, including ribosomal proteins and elongation factors, were underproduced in the presence of one or both EPS. At the same time a translation initiation inhibitor was overproduced in the presence of EPS R1 (**Table 2**). These metabolic changes suggest a modification of the metabolism of amino acids, proteins, and peptides in *B. fragilis* when the microorganism is grown in the presence of bifidobacterial EPS fractions as compared to glucose.

Other proteins whose production was affected by carbohydrates available in the culture medium include: (i) the large subunit of the methylmalonil-CoA mutase-enzyme participating in the synthesis of propionate from succinate, the production of which was enhanced with EPS fractions, (ii) the major outer membrane protein OmpA which was overproduced only with EPS R1, and (iii) ferritin which was overproduced in the presence of both EPSs (**Table 2**). Additionally, several hypothetical proteins of unknown function in *B. fragilis* were differentially produced in the presence of one or two EPS in the culture medium. Possibly, proteins BF2494, BF2537, and BSGH_4061 according to BLAST homology with membrane proteins with TRP domain, a histidine kinase membrane protein and heat-shock protein respectively, in other *Bacteroides* species (data not shown).

Changes in Gene Expression by RT-qPCR

The relative expression of genes coding for some proteins selected on the basis of the 2D-DIGE experiments was further analyzed by qRT-PCR (Supplementary Figure S1). The genes for the NAD(P)H-dependent glutamate dehydrogenase (*gdhB*) and the acetolactate synthase (*ilvB*) showed downregulation in the presence of both EPSs, whereas the gene coding for the major outer membrane protein OmpA (*ompA*) and the pyruvate phosphate dikinase (*ppdK*) displayed increased expression under the same conditions. These results are in agreement with the differential protein production found by proteomic analyses. The transketolase gene (*tktB*) appeared upregulated in the presence of the EPS E44 and slightly downregulated with EPS R1, which partly supports the behavior found for the corresponding protein in 2D-DIGE experiments. However, in spite of the clearly higher molar proportions of propionate obtained by us in cultures of *B. fragilis* in the presence of EPS E44 and R1 and the enhanced production of the methyl-malonyl-CoA mutase enzyme in such conditions, only a moderate overexpression and a slight underexpression of the corresponding gene (*mutB*) were respectively obtained in cultures of *B. fragilis* with EPS E44 and EPS R1 at the OD_{600} max of cultures. Attempts to analyze the expression of *mutB* at earlier points during growth proved unsuccessful due to the lack of sufficient RNA for RT-qPCR determinations (data not shown).

In addition, we assessed the expression of three genes playing a key role in the entry of PEP/pyruvate into the succinate-propionate pathway in *Bacteroides* (**Figure 4** and Supplementary Figure S1) and which did not display detectable changes in 2D-DIGE gels. One of them, the PEP carboxykinase (*pckA*) is responsible for the carboxylation of PEP to oxaloacetate with ATP formation. Pyruvate carboxylase (*pyc*) catalyzes the synthesis of oxaloacetate by carboxylation of pyruvate with ATP consumption, and malate dehydrogenase (*mdh*) catalyzes the formation of malic acid from pyruvate with consumption of reducing power. *PckA* was underexpressed in the presence of both EPS, whereas *pyc* was overexpressed and a slight overexpression or underexpression was respectively found for *mdh* with EPS E44 and R1.

Intracellular Redox Balance

The redox ratio was significantly lower ($P < 0.05$) in cultures added with EPS E44 and R1 than in cultures in the presence of glucose, indicating a more reduced intracellular state of *B. fragilis* in the former two conditions. The NAD(P)H-associated fluorescence was significantly higher ($P < 0.05$) in cultures of *B. fragilis* ran with EPS fractions than in cultures with glucose whereas no significant differences ($P > 0.05$) were found in the fluorescence associated to FAD between cultures with EPS and glucose (**Table 3**). This indicated that the lower redox ratio found in our cultures when *B. fragilis* was grown in the presence of EPS was mainly due to an increase of the pool of intracellular NAD(P)H rather than to variations in FAD intracellular levels.

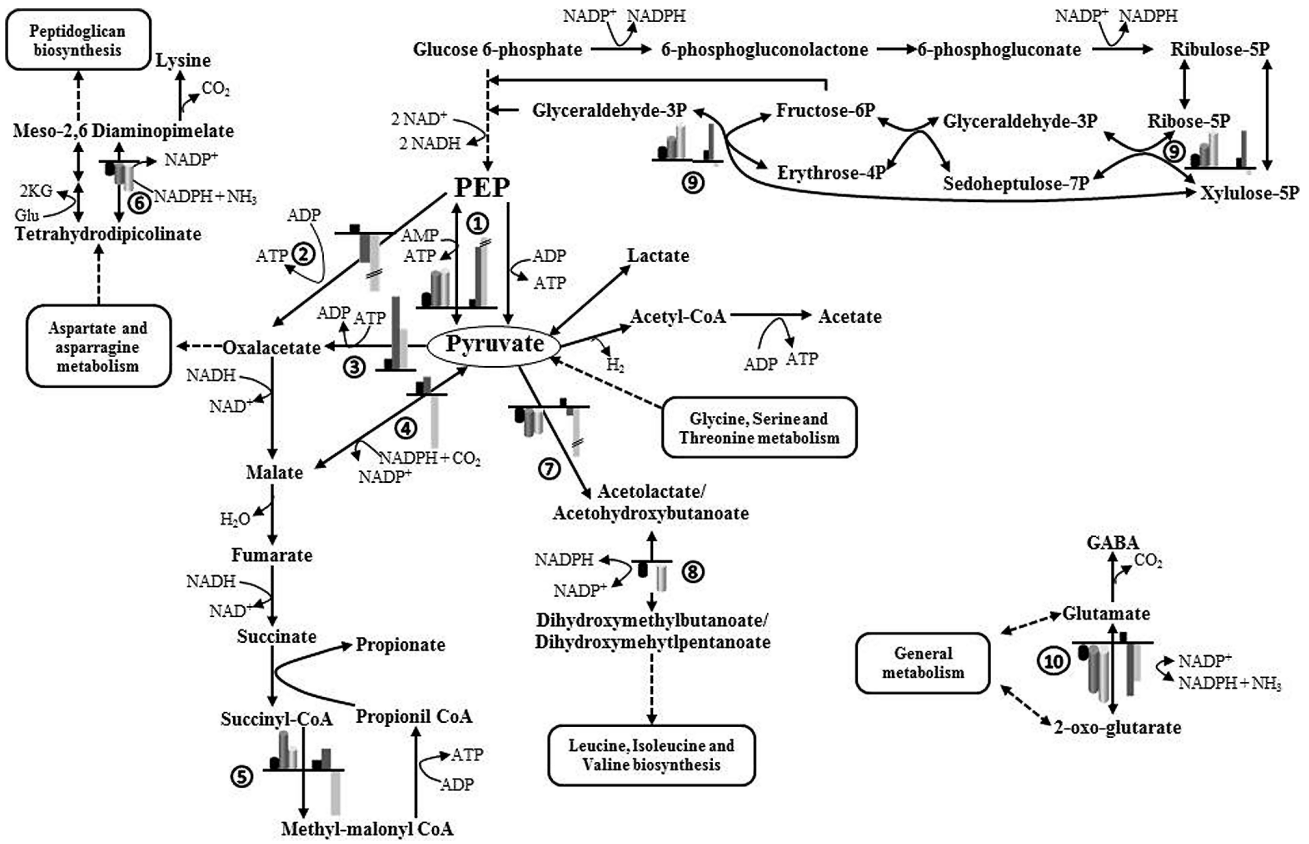

FIGURE 4 | Schematic representation of the main metabolic pathways affected by the availability of carbohydrates added to the culture medium (glucose, EPS E44, and EPS R1) during growth of *B. fragilis*. Production ratios of enzymes catalyzing different steps in the presence of EPS with respect to glucose are indicated by cylindrical bars. Fold changes in the expression of genes in the presence of EPS with respect to glucose are indicated by rectangular bars. Black bars, glucose; dark gray bars, EPS E44; light gray bars, EPS R1. Ratios greater than sevenfold are shown with a height break. Enzymes: (1) pyruvate phosphate dikinase, (2) PEP carboxykinase, (3) pyruvate carboxylase, (4) malate dehydrogenase, (5) methyl-malonylCoA mutase, (6) diaminopimelate dehydrogenase, (7) acetolactate synthase, (8) ketol-acid reductoisomerase, (9) transketolase, (10) NADPH-dependent glutamate dehydrogenase. 2KG, 2-oxoglutarate; Glu, glutamate; PEP, phosphoenolpyruvate.

TABLE 3 | Intracellular redox ratios of NAD(P)H- and FAD fluorescence associated with *B. fragilis* grown in the presence of additional glucose, EPS E44 and EPS R1.

	Glucose	EPS E44	EPS R1
Redox ratio	0.70 ± 0.03^{c}	0.59 ± 0.06^{b}	0.46 ± 0.09^{a}
Fluorescence (NAD(P)H)	2.89 ± 0.99^{a}	7.07 ± 0.93^{b}	9.21 ± 0.90^{c}
Fluorescence (FAD)	6.30 ± 1.71	10.38 ± 2.97	8.31 ± 3.48

Letters indicate significant differences between cultures with different carbohydrates ($P < 0.05$). The redox ratio was calculated according to the formula: $\frac{FAD^{+}}{FAD^{+}+NAD(P)H}$.

Discussion

The majority of *B. fragilis* metabolic studies go back 2–3 decades ago, and in those studies the influence of external nutritional conditions were not considered. Under the culture conditions used in the present study, *B. fragilis* was able to grow in the presence of glucose and bifidobacterial EPS, the pH decrease in the culture medium being more pronounced with glucose than with the bacterial polymers. The slow growth of *B. fragilis* in the presence of EPS and the need of enough biomass and comparable cell counts for proteomic analyses, prompted us to choose late exponential phase for most of the experiments in this study (growth curves shown in Rios-Covian et al., 2013).

Molar proportions of propionic acid in cultures of *B. fragilis* in the presence of EPS or in the absence of additional carbohydrates were considerably higher than in cultures with glucose, which occurred with a concomitant reduction in the proportion of succinic, formic, and lactic acids. These results essentially confirmed our previous findings and those of other authors indicating that the ratio of propionic to succinic acid in cultures of *Bacteroides* at advanced stages of growth is higher in complex carbon sources or under carbohydrate-shortage conditions than in medium with glucose (Kotarski and Salyers, 1981; Rios-Covian et al., 2013; Adamberg et al., 2014). In addition, the higher production of SCFA and organic acids in cultures with EPS compared to cultures without carbohydrate supplementation suggests a possible utilization of these polymers as fermentable substrates (Rios-Covian et al., 2013).

The α-D-glucosidase is one of the most abundant glycoside hydrolases produced by *B. fragilis*; the enzyme, located in the periplasmic space (Berg et al., 1980), catalyzes the hydrolysis of terminal, non-reducing (1→4) linked α-D glucose from complex carbohydrates. We found that the α-glucosidase activity was virtually absent both in culture supernatants and in CFEs when grown in glucose or in the absence of additional carbohydrates, but was present in CFEs from *B. fragilis* cultured in the presence of EPSs. The EPS 44 fraction contains two polymers of different molar mass which are composed of glucose and galactose in proportion 1:1 (Salazar et al., 2009a) whereas EPS R1 is formed by three polymers of different molar mass which are composed by glucose, galactose, and rhamnose in proportions 1:1:1.5 (Ruas-Madiedo et al., 2010). Since both EPS fractions contain glucose in their composition (Salazar et al., 2009a; Ruas-Madiedo et al., 2010), α-glucosidase may be involved in the utilization of these EPS fractions by *B. fragilis*. Proteomics confirmed the overproduction of the α-glucosidase in the presence of both EPSs and also revealed that galactokinase, another enzyme related to the metabolism of carbohydrates, was overexpressed in the presence of EPS R1. The overproduction of these two enzymes suggests an expansion in the metabolic ability of *B. fragilis* to take advantage of complex carbohydrates when the microorganism is grown in the absence of readily fermentable carbohydrates. To this respect it is worth mentioning that galactose is one of the most abundant monosaccharides in intestinal mucin (Robbe et al., 2003) whereas starch (backbone of 1→4 α-D-glucose) is the main source of complex carbohydrates in the human diet.

Amino Acids Metabolism

Bacteroides can produce BCFA from amino acids and proteins (Macfarlane et al., 1991; Smith and Macfarlane, 1998) and recent data showed that *B. fragilis* can release them in the presence of EPS (Rios-Covian et al., 2013). Remarkably, isovaleric and isobutyric acids, originating from the catabolism of leucine and valine respectively, were formed in *B. fragilis* cultures with EPS but not glucose. Our experimental results also point to an enhanced consumption of the pool of amino acids present in the culture medium and a higher release of ammonia in the presence of EPS as compared to glucose. The high removal of asparagine from supernatants and the absence of residual asparagine and alanine in CFEs indicated the efficient conversion of asparagine to aspartic acid rendering alanine that is finally transformed to pyruvic acid, under all culture conditions tested. An enhanced removal of threonine from the culture medium also occurred in the presence of EPS, whereas glycine accumulated at lower levels than in the presence of glucose. Glycine and serine are intermediate in the degradation of threonine toward their incorporation at the level of pyruvate; therefore these three amino acids may serve as a source of pyruvate for *B. fragilis* grown in the presence of EPS. Moreover, the enzymes ketol-acid reductoisomerase and acetolactate synthase, that participate in the biosynthetic pathway of isoleucine, leucine (amino acids removed from the culture medium by *B. fragilis* under all conditions) and valine from pyruvate were found to be underproduced in EPS as compared to glucose. These facts suggest that in *B. fragilis* the metabolism of some amino acids rendering pyruvate, a key intermediate of central catabolism, was activated whereas the synthesis of those using pyruvate as a precursor may be partly inhibited in the presence of bacterial EPS. Interestingly, threonine is a major component of the human intestinal mucin, together with asparagine and serine (Aksoy and Akinci, 2004). This result points out an adaptation of *B. fragilis* metabolism for improving utilization of intestinal mucin, as previously reported by Adamberg et al. (2014) for *Bacteroides thetaiotaomicron* subjected to amino acid starvation.

A clear underproduction of the enzyme NAD(P)H-dependent glutamate dehydrogenase was found in the presence of EPS. *B. fragilis* possesses two distinct glutamate dehydrogenases that play a fundamental role in nitrogen assimilation (Yamamoto et al., 1987; Baggio and Morrison, 1996). The NAD(P)H-dependent glutamate dehydrogenase catalyzes the assimilation of ammonia by reductive amination of α-ketoglutarate to form L-glutamate, whereas the NADH-dependent glutamate dehydrogenase catalyzes the reverse reaction. The NADH-dependent enzyme displays a basal activity that increases at high organic nitrogen concentration whereas in such conditions the NAD(P)H-dependent enzyme was inhibited (Abrahams and Abratt, 1998). In addition, glutamic acid is a metabolic intermediate in the synthesis of different amino acids and other compounds, its concentration in CFE being the highest found by us under all conditions assayed. It is then plausible that the catabolic reaction in the direction of ammonia and α-ketoglutarate formation from glutamate would predominate in cultures with EPS, as compared to cultures performed with glucose. The α-ketoglutarate formed could then serve as a substrate for transamination reactions in *B. fragilis* (Abrahams and Abratt, 1998). On the other hand, GABA was present in significantly lower levels in the culture supernatants of *B. fragilis* incubated with additional EPS, as compared with the cultures in the presence of glucose. GABA is formed from glutamate in a single reaction step mediated by a glutamate decarboxylase enzyme. An acid resistance mechanism has been described in some intestinal bacteria in which a molecule of extracellular glutamate is antiported with an intracellular proton and converted to GABA that is subsequently exchanged for another extracellular glutamate (Cotter et al., 2001). Likely, under mild acidic conditions occurring in cultures with EPS as compared to glucose, the glutamate decarboxylase pathway is not being used by *B. fragilis,* thus GABA is not formed.

Aspartic acid is a precursor in the biosynthetic pathway of lysine and meso-diaminopimelic acid. Lysine takes part of the pentapeptide bridge of the peptidoglycan in Gram-positive bacteria, and is replaced by meso-diaminopimelic acid in Gram negatives. *B. fragilis* has two redundant biosynthetic pathways for the synthesis of meso 2,6-diaminopimelate: one is catalized by the oxidorreductase diaminopimelate dehydrogenase with consumption of ammonia and NADPH and the other occurs in two steps, catalyzed by an aminotransferase and an epimerase, respectively. The diaminopimelate dehydrogenase was found underproduced by proteomic analyses when *B. fragilis* was grown in the presence of EPS, thus favoring in such conditions the alternative route which generates a molecule of α-ketoglutarate from glutamate to form 2,6-diaminopimelate (Hudson et al.,

2011). The α-ketoglutarate could then serve as substrate for cellular transamination reactions, as commented before. Our results also support the idea that the amino acids are more efficiently removed by *B. fragilis* from the culture medium under shortage conditions of readily fermentable carbohydrates, as would be the case when this microorganism is incubated with EPS. In this respect, the strong influence that available carbohydrates exert on peptide and amino acids metabolism in *Bacteroides* and other intestinal bacteria is known (Macfarlane et al., 1991; Smith and Macfarlane, 1998; Adamberg et al., 2014).

Finally, some proteins involved in translation, ribosomal structure, and biogenesis were underproduced whereas a translation initiation inhibitor was upregulated in *B. fragilis* in the presence of EPS as compared to glucose, indicating a slowdown of the protein synthesis in this bacterium. Under these conditions the intracellular pool of amino acids can be maintained by transamination reactions involving α-ketoacids and amino acids collected from the culture medium.

Energetics, Metabolism, and Redox Balance

Using proteomics we could not find variations in the production of glycolytic enzymes. However, overproduction of pyruvate phosphate dikinase was confirmed by proteomics and gene expression analyses. This enzyme catalyzes the interconversion of PEP and pyruvate, with ATP formation in the forward direction. Although pyruvate phosphate dikinase functions in the gluconeogenesis direction in several organisms, the pyruvate formation seems to be favored at moderate acidic conditions in *Bacteroides symbiosus* (optimum pH in the forward direction 6.6 and 7.2-7.8 in the reverse direction; Reeves, 1971), as may also occur in some parts of the human colon ecosystem (Vertzoni et al., 2010). The dominant route of microbial propionate synthesis in the gut is the succinate–propionate pathway via formation of methyl-malonyl-CoA, which is present in *Bacteroides* (Reichardt et al., 2014). In our case, it seems that when *B. fragilis* is growing in glucose, a significant part of PEP would be converted to oxaloacetate, malate, and pyruvate being then formed by decarboxylation of oxaloacetate (Macy et al., 1978). However, we found that the gene coding for PEP carboxykinase (catalyzing carboxylation of PEP to oxaloacetate) was under-expressed when *B. fragilis* was grown in EPS as compared to glucose; in such conditions the pyruvate carboxylase (catalyzing carboxylation of pyruvate to oxaloacetate) was over-expressed and the malate dehydrogenase displayed slight variation in its expression in cultures with EPS when compared to cultures with glucose. Taking into account that an increase in the degradation of amino acids leading to pyruvate and a partial inhibition of the synthesis of amino acids from pyruvate seems to occur in *B. fragilis* cultured in the presence of EPS, a relative accumulation of pyruvate and a relative shortage of PEP originating from carbohydrates may happen under such conditions. Therefore, it is plausible that in the presence of EPS a significant part of oxaloacetate would be formed from pyruvate. The pentose-phosphate pathway generates pentoses and reducing power in the form of NADPH. In the presence of glucose, the succinate-propionate pathway keeps the intracellular redox balance of NAD^+/NADH + H^+ through the reoxidation of two moles of NADH generated in the conversion of glucose to PEP in glycolysis. However, under conditions of carbohydrate shortage and enhanced amino acid removal from the medium, as we propose occurs when *B. fragilis* grows in a BM with additional EPS, a redox imbalance may happen due to insufficient reducing power supply from glycolysis. Under such conditions, the main input for intermediates of the succinate–propionate pathway would be the amino acids incorporated at the level of pyruvate and therefore a relative discompensation between NADH/NADPH may occur. The flux enhancement through the pentose-phosphate pathway leads to NADPH synthesis and overexpression of transketolase in cultures with EPS, providing the additional reducing power needed to maintain the cellular redox balance while accomplishing essential metabolic functions. The lower redox ratio associated with higher levels of NADH that we found in cells of *B. fragilis* grown with EPS, agrees with this hypothesis. Moreover, a metabolic redirection of NADH to NADPH has been shown in *Pseudomonas fluorescens* under oxidative stress (Singh et al., 2008). The metabolic ability to inter-convert both nicotinamide dinucleotides, although not yet demonstrated in *Bacteroides*, may ensure keeping the intracellular NADH and NADPH pools as an adaptation to external stimuli.

The Unknown Balance between Beneficial and Detrimental Effects in the Intestinal Ecosystem

A ferritin was overproduced in *B. fragilis* grown in the presence of EPS as compared to glucose. Ferritin-like proteins sequestrate iron in response to the presence of oxygen, playing an important role in the pathogenicity of this bacterium (Rocha and Smith, 2013). On the other hand, the major outer membrane protein OmpA was overproduced in cultures of *B. fragilis* grown with EPS R1. In other microorganisms, OmpA functions as a porin that enables the passage of nutrients and different compounds into the cell (Reeves et al., 1996, 1997). OmpA has also been implicated in cell functions related with pathogenicity (Soulas et al., 2000; Smith et al., 2007). In *B. fragilis*, the involvement of OmpA1 in maintaining cell structure as well as in the release of cytokines by murine splenocytes has been demonstrated (Magalashvili et al., 2008; Wexler et al., 2009). Whether OmpA overproduction is related with an improved ability of *B. fragilis* to obtain nutrients from the environment, and whether this is related with beneficial or detrimental effects for the host is, at present, unknown.

Conclusion

The results presented provide an insight into the physiological and molecular mechanisms that allow *B. fragilis* to adapt to an environment where EPSs are the main source of carbohydrates. Protein expression with different functions was modulated, and the intracellular and extracellular pattern of given amino acids, redox balance, and α-glucosidase activity were affected by the external environment. Three main events, namely the activation of amino acid catabolism, enhancement of the transketolase reaction from the pentose-phosphate pathway and the activation of the succinate–propionate pathway for propionic formation,

suggest a metabolic shift in this bacterium toward the generation of more reducing power, and to optimize the ATP yield. These changes would represent a metabolic adaptation of *B. fragilis* to take advantage of carbohydrates and proteins available in the intestinal environment, such as mucin. Recent results from our group indicated that in the presence of EPS, propionic acid production increased in human fecal cultures (Salazar et al., 2008), whereas the growth of bifidobacteria was enhanced by *B. fragilis* (Rios-Covian et al., 2013). Both effects are generally considered as health promoters in the human gut, but other potentially harmful effects from the couple EPS/*B. fragilis* cannot be ruled out and deserve further investigation. Our work has revealed a number of intriguing topics for future research regarding the relationship of probiotics and prebiotics with the symbiont or mutualistic microbial populations of the human intestinal ecosystem.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00825

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Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health

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The colon is inhabited by a dense population of microorganisms, the so-called "gut microbiota," able to ferment carbohydrates and proteins that escape absorption in the small intestine during digestion. This microbiota produces a wide range of metabolites, including short chain fatty acids (SCFA). These compounds are absorbed in the large bowel and are defined as 1-6 carbon volatile fatty acids which can present straight or branched-chain conformation. Their production is influenced by the pattern of food intake and diet-mediated changes in the gut microbiota. SCFA have distinct physiological effects: they contribute to shaping the gut environment, influence the physiology of the colon, they can be used as energy sources by host cells and the intestinal microbiota and they also participate in different host-signaling mechanisms. We summarize the current knowledge about the production of SCFA, including bacterial cross-feedings interactions, and the biological properties of these metabolites with impact on the human health.

Keywords: short chain fatty acids, diet, human health, intestinal microbiota, cross feeding

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INTRODUCTION

The gut microbiota influences our health and nutritional stage via multiple mechanisms, and a mounting body of evidence recognizes that microbial metabolites have a major influence on host physiology. Short chain fatty acids (SCFA) are volatile fatty acids produced by the gut microbiota in the large bowel as fermentation products from food components that are unabsorbed/undigested in the small intestine; they are characterized by containing fewer than six carbons, existing in straight, and branched-chain conformation. Acetic acid (C2), propionic acid (C3), and butyric acid (C4) are the most abundant, representing 90–95% of the SCFA present in the colon. The main sources of SCFA are carbohydrates (CHO) but amino acids valine, leucine, and isoleucine obtained from protein breakdown can be converted into isobutyrate, isovalerate, and 2-methyl butyrate, known as branched-chain SCFA (BSCFA), which contribute very little (5%) to total SCFA production. The aim of the present mini-review is to summarize the current knowledge about SCFA production, including bacterial cross-feedings interactions, and the biological properties of these metabolites with impact in human health.

MECHANISMS OF SCFA PRODUCTION

Metabolic Routes

The main end products resulting from the CHO catabolism of intestinal microbes are acetate, propionate, and butyrate. Lactate, although is not a SCFA, is also produced by some members of the microbiota, such as lactic acid bacteria, bifidobacteria, and proteobacteria, but under normal physiological conditions it does not accumulate in the colon due to the presence of some species, such as *Eubacterium hallii*, that can convert lactate into different SCFA (Flint et al., 2015).

Acetate is the most abundant SCFA in the colon and makes up more than half of the total SCFA detected in feces (Louis et al., 2007). Two main metabolic routes have been described for acetate production by the gut microbiota (**Figure 1**). The majority of acetate is produced by most enteric bacteria as a result of CHO fermentation. In addition, approximately one-third of the colonic acetate is coming from acetogenic bacteria, which are able to synthesize it from hydrogen and carbon dioxide or formic acid through the Wood–Ljungdahl pathway (Miller and Wolin, 1996; Louis et al., 2014).

Propionate and butyrate metabolism have received much attention during the last years, mainly due to the connection between low levels of butyrate and propionate bacterial producers and some diseases in which inflammatory processes are involved. For instance, butyrate producers are normally low in ulcerative colitis (Machiels et al., 2014) and reduced levels of propionate producers have been detected in children at risk of asthma (Arrieta et al., 2015).

Three different pathways are used by colonic bacteria for propionate formation: succinate pathway, acrylate pathway, and propanodiol pathway (Reichardt et al., 2014) (**Figure 1**). The succinate route utilizes succinate as a substrate for propionate formation and involves the descarboxylation of methylmalonyl-CoA to propionyl-CoA. This pathway is present in several *Firmicutes,* belonging to the *Negativicutes* class, and in *Bacteroidetes*. In the acrylate pathway lactate is converted to propionate through the activity of the lactoyl-CoA dehydratase and downstream enzymatic reactions; this route appears to be limited to a few members of the families *Veillonellaceae* and *Lachnospiraceae* (Flint et al., 2015). In the propanodiol pathway, characterized by the conversion of deoxy-sugars to propionate, the CoA-dependent propionaldehyde dehydrogenase, that converts propionaldehyde to propionyl-CoA, has been suggested as a marker for this route. This metabolic pathway is present in bacteria which are phylogenetically distant, including proteobacteria and members of the *Lachnospiraceae* family (Louis et al., 2014; Reichardt et al., 2014). The relative abundance of *Bacteroidetes* has also been linked to the total fecal propionate concentration, suggesting that the succinate pathway is the dominant route within the gut microbiota (Salonen et al., 2014).

Two different pathways for butyrate production are known in butyrate-producing bacteria (**Figure 1**). The butyrate kinase pathway employs phosphotransbutyrylase and butyrate kinase enzymes to convert butyryl-CoA into butyrate (Louis et al., 2004). This route is not common among members of the gut microbiota and is mainly limited to some *Coprococcus* species (Flint et al., 2015). In contrast, the butyryl-CoA: acetate CoA-transferase pathway, in which butyryl-CoA is converted to butyrate in a single step enzymatic reaction, is used by the majority of gut butyrate-producers (Louis et al., 2010), including some of the most abundant genera of the intestinal microbiota, such as *Faecalibacterium*, *Eubacterium,* and *Roseburia*. Remarkably, the production of butyrate and propionate by the same bacterium is not common and only a few anaerobes, such as *Roseburia inulinivorans* and *Coprococcus catus,* are able to produce both (Louis et al., 2014).

Cross-Feeding Mechanisms

Bacterial cross-feeding has a huge impact on the final balance of SCFA production and the efficient exploitation of the substrates that reach the human gut. These mechanisms consist either in the utilization of end products from the metabolism of a given microorganism by another one, called metabolic cross-feeding (**Figure 1**), and/or the utilization by one microorganism of the energy rich complex CHO breakdown products formed by another one, called substrate cross-feeding (Belenguer et al., 2006; Flint et al., 2007). A recent *in silico* study showed that mutualism cross-feeding interactions were promoted by anoxic conditions, which are more common in the large intestine than in the small one (Heinken and Thiele, 2015).

Microorganisms that are not capable of using complex CHO may proliferate by taking advantage of substrate cross-feeding, using breakdown compounds produced by hydrolytic bacteria. This is the case of some *Bifidobacterium* species that are not able to use inulin-type fructans (ITF) but can grow by cross-feeding of mono- and oligosaccharides released by primary inulin degraders in fecal cultures added with inulin as carbon source (Rossi et al., 2005; Salazar et al., 2009). Other example is the degradation of agaro-oligosaccharides (AO), which is more effective when *Bacteroides uniformis* and *Escherichia coli* are grown in co-culture than in separated monoculture (Li et al., 2014). In the same study the authors suggest the utilization of agarotriose, an intermediate in the degradation of AO, by *Bifidobacterium adolescentis* and *Bifidobacterium infantis.* In another work, it was demonstrated that *Roseburia* sp. strain A2-183 is unable to use lactate as carbon source, but when it is co-cultured with *B. adolescentis* L2-32 in the presence of FOS or starch, produces butyrate (Belenguer et al., 2006).

Although, there are a lot of *in vitro* studies pointing to metabolic cross-feeding it was not until recently that was demonstrated *in vivo* by using stable isotopes of acetate, propionate and butyrate perfused into the caecum of mice (Den Besten et al., 2013a). This study evidenced that the bacterial cross-feeding occurred mainly from acetate to butyrate, at lower extent between butyrate and propionate, and almost no metabolic flux exists between propionate and acetate. *In vitro* utilization of acetate by *Faecalibacterium prausnitzii* and *Roseburia sp.* has been evidenced (Duncan et al., 2002, 2004b). Prediction of metabolic fluxes between *F. prausnitzii* A2-165 and *B. adolescentis* L2-32 in co-culture has been reported in a

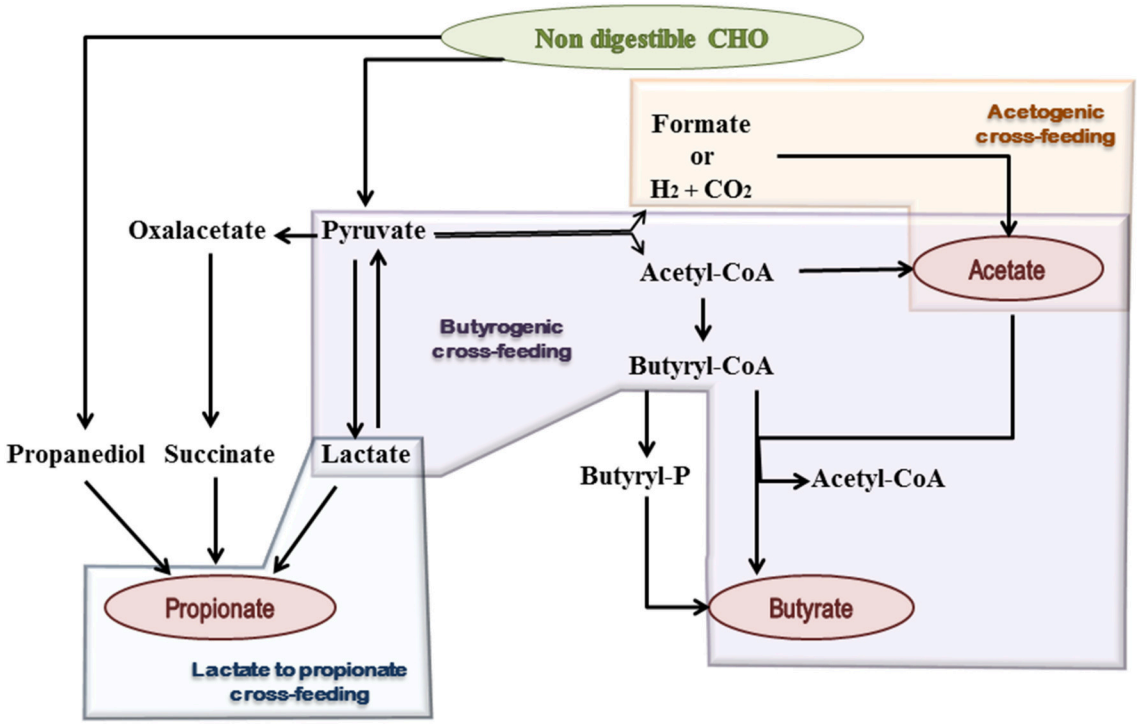

FIGURE 1 | Schematic representation of microbial metabolic pathways and cross-feeding mechanisms, contributing to SCFA formation in the human gut. Shaded geometric shapes summarize routes of formation for each of the three main SCFA: acetate, propionate, and butyrate.

computational model (El-Semman et al., 2014) and was recently demonstrated *in vitro* (Rios-Covián et al., 2015). Moreover, a recent animal study suggests that *F. prausnitzii* is able to use the acetate produced by *Bacteroides thetaiotaomicron in vivo*, this interaction having a significant impact in the modulation of the intestinal mucus barrier (Wrzosek et al., 2013). Although lactate is not a SCFA, it is used by some butyrate and propionate producing bacteria, avoiding metabolic acidosis in the host (El Aidy et al., 2013). Several *in vitro* studies confirm that lactate and/or acetate produced by *Bifidobacterium* when grown in oligofructose, is used by members of *Roseburia, Eubacterium,* and *Anaeroestipes* genera (Duncan et al., 2004a,b; Belenguer et al., 2006; Falony et al., 2006). Members of *Veillonella* and *Propionibacterium* are capable of transforming lactate to propionate *in vitro* (Counotte et al., 1981). H_2 plays an important role in cross-feeding as well. Co-cultures of *Roseburia intestinalis* with the methanogen *Methanobrevibacter smithii* and the acetogen *Blautia hydrogenotrophica,* resulted in a decrease of final H_2 and the production of CH_4 and acetate. The acetate formed is used by *R. intestinalis* to produce butyrate (Chassard and Bernalier-Donadille, 2006). *Ba. thetaiotaomicron* bi-associated mice with *Bl. hydrogenotrophica* showed higher levels of acetate in caecal contents and lower NADH/NAD^+ ratio; the removal of H_2 by *B. hydrogenotrophyca* in this case allows *Ba. thetaoitaomicron* to regenerate NAD+ (Rey et al., 2010).

IMPACT OF DIET ON GUT MICROBIOTA COMPOSITION AND SCFA PRODUCTION

Diet affects the gut microbiota composition and activity, and therefore the profile of SCFA and BSCFA synthesized, this having a deep impact on human health (Brussow and Parkinson, 2014; Louis et al., 2014). The first work linking the long-term diet style with the so-called human "enterotypes" was published in 2011 (Wu et al., 2011) but it has been also demonstrated that short-term diets can alter the human gut microbiome (David et al., 2014). The amount and relative abundance of SCFA may be considered as biomarkers of a healthy status (**Table 1A**). For example, high fiber-low fat and meat diets are characterized by the presence of higher amounts of fecal SCFA than diets with reduced fiber intake (De Filippo et al., 2010; Cuervo et al., 2013; Ou et al., 2013). A reduction in fecal butyrate has been found in patients with colorectal adenocarcinoma (Chen et al., 2013), whereas obesity has been related with increases in total fecal SCFA concentration (Fernandes et al., 2014; Rahat-Rozenbloom et al., 2014) which tend to decrease following an anti-obesity treatment (Patil et al., 2012). These epidemiological data have been further supported by dietary intervention studies carried out with different human populations (**Table 1B**). Prebiotic substrates that selectively promote the growth of beneficial microbiota also induce changes in SCFA production of healthy individuals (Lecerf et al., 2012) and in patients with irritable

bowel syndrome or those receiving enteral nutrition (Majid et al., 2011; Halmos et al., 2015). Interestingly, the consumption of dairy products fermented with beneficial bacteria also modifies the intestinal microbiota toward more butyrate producers in comparison to chemically-acidified milk (Veiga et al., 2014). Finally, dietary intervention studies carried out with different overweight and obese populations seemed to be effective in lowering the high levels of fecal SCFA associated with the obesity status (Salazar et al., 2015).

Although animal and human trials provide the best models for studying the influence of diet on the gut microbiota, *in vitro* fecal cultures constitute simpler approaches for investigating the interactions of diet and food components with the intestinal microbiota. Available *in vitro* models range from simple batch fermentation (Salazar et al., 2009; Arboleya et al., 2013b) to complex multi-stage continuous culture systems. The SHIME (Van Den Abbeele et al., 2010) and SIMGI models (Barroso et al., 2015) simulate the digestion from stomach to colon whereas the EnteroMix (Makivuokko et al., 2005) and the Lacroix models mimic the entire colonic process. TIM-2 reproduces the proximal colon and incorporates a dialysis membrane that simulates absorption of microbial metabolites by the body (Minekus et al., 1999). A microbial bias regarding butyrate and propionate producers occurs with some of these models (Van Den Abbeele et al., 2010), that could be alleviated by incorporating a simulation of the intestinal mucosa surface (Van Den Abbeele et al., 2013a). Labelling substrates with the stable isotope ^{13}C makes possible to link the fermentation with specific members of the microbiota and to quantify production of metabolites (Maathuis et al., 2012) whilst the mathematical modeling is becoming a useful tool to study microbe-diet-host interactions (Shoaie et al., 2015).

When studying *in vitro* the influence of dietary components on microbial composition, the main aim usually is to increase beneficial bacteria and to enhance the production of SCFA whereas minimizing the synthesis of BSCFA. The fermentation of different substrates has been evaluated, ITF being the most studied (Sivieri et al., 2014). Starch (Fassler et al., 2006), arabinans, arabinoxylans (Van Den Abbeele et al., 2013b), galactooligosaccharides (Rodriguez-Colinas et al., 2013), xylitol (Makelainen et al., 2007), and lactulose (Cardelle-Cobas et al., 2009) have been also considered. The influence of polyphenols on the gut microbiota metabolism is currently receiving considerable attention (Valdés et al., 2015). Different microbial fermentation patterns can be obtained depending on physico-chemical characteristics of the substrates, speed of fermentation and the microbial populations involved in the process (initial breakdown of long polymers, direct fermentation of substrates, and cross-feeding interactions; Hernot et al., 2009; Zhou et al., 2013; Puertollano et al., 2014). Probiotics and their extracellular components (exopolysaccharides), can also act as modulators of SCFA microbial formation (Salazar et al., 2009; Van Zanten et al., 2012). In addition, a large number of studies highlight the influence of different foods and long-term diets on the intestinal microbiota activity and specifically, over the pattern of SCFA (Yang and Rose, 2014; Costabile et al., 2015).

The basal microbiota composition has also a profound influence on the final effects exerted *in vitro* by diet on microbial populations and metabolic activity (Arboleya et al., 2013a; Souza et al., 2014). In this regard, it has been found a different response to probiotics and prebiotics by the microbiota of individuals from different groups of age (Arboleya et al., 2013a; Likotrafiti et al., 2014), or between obese and lean people (Yang et al., 2013).

BIOLOGICAL EFFECTS OF SCFA

One of the health effects attributed to the production of SCFA is the concomitant reduction of the luminal pH, which by itself inhibits pathogenic microorganisms and increases the absorption of some nutrients (Macfarlane and Macfarlane, 2012). Acetate has been found to be a key player in the ability of bifidobacteria to inhibit enteropathogens (Fukuda et al., 2011). Moreover, butyrate fuels the intestinal epithelial cells and increases mucin production which may result in changes on bacterial adhesion (Jung et al., 2015) and improved tight-junctions integrity (Peng et al., 2009). Thus, the production of SCFA seems to play an important role in the maintenance of the gut barrier function.

After their production, SCFA will be absorbed and used in different biosynthetic routes by the host (Den Besten et al., 2013b). During the intestinal absorption process part of the SCFA, mainly butyrate, will be metabolized by the colonocytes (Pryde et al., 2002) whilst the rest will be transported by the hepatic vein and go into the liver, where they will be metabolized (Den Besten et al., 2013b). These SCFA will enter diverse CHO and lipid metabolic routes; propionate will mainly incorporate into gluconeogenesis whilst acetate and butyrate will be mostly introduced into the lipid biosynthesis. The involvement of SCFA in energy and lipid metabolism attracted the attention of researchers toward the potential role of SCFA in the control of metabolic syndrome. A reduction in obesity and insulin resistance in experimental animals on high-fat diet after dietary supplementation with butyrate has been observed (Gao et al., 2009). This protective effect of SCFA on the high-fat diet-induced metabolic alterations seems to be dependent on down-regulation of the peroxisome proliferator-activated receptor gamma (PPARγ), therefore promoting a change from lipid synthesis to lipids oxidation (Den Besten et al., 2015). Interestingly although the three main intestinal SCFA have a protective effect on diet-induced obesity, butyrate and propionate seem to exert larger effects than acetate (Lin et al., 2012). Different mechanisms have been proposed to explain these effects, the activation of signaling pathways mediated by protein kinases, such as AMP-activated protein kinase (Gao et al., 2009; Peng et al., 2009; Den Besten et al., 2015) or mitogen-activated protein kinases (MAPK; Jung et al., 2015), being a common observation. Butyrate and propionate, but not acetate, have been reported to induce the production of gut hormones, thus reducing food intake (Lin et al., 2012). Acetate has also been found to reduce the appetite, in this case through the interaction with the central nervous system (Frost et al., 2014). However, in spite of these promising animal data, controlled human intervention studies are still needed before drawing firm conclusions (Canfora et al., 2015).

TABLE 1 | (A) Epidemiological studies, carried out since 2010, showing the impact of diet on SCFA produced by the gut microbiota. The shaded areas indicate a change in the populations analyzed in terms of their health status. D, days; y, year. (B) Intervention studies, carried out since 2010, showing the impact of diet on SCFA produced by the gut microbiota. The shaded areas indicate a change in the populations analyzed in terms of their health status. D, day; w, week; m, month; y, year.

(A)

Subjects, age (*n*)	Parameters determined	Main results	References
•European children, 1–6 y (15) •Burkina Faso (BF) (rural) children (15)	3-d dietary questionnaire (from EU parents) and interview on diet (from BF mothers), fecal samples	**BF children**: ↑SCFA;↑*Bacteroidetes*, ↓*Firmicutes*, ↓*Enterobacteriaceae*; unique *Prevotella*, *Xylanibacter* (lacking in EU)	De Filippo et al., 2010
•Healthy African Americans, 50–65 y (12) •Healthy South Africans (12)	Fresh fecal samples, microbiota and SCFA analysis, cancer biomarkers	**Native Africans**: ↑SCFA, total bacteria, major butyrate-producing groups, dominance of *Prevotella* **African-Americans**: dominance of *Bacteroides*	Ou et al., 2013
•Healthy elderly, 76–95 (32)	Food frequency questionnaire, fecal SCFA analysis	**Correlation fiber and SCFA**: Potato intake with total SCFA and apple with propionate	Cuervo et al., 2013
•Overweight (OWO) (11) •Lean (11)	3-d diet record, fresh fecal sample, SCFA absorption measure	**OWO**: ↑Age-adjusted fecal SCFA concentration, not due to higher absorption rate	Rahat-Rozenbloom et al., 2014
•Overweight (OWO) (42) •Lean (52)	3-d diet records, physical activity questionnaires, fecal samples	**OWO:** ↑ SCFA; dietary intakes and physical activity levels did not differ	Fernandes et al., 2014
•Indian individuals, 21–62 y (20): lean (5), normal (5), obese (5), surgically treated obese (5)	Fresh fecal samples, microbiota, and SCFA analysis	**Obese**: ↑ SCFA,↑*Bacteroides* **Treated-obese**: ↓SCFA ↓*Bacteroides*	Patil et al., 2012
•Advanced colorectal adenoma patients (A-CRA) (344) •Healthy control (344)	Dietary fiber intake, fecal SCFA, and microbiota analysis	**A-CRA group**: ↓SCFA production, ↓butyrate and butyrate-producing bacteria	Chen et al., 2013
•Celiac disease (CD) patients: normal diet, 13–60 y (10) and gluten-free, 21–66 y (11) •Healthy, 24–42 y (11)	Fresh fecal samples, microbiota, and SCFA analysis	**Untreated CD and treated CD**: ↑ SCFA than healthy **Treated CD patients**: ↓*Lactobacillus* and *Bifidobacterium* diversity	Nistal et al., 2012

(B)

Subjects, age (*n*)	Intervention diet (period)	Main outcomes	References
•Healthy African Americans, 50–65 y (20) •Healthy South Africans, 50–65 y (20)	Own diet (2 w) followed by exchange to high-fiber, low-fat African-style (2 w) Own diet (2 w) followed by high-fat, low-fiber Western-style (2 w)	**African style diet**: ↑ butyrate; reciprocal changes in colon cancer risk biomarkers	O'keefe et al., 2015
•Healthy volunteers (23)	Cross-over: high red meat (HRM) diet vs. HRM plus butyrylated high-amylose maize starch (HAMSB) (4/4 w wash-out)	**HRM+HAMSB diet**:↑ excretion of SCFA and microbiota composition changes	Le Leu et al., 2015
•Healthy active volunteers (51)	Parallel-groups: butyrylated high amylose maize starch (HAMSB) vs. low-AMS (28 d)	**HAMSB diet**:↑free, bound and total butyrate and propionate	West et al., 2013
•Healthy volunteers, 20–50 y (17)	Cross-over: whole-grain (WG) vs. refined grain (2/5 w wash out)	**WG diet**: ↑acetate and butyrate	Ross et al., 2013
•Healthy volunteers, 18–85 y (63)	Cross-over: wheat bran extract (WBE) (3 or 10 g WBE) vs. placebo (0 g WBE; 3 w, 2 w wash-out)	**Daily intake of 10 g WBE**:↑bifidobacteria;↑ fecal SCFA and ↓ fecal pH	Francois et al., 2012
•Healthy volunteers, 18–24 y (60)	Parallel-groups: xylo-oligosaccharide (XOS) vs. inulin-XOS mixture (INU-XOS) vs. placebo (maltodextrin; 4 w)	**XOS**: ↑bifidobacteria and butyrate, and ↓acetate **INU-XOS**: ↑SCFA and propionate, and maintain acetate level	Lecerf et al., 2012
•Ulcerative colitis (UC) remission patients (19) •Healthy volunteers (10)	Cross-over: Australian diet vs. plus wheat bran-associated fiber and high amylose-associated resistant starch (8 w)	**Intervention diet**: did not correct the low gut fermentation in patients with UC	James et al., 2015
•Irritable bowel syndrome (IBS) with constipation woman, 20–69 y (32)	Parallel-groups: Milk acidified product (MP) vs. Fermented Milk product (FMP) (4 w)	**FMP**:↑potential butyrate producers, and ↑Total SCFA *in vitro*↑butyrate	Veiga et al., 2014

(Continued)

TABLE 1 | Continued

(B)

Subjects, age (*n*)	Intervention diet (period)	Main outcomes	References
•IBS patients (27) •Healthy volunteers (6)	Cross-over: Australian diet vs. low FODMAP (Fermentable Oligo-, Di-, Mono-saccharides And Polyols) diet (21/21 d wash-out)	**Australian diet**:↑ relative abundance *Clostridium* cluster XIVa (butyrate-producer) **Low FODMAP diet**:↓total bacterial abundance	Halmos et al., 2015
•Cow's milk protein allergy infants (16) Healthy infants (12)	Cross-over:hydrolysed whey protein formula (eHF) without lactose vs. eHF containing 3.8% lactose (2 m)	**Addition of lactose**: ↑SCFA**;** ↑LAB and bifidobacteria; ↓*Bacteroides*/clostridia	Francavilla et al., 2012
•Obese women 18–65 y (30)	Parallel-groups: ITF vs. placebo (maltodextrin) (3m)	**ITF**:↓ total SCFA, acetate and propionate; ↑bifidobacteria	Salazar et al., 2015
•Obese men, 27–73 y (14)	Cross-over: high type III resistant starch (3 w) or high in wheat bran (3 w) and ended with weight-loss (low fat and carbohydrate, high protein, 3 w)	**Diet**: only explain 10% total variance in microbiota; amount of propionate correlated with *Bacteroidetes*	Salonen et al., 2014
•Obese volunteers, 45–77 y (6)	Cross-sectional: strict vegetarian diet (1 m)	↓SCFA;↓*Firmicutes/Bacteroidetes* ratio; ↑*Clostridium* clusters XIVa-IV; ↓*Enterobacteriaceae*	Kim et al., 2013
•Obese men, 21–74 y (17)	Cross-over: high-protein moderate-carbohydrate (HPMC) vs. high-protein low-carbohydrate (HPLC) (maintenance diet 7 d, 4 w)	**HPMC and HPLC diets**: ↑BSCFA (respect maintenance diet) **HPLC diet**: ↓butyrate and ↓*Roseburia/E.rectale*	Russell et al., 2011
•High Metabolic Syndrome risk volunteers (88)	Parallel-groups: High saturated fat (HS) vs. high monounsaturated fat (MUFA)/high glycaemic index (GI) (HM/HGI) vs. high MUFA/low GI (HM/LGI) vs. high carbohydrate (CHO)/high GI (HC/HGI) vs. and high CHO/low GI (HC/LGI) (24 w)	**High carbohydrate diets** (regardless GI):↑saccharolytic bacteria (including *Bacteroides* and *Bifidobacterium*) **High fat diets**:↓bacterial numbers **High saturated fat diet**:↑excretion of SCFA	Fava et al., 2013
•Hospitalized patients under enteral nutrition (41)	Parallel-groups: standard enteral formula vs. standard formula enriched FOS and fiber (12 d)	**FOS/fiber-enriched formula**: ↑butyrate	Majid et al., 2011

It has also been observed that SCFA protect against the development of colorectal cancer (CRC), with most studies focusing on butyrate (Canani et al., 2011; Keku et al., 2015). Butyrate promotes colon motility, reduces inflammation, increases visceral irrigation, induces apoptosis, and inhibits tumor cell progression (Zhang et al., 2010; Canani et al., 2011; Leonel and Alvarez-Leite, 2012; Keku et al., 2015), all of these properties being beneficial in CRC prevention. In cancerous colonocytes, due to the Warburg effect, butyrate accumulates, which increases its activity as inhibitor of histone deacetylation, promoting apoptosis of CRC cells. Interestingly, a recent animal study suggests that the protective effect of dietary fiber upon CRC is dependent on the production of butyrate by the microbiota (Donohoe et al., 2014).

In addition, butyrate and propionate have also been reported to induce the differentiation of T-regulatory cells, assisting to control intestinal inflammation; this effect seems to be mediated via inhibition of histone deacetylation (Donohoe et al., 2014; Louis et al., 2014). This control of intestinal inflammation may result beneficial in terms of gut barrier maintenance, reducing the risk of inflammatory bowel disease or CRC. Unlike what happens with the three main intestinal SCFA, acetate, propionate, and butyrate, little is known about the potential health effects of other SCFA.

CONCLUDING REMARKS

The main role of diet is to provide enough macro- and micronutrients to fulfill daily requirements and well-being. However, during the last decades the association between dietary intake and physiology has been increasingly-recognized, although many of the molecular and immunological aspects by which dietary components could influence human health remain still largely unknown. Bacterial fermentation of CHO and proteins produces SCFA which emerge as major mediators in linking nutrition, gut microbiota, physiology and pathology. Many biological effects seem to be mediated by these bacterial metabolites but a conclusive proof is not available for many of the health claims made for SCFA. Promising *in vitro* and animal studies have been published but they cannot be easily extrapolated to the human situation. The design of improved approaches combining *in vitro*, *in vivo,* and "omics" technologies should be carried out, with emphasis in human intervention trials, to explore the mechanisms of production and action of SCFA, thus opening the possibility to find strategies for developing personalized nutrition.

AUTHOR CONTRIBUTIONS

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

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