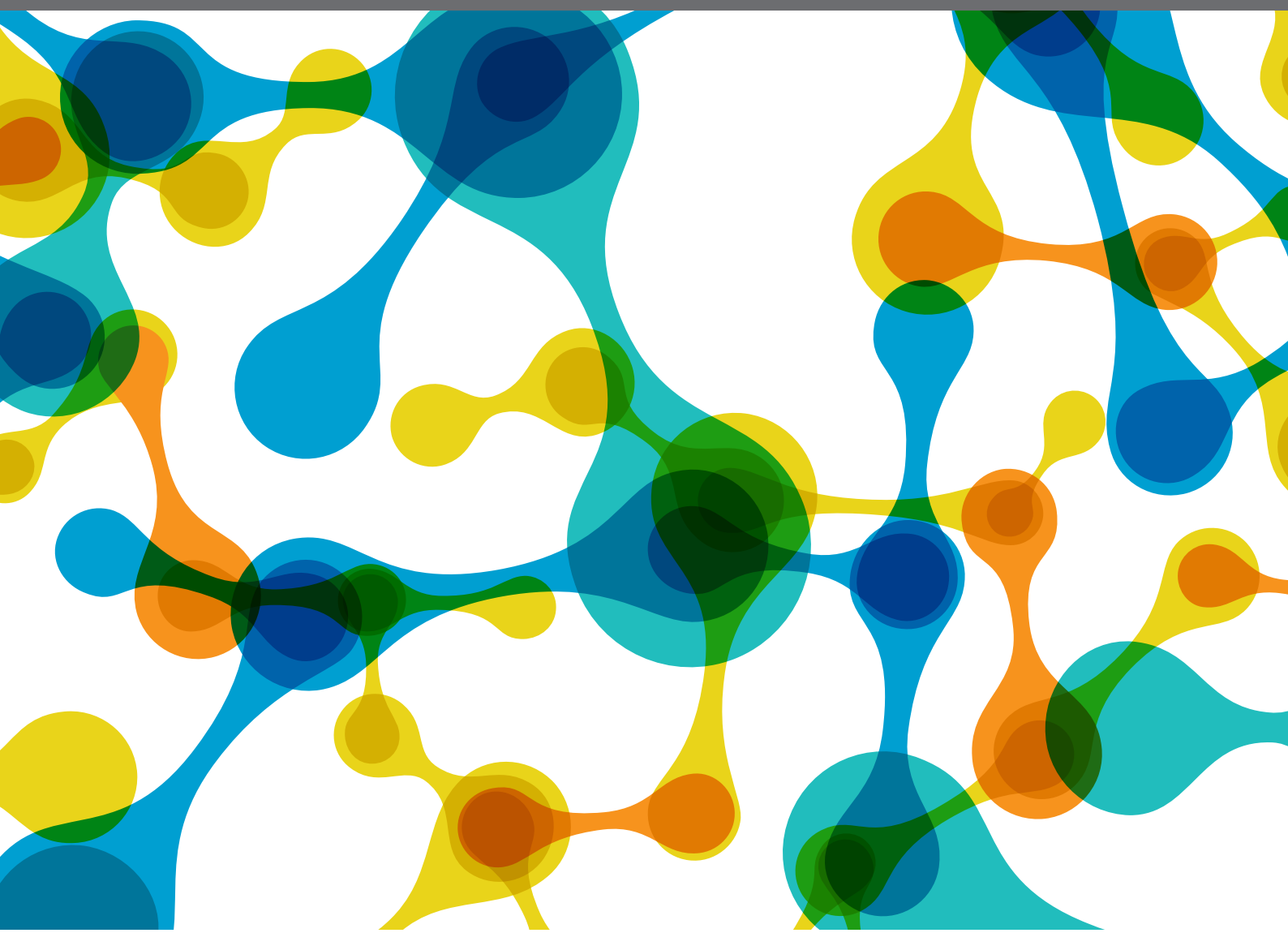
XENOBIOTICS AND THE GUT MICROBIOME IN HEALTH AND DISEASE

EDITED BY: Stephen J. Pandol and Zhaoping Li
PUBLISHED IN: Frontiers in Physiology, Frontiers in Microbiology and Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88963-082-0
DOI 10.3389/978-2-88963-082-0

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XENOBIOTICS AND THE GUT MICROBIOME IN HEALTH AND DISEASE

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Citation: Pandol, S. J., Li, Z., eds. (2019). Xenobiotics and the Gut Microbiome in Health and Disease. Lausanne: Frontiers Media. doi: 10.3389/978-2-88963-082-0

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frontiers in Physiology

DATA REPORT
published: 27 November 2018
doi: 10.3389/fphys.2018.01689

Complete Genome Sequencing of *Lactobacillus plantarum* ZLP001, a Potential Probiotic That Enhances Intestinal Epithelial Barrier Function and Defense Against Pathogens in Pigs

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Keywords: ***Lactobacillus planatrum*, complete genome sequencing, intestinal barrier, probiotic, comparative genome analysis**

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: *21 August 2018*
Accepted: *09 November 2018*
Published: *27 November 2018*

Citation:
Zhang W, Ji H, Zhang D, Liu H, Wang S, Wang J and Wang Y (2018) Complete Genome Sequencing of Lactobacillus plantarum ZLP001, a Potential Probiotic That Enhances Intestinal Epithelial Barrier Function and Defense Against Pathogens in Pigs. Front. Physiol. 9:1689. doi: 10.3389/fphys.2018.01689

INTRODUCTION

The mammalian gastrointestinal tract is a heterogeneous ecosystem with the most abundant, and one of the most diverse, microbial communities. The gut microbiota, which may contain more than 100 times the number of genes in the human genome, endows the host with beneficial functional features, including colonization resistance, nutrient metabolism, and immune tolerance (Bäckhed, 2005). Dysbiosis of gut microbiota may result in serious adverse consequences for the host, such as neurological disorders, cancer, obesity, malnutrition, inflammatory dysregulation, and susceptibility to pathogens (Turnbaugh et al., 2006; Malo et al., 2010; Wang T. et al., 2012; Subramanian et al., 2014; Sampson et al., 2016).

Lactic acid bacteria (LAB) function as probiotics. *Lactobacillus plantarum*, an important member of the family of LAB, is commonly found in fermented food and as a commensal bacterium in the gut microbiota. The beneficial effects of probiotics are strain-specific (Ramos et al., 2013). Some *L. plantarum* strains were demonstrated to confer various beneficial properties by improving growth performance and promoting gut health, and has been used for the prevention or treatment of various diseases, including inflammatory bowel disease [strain Lp91; (Duary et al., 2012)], uremia [strain AD3; (Patra et al., 2018)] and liver damage [strain C88; (Duan et al., 2018)] in humans and animals. The beneficial effects of *L. plantarum* are associated with the regulation of the immune response [strain 426951; (Soltani et al., 2017)], maintenance of gut microbiota homeostasis [strain ZDY04; (Qiu et al., 2018)], and enhancement of epithelial barrier function [strain 299v; (Barnett et al., 2018)]. In our previous study, *L. plantarum* ZLP001 strain isolated from the gastrointestinal mucosa of a healthy weaned piglet was demonstrated to exhibit high antioxidant ability, and the dietary supplementation of *L. plantarum* ZLP001 was shown to improve growth performance and antioxidant status of weaned piglets (Wang J. et al., 2012). *L. plantarum* ZLP001 inhibits growth and adhesion of enterotoxigenic *Escherichia coli* and enhances host defense by strengthening intestinal epithelial barrier function and innate immune response to secret antimicrobial peptides (Wang J. et al., 2018).

Lactobacillus plantarum is a highly flexible and versatile species and has one of the largest genomes among the known LAB. Consistent with the diversity of probiotic functions, complete genome sequencing analysis revealed the genomic diversity and environment specialization of

L. plantarum strains isolated from different niches, including plant (Liu et al., 2015), dairy (Zhang et al., 2015), or vegetable (Crowley et al., 2013) fermentations, as well as saliva (Kleerebezem et al., 2003) and gut (Li et al., 2013). To gain a better insight into the beneficial effects on gut health in piglets, the complete genome of *L. plantarum* ZLP001 was sequenced and a comparative genome analysis study was conducted between *L. plantarum* ZLP001 and other available *L. plantarum* genomes.

MATERIALS AND METHODS

Bacterial Growth and DNA Extraction

Lactobacillus plantarum ZLP001 from single colony was grown in De Man, Rogosa, and Sharpe broth (Oxoid, Hampshire, UK) for 18 h at 37°C under microaerophilic condition. Total genomic DNA was extracted and purified using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

Genome Sequencing, Assembly, and Annotation

A 8–12 kb DNA library was constructed and sequenced using single molecular real-time (SMRT) sequencing technology with C4 chemistry and P6 DNA polymerase on the PacBio RS II system (Pacific Biosciences, Menlo Park, CA) by Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China). Raw sequence data were filtered using SMRT Analysis v2.3.0. A total of 73,238 subreads with a median length of 8,661 bp were obtained for *de novo* assembly using the SOAPdenovo v2.04 (Luo et al., 2012). Genes were predicted using Glimmer v3.02 (www.cbcb.umd.edu/software/glimmer) and the corresponding function annotation was completed by blasting genes against Cluster of Orthologous Groups of proteins (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Tandem repeats were predicted using Tandem Repeat Finder v4.04, and the minisatellite and microsatellite DNAs were selected based on the number and length of repeat units. In addition, rRNA, tRNA, and sRNA were predicted using rRNAmmer v1.2, tRNAscan v1.23, and Rfam v10.1, respectively. Genome visualization was performed using Circos v0.69-6. Possible prophage sequences were searched using PHAST (http://phast.wishartlab.com/index.html), and clustered regularly interspaced short palindromic repeats (CRISPR) were predicted using MinCED 3 (https://sourceforge.net/projects/minced/). Carbohydrate active enzymes (CAZymes) were searched against the CAZy database (http://www.cazy.org/).

Phylogenetic and Ortholog Clustering Analyses

For phylogenetic and comparative genome analyses, a total of 18 complete genome sequences of *L. plantarum* strains were obtained from the NCBI database (**Table 1**). An orthologous gene set was built to identify the core-genome and pan-genome sizes using OrthoMCL package v2.0 (Li et al., 2003). All predicted protein sequences were merged together and compared with each other using BLASTP algorithm, with an *E*-value cutoff of 1e−5 and a percent match $\geq$ 50%. All homologous protein pairs were parsed and grouped into orthologous families by cluster tool MCL, with an inflation value of 1.5.

The phylogenetic tree based on 16S rRNA gene sequences was constructed using the neighbor-joining method by MEGA software. The predicted amino acid sequences of each single copy orthologous gene family were aligned using MAFFT v7 (https://mafft.cbrc.jp/alignment/software/). The individual alignments were concatenated into a string of amino acid sequence alignment and the concatenated alignment data were submitted to RAxML (https://github.com/stamatak/standard-RAxML) to build phylogenomic trees with the maximum-likelihood algorithm. The bootstrap method of 1,000 bootstrap repetitions was used to assess tree reliability.

Data Accession Number

The raw and assembled sequence data for *L. plantarum* ZLP001 genome have been deposited at SRA database under the accession number PRJNA381357 (SRP102895) and GenBank under the accession number CP021086, respectively. The strain has been deposited at the China General Microbiological Culture Collection Center (CGMCC no. 7370).

RESULTS AND DISCUSSION

General Genome Features of *L. plantarum* ZLP001 Genome

As shown in **Figure 1A**, the complete genome of *L. plantarum* ZLP001 contained a single circular chromosome of 3,164,369 bp with a GC content of 44.65% and seven plasmids, namely, A (67,802 bp), B (48,418 bp), C (31,389 bp), D (27,860 bp), E (16,139 bp), F (15,258 bp), and G (13,837 bp), with an average GC content of 42.05%. A total of 3,264 protein-coding sequences (CDSs) were identified. Of these, 3,104 genes with an average length of 886 bp were on the chromosome that occupied 83.28% of the genome and 77, 56, 34, 33, 17, 22, and 21 CDSs with an average length of 614 bp were found in the plasmid ZLP001 plasmid A to plasmid G, respectively. The chromosome contained 16 rRNAs, 69 tRNAs, 91 tandem repeats, 56 minisatellite DNAs, and 7 microsatellite DNAs. A total of 10 sRNAs, 47 tandem repeats, and 27 minisatellite DNAs were found in the plasmids.

Functional Classification

A total of 1,603 CDSs were classified into 39 KEGG functional categories, mainly functioning in replication and repair, carbohydrate metabolism, amino acid metabolism, and signal transduction (**Figure 1B**). Furthermore, 1,783 CDSs were specifically assigned to clusters of COG comprising 20 categories (**Figure 1B**). Most genes were classified into function categories for carbohydrate transport and metabolism (200 genes), amino acid transport and metabolism (199 genes), replication, recombination, and repair (150 genes), translation, ribosomal structure, and biogenesis (141 genes), transcription (120 genes), and inorganic ion transport and metabolism (101 genes).

TABLE 1 | Genome summary of *Lactobacillus plantarum* strains.

Strain	Source	Size (Mb)	CDS	GC%	GenBank No.	Probiotic property	References
ZLP001	Weaned piglet gut	3.16	3,264	44.37	CP021086	Inhibit *E. coli* adhesion and maintain tight junction barrier in pigs	Wang J. et al., 2018
WCFS1	Human saliva	3.31	3,042	44.50	AL935263.2	Increase leptin and T cell level to ameliorate high fat diet-induced pathology in mice	Ivanovic et al., 2015
JDM1	Human intestinal tract	3.20	2,948	44.66	CP001617.1	No report	
ST-III	Kimchi	3.25	3,013	44.58	CP002222.1	Inhibit growth of *Streptococcus mutans* from children with active caries	Lin et al., 2017
ZJ316	Healthy newborn infant feces	3.20	3,159	44.65	CP004082.1	Improve pig growth and pork quality	Suo et al., 2012
P-8	Fermented raw cow milk	3.03	3,140	44.80	CP005942.2	Improve growth performance and activate intestinal immune response in chickens	Wang et al., 2015
16	Malt production steep water	3.04	2,787	44.74	CP006033.1	Possess potent antifungal activity	Crowley et al., 2012
5-2	Fermented soybean	3.24	3,114	44.70	CP009236.1	Possess high autoaggregation properties and inhibit growth of *Gardnerella vaginalis*	Pessoa et al., 2017
B21	Vietnamese sausage	3.28	2,930	44.47	CP010528.1	Produce bacteriocins against a wide range of Gram-positive bacteria	Golneshin et al., 2015
LZ95	Newborn infant fecal	3.32	2,951	44.49	CP012122.1	Produce extracellular vitamin B12	Li et al., 2017
HFC8	Human gut	3.41	3,447	44.33	CP012650.1	No report	
CCUG 36733	Human oral samples	3.05	2,542	44.38	CP014228.1	No report	
LZ227	Raw cow milk	3.13	3,262	44.71	CP015857.1	Produce both riboflavin and folate	Li et al., 2016b
LZ206	Raw cow milk	3.21	2,837	44.64	CP015966.1	Possess antimicrobial activity against various pathogens	Li et al., 2016a
KLDS1.0391	Fermented dairy products	2.90	2,902	44.70	CP019348.1	No report	
LPL-1	Fermented fish	3.19	2,932	44.65	CP021997.1	Produce class IIa bacteriocin against *Listeria monocytogenes* 54002	Wang Y. et al., 2018
BDGP2	Drosophila melanogaster gut	3.41	3,148	44.24	CP023174.1	No report	
10CH	Cheese	3.31	3,192	44.51	CP023728.1	Possess antimicrobial activity against various pathogens	El Halfawy et al., 2017
LQ80	Fermented liquid feed for pigs	3.23	3,186	44.66	CP028977.1	Enhance immune response with increased immunoglobulin A, M and G in pigs	Mizumachi et al., 2009

Phylogenetic Relationships Among *L. plantarum* Strains

As shown in the phylogenetic tree constructed based on the 16S rRNA gene sequence, *L. plantarum* members were grouped and distinguishably separated from other *Lactobacillus* genus strains (**Figure 1C**). The strain ZLP001 displayed more than 99% similarity with other *L. plantarum* strains based on the 16S rRNA gene sequence. *L. plantarum* genomes were difficult to distinguish by 16S rRNA gene sequence similarity. To further understand the phylogenetic relationship among *L. plantarum* strains, a phylogenetic tree based on the single copy orthologous gene families was built (**Figure 1D**). A total of 553 single copy orthologous gene families were reported. *L. plantarum* ZLP001 showed a close relationship with the strains BDGP2, JDM1, and LZ95, but was located on a relatively standalone branch. This observation suggests that the strain ZLP001 has a distinguishing pattern of genomic evolution in order to adapt to the gut environment.

Core- and Pan-Genomes of *L. plantarum* Strains

OrthoMCL results showed that 19 genomes had a pan genome size of 6,598 orthologous gene families and a core genome size of 596 (9.03%) orthologous gene families (**Figure 1E** and **Table S1**). A total of 4,141 orthologous gene sets were constructed. The pan genome size was 1.36 times the average size of these 19 genomes and the core genome constituted 19.59% of each *L. plantarum* genome. A total of 2,597 (39.36%) strain-specific genes were found, and the number of strain-specific genes ranged from 13 genes only in strain 10CH to 1,306 genes unique to strain CCUG36733. A total of 65 genes were unique to ZLP001 strain (**Table S2**), including 30 genes encoding hypothetical proteins. The formation of a large gene pool for these *L.*

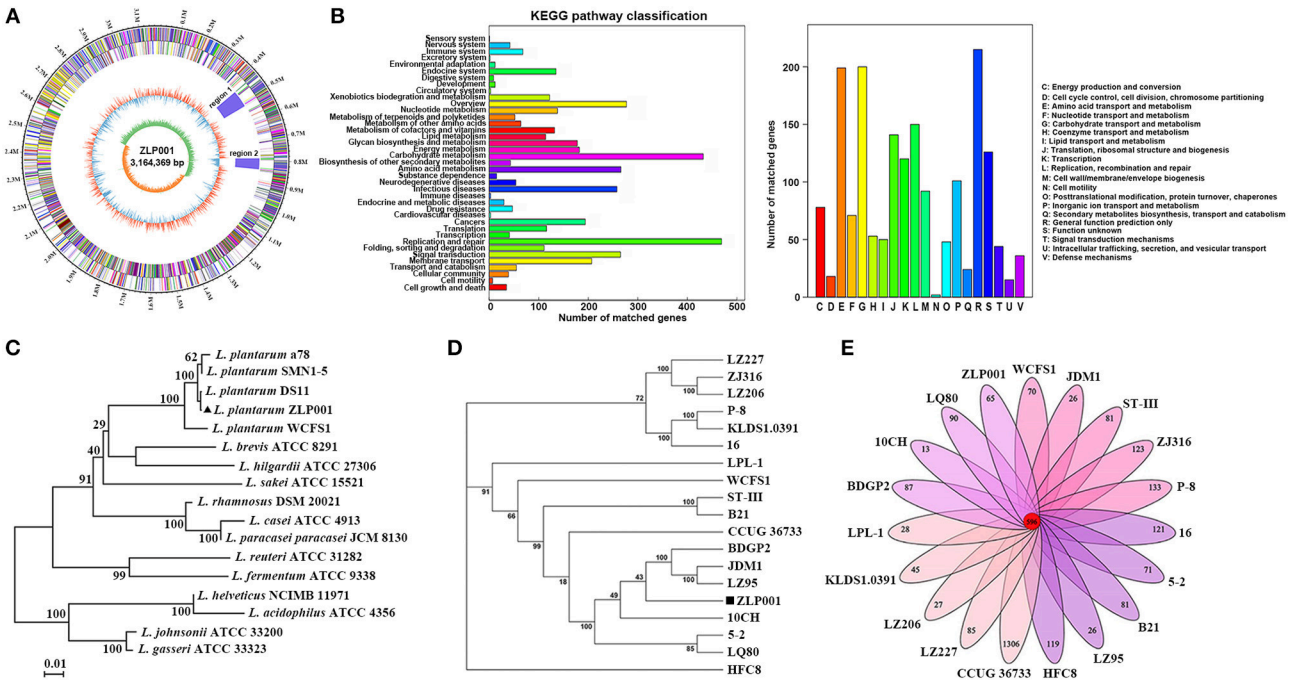

FIGURE 1 | Genome features of *L. plantarum* ZLP001. **(A)** Circular genomic map of *L. plantarum* ZLP001. The circular map was generated using Circos and contains seven circles. Marked information is displayed from the outer circle to the innermost circle as follows: Genome size, CDSs on the forward stand, CDSs on the reverse stand, prophage regions, rRNA and tRNA, GC content, and GC skew. **(B)** Gene number of KEGG and COG categories. **(C)** The neighbor-joining tree of *L. plantarum* ZLP001 based on the 16S rRNA gene sequence. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replications. Bar, 0.01 nucleotide substitution per site. **(D)** Phylogenetic tree of 19 *L. plantarum* strains. The phylogenetic tree was built based on aligned concatenated sequences of single copy orthologous gene families. The bootstrap support value before each node represents the confidence degree of each branch. **(E)** Numbers of orthologous gene families and unique genes among 19 *L. plantarum* strains. The Venn diagram shows the number of orthologous gene families of the core genome (the center part) and the numbers of unique genes of each genome. The different colors indicate different sampling areas of the strains as indicated. The orthologous gene families were determined by OrthoMCL software with an inflation value of 1.5.

plantarum members implies their open pan genome structures. This property, to a certain extent, endows these members with the capability of adaptation to the surrounding environments or their hosts.

Lifestyle Adaptation to Stress

Lactobacillus plantarum ZLP001 carries several genes encoding stress-related proteins. ZLP001 encoded genes for Na^+:H^+ antiporter and choloylglycine hydrolase, providing the evidences of the tolerance of *L. plantarum* ZLP001 to low pH and bile salt in the gastrointestinal environment. *L. plantarum* ZLP001 also carried various genes encoding heat stress proteins, including heat shock protein 10 (Hsp20, gene 0190, gene 1178, gene 2786, and gene 2876) and Hsp33 (gene 0295, gene 0414, gene 1058, and gene 2105), heat shock protein Htpx (gene 0076, gene 0632, gene 1622, gene 1079, and gene 2136), and molecular chaperone DnaK (gene 0299, gene 0873, gene 0901, gene 1193, and gene 1501). The whole genome analysis suggests the strong potential of *L. plantarum* ZLP001 proliferation and genetic tolerance and lifestyle adaptation to detrimental stress in the gut.

Transport and Secretion System

Lactobacillus plantarum ZLP001 genome harbored 306 genes related to transport system, which mainly constitutes the phosphotransferase system (PTS) and ATP-binding cassette (ABC) transporter system (**Table S3**).

Of these transporters, 54 genes were related to the genomic PTS system. *ptsH* (gene 1521) encoded for the phosphocarrier protein HPr, which delivered phosphoryl groups from phosphoenol pyruvate to PTS EII enzymes (EIIs). A total of 10 complete phosphoenolpyruvate-dependent PTS EII complexes were present in ZLP001 genome that were involved in the transport of carbon sources, including β-glucosides, cellobiose, fructose, galactitol, glucose, mannitol, mannose, N-acetylgalactosamine, sorbitol, and sucrose. These sugar PTS systems have the ability to import more than one substrate and expand the carbon transport capacity of *L. plantarum*.

A total of 252 genes involved in the genomic ABC transporter system components were found in ZLP001 genome. Many of these ABC importers transport inorganic ions, peptides, and amino acids, whereas the substrate specificity of most of the exporters was unknown, as described for *L. plantarum* WCSF1 (Kleerebezem et al., 2003) and 5–2 (Liu et al., 2015). The ZLP001 chromosome harbored 10 transporters for the uptake of branched-chain amino acids, including an ABC transporter encoded by the *livHKM* genes (gene 3099 to gene 3102), which were more than those reported in 5-2 genome (5 transporters). Several major facilitator superfamily transporters were also found

in ZLP001 genome. Two complete glutamine-specific systems (gene 0770 to gene 0771 and gene 1837 to gene 1840) were found in ZLP001 genome, which are lesser than those reported in the genomes of *L. plantarum* WCSF1 and 5-2. However, one gene (gene 0854) encoding nitrate ABC transporter was unique in the ZLP001 genome. Among LAB, nitrite reduction was demonstrated to occur in *L. plantarum* (Paik and Lee, 2014). *L. plantarum* ZLP001 may play an important role in the regulation of nitrogen metabolism via glutamine synthetase and nitrite reduction.

The Sec-SRP secretion system was found on the chromosome of *L. plantarum* ZLP001 genome, including the signal-recognition particle proteins Ffh (gene 1224), membrane protein YidC (gene 1301 and gene 2562), and the component SecA (gene 1968), SecE (gene 2015 and gene 2026), SecG (gene 1917), SecY (gene 1672), and YajC (gene 0596). In addition, the type IV secretion system was present in the plasmids of *L. plantarum* ZLP001, comprising three VirD4 proteins in plasmid F (encoded by gene 0001 and gene 0019) and plasmid G (encoded by gene 0015).

Mobile Genetic Element Analysis

The ZLP001 genome contained two intact prophage elements (**Figure 1A** and **Table S4**). One prophage region resembled Lactob_Lj965 (82.1 kb, region 1) with a GC content of 42.27%, while the other resembled Lactoc_lato (52.7 kb, region 2) with a GC content of 40.18%. The closest related phage for *L. plantarum* was phig1e (Desiere et al., 2002). Integrases are useful markers for prophages in bacterial genomes. Three integrases (genes 0417, 0040, and 0801) were identified in the prophage region 1 and region 2. Prophage region 1 extended from 437,762 to 519,876 bp and contained 105 CDSs, with a complete prophage element from gene 0417 (phage integrase) to gene 0521. Prophage region 2 extended from 786,842 to 839,563 bp and carried 60 CDSs, with a complete prophage element from gene 0801 (phage integrase) to gene 0860. Consistent with the findings reported for *L. plantarum* WCSF1 and 5–2, the two intact prophage elements carried the entire packaging/head/tail gene clusters and lysis cassette (14), DNA packaging genes (encoding small and large terminase, portal protein), and head genes (encoding major head protein), as well as tail genes (Liu et al., 2015).

Lactobacillus plantarum ZLP001 genome contained 22 CRISPR loci (CRISPR 1 to CRISPR 22), including 9 CRISPR loci in the chromosome and 13 CRISPR loci in the plasmids (**Table S5**). The detected CRISPR/CRISPR-associated (Cas) system in plasmid C was type II-A (four cas genes; cas 1, cas 2, cas 9, and csn 2), consistent with the previously described CRISPR loci characteristic of *L. rhamnosus* Pen (Jarocki et al., 2018).

CAZymes

The analysis of CAZymes showed that the ZLP001 genome contained 119 genes in the five CAZymes gene families (**Table S6**) as follows: 18 carbohydrate esterase (CE) genes, 13 carbohydrate-binding modules (CBMs), 32 glycosyl transferase (GT) genes, 50 glycoside hydrolase (GH) genes, and 6 auxiliary activity (AA) genes. These numbers of CAZymes were relatively smaller than those for *L. plantarum* KLDS1.0391. *L. plantarum* KLDS1.0391 harbors only 14 CEs, 21 CPMs, 23 GTs, 34 GHs, and 2 AAs (Jia et al., 2017). GTs may catalyze the transfer of sugars from the activated donor molecules to specific acceptors and are essential for the formation of surface structures recognized by the host immune system (Mazmanian et al., 2008). The higher number of genes encoding CAZymes in *L. plantarum* ZLP001 genome is suggestive of its probiotic potential for pathogen defense and immune stimulation.

Genes Related to Antioxidative Capacity

Excessive accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) during cellular metabolism results in oxidative stress and leads to oxidative damage to proteins, lipids, and nucleic acids. *L. plantarum* ZLP001 encoded genes for some proteases involved in stress response, such as the ATP-dependent intracellular proteases ClpP (gene1927) and HslV (gene1042), which prevent aberrant damage to proteins (**Table S7**). LAB species with a complete glutathione (GSH) system may directly detoxify hydrogen peroxide and lipid peroxyl radicals through the regulation of the protein dithiol/disulfide balance (Pophaly et al., 2012). We identified a series of genes for GSH redox reaction, including *gpx* coding GSH peroxidase (gene 2371) and *gor* coding GSH reductase (genes 1060, 1539, 2244, and 2804). GSH-dependent disulfide reductions are also catalyzed by the thioredoxin system and glutaredoxin. The thioredoxin system provides electrons to thiol-dependent peroxidases to remove ROS and RNS at high reaction rates (Lu and Holmgren, 2014). *L. plantarum* ZLP001 harbored the genes related to the complete thioredoxin system, including five *trxA* genes coding thioredoxin (genes 0579, 0606, 2352, 2358, and 2727), one *trxB* gene encoding thioredoxin reductase (gene 1948), and one *tpx* gene coding thiol peroxidase (gene 0564). The gene *nrdH* encoding glutaredoxin (gene 2011) was also identified.

Bacteria regulate the levels of ROS and RNS with enzymatic and non-enzymatic cellular defense mechanisms. The gene *kat* (gene 2622) encoding catalase was shown to catalyze the decomposition of hydrogen peroxide to non-toxic water. *L. plantarum* ZLP001 genome encoded various nicotinamide adenine dinucleotide (NADH) oxidation-related proteins, including three *nox2* genes encoding NADH oxidase (genes 0956, 1949, and 2717) and two *npr* genes encoding NADH peroxidase (genes 0354 and 1370). Catalase and NADH oxidase/peroxidase are directly implicated in hydrogen peroxide and ROS degradation. Consistent with the observations in other *L. plantarum* strains (Kleerebezem et al., 2003; Liu et al., 2015), *L. plantarum* ZLP001 had no genes encoding the enzyme superoxide dismutase. In *L. plantarum*, nonheme type II catalase contains a dinuclear manganese active site. Crystallography analysis showed that the manganese active sites include $\mu_{1,3}$-bridging glutamate carboxylate residues that appears to be unique to *L. plantarum* and are involved in the transfer of the peroxidic protons to active site bases (Barynin et al., 2001). We found that *L. plantarum* ZLP001

carried the gene *aspB* (genes 0071, 1136, and 2904) encoding aspartate aminotransferase, while the same gene was absent from other sequenced *L. plantarum* strains such as *L. plantarum* strain WCFS1 (Kleerebezem et al., 2003), JDM1 (Zhang et al., 2009), and ZJ316 (Li et al., 2013). Aspartate aminotransferase catalyzes the production of glutamate from oxoglutarate, which could enhance the activity of manganese active site in catalase and strengthen *L. plantarum* ZLP001 tolerance to oxidative stress.

Lactobacillus plantarum ZLP001 is a potential probiotic with antioxidative capacity and is known to enhance the intestinal epithelial barrier function and defense against pathogens. The present study provides a genomic overview and reports the distinguishing gene features of ZLP001 by comparative genomic analysis of 18 related strains. The primary finding of this study requires further confirmation through *in vivo* and *in vitro* studies. The complete genome sequence of *L. plantarum* ZLP001 may improve our understanding of the probiotic effects of *L. plantarum* ZLP001 and extend its potential applications in humans and animals.

AUTHOR CONTRIBUTIONS

WZ and HJ conceived and designed the experiments. SW, JW, and YW performed the ZLP001 cultivation, and DNA extraction. WZ, DZ, and HL performed the genome analysis. WZ and HJ prepared the manuscript.

FUNDING

This work was supported financially by the National Key Research and Development Program of China (Project no. 2017YFD0502200), Beijing Innovation Consortium of Agriculture Research System (Grant no. BAIC02-2018) and the Special Program on Science and Technology Innovation Capacity Building of BAAFS (Grant no. KJCX20161503).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01689/full#supplementary-material

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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.

frontiers in Physiology

REVIEW
published: 31 October 2018
doi: 10.3389/fphys.2018.01534

Mouse Microbiota Models: Comparing Germ-Free Mice and Antibiotics Treatment as Tools for Modifying Gut Bacteria

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As the intestinal microbiota has become better appreciated as necessary for maintenance of physiologic homeostasis and also as a modulator of disease processes, there has been a corresponding increase in manipulation of the microbiota in mouse models. While germ-free mouse models are generally considered to be the gold standard for studies of the microbiota, many investigators turn to antibiotics treatment models as a rapid, inexpensive, and accessible alternative. Here we describe and compare these two approaches, detailing advantages and disadvantages to both. Further, we detail what is known about the effects of antibiotics treatment on cell populations, cytokines, and organs, and clarify how this compares to germ-free models. Finally, we briefly describe recent findings regarding microbiota regulation of infectious diseases and other immunologic challenges by the microbiota, and highlight important future directions and considerations for the use of antibiotics treatment in manipulation of the microbiota.

Keywords: antibiotics, microbiota, gnotobiotic, microbiome, immunity

OPEN ACCESS

Edited by:
Stephen J. Pandol, Cedars-Sinai Medical Center, United States

Reviewed by:
Aline Silva Miranda, Universidade Federal de Minas Gerais, Brazil
Breck A. Duerkop, University of Colorado, United States

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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: *03 August 2018*
Accepted: *11 October 2018*
Published: *31 October 2018*

Citation:
Kennedy EA, King KY and Baldridge MT (2018) Mouse Microbiota Models: Comparing Germ-Free Mice and Antibiotics Treatment as Tools for Modifying Gut Bacteria. Front. Physiol. 9:1534. doi: 10.3389/fphys.2018.01534

INTRODUCTION

Over the past several decades, there has been a dramatic increase in both scientific and popular interest in the effects of the intestinal microbiota on human health. The microbiota, consisting of the bacteria, viruses, fungi, and archaea that inhabit different niches in the human body, has been implicated in regulation of inflammatory, infectious and metabolic diseases, and appears to play a critical role in potentially causing, propagating, or preventing human illnesses (Lai et al., 2014; Norman et al., 2014; Palm et al., 2015). With the surge of enthusiasm to understand this new and massively complex factor in human health has come the need to effectively model it. Specifically, the development of small animal models of the microbiota permits testing of subsets of the microbiota as causative vs. correlative factors in disease states, as well as offering a system to uncover putative therapeutics.

Two main methods have emerged to explore the effects of the microbiota on physiology and disease in mice: germ-free models and antibiotics treatment regimens. Both approaches have strengths and weaknesses. Here we will discuss commonly used regimens and methods to deplete the microbiota, the effects of these approaches on host physiology including cellular composition, signaling pathways, and organ function, and briefly describe what has been found using these two different methods to model the effects of the microbiota on human disease.

GERM-FREE AND ANTIBIOTICS TREATMENT MODELS

Germ-free mice are bred in isolators which fully block exposure to microorganisms, with the intent of keeping them free of detectable bacteria, viruses, and eukaryotic microbes. Initially conceptualized by Louis Pasteur in 1885, colonies of germ-free rodents were first established in the 1940s (Yi and Li, 2012; Al-Asmakh and Zadjali, 2015). Germ-free mice allow for study of the complete absence of microbes or for the generation of gnotobiotic animals exclusively colonized by known microbes. However, generating and maintaining these mice requires specialized facilities, and the cost, labor, and skills required to maintain them can make these models inaccessible to many researchers. Germ-free mice must be monitored regularly for contamination using a combination of culturing, microscopy, serology, gross morphology, and sequencing-based detection techniques (Fontaine et al., 2015; Nicklas et al., 2015). For example, Charles River, one common germ-free vendor, routinely uses a serologic assay to test for viral contamination including murine norovirus, mouse rotavirus, and mouse cytomegalovirus; PCR (both 16S and pathogen-specific), microscopy, and culturing to test for bacteria; and gross examination of animals to test for parasites (Charles River Germ-Free Mouse Report). Additionally, any unique mouse strain to be studied under germ-free conditions must be rederived in these facilities, and this limits the number of different genotypes that are feasible to study. Further, maintenance of mice in isolators may make it impractical or challenging to conduct some studies (for example, behavioral testing or pathogen infections).

An alternate method that has emerged to avoid some of these complications has been the use of antibiotics treatment (**Figure 1**). Treatment with broad-spectrum antibiotics is commonly used to deplete the gut microbiota of mice, and can be readily applied to any genotype or condition of mouse. Unlike germ-free conditions, under which complete sterility is maintained throughout life, antibiotics can deplete bacterial populations in mice which were normally colonized since birth. Germ-free animals are broadly impaired in many aspects of development and early immune education, whereas antibiotics treatment in adult mice specifically allows for study of the role of bacteria in maintaining cell functionality and signaling pathways after development. Alternatively, some studies deliver antibiotics in drinking water to pregnant dams to limit maternal transfer of microbes and then maintain the cage on the regimen during weaning to study the effects of bacterial depletion early in development (Lamousé-Smith et al., 2011; Deshmukh et al., 2014; Gonzalez-Perez et al., 2016; Li et al., 2017).

Due to differences in mechanism of action, antibiotics can selectively deplete different members of the microbiota. For example, metronidazole and clindamycin both target anaerobes, vancomycin is only effective against gram-positive bacteria, and polymyxin B specifically targets gram-negative bacteria (Atarashi et al., 2011; Schubert et al., 2015). Individual antibiotics can be used to shift the composition of the gut microbiota in order to identify classes of bacteria relevant to different phenotypes (Schubert et al., 2015; Zackular et al., 2016). In contrast, a cocktail of different classes of antibiotics can be used to broadly deplete the gut microbiota. Researchers have used various regimens which differ in antibiotic combination, dose, and length of treatment (**Table 1**). All of these combinations broadly target Gram-positive, Gram-negative, and anaerobic bacteria. Often, antibiotics are diluted in drinking water and mice are allowed to drink *ad libitum* throughout the course of treatment; therefore, actual delivered doses can vary. Some protocols additionally include antifungals in the cocktail to avoid fungal overgrowth during treatment (Reikvam et al., 2011; Grasa et al., 2015; Zákostelská et al., 2016). Many also add sweeteners such as sugar, Splenda, or Kool-aid to mask any bitterness and ensure mice drink the antibiotics-containing water (Abt et al., 2012; Baldridge et al., 2015; Emal et al., 2017). However, there are reports of mice avoiding water and becoming dehydrated when provided antibiotics in this manner (Hill et al., 2010; Reikvam et al., 2011; Zákostelská et al., 2016). Daily oral gavage can prevent dehydration and allow delivery of a precise dose of antibiotics, so this method is sometimes used alone or in combination with delivery in drinking water, though it is more labor-intensive (Kuss et al., 2011; Reikvam et al., 2011).

Validation of bacterial depletion can be performed with culture-based methods by assessing the colony-forming units (CFUs) from fecal samples plated in aerobic and/or anaerobic conditions on non-selective media. However, this method only accounts for cultivatable microbes. Quantitative PCR of the gene encoding 16S rRNA allows for culture-independent assessment of gastrointestinal bacterial load. Broad-spectrum antibiotics treatment can decrease bacterial load by multiple orders of magnitude in 2 weeks of treatment or less (Baldridge et al., 2015; Gonzalez-Perez et al., 2016; Brown et al., 2017). Both germ-free and antibiotics-treated mice allow for the introduction of microbes in which the contributions of defined bacterial species or consortia can be studied (Tan et al., 2016; Staley et al., 2017). Although some phenotypes seen with antibiotics treatment are attributed to removal of a single bacterial species, many differences that occur are due to broad decreases in bacterial load. Consistent with this, treating bacterially-depleted mice with conserved pattern recognition receptor ligands such as flagellin (Ichinohe et al., 2011; Oh et al., 2014) or CpG dinucleotides (Ichinohe et al., 2011; Hill et al., 2012) can restore some defects, even in the absence of microbiota restoration.

Although most studies attribute phenotypic differences after antibiotics treatment to the depletion of gut microbes, some studies have assessed how regimens affect commensal populations at other sites. Oral antibiotic regimens can decrease culturable bacteria in the respiratory tract (Ichinohe et al., 2011; Abt et al., 2012; Brown et al., 2017) and the vagina of mice (Oh et al., 2016), but do not affect skin bacterial communities (Naik et al., 2012). Importantly, though rarely quantified, antibiotics treatment also likely drastically affects bacteriophage populations, though there is debate in the literature about whether phage play important roles in transfer of antibiotic resistance genes (Modi et al., 2013; Enault et al., 2017; Górska et al., 2018). Antibiotics treatment can allow for the outgrowth of commensal fungal species, potentially confounding results as

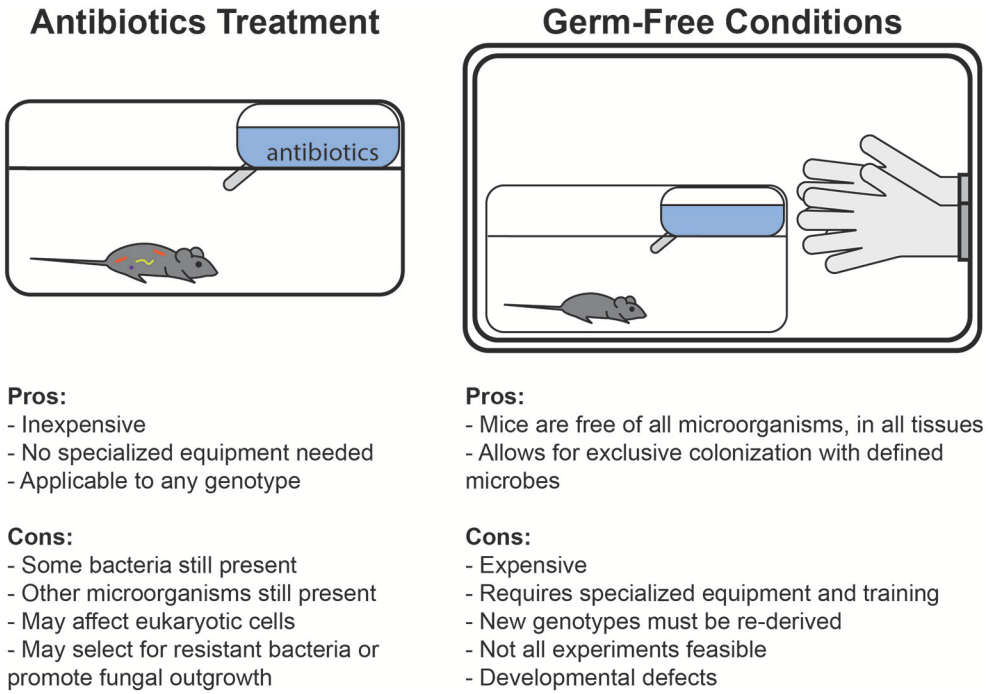

FIGURE 1 | Comparison of the advantages and disadvantages of germ-free and antibiotics-treated mouse model systems.

these organisms can alter immune function, hence the inclusion of antifungals in some antibiotics treatment regimens (Noverr et al., 2004; Kim et al., 2014). An increasing appreciation for important roles for the virome and mycobiome may lead to enhanced interrogation of these effects in the future, as well as the potential impact of antibiotics on eukaryotic viruses and archaea (Norman et al., 2014). An important final potential disadvantage of antibiotics treatment can be the evolution or development of antibiotic-resistant bacteria and their subsequent selection and outgrowth in mouse intestines (Zhang et al., 2013; Morgun et al., 2015). Depending upon the starting bacterial composition, antibiotic cocktail, duration of treatment, and phenotypic read-out, antibiotic-resistance may confound findings in experiments, especially if resistant bacteria are present in only a subset of tested mice. Longitudinal analysis of bacterial populations in all experimental groups can aid in detection of resistance and analysis of whether resistant bacteria may affect experimental results.

Mice on antibiotics are not completely cleared of bacteria, but significant reductions in bacterial load are associated with shifts in cell populations, signaling pathways, and organ morphology, with results often paralleling what is seen in germ-free mice.

EFFECTS OF THE MICROBIOTA ON CELL POPULATIONS AND CYTOKINES

Although many aspects of murine physiology are affected by microbial populations, the effects of antibiotics treatment on immune cell populations are some of the most well-studied (**Figure 2**). The immune system constantly responds to both pathogenic and commensal microbial populations, and shifts after antibiotics treatment reflect the dependence of cell populations and function on bacterial signals. While results of cell composition analysis are not uniform across studies, we will describe the most prominent and consistent observations (see **Table 2** for exact findings by different groups).

MYELOID CELLS

Innate immune cells lack antigen-specific receptors, responding instead to broadly conserved microbial patterns. As innate cells continuously interface with the microbial populations constituting the microbiota, sensing of these microbes via pattern recognition receptors is essential for maintenance of normal host physiology (Chu and Mazmanian, 2013; Fawkner-Corbett et al., 2017).

Myeloid cell populations, which include macrophages, monocytes, and granulocytes, are broadly decreased in systemic sites after antibiotics treatment, similar to what is seen in germ-free mice (Khosravi et al., 2014). Although monocytes are generally not diminished in the bone marrow or peripheral blood of mice receiving antibiotics (Zhang et al., 2015; Josefsdottir et al., 2017), these cells have a reduced migratory capacity consistent with their decreased presence in peripheral tissues (Zhang et al., 2015; Emal et al., 2017). In contrast, the effects of antibiotics treatment on inflammatory monocytes and macrophages are more variable, with some groups reporting decreases in blood,

TABLE 1 | Broad-spectrum antibiotics treatment regimens.

Method	Antibiotics	Concentration	Duration	Additions	References
Drinking water (*ad libitum*)	Vancomycin + metronidazole	0.5–1.0 g/L each	10 weeks		Atarashi et al., 2008
	Ciprofloxacin + metronidazole	1 g/L each	2 weeks		Josefsdottir et al., 2017
	Vancomycin + ampicillin + polymixin	0.1–1.0 g/L each	4 weeks		Kim et al., 2017
	Vancomycin + neomycin + metronidazole	0.5–1.0 g/L each	7 days		Brandl et al., 2008; Kinnebrew et al., 2010
			2 weeks	Kool-Aid	Josefsdottir et al., 2017
	Streptomycin + colistin + ampicillin	1–5 g/L each	6 weeks	2.5% sucrose	Sawa et al., 2011
	Ampicillin + neomycin + streptomycin + vancomycin	0.5–1.0 g/L each	4–5 weeks		Khosravi et al., 2014
	Cefoxitin + gentamicin + metronidazole + vancomycin	1 g/L	10 days		Ganal et al., 2012
	Gentamicin + ciprofloxacin + streptomycin + bacitracin	0.15–2 g/L each	4 weeks	3% sucrose	Yan et al., 2016
	Vancomycin + neomycin + kanamycin + metronidazole	0.5–1.0 g/L each	3 weeks		Gury-BenAri et al., 2016
	Vancomycin + ampicillin + kanamycin + metronidazole	0.5–1.0 g/L each			Levy et al., 2015
	Vancomycin + neomycin + ampicillin + metronidazole	0.35–1.0 g/L each	7 days	3% sucrose, 1% glucose, or Kool-aid	Ochoa-Repáraz et al., 2009
			2 weeks		Hägerbrand et al., 2015; Hashiguchi et al., 2015; Knoop et al., 2015; Brown et al., 2017; Emal et al., 2017; Josefsdottir et al., 2017; Steed et al., 2017; Burrello et al., 2018; Thackray et al., 2018
			3 or more weeks		Rakoff-Nahoum et al., 2004; Ivanov et al., 2008; Vaishnava et al., 2008; Ichinohe et al., 2011; Ismail et al., 2011; Yoshiya et al., 2011; Naik et al., 2012; Corbitt et al., 2013; Diehl et al., 2013; Balmer et al., 2014; Mortha et al., 2014; Oh et al., 2014; Johansson et al., 2015; Wu et al., 2015; Zhang et al., 2015; Park et al., 2016; Yan et al., 2016; Cervantes-Barragan et al., 2017; Ge et al., 2017; Li et al., 2017; Durand et al., 2018
			3 4-day treatments with 3 day rests		Adami et al., 2018
Gavage	Vancomycin + neomycin + ampicillin + metronidazole + gentamicin	200 μl of 0.5–1.0 g/L each by daily gavage	3 day		Kelly et al., 2015
			10 days		Hill et al., 2010
	Bacitracin + neomycin + streptomycin	200 mg/kg body weight	3 days		Sayin et al., 2013; Wichmann et al., 2013; Fernández-Santoscoy et al., 2015

(Continued)

TABLE 1 | Continued

Method	Antibiotics	Concentration	Duration	Additions	References
	Neomycin + bacitracin	20 mg each in 200 μl by daily gavage	7 days	Pimaricin (anti-fungal), adjusted pH to 4	Grasa et al., 2015
Combination	Ampicillin by drinking water; vancomycin + neomycin + metronidazole by gavage	1.0g/L in water 10 ml/kg of 5–10 g/L by gavage every 12 h	10–21 days	Amphotericin B (anti-fungal)	Reikvam et al., 2011; Hintze et al., 2014
	Vancomycin + neomycin + ampicillin + metronidazole	10 mg each by daily gavage 0.5–1.0 g/L each in water	5 days gavage followed by 7–10 days drinking water		Kuss et al., 2011
	Kanamycin + gentamicin + colistin + metronidazole + vancomycin	200 μl of 0.35–4 mg/ml by daily gavage, and mixed 2:100 into drinking water	7 days gavage followed by administration in water		Bashir et al., 2004; Stefka et al., 2014
	Metronidazole + colistin + streptomycin by gavage, vancomycin by drinking water	0.3–2 mg each by daily gavage, and 0.25 mg/ml in water	2 weeks	Amphotericin B (anti-fungal)	Zákostelská et al., 2016
	Oral streptomycin + ampicillin in drinking water	20 mg/mouse orally and 1 g/L in drinking water	1–2 weeks		Kim et al., 2018
	Streptomycin by gavage, followed by vancomycin + neomycin + ampicillin + metronidazole by drinking water	100 mg/mouse for single gavage and 0.5–1.0 g/L in drinking water	single gavage followed by >7 days drinking water	1% sucrose	Kernbauer et al., 2014

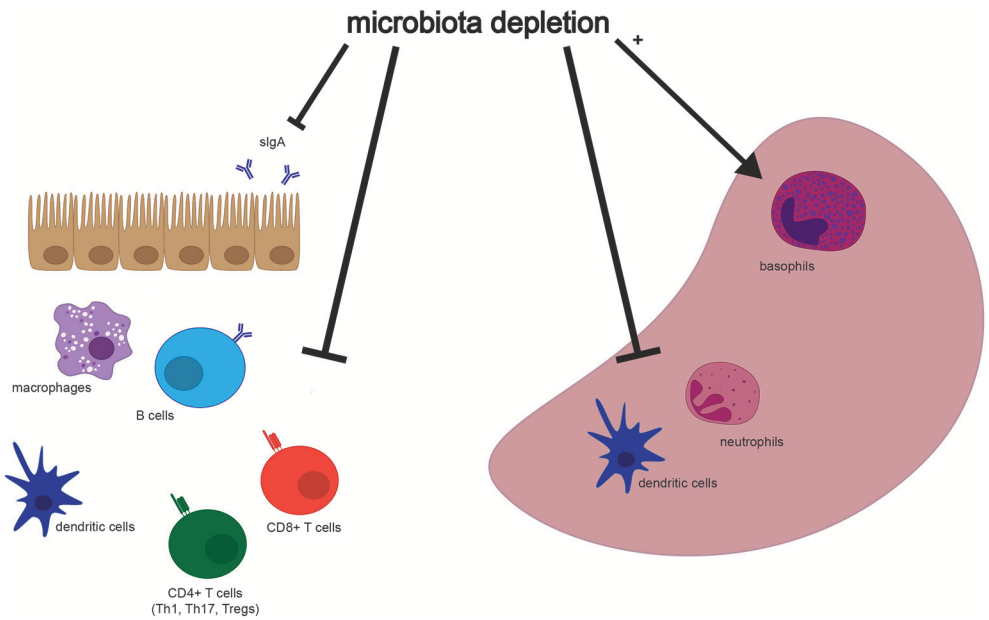

FIGURE 2 | Selected effects of microbiota depletion on cells in the gastrointestinal tract and spleen. Populations of different cell types are altered in association with depletion of the microbiota in both the gastrointestinal tract **(Left)** and the spleen **(Right)**. Both secretory IgA (sIgA) and immune cell types are depleted in the intestine, while dendritic cells and neutrophils are depleted, but basophils are enriched, in the spleen. Please see **Table 2** for more detailed findings by different groups and in other tissues.

bone marrow, and peripheral tissues (Zhang et al., 2015; Hergott et al., 2016; Ekmekciu et al., 2017) and others reporting no significant differences at baseline or after infection (Abt et al., 2012; Oh et al., 2016; Brown et al., 2017; Robak et al., 2018). Even when cell numbers are similar, macrophages are often less mature after antibiotics treatment, impairing their responses to

TABLE 2 | Effects of the microbiota on cell populations.

	Broad-spectrum antibiotics	References	Germ-Free	References
Monocytes	↓ in spleen similar in blood, BM ↓ inflammatory monocytes in BM, spleen, blood Similar inflammatory monocytes in vaginal mucosa after HSV-2 infection, in lung after flu infection	Abt et al., 2012; Balmer et al., 2014; Zhang et al., 2015; Oh et al., 2016; Josefsdottir et al., 2017	↓ or similar in blood ↓ in BM, spleen ↓ inflammatory monocytes in spleen, similar but trend ↓ in BM; similar in SI, colon Similar inflammatory monocytes in mesenteric LN after *S. typhimurium* infection	Balmer et al., 2014; Khosravi et al., 2014; Fernández-Santoscoy et al., 2015; Zhang et al., 2015; Hergott et al., 2016; Tan et al., 2016
	Increased inflammatory monocyte turnover and apoptosis in bloodstream; decreased migratory capacity of BM monocytes	Hergott et al., 2016; Emal et al., 2017		
Macrophages	↓ in SI, colon; ↓ or similar in spleen, liver; similar in PP, mesenteric LN, cervical LN, kidney, lungs; ↑ in BM Similar in lungs after *P. aeruginosa* or flu infection	Ochoa-Repáraz et al., 2009; Abt et al., 2012; Corbitt et al., 2013; Zhang et al., 2015; Ekmekciu et al., 2017; Emal et al., 2017; Robak et al., 2018	↓ or similar in spleen ↓ in liver	Ganal et al., 2012; Corbitt et al., 2013; Khosravi et al., 2014
	Less mature in kidney, liver, spleen; decreased cytokine production in lung after respiratory infection	Abt et al., 2012; Brown et al., 2017; Emal et al., 2017		
Dendritic cells (DCs)	↓ mDCs, pDCs in spleen; ↓ activated DCs in SI, colon, mesenteric LN, spleen; ↓ CD103+ DCs in lung; ↓ or similar CD103+ DCs in mesenteric LN Similar in lung/mediastinal LN after flu infection, similar in vaginal mucosa before or after HSV-2 infection	Abt et al., 2012; Hägerbrand et al., 2015; Oh et al., 2016; Ekmekciu et al., 2017; Thackray et al., 2018	↓ in spleen; ↓ or similar in mesenteric LN Similar in skin; similar cDCs in spleen	Walton et al., 2006; Naik et al., 2012; Hägerbrand et al., 2015
	Similar antigen-presentation abilities Similar surface markers in lung, altered in mesenteric LN, PP Impaired type 1 IFN production and priming of CD8 T cells after flu infection	Ochoa-Repáraz et al., 2009; Ichinohe et al., 2011; Abt et al., 2012; Ganal et al., 2012; Thackray et al., 2018	Decreased maturity but similar antigen presentation abilities impaired type 1 IFN production	Walton et al., 2006; Ganal et al., 2012
Granulocytes	↓ total in BM; similar in blood	Balmer et al., 2014; Josefsdottir et al., 2017	↓ total in BM; similar in blood	Balmer et al., 2014
Neutrophils	↓ in BM, spleen, blood; similar in liver, BALF, vaginal mucosa; ↑ in lung after *P. aeruginosa*, flu, *S. pneumoniae* or *K. pneumoniae* infection	Abt et al., 2012; Zhang et al., 2015; Hergott et al., 2016; Oh et al., 2016; Brown et al., 2017; Li et al., 2017; Robak et al., 2018	↓ or similar in spleen, BM ↓ blood similar in mesenteric LN after *S. typhimurium* infection ↓ in lung after *K. pneumoniae* infection	Fagundes et al., 2012; Khosravi et al., 2014; Fernández-Santoscoy et al., 2015; Sturge et al., 2015; Zhang et al., 2015; Hergott et al., 2016; Josefsdottir et al., 2017
	Decreased accumulation in BM or blood of neonates Increased turnover and apoptosis in bloodstream; Fewer aged neutrophils in blood	Deshmukh et al., 2014; Zhang et al., 2015; Hergott et al., 2016	Decreased accumulation in BM or blood of neonates Fewer aged neutrophils in blood	Deshmukh et al., 2014; Zhang et al., 2015
	Similar phagocytosis/reactive oxygen species production, adhesion in neonates; impaired neutrophil extracellular trap formation *in vitro*	Deshmukh et al., 2014; Zhang et al., 2015		
Basophils, eosinophils, mast cells	↑ basophils in blood, spleen Similar mast cells, eosinophils in blood ↑ eosinophils in inguinal subcutaneous adipose tissue, vaginal mucosa	Hill et al., 2012; Suárez-Zamorano et al., 2015; Oh et al., 2016	↑ basophils in blood, spleen similar eosinophils and mast cells in skin	Hill et al., 2012; Naik et al., 2012
	↑ eosinophils in lung/BALF after allergen exposure	Hill et al., 2012; Adami et al., 2018		

(Continued)

TABLE 2 | Continued

	Broad-spectrum antibiotics	References	Germ-Free	References
Lymphocytes	Similar in spleen; ↓ in lung, liver	Cheng et al., 2014		
αβ T cells	↓ in PP, mesenteric LN, cervical LN, SI, colon Similar or ↑ in BM Similar in blood, liver ↓ or ↑ in spleen	Ochoa-Repáraz et al., 2009; Zhang et al., 2015; Ekmekciu et al., 2017; Josefsdottir et al., 2017; Li et al., 2017	↓ in SI, blood, spleen Similar in skin ↑ in BM	Naik et al., 2012; Kernbauer et al., 2014; Zhang et al., 2015
CD4 T cells	↓ in PP, cervical LN, SI, colon, spleen, blood Similar in BM Similar or ↓ in mesenteric LN, SI Similar or ↓ or ↑ in spleen ↓ % CD4+ memory cells in SI, colon, mesenteric LN, spleen	Ivanov et al., 2008; Ochoa-Repáraz et al., 2009; Sawa et al., 2011; Kernbauer et al., 2014; Ekmekciu et al., 2017; Josefsdottir et al., 2017; Burrello et al., 2018; Thackray et al., 2018	↓ in SI, mesenteric LN, BM ↓ or similar in colon, spleen Similar, blood, cutaneous LN	Huang et al., 2005; Mazmanian et al., 2005; Atarashi et al., 2008; Sawa et al., 2011; Naik et al., 2012; Sjögren et al., 2012; Smith et al., 2013; Kernbauer et al., 2014
	Impaired activation after HSV-2 infection	Oh et al., 2016		
Th1 cells (Ifnγ+)	↓ in SI, colon Similar in mesenteric LN, spleen, vaginal mucosa or draining LNs, skin	Naik et al., 2012; Kernbauer et al., 2014; Oh et al., 2016; Ekmekciu et al., 2017	↓ in SI, mesenteric LN, colon, skin Similar or ↓ in mesenteric LN Similar in cecal patch, colon ↓ in draining lymph nodes after EAE induction?	Zaph et al., 2008; Lee et al., 2011; Naik et al., 2012; Kernbauer et al., 2014
	↓ IFNγ response to flu Similar IFNγ response to OVA, respiratory HSV-2, *L. pneumophila* ↑ IFNγ response to *Salmonella* in mesenteric LN, SI	Ichinohe et al., 2011; Diehl et al., 2013; Kim et al., 2018		
Th2 cells (IL4+)	↑ in mediastinal LN after allergen exposure	Hill et al., 2012		
Th17 cells (IL17+, Rorc+)	↓ in SI, colon, mesenteric LN, spleen Similar in skin, liver	Atarashi et al., 2008; Ivanov et al., 2008; Sawa et al., 2011; Naik et al., 2012; Ekmekciu et al., 2017; Li et al., 2017	↓ in colon, cecum, mesenteric LN, skin Similar or ↓ in PP Similar or ↓ or ↑ in SI ↑ in cecal patch, colon	Atarashi et al., 2008; Ivanov et al., 2008; Zaph et al., 2008; Sawa et al., 2011; Naik et al., 2012; Kernbauer et al., 2014; Tan et al., 2016
T regulatory cells (FoxP3+)	↓ in colon Similar or ↓ in SI, spleen, PP ↓ or ↑ in mesenteric LN Similar in BM, liver ↑ in cervical LN, lung	Ivanov et al., 2008; Ochoa-Repáraz et al., 2009; Ichinohe et al., 2011; Mortha et al., 2014; Ekmekciu et al., 2017; Josefsdottir et al., 2017; Li et al., 2017; Thackray et al., 2018	↓ in PP, colon Similar in spleen, mesenteric LN, peripheral LN, cutaneous LN, cecal patch, colon, blood, thymus ↑ in SI, skin; ↑ in draining LN and spleen after EAE induction ↓ Rorγt+ T_{regs} in colon, SI, MLN; similar or ↓ Helios+, similar Gata3+ T_{regs} in colon	Ivanov et al., 2008; Zaph et al., 2008; Lee et al., 2011; Naik et al., 2012; Smith et al., 2013; Ohnmacht et al., 2015; Durand et al., 2018
CD8+ T cells	↓ in SI, colon, blood Similar or ↑ in mesenteric LN ↓ or ↑ in spleen ↑ in PP, cervical LN, BM	Ochoa-Repáraz et al., 2009; Kernbauer et al., 2014; Ekmekciu et al., 2017; Josefsdottir et al., 2017; Thackray et al., 2018	↓ in mesenteric LN Similar in SI, colon, blood, cutaneous LN, spleen	Huang et al., 2005; Naik et al., 2012; Kernbauer et al., 2014
	Similar IFNγ+ in SI, vaginal mucosa or draining LNs Impaired response to flu, vaginal HSV-2; similar response to OVA, respiratory HSV-2, *L. pneumophila*	Kernbauer et al., 2014; Oh et al., 2016 Ichinohe et al., 2011,?; Abt et al., 2012; Oh et al., 2016	↓ IFNγ+ in SI, colon, mesenteric LN	Kernbauer et al., 2014

(Continued)

TABLE 2 | Continued

	Broad-spectrum antibiotics	References	Germ-Free	References
CD4+CD8aa+ cells	↓ in SI epithelium	Cervantes-Barragan et al., 2017	↓ in SI epithelium	Cervantes-Barragan et al., 2017
γδ T cells	Similar IL-17+ in SI ↓ IL-17+ in liver	Ivanov et al., 2008; Li et al., 2017	Similar in skin Similar or ↑ in SI ↓ IL-17+ in SI, skin, liver	Bandeira et al., 1990; Ivanov et al., 2008; Ismail et al., 2011; Naik et al., 2012; Li et al., 2017
	Less activated and more apoptotic in liver, ↓ production of antimicrobials in SI	Ismail et al., 2011; Li et al., 2017	↓ production of antimicrobials in SI, less activated in liver, diminished response to mucosal injury in colon	Ismail et al., 2009, 2011; Li et al., 2017
NK T cells	Similar or ↓ in spleen Similar in PP, cervical LN, mesenteric LN, liver ↑ in colon	Ochoa-Repáraz et al., 2009; Li et al., 2017; Burrello et al., 2018	↑ in colon	Kernbauer et al., 2014
B cells	↓ in SI, colon, PP Similar or ↓ in spleen, blood, BM Similar in mesenteric LN, cervical LN, liver	Ochoa-Repáraz et al., 2009; Yoshiya et al., 2011; Zhang et al., 2015; Ekmekciu et al., 2017; Josefsdottir et al., 2017; Li et al., 2017; Thackray et al., 2018	↓ in blood Similar in spleen ↑ in BM ↓ IgA, IgG production in SI	Kernbauer et al., 2014; Zhang et al., 2015
Antibodies	Similar IgM, IgG in BALF, IgG in serum, ↑ in serum after allergen exposure ↓ IgA in BALF, blood, feces ↑ IgE in serum at baseline, after allergen exposure	Atarashi et al., 2008; Hill et al., 2012; Oh et al., 2014; Stefka et al., 2014; Uchiyama et al., 2014; Adami et al., 2018; Lynn et al., 2018; Robak et al., 2018	Similar IgG in serum, ↑ after allergen exposure ↓ IgA in feces ↑ IgE in serum at baseline, after allergen exposure	Atarashi et al., 2008; Hill et al., 2012; Oh et al., 2014; Stefka et al., 2014
	↓ antigen-specific response to vaccines in neonates, not adults ↓ flu-specific IgG, IgA after infection, IgG early after flu vaccine ↑ *Salmonella*-specific IgG in blood and IgA in feces ↑ rotavirus-specific IgA in serum, feces, only at later times after infection	Ichinohe et al., 2011; Lamousé-Smith et al., 2011; Abt et al., 2012; Diehl et al., 2013; Oh et al., 2014; Uchiyama et al., 2014; Li et al., 2017; Lynn et al., 2018	↓ Ova-specific IgG in response to Ova immunization at all ages tested ↓ flu-specific IgM in serum after infection, IgG early after flu vaccine ↑ rotavirus-specific IgA, IgG in serum, only at later time points	Lamousé-Smith et al., 2011; Abt et al., 2012; Oh et al., 2014; Uchiyama et al., 2014
Innate lymphoid cells (ILCs)	↓ ILC3s and ILC1s in PP ↑ ILC3s in terminal ileum PP Similar or ↑ ILC3s in SI LP ↑ ILC2 in vaginal mucosa ↓ GM-CSF$^+$ ILC3s in colon ILC1 and ILC2 expression becomes more ILC3-like	Sawa et al., 2011; Mortha et al., 2014; Hashiguchi et al., 2015; Gury-BenAri et al., 2016; Oh et al., 2016; Kim et al., 2017	↑ ILC2s in SI; similar activation Similar or ↑ ILC3s in SI Similar ILC1 in SI	Sawa et al., 2011; Kernbauer et al., 2014; Gury-BenAri et al., 2016; Schneider et al., 2018
Natural killer (NK) cells	↓ in spleen Similar in PP, mesenteric LN, cervical LN, liver	Ochoa-Repáraz et al., 2009; Li et al., 2017	Similar in spleen	Ganal et al., 2012
	Impaired cytotoxicity and cytokine production in spleen	Ganal et al., 2012	Impaired cytotoxicity and cytokine production in spleen	Ganal et al., 2012

BALF, bronchoalveolar lavage fluid; BM, bone marrow; EAE, experimental autoimmune encephalomyelitis; LN, lymph node; PP, Peyer's patch; SI, small intestine.

pathogens (Abt et al., 2012; Brown et al., 2017; Emal et al., 2017).

Bulk granulocytes decrease in the bone marrow of antibiotics-treated mice, though their numbers in peripheral blood are similar (Balmer et al., 2014; Josefsdottir et al., 2017). Neutrophils decrease in bone marrow and in peripheral sites, with an increased rate of apoptosis and decrease in aged neutrophils in the bloodstream after microbiota depletion (Deshmukh et al.,

2014; Zhang et al., 2015; Hergott et al., 2016). However, neutrophil populations are not diminished at the site of infection after pathogen exposure in antibiotics-treated mice (Abt et al., 2012; Oh et al., 2016; Brown et al., 2017; Robak et al., 2018). In contrast to neutrophils, the proliferation of basophil precursors in the bone marrow is increased after antibiotics treatment, associated with increased basophils in the periphery and an enhanced response to allergen exposure (Hill et al., 2012). Similarly, eosinophils in various tissues are enhanced at baseline (Suárez-Zamorano et al., 2015; Oh et al., 2016), and in the lung after inhaled allergen exposure (Hill et al., 2012). These alterations in granulocyte populations are consistent with a shift from type 1 to type 2 immune responses after depletion of the commensal microbiota.

Various dendritic cell subsets are also reduced after antibiotics treatment at both mucosal and systemic sites (Ichinohe et al., 2011; Ekmekciu et al., 2017; Thackray et al., 2018), although these differences may not be apparent after infection (Abt et al., 2012; Oh et al., 2016). Differences in dendritic cell numbers have not been reported in germ-free mice, though impairment in priming has been seen (Walton et al., 2006; Ganal et al., 2012).

Reductions in innate immune cell number and function, characteristic of both germ-free mice and antibiotics-treated mice, may be explained by diminished cytokine and chemokine levels, which are necessary for normal cell recruitment, differentiation, and functionality (Mortha et al., 2014; Brown et al., 2017). Reductions in myeloid populations are likely not explained by decreases in progenitor populations: although antibiotic exposure beginning *in utero* can reduce postnatal granulocytosis (Deshmukh et al., 2014), treatment in adult mice does not reduce myeloid progenitor populations in the bone marrow (Josefsdottir et al., 2017; Thackray et al., 2018).

LYMPHOID CELLS

In contrast to what is seen with myeloid progenitors, common lymphoid progenitors are reduced in the bone marrow after microbiota depletion (Josefsdottir et al., 2017; Thackray et al., 2018), consistent with what is seen in some, but not all, germ-free models (Balmer et al., 2014; Iwamura et al., 2017). Total lymphocytes are similarly reduced in the peripheral blood after antibiotics treatment (Josefsdottir et al., 2017).

The effects of the microbiota in regulating differentiated T cell populations has been widely explored, but results found are somewhat variable. αβ T cells generally decrease in peripheral organs (Ochoa-Repáraz et al., 2009; Zhang et al., 2015; Ekmekciu et al., 2017), although not in the bone marrow or blood (Zhang et al., 2015; Josefsdottir et al., 2017). Similarly, many reports suggest that CD4+ T helper cells decrease in tissues (Ochoa-Repáraz et al., 2009; Kernbauer et al., 2014; Ekmekciu et al., 2017; Josefsdottir et al., 2017; Thackray et al., 2018), as do CD4+ T memory cells (Ekmekciu et al., 2017), although others see either no difference or increases in specific tissues (Ivanov et al., 2008; Sawa et al., 2011; Ekmekciu et al., 2017; Josefsdottir et al., 2017; Burrello et al., 2018). Th1 cells tend to decrease in the gastrointestinal tract (Naik et al., 2012; Kernbauer et al., 2014; Ekmekciu et al., 2017), but not in extra-intestinal tissues (Naik et al., 2012; Oh et al., 2016; Ekmekciu et al., 2017), whereas Th17 cells decrease in most tissues studied (Atarashi et al., 2008; Ivanov et al., 2008; Sawa et al., 2011; Naik et al., 2012; Ekmekciu et al., 2017). The effects of microbial depletion on Th2 cells are less well-studied, although they have been seen to increase in lymph nodes after allergen exposure (Hill et al., 2012). Results with regulatory T cells are inconsistent across studies, with some citing decreases in different tissues (Ochoa-Repáraz et al., 2009; Mortha et al., 2014; Ekmekciu et al., 2017; Thackray et al., 2018), others seeing similar numbers regardless of antibiotics treatment (Ivanov et al., 2008; Ichinohe et al., 2011; Josefsdottir et al., 2017; Li et al., 2017), and still others seeing increases in some sites (Ochoa-Repáraz et al., 2009; Ichinohe et al., 2011).

Similar to CD4+ T cells, cytotoxic CD8+ T cells generally decrease in the intestine after antibiotics, though results at other sites are more varied (Ochoa-Repáraz et al., 2009; Kernbauer et al., 2014; Ekmekciu et al., 2017; Josefsdottir et al., 2017; Thackray et al., 2018). Proinflammatory cytokine production from cytotoxic T cells is not diminished at baseline after antibiotics treatment, but has been reported to decrease in response to infection with some pathogens (Ichinohe et al., 2011; Abt et al., 2012; Kernbauer et al., 2014; Oh et al., 2016).

Reports of B cell population shifts are varied, with some groups seeing declines in the blood, bone marrow, and tissues after antibiotics treatment, and others noting similar numbers regardless of microbiota depletion (Ochoa-Repáraz et al., 2009; Yoshiya et al., 2011; Zhang et al., 2015; Ekmekciu et al., 2017; Josefsdottir et al., 2017; Li et al., 2017; Thackray et al., 2018). Likewise, shifts in antibody responses are inconsistent—in general, total IgG and IgM levels remain similar in different sites analyzed, but secretory and serum IgA levels tend to decrease and serum IgE levels increase after microbiota depletion (Hill et al., 2010; Oh et al., 2014; Uchiyama et al., 2014; Adami et al., 2018; Lynn et al., 2018; Robak et al., 2018). Antigen-specific response to infection or vaccination vary by pathogen, mouse age, and time point after exposure analyzed, but neonates in particular generally produce a less robust response to vaccination after exposure to antibiotics (Ichinohe et al., 2011; Lamousé-Smith et al., 2011; Abt et al., 2012; Diehl et al., 2013; Oh et al., 2014; Uchiyama et al., 2014; Li et al., 2017; Lynn et al., 2018).

Innate-like lymphocytes (including CD8αα+ T cells, γδ T cells, NK T cells) and innate lymphoid cells (ILCs, including natural killer cells) localize to barrier sites and are influenced by the presence of commensal microbes (Constantinides, 2018). Double-positive CD4+CD8αα+ T cells serve regulatory functions in the small intestinal epithelium and are diminished in antibiotics-treated mice, associated with a reduction in the bacterium *Lactobacillus reuteri* which induces this cell type (Cervantes-Barragan et al., 2017). γδ T cells are present in epithelial tissues, mediating tissue repair and monitoring microbial populations. Although studies in germ-free mice suggest that the microbiota is dispensable for these cells to home to the intestine or skin (Bandeira et al., 1990; Ismail et al., 2011; Naik et al., 2012), both germ-free and antibiotics-treated models indicate that microbial colonization is necessary for normal activation and production of antimicrobial compounds by these

TABLE 3 | Effects of the microbiota on cytokine signaling.

	Broad-spectrum antibiotics	References	Germ-Free	References
IL-1 family cytokines	Similar IL-1β in jejunum, colon, BM, BALF, liver; similar IL-1α in jejunum, BALF, liver ↓ pro-IL-1 in lung ↓ IL-1β, IL-1α in vaginal washes after HSV-2 infection; ↓ IL-1β in BALF after flu infection ↓ Pro-IL-18 in BALF, ↓ IL-18 in colon trend ↓ IL-18 in vaginal washes after HSV-2 infection ↑ IL-33 in vaginal mucosa	Ichinohe et al., 2011; Abt et al., 2012; Levy et al., 2015; Suárez-Zamorano et al., 2015; Oh et al., 2016; Yan et al., 2016; Li et al., 2017; Robak et al., 2018	↓ IL-1 in BM ↓ IL-1β in SI, colon, trend ↓ BM ↓ IL-1α in skin ↓ IL-18 in colon ↑ IL-33 in colon, SI	Naik et al., 2012; Shaw et al., 2012; Sjögren et al., 2012; Singh et al., 2014; Levy et al., 2015; Ohnmacht et al., 2015; Yan et al., 2016
Th1 cytokines	Similar IL-2 in liver ↓ IFNγ in SI, in vaginal mucosa after HSV-2 infection; similar IFNγ in SI, colon, vaginal mucosa at baseline ↓ or similar TNFα in colon, trend ↓ in BM, ↓ in lung after flu infection; similar TNFα in vaginal washes, SI, BALF ↓ IL-12 in spleen after LCMV-infection; similar IL-12 in colon, SI, vaginal washes with or without HSV-2 infection	Hill et al., 2010; Abt et al., 2012; Suárez-Zamorano et al., 2015; Oh et al., 2016; Yan et al., 2016; Ekmekciu et al., 2017; Li et al., 2017; Burrello et al., 2018; Robak et al., 2018	↓ TNFα in colon, BM, WAT; similar in popliteal LN; ↓ in lung after *K. pneumoniae* infection ↑ IL-12β, similar IL-12α in colon ↓ IFNγ, TNFα in skin, similar IFNγ in popliteal LN, spleen after *Leishmania* infection ↓ IFNγ in draining LN after EAE induction	Oliveira et al., 2005; Zaph et al., 2008; Lee et al., 2011; Caesar et al., 2012; Fagundes et al., 2012; Naik et al., 2012; Sjögren et al., 2012; Yan et al., 2016
Th2 cytokines	↑ IL-4 in inguinal subcutaneous adipose tissue, in mediastinal LN after allergen exposure; similar in SI, vaginal washes ↑ IL-5 in inguinal subcutaneous adipose tissue, vaginal mucosa; similar in SI Similar IL-6 in SI, BM, vaginal washes, BALF, liver; ↑ in BALF after *P. aerigunosa* infection; similar or ↓ in colon; ↓ in BALF after flu infection, in spleen after LCMV infection Similar IL-10 in spleen, lung; similar or ↓ in SI; ↓ in colon Similar IL-13 in SI; ↑ in inguinal subcutaneous adipose tissue, in mediastinal LN after allergen exposure	Rakoff-Nahoum et al., 2004; Abt et al., 2012; Hill et al., 2012; Suárez-Zamorano et al., 2015; Oh et al., 2016; Yan et al., 2016; Ekmekciu et al., 2017; Li et al., 2017; Burrello et al., 2018; Robak et al., 2018	Similar IL-6 in colon; similar or ↑ in BM; similar or ↓ in SI Similar IL-10 in colon; ↓ IL-10 in WAT Similar IL-13 in colon ↑ IL-10 in lung after *K. pneumoniae* infection Similar IL-4 in popliteal LN, spleen after *Leishmania* infection	Oliveira et al., 2005; Zaph et al., 2008; Caesar et al., 2012; Fagundes et al., 2012; Shaw et al., 2012; Sjögren et al., 2012; Ohnmacht et al., 2015; Yan et al., 2016
Th17 cytokines	↓ IL-22 in SI, colon Similar IL-17 in lung; similar or ↓ in SI, colon; ↓ in liver; ↓ in lung after *S. pneumoniae* or *K. pneumoniae* infection	Hill et al., 2010; Deshmukh et al., 2014; Suárez-Zamorano et al., 2015; Brown et al., 2017; Ekmekciu et al., 2017; Li et al., 2017; Burrello et al., 2018	↓ IL-17 in SI; ↑ IL-17 in colon ↓ IL-17 in draining LN after EAE induction	Ivanov et al., 2008; Zaph et al., 2008; Deshmukh et al., 2014

BALF, bronchoalveolar lavage fluid; BM, bone marrow; EAE, experimental autoimmune encephalomyelitis; LN, lymph node; SI, small intestine; WAT, white adipose tissue.

cells (Ivanov et al., 2008; Ismail et al., 2009, 2011; Naik et al., 2012; Li et al., 2017). Levels of NK T cells are generally similarly maintained in tissues after antibiotics treatment, though their activation has not been well-studied (Ochoa-Repáraz et al., 2009; Li et al., 2017; Burrello et al., 2018). Likewise, ILCs have not been extensively evaluated after antibiotics treatment, although multiple studies report shifts in the representation or function of ILC subsets at mucosal surfaces (Ochoa-Repáraz et al., 2009; Sawa et al., 2011; Ganal et al., 2012; Mortha et al., 2014; Hashiguchi et al., 2015; Gury-BenAri et al., 2016; Oh et al., 2016; Kim et al., 2017; Li et al., 2017).

CYTOKINES

Although shifts in cytokine levels after antibiotics treatment are variable (**Table 3**), most studies that report differences describe a shift away from proinflammatory cytokines. Many associate microbiota depletion with decreases in the production of IL-1

family cytokines, Th1 cytokines such as IFNγ and TNFα, and IL-17 family cytokines. The production of these cytokines is often similar or reduced specifically in the gastrointestinal tract in naïve animals, but the diminished response becomes apparent after challenge with a pathogen, often at the site of infection. For example, although IL-1 family cytokine levels are similar, pro-IL-1 and pro-IL-18 are reduced in the vaginal mucosa and lung in antibiotics-treated mice at baseline, associated with a reduced production of IL-1 family cytokines in response to infection at each site (Ichinohe et al., 2011; Abt et al., 2012; Oh et al., 2016; Robak et al., 2018). Similarly, type 1 cytokines such as IFNγ and TNFα are generally present at similar levels in tissues after antibiotics treatment in uninfected mice but reduced at the site of infection in microbiota-depleted mice (Abt et al., 2012; Oh et al., 2016; Robak et al., 2018). Inflammatory Th17 cytokines such as IL-17 and IL-22 are generally reduced in the intestines even at baseline in antibiotics-treated mice (Hill et al., 2010; Deshmukh et al., 2014; Ekmekciu et al., 2017), whereas differences in tissues such as the lung become apparent after infection at that site (Deshmukh et al., 2014; Suárez-Zamorano et al., 2015). In parallel with this decrease in inflammatory cytokines, some reports suggest that there is an increase in the expression of Th2 family cytokines such as IL-4, IL-5, and IL-13, especially after allergen exposure, consistent with a shift from Th1 to Th2-type immunity after microbiota depletion (Hill et al., 2010; Suárez-Zamorano et al., 2015; Oh et al., 2016).

Broadly, there is reasonable concordance between germ-free and antibiotics treatment mouse models in alterations of cellular compartments and cytokines. However, different groups have reported disparate findings with different antibiotics treatment regimens, making it challenging to definitively categorize microbiota-mediated modulatory effects. We propose that distinct starting microbiota composition and distinct regimens likely underlie this variability, and highlight this as an area of much-needed standardization.

MICROBIOTA EFFECTS AT THE ORGAN LEVEL

In addition to shifts in cell populations and signaling pathways, antibiotics treatment has been seen to affect organ morphology more broadly, both in the gastrointestinal tract as well as in extra-intestinal organs (**Table 4**). As the bulk of commensals reside in the gastrointestinal tract where they assist with digestion and interact closely with epithelial cells, it is not surprising that many changes are seen in intestinal physiology after microbial depletion. The length of the whole intestine or the colon is not affected, but the cecum becomes dramatically larger, transit time increases, and fecal pellet frequency and consistency can be altered (Grasa et al., 2015; Suárez-Zamorano et al., 2015; Ge et al., 2017). Moreover, villi become narrower (Kernbauer et al., 2014), cellular proliferation decreases (Reikvam et al., 2011; Ekmekciu et al., 2017), and features such as tuft cells (Wilen et al., 2018) or goblet-cell antigen passages (Knoop et al., 2015) are affected in specific regions of the gastrointestinal tract. Immune function in the intestines is also affected, as the production of antimicrobial peptides is reduced (Brandl et al., 2008; Vaishnava et al., 2008; Kinnebrew et al., 2010; Reikvam et al., 2011), Paneth cells granules are diminished (Kernbauer et al., 2014), Peyer's patches become less abundant and decrease in cellularity (Reikvam et al., 2011; Grasa et al., 2015; Hashiguchi et al., 2015), expression of Toll-like receptors is altered (Grasa et al., 2015), and tolerance to the commensal intestinal microbiota is impaired (Kim et al., 2018).

Non-gastrointestinal organs also depend on ongoing bacterial signals to maintain normal morphology. The spleen (Ochoa-Repáraz et al., 2009; Reikvam et al., 2011; Yoshiya et al., 2011; Zhang et al., 2015; Josefsdottir et al., 2017; Thackray et al., 2018), thymus (Josefsdottir et al., 2017), and lymph nodes (Ichinohe et al., 2011; Durand et al., 2018) may decrease in size and/or cellularity after antibiotics treatment. Liver regeneration is impaired in antibiotics-treated mice (Wu et al., 2015) and bile acid synthesis is altered (Sayin et al., 2013; Zhang et al., 2014). Additionally, fat pads diminish and bone mass increases, consistent with a role for the microbiota in maintaining normal body composition (Suárez-Zamorano et al., 2015; Yan et al., 2016). There have been a number of intriguing studies recently exploring the role of the microbiota in regulating brain function and behavior via the gut-brain axis; this complex topic has been recently reviewed elsewhere (Cryan and Dinan, 2012; Liu and Zhu, 2018).

MICROBIOTA REGULATION OF IMMUNE CHALLENGES

As might be expected, given the systemic and tissue-specific differences in immune function, antibiotics-treated mice are more susceptible to a variety of pathogens. For example, microbiota-depleted mice are more susceptible to bacterial pathogens such as vancomycin-resistant enterococcus, *Salmonella*, and *Clostridium difficile* in the gastrointestinal tract (Kinnebrew et al., 2010; Fernández-Santoscoy et al., 2015; Theriot et al., 2016), a variety of pneumonia-causing bacteria in the respiratory tract (Brown et al., 2017; Robak et al., 2018), and systemic *Escherichia coli* (Deshmukh et al., 2014). Additionally, after antibiotics treatment, mice are impaired in their response to vaginal HSV-2 (Oh et al., 2016), flaviviruses (Thackray et al., 2018), influenza (Ichinohe et al., 2011; Abt et al., 2012), and cutaneous *Leishmania* (Naik et al., 2012). However, microbiota-depleted mice actually become less susceptible to enteric viral pathogens including murine norovirus and poliovirus (Kuss et al., 2011; Uchiyama et al., 2014; Baldridge et al., 2015), possibly due to direct interactions between viral pathogens and enteric bacteria or due to loss of specific cell types required for viral infection. Antibiotics-treated mice are additionally impaired in their development of tolerance to food antigens (Bashir et al., 2004; Kim et al., 2018) and are more prone to allergic diseases (Hill et al., 2012; Adami et al., 2018).

Many of the effects after antibiotics treatment in mice are consistent with what is seen in germ-free models, suggesting that these are dependent on regular signals from the microbiota.

TABLE 4 | Effects of the microbiota on individual organs.

	Broad-spectrum antibiotics	References	Germ-Free	References
Whole intestine	Similar length, ↑ transit time	Grasa et al., 2015; Ge et al., 2017		
Small intestine	↓ transit time similar apoptotic cells, fewer proliferating cells ↓ RegIIIγ and RegIIIβ production ↓ number of PP; ↓ cells in PP ↓ villus width, ↓ T cells/vilus ↓ granules/Paneth cell altered expression of TLRs similar tuft cells	Brandl et al., 2008; Vaishnava et al., 2008; Kinnebrew et al., 2010; Reikvam et al., 2011; Wichmann et al., 2013; Kernbauer et al., 2014; Grasa et al., 2015; Hashiguchi et al., 2015; Park et al., 2016; Ekmekciu et al., 2017; Durand et al., 2018; Schneider et al., 2018; Wilen et al., 2018	↓ transit time fewer proliferating cells ↓ RegIIIγ, RegIIIβ production ↓ villus width, ↓ T cells/vilus, ↓ cells in LP ↓ cells in PP ↓ mucus thickness, attachment to epithelium; mucus more attached ↓ granules/Paneth cell, ↓ lysozyme+ cells/crypt similar tuft cells ↑ bile acids	Vaishnava et al., 2008; Sayin et al., 2013; Wichmann et al., 2013; Kernbauer et al., 2014; Schütte et al., 2014; Johansson et al., 2015; Park et al., 2016; Durand et al., 2018; Schneider et al., 2018
Cecum	↑ size ↑ villus length and width ↓ SCFAs decreased thickness of muscularis propria	Hill et al., 2010; Corbitt et al., 2013; Kelly et al., 2015; Park et al., 2016; Yan et al., 2016	↑ size ↑ villus length and width ↓ SCFAs, bile acids Decreased thickness of muscularis propria	Hill et al., 2010; Corbitt et al., 2013; Sayin et al., 2013; Smith et al., 2013; Yan et al., 2016
Colon	Similar length, ↑ transit time ↓ RegIIIγ and RegIIIβ, other anti-microbial factors ↓ epithelial regeneration, ↓ proliferating cells Similar mucus penetrability Altered expression of TLRs ↓ tuft cells ↓ SCFAs Formation of goblet-cell antigen passages	Reikvam et al., 2011; Wichmann et al., 2013; Grasa et al., 2015; Johansson et al., 2015; Knoop et al., 2015; Ekmekciu et al., 2017; Ge et al., 2017; Wilen et al., 2018	↓ RELMβ, other anti-microbial factors ↓ crypt height Similar mucus thickness, attachment to epithelium; decreased impenetrable mucus Similar tuft cells ↓ SCFAs, bile acids Formation of goblet-cell antigen passages	He et al., 2003; Matsumoto et al., 2012; Sayin et al., 2013; Wichmann et al., 2013; Kernbauer et al., 2014; Johansson et al., 2015; Knoop et al., 2015; Levy et al., 2015; McKinley et al., 2017
Lymph nodes	Similar or ↓ cellularity ↓ size and cellularity after flu infection	Ichinohe et al., 2011; Durand et al., 2018	↓ or similar cellularity Altered structure	Bauer et al., 1963; Manolios et al., 1988; Kernbauer et al., 2014; Zhang et al., 2015; Durand et al., 2018
Spleen	Similar or ↓ weight ↓ cellularity, fewer leukocytes	Ochoa-Repáraz et al., 2009; Reikvam et al., 2011; Yoshiya et al., 2011; Grasa et al., 2015; Suárez-Zamorano et al., 2015; Zhang et al., 2015; Josefsdottir et al., 2017; Thackray et al., 2018	Similar cellularity, similar lymphocytes Altered structure	Bauer et al., 1963; Mazmanian et al., 2005; Zhang et al., 2015
Thymus	↓ weight	Josefsdottir et al., 2017	Similar cellularity	Nakajima et al., 2014
Liver	Similar or ↓ weight Impaired regeneration Altered bile acid production	Corbitt et al., 2013; Sayin et al., 2013; Zhang et al., 2014; Wu et al., 2015; Yan et al., 2016	Similar weight Impaired regeneration Altered bile acid production	Cornell et al., 1990; Corbitt et al., 2013; Sayin et al., 2013; Yan et al., 2016
Fat	↓ weight of abdominal fat pads ↓ inguinal and perigonadal adipose tissue	Suárez-Zamorano et al., 2015; Yan et al., 2016	↓ weight of abdominal fat pads ↓ % body fat	Caesar et al., 2012; Yan et al., 2016
Bone	↑ bone mass	Yan et al., 2016	↑ bone mass vs. short-term SPF colonized, ↓ bone mass/length vs. long-term SPF colonized ↑ bone mass vs. conventional	Sjögren et al., 2012; Yan et al., 2016

PP, Peyer's patch; TLR, Toll-like receptor; LP, lamina propria; SCFA, short-chain fatty acid; SPF, specific-pathogen-free.

However, it is important to note that antibiotics can have effects on eukaryotes independently of the microbiota, as treatment of germ-free mice with antibiotics can replicate some findings seen when treating normally colonized mice (Han et al., 2015; Gopinath et al., 2018). Replicating key findings in germ-free mice can help confirm that the differences seen after antibiotics treatment are indeed caused by microbial depletion.

CONSIDERATIONS FOR THE FUTURE

While antibiotics treatment offers an inexpensive and accessible alternative to germ-free models, results obtained using these regimens come with the caveats of potential off-target drug effects and incomplete or inconsistent ablation of microbes. Additionally, because so many groups use distinct treatment regimens and mouse microbial populations may be institution-specific, antibiotics studies are much more challenging to compare than germ-free mouse studies.

We suggest that some standardization of antibiotics treatment regimens would be helpful; for example, if a standard cocktail were employed to demonstrate an initial finding, this could be compared to other studies, and subsequent follow-up experiments could be done with modified cocktails as necessary. Additionally, we suggest that at least a limited assessment of the replicability of findings in antibiotics-treated mice and germ-free mice would be of high value for most studies, to rule in or out potential off-target drug effects or developmental differences between germ-free and standard pathogen-free mice that may be important for a phenotype. Finally, it will be critical for investigators to ensure that microbial loads are consistently monitored in both antibiotics treatment and germ-free models to identify any effects of contaminants or antibiotic-resistant microbes.

Ensuring that we are able to interpret the contribution of an individual study to the field of microbiota research will require careful planning and execution of these experiments on the part of investigators. As we continue to uncover additional health and disease states in which the microbiota plays a role, the use of these models will become increasingly common.

AUTHOR CONTRIBUTIONS

EK and MB wrote and edited the manuscript. KK edited the manuscript.

ACKNOWLEDGMENTS

MB was supported by NIH grant K22 AI127846, Digestive Diseases Research Core Centers P30 DK052574, and the Global Probiotics Council's Young Investigator Grant for Probiotics Research. KK was supported by NIH grants R01 HL136333 and R01 HL134880. EK was supported by NSF Graduate Research Fellowship DGE-1745038.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 31 July 2018
doi: 10.3389/fimmu.2018.01765

Antitumor Activity of Extract From the Sporoderm-Breaking Spore of *Ganoderma lucidum*: Restoration on Exhausted Cytotoxic T Cell With Gut Microbiota Remodeling

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OPEN ACCESS

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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: 15 November 2017
Accepted: 17 July 2018
Published: 31 July 2018

Citation:
Su J, Su L, Li D, Shuai O, Zhang Y, Liang H, Jiao C, Xu Z, Lai Y and Xie Y (2018) Antitumor Activity of Extract From the Sporoderm-Breaking Spore of Ganoderma lucidum: Restoration on Exhausted Cytotoxic T Cell With Gut Microbiota Remodeling. Front. Immunol. 9:1765. doi: 10.3389/fimmu.2018.01765

As breast cancer is the leading cause of cancer-related deaths in women population worldwide, ongoing endeavor has been made for alternative regimens with improved efficacy but fewer adverse effects. Recently, active components from the spores of *Ganoderma lucidum* have attracted much attention for their versatile biological activities owing to the advance in sporoderm-breaking technology. Here, anticancer potential of an extract derived from the sporoderm-breaking spores of *G. lucidum* (ESG) was explored in a 4T1-breast cancer xenograft mice model. Results showed that ESG was able to suppress 4T1 tumor growth *in vivo* rather than *in vitro*. Flowcytometry analysis revealed that ESG could significantly increase both cytotoxic T cell (Tc) population and the ratio of Tc to helper T cell (Th) in peripheral blood of the tumor-bearing mouse; similar promotion on Tc was also found in tumor-infiltrating lymphocyte. Moreover, ESG evidently down-regulated the two immune checkpoints, programmed cell death protein-1 (PD-1, in the spleen) and cytotoxic T lymphocyte antigen-4 (CTLA-4, in the tumor), suggesting that ESG could effectively restore the T cell paradigm by recovering the exhaustion status *via* suppressing the co-inhibitory checkpoints. By 16S rRNA gene sequence analysis on the fecal microbiota, it was found that ESG would remodeling the overall structure of the samples from tumor-bearing mice toward that of the normal counterparts, including 18 genera in 5 phyla, together with regulations on several genes that are responsible for signaling pathways involved in metabolism, cellular processes, and environmental information processing. Collectively, this study demonstrated that ESG would serve as a natural anticancer adjuvant *via* a restoration on the exhausted Tc, highlighting important clinical implications for the treatment of breast cancer.

Keywords: spore of *Ganoderma lucidum*, cytotoxic T cells, exhaustion, immune checkpoints, gut microbiota

INTRODUCTION

Breast cancer is one of the most frequently diagnosed cancers and leading causes of cancer-related death both worldwide (1, 2). It is a highly heterogeneous disease. Besides the multiple signaling pathways that mediate its initiation and progression, accumulating epidemiology studies have proposed several suspected risk factors for breast cancer, including exposures to cancerigenic substances,

certain lifestyles (such as lack of physical exercise, intemperance), aging and family history (3). To date, surgical resection, adjuvant chemotherapy, radiotherapy, and hormone therapy represent the main treatment options for early-stage breast cancer (4), but they are still unsatisfied due to the emerging drug resistance and adverse toxic effects (5, 6). Even more disturbing, triple-negative breast cancer does not respond to hormone therapies (7).

Despite the slow progress in the above regimes, recent success of cancer immunotherapy utilizing immune checkpoint blockade has reformed the treatment algorithms in various aggressive neoplastic diseases (8, 9). Immunotherapy is based upon the "3E immunoediting theory" (Eliminate, Equilibrium, and Escape) (10). It reminds that immune surveillance stands at the very heart of the fighting against cancer, but T cells would be exhausted and have decreased effector function and proliferative capacity during cancer progression, due the overexpression of immune checkpoints, and the interaction with their ligands (11, 12). So far, immunotherapy targeting programmed cell death protein-1 (PD-1)/programmed death-ligand 1 (PD-L1) has been on it way of clinical trials (13) for breast cancer, although optimal selection of ideal candidates to the immune therapy remains a challenge. On the other hand, increasing evidence highlights the cardinal role of gut microbiota in tumour genesis (14, 15) and that in the outcomes of chemotherapy (16) and immunotherapy (17, 18), due to their intrinsic capacity of drug metabolism and the influence on host metabolizing homeostasis (19, 20).

Ganoderma lucidum (Leyss. ex Fr.) Karst. is a valuable medical macrofungi that has long been used in traditional Chinese medicine for health and longevity for thousands of years (21). In addition to numerous benefit for the treatment of allergy, cardiovascular diseases, diabetics (22, 23), ample evidence have revealed that *G. lucidum* exerts anticancer effects not only *via* direct cytotoxicity approaches, such as cell cycle arrest (24), apoptosis induction (25), and migration inhibition (26), but also, more importantly, through several ways of immune enhancement (27–29). Recently, although active components from fruiting body or mycelia are still hotspots of *G. lucidum* studies, those from the spores of *G. lucidum* (SG) have attracted much attention for their versatile biological activities owing to the advance in sporoderm-breaking technology. Studies reported that SG displayed anticancer potentials against Sarcoma 180 and HCT116 (30–32). Moreover, it has been found that polysaccharide content in SG was higher than that in fruiting body (33) and that sporoderm-breaking would potentiate the immunoregulation activity of spore (32, 34, 35). These studies suggest that SG may serve as promising anticancer agent for cancer therapy. In this study, anticancer potential of an extract from the sporoderm-breaking SG, for the first time, was investigated in a breast cancer xenograft mice model. The exploration focused on the interaction between T cell restoration and gut microbiota, to make a comprehensive interception for the anticancer activity of the sporoderm-breaking SG.

MATERIALS AND METHODS

Animals

BALB/c mice (female, 18–22 g, aged 6–8 weeks) were provided by Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, China). All animals were housed at 20 ± 2°C with a humidity of 50 ± 5% in a 12 h light/dark cycle with food and water *ad libitum*. The animals were acclimatized for 7 days, and the experiment was performed according to the Guidelines of Guangdong Institute of Microbiology Laboratory Animal Center, Guangdong Institute of Microbiology Laboratory Animal Ethics Committee. The experimental protocols were approved by the Guangdong Institute of Microbiology Laboratory Animal Ethics Committee.

Preparation for Extract of the Sporoderm-Breaking Spore of *G. lucidum* (ESG)

The sporoderm-breaking SG was provided by Guangdong Yuewei Edible Fungi Technology Co. Ltd. It was extracted with boiling water (15 L/kg) for 2 h and concentrated under vacuum. The concentrated extract was subjected to two to three cycles of precipitation with anhydrous ethanol at a final ethanol percentage of 85%. The obtained precipitate was dissolved in water and dialyzed with 3,500 Da dialysis tube (MWCO). Content in the 3,500 Da dialysis tube was then dialyzed in 100 kDa dialysis tube (MWCO). The dialysate was pooled, concentrated, and lyophilized, to obtain ESG with a yield of 0.4%.

Characteristics analysis for ESG was performed as described by Qiao et al. (36) with mild modification. Results showed that sugar content of ESG was 50% (determined by the phenol–sulfuric acid method using glucose as standard), while protein was hardly detected by BCA methods with the BCA protein kit (Kang wei shiji Co. Ltd., Beijing, China), suggesting that ESG is rich in polysaccharide. Weight average molecular weight (M_w) by size-exclusive high performance liquid chromatography showed that polysaccharide in ESG was about 3.6 kDa (Table S1 and Figure S1 in Supplementary Material). Monosaccharide composition analysis by gas chromatograph showed that ESG was mainly made up of glucose (Figure S2 in Supplementary Material).

Cell Culture

Murine metastatic breast cancer 4T1-cell line was obtained from the Cell bank of Chinese Academy of Sciences, Shanghai, China. 4T1 cells were cultured in high glucose DMEM medium (4.5 mg/mL, Gibco, NY, USA) supplemented with 10% fetal bovine serum (Gibco, NY, USA) and 1% penicillin/streptomycin (Gibco, NY, USA) and maintained in humidified incubators at 37°C under an atmosphere of 5% CO_2.

Cytotoxicity Assay

4T1 cells were seeded in a 96-well plate at a density of 1.25×10^4 cells/mL (sextuple wells in each group) and treated with ESG in complete DMEM medium at multiple concentrations (12.5, 25, 50, 100, and 200 μg/mL) for 24 and 48 h. Then, the medium was replaced with 100 μL of complete DMEM medium containing 0.5 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) for another 4-h incubation. At last, the medium was discarded, and 150 μL of DMSO was added to dissolve the formazan. The optical density was measured at 490 nm on a microplate reader.

4T1-Breast Cancer Xenograft Model Induction

To induce the breast cancer xenograft model, female BALB/c mice were implanted with 4T1 tumor cells by subcutaneous injection at the right forleg armpit (0.1 mL/mouse, 2×10^5 cells/mouse), and then they were randomly divided into Model group, paclitaxel group (PTX, Hainan Quanxing Pharmaceutical Co. Ltd., Hainan, China), and the ESGH group (400 mg/kg), ESGL group (200 mg/kg), eight for each. On the same day, control animals (Normal group, eight mice) received an injection of 0.1 mL of complete DMEM medium at the similar site. Over the following 21 days, mice in the PTX group were administrated with PTX at a dose of 15 mg/kg (i.p.) twice a week; mice of the ESG groups were given different doses of ESG (i.g.) every day. Normal group and Model group received equal volume of water.

Tumor Measurement and Histology Observation by Hematoxylin–Eosin Staining

Throughout the 21-day administration, tumor volume was measured with an electronic vernier caliper every 3 days since sixth day. Tumor volume was calculated as $V = a \times b^2/2$, where a indicates the longer diameter and b indicates the shorter diameter. 24 h after the last administration, all animals were sacrificed by cervical dislocation. Tumors, spleens, and peripheral blood were harvested for further analysis.

Tumors were weighted, photographed, cut into several segments, and then stored according to different purposes once they were harvested. One segment of the tumor was fixed in 4% neutral formalin (in PBS), embedded by paraffin, and stained with hematoxylin–eosin (HE). The stained sections were observed and photographed under a light microscope (with 200× magnification).

Peripheral Blood Lymphocyte Analysis by Flow Cytometry

Peripheral blood was collected from the orbital vein plexus with EDTA-Li micro-anticoagulant tube. 50 μL of blood was stained with FITC anti-mouse CD3 (0.125 μg/test), APC anti-mouse CD4 (0.0625 μg/test), PE anti-mouse CD8 (0.125 μg/test, Invitrogen, Thermo Fisher Scientific, San Diego, CA, USA) at 4°C in dark for 30 min, and then erythrocytes were lysed in ACK Lysis Buffer for 10 min. Following by two washes with pre-cold PBS, T cell subsets in the peripheral blood were enumerated with a FACS Canto II cytometer (BD, NY, USA), and the data were analyzed by Diva software (version 6.1.3).

Tumor-Infiltrating Lymphocyte (TIL) Isolation and Analysis

Tumor segment kept in cold PBS was used for TIL isolation and analysis. In brief, they were minced and digested in 3 mL digestive medium, which was mainly composed of basic RPMI-160 medium supplemented with 0.1% Type IV collagenase (Invitrogen, Thermo Fisher Scientific, Grand Isle, NY, USA), 350 U/mL DNAse I (Roche, Basel, Switzerland), and 1% penicillin–streptomycin. Then, it was ground in pre-cold PBS by passing through a 70 μm strainer, washed with PBS, and resuspended in basic RPMI-160 medium. TILs from the obtained cell suspension were separated with Mouse 1× Lymphocyte Separation Medium (Dakewe Biotechnology Co. Ltd., Shenzhen, China) according to the manufacture's instruction. TILs were stained with FITC anti-mouse CD3 (0.125 μg/test), APC anti-mouse CD4 (0.0625 μg/test), and PE anti-mouse CD8 (0.125 μg/test) at 4°C in dark for 30 min. After two washes with pre-cold PBS, T cell subsets in TIL were enumerated with a FACS Canto II cytometer, and the data were analyzed by Diva software (version 6.1.3).

Programmed Cell Death Protein-1 (PD-1) Protein Content Determination by ELISA

About 100 mg of spleen tissue was cut into pieces, homogenized in ice-cold PBS with proteinase inhibitor cocktail (Roche, USA) for 30 s, centrifuged at 18,000 × *g* for 20 min at 4°C. Protein content in the supernatant was quantified with BCA protein kit (Kang wei shiji Co. Ltd., Beijing, China). PD-1 protein content in spleen was measured using the Mouse PD1 ELISA Kit (Abcam, Cambridge, UK, Cat: ab210971) according to the manufacturer's instruction.

Immunohistochemistry (IHC) for Cytotoxic T Lymphocyte Antigen-4 (CTLA-4)

Immunohistochemistry for CTLA-4 was performed with the formalin-fixed, paraffin-embedded tumor segment. Briefly, the slides were deparaffinized. Antigen retrieval was carried out by incubation in EDTA buffer (pH 8.0) *via* microwave heating. After being washed with PBS, endogenous peroxidase in the section was blocked with 3% H_2O_2 in dark for 25 min. Sections were blocked with 3% normal non-immune serum and then incubated with primary antibody against mouse CTLA-4 (1:100, Lifespan Biosciences, London, UK) at 4°C overnight, and then with HRP-conjugated secondary antibodies at room temperature for 50 min. Finally, the sections were stained with DAB substrate and counterstained with hematoxylin. The mean density of positive area was calculated as ratio of integrated optical density to the total pixel of each picture (IOD/10^6 pixel), which was analyzed by Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Total RNA Extraction and Quantitative Real-Time PCR

Total RNAs from spleen and tumor were extracted with TRIzol according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, Grand Isle, NY, USA). 0.1 μg of total RNA was reverse transcribed using the PrimeScript™ RT reagent kit (Takara Bio, Inc., Shiga, Japan) following the supplier's protocol. The reactions were incubated at 35°C for 15 min, then at 85°C for 5 s, and the products were stored at 4°C. The PCR primer sequences are listed in **Table 1**. Real-time PCR reactions were performed with SYBR® Premix Ex Taq™ II (Takara Bio, Inc., Shiga, Japan), and the reaction program in an StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Grand Isle, NY, USA) was as follows: a precycling stage at 95°C for 30 s, then

TABLE 1 | Primers for real-time PCR.

Gene name	GenBank accession.		Primer	Product length (bp)
pd1	NM_008798.2	Sense	TTTGAGCCAACCCGTCCAGGAT	90
		Antisense	CGCCGTGTGTCAAGGATGTTCA	
ctla4	NM_001281976.1	Sense	GAGGTCTGTGCCACGACATTCA	190
		Antisense	CGTTGCCCATGCCCACAAAGTA	
gapdh	NM_008084	Sense	AAATGGTGAAGGTCGGTGTGAAC	90
		Antisense	CAACAATCTCCACTTTGCCACTG	

40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s. Fluorescence was measured at the end of each annealing step, and the melting curves were monitored to confirm the specificity of the PCR products. The $2^{-\Delta\Delta Ct}$ method was used to determine the mRNA expression levels of *pd1* and *ctla4* relative to control gene *gapdh*.

16S rRNA Gene Sequence Analysis of Gut Microbiota in Fecal Samples

Sequencing service was provided by Personal Biotechnology Co., Ltd., Shanghai, China. Total DNA was isolated from the fecal samples as previously reported with some modification (37). Briefly, OMEGA Soil DNA Kit (OMEGA, US) was used following the manufacturer's recommendations. The bacterial 16S rRNA gene V3–4 region was amplified by PCR using the forward primer (5′-AYTGGGYD TAAAGNG-3′) and the reverse primer (5′-TACNVGGGTATCTAATCC-3′). The PCR products were separated by gel electrophoresis and purified using the AP-GX-500 DNA Gel Extraction Kit (Axygen, Corning, USA). Library was build up with the obtained products and then sequenced on a MiSeq sequencing platform (Illumina, USA) as described by Zhao et al. (38).

Bioinformatics Analysis

The trimmed and assembled sequences from each sample were aligned to the Greengene 16S rRNA database set 10 using the best hit classification option to classify the taxonomy abundance in QIIME[1] (39). Bacterial operation taxonomic units (OTU) were generated using the uclust function in QIIME.[2] A Venn diagram was generated to compare OTUs between groups. The following statistics were performed by R software. ACE, Chao, Simpson, and Shannon indices were calculated for α-diversity evaluation. Principal component analysis (PCA) and UniFrac distance-based Nonmetric Multidimensional Scaling (NMDS) were employed to assess β-diversity. Hierarchical clustering analysis of the OTUs presented by heatmap was performed using the heatmap package v1.0.7 running in R v3.2.1.[3] Taxon-based analysis and LefSe analysis were applied to identify different taxa microbes among lines using the default parameters (40).

Microbial functions were predicted using PICRUSt (41). The OTUs were mapped to gg13.5 database at 97% similarity by QIIME's command "pick_closed_otus." The OTUs' abundance was normalized automatically using 16S rRNA gene copy numbers from known bacterial genomes in Integrated Microbial Genomes. The predicted genes and their functions were aligned to Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and differences among groups were compared through software STAMP[4] (42). Two-side Welch's *t*-test and the Benjamini–Hochberg FDR ($p < 0.05$) correction were used in two-group analysis.

Statistics

Statistical analysis was performed with SPSS 22 (IBM Corp., NY, USA). Datasets from each experiment were subjected to normal distribution test first. If according with the normal distribution, the data was analyzed by one-way analysis of variance (ANOVA), following by pairwise comparison with different parametric test depending on test for homogeneity of variance, otherwise the data was compared by Kruskal–Wallis *H* Test. In ANOVA, *post hoc* LSD test was applied for difference analysis under homogeneity of variance, if not, a Dunnett's test would be applied. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared with Normal group; $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with Model group.

RESULTS

ESG Inhibited Tumor Growth

In **Figure 1A**, it was found that viability of 4T1 cells was not affected by either 24 or 48 h treatment of ESG (12.5–200 μg/mL) *in vitro*, implying that the antitumor activity of ESG was not mediated by a directly cytotoxicity. However, ESG exhibited a favorite antitumor activity in the xenograft model. Two mice of the PTX group died during the experiment. As shown in **Figures 1B,C**, tumor volume of Model group kept increasing throughout the 21-day experiment. By comparison, tumor volumes of the PTX group and ESG groups (400 and 200 mg/kg) showed remarkable reduction since the 15th day, although they also kept increasing in the earlier days. Finally, tumors of the Model group weighted 512 ± 45 mg, while those of PTX group and ESG groups (400 and 200 mg/kg) were significantly decreased to 193 ± 45 mg ($p < 0.01$), 387 ± 23 mg ($p < 0.05$), and 450 ± 41 mg, respectively, which were in accordance with the depressed volume. Moreover, histology analysis by HE staining revealed that tumors from Model group exhibited a homogeneous distribution of viable cells, while those of PTX group and ESG groups showed significant indication of

[1] http://qiime.org/index.html (Accessed: July 25, 2017).
[2] http://qiime.org/scripts/pick_otus.html (Accessed: July 25, 2017).
[3] http://www.r-project.org (Accessed: July 25, 2017).
[4] http://kiwi.cs.dal.ca/Software/STAMP (Accessed: July 25, 2017).

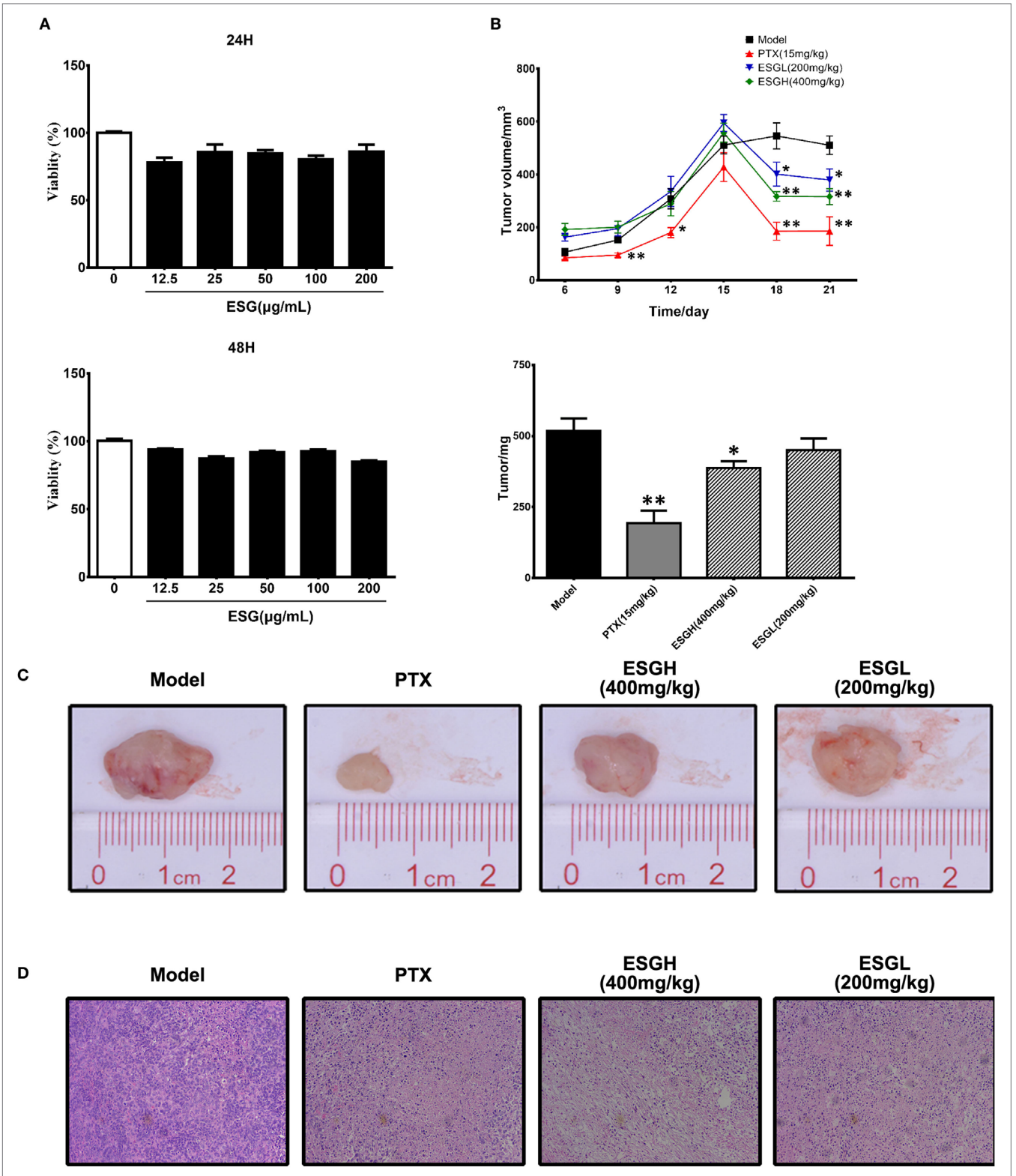

FIGURE 1 | Effect of ESG on 4T1-xenograft model. **(A)** Effect of ESG on the viability of 4T1 cell *in vitro* (48 and 72 h). **(B)** Tumor volume changes and tumor mass. **(C)** Representative images for tumor from each group. **(D)** Representative hematoxylin–eosin staining images for tumor tissue from each group (200× magnification). Values were represented the means ± SEM (n = 6–8). *$p < 0.05$ and **$p < 0.01$ versus Model group.

necrosis, including shrinkage of the cells, nuclear condensation, and fibrosis (**Figure 1D**).

ESG Recovered Proportions of Tc Cells Both in Peripheral Blood and Tumor Microenvironment (TME)

Since the antitumor activity of ESG was independent of cytotoxicity, we analyzed the impact of oral administration of ESG on immune surveillance system by flowcytometry (FCM) as it plays a cardinal role in the suppression of tumor. Results showed that in peripheral blood (**Figure 2**), percentage of T cell (CD^{3+}) of Model group was obviously lower than those of Normal group ($p < 0.01$), and distinct decreases were also found in the proportions of the two main subsets ($p < 0.01$), helper T cell (Th, $CD3^+CD4^+$) and cytotoxic T cell (Tc, $CD3^+CD8^+$). PTX made a more serious lost on T cell and Th cell when comparing with those of Model group ($p < 0.01$ and $p < 0.05$, respectively). On the other hand, although failing to lift the amount of T cell or that of the Th subset, ESG (200 mg/kg) prominently increased the percentage of Tc cell in the circulatory system ($p < 0.05$). Moreover, the ratio of Tc/Th was obviously elevated after ESG treatment (400 and 200 mg/kg, $p < 0.01$).

To make a more detail observation for the immune surveillance system in the TME, TIL was also determined by FCM. It was found that, although percentage of Th cell was decreased ($p < 0.01$), ESG brought out an evident increase on the Tc cell proportion in the TME ($p < 0.05$) accompanying with a higher Tc/Th ratio ($p < 0.01$), which was quite corresponding to the results of peripheral blood (**Figure 3**). Nevertheless, this promotion on Tc cell by ESG did not exist when ESG directly acted on splenocytes *in vitro* (Figure S3 in Supplementary Material).

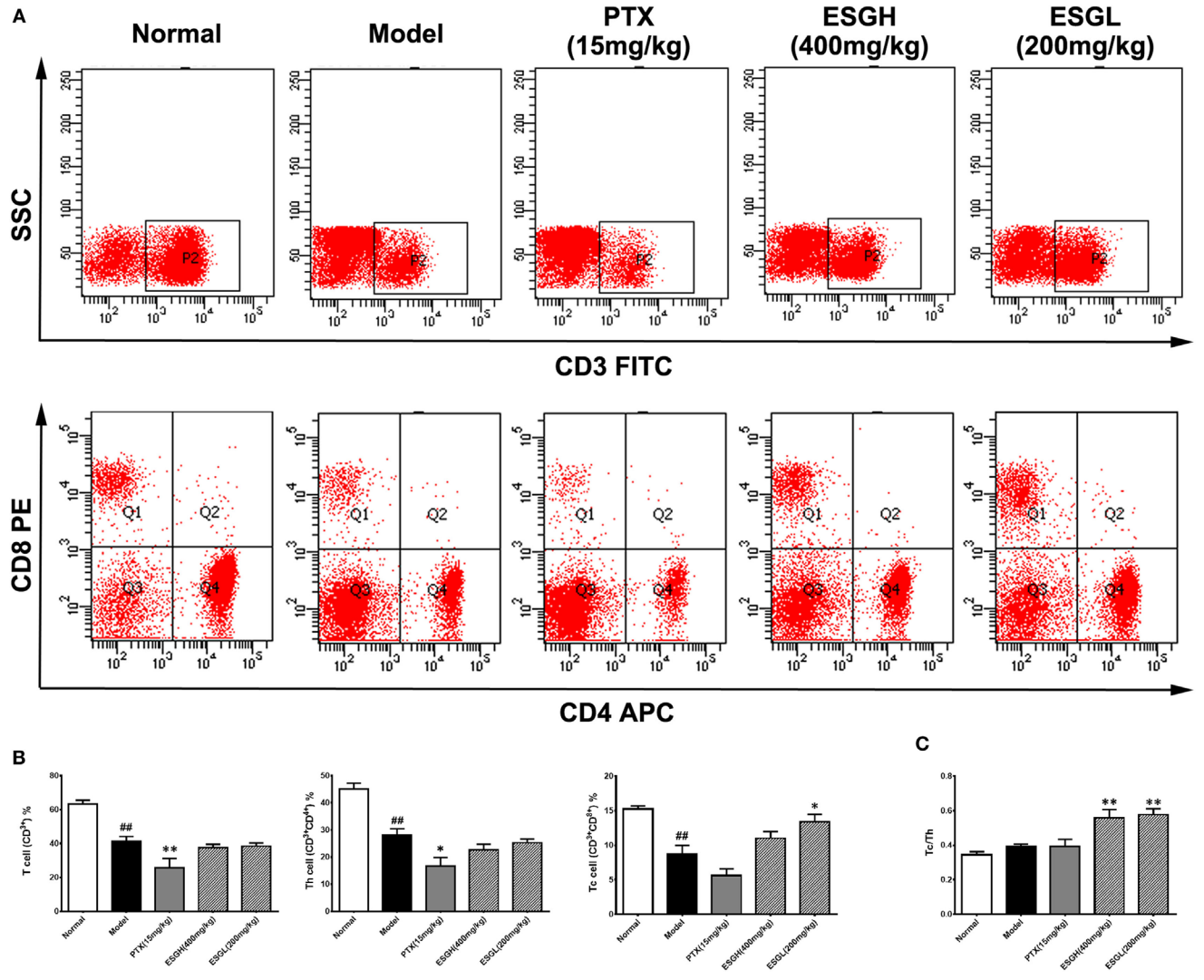

FIGURE 2 | Effect of ESG on T cell subsets in peripheral blood. **(A)** Representative scatter diagram. **(B)** Quantitative analysis for T cell subsets in peripheral blood. **(C)** Ratio of Tc/Th in peripheral blood. Values were represented the means ± SEM ($n = 6$–8). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus Normal group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus Model group.

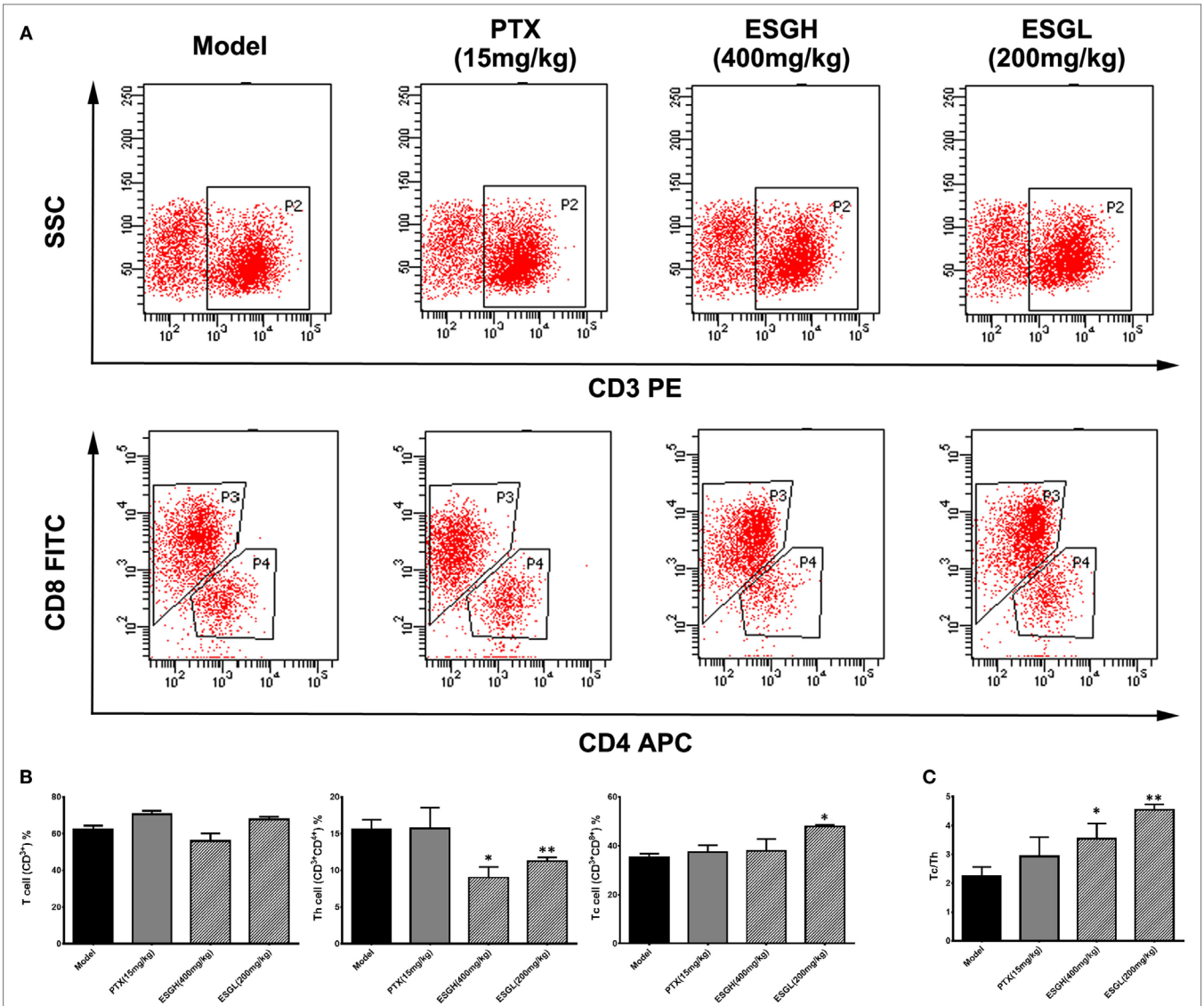

FIGURE 3 | Effect of ESG on T cell subsets in tumor-infiltrating lymphocyte (TIL). **(A)** Representative scatter diagram. **(B)** Quantitative analysis for T cell subsets in TIL. **(C)** Ratio of Tc/Th in TIL. Values were represented the means ± SEM (n = 6–8). *$p < 0.05$ and **$p < 0.01$ versus Model group.

ESG Suppressed the Immune Checkpoints

To further evaluate the enhancement of ESG on the T cell status, expressions of the two immune checkpoints, PD-1 and CTLA-4, were explored. Results from **Figure 4** displayed that 4T1-cell xenograft significantly upregulated the mRNA expressions of *pd1* in spleen ($p < 0.01$), while decreasing the protein level of PD-1 ($p < 0.01$). In comparison, both PTX and ESG obviously downregulated the *pd1* gene expression ($p < 0.01$), but not affecting the protein level of PD-1. In tumor tissue, PTX evidently increased PD-1 protein expression ($p < 0.05$), while the mRNA level was almost the same as those from the Model group. By contrast, ESG exhibited obvious inhibition on PD-1, especially that the 400 mg/kg ESG made strong suppressions on both levels of mRNA and protein ($p < 0.05$ and $p < 0.01$, respectively).

Regarding to CTLA-4, tumor xenograft made an evident upregulation on mRNA and protein level in spleen, while both treatments of PTX and ESG decreased them significantly (**Figure 5**). In tumor (**Figure 6**), PTX was intended to decrease mRNA level of *ctla4* but to increase its protein expression. By contrast, both dosages of ESG were able to evidently downregulate *ctla4* mRNA ($p < 0.05$), whereas not affecting the protein expression of CTLA-4 apparently.

ESG Modulated the Gut Microbiota

Overall Structural Modulation of Gut Microbiome After ESG Treatment

Given that gut microbiota has been recognized as a pivotal assistant in chemotherapy and immunotherapy, we investigated its possible involvement in the efficacy of ESG in this part. As the

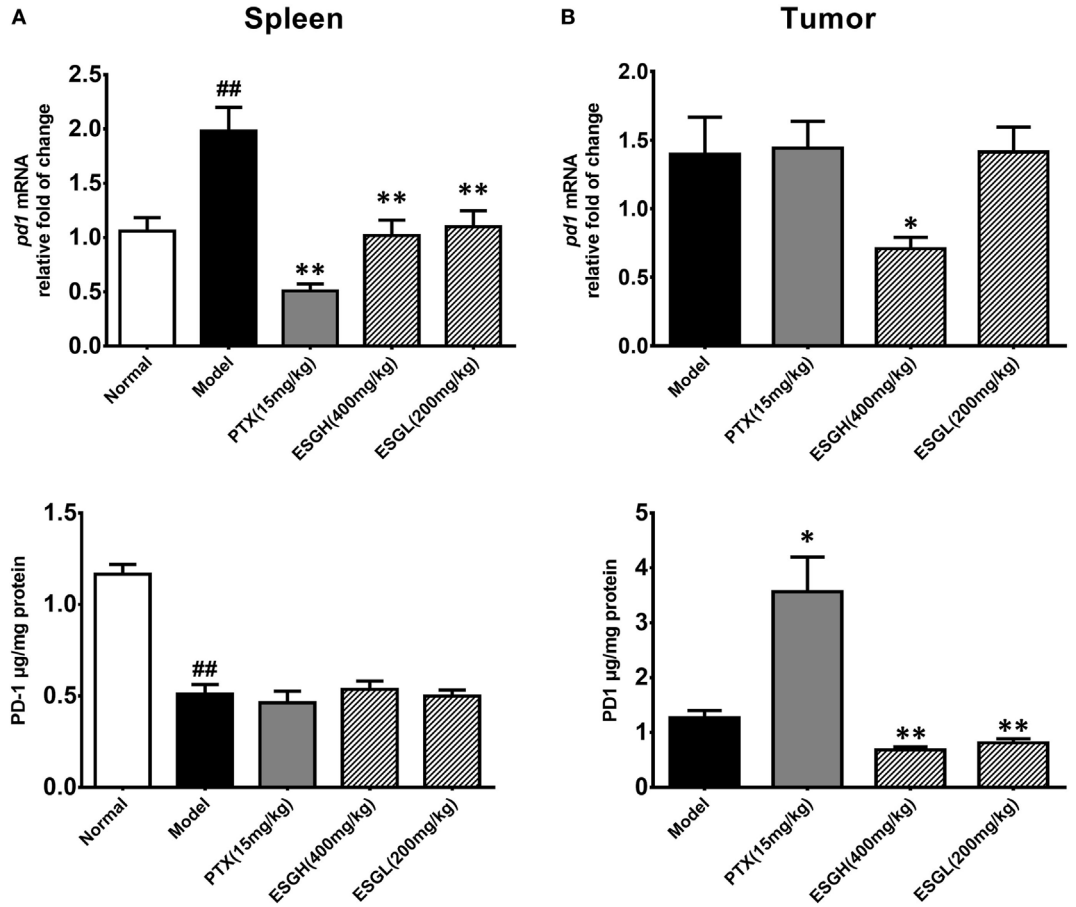

FIGURE 4 | Effect of ESG on PD-1 in spleen **(A)** and tumor **(B)**. The upper panels are results of quantitative real-time PCR for *pd1*. The lower panels are results of ELISA for PD-1 protein. Values were represented the means ± SEM (n = 6–8). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus Normal group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus Model group.

above data indicated that ESGH group (400 mg/kg) exhibited better antitumor effect in the 4T1-xenograft model, gut microbiota in fecal samples from the Normal group, Model group, and ESGH group were analysis by the Illumina Miseq sequencing system. A total of 903,411 sequences were obtained from all the fecal samples, with an average of 34,962 sequences per sample (33,526–43,436 sequences). The high-quality sequences were then delineated into 25,394 OTUs at a similarity cutoff of 97% as previously reported (43). Common OTU analysis presented by Venn diagram indicated that there existed 889 unique OTUs in Normal group, 723 in Model group, and 541 in ESGH group (400 mg/kg), respectively, while 1,480 common OTUs were identified in all samples (**Figure 7A**).

Microbiota community diversity was first evaluated by α-diversity analysis employing indices including Chao1, ACE, Shannon, and Simpson. Chao1 and ACE indices are estimators for community richness (44, 45). Shannon and Simpson indices represent community diversity and uniformity (46). As depicted by the data, gut microbiome of the ESGH group demonstrated significant reduced richness with lower Chao1 and ACE indexes ($p < 0.01$), compared with that of the Normal group (**Figure 7B**), while overall diversity was not affected. Rarefaction curve (**Figures 7C–D**) also presented a significant difference in the richness (Chao1), but not diversity (Shannon), among the three groups in this study.

β-Analysis was used to compare the similarity of overall community structure, which employed several unsupervised multivariate statistical assessments, including PCA and UniFrac NMDS. Both PCA (**Figure 8A**) and UniFrac NMDS (**Figure 8B**) displayed a marked structure shift in samples of Model group in contrast to those of Normal group; while after the 3-week treatment, gut microbiome from the tumor-bearing mice of ESGH group was restored to be similar with that of Normal group. UniFrac distances analysis (both weighted and unweighted, **Figure 8C**) made a further confirmation on the structure remodeling by ESG, as indicated by the significant intergroup difference between Normal group and Model group, as well as that between ESGH group and Model group ($p < 0.01$).

Shifts in Community Membership After ESG Treatment

Taxon-based analysis revealed marked differences at both phylum and genus levels among Normal, Model, and ESGH samples.

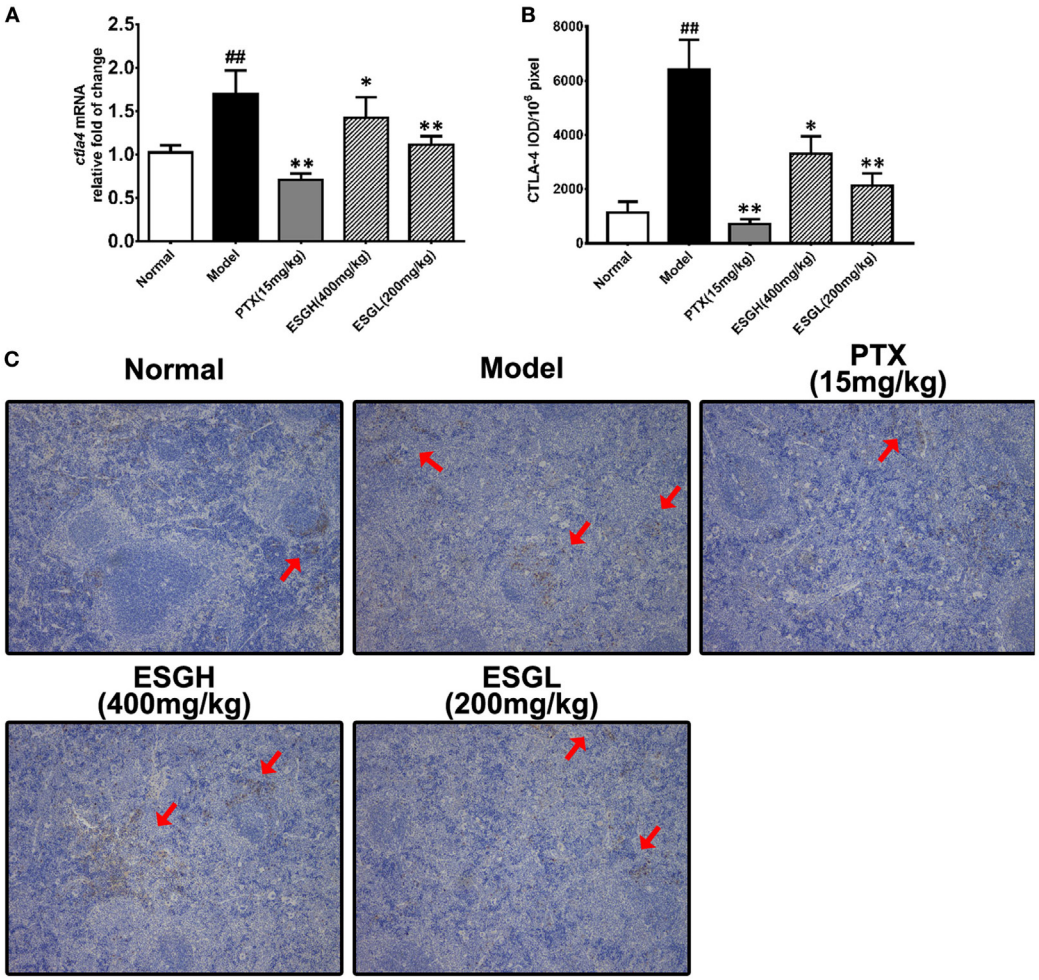

FIGURE 5 | Effect of ESG on cytotoxic T lymphocyte antigen-4 (CTLA-4) in spleen. **(A)** Quantitative real-time PCR for *ctla4*. **(B)** Immunohistochemistry (IHC) for CTLA-4 in spleen. The mean density of positive area was calculated as ratio of integrated optical density to the total pixel of each picture (IOD/10^6 pixel). **(C)** Representative IHC image (400×) for CTLA-4 in spleen. Values were represented the means ± SEM (n = 6–8). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus Normal group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus Model group.

Overall, a total of nine phyla were shared by samples from all groups (**Figure 9A**). Of them, *Firmicutes* and *Bacteroidetes* compromised over 90% of the total classified sequences. Relative abundances of *Actinobacteria, Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, and *Proteobacteria* displayed significant differences in the three groups. In particular, ESGH treatment significantly raised the relative abundances of *Firmicutes* ($p < 0.05$) and *Proteobacteria* ($p < 0.05$) but reduced those of *Actinobacteria* ($p < 0.01$), *Bacteroidetes* ($p < 0.01$), and *Cyanobacteria* ($p < 0.01$), which apparently reversed the community shift induced by tumor xenograft (**Figure 9B**). At genus level, a total of 61 genera were identified from all samples (Data Sheet 2 in Supplementary Material), and hierarchical clustering analysis presented by heatmap showed that fecal microbiota from the Model group exhibited obvious community shift compared with those of Normal group and ESGH group (**Figure 10A**). Although LefSe analysis indicated no genus is specific for any group, there were 18 genus exhibited obvious differences among them. Briefly, three genera (*Helicobacter*, *Rikenella*, and *Turicibacter*) were evidently higher in both Normal group and ESGH group than those in Model group, which have been reported to be positively related to the enhanced immune response (47–50); while another 15 genera (*Acinetobacter*, *Arthrobacter*, *Bacillus*, *Bacteroides*, *Blautia*, *Brevundimonas*, *Clostridium*, *Coprobacillus*, *Corynebacterium*, *Facklamia*, *Jeotgalicoccus*, *Parabacteroides*, *Prevotella*, *Sporosarcina*, *Staphylococcus*, and *Streptococcus*) were lowered in both Normal group and ESGH group in contrast to those in Model group (**Figure 10B**).

Microbiome Function Regulation by ESG Treatment

Via comparing the sequencing data with those collected in KEGG pathway database by PICRUSt (**Figure 11**), it was found that tumor xenograft significantly upregulated abundances of several genes that are responsible for five metabolism pathways, including "biosynthesis of other Secondary metabolites," "energy metabolism," "enzyme families," "glycan biosynthesis and

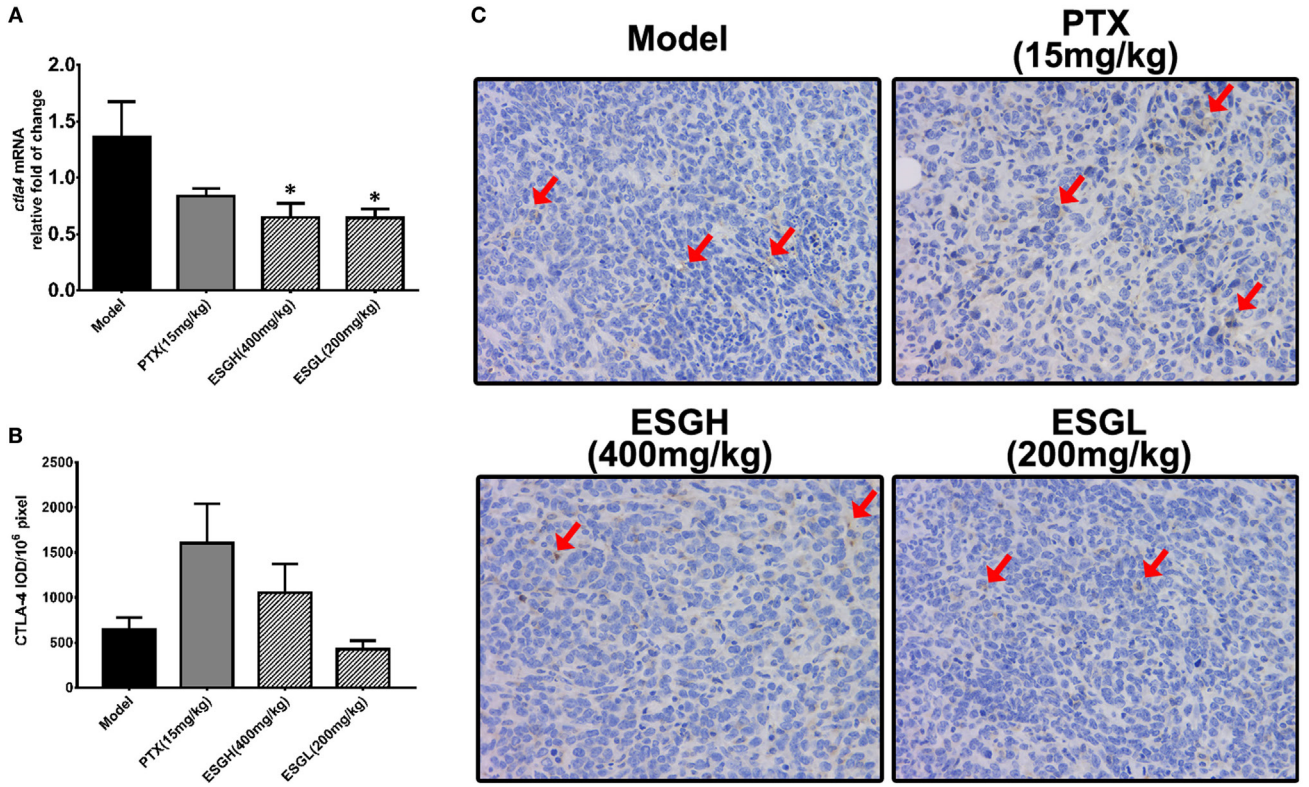

FIGURE 6 | Effect of ESG on cytotoxic T lymphocyte antigen-4 (CTLA-4) in tumor. **(A)** Quantitative real-time PCR for *ctla4*. **(B)** Immunohistochemistry (IHC) for CTLA-4 in tumor. The mean density of positive area was calculated as ratio of integrated optical density to the total pixel of each picture (IOD/10^6 pixel). **(C)** Representative IHC image (400×) for CTLA-4 in tumor. Values were represented the means ± SEM (n = 6–8). *$p < 0.05$ and **$p < 0.01$ versus Model group.

metabolism," and "metabolism of cofactors and vitamins," and a cellular processes pathway ("transport and catabolism"), but downregulated genes involved in the "cell mobility" and "signal transduction" pathways. After ESGH treatment, changes in the genes that referred to most of these pathways were evidently reversed, such as "transport and catabolism," "enzyme families," "glycan biosynthesis and metabolism," and "biosynthesis of other Secondary metabolites," indicating that together with the structural modulation, ESG could regulate the metabolic activities of the gut microbiota to promote the tumor immune surveillance.

DISCUSSION

With the great success of paclitaxel in cancer chemotherapy, increasing attention has been paid for the natural compounds in prevention and treatment of cancer. So far, anticancer candidates derived from natural products, including alkaloids, saponins, polysaccharides, terpenoids, and flavonoids, have been extensively studied in laboratories and clinical investigations. More recently, potential of the active constituents from sporoderm-broken SG was also explored. Na et al. (30) found that the aforementioned polysaccharide exerted direct cytotoxicity on HCT116 *via* cell cycle arrest and apoptosis, but at a relative high concentration (1.25–7.5 mg/mL) (30); while Wang et al. (31) showed that a commercial polysaccharide from SG suppressed Sarcoma 180 only *in vivo via* the stimulation of NK cells, T cells, and macrophages (31).

In this study, anticancer potential of the polysaccharide-rich ESG was investigated in a murine 4T1-breast cancer xenograft model. Results showed that ESG effectively inhibited tumor growth both in terms of volumes and mass, accompanying with a significant necrosis in the tumor tissue; whereas this suppression was not mediated by a directly cytotoxicity as proved by the *in vitro* experiment. Numerous studies have demonstrated the importance of adaptive T cell-mediated cytotoxic responses as a dominant mechanism of host antitumor immune responses (51, 52), and TILs nesting in and around neoplastic cells have showed potential clinical implications, especially that the presence of $CD8^+$ TIL is strongly associated with favorable prognosis in every solid human cancer studied virtually (53). Specifically, breast cancer patients with higher numbers of $CD8^+$ TIL in the tumors are more likely to gain better outcomes concerning survival (54, 55). Tc cells ($CD8^+$ T cells) are the most effective elements for tumor destruction (56). Spontaneously, these antitumor effector cells are first activated by the tumor cell-expressing surface molecules, such as calreticulin, tumor antigens in context of MHC class I molecules, and/or NKG2D ligands; then they lead apoptosis or inhibition on proliferation and angiogenesis to destroy the tumor tissue, mainly *via* increasing the cytotoxic factors within TME (including perforin, granzymes, IFN-α/β/γ,

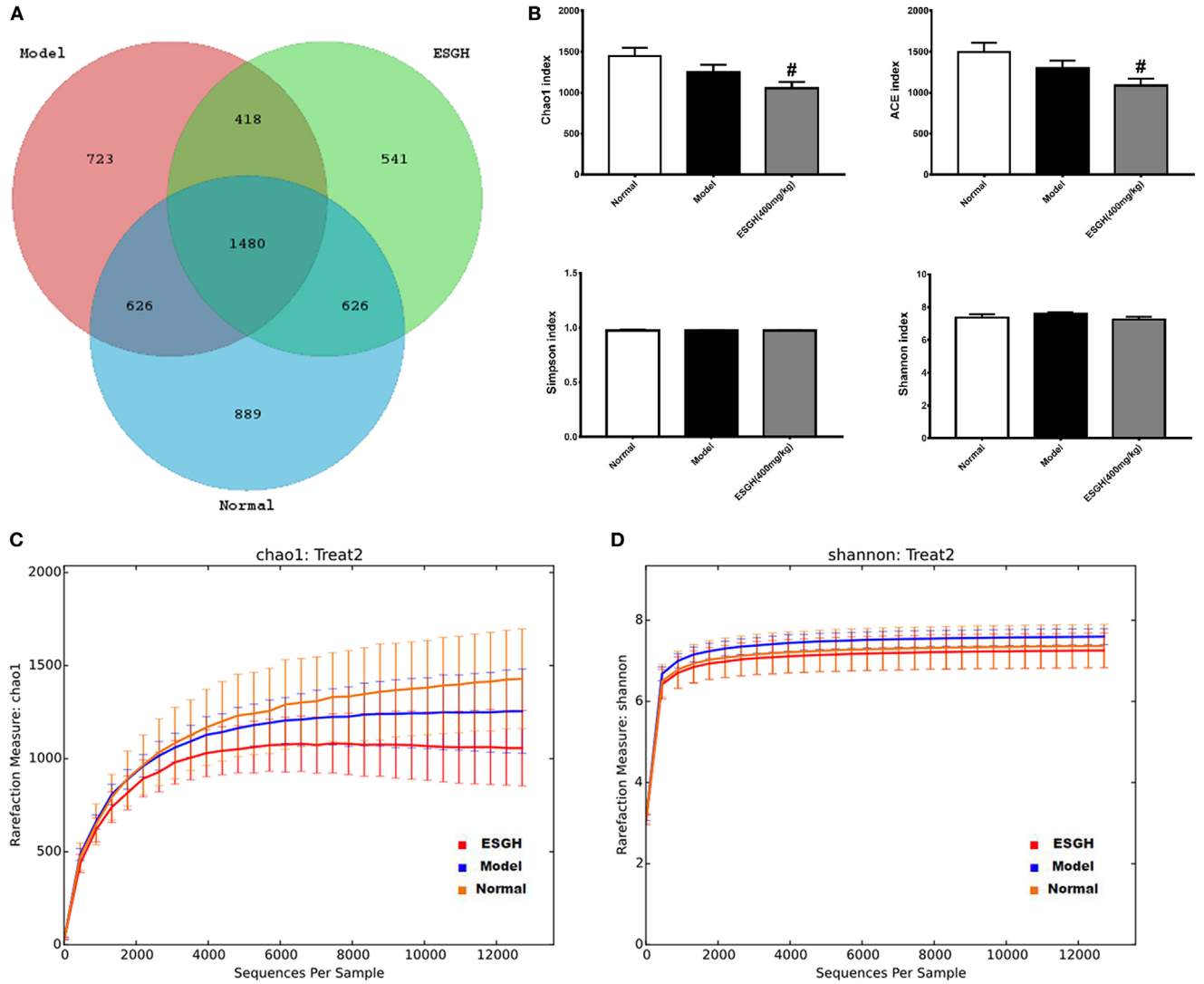

FIGURE 7 | α-Diversity analysis on the fecal microbiota. **(A)** Venn diagram. **(B)** Alpha diversity indices comparison. **(C,D)** Rarefaction curve. Values were represented the means ± SEM ($n = 8$). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus Normal group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus Model group.

IL-1, IL-12, and TNF-α) (57). Despite not being an antitumor enforcer, Th cells ($CD4^+$ T cells) have multiple impacts on the antitumor effect of Tc cells depending on the specific functions of various subsets, such as Th1, Th2, and regulatory Th cells (Treg). By secreting interferon-γ, Th1 cells are essential for the activation of Tc cells and have been shown to correlate with favorable survival in breast cancer (58). However, Treg cells are able to dampen the immune system so as to limit excessive immune responses that can cause collateral damage to normal tissue, which, on the other hand, resulted in a weaken on the antitumor function of Tc cells (58–61). In view of this, we hypothesized that the tumor control of ESG would be a restoration on the T cell paradigm. FCM data indicated that in the peripheral blood, percentage of Tc cells was increased by ESG treatment, and consequently, the ratio of Tc/Th was increased on account of the unchanged total T cell proportion. Intriguingly, $CD8^+$ TILs of the tumor-bearing mice, together with the ratio of Tc/Th with TME, displayed a correspondent increase in response to ESG treatment. Moreover, it was found that ESG evidently increased the production of TNF in serum of the tumor-bearing mice but did not affect any other cytokines that are characteristic for Th1, Th2, or Th17 (Figure S4 in Supplementary Material). These collective results verified our hypothesis that by promoting the differentiation of T cell toward Tc, ESG employed Tc cells to potentiate the tumor immune surveillance, thereby effectively suppressing tumor growth. However, although ESG did not affect neither Th cell proportion in peripheral blood nor that in TME, its impact on Th cell subsets would be explored in the future due to their possible influence in tumor immune surveillance.

As depicted by the "3E immunoediting" theory, despite host antitumor immune responses stand the very heart of self-surveillance, the spontaneous tumor immunity, especially

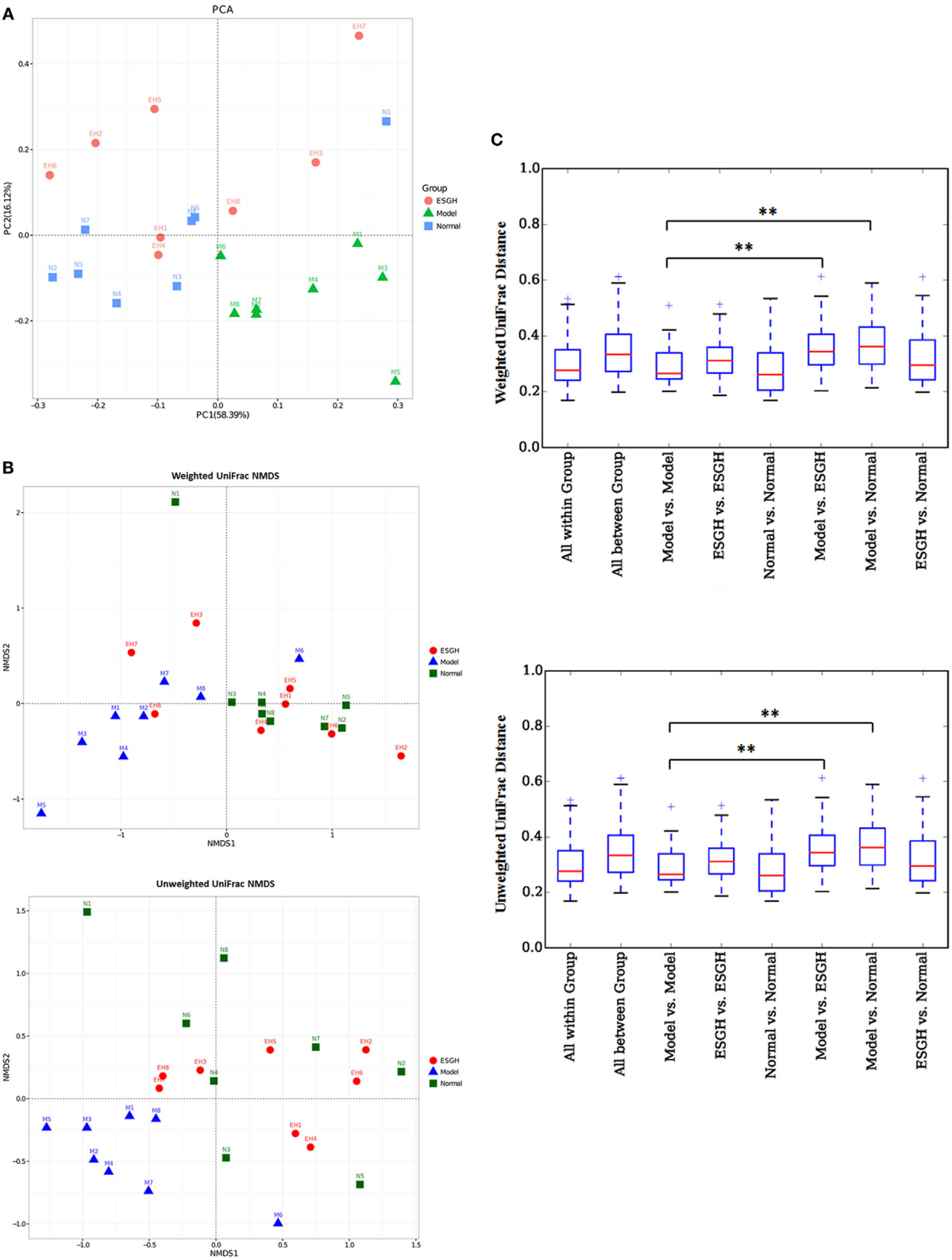

FIGURE 8 | β-Diversity analysis on fecal microbiota. **(A)** Principal component analysis. The percent variation explained by each principal coordinate was indicated on the axes. **(B)** UniFrac distance-based Nonmetric Multidimensional Scaling (NMDS). **(C)** UniFrac distance analysis. Boxes are the interquartile range; median values are bands within the boxes; whiskers are 1.5 times the IQR; and open circle is an outlier value. There would be statistic difference between two groups when the intergroup distance is evidently higher than the within-group distance. *$p < 0.05$ and **$p < 0.01$ versus the distance of "Model versus Model" ($n = 8$).

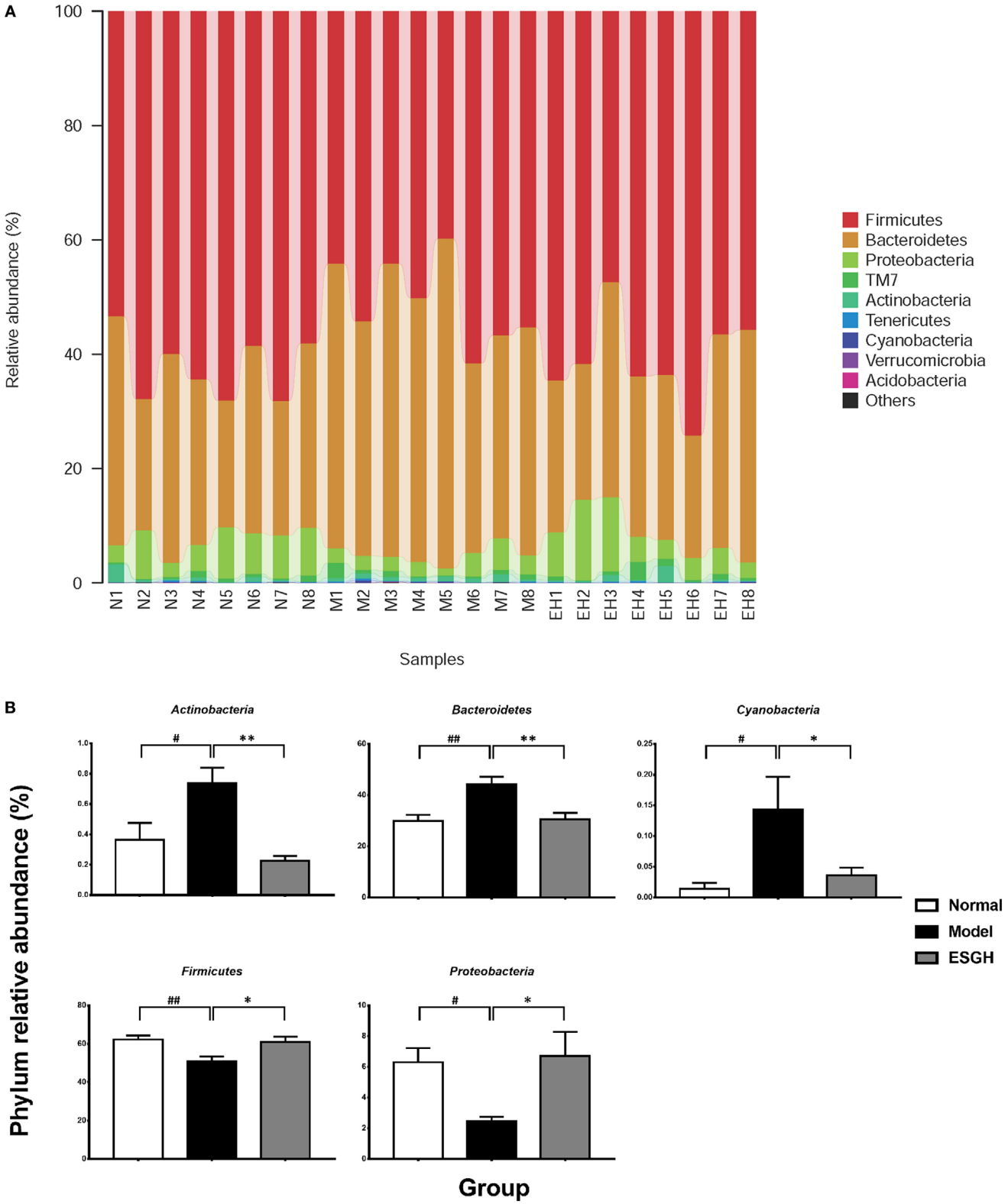

FIGURE 9 | Taxonomy analysis on level of phyla. **(A)** Relative abundance of all detected phylum from each sample. **(B)** Significant intergroup differences were found in five phyla. Values were represented the means ± SEM ($n = 8$). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus Normal group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus Model group.

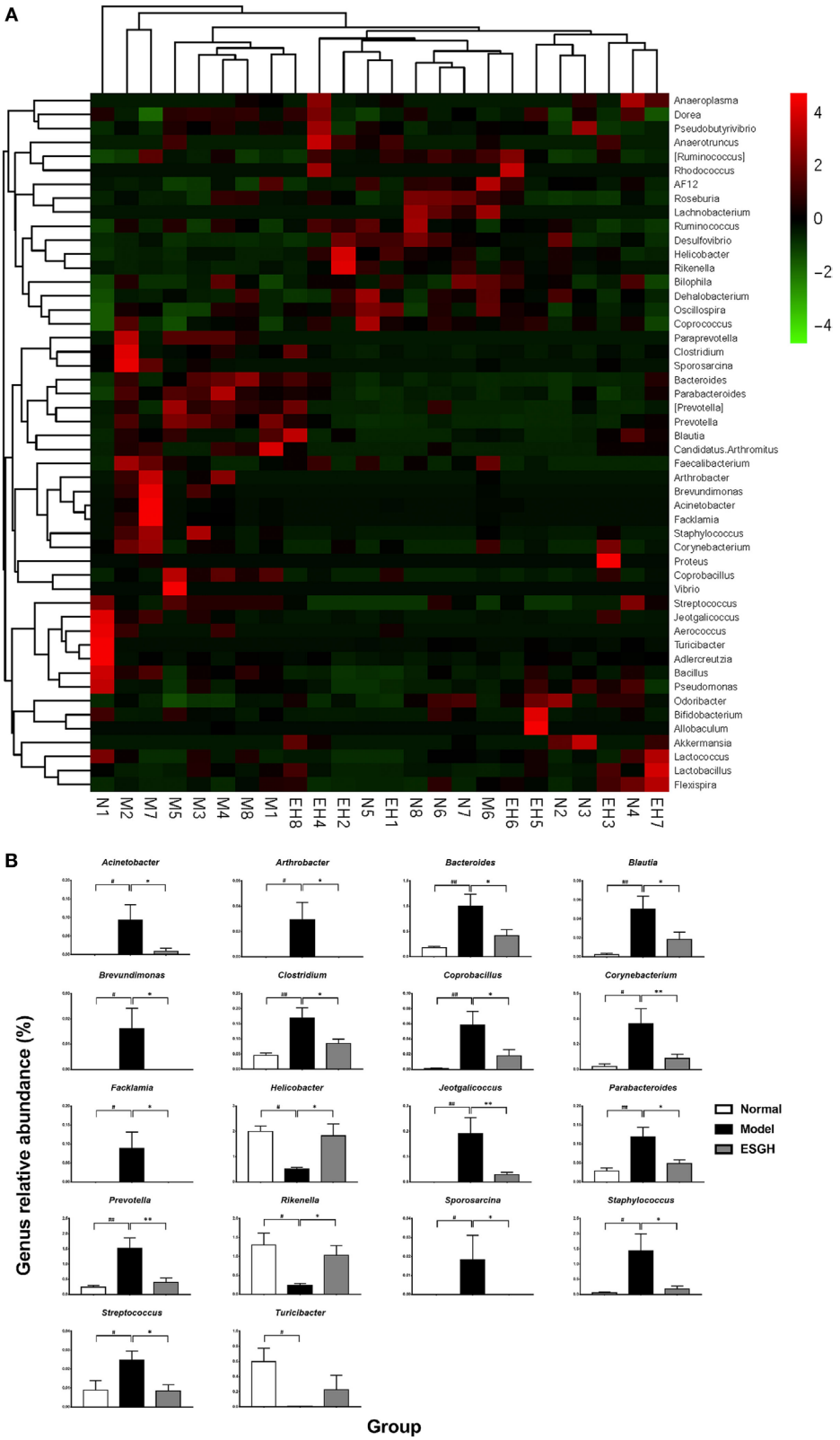

FIGURE 10 | Taxonomy analysis on level of genera. **(A)** Heat map showing the relative abundance of major genera ranking top 50 from each sample. M, N, and EH denote Model group, Normal group, and ESGH groups, respectively. **(B)** Significant intergroup differences were found in 18 genera. Values were represented the means ± SEM ($n = 8$). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus Normal group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus Model group.

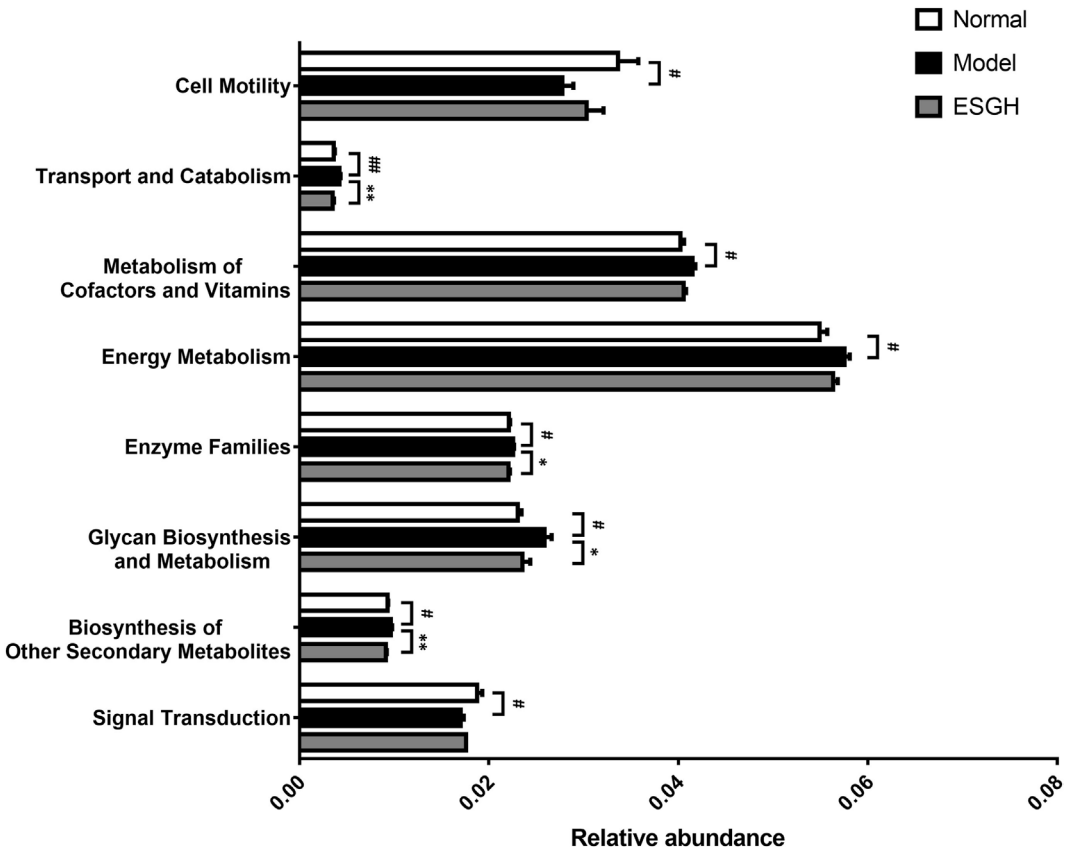

FIGURE 11 | Microbiome function prediction according to Kyoto Encyclopedia of Genes and Genomes pathway database. Values were represented the means ± SEM ($n = 8$). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus Normal group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus Model group.

the dominant T-cell response, would not only be seized up by the tumor cell-driving "escape strategy" but also be limited in the setting of standard treatments (10, 52). Since derived from normal cells, tumor cells can develop several survival features to get immune tolerance, including reduced tumor antigens (CA15-3, CEA, PCNA, etc.), increased resistance or survival (STAT-3 or anti-apoptotic molecule Bcl2), or development of an immunosuppressive TME (releasing cytokines such as VEGF, TGF-β, or expressing immunoregulatory molecules such as IDO, PD-L1, Tim-3/galectin-9, and CTLA-4/CD80) (57). On the other hand, T-cell paradigm turns out to be anergy and exhaustion during cancer progression, due to the activation of membrane co-inhibitory signaling pathways, such as PD-1/PD-L1, and CTLA-4/B7 (11, 12), which finally results in decreased effector function and proliferative capacity. As demonstrated by our data, PTX apparently inhibited PD-1 and CTLA-4 in spleen but upregulated PD-1 in the tumor, implying a possible induction of anergy on the tumor immune surveillance. By contrast, ESG could evidently reduce the mRNA levels of *pd1* and *ctla4*, both in tumor tissue and spleen. Although effect of ESG on the protein expressions was not exactly consistent with that on mRNA level, it was also found that protein expression of PD-1 was significantly suppressed in tumor, whereas that of CTLA-4 was only down-regulated in spleen.

Indeed, growing evidence reveals the correlation between mRNA and protein abundances in the cell is notoriously poor (62). This is because there exists the translational control on the process of protein translation from mRNA, involves mRNA degradation induced by microRNA and siRNA, initiation codon scanning, ribosome assemble, and so on, and the initiation phase is often the most regulated part (63–65). Thus, translational control results in an unequal protein translation from the transcribed mature mRNA, which enables the organisms to adapt to the changed circumstances *via* a quick regulation on the protein biosynthesis in each cell. As showed in the data, when compared with the normal control, protein level of PD-1 in spleen was reduced in the tumor-bearing mice, although the mRNA expression of PD-1 was upregulated. By contrast, PD-1 protein of ESG groups was evidently decreased, while their mRNA levels were equal to that of normal control group. Similar poor correlation between the mRNA and protein levels of PD-1 also exhibited in tumor. These results suggested that ESG would make profound regulations on the translation control of PD-1 and CTLA-4, so as to suppress this immune checkpoints signaling. Together with the previous results, it would be rationally to speculate that ESG could effectively restore the T cell paradigm by reversing the anergy and exhaustion status *via* suppressing the co-inhibitory checkpoints, thereby resulting in a favorite control

on breast cancer. Furthermore, impacts on the tumor immune surveillance, such as co-inhibitory signaling pathway interaction (PD-1/PD-L1 and CTLA-4/CD86) and tumoral immunological balances (such as CD28: B7 binding versus CTLA-4: B7 binding), would be explored to make a more comprehensive exploration for the antitumor activity of ESG.

One more issue to be noteworthy is that, ESG did not exhibit obvious promotion on Tc cells differentiation *in vitro*. As ESG was administrated orally in this work, it is highly probable that a regulation on gut microbiota by ESG promotes the restoration on the exhausted Tc cells. Besides the cardinal role in the development and efficiency of immune system, microbiota also interact with gut mucosal surfaces thereby intervene the therapeutic responses for tumors occurring outside of the intestinal tract (16, 66). Specifically in breast cancer, increasing data have shown that gut microbiome is involved with all potential related factors of breast cancer, including immune regulation, metabolism of endogenous and exogenous substances, obese status, and so forth. $CD8^+$ TIL has been found to be positive related to a better outcomes concerning survival in breast cancer patients (54), and an optimization on the gut microbiota community brings about a promotion on $CD8^+$ T cell-mediated immunity, thereby indicating a better prognosis and an effective outcome of immunotherapy (14, 67). As demonstrated by our data, in despite of the reduced richness in the microbiome of the tumor-bearing mice (α-diversity), mice receiving ESG treatment shared a parallel microbiome structure as those of the normal counterparts when compared with the mice of Model group (β-diversity). Furthermore, ESG was able to reverse the microbiota community shift caused by tumor xenograft, of which 5 phyla (*Actinobacteria, Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, and *Proteobacteria*) and 18 genera were significantly affected. Among the influenced genera, *Helicobacter*, *Rikenella*, and *Turicibacter* were the enriched ones that accounted over 0.5%, and especially, both *Helicobacter* and *Rikenella* were restored up to about 1%. Despite as pathogenicities to certain inflammatory-related carcinoma and autoimmune disorders, it has been revealed that *Helicobacter* and *Rikenella* were positively correlated to enhanced immune response (47, 48). *Turicibacter* was also strongly associated with immune functions, as indicated by the fact that *Turicibacter* populations within the gastrointestine would be almost abolished both in innate and adaptive immunodeficiency mouse models (49, 50). On the other hand, *Bacteroides* and other 14 genera were declined by ESG treatment. Typically, *Bacteroides* took up the biggest population (1% or so) among them, and it has been considered as a beneficial commensal when retained to a proper quantity in the gut, otherwise inducing bacteremia and abscess formation in multiple body sites (68). Nevertheless, increased abundance of *Bacteroides* is associated with immunosuppression (69) and carcinogenesis (70). Overall, ESG made a beneficial alterations on the gut microbiome community, i.e., to enrich the immunocompetence-related genera (*Helicobacter*, *Rikenella*, and *Turicibacter*) and to decline certain immunosuppressive-related ones (such as *Bacteroides*), resulting in a restoration on the dysbiosis induced by cancer xenograft.

Indeed, gut microbiota not only experiences long-term coevolution with host (71) but also influences the host immunity (72, 73). Especially, lifestyle, including diets and medicine intake, contributes greatly to the modification on gut microbiota (71). On the other hand, microbial utilization of complex polysaccharides is a major driving force in shaping the composition of the human gut microbiota (74, 75). In our works, it was found that ESG is mainly composed of polysaccharide made up of glucose, and it restored several pathways involving metabolism ("biosynthesis of other Secondary metabolites," "energy metabolism," "enzyme families," "glycan biosynthesis and metabolism," and "metabolism of cofactors and vitamins"), cellular processes ("transport and catabolism" and "cell mobility"), and environmental information processing ("cell mobility"). So it is probable that by oral administration, ESG was able to directly affect the growth homeostasis within the microbiota *via* regulating its metabolism mode, and possibly, serves as selective growth promoters to certain species. Therefore, microbiota community of the ESG-treatment samples was evidently shifted with lowered community richness (with reduced Chao1 and ACE indexes) but unaltered diversity. Nevertheless, the specific organisms affected by ESG have to be figured out and confirmed *via* experiment on germ-free animal models. Moreover, several studies revealed that natural polysaccharides would take several weeks to influence the gut microbiota (76, 77). It is probable that the remodeling by ESG may take several days to shift the community membership and to change the metabolism characteristics. Hence, tumor growth was not suppressed until the tumor immune surveillance was powerful enough by a long-term ESG treatment. Taken together, given that the promotion by ESG on Tc-mediated tumor suppression would only achieve by oral administration, it is feasible that the modulation on gut microbiome community would contribute greatly to ESG's tumor control by restoring the exhausted Tc cells.

Of course, there are some more affairs to be taken in account for the antitumor activity of ESG, such as oxidative stress and aerobic metabolism (also namely Warburg effect). Oxidative stress has a controversial association with cancer, which is mainly mediated by reactive oxidative species (ROS). The elevated levels of ROS not only increases gene mutations and genomic instability (78, 79) but also inactivates the phosphatases within cancer cells, so their target proteins responsible for proliferation are kept activated, resulting in an uncontrolled cell growth (80). In these view of oxidative stress, Chikara et al. have reviewed the antioxidant potential of phytochemicals in cancer chemoprevention and treatment (81). However, activation of oxidation (such as by increasing ROS) contributes to autophagy and apoptosis in cancer cells, even though it is crucial for normal-to-cancerous cell transformation and cancer development (82, 83). Therefore, whether the antioxidant effect contributes to the anticancer activity depends on the various types of compounds. For instance, it is reported that ganoderic acids induce apoptosis in human cervical cancer HeLa cells *via* increasing the generation of intracellular ROS (84); while a *G. lucidum* extract elicited antitumor effects by suppressing cell growth and inducing antioxidative/detoxification activity in human ovarian OVCAR-3 cells (85). Regarding Warburg effect, it is indeed a metabolic nature of TME shifting from oxidative phosphorylation to aerobic glycolysis (86). It is critical for cancer progress and immune escape and characterized by increased glucose uptake and accumulation of lactate,

accompanying with the upregulations of transporters, glycolytic enzymes, and the responsible signaling pathway proteins (87, 88). Therefore, pathways and activities involved in Warburg effect have been considered as novel targets in cancer therapy, such as metformin that acts via reducing the expression of monocarboxylate transporter 4 on cancer-associated fibroblasts (89). Although no researches report the impact on Warburg effect, it has revealed an antioxidant effect of the spore of *G. lucidum* in model of ischemia/reperfusion and streptozotocin-induced neuron damage (90, 91). In the future, we would pay attention to the possible role of antioxidant effect (or Warburg effect) by ESG in its anticancer activity.

Collectively, this study revealed that a polysaccharide-rich extract from sporoderm-breaking spore of *G. lucidum* (ESG) would serve as natural candidate for breast cancer treatment. The underlying mechanism of ESG was probably contributed to a suppression on co-inhibitory signaling (PD-1 and/or CTLA-4) and a consequent restoration on the exhausted Tc cells, to which the gut microbiome remodeling made a great contribution. Especially, such regulation involved not only changes in microbiota structure and community membership but also alternations in several metabolism pathways within the microbiome. So far, it has been found that, there is an intricate relationship between the biology of T cells (mainly regulatory T cells and Tc cell), and the various metabolites produced by host and commensal microbes, such as vitamins and short chain fatty acids (67, 92). Hence in all probability, regulation on the metabolism of gut microbiota would be the pivotal of ESG to enhance the Tc-mediated tumor surveillance against breast cancer. On the other hand, toxicity test is demanded to make comprehensive evaluation on its safety, although no article has yet reported the adverse effect of the spore of *G. lucidum*, except one suggests that an extract from fruit body of *G. lucidum* has lethal and sub-lethal effects on zebrafish embryos (93). And rigor pharmacokinetics study, as well as multi-omics investigations, for ESG will be applied to intercept the underlying mechanism of its immune-regulation activity, as well as the potential in cancer treatment.

ETHICS STATEMENT

The experiment was performed according to the Guidelines of Guangdong Institute of Microbiology Laboratory Animal Center, Guangdong Institute of Microbiology Laboratory Animal Ethics Committee. The experimental protocols were approved by the Guangdong Institute of Microbiology Laboratory Animal Ethics Committee.

AUTHOR CONTRIBUTORS

Conceived and designed the experiments; drafted and revised the manuscript: JS, YL, and YX. Performed the experiments: JS, LS, DL, OS, YZ, HL, and ZX. Analyzed the data: JS, LS, DL, and CJ.

ACKNOWLEDGMENTS

We thank Dr. Shijie Li, Ms. Cuiyun Mo, and Ms. Lixia Gan for the help in characteristics analysis for ESG. This work was financially supported by the High-level Leading Talent Introduction Program of Guangdong Academy of Science (No. 2016GDASRC-0102), the Natural Science Foundation of Guangdong Province (No. 2015A030313711), Guangdong Province Science and Technology Project (No. 2016A030303041), and Guangzhou Science and Technology Planning Project (No. 2016201604030072, No. 201707020022).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01765/full#supplementary-material.

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Conflict of Interest Statement: All coauthors 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.

frontiers in Physiology

REVIEW
published: 10 July 2018
doi: 10.3389/fphys.2018.00888

Flux, Impact, and Fate of Halogenated Xenobiotic Compounds in the Gut

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Humans and their associated microbiomes are exposed to numerous xenobiotics through drugs, dietary components, personal care products as well as environmental chemicals. Most of the reciprocal interactions between the microbiota and xenobiotics, such as halogenated compounds, occur within the human gut harboring diverse and dense microbial communities. Here, we provide an overview of the flux of halogenated compounds in the environment, and diverse exposure routes of human microbiota to these compounds. Subsequently, we review the impact of halogenated compounds in perturbing the structure and function of gut microbiota and host cells. In turn, cultivation-dependent and metagenomic surveys of dehalogenating genes revealed the potential of the gut microbiota to chemically alter halogenated xenobiotics and impact their fate. Finally, we provide an outlook for future research to draw attention and attract interest to study the bidirectional impact of halogenated and other xenobiotic compounds and the gut microbiota.

Keywords: xenobiotics, halogenated compounds, gut microbiota, xenobiotic-microbiota interaction, dehalogenation genes, metagenomics

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: *29 April 2018*
Accepted: *20 June 2018*
Published: *10 July 2018*

Citation:
Atashgahi S, Shetty SA, Smidt H and de Vos WM (2018) Flux, Impact, and Fate of Halogenated Xenobiotic Compounds in the Gut. Front. Physiol. 9:888. doi: 10.3389/fphys.2018.00888

INTRODUCTION

The term xenobiotic is usually used in the context of environmental pollutants to refer to synthetic compounds produced in large volumes for industrial, agricultural and domestic use (Atashgahi et al., 2018c). Xenobiotics can enter the environment at high (μg/L to mg/L range) or at "micropollutant" concentrations (ng/L to μg/L range) (Schwarzenbach et al., 2006; Meckenstock et al., 2015). One important group of xenobiotics comprise halogenated compounds with diverse sources and sinks. Halogenated organic compounds, organohalogens, are usually synthesized for industrial, agricultural and pharmaceutical applications (Häggblom and Bossert, 2003). It has also been shown that over 5000 organohalogens are naturally produced from biogenic and geogenic sources (Gribble, 2010). Inorganic halogenated compounds such as chlorine dioxide, hypochlorite, and chlorite are commonly applied as bleaching agents and disinfectants (Liebensteiner et al., 2016). In turn, halogenated compounds can be used as carbon sources, electron donors and acceptors by a diverse array of aerobic and anaerobic microorganisms in growth-dependent and co-metabolic modes (Janssen et al., 2001; Van Pée and Unversucht, 2003; Schneidewind et al., 2014; Peng et al., 2017). As such, microbial degradation represents an important sink of halogenated compounds.

Xenobiotics are also considered as chemical substances from natural or synthetic sources found within an organism that are not naturally produced by the organism or expected to be present. As such, the human body is exposed to variety of (halogenated) xenobiotic

compounds, such as persistent organic compounds (POPs), pesticides, pharmaceuticals and personal care products (PPCPs), and food additives. Site-specific microbiomes associated with the gut, skin, or respiratory tract are the first to encounter xenobiotics and mediate "first pass" metabolism prior to compound absorption to internal organ systems (Dietert and Silbergeld, 2015). Among these portals of entry, most interactions between xenobiotics and the human microbiota occur within the human gut (Sousa et al., 2008; Dietert and Silbergeld, 2015). The gut microbiota is a diverse and dense microbial community composed of bacteria, fungi, archaea, and viruses (Li et al., 2014; Nielsen et al., 2014). Its immense metabolic diversity is encoded by the intestinal metagenome, that contains genetic information for multiple xenobiotic detoxification and sequestration functions (Haiser and Turnbaugh, 2013; Spanogiannopoulos et al., 2016). The anoxic environment of the gut is well-suited for a reductive and hydrolytic metabolism. This will generate non-polar low-molecular weight by-products that can be absorbed by the host cells. In contrast, the readily absorbed non-polar xenobiotics are transported and metabolized in the liver by a rich collection of oxidative and conjugative enzymes. Such hepatic metabolism will generate hydrophilic, polar and high-molecular weight metabolites. The latter are secreted via the bile and reach the gut where they can be re-metabolized by reductive and hydrolytic enzymes (Sousa et al., 2008; Claus et al., 2016; Koppel et al., 2017). Thus, xenobiotic metabolism by gut microbiota can exert a profound influence on the toxicity and bioavailability of xenobiotics entering the gut via different routes. The outcome of xenobiotic metabolism may be beneficial (Shin et al., 2013), detrimental or even lethal (Okuda et al., 1998) to the host. In turn, exposure to xenobiotics can alter gut microbiota composition and change metabolic activity (Maurice et al., 2013). This may increase predisposition to various diseases (Wang et al., 2011; Lee et al., 2014; Lu et al., 2015).

Extensive research of the last decades has provided insight into the metabolism of halogenated xenobiotics and opened avenues to harness the metabolic machinery of microbes for bioremediation (Smidt and de Vos, 2004; Sutton et al., 2015; Atashgahi et al., 2017, 2018c; Weatherill et al., 2018). In contrast, much less is known about the flux, impact, and fate of halogenated xenobiotics in host-associated ecosystems like the human gut. As such, there is only very limited information about specific microorganisms, genes, and enzymes responsible for halogenated xenobiotic metabolism in the human gut. This contrasts with the enormous and expanding interest in understanding the role of the gut microbiome in health and disease.

Here, we address the present state of the art on the flux, impact and fate of halogenated compounds in the gut. We first provide an overview of the *flux* of halogenated compounds to highlight their environmental sources and diverse exposure routes of human microbiota to these compounds. Subsequently, we provide an overview of the *impact* of halogenated compounds on the structure and function of the gut microbiota and host cells. Lastly, we review the *fate* and metabolism of halogenated compounds in the gut based on published experimental data and a metagenomic survey of the dehalogenation genes in gut microbiome. In a larger context, we provide a rationale for studying the bidirectional impact of (halogenated) xenobiotic compounds and the gut microbiota, i.e., toxicant-microbiota interactions.

FLUX

The main sources of halogenated (micro)pollutants are from industry (e.g., POPs), agriculture (e.g., pesticides), domestic use (e.g., PPCPs) and disinfection by-products (DBPs). Some examples of the halogenated (micro)pollutants that are discussed in this review are shown in **Figure 1.** Humans come into contact with halogenated xenobiotics through: (i) oral exposure (eventually ending up in the gut), (ii) inhalation (via nose and lungs), (iii) dermal exposure (through the skin), and (iv) ocular exposure (through the eyes) (**Figure 2**). Oral ingestion is the main exposure route of the general population to halogenated xenobiotics. This is especially the case for the chronic exposure to micropollutant concentrations of residues in food, vegetables, fruits, and drinking water (Boxall et al., 2006, 2012; Damalas and Eleftherohorinos, 2011). In contrast, dermal/inhalation exposure due to showering, bathing, and swimming through daily-life and/or recreational activities is a more important route of exposure to DBPs than oral exposure (Villanueva et al., 2006a,b). Inhalation, dermal and ocular routes are more relevant to occupational exposure of workers in- or nearby residents of-industrial production plants, farms or greenhouses that produce and use halogenated xenobiotics (Damalas and Eleftherohorinos, 2011; Besis and Samara, 2012).

Raw sewer and treated effluents from wastewater treatment plants play a key role in the spread of halogenated compounds in the water cycle (**Figure 2**). Most current waste- and drinking water treatment plants are not (optimally) designed for the removal of halogenated compounds or their transformation products (Heidler and Halden, 2009; Noguera-Oviedo and Aga, 2016). The latter is particularly relevant as sometimes the transformation products can be more toxic than the parent compounds. For example, perfluorooctanesulfonic acid (compound number **1** in **Figure 1**) is a POP that can also be produced through biotransformation of other synthetic chemicals such as perfluoroalkyl acids in wastewater treatment plants (Guerra et al., 2014). Similarly, biodegradation of clofibric acid (**9**) leads to production of the more toxic 4-chlorophenol (Salgado et al., 2012). Trace concentrations of such contaminants of emerging concern may eventually end up in finished drinking water due to their toxicity and persistence (Benotti et al., 2008).

Biosolids from the treated sewage sludge represent concentrated sources of hydrophobic organohalogens such as the flame retardant polybrominated diphenyl ethers (**2**), and the biocidal compounds triclosan (**18**) and triclocarbon (**19**). These compounds can desorb and contaminate soil and water, once biosolids are applied as fertilizer (Andrade et al., 2015) (**Figure 2**). Similarly, organohalogen antibiotics such as fluoroquinolones added to animal feed for disease prevention or for growth promotion may end up in biosolids from livestock production (Martínez-Carballo et al., 2007). Once applied as

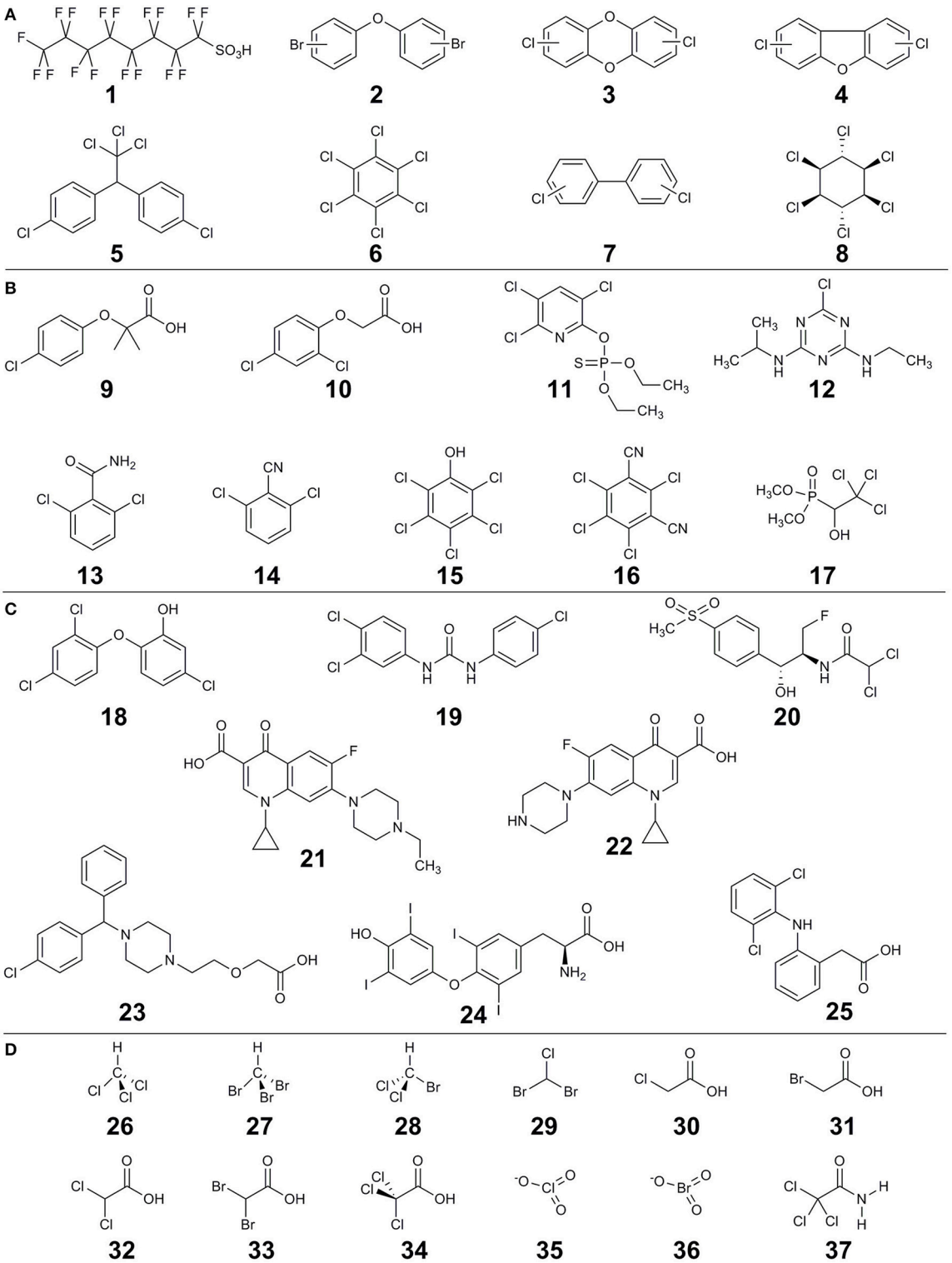

FIGURE 1 | Examples of halogenated compounds discussed in this review. **(A)** persistent organic compounds (POPs), **(B)** pesticides, **(C)** pharmaceuticals and personal care products (PPCP) and **(D)** disinfection by-products (DBPs). Compounds are: perfluorooctanesulfonic acid (**1**), polybrominated diphenyl ethers (**2**), polychlorinated dibenzo-*p*-dioxins (**3**), polychlorinated dibenzofurans (**4**), dichlorodiphenyltrichloroethane (**5**), hexachlorobenzene (**6**), polychlorinated biphenyls (**7**), γ-hexachlorocyclohexane (lindane) (**8**), clofibric acid (**9**), 2,4-dichlorophenoxyacetic acid (**10**), chlorpyrifos (**11**), atrazine (**12**), 2,6-dichlorobenzamide (**13**), 2,6-dichlorobenzonitrile (**14**), pentachlorophenol (**15**), chlorothalonil (**16**), trichlorfon (**17**), triclosan (**18**), triclocarbon (**19**), florfenicol (**20**), enrofloxacin (**21**), ciprofloxacin (**22**), cetirizine (**23**), thyroxine (L) (**24**), diclofenac (**25**), chloroform (**26**), bromoform (**27**), bromodichloromethane (**28**), chlorodibromomethane (**29**), chloroacetic acid (**30**), bromoacetic acid (**31**), dichloroacetic acid (**32**), dibromoacetic acid (**33**), trichloroacetic acid (**34**), chlorate (**35**), bromate (**36**), trichloroacetamide (**37**).

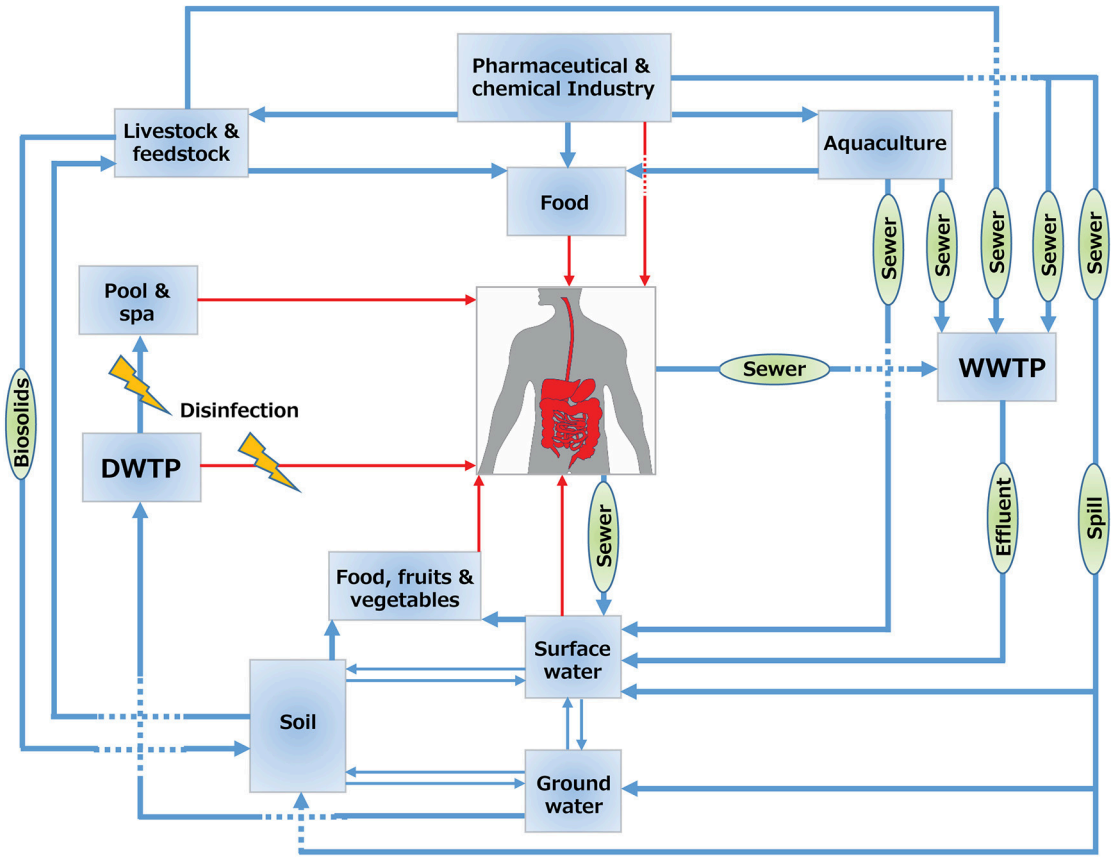

FIGURE 2 | Flux of halogenated compounds. Blue arrows show the flux in the environment and red arrows show the flux to humans. WWTP, wastewater treatment plant; DWTP, drinking water treatment plant.

manure for fertilization of arable land, these compounds can reach humans via different routes. For example, veterinary organohalogen drugs such as florfenicol (**20**) and enrofloxacin (**21**) can be taken up by plants such as lettuce (Boxall et al., 2006).

Recent non-targeted analysis has further highlighted the environmental footprint of organohalogens. For example, monitoring of bottlenose dolphins has identified 327 organohalogens of synthetic as well as natural origins (Shaul et al., 2015). This indicates severe bioaccumulation of organohalogens in marine food webs. A similar non-targeted screening of the sediments of Lake Michigan has identified 1,593 organobromine compounds, many of which were not known previously (Peng et al., 2015). The deposited organohalogens in sediments can be chronically released (Yamashita et al., 2000) and bioaccumulate in organisms in food webs and thereby reach humans.

POPs

POPs are long lived organic compounds that resist biological, chemical, and photolytic degradation. They exhibit high lipid solubility and hence bioaccumulate in fatty tissues and become concentrated as they move up the food chain. POPs are dominated by organohalogens such as polychlorinated dibenzo-*p*-dioxins and furans (**3**, **4**), dichlorodiphenyltrichloroethane (**5**), hexachlorobenzene (**6**), polychlorinated biphenyls (**7**), γ-hexachlorocyclohexane (lindane, **8**), perfluorooctanesulfonic acid, polybrominated diphenyl ethers, etc.

Although application of organochlorine pesticides and polychlorinated biphenyls has been restricted since the late 1970s, they are still among the most ubiquitous and concerning environmental pollutants due to their persistence, toxicity and bioaccumulation (Xu et al., 2017). Moreover, electronic waste (e-waste) continues to produce POPs such as polychlorinated dibenzo-*p*-dioxins and furans, polybrominated diphenyl ethers, and polychlorinated biphenyls. The e-waste recycling industry in developing countries has drawn the world's attention as a new source of environmental contamination by POPs (Leung et al., 2007; Zhang et al., 2010). POPs can also be produced naturally. For example, polychlorinated dibenzo-*p*-dioxins and furans are formed as by-products of high-temperature processes, such as volcano outbursts, forest fires and waste incineration. The endocrine-disrupting flame retardant polybrominated diphenyl ethers have natural counterparts that are produced by marine sponges (Agarwal et al., 2017) at levels that can exceed 10% of the sponge tissue dry weight (Unson et al., 1994). Naturally

produced organohalogens were reported to bioaccumulate in marine mammals (Vetter et al., 2002; Teuten and Reddy, 2007) and humans (Wan et al., 2010; Wang et al., 2012) indicating human contact through the marine food web. Accordingly, POPs can come into contact with humans primarily through dietary intake including fatty fish, red meat and poultry (Kiviranta et al., 2004; Schecter et al., 2010), but also through inhalation and dermal absorption (Besis and Samara, 2012).

Pesticides

Pesticides such as herbicides, insecticides, and fungicides have been used since the 1940s for agricultural and non-agricultural purposes (Schwarzenbach et al., 2010). Chlorinated phenoxy acid herbicides such as 2,4-dichlorophenoxyacetic acid (**10**), the broad-spectrum chlorinated organophosphate chlorpyrifos (**11**) and the herbicide atrazine (**12**) are among the most intensively used pesticides worldwide (Bradberry et al., 2000; Arias-Estévez et al., 2008; John and Shaike, 2015). Pesticide contamination from specific point sources (μg/L to mg/L range) can be due to accidental releases at manufacturing plants, spills on farm yards and from wastewater treatment plant effluent (Vandermaesen et al., 2016). In contrast, diffuse contamination (ng/L to μg/L range) originates from actual pesticide application that results in large-scale contamination of groundwater through leaching, and contamination of surface water through runoff, erosion, drainage, and drifting (Holvoet et al., 2007). Moreover, contamination can be due to transformation products. For example, 2,6-dichlorobenzamide (**13**) is a highly mobile and persistent groundwater pollutant that originates from transformation of the widely used herbicide dichlobenil (2,6-dichlorobenzonitrile) (**14**) (Horemans et al., 2017). Besides oral ingestion as the main route of exposure, human contact with pesticides could be through: (i) inhalation by breathing the mobile pesticides e.g., during on farm pesticide spraying, (ii) dermal, and (iii) ocular routs e.g., during accidental splashing or spraying pesticides on unprotected skin/eyes of agricultural workers and workers in the pesticide industry (Damalas and Eleftherohorinos, 2011).

PPCPs

Pharmaceuticals are used to treat or prevent disease or as feed additives in animal farming, whereas personal care products are used in personal hygiene and for beautification, and include products such as shampoos, toothpastes, moisturizers, deodorants, lipsticks, perfumes, etc (Boxall et al., 2012). Whereas the main route of PPCPs release into environment is generally excretion to the sewage system following use, manufacturing facilities can be important local point sources (Ebele et al., 2017) (**Figure 2**). For example, effluent from a wastewater treatment plant of a major drug manufacturer contained ciprofloxacin (**22**) at a concentration of up to 31 mg/L, that exceeds levels toxic to some bacteria by over 1000-fold (Larsson et al., 2007). A subsequent study found ciprofloxacin (up to 6.5 mg/L) and cetirizine (**23**) (up to 1.2 mg/L) in two lakes in the same region impacted by the wastewater treatment plant effluent (Fick et al., 2009). These mg/L concentrations are 100,000 to 1 million times higher than reported levels of fluoroquinolones in surface waters contaminated by effluents from wastewater treatment plants (Kolpin et al., 2002; Xiao et al., 2008).

Triclosan and triclocarban are broad-spectrum phenolic organochlorine biocides with activity against both bacteria and fungi. These chemicals are found in a wide variety of consumer products, including soaps, detergents, toothpaste, medical devices, plastics, and textiles (Pycke et al., 2014). As a result of common and widespread use, humans are exposed to these chemicals via different routes including absorption (e.g., soaps, toothpaste), ingestion (e.g., drinking water, food), inhalation (e.g., aerosols, dust), and injection/implantation (e.g., medical sutures and devices) (Halden, 2016). For example, according to a survey in 2003-2004, triclosan was found in about three-quarters of urine samples analyzed in the USA at concentrations of 2.4–3790 μg/L (Calafat et al., 2008). A later analysis of triclosan, triclocarban, thier metabolites and by-products in maternal urine and cord plasma in an urban population in the USA has shown widespread fetal exposure to these compounds (Pycke et al., 2014). Triclosan and triclocarban have been detected in aquatic environments such as groundwater, drinking water, wastewater, sewage sludge, and in some food sources representing environmental sources, besides direct consumer-product use (Lindström et al., 2002; Singer et al., 2002; Halden and Paull, 2005).

DBPs

Water disinfection during the production of drinking water has been widely implemented to protect human health against waterborne diseases like cholera, typhoid, dysentery, etc (Richardson and Ternes, 2018). To this end, strong oxidants such as free chlorine, chlorine dioxide, chloramines, and ozone are used, which efficiently kill pathogens. However, disinfectants can unintentionally form DBPs by further reacting with other constituents found in waters i.e., natural organic matter, anthropogenic organic contaminants, and halide ions (chloride, bromide, iodide) (Richardson et al., 2007; Gonsior et al., 2014; Postigo and Richardson, 2014). Moreover, DBPs in pool and spa waters are formed by the reaction of disinfectants with organic matter including natural organic matter from source water and human inputs such as urine, sweat and PPCPs (Daiber et al., 2016; Jmaiff Blackstock et al., 2017). For instance, more than 100 DBPs were recently found in swimming pools and hot tubs, and organic extracts from those samples were more mutagenic than the corresponding tap water extracts (Daiber et al., 2016). Although ∼700 DBP have been identified, only three classes are regularly monitored: trihalomethanes (e.g., chloroform, bromoform, bromodichloromethane, chlorodibromomethane) (**26**–**29**), haloacetic acids (e.g., chloro-, bromo-, dichloro-, dibromo-, and trichloro-acetic acid) (**30**–**34**), and oxyhalides (e.g., chlorate and bromate) (**35**–**36**) (Richardson and Ternes, 2018). People are exposed to a diverse range of DBPs by drinking, uptake through the skin upon contact, and inhalation of volatile DBPs e.g., in indoor swimming facilities (Gonsior et al., 2014; Daiber et al., 2016). Epidemiological studies have reported a relation between human ingestion of drinking water containing DBPs and increased spontaneous abortions, stillbirth,

birth defects and bladder cancer in particular (Richardson et al., 2007).

IMPACT

The negative impact of halogenated compounds has been known since long from a toxicological point of view. However, the role of the (gut) microbiota has not been well-incorporated into the study of interactions between environmental exposures and health outcomes (Dietert and Silbergeld, 2015).

The impact of halogenated compounds on gut microbiota has mostly been studied using rodent models (**Table 1**). For example, dietary exposure to 2,3,7,8-tetrachlorodibenzofuran has been shown to induce inflammation and decrease the *Firmicutes* to *Bacteroidetes* ratio in mice (Zhang L. et al., 2015). A similar decreased *Firmicutes/Bacteroidetes* ratio in mice was reported due to exposure to trichloroacetamide (**37**) (Zhang L. et al., 2015) or chlorpyrifos (**11**) (Zhao et al., 2016), and in juvenile goldfish due to exposure to pentachlorophenol (**15**) (Kan et al., 2015) (**Table 1**). In contrast, the *Firmicutes/Bacteroidetes* ratio increased in mice after 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure (Lefever et al., 2016). Changes in the *Firmicutes/Bacteroidetes* ratio was first observed in obesity studies and was subsequently addressed by various studies, confirming or challenging its impact, or pointing to technical artifacts (Ley et al., 2005; Schwiertz et al., 2010; Bahl et al., 2012). Nevertheless, these ratios may in any case indicate different levels of short chain fatty acids production, pH and activity in the gut, and hence could be meaningful when methodological bias is excluded (Duncan et al., 2009; Kolmeder et al., 2015). Accordingly, treatment of mice with 2,3,7,8-tetrachlorodibenzofuran enriched *Butyrivibrio* spp., common butyrate-producing gut microbes, coupled with elevation of butyrate and propionate in feces and cecal contents (Zhang L. et al., 2015). In another study, oral exposure to polychlorinated biphenyls decreased the overall abundance of bacterial species in mice gut microbiota primarily by decreasing the levels of *Proteobacteria* (Choi et al., 2013). Interestingly, exercise attenuated alterations of mice gut microbiota composition (Choi et al., 2013). Exercise has been shown to increase beneficial metabolites, such as butyrate in the rat cecum (Matsumoto et al., 2008).

Organohalogen POPs accumulate in adipose tissue in humans because of their lipophilicity. These POPs have been shown to bind to aryl hydrocarbon receptor (AHR), that is a transcription factor involved in the regulation of biological responses to planar aromatic (aryl) hydrocarbons (Arsenescu et al., 2008). This xenobiotic sensor modulates the activity of immune and nonimmune cells in the gut, and may represent an important link between the environment and immune system perturbations (Monteleone et al., 2012). In line with this, *ahr*-knockout ($Ahr^{-/-}$) mice did not show large shifts in gut microbial composition in response to 2,3,7,8-tetrachlorodibenzofuran exposure (Zhang L. et al., 2015). Modulation of gut microbiota by AHR was proposed to play an important role in the induction of obesity by chronic POP exposure (Myre and Imbeault, 2014). For instance, exposure to polychlorinated biphenyls has been shown to impair glucose homeostasis in mice (Baker et al., 2013). Furthermore, higher body burden of dioxins, and polychlorinated biphenyls was reported in obese people as opposed to lean people (Kim et al., 2011). A comprehensive literature review on the impact of chlorinated POPs in humans showed that rather than a few individual POPs, background exposure to low-dose POP mixtures may promote type 2 diabetes and obesity (Lee et al., 2014). In another survey, evaluation of 72 epidemiological studies revealed the strongest positive correlation of diabetes with organochlorine than non-organochlorine POPs (Taylor et al., 2013).

In a recent *in vitro* study, the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) model was inoculated with feces from healthy humans, and subsequently exposed to chronic and low-doses of the insecticide chlorpyrifos (**11**). This induced major changes in the microbial community, in particular, increased numbers of *Enterococcus* and *Bacteroides* spp., and decreased numbers of lactobacilli and bifidobacteria, the latter including probiotics commonly associated with health benefits (Joly et al., 2013). Compositional shifts in intestinal bacterial community structure and distortion of their metabolic functions were similarly reported due to exposure of rats to chlorpyrifos (Zhao et al., 2016; Fang et al., 2018) (**Table 1**). In another study, oral exposure of bees to the organochlorine fungicide chlorothalonil (**16**) induced microbial changes, increased putative genes for oxidative phosphorylation and declined sugar metabolism and peptidase potential (Kakumanu et al., 2016). In contrast to these reports, early-life exposure to the estimated environmental concentration of atrazine (**12**) (200 μg/L) did not affect gut bacterial diversity or community composition of tadpoles (*in vivo* or *in vitro*) or adult frogs (Knutie et al., 2018). Discrepancies may arise due to dose, timing, route of exposure, host type and metabolism, and the applied chemical differences.

Besides the known impact of (halogenated) antibiotics in inducing major but partly reversible changes in gut microbiota composition (Dethlefsen and Relman, 2011; Vrieze et al., 2014), an increasing number of studies have shown microbiota perturbations by non-antibiotic biocides. For example, a recent comprehensive screening of more than 1,000 non-antibiotic drugs against 40 representative gut bacterial strains showed that 24% of the drugs with human targets inhibited the growth of at least one strain *in vitro* (Maier et al., 2018). Some intensively used organohalogen drugs such as thyroxine (L) (**24**), a medication used to treat thyroid hormone deficiency, and the anti-inflammatory diclofenac (**25**) were among the tested compounds (Maier et al., 2018). In another study, adolescent rats receiving triclosan orally at levels comparable to human exposures showed lower gut microbiota diversity and more noticeable compositional changes, whereas these differences were diminished in adult rats (Hu et al., 2016). Moreover, triclosan exposure was reported to reduce alpha diversity in the gut microbiota of rats (Kennedy et al., 2016; Gao et al., 2017) (**Table 1**). In contrast, triclosan exposure experiments in humans have not shown major perturbations in the gut and oral microbiota (Poole et al., 2016; Ribado et al., 2017) (**Table 1**).

TABLE 1 | Impact of halogenated compounds on gut microbiota and host (where applicable).

Compound, concentration and duration of use	Compound type	Study model	Impact on gut microbiota	Impact on host	References
2,3,7,8-tetrachlorodibenzofuran, 24 μg/kg for 5 days	POP	C57BL/6J mice ($Ahr^{+/+}$) and C57BL/6J congenic mice ($Ahr^{-/-}$)	Decreased *Firmicutes*/*Bacteroidetes* ratio; enriched *Butyrivibrio* spp. and depleted *Oscillibacter* spp. in cecal contents; production of short chain fatty acids like butyrate	Altered bile acid metabolism; significant inflammation and host metabolic disorders as a result of activation of bacterial fermentation; altered hepatic lipogenesis, gluconeogenesis and glycogenolysis in an Ahr-dependent manner	(Zhang L. et al., 2015)
Polychlorinated biphenyls (PCB) congeners (PCB153, PCB138, and PCB180) total dose of 150 μmol/kg for 2 days	POP	C57BL/6 mice	Decreased overall abundance of bacterial species; decreased levels of *Proteobacteria*; exercise attenuated PCB-induced alterations of gut microbiota composition; abundant *Lactobacillales* and depleted *Erysipelotrichaceae* bacterium C11_K211 (*Tenericutes* phylum) in the exercised group	Exercise provided protection against PCB-induced changes in the gut microbiota than sedentary mice.	Choi et al., 2013
2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 0-30 μg/kg every 4 days for 28 and 92 days	POP	C57BL/6 mice	Significant increase of fourteen antimicrobial resistance genes and mobile genetic elements genes typically observed in *Enterobacteriaceae*	Increased hepatic fat accumulation; depletion of immune cell expression and populations of macrophage and dendritic cells in the intestinal lamina propria	Fader et al., 2015; Stedtfeld et al., 2017
2,3,7,8-tetrachlorodibenzo-*p*-dioxin, biweekly with a dose of 6 μg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin /kg for 26 weeks	POP	CD-1 mice	Increased *Firmicutes*/*Bacteroidetes* ratio; increased *Lactobacillaceae* and *Desulfovibrionaceae*, and decreased *Prevotellaceae* and ACKM1	Liver toxicity, polydipsia (excessive thirst), polyphagia (increased appetite) and prediabetes	Lefever et al., 2016
Chlorpyrifos, 1 mg everyday for 30 days	Pesticide	Human Intestinal Microbial Ecosystem (SHIME)	Compositional change in the microbial community; increased numbers of *Enterococcus* and *Bacteroides* spp. and decreased numbers of lactobacilli and bifidobacteria		Joly et al., 2013
0.3 or 3 mg chlorpyrifos/kg bodyweight/day or for 9 weeks in rats fed a normal (NF) or high fat (HF) diet	Pesticide	Wistar rats	Reduced relative abundance of *Aerococcus*, *Brevundimonas*, and *Trichococcus* in NF-fed rats, and *Olsenella*, *Clostridium* sensu stricto 1, *Amphibacillus*, *Enterorhabdus*, and *Alloprevotella* in HF-fed rats	Pro-obesity phenotype in NF-fed rats; significantly reduced serum insulin, C-peptide, and amylin concentrations in NF- and HF-fed rats; no impact on serum glucose and lipid profiles	Fang et al., 2018
1 mg chlorpyrifos /kg bodyweight in corn oil once daily for 30 days	Pesticide	*Mus musculus* mice	Decreased *Firmicutes*/*Bacteroidetes* ratio; reduced relative abundance of *Lactobacillaceae* and increased relative abundance of *Bacteroidaceae*	Alterations of urine metabolites related to the metabolism of amino acids, energy, short chain fatty acids, phenyl derivatives and bile acids	Zhao et al., 2016
Chlorothalonil, 10 μg/L in a 30% sucrose solution for 6 weeks	Pesticide	Honey bees *(Apis mellifera)*	Perturbed bacterial communities but not fungal communities; reduced relative abundance of *Lactobacillaceae* and increased relative abundance of *Enterobacteriaceae* and *Caulobacteraceae*; increased putative genes for oxidative phosphorylation and declined genes for sugar metabolism and peptidase		Kakumanu et al., 2016
Pentachlorophenol, 0–100 μg/L for 28 days	Pesticide	Goldfish (*Carassius auratus*)	Decreased *Firmicutes*/*Bacteroidetes* ratio; increased relative abundance of *Bacteroides* and decreased relative abundance of *Chryseobacterium*, *Microbacterium*, *Arthrobacter* and *Legionella*	Accumulation of PCP in the fish intestinal tract in a time- and dose-dependent manner; reduced fish body weight and liver weight; antioxidant system disturbance	Kan et al., 2015

(Continued)

TABLE 1 | Continued

Compound, concentration and duration of use	Compound type	Study model	Impact on gut microbiota	Impact on host	Reference
Triclosan at 0.05 mg/kg body weight, administration through milk until 28 days and afterwards through oral gavage three times a week till day 181	PPCP	Sprague Dawley rats	Decreased *Firmicutes* /*Bacteroidetes* ratio; increased *Deltaproteobacteria* and *Lactobacillus,* increased *Lachnospiraceae*	Reduction in the bodyweight in adolescent rats	Hu et al., 2016
Triclosan in water solution (2 mg/L) for 13 weeks	PPCP	C57BL/6 mice	Decreased alpha diversity; depletion of *Turicibacteraceae*, *Christensenellaceae* and *Clostridiales*; enrichment of gut bacterial genes related to triclosan resistance, stress response, antibiotic resistance and heavy metal resistance		Gao et al., 2017
Triclocarbon, supplemented in feed (0.1% w/w) for 12 days	PPCP	Sprague Dawley rats	Significantly reduced phylogenetic diversity of gut among exposed dams and neonates during gestation and lactation; dominance of *Enterobacteriaceae*		Kennedy et al., 2016
Commercially available wash products either containing or not containing triclosan for 1 year	PPCP	Humans: 39 pairs of mothers and babies	No global reconstruction or loss of microbial diversity of either infant or maternal gut microbiotas; broadly antibiotic-resistant species from the phylum *Proteobacteria* were enriched in stool samples from mothers		Ribado et al., 2017
Triclosan-containing PPCP (4 months) and non-triclosan-containing PPCP (4 months)	PPCP	Humans (16 persons)	No differences in microbiota composition, species richness and overall diversity of the stool, molar, or incisor	Higher urinary concentrations of triclosan in all volunteers during the triclosan period; no differences in metabolic or endocrine markers, or weight	Poole et al., 2016
Chloroacetate, bromoacetate, dichloroacetate, dibromoacetate, trichloroacetate, tribromoacetate, or bromochloroacetate; 1 gm/ml of each compound in selective gorwth media	DBP	Incubations of CDF rat cecal microbiota	Toxic impacts on cecal microbiota especially to the enterococci; increased activities for β-glucuronidase, β-galactosidase, β-glucosidase, azoreductase, nitroreductase, dechlorinase, and dehydrochlorinase that can affect the biotransformation of co-exposed compounds		Nelson et al., 2001
Trichloroacetamide, 50, 500 and 5000 μg/l for 90 days	DBP	*Mus musculus* mice	Decreased *Firmicutes*/*Bacteroidetes* ratio with an increase in the concentration of trichloroacetamide; Increased relative abundance of *Bacteroidaceae*, *Porphyromonadaceae*, *Sphingobacteriaceae*, *Aerococcaceae*, and *Erysipelotrichaceae* and decreaseed relative abundance of *Bacillaceae*, *Heliobacteriaceae*, *Syntrophomonadaceae*	Disruption of the host metabolism, weight loss, altered choline metabolites in urine samples; decreased urine tyrosine and intestinal lesions; disordered amino acid and lipid metabolism, alterations in the serum metabolome, including altered choline, trimethylamino oxide, as well as hepatotoxicity and cytotoxicity	(Zhang et al., 2013; Zhang Y. et al., 2015)

Since humans experience much lower triclosan exposures in products such as soap and toothpaste that are rinsed off immediately, the impacts observed in high-dose and acute animal exposures might not be observed in humans. However, a positive correlation was reported between the exposure to triclosan and the occurrence of *Staphylococcus aureus* as an opportunistic pathogen in the human nasal microbiota (Syed et al., 2014). There are also concerns regarding contribution of non-antibiotic antimicrobials to antibiotic resistance due to cross-resistance (Hartmann et al., 2016; Maier et al., 2018). For example, prolonged exposure to triclosan was associated with developing resistance and cross-resistance to ampicillin and/or ciprofloxacin in *S. aureus* and *Escherichia coli* (Wesgate et al., 2016). Exposure of a susceptible *Pseudomonas aeruginosa*

strain to triclosan has been shown to select multidrug-resistance mediated by multidrug efflux pumps (Chuanchuen et al., 2001). Similarly, abundance of several multidrug-resistance efflux pump genes was reported to significantly increase after triclosan exposure (Gao et al., 2017). Recent studies on the mouse gut indicated the selective pressure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in promoting blooms of *Enterobacteriaceae,* that harbor antimicrobial resistance genes (Stedtfeld et al., 2017) (**Table 1**). Similar increased levels of *Enterobacteriaceae* were reported in: (i) the gut of honey bees exposed to the organochlorine fungicide chlorothalonil (**16**) (Kakumanu et al., 2016), and (ii) the human gut due to gastrointestinal infection (Lupp et al., 2007) and antibiotic therapies (Sekirov et al., 2008). Finally, DBPs have the potential to select for antibiotic resistance (Li et al., 2016; Zhang et al., 2016). These reports indicate similar impacts despite different types of stressors and hosts.

FATE

Extensive studies have been performed to understand the fate of xenobiotics by oxidative and conjugative enzymes in the liver (Zanger et al., 2008; Zanger and Schwab, 2013). Among the hepatic enzymes, cytochrome P-450s are the major oxidative enzymes for transformation of (halogenated) lipophilic xenobiotics and drugs e.g., PCDDs (Hu and Bunce, 1999) and diclofenac (Leemann et al., 1993). In contrast, much less is known about the dehalogenation and/or degradation of halogenated xenobiotics by intestinal microbiota that employ hydrolytic and reductive mechanisms (Sousa et al., 2008).

Cultivation-Dependent View

Understanding of the fate of halogenated xenobiotics using cultivation-based studies has been derived from the exposure of the intestinal contents or specific microbial isolates of intestinal origin. For example, incubation of chloramphenicol, that contains a nitrobenzene group and an amide of dichloroacetic acid, with human fecal bacteria led to the hydrolysis of the amide linkage and reduction of the nitro group to an amine on the aromatic ring (**Figure 3A**) (Holt, 1967). Nitroreductases reducing nitro ($-NO_2$) functional groups to the corresponding amines are an important group of enzymes identified for the gut microbial xenobiotic metabolism (Rickert et al., 1981; Claus et al., 2016). Another example of nitroreductive metabolism of organohalogens was shown for clonazepam, a medication used to prevent and treat seizures and panic disorder, that was converted to 7-aminoclonazepam by rat intestinal lumen microbiota (**Figure 3B**) (Elmer and Remmel, 1984). In contrast to such amine group formation, amine group removal by human intestinal microbiota was reported for the anti-fungal 5-fluorocytosine (**Figure 3C**) (Harris et al., 1986; Vermes et al., 2003). Susceptible fungi contain a cytosine deaminase which converts 5-fluorocytosine to 5-fluorouracil (**Figure 3C**). The latter is further metabolized to 5-fluorodeoxyuridylic acid, an inhibitor of thymidylate synthetase and subsequently DNA synthesis (Vermes et al., 2000). Although human host cells lack the deaminase enzyme, 5-fluorocytosine conversion to 5-fluorouracil by the human intestinal microbiota plays an important role in the development of hematologic and gastrointestinal toxicity (Harris et al., 1986). Co-administration of 5-fluorocytosine with the antiviral drug sorivudine led to 18 acute deaths due to an unknown lethal gut microbial metabolism (Okuda et al., 1998). Further research has revealed that intestinal *Bacteroides* species, namely *Bacteroides vulgatus*, *B. thetaiotaomicron, B. fragilis, B. uniformis,* and *B. eggerthii* can convert sorivudine to (E)-5-(2-bromovinyl)uracil (Nakayama et al., 1997) (**Figure 3D**), whereas the latter was barely detected in the plasma of germ-free rats (Ashida et al., 1993). A key liver enzyme that regulates the systemic 5-fluorocytosine level is subsequently inactivated by (E)-5-(2-bromovinyl)uracil, leading to toxic levels of 5-fluorocytosine and death in rats and humans (Okuda et al., 1998). This is an important example of the role of gut microbiota in toxification processes.

Reductive dehalogenation of dichlorodiphenyltrichloroethane to dichlorodiphenyldichloroethane (**Figure 3E**) has been shown in anoxic incubations of the strictly anaerobic human intestinal bacterium *Eubacterium limosum* (Yim et al., 2008) and rat intestinal microbiota (Mendel and Walton, 1966). However, it is not known if this is a bioactivation or detoxification mechanism as dichlorodiphenyldichloroethane is still an endocrine disruptor (Claus et al., 2016). Reductive dehalogenation can be mediated co-metabolically by vitamin B_{12} (cobalamin) that is synthesized by some human gut microbes (Degnan et al., 2014). In contrast, metabolic reductive dehalogenation is mediated by specific bacterial groups that can use organohalogens as their terminal electron acceptors (Smidt and de Vos, 2004; Atashgahi et al., 2016). A co-culture of *Clostridium perfringens* and *C. beijerinckii* was also shown to reductively dehalogenate hexachlorobiphenyl to pentachlorobiphenyl (**Figure 3F**) and tetrachlorobiphenyl to trichlorobiphenyl (De et al., 2006). Gut microbiota was also shown to be involved in generation of methylsulfone ($MeSO_2$) metabolites from polychlorinated biphenyls (PCBs) through a series of reactions in combination with the host cells (**Figure 3G**) (Bakke et al., 1982; Brandt et al., 1982). $MeSO_2$–PCBs can bind to specific proteins and accumulate in the lipophylic tissues with adverse effects (Shigematsu et al., 1978). Exposure to 2,3,7,8-tetrachlorodibenzofuran has been shown to enhance the level of *Flavobacteria* in the gut of mice (Zhang L. et al., 2015). These bacteria are reported to possess glutathione-dependent reductive dehalogenase activity (Xun et al., 1992), although 2,3,7,8-tetrachlorodibenzofuran dehalogenation by the gut microbiota has not been shown yet.

Intestinal lactobacilli have been reported to degrade organohalogens *in vitro*. For instance, *Lactobacillus lactis, L. fermentum, L. plantarum, E. coli,* and *Enterococcus faecalis* were tested for chlorpyrifos degradation potential. The results indicated that besides *E. coli*, *L. lactis* and *L. fermentum* could grow in the presence of over 1.5 mg/mL chlorpyrifos (Harishankar et al., 2013). Similarly, four lactic acid bacteria isolated from kimchi fermentation in the presence of 200 mg/L chlorpyrifos were reported to use this pesticide as the sole source of carbon and phosphorus (Cho et al., 2009). Moreover, lactic acid bacteria seeded to skimmed milk were shown to degrade the insecticide trichlorfon (**17**) (Zhao and Wang, 2012).

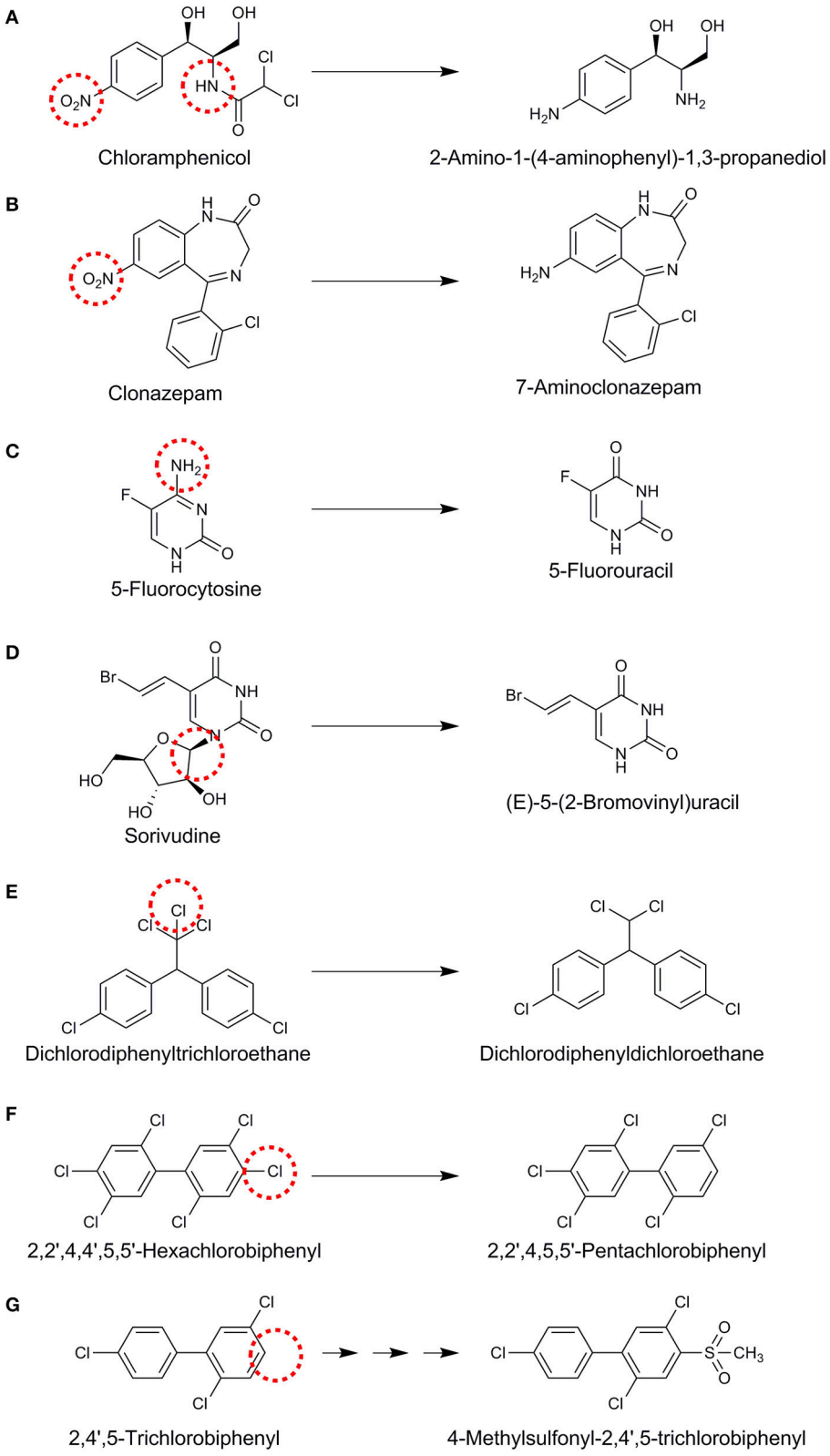

FIGURE 3 | Proposed reactions for different organohalogens **(A–G)** mediated by gut microbiota. Reaction sites are indicated with red dashed circles.

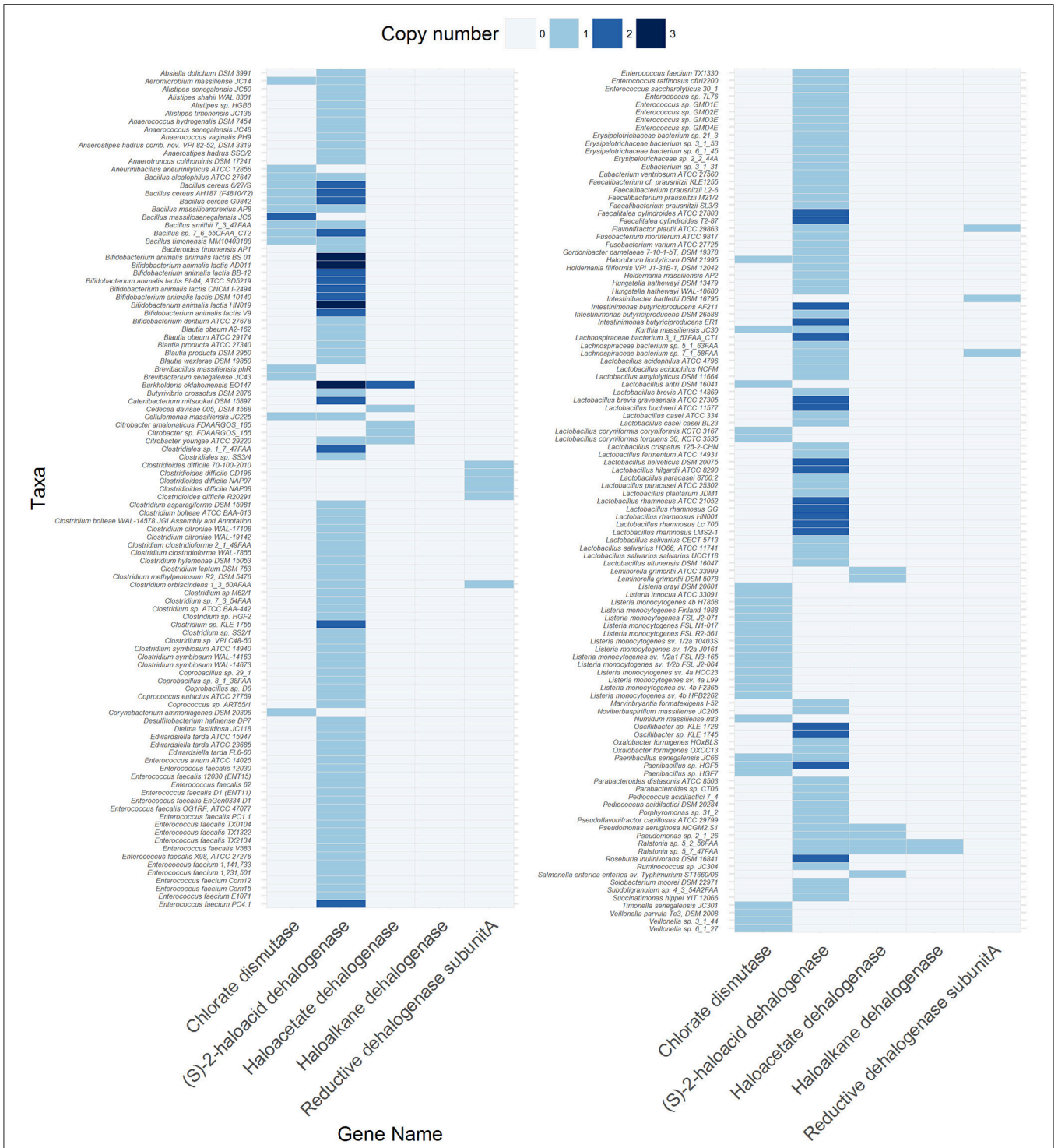

FIGURE 4 | Genes encoding different dehalogenases found in 216 out of 670 bacterial and archaeal genomes of gut origin. The code to reproduce the figure is available at https://github.com/mibwurrepo/Atashgahi-et-al.-XenobioticReview2018.

However, the actual impact of lactobacilli on organohalogen fate in the gut is not known, especially since this bacterial group seems particularly sensitive to organohalogen exposure (**Table 1**).

Cultivation-Independent View

As a complementary approach to cultivation, metagenomic approaches can also be used to infer xenobiotic metabolism potential by the gut microbiota (Haiser and Turnbaugh,

2013; Spanogiannopoulos et al., 2016). Due to environmental persistence and toxicity, great attention has been given to understand microbial transformation of halogenated xenobiotics in environmental studies with the end goal of bioremediation. Indeed a variety of microbial dehalogenation mechanisms have been described that can remove halogens from organic compounds by oxidation, reduction and substitution mechanisms that are employed in co-metabolic and/or energy-yielding modes (van Pée, 1996; Fetzner, 1998; Janssen et al., 2001; Smidt and de Vos, 2004).

Although a metagenomic view of xenobiotic metabolism in the gut has been provided (Haiser and Turnbaugh, 2013; Spanogiannopoulos et al., 2016), specific information on the prevalence and diversity of known dehalogenase-encoding genes in the gut metagenome is lacking. Therefore, we surveyed the Joint Genome Institute Integrated Microbial Genomes & Microbiome System (JGI-IMG/MER) database for the occurrence of different dehalogenating gene classes (Table S1) in 670 bacterial and archaeal genomes from fecal origin (Table S2), 254 metagenomes obtained from human fecal samples (Table S3) and 86 metagenomes obtained from gut/rumen fluid/fecal samples of animals (Table S4). The metadata and criteria used for the selection of genomes and metagenomes are listed in Tables S2–4. The codes used for the analysis and visualization are provided in supplementary information. The results showed that at least one dehalogenating gene was present in 32.2% of the bacterial and archaeal genomes, and in 61 and 75.6% of the metagenomes derived from human and non-human origins, respectively (Tables S5–S7). Among these, five types of genes were found in the bacterial genomes (**Figure 4**, Table S5) and human metagenomes (Table S6), and six types of genes in non-human metagenomes (Table S7). Four types of genes were shared among the three datasets and were predicted to code for (S)-2-haloacid dehalogenase (EC:3.8.1.2), haloacetate dehalogenase (EC:3.8.1.3), haloalkane dehalogenase (EC:3.8.1.5), and reductive dehalogenase (EC:1.21.99.5). In contrast, a canonical gene for chlorate dismutase (EC: 1.13.11.49) was not found in the human metagenomes whereas that for atrazine chlorohydrolase (EC:3.8.1.8) was absent from the bacterial genomes. Atrazine chlorohydrolase catalyzes the conversion of the herbicide atrazine to hydroxyatrazine, the first step in the atrazine degradation pathway (Mandelbaum et al., 1995).

A gene for (S)-2-haloacid dehalogenase was the most abundant in both the genome (214 genes) and metagenome (2612 genes in human and 4549 genes in non-human) datasets indicating exposure of gut microbiota to haloacids that are common DBPs (Richardson et al., 2007). Many of the known core gut genera, such as *Faecalibacterium*, *Blautia*, *Roseburia*, *Alistipes*, *Eubacterium* (Shetty et al., 2017) harbor a (S)-2-haloacid dehalogenase gene in their genome (**Figure 4**, Table S5). The encoded enzyme belongs to the family of hydrolases, acting specifically on halide bonds in α-substituted haloacids (Janssen et al., 2001). Interestingly, a (S)-2-haloacid dehalogenase encoding gene co-occurred with that of chlorite dismutase in 14 microbial genomes (**Figure 4**, Table S5). Chlorite dismutase mediates the last step in chlorate reduction splitting chlorite to chloride and oxygen (Liebensteiner et al., 2016). An environmental bacterium, *Pseudomonas chloritidismutans* AW-1^T, which similarly harbored these two genes, was recently shown to concurrently degrade haloacids and chlorate as the electron donor and acceptor, respectively (Peng et al., 2017). This is an interesting finding considering that haloacids and chlorate are common DBPs in drinking water resources (Richardson et al., 2007) that might be degraded by the gut microbes harboring these genes. Further, the oxygen produced from chlorite dismutation can be used for degradation of haloacids (Peng et al., 2017) or other organic compounds (Oosterkamp et al., 2013; Atashgahi et al., 2018b) in an "intra-aerobic" pathway. If functional, this can be an important metabolism in the gut environment where oxygen is largely unavailable to serve as a terminal electron acceptor.

The well-studied reductive dehalogenase genes were more abundant in the non-human than in the human metagenomes (Table S7). Interestingly, of the 59 reductive dehalogenase genes found in the non-human metagenomes, 53 were from the rumen content of sheep, goat and cow. This implies that the rumen content of ruminant farm animals is an appropriate environment for the reductive dehalogenation metabolism that represents useful but largely unexplored sources for future enrichment/isolation of organohalide-respiring bacteria. These bacteria reductively dehalogenate organohalogens by replacing the halogen substitutes with hydrogen in a process known as organohalide respiration (Smidt and de Vos, 2004; Atashgahi et al., 2016). This metabolism usually reduces the toxicity of organohalogens and makes the otherwise chemically locked organohalogens available to other microbial metabolisms such as fermentative and aerobic degradation. Organohalide-respiring bacteria have only been found in pristine and contaminated environments impacted by natural or anthropogenic organohalogens (Atashgahi et al., 2018a). The activity of these microbes in host-associated ecosystems and their potential impacts on the organohalogen fate especially in the gut remains largely unknown. There is only one study showing reductive dehalogenation of polychlorinated biphenyls by the co-culture of *Clostridium perfringens* and *C. beijerinckii* as prominent species in the human gut (De et al., 2006) (**Figure 3F**). Interestingly, we found six reductive dehalogenase genes in the genomes of *Clostridia* (**Figure 4**, Table S5). This may point to an unrecognized reductive dehalogenation potential in the gut microbiota. Of interest, the gut isolate strain DP7, belonging to the genus *Desulfitobacterium* (*Clostridia*) that is known for its active organohalogen respiration metabolism (Kruse et al., 2017), was reported to lack reductive dehalogenation activity (van de Pas et al., 2001) and the corresponding genes (Kruse et al., 2017).

CONCLUSIONS AND OUTLOOK

The toxicant-microbiota interaction has emerged in recent years as one of the novel concepts from the intensive research on the human microbiome. The gut microbiota constitutes a critical zone for xenobiotic (de)toxification and sequestration at the interphase between the external environment and our mucosal epithelial cells. An important class of toxicants are

halogenated compounds from anthropogenic and natural sources that come into contact with the human gut and other body parts mainly by ingestion of, or exposure to contaminated food and water. Although canonical toxicological approaches using short-term high-dose exposure experiments are informative about the toxicity and impact of halogenated compounds, they do not represent scenarios of chronic exposure to low-level xenobiotic cocktails throughout life. The ingested concentrations of the halogenated xenobiotics in food and water resources are in most cases below the regulatory thresholds. However, little is known about the physiological impact, reactivity, bioaccumulation in food chains, additive/cumulative toxicity of these emerging contaminants, and their (bio)transformation products. For example, even at trace concentrations, mixtures of biocides, antibiotics, and heavy metals have the potential to contribute to the emergence, maintenance and transmission of antibiotic-resistant and disease-causing bacteria (Gullberg et al., 2014; Pal et al., 2015). Therefore, future studies are necessary to reveal the impact of halogenated (micro)pollutants on the gut microbial community membership, gene expression, physiology, metabolite profile, antibiotic resistance genes, and also parallel impacts on the host. Long-term incubations of the gut contents/isolates with environmentally relevant doses and diversity of halogenated compounds, in combination with other micropollutants, should aid in understanding the actual consequences of chronic low-dose exposures.

Even less information is available on the specific microorganisms responsible for halogenated xenobiotic metabolism, the molecular mechanisms and biotransformation pathways involved that can either diminish or enhance the toxicity. The metagenomic approach described here showed that genes involved in dehalogenation are widespread among gut bacteria, and this may impact flux, toxicity, bioavailability and fate of halogenated compounds. Future cultivation and omics experiments are necessary to test the actual metabolism of the halogenated compounds by the gut microbiota. To this end, we can immensely benefit from the wealth of knowledge gained about the metabolism of halogenated xenobiotic compounds in terrestrial and aquatic environments (Janssen et al., 2001; Smidt and de Vos, 2004; Atashgahi et al., 2018a) and high-throughput cultivation of the gut microbiota (Ingham et al., 2007; Lagier et al., 2016). These approaches should be coupled with untargeted metabolomics using high-resolution mass spectroscopy to identify xenobiotics and biotransformation products. Untargeted metabolomics has the potential to aid in determination of pathways and mechanisms of action (Warth et al., 2017).

Given the immense potential of gut microbiota to alter the chemical structure and bioactivity of xenobiotics with beneficial (Shin et al., 2013) or severely detrimental impacts (Okuda et al., 1998), assessments of xenobiotic metabolism should be an integral part of designing drugs and chemicals such as PPCPs and pesticides, informing toxicology risk assessment, improving nutrition, and guiding personalized medicine.

AUTHOR CONTRIBUTIONS

SA, HS and WMdV have designed the study, SAS has performed the metagenomic analysis and all authors wrote the manuscript.

ACKNOWLEDGMENTS

This research is supported by the Soehngen Institute of Anaerobic Microbiology (SIAM) Gravitation grant (024.002.002) and Spinoza Award of the Netherlands Organization for Scientific Research (NWO) to WMdV. We thank the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) for maintaining and curating the genomic and metagenomic, and making available via the IMG system.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00888/full#supplementary-material

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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.

frontiers in Microbiology

OPINION
published: 06 July 2018
doi: 10.3389/fmicb.2018.01510

Gut Microbiota as a Missing Link Between Nutrients and Traits of Human

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Keywords: gut microbiota, nutrients, human traits, diet, disease

CONTRIBUTION OF GUT MICROBIOTA IN DETERMINING HUMAN DISEASES

With the development of next generation sequencing (NGS) technology, the role of gut microbiota in human health has been extensively studied. Metagenome sequencing analysis, which is based on NGS, and subsequent statistical analysis showed that the relationship between gut microbiota and humans is not merely commensal but rather a mutualistic relationship (Chen et al., 2013; Jandhyala et al., 2015). Recent advances in the field of gut microbiota are elucidating our understanding of human biology.

The microbiota of the human gut is a massive and complex microbial community consisting of 100 trillion microbes in the intestine. The gut microbiota is essential to the health and well-being of the host (Marchesi et al., 2016). Although interactions between gut microbiota and its host have negative effects in some cases, these interactions positively affect the host in most cases. It is now clear that the gut microbiota contributes significantly to the traits of humans as much as our genes, especially in the case of atherosclerosis, hypertension, obesity, diabetes, metabolic syndrome, inflammatory bowel disease (IBD), gastrointestinal tract malignancies, hepatic encephalopathy, allergies, behavior, intelligence, autism, neurological diseases, and psychological diseases (Chen et al., 2013; Nguyen et al., 2017; Zhang et al., 2017; **Table 1**). It has also been found that alteration of the composition of the gut microbiota in its host affects the behavior, intelligence, mood, autism, psychology, and migraines of its host through the gut-brain axis (Chen et al., 2013). Thus, the effect of the gut microbiota on human phenotypes has become a booming area of research and presents a new paradigm of opportunities for medical and food applications.

THE GUT MICROBIOTA OF HUMANS FLUCTUATES IN RESPONSE TO NUTRITIONAL UPTAKE RATHER THAN REMAINING STABLY IMMUTABLE THROUGHOUT LIFE

Recent studies have elucidated that the gut microbiota plays essential roles in the health and well-being of its host, and whether the composition of the gut microbiota fluctuates or stays constant throughout the lifetime of its host has become one of the main questions to ponder in the scientific community. The prevailing opinion has been that the gut microbiota develops rapidly right after birth and fluctuates only until it matures, which usually takes ∼2 years after birth (Koenig et al., 2011). Once the gut microbiota is established, its composition remains stably immutable throughout life. However, recent evidence shows that this opinion is wrong, and the composition of gut microbiota can fluctuate during the lifetime of its host (Wu et al., 2011; David et al., 2014).

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *01 December 2017*
Accepted: *18 June 2018*
Published: *06 July 2018*

Citation:
Chung H-J, Nguyen TTB, Kim H-J and Hong S-T (2018) Gut Microbiota as a Missing Link Between Nutrients and Traits of Human. Front. Microbiol. 9:1510. doi: 10.3389/fmicb.2018.01510

It is observed that the growth of almost all microbial organisms is very sensitive to their ambient nutrients. Additionally, considering the diversity and number of microbes in the gut microbiota, it would be more reasonable to speculate that the composition of the gut microbiota could constantly fluctuate, reflecting the diet of its host. Wu et al. recently showed that the long-term consumption of different diets, such as plant-based diets or animal-based diets, drastically altered the composition of gut microbiota, even at the phylum level in the taxonomic hierarchy (Wu et al., 2011). Vegetarian diets consist of fibers containing resistant starch and non-starch polysaccharides. Interestingly, numerous studies have shown that vegetarian diets increased the abundance of carbohydrate-degrading microbes, such as *Prevotella, Roseburia, Eubacterium rectale,* and *Ruminococcus bromii*, in their gut microbiota (Wu et al., 2011; David et al., 2014). In contrast, western diets high in protein and fat, which promote chylomicron and bile acids, increase the abundance of bile acid-tolerant microbes, such as *Alistipes, Bilophila*, and *Bacteroides*, in their gut microbiota (David et al., 2014). A defined food consumption experiment by David et al. even showed that the composition of gut microbiota is promptly affected by the dietary fluctuations within a day. Even cyclical shifts in daily feeding or fasting affected the increase of specific genera in the gut microbiota (David et al., 2014). These studies clearly show that the composition of the human gut microbiota constantly fluctuates in response to the nutritional composition of the diet rather than remaining stably immutable throughout life.

NUTRIENTS AFFECT THE COMPOSITION OF THE GUT MICROBIOTA, AND BOTH MODIFIED GUT MICROBIOTA AND NUTRIENTS AFFECT HUMAN TRAITS TOGETHER

Nutrients are dietary components that an organism metabolizes for survival and growth. Nutrients are substances that provide energy and/or form a component of body tissues. Higher organisms, such as humans, intake nutrients in their diets to maintain the precisely functioning metabolic machinery affecting the health and well-being of the organism. Because nutrients are essential substances for sustaining life, there is much less genetic variation in the genes involved in processing nutrients compared to other genes in humans (Fraser, 2015). Considering that nutrients absorbed by an organism are precisely processed by the well-orchestrated metabolic machinery in the bodies of organisms, diets have a limited ability in terms of affecting the traits of human. However, epidemiological research has proven that diet significantly affects human traits (Sharief et al., 2011; Boada et al., 2016). The quantity of calories and dietary patterns are key determinants of the anthropometric quantitative traits, which are especially reflected in the positive height trend in the developed countries (Jelenkovic et al., 2016). In the context of the nature of nutrients, an association between anthropometric quantitative traits and nutrients is expected. Interestingly, the effect of nutrients on human traits is not limited

TABLE 1 | Effect of the gut microbiota on human diseases.

Disease/ disorder	Implicated microbiota	Potential role of the microbiome
METABOLIC DISEASES		
Obesity	Firmicutes/Bacteroidetes ratio Prevotellaceae Eubacterium Faecalibacterium Roseburia	Significant changes in gut microbiota are associated with increased obesity
Type II diabetes	Bacteroidetes/Firmicutes Bacteroides-Prevotella *Eggerthella lenta* *Clostridia* *Eubacterium rectale* *Faecalibacterium prausnitzii* *Roseburia intestinalis* *Roseburia inulinivorans*	Shifts in gut microbiota are associated with increases in plasma glucose concentrations
Hypertension	*Prevotella* *Klebsiella* *Bifidobacterium* *Butyrivibrio* *Coprococcus* *Faecalibacterium* *Roseburia*	Gut dysbiosis increases hypertension
IMMUNE DISEASES		
IBD	Bacteroidetes *Lachnospiraceae* *Actinobacteria* *Proteobacteria* *Clostridium leptum* *Clostridium coccoides* *Faecalibacterium prausnitzii* Firmicutes/Bacteroidetes ratio *Bifidobacteria*	Immune response to the gut microbial community Composition of the gut microbiota contributes to inflammation
Allergies	*Lactobacillus* spp. *Bifidobacterium adolescentis* *Clostridium difficile*	Early colonization with *Lactobacillus* is associated with decreased allergies Early colonization with more diverse microbiota might prevent allergies
Celiac disease	*Bacteroides vulgatus* *Escherichia coli* *Clostridium coccoides*	High diversity in Celiac disease patients vs. control
Type I diabetes	*Bacteroides* *Streptococci* *Clostridium cluster IV and XIVa*	Interaction between the gut community and the innate immune system may be a predisposing factor for diabetes
Rheumatoid arthritis	Bifidobacteria *Bacteroides* *Porphyromonas* *Prevotella* *Bacteroides fragilis* *Eubacterium rectale* *Clostridium coccoides*	Treg-promoting organisms depleted; overgrowth of bacteria that induce Th17 cell populations, leading to inflammation Intestinal microbes associated with etiology
Atopy and asthma	Bifidobacteria *Bacteroides* *Staphylococcus* spp. *Streptococcus* spp. Enterobacteria *Clostridium difficile*	Pre- and post-natal microbial exposure appear key to appropriate immune development Mode of delivery and nutrient uptake are important factors for GI community development and protection against subsequent atopic disease development
AUTISM		
Autism	*Clostridial species*	Increased bacterial diversity in the feces of autistic children compared to control
PSYCHOLOGICAL DISEASE		
Anxiety and depression	*Lactobacillus reuteri* *Lactobacillus rhamnosus* *Bifidobacterium infantis*	Decreased anxiety and stress-induced increase of corticosterone

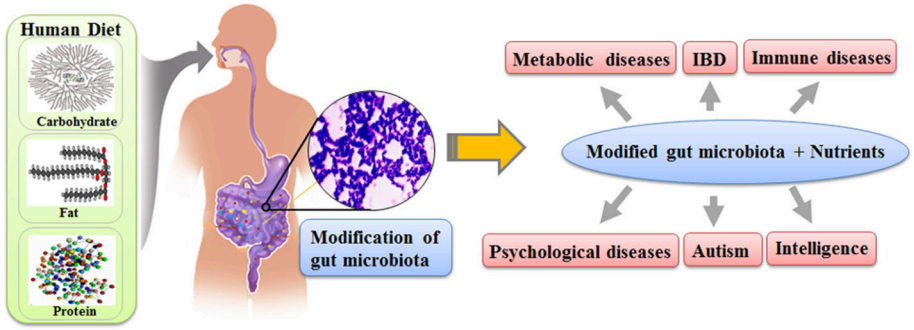

FIGURE 1 | The schematic diagram on how nutrients affect the human traits through gut microbiota.

to anthropometric quantitative traits. Studies on monozygotic twins show that nutrients strongly affect various metabolic diseases and immune diseases (Rissanen et al., 2002; Spehlmann et al., 2012). Epidemiological research has shown that nutrients are considered a fundamental factor along with genetics in the development and/or prevention of rheumatoid arthritis, multiple sclerosis, asthma, and allergies (Sharief et al., 2011; Thorburn et al., 2014). In addition, numerous studies have proven an association between cancer and nutrients (Boada et al., 2016). Because of the clear association between cancer and nutrients, the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) even declared that hydrogenated oils, potato chips, processed meats, red meats, farmed salmon, and refined sugar are associated with various cancers. These results definitely indicate that human traits are also strongly shaped by nutrients. In this sense, the opinion of "we are what we eat" by the general public would be scientifically wise. Despite the expectation that nutrients only slightly affect human traits, it is surprising to observe that nutrients seem to affect human traits as much as our own genes, which suggests that there might be a strong link between nutrients and human traits.

Recent scientific evidence regarding the gut microbiota makes it possible to explain the link between nutrients and human traits. The gut microbiota not only directly interacts with the somatic cells of its host to affect the traits of human, as in the case of training immunological networks, but also generates various chemicals, which can directly modify the biochemical pathways of humans. The composition of the gut microbiota fluctuates based on the nutrient uptake of its host, and the composition of the gut microbiota affects various human traits as much as our genes (**Figure 1**). Therefore, it is reasonable to speculate that the effect of nutrients on human traits would be the combined results from both the gut microbiota modified by the nutrient uptake and the nutrients themselves. We believe that the gut microbiota is the missing link between nutrients and modifications of human traits.

AUTHOR CONTRIBUTIONS

S-TH conceived the idea and designed the structure of the manuscript. H-JC, TN, and S-TH drafted the manuscript, table and figure. All authors have critically read, corrected, and approved the final version of the manuscript and agree with the opinions expressed here.

FUNDING

This research was financially supported by the Global accompanied growth R&BD program (N042600010) through the Ministry of Trade, Industry and Energy (MOTIE, Korea).

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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.

frontiers in Microbiology

HYPOTHESIS AND THEORY
published: 26 June 2018
doi: 10.3389/fmicb.2018.01379

The Microbiological Memory, an Epigenetic Regulator Governing the Balance Between Good Health and Metabolic Disorders

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If the transmission of biological information from one generation to the next is based on DNA, most heritable phenotypic traits such as chronic metabolic diseases, are not linked to genetic variation in DNA sequences. Non-genetic heritability might have several causes including epigenetic, parental effect, adaptive social learning, and influence of the ecological environment. Distinguishing among these causes is crucial to resolve major phenotypic enigmas. Strong evidence indicates that changes in DNA expression through various epigenetic mechanisms can be linked to parent-offspring resemblance in terms of sensitivity to metabolic diseases. Among non-genetic heritable traits, early nutrition could account for a long term deviant programming of genes expression responsible for metabolic diseases in adulthood. Nutrition could shape an inadequate gut microbiota (dysbiosis), triggering epigenetic deregulation of transcription which can be observed in chronic metabolic diseases. We review herein the evidence that dysbiosis might be a major cause of heritable epigenetic patterns found to be associated with metabolic diseases. By taking into account the recent advances on the gut microbiome, we have aggregated together different observations supporting the hypothesis that the gut microbiota could promote the molecular crosstalk between bacteria and surrounding host cells which controls the pathological epigenetic signature. We introduce for the first time the concept of "microbiological memory" as the main regulator of the epigenetic signatures, thereby indicating that different causes of non-genetic heritability can interact in complex pathways to produce inheritance.

Keywords: metabolic diseases, infectious diseases, microbiome, diet, dysbiosis, microbiological memory, epigenetic programming, gene expression

OPEN ACCESS

Edited by:
Stephen J. Pandol, Cedars-Sinai Medical Center, United States

Reviewed by:
Aaron Lerner, AESKU.KIPP Institute, Germany
Miguel Gueimonde, Instituto de Productos Lácteos de Asturias (IPLA), Spain

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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *20 April 2018*
Accepted: *06 June 2018*
Published: *26 June 2018*

Citation:
Devaux C and Raoult D (2018) The Microbiological Memory, an Epigenetic Regulator Governing the Balance Between Good Health and Metabolic Disorders. Front. Microbiol. 9:1379. doi: 10.3389/fmicb.2018.01379

GENETIC AND NON-GENETIC HERITABILITY

Although the genetic material of an individual can be considered as a highly stable element that may slowly evolve from one generation to the next by random mutations and selection pressure of the environment, the way this genetic material is used within the cell as part of the transcription process can vary in astonishingly different levels of plasticity. Beyond the outdated tendency assuming that

only the DNA sequence is inherited across generations, data issued from genome-wide association (GWA) studies have shown that the most common human diseases transmitted by parents to their offspring, could not be explained by common genetic variants (Maher, 2008). This paved the way for the search for mechanisms that could account for the intergenerational transmission of phenotypic traits proved to be different from simple DNA sequences. The term "inclusive heritability" encompasses the genetic and non-genetic dimensions of inheritance. Several causes of non-genetic inheritable traits can be assigned to parental effect, adaptive social learning, influence of the ecological environment, and/or epigenetics (Danchin et al., 2011). Epigenetic modifications regulate through switch on or off the intensity and timing of gene expression without changes to the underlying DNA sequence. Epigenetic control plays a crucial role during embryologic development in the silencing of genes not required in specific cells (Wolffe and Matzke, 1999). Epigenetic mechanisms include DNA methylation (cytosine methylation at the CpG/cytosine-phosphate-guanine dinucleotide residues), histone modifications (histone packaging through acetylation, methylation, phosphorylation, biotinylation, ubiquitination, and ADP-ribosylation), chromatin remodeling (modification of chromatin architecture and restructuration of nucleosomes), and transcriptional or translation interference by non-coding RNA (ncRNAS including microRNAs, miRNAs involved in transcriptonal silencing; short -<30 nts- interfering RNAs, siRNAs involved in post-transcriptional silencing and chromatin condensation; piwi-interacting RNAs, piRNAS acting on transposon silencing; and long ->200 nts- non-coding RNA, lncRNAs influencing gene expression by forming complexes with chromatin-modifying proteins) (Egger et al., 2004; Peschansky and Wahlestedt, 2014). In recent years evidence was accumulated suggesting that epigenetic modifications can play a major role in the etiology of chronic diseases such as depression, obesity, and cardiovascular diseases (CVD) and this discovery was regarded as a scientific breakthrough (Shenderov and Midtvedt, 2014). More intriguing though was the observation that pathologic metabolic processes can be inheritable within a family, leading to the conclusion that epigenetic modifications could be inherited during cell division. A growing number of studies suggested that maternal and neonatal diet strongly influences epigenetic processes linked to chronic metabolic diseases (Choi and Friso, 2010). This prompted us to question whether the epigenetic changes can be somatically inherited and stays constant from one generation to another within the patient's family or if it is the cell molecular microenvironment that remains stable between parents and children. According to the second hypothesis, the molecular microenvironment -let us provisionally say microbiota antigens- would lead to the conservation of such epigenetic signatures, making them appear heritable although potentially reversible. We hypothesize that early nutrition could shape an inadequate gut microbiota impairing microbiota antigens which, in turn, could account for a long term deviant programming of genes expression and progress toward chronic metabolic diseases during adulthood. We propose that the diversity of bacteria species defining the gut microbiota can be partly shared within patient's family (because they shared the same diet for years) and that the molecular crosstalk between the microbiota, the trillions of bacteria that live within us, and host cells, shapes the epigenetic modifications found in the host and the offspring.

GUT COMMENSAL MICROBIOTA AND RISKS FACTORS

Since a few decades, microbiologists have been convinced that our microbiota shapes gene expression. Yet, we are at the very first phase of discovery of mechanisms that lead bacterial species to alter expression of eukaryotic genes of the host and trigger metabolic diseases. Entering deeper into these multifactorial mechanisms could consist in investigating the molecular crosstalk between the microbiota and hosts cells.

Although the accumulation of data on the composition of the microbiota is becoming more precise every day, thanks to culturomics (Lagier et al., 2016), and genomics, there is still a long way to go to link microbiota and chronic metabolic diseases (sometimes called "civilization diseases"). However, it no longer remains questionable whether or not dysbiosis impacts cellular genes expression and progression toward metabolic diseases. In the 1990s, it became evident that prokaryotes played an important role in shaping gene expression in eukaryotic cells (Bry et al., 1996). Researchers have reported the importance of the fetal environment in the womb, and of postnatal colonization of the gut by commensal bacteria. Between the first and third trimester of pregnancy, there are shifts in maternal microbiota composition expected to confer evolutionary advantages for fecundity and infant survival (Koren et al., 2012). It is likely that the microbial colonization starts in the amniotic fluid and placenta and that the maternal gut supports the development of a prenatal microbiota (Collado et al., 2016). During vaginal delivery, the infant probably gain bacteria colonizing the maternal birth canal. To support this hypothesis, it could be argued that neonates born by C-section delivery exhibit aberrant gut colonization patterns (Dominguez-Bello et al., 2010). *Enterococcus faecalis*, *Staphylococcus epidermidis,* and *Escherichia coli* have been isolated from the meconium of healthy neonates, indicating that the fetus are far from being germ-free (Nicholson et al., 2012). Microbial contact during pregnancy and breastfeeding should also account for an important role in the selection of microbiota; during this early period of life, the infant's gut will be colonized by health-promoting bacteria such as *Bifidobacterium* and *Lactobacillus* (Rautava et al., 2012). In the initial days of life, the gut microbiota is unstable and of low diversity (Arrieta et al., 2014). A microbiota with adult-like complexity is expected to be achieved at age of 3 years, when the infant's diet evolves toward that of adult individuals (Yatsunenko et al., 2012). Methanogenic archaea (e.g., *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*), that reduce hydrogen levels via production of methane thereby stimulating food fermentation, are not found during infancy while omnipresent in school-aged children (Van de Pol et al., 2017) (**Figure 1**). Interestingly, it was evidenced that Kwashiorkor patients (children with severe

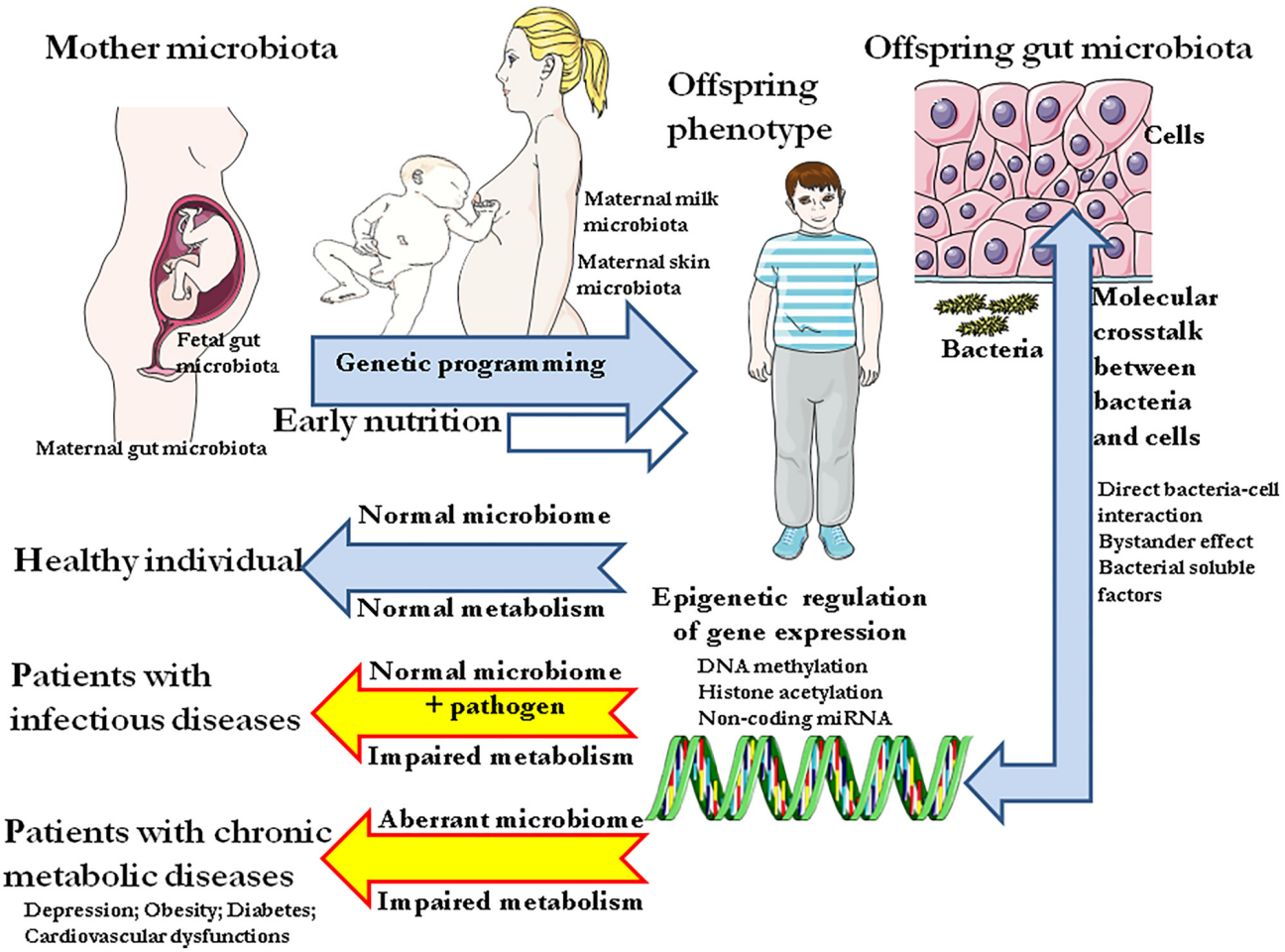

FIGURE 1 | Schematic representation of the colonization of the gut by commensal bacteria since the fetal environment in the womb up to early period of life and from infants to adult individuals. The microbiota of infants and adults is essential to physiological metabolic processes (digestion) and should be capable of supplying the host with metabolic precursors, bioactive molecules neither primarily present in the diet nor produced by the host itself. The absorption of nutriments takes place at the level of the intestinal barrier, a vast epithelium surface of about 400 m^2 maintained by tight junction between cells. Through constant molecular crosstalk with host cells, the gut microbiota can modulate the host metabolism by epigenetic regulation of cellular genes. Consequently, altered microbiota composition will provide aberrant signal to host cells resulting into metabolic disease.

acute malnutrition) lack methanogenic bacteria (Million et al., 2016).

In adults, bacteria concentrations range from 10^1–10^3 per gram to 10^{10}–10^{11} per gram in the upper intestines and colon respectively, and they reach their highest biomass in the distal gut (Hooper et al., 2001; Gill et al., 2006; Hugon et al., 2013). They are essential to physiological metabolic processes such as digestion and absorption of nutriments (**Figure 2**). A recent study has reported metagenomical and metatranscriptomical data providing evidence that the gut microbiome remains stable over time and that within a person, taxonomic and functional variation remain consistently lower than between persons (Mehta et al., 2018). A stable gut microbiota results from a long co-evolution process aimed at supplying the host with numerous metabolic precursors, bioactive molecules, cofactors, and signaling molecules, neither primarily present in the diet nor produced by the host itself. Within human bodies *Homo sapiens* DNA is estimated to account for less than 10% of the total DNA because of the incredibly large numbers of microorganisms that reside in (and on) humans, primarily within the gut (Human Microbiome Project Consortium, 2012). The characterization of microbiota has implied drawing maps of the most common elements of the gut microbiota. A considerable bacterial genetic diversity contributes to the microbiome's steady state; with over 1,000 species and 7,000 strains at the human populations level, the human gut microbiota is an extremely complex ecosystem in which the phyla *Firmicutes* (species such as *Lactobacillus*, *Clostridium*, *Enterococcus*) and *Bacteroidetes* (species such as *Bacteroides*) account for the majority, though other phyla such as *Proteobacteria* (*Escherichia coli*), *Actinobacteria* (*Bifidobacteria*), *Cyanobacteria*, *Fusobacteria,* and *Verrucomicrobia* are also present in low abundance (Eckburg et al., 2005; Qin et al., 2010). Environmental selection and competitive exclusion between

microbes during gut colonization, are expected to be the major driving forces that shape the core microbial diversity whereas stochastic factors and *in situ* evolution are likely at the origin of inter-subject variability among groups of people living in similar environments (Walter and Ley, 2011) (**Figure 3**). At the individual level, only a few hundred bacterial species are found. With its extremely high concentration of bacteria and bacteriophages, optimal temperature, the gut provides ideal conditions for horizontal gene transfer (HGT) (Lerner et al., 2017a). HGT enables the transfer of genetic materials by transformation (uptake of foreign genetic material), transduction (transfer of microbial DNA from one bacterium to another by a viral intermediate), and conjugal transfer (transfer of DNA via a mobile genetic elements). HGT, allows a rapid acquisition of a new function important to pass through the natural selection. The most documented HTG refers to dissemination of antibiotic resistance genes among bacteria. Yet, HTG from successfully adapted members of an ecosystem to other members of the ecological niche is common and likely increases the fitness of the recipients.

Advanced culturomics had made possible the search for new bacterial species in cohorts of individuals with a metabolic disease; namely *Urmitella timonensis*, *Bacillus andreraoultii, Blautia marasmi*, *Lachnoclostridium pacaense*, *Bacillus marasmi*, and *Anaerotruncus rubiinfantis* which were recently isolated from stool samples of undernourished African children (Pham et al., 2017). Several studies have established a relationship between the gut microbiota and the host's metabolism (Bäckhed et al., 2004; Ley et al., 2005; Turnbaugh et al., 2006; Tidjani Alou et al., 2017). Other results point punctually to link a bacterial species with a metabolic disease; for example, after caloric restriction, high abundance of *Akkermansia muciniphila* and microbiome richness correlate with a healthier metabolic status in overweight/obese adult patients (Dao et al., 2016). In addition, a study that explored the fecal samples of 416 twin pairs in the United Kingdom found evidence that *Christensenella minuta* favored a low body mass index (Goodrich et al., 2014). The children born to malnourished Dutch women during World War II, were more likely to have chronic diseases like depression and diabetes, demonstrating the great correlation existing between the diet of a pregnant mother and expression of genes in her offspring (Choi and Friso, 2010). Since the pioneering works linking the microbiome to metabolic diseases, the search for the "microbial signature" of a metabolic disease has lead to an intense international scientific competition.

In parallel, major breakthroughs regarding the epigenetic signature of metabolic diseases have been made. Indeed, people who were exposed to famine *in utero* had a lower degree of methylation of a gene implicated in insulin metabolism (the insulin-like growth factor II gene) than their unexposed siblings (Heijmans et al., 2008). Currently, the number of scientific reports indicating that patients with metabolic diseases exhibit a particular epigenetic signature is steadily increasing. These data are opening new avenues for the investigation of relationships between microbiota and epigenetic programming in health and disease.

LESSONS FROM CLINICS AND ANIMAL MODELS: EVIDENCE OF LINKS BETWEEN GUT MICROBIOTA AND METABOLIC DISEASES

Emotional Disorders and Depression

Central nervous system (CNS) has long been considered an immune-privileged site capable of unidirectional signaling to the gut. It recently became obvious that the gut microbiome is also able to influence the brain via an array of multichannel sensing and trafficking pathways (Lerner et al., 2017b). Emerging evidence suggests that the gut microbiota may play a role in shaping cognitive networks that control the genesis of emotional and neurodevelopmental disorders such as depression, autism spectrum disorders, and schizophrenia (Kelly et al., 2017). Increased abundance of *Lactobacillus*, *Bifidobacterium* and *Ascomycota* in the oropharyngeal microbiota has been documented in patients with schizophrenia compared to healthy controls (Castro-Nallar et al., 2015). Maternal infection during pregnancy can be associated with the development of neurodevelopmental disorders (Jiang et al., 2015). Increased risk for mood, anxiety and depression disorders was reported after antibiotic exposure that alter the microbiome composition (Lurie et al., 2015). Clinically depressive episodes in humans are known to refer to dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (Barden, 2004). Using germ-free mice, a direct link between microbiota and the operation of the HPA axis was established based on the corticosterone and adrenocorticotrophin response to restraint stress (Sudo et al., 2004). In rats submitted to maternal separation stress, treatment with *Lactobacillus* spp. probiotics reduced stress responses (Gareau et al., 2007). Beside the modulation of the HPA axis, microbiota including bacteria such as *Citrobacter rodentium*, *Campylobacter jejuni, Bifidobacterium infantis,* or *E. coli*, may directly influence the CNS function and have a direct effect on c-FOS activation in cells from CNS (Foster and McVey Neufeld, 2013). A meta-analysis of infectious agents associated with schizophrenia has revealed that among others, *Chlamydophila pneumoniae* and *Chlamydophila psittaci* might be inducers of the disease (Arias et al., 2012). Another study indicated that *Lactobacillus*, *Tropheryma*, *Halothiobacillus*, *Saccharophagus*, *Ochrobactrum*, *Deferribacter*, and *Halorubrum* were increased whereas *Anabaena*, *Nitrosospira* and *Gallionella* were decreased during the first episode of schizophrenia (Schwarz et al., 2018). Species such as *Bacteroides vulgatus, Clostridium histolyticum* and *Clostridium bolteae* were reported to be over-represented in the gut microbiota of autists (Parracho et al., 2006; Finegold et al., 2010). SCFAs neurohormonal molecule (butyrate, acetate and propionate) are known to be produced by bacteria such as *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Clostridium,* and *Prevotella* (Macfarlane and Macfarlane, 2012). Recently, a placebo-controlled trial showed that treatment with probiotic *Bifidobacterium longum* reduces depression scores but not anxiety, and increases the quality of life in patients with irritable bowel syndrome (Pinto-Sanchez et al., 2017). These observations indicate that the gut microbiota can act on the CNS.

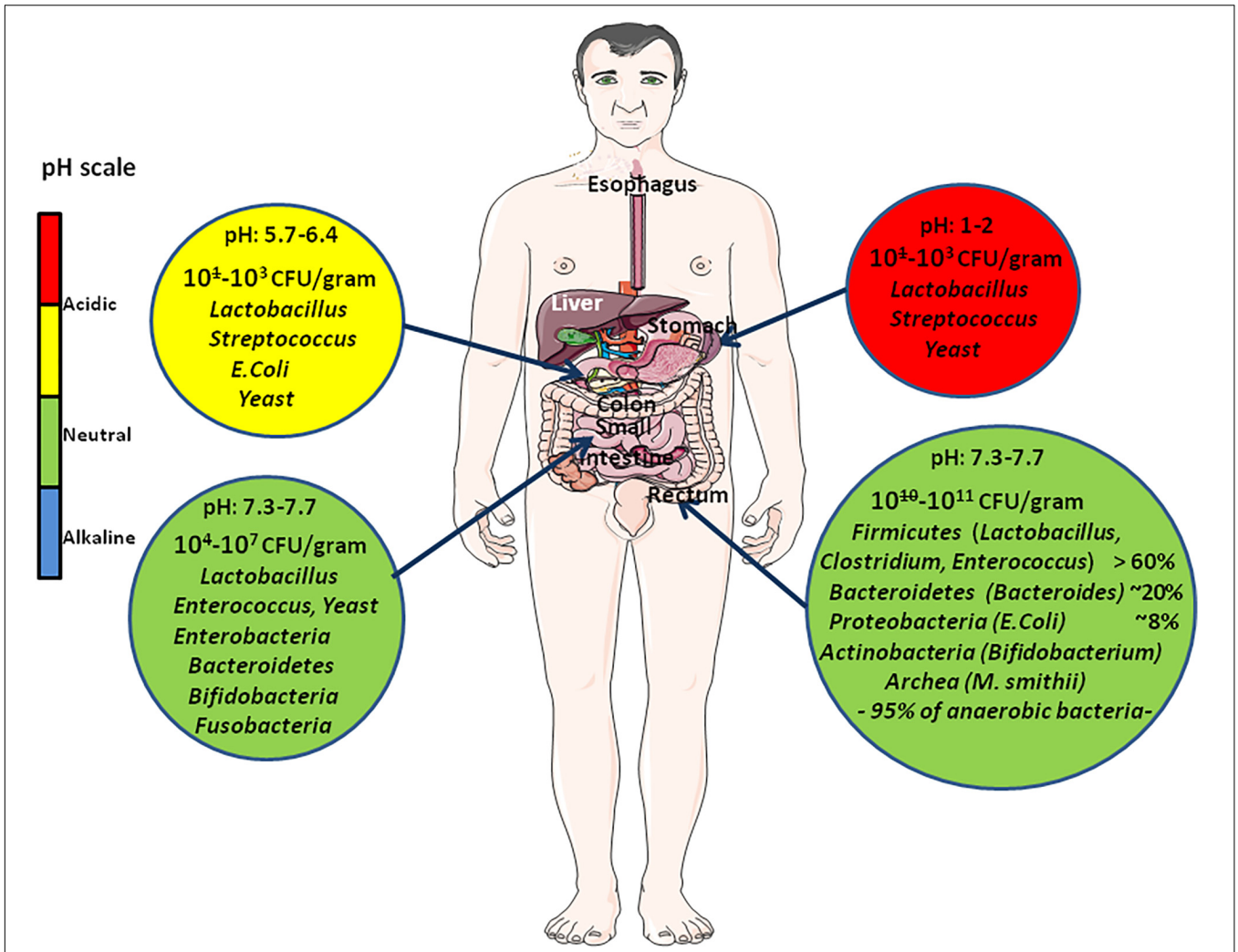

FIGURE 2 | Quantitative and qualitative variation of intestinal flora all along the digestive tract. This diagram illustrates the variations in pH (that changes from acidic in the stomach to almost neutral or slightly alkaline in the intestine) and usual composition of the microbiome at different levels (stomach, small intestine, colon) of the digestive tract. The abundance of microbes is indicated as the number of colony forming unit (CFU) per gram. Due to the intimate crosstalk between gut microbiota and human cells, the gut microbiome integrity is of prime significance to human health.

Inflammatory Bowel Disease (IBD), Crohn's Disease (CD), Ulcerative Colitis, and Celiac Disease

The study of a cohort from Denmark has revealed that the incidence of IBD is significantly increased in patients born by C-section delivery and exhibiting aberrant gut colonization patterns, compared to controls born by vaginal delivery (Sevelsted et al., 2015). Enterotoxigenic *Bacteroides fragilis* has been suggested to be associated with acute and persistent diarrheal disease in patients with IBD and the oral intake of yogurt containing *Bifidobacterium longum* BB536 improves the clinical status of the patients (Odamaki et al., 2012). Using a model of mice infected with enterotoxigenic *B. fragilis*-inducing colitis, it was evidenced that induction of colonic hyperplasia and colorectal cancer is associated to the STAT-3 signaling pathway and a pro-inflammatory Th17 response (Wu et al., 2009). Very recently, by using the adenomatous polyposis coli tumor suppressor gene heterozygous APC^{Min} mice infected with enterotoxigenic *B. fragilis,* it was demonstrated that progression to cancer involves a multistep cascade requiring IL-17-dependent activation of NF-κB and STAT-3 signaling in colic epithelial cells that trigger chemokines expression which in turn activates mucosal Th17 response and distal colon tumorigenesis (Chung et al., 2018). A reduction in the diversity of microbiota species was recently reported in pediatric patients suffering from ulcerative colitis or CD. The microbiota of these young patients showed a low abundance of *Lactobacillus*, *Bifidobacteria*, *Eubacterium rectale,* and *Faecalibacterium prausnitzii* (Knoll et al., 2017). Fecal transplantation in the treatment of ulcerative colitis was recently considered as a promising rescue approach (Uygun et al., 2017). Interestingly, *Faecalibacterium prausnitzii* was shown to prevent the occurrence of experimentally induced

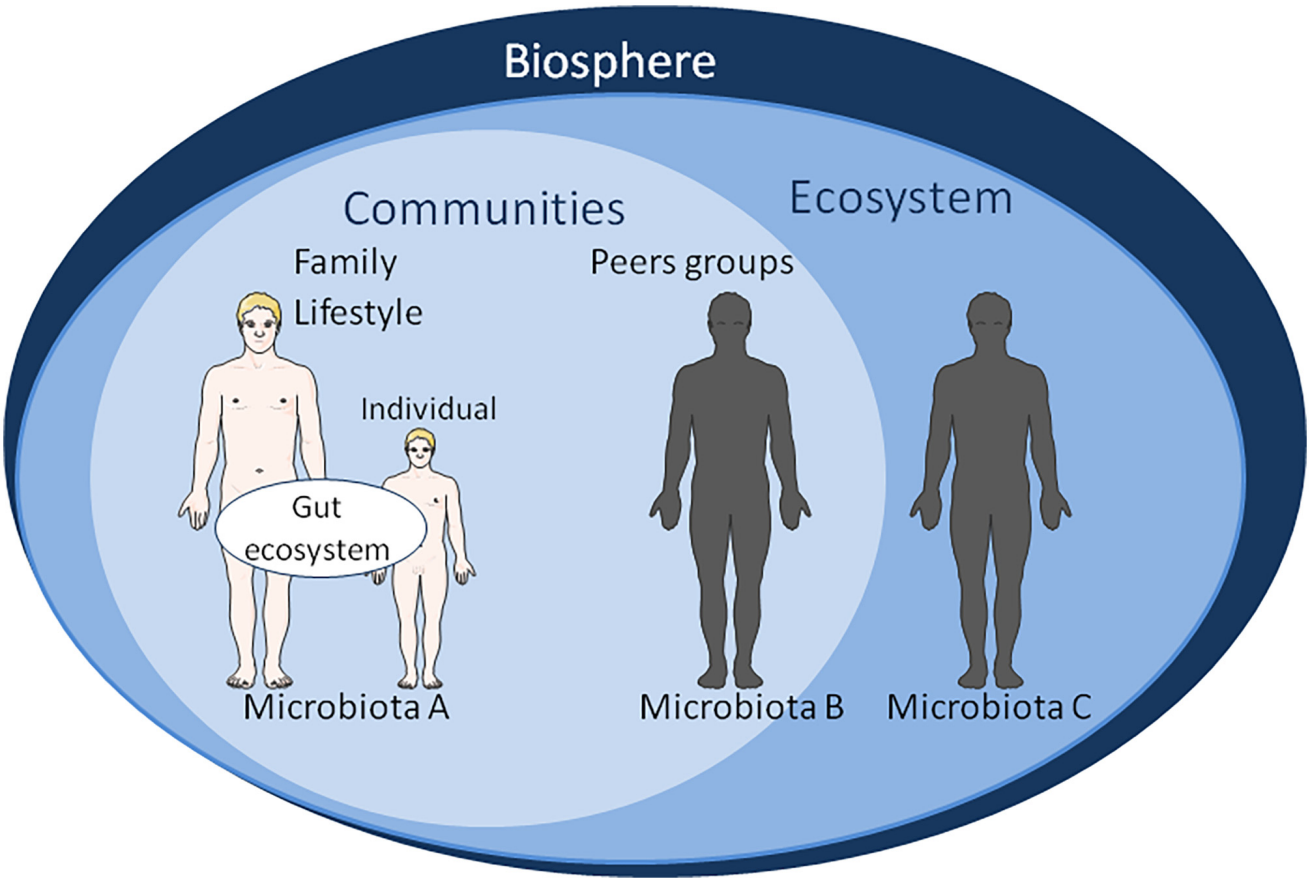

FIGURE 3 | Schematic representation of factors influencing the gut microbiota ecosystem. At the level of biosphere: natural ecosystems, biodiversity, water resource, climate change. At the level of ecosystem: damage and loss of natural environment, biodiversity loss, land degradation, freshwater decline, air water pollution, population displacement. It is worth noting that the appearance of human on the planet has extensively changed the biosphere within a very short time on the evolution scale. At the level of community: human-made environment, natural ecosystems area, water resource, agricultural area, urban area, economic growth, sick-care system, responsible consumption, gender equality. Gut microbiomes from urbanized and pre-agricultural populations do not show the same bacterial strains' diversity; At the level of family lifestyle: diet behavior, sexual behavior, psycho-socio-economic environment, physical environment, reduced environmental microbial biodiversity, reduced human contact with microbes, spiritual, culture. At the individual level: diet supplemented with probiotics or antibiotics, dysbiosis, systemic immune dysregulation, inflammatory disease, tissue damage, emotional, cognitive. The main vectors for transmission of biological information from one generation to the next are: (a) DNA sequence inheritance (mainly vertical inheritance from generation n to generation n+1). Genetic inheritance is vertical in humans. However, it is known that HGT is possible among microorganisms (e.g., bacteria and virus) and between microorganisms and plants; (b) Epigenetic inheritance (vertical inheritance). Phenotypic variation results from changes in DNA expression without changes of DNA sequence. We hypothesize that the molecular crosstalk between the "microbiological memory" and host cells shape epigenetic inheritance; (c) Parental effect (vertical inheritance). It refers to effects that parents have on the phenotypic traits of their offspring independently of the offspring's own genotype; the offspring's fitness is affected. It might be considered that nutrition shaping the microbiota of the offspring participates to non-genetic inheritance; (d) Adaptive social learning (involves both vertical and horizontal inheritance). It refers to information learnt from others and culturally transmitted that influences behavior. The human diet is usually mixed with meat, fruit, vegetables, water; the relative proportions of these compounds in diet have an impact on the microbiota. For various reasons, some families have a regular high-fat diet and/or very high sugar diet. Some groups of people choose to adopt a lacto-ovo-vegetarian diet, other are limited to lacto-vegetarian diet, and finally some even claim to be vegan. All these eating behavior alter the microbiota; and (e) Ecological environment inheritance (mainly vertical inheritance). Individuals act on their environment (named "environmental anthropization") in such a way that it affects their fitness, thereby altering the selection pressures acting on them. We consider that "microbiological memory" belongs to this category of non-genetic inheritable phenotypic traits.

inflammatory colitis in mice (Sokol et al., 2008). Adherent-invasive *Escherichia coli* are more frequently encountered in the intestinal mucosa of patients with CD than in healthy individuals (Darfeuille-Michaud et al., 2004). Moreover, it was reported that the stress response chaperon Gp96 is abnormally expressed at the apical surface of epithelial cells from the ileum of CD patients and acts as receptor for the outer membrane protein A (OmpA) of adherent-invasive *E. coli* thereby promoting colonization (Rolhion et al., 2010). Chronic inflammation linked to microbial infections can sometimes evolve toward cancer, as it has been demonstrated for gastric cancer associated to *Helicobacter pylori* infection (Sears and Pardoll, 2011), or *Coxiella burnetii*-induced lymphoma (Melenotte et al., 2016). Celiac disease, a polygenic autoimmune inflammatory disorder of the small intestine induced in genetically susceptible individuals following ingestion of gluten (a mixture of prolamin proteins

present in wheat, rye, and barley) (Balakireva and Zamyatnin, 2016), could be included as a disease associated with the gut microbiota composition. Specific amino acid sequences in gluten activate T cells, which triggers inflammation. While gluten plays a major role in the induction of celiac disease, infection like *Campylobacter jejuni* in adults are associated with an increased risk of celiac disease, and a possible association between celiac disease and bacterial transglutaminase was also suggested (Lerner and Matthias, 2015). These data demonstrate that the gut microbiota can trigger inflammation and autoimmune diseases.

Obesity (OBS)

A large Danish cohort investigation (Le Chatelier et al., 2013) revealed that non-obese and obese individuals differed in the richness of gut bacteria (58 species differing in abundance), and that people with low bacterial richness were characterized by weight gain over time, a marked overall adiposity, insulin resistance and dyslipidaemia. A study performed on a Danish cohort of 28,000 mother-child pairs, revealed that antibiotic exposure of children during the first 6 months of life was associated with increased risk of being overweight at age of 7 years (Ajslev et al., 2011). Another study investigating the effects of macrolide use in a cohort of Finnish children also concludes that early life antibiotic use resulted in disruption of the microbiome, long-lasting shift in microbiota composition with a depletion of *Actinobacteria*, increase in *Bacteroidetes* and *Proteobacteria*, and an increased risk for metabolic diseases (Korpela et al., 2016). Abnormal weight gain was a side effect observed in patients with long-term doxycycline and hydroxychloroquine treatment (Angelakis et al., 2014). It is worth noting that during the last 60 years, there has been a widespread use of antibiotics, such as tetracycline and penicillin, as food additives in mammalian livestock (pigs, cows, and sheep), poultry, and farmed fishes, with the multiple objectives of preventing and treating infectious diseases as well as promoting growth (Taylor and Gordon, 1955). Increase in *Proteobacteria* (*E. coli*) in pig gut microbiota was found after 2 weeks of a diet supplemented with antibiotic (Looft et al., 2012). The mechanism of weight gain in agricultural animals receiving food supplemented with antibiotics is not fully understood although it is likely the consequence of low bacterial richness in the gut microbiota. A sheep animal model showed that feeding mature females with food poor in folate, vitamin B12 and methionine during the periconceptional period induced obesity in adult offspring (Sinclair et al., 2007). In models of diet-induced OBS and genetically modified (ob/ob) mice, administration of a broad-spectrum antibiotic reduced weight gain (Cani et al., 2008) and improved glucose tolerance (Membrez et al., 2008). Finally, a very interesting observation is that a patient successfully treated with fecal microbiota transplantation for recurrent *Clostridium difficile* infection, developed new-onset OBS after receiving stools from a healthy but overweight donor (Alang and Kelly, 2015). It makes no doubt that there is a link between gut microbiota and weight.

Diabetes Mellitus (DM)

Decline in abundance of the bacterial phyla *Firmicutes* and increase of *Bacteroidetes* in the gut microbiota, were correlated with the progression of type 1 DM, an autoimmune disorder that involves β-cell inflammation and destruction (Giongo et al., 2011). A case control study revealed that the gut microbiota of children with type 1 DM differs from that of healthy children with an increase in the abundance of *Bacteroidetes*, *Clostridium* spp. and *Veillonella* and reduction in *Lactobacillus*, *Bifidobacterium*, *Blautia*, and *Prevotella* (Murri et al., 2013). This observation corroborates an analysis comparing gut microbiota for type 1 DM, indicated an increase in the abundance of *Bacteroides* and *Akkermansia* and decrease in *Prevotella* in subject at high risk for diabetes (Alkanani et al., 2015). Decreased abundance of *Blautia* was also reported in a case-control study that examined feces from Chinese children with type 1 DM. In this study, a decreased abundance of *Lachnospira*, *Dialister*, and *Acidaminococcus* in the feces was also observed (Qi et al., 2016). It is known that maternal high fat feeding results in offspring with exacerbated adiposity and altered expression of proteins involved in hepatic insulin sensitivity (Buckley et al., 2005). In a rat animal model, feeding a protein-restricted diet to pregnant rats induced hypomethylation of the glucocorticoid receptor (Lillycrop et al., 2005), increased glucocorticoid receptor expression, and reduced the expression of the 11b-hydroxysteroid dehydrogenase type II enzyme which inactivates corticosteroids in the liver, lung and brain of the offspring (Bertram et al., 2001). In the liver, a higher expression of the glucocorticoid receptor increases phosphoenolpyruvate carboxykinase expression and activity and gluconeogenesis contributing to insulin resistance (Burns et al., 1997). Giving a protein-restricted diet to pregnant rats also increased glucokinase expression in the liver of the offspring and the capacity for glucose uptake (Bogdarina et al., 2004). Since five decades, converging evidence suggests that the frequency of type 1 DM is increasing in industrialized country to become a public health problem. Viral, environmental, chemical, life-style factors were suggested in the search for establishing causality. The hygiene hypothesis (Bach and Chatenoud, 2012) claims that the decline in infectious diseases resulting from better hygiene and medical care plays a major role in emerging metabolic/autoimmune diseases. For example, Tuberculosis is more frequent in the southern countries compared with those of the north and a negative correlation between the frequency of *Mycobacterium tuberculosis*-induced disease and type 1 DM has been clearly established (Airaghi and Tedeschi, 2006). According to the hygiene hypothesis, the difference in social and economic development between Finland and Russia which are neighboring countries, also account for the higher type 1 DM incidence in Finland compared with Russia (Kondrashova et al., 2007). The gut microbiota is modified in patients with type 1 DM.

Cardiovascular Diseases

Almost four decades ago, in studying coronary artery disease death rates among 5,654 men born during 1911–1930 in Hertfordshire, United Kingdom, Barker and his colleagues observed that birth weight was inversely correlated with increased early death from ischemic heart disease (Barker et al., 1989). Furthermore, birth weight and rates of growth in the first 3 years of life have also been associated with

adult onset of hypertension (Thompson, 2007). Yet, the Barker's hypothesis is not supported by evidence from low-income countries, where intrauterine growth retardation and low birth weight are common but hypertension and coronary heart disease are less prevalent than in high-income countries; it could probably be explained by marked variations in microbiota composition between individuals from the north and those from the south. There has been evidence suggesting that alterations of gut microbiota may be associated to CVD (Tang et al., 2013). A study using high-throughput sequencing that compared the gut microbiota of 29 patients with coronary heart disease and that of 35 healthy volunteers indicated that the diversity and compositions of gut microbiota were different; the proportion of *Bacteroidetes* was lower and that of *Firmicutes* was higher in patients from the coronary heart disease group (Cui et al., 2017a). It was reported that the gut microbiota-dependent metabolite, trimethylamine *N*-oxide, whose levels are markers of CVD, could promote atherosclerosis (Tang and Hazen, 2014). A study consisting in the modulation of microbiota by supplementing probiotic *Lactobacillus plantarum* in the diet of a cohort of patients indicated that after treatment atherogenic parameters including LDL-cholesterol, were significantly reduced (Wu et al., 2017). These results were confirmed by the study of animal models. It was reported that lipoteichoic acid from *Lactobacillus plantarum* inhibits the inflammation causing atherosclerosis plaques by inhibiting NF-κB and activation of MAP kinases (Kim et al., 2013). A murine animal model showed that feeding mice with dietary supplementation of L-carnitine and choline, the precursor of trimethylamine *N*-oxide, accelerated atherosclerosis (Koeth et al., 2013). Interestingly, a study performed on mice genetically deficient in Toll-like receptor (TLR, a component of the anti-infectious innate immunity expressed in gut mucosa), indicated that these deficient mice developed metabolic syndrome, with body masses that were 20% greater than those of wild type mice, hyperlipidemia (increased serum level of triglycerides and cholesterol) and hypertension (Vijay-Kumar et al., 2010). Moreover, grafting "germ-free" mice with the microbiota from mice lacking TLR confers the sensitivity to metabolic syndrome to these mice. It was also found that deficiency of either TLR4 or Myd88 attenuates the high-fat diet-induced atherosclerosis and inflammation in apolipotrein E-deficient mice (Michelsen et al., 2004). It is also worth noting that a link between *Porphyromonas gingivalis* periodontal infections and CVD has been established (Pussinen et al., 2007). These reports evidence that a particular gut microbiota may be associated to CVDs.

FOOD SHAPES THE METABOLOME BY MODIFYING THE MICROBIOME

Metagenomic studies of the intestinal microbiome, have demonstrated that it is a dynamic entity, influenced by different factors, including diet and antibiotic treatment (**Figure 4**). During feeding, a very fast diet-microbiota crosstalk is established and it rapidly and reproducibly alters the human gut microbiota (David et al., 2014). Although bacteria essential to life are shared among people (Arumugam et al., 2011), there is a great diversity of bacterial species in the gut between related and non-related individuals. It seems that the presence or absence of some species in the commensal bacterial population predisposes to a risk of metabolic illness. This opens new avenues for research which is probably the very beginning of a scientific (and pharmaceutical) revolution. Studies using young (3 weeks old) "germ-free" mice, have shown that mice grafted with microbiota from mice treated with a low dose penicillin gained more weight and fat mass than mice grafted with microbiota from control animals. Also, mice grafted with microbiota from penicillin-treated mice showed reduced gut bacteria richness affecting species such as *Lactobacillus*, *Allobaculum*, *Rikenellaceae*, and *Candidatus arthromitus,* suggesting these bacteria might have a protective role in shaping adult metabolism (Cox et al., 2014).

Given that antibiotics are widely used for promoting growth in livestock, poultry and aquaculture, increasing levels of human food contamination by antibiotics have been reported. Meat and milk from these animals might contain traces of antibiotics which could modify the gut microbiota during early life (more vulnerable to change than later in life) and select antibiotic-resistant bacteria, with consequences on host cell metabolism and human health (i.e., increasing the risk of OBS in the general population) (Cox and Blazer, 2015). Studies in a "germ-free" chicken animal model, have shown that antibiotics alone demonstrated no growth-promoting effect (Coates et al., 1963), suggesting that antibiotics do not directly change the host's metabolism but that the metabolic effects observed with antibiotic treatment in "normal" animals are driven by changes in their microbiota. In addition the effects of antibiotic treatment in the diet are not always predictable and their use is empirical. The administration of low doses of penicillin or oxytetracycline to mice was found to result in weight gain whereas high doses antibiotics resulted in weight loss (Dubos et al., 1963). It has also been claimed that *Lactobacillus* spp., *Streptococcus* spp., *Bacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp. and other microbial species added in poultry feed, positively affect growth performance (Smith, 2014). Although many infant formulas already contain probiotics, it is likely that improving the knowledge on the function of "good" and "bad" bacteria will probably produce impact on the use of probiotics in human nutrition.

Moreover, food commensal bacteria are a potential important source for HGT (Wang et al., 2006), including probiotic bacteria which act as reservoir for antibiotic resistant determinants (Wong et al., 2015). It is estimated that about a quarter of all food production (e.g., ham, cheese, yogurt) involves bacterial fermentation processes using lactic acid bacteria and other microbial and fungal strains, opening the possibility of HGT among the bacteria of foodstuff (Aminov, 2010). In addition, microbial transglutaminases, which are functionally close to endogenous autoantigens involved in celiac disease, are extensively used as food additives. These molecules are used as cross-linking agents of proteins aimed at improving

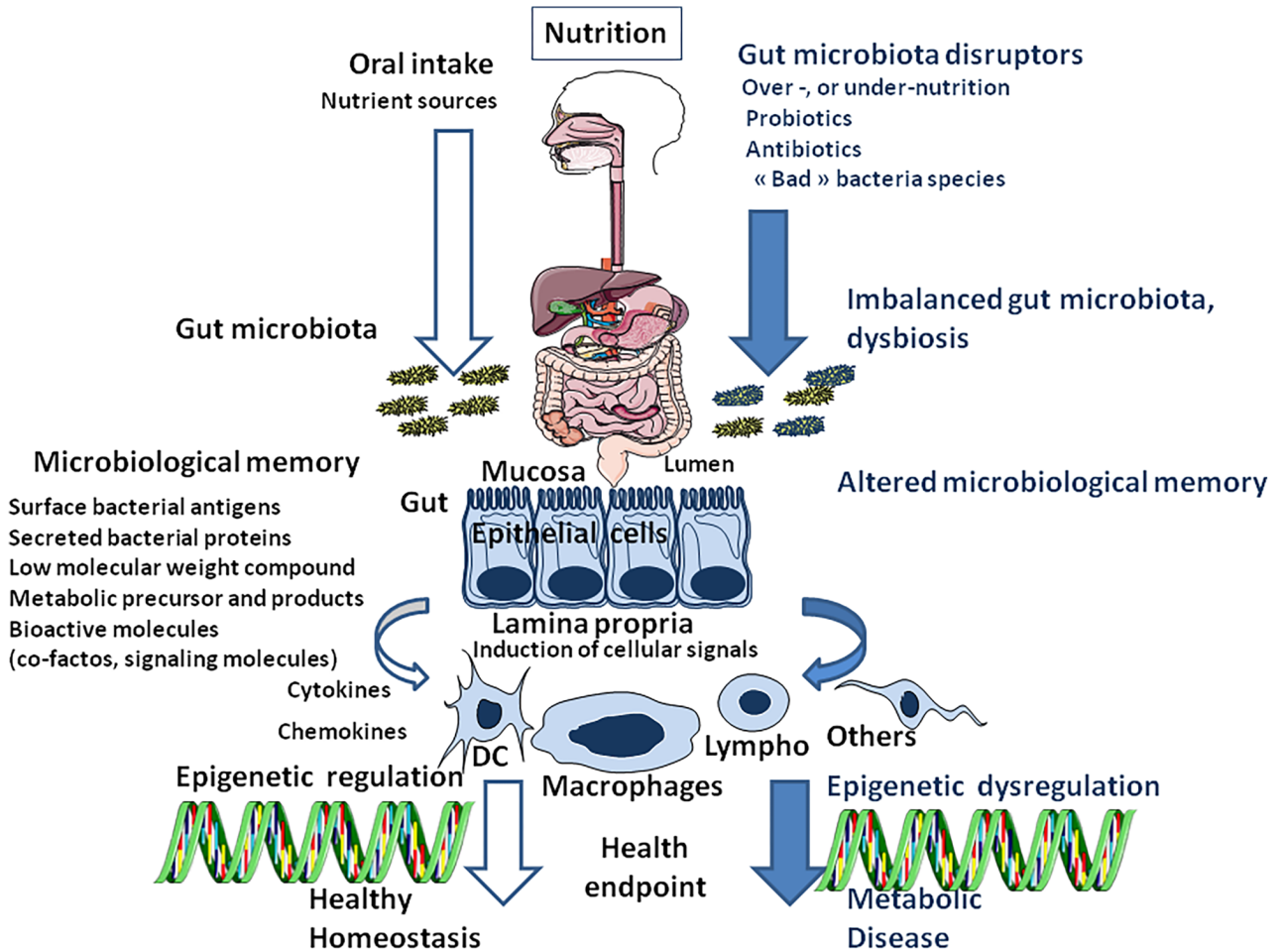

FIGURE 4 | Schematic representation of nutrition/oral intake and the result on gut bacteria composition (microbiota) depending on the absence **(left)** or presence **(right)** of gut microbiota disruptors. Next, the cell microenvironment (including all surface antigens, secreted proteins and low-molecular-weight compounds from bacteria and bioactive molecules supplied through the diet and processed by the gut microbiota), named microbiota memory, trigger the activation of cellular signaling pathways that regulate gene transcription in different cells types [DC: Dendritic cells; Lympho: lymphocytes (B and T-cells);Macrophages; Others: other cell types]. Epigenetic programming is the result of a molecular crosstalk between gut prokaryotes (microbiota metabolome) and eukaryotic cells. When stable, this continuous reshaping of cellular genes appears as an epigenetic signature.

the texture of food products, and it has been suggested that microbial transglutaminases may be involved in the development of celiac disease (Lerner et al., 2017c). The increase of autoimmune diseases in humans is difficult to explain by genetic changes which may have occurred during the recent and relatively short period of human evolution. Thus, the role of introduction of industrially processed food in the onset of autoimmune diseases becomes increasingly recognized (Arleevskaya et al., 2017). It is currently hypothesized that industrially processed food may result in substantial loss of gut bacterial diversity contributing to these contemporary diseases (Mancabelli et al., 2017). Indeed hygienic behaviors which probably reduce microbial diversity associated with human gut colonization in early life, may compromise the establishment of appropriated immune responses thereby reducing the benefits of measure aimed at improving human health (Mulder et al., 2011). In particular, the long-term effect of antibiotic therapies in children under the age of 3 years should be evaluated.

EPIGENETIC PROGRAMMING/ REPROGRAMMING/MEMORY AND MICROBIOTA

Epigenetic programming is likely the integrated result of interactions derived from global metabolism, microbiota, immune system activation, and external factors such as diet (macro- and micro-nutriments), pharmaceuticals (particularly antibiotics), and environmental factors (pH, oxygen, temperature). Epigenetic developmental plasticity allows for a complex organism to adapt to microenvironmental signals, especially during early life, thereby increasing its fitness. The links between epigenetic programming and nutriments or

microbiota have been investigated quite recently (Gallou-Kabani and Junien, 2005). Short chain fatty acids produced by bacteria have emerged as one clear link allowing microbiota to intersect with host epigenetic (Woo and Alenghat, 2017). Yet, most of the molecular mechanisms driving epigenetic modifications remain to be further explored. Food- or microbiota-derived folate (a water soluble B-vitamin), choline, betain and vitamin B12 might contribute to generating 6-methyltetrahydrofolate which is the methyl group donor for synthesis of SAM, a molecule involved in DNA methylation. Among others, *Lactobacillus* and *Bifidobacteria* produced folate, thereby affecting DNA methylation through regulation of methyl-donor (Rossi et al., 2011). In a sheep animal model, it was found that feeding mature females with food free of folate, vitamin B12 and methionine during the periconceptional period induced obesity in adult offspring and altered their methylation status (Sinclair et al., 2007). It has been suggested that folate may represent a good candidate for controlling DNA methylation since aberrant reprogramming of DNA methylation was observed after low dietary folate (Steegers-Theunissen et al., 2009). Histone methylation was also shown to be associated with OBS; the loss of Jhdm2a demethylase in mice results in OBS and hyperlipidemia (Tateishi et al., 2009).

An intriguing study reported that distinct DNA methylation profiles were found in blood samples from women 6 months after delivery, the result depended on the predominance of either *Firmicutes*, *Bacteroidetes,* and *Proteobacteria* in their fecal microbiota during pregnancy (Kumar et al., 2014). It was reported that inadequate intakes of long-chain polyunsaturated fatty acids (LCPUFAs) during pregnancy, may result in aberrant DNA methylation patterns in the offspring, thereby influencing their health (Khot et al., 2017). Increased maternal serum levels of vitamin B12 during pregnancy were found to correlate with a decrease in DNA methylation in newborns (McKay et al., 2012). Food might also provide molecules known to interfere with DNA methylation such as genistein. Butyrate that is produced by certain classes of bacteria, is a ligand for a subset of G protein-coupled receptors and also acts as a potent inhibitor of histone deacetylase (HDAC) (Bourassa et al., 2016). It was shown that mice with an intestinal epithelial cell specific deletion of the epigenome-modifying enzyme HDAC3 demonstrated alterations in the composition of the intestinal microbiota (Alenghat et al., 2013). It was also reported that most HDAC were downregulated in female mice with a high-fat diet (Panchenko et al., 2016). In addition, butyrate is capable of suppressing the activation (nuclear translocation) of NF-κB, a nuclear factor known to play a major role in immune response to infection (Iwai, 2012). It was reported that premalignant stages of gastric cancer associated to *Helicobacter pylori* were characterized by altered DNA methylation (Park et al., 2009) and histone acetylation patterns (Dinz et al., 2010). The relationship between *Helicobacter*-induced chronic inflammation, DNA damage and colon cancer was investigated using a model of $Rag2^{-/-}$ mice infected by *Helicobacter hepaticus*. This showed that 5-Cl-dC mimics 5-methyl-dC induces inappropriate increases in CpG methylation that can silence tumor suppressor genes and initiate the carcinogenesis (Mangerich et al., 2012). Moreover, the most common gene mutated in colorectal cancer is the adenomatous polyposis coli (APC) tumor suppressor gene and the mechanisms of *APC* inactivation include hypermethylation of CpG sites in APC promotor and decreased translation due to inhibition by microRNA (Wu et al., 2012). Recently wild-type and TLR 2 knockout ($Tlr2^{-/-}$) mice were used to examine the dynamic interplay between host and gut microbiota. DNA methylation patterns of the genes involved in immune response was found affected along with an altered composition of the mucosal microbiota (Kellermayer et al., 2011). It was reported that genetically modified gut TLR2 ligand lipoteichoic acid (LTA)-deficient *Lactobacillus acidophilus* suppress inflammation and protect mice against colitis (Mohamadzadeh et al., 2011) and colon cancer (Khazaie et al., 2012). By using a rat animal model it was observed that various phenotypic characteristics (OBS, cancer risk) could be obtained by changing the amount of vitamins, choline, betain and selenium in the diet of pregnant rat, and these changes were associated with increased CpG methylation of eukaryotic DNA (Delage and Dashwood, 2008). A mouse model, showed that gestational exposure to *Acinetobacter lwoffii*, triggers modulation of histone acetylation protecting the offspring from the development of asthma-like diseases (Brand et al., 2011). Overnutrition was reported to regulate the synthesis of several miRNAs whose expression control metabolism (Cui et al., 2017b). Finally, it was reported that specific diets (tea polyphenols, soybean genistein, or plant food isothiocyanates), might inhibit the development of cancer by reducing DNA hypermethylation in critical genes associated with cancer such as *p16 Ink* or *RAR*β (Fang et al., 2007). Since the diet strongly influences the composition of the microbiota, it is likely that the observed effects reflect, at least partly, the composition of the gut microbiota and that of their bioactive compound on cellular gene expression.

DISCUSSION

The gut microbiota is composed of diverse populations of commensal bacteria species that, together with salivary proteases, stomach secretions and gastric acidity, represent the first barriers of protection against colonization and invasion by pathogens (Pamer, 2016). During the past decades, the attention of infectious diseases physicians was focused on the identification of unique pathogens accidentally introduced into patients and causative agents of diseases. When the commensal bacteria failed to protect the host against pathogens and the immune system turned to be unsuccessful in eradicating the aliens on its own, the obligatory next step for the clinicians is identification of antibiotics to which the pathogen could be sensitive, for therapeutic use. Most frequently an antibiotic treatment intended to suppress adverse effects of the pathogens is sufficient to cure infected patients. When antibiotic treatments fail (e.g., antibiotic-resistant *Clostridium difficile* infection), fecal microbiota transplantation frequently shows its usefulness (Hocquart et al., 2017). This pragmatic vision of a fight against "bad" microorganisms has often been

sufficient and successful in eradicating infectious pathogens such as OXA-48 carbapenemase-producing *Klebsiella pneumonia* or vancomycin-resistant *enterococci* (Lagier et al., 2015; Davido et al., 2017).

Although this "old-fashioned pathogen-suppression strategy" is clearly crucial in clinical treatment of patients suffering from infectious diseases, approaching bacteria-induced chronic metabolic disease with this perspective comes down to drastically simplify the problem. It deliberately ignored hundreds of commensal bacteria species whose presence in individuals is nothing less than essential to life as the result of the coevolution of hosts and their commensal microbiota. At the beginning of the XXIth century, the Human Microbiome Project from the National Institutes of Health (NIH HMP Working Group Peterson et al., 2009), was designed to repel the frontiers of microbiology by providing data, tools and resources aimed at understanding the role of changes in the resident microbiome in disease and health. During early postnatal life, humans are colonized by commensal intestinal bacteria, the gut microbiota is unstable during the first days of life and develops an adult-like complexity by age of 3 years. According to the Barker's and the hygiene hypothesis, one might expect that common adult metabolic diseases are the results of how multiple gene were turned off or on to optimize perinatal and early adult life. Interestingly, the period of life during which epigenetic DNA imprinting is the most active overlap this early 3 years period (Bhutta et al., 2013). In healthy individuals, the gut microbiota forms stable communities of bacteria belonging to different species that display particular compositional and functional characteristics. Moreover, the microbiota remains relatively stable across generations of individuals within a family. In adults, bacterial concentrations range from 10^1–10^3 per gram in the upper intestines to 10^{10}–10^{11} per gram in the colon. These bacterial concentrations should be compared to the 10^6–10^7

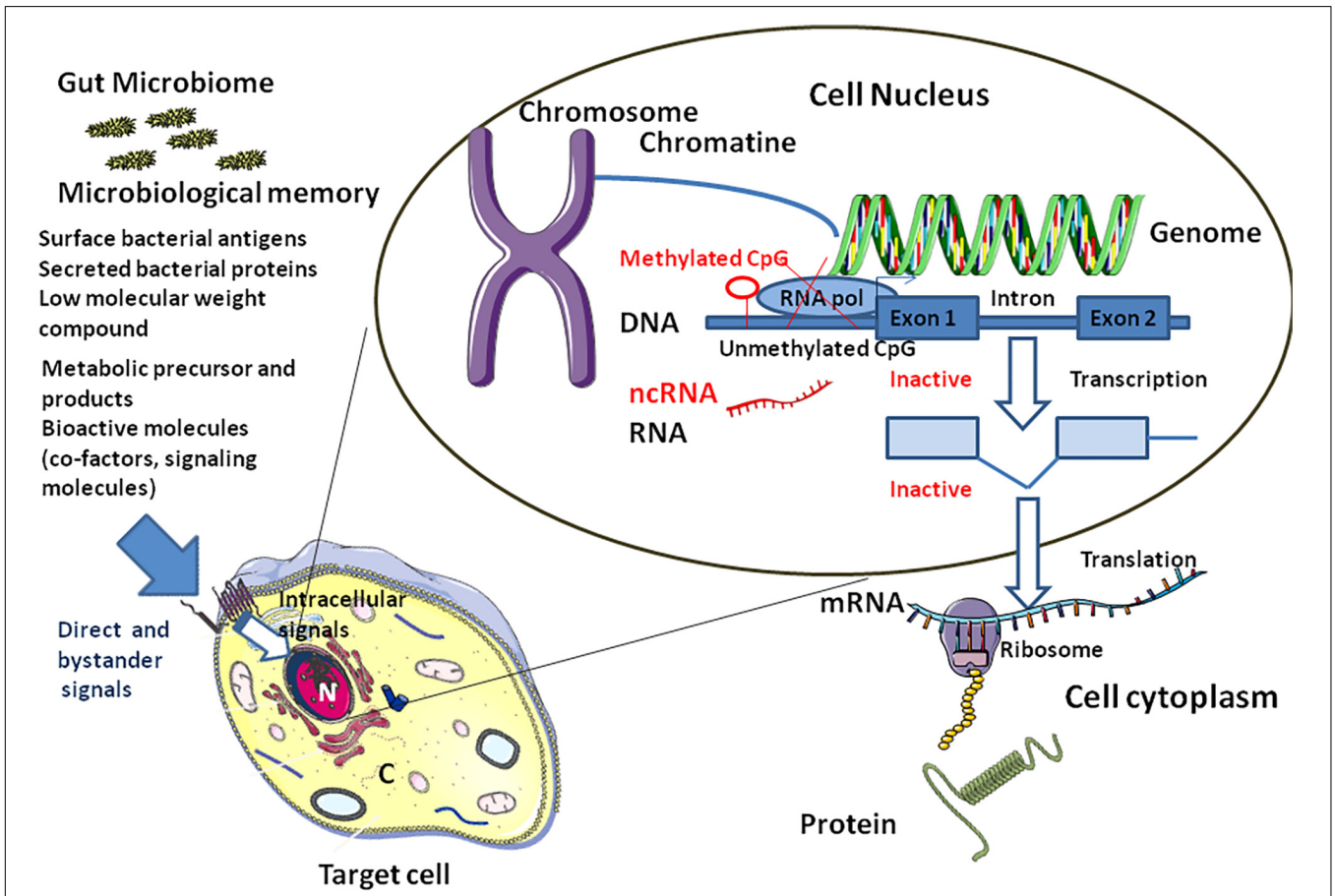

FIGURE 5 | Link between microbiota and epigenetic modifications. The "microbiological memory" will deliver direct and bystander signals to the target cell (left panel). After cell surface interaction (e.g., soluble bacterial compound interacting with a cell surface receptor complex), intracellular signal activation pathways will be modulated (e.g., phosphorylation of cytoplasmic proteins by kinases, nuclear translocation of transcription factors,...), thereby influencing the balance between activation and genetic silencing of transcription by DNA methylation and ncRNAs. Nucleosomes (not shown) comprises an octamer of histones and double-stranded DNA. When CpG dinucleotides are unmethylated in the gene promoter region, the RNA polymerase (RNA pol) can bind and activate transcription **(right)**. Methylation of CpG dinucleotides (red symbols) by DNA methyl transferases will recruits a histone deacethylase (HDAC)/histone methyl transferase (HMT) complex which in turn will remove acethyl groups from histones and methylate specific residues the overall effect being silencing of transcription. Transcriptional and/or translational interference by non-coding RNA (ncRNAs) is also illustrated.

Lactobacillus spp. contained per gram of yogurt (Angelakis et al., 2011). This indicates that diets could theoretically modify the composition of the gut microbiota daily, thus changing the molecular microenvironment of host cells and consequently reshaping the eukaryotic genes by epigenetic reprogramming (Shenderov, 2012). Faced with these observations, it seem reasonable to consider that it is the relative stability of the diet that maintains the balance of the gut microbiota over time.

More recently, increasing evidence from metagenomic studies was reported indicating that the diversity of the intestinal microbiome differs from one individual to another, and that dysbiosis might determine risk factors. Microbial surface antigens and metabolic end-products can activate signaling pathways resulting in the modulation of the cells' metabolome. It is worth noting that the lipopolysaccharide (LPS) receptor TLR4 which binds Gram-negative bacteria LPS, was found to promote diet-induced metabolic syndrome (Shi et al., 2006; Cani et al., 2008). This observation suggests that the molecular crosstalk between gut bacteria and host cells activates signaling pathways in host cells which will ultimately modulates gene expression through DNA-binding proteins activation and epigenetic modifications. LPS which is continuously produced within the gut by the death of Gram-negative bacteria and is carried into intestinal capillaries through a TLR4-dependent mechanism (Neal et al., 2006), is known to trigger the secretion of proinflammatory cytokines when it binds to the CD14/TLR4 receptor complex (Wright et al., 1990). In favor of the molecular crosstalk hypothesis, it was found that levels of *Bifidobacterium* spp. were reduced, whereas plasma LPS increased in mice given a high-fat diet for 4 weeks. Also, LPS infusion in wild type mice mimics the phenotype of mice given a high-fat diet (Backhed et al., 2007). Moreover, mice given a high-fat diet for 8 weeks developed a LPS receptor-dependent vascular inflammation (higher thoracic aorta IκBα-phosphorylation, ICAM, IL-6). Finally, it was reported that the mechanism underlying the resistance to diet-induced obesity in germ-free mice, depends on the activation of AMP-activated protein kinase (Cani et al., 2007). Obviously, bacteria have acquired multiple systems to expose proteins at their surface and to release soluble factors in the extracellular environment, over the course of evolution. More global approaches are therefore required to study the metabolome from gut bacteria and deciphering their functional participation in cellular gene modulation linked to chronic diseases (Maffei et al., 2017). Within families of patients with chronic metabolic diseases, bacterial induction of phenotypic changes in the offspring that persist for life implies stable modifications to gene transcription and altered activities of metabolic pathways. It has been known for a long time that patients with bowel disease had increased risks of colorectal cancer, starting with the formation of flat lesions or polyps protruding from the bowel wall that progress toward dysplastic adenomas and colic carcinoma (Couturier-Maillard et al., 2013). Recent studies indicate that it is feasible to rescue an experimental model of colic cancer by oral treatment using genetically engineered bacteria. This is only one among numerous demonstrations that reconstitution of a favorable microenvironment may be advantageous for treatment of metabolic diseases. Interventional changes in the composition of the microbiota (oral administration of commensal bacteria) can lead to reshape the prokaryotic molecular effectors of the eukaryotic microenvironment. This binary classification opposing bacteria that favor the development of diseases to others that protect against their occurrence is likely oversimplified since it ignores the dual behavior of certain pathobionts. It was recently reported that the translocation to the liver and other systemic tissues of *Enterococcus gallinarum* that usually live as a symbiont in the gut, can promote autoimmunity in mice and humans (Vieira et al., 2018), opening a new avenue for research. Moreover, it was recently shown that efficacy of cancer immunotherapy with immune checkpoint antibodies requires specific gut microbiota and is decreased by antibiotic treatments (Zitvogel et al., 2018).

We are just beginning to understand that the intestinal microbiota is a dynamic system that readjusts daily according to nutritional intake. Yet, little is known about the distant effects of these bacteria on eukaryotic epigenetic regulation. Recently, differential DNA methylation and covalent modification of histones that regulate gene transcription were associated to nutrition. Both under- and over-nutrition during pregnancy and/or lactation were shown to induce stable modifications of the offspring and termed "fetal programming" with a connotation of genetic inheritance. The link between microbiota and epigenetic modifications should be further investigated. Here, we hypothesize that when epigenetics shows inherited characteristics (so-called "epigenetic programming"), it is actually the cell microenvironment (bacterial surface antigens and secreted proteins, low-molecular-weight compound from bacteria and bioactive molecules supplied through the diet and processed by the gut microbiota), that remains constant from one generation to the next. We propose the term "microbiological memory" to account for this microbiota-shaped microenvironment (**Figure 5**). Microbiological memory would remain stable when diet and microbiota remain almost unchanged. According to this model, what is currently known as epigenetic programming is probably nothing more than a non-genetic inheritable signature resulting from the molecular crosstalk between gut prokaryotes (microbiota metabolome) and eukaryotic cells. This crosstalk would trigger a continuous reshaping of cellular genes through activation of signaling pathways in host cells, thereby controlling the epigenetic signature. It should be emphasized that this remains a hypothesis and, as such, has to be verified in future studies. While epigenetic can be investigated in a more or less straightforward manner by studying diseases' signature, the influence of the microbiological memory is much more difficult to decipher because of the involvement of many variables. Studies are very much needed to discriminate what is causal and what is co-occurrence in the trinomial diet-microbiota-epigenetics.

AUTHOR CONTRIBUTIONS

CD and DR contributed to the conception of the manuscript. CD wrote the manuscript.

FUNDING

This study was supported by IHU Méditerranée Infection, University of Marseille, and CNRS, France. This work has benefited from the French State support, managed by the "Agence Nationale de la Recherche," including the "Programme d'Investissement d'Avenir" under the reference Méditerranée Infection 10-1AHU-03.

ACKNOWLEDGMENTS

We thank Prof. Jean-Christophe Lagier for helpful discussions and Pierre Pontarotti for critical reading of the manuscript. Figures were designed using the Servier Medical Art supply of images available under a Creative Commons CC BY 3.0 license. We thank Magdalen Lardière for English editing.

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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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 June 2018
doi: 10.3389/fmicb.2018.01258

Comparative Analysis of Gut Microbiota Changes in Père David's Deer Populations in Beijing Milu Park and Shishou, Hubei Province in China

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 02 April 2018
Accepted: 24 May 2018
Published: 12 June 2018

Citation:
Zhang M, Shi M, Fan M, Xu S, Li Y, Zhang T, Cha M, Liu Y, Guo X, Chen Q, Li Y, Zhang S, Hu D and Liu S (2018) Comparative Analysis of Gut Microbiota Changes in Père David's Deer Populations in Beijing Milu Park and Shishou, Hubei Province in China. Front. Microbiol. 9:1258. doi: 10.3389/fmicb.2018.01258

This study used 16S rRNA high-throughput sequencing technology to examine the differences in gut microbiota between the Père David's deer populations in the Beijing and Shishou areas of China in order to understand the effects of *ex situ* conservation on the intestinal microflora in the Père David's deer.

Results: On the phylum level, the main bacteria found in the Père David's deer populations from both areas were similar: *Firmicutes* and *Bacteroidetes*. However, the relative abundances of the two groups were significantly different. Alpha diversity results indicated that there was a difference in the evenness of the microflora between the two groups, and the beta diversity results further indicated that there was a significant difference in the microflora structure between the two groups.

Conclusions: During the *ex situ* conservation process of the Père David's deer, their food sources may change, resulting in differences in the gut microbiota. The intestinal microflora in the Père David's deer from the same area are clustered. Therefore, the impact of changes in food on the gut microbiota of the Père David's deer should be taken into consideration during *ex situ* conservation.

Keywords: Père David's deer, gut microbiota, 16s rRNA gene, high-throughput sequencing, Bacteroidetes, Firmicutes

INTRODUCTION

The Père David's deer (*Elaphurus davidianus* or milu) first appeared in the early Pleistocene epoch and was a typical deer in the northern regions of China. It became extinct in China around 1900, but was bred again at the Beijing Milu Ecological Research Center in 1985 (Zhong et al., 2015). The Père David's deer is currently considered an endangered species (Bai et al., 2012). Initially, the number of Père David's deer in China was 79, but today there are more than 6,000 Père David's deer spread over 70 places throughout the country. The Père David's deer has increased significantly in number and become the representative species for the restoration and revival of endangered species in China (Zhang et al., 2012). The deer has been breeding in *ex situ* conservation areas, such as Dafeng, Jiangsu Province, Shishou, Hubei Province, Cixi Wetland, Zhejiang Province, Qianshan Deer Farm in the city of Liaoyang, and Mulan Weichang in Hebei Province (Zhang and Zhang, 2013). To better protect the re-introduced population and eventually release the captive deer, many

domestic and foreign studies have focused on the feeding and management (Chen et al., 2004; Li K. et al., 2007; Meng et al., 2010), genetic breeding (Li et al., 2005a,b; Ding et al., 2009), and populations (Jiang et al., 2001; Yang et al., 2007; Wang et al., 2009) of the Père David's Deer. As their population increases, the incidence of disease has also risen. Bacteria such as *Clostridium perfringens* (Yang et al., 2004; Li C. X. et al., 2007; Zhong et al., 2007), pathogenic *Escherichia coli* (Wang et al., 1991; Zhong et al., 2007), *Clostridium septicum* (Ding, 2004), and *Pasteurella* (Yang et al., 2004) are a serious threat to the survival of the deer, and they normally infect Père David's deer in combinations. Zhang et al. (1997) analyzed the death patterns of the Père David's deer and found that the peak period of deaths was in the seasons of winter and spring. They considered digestive tract diseases to be the primary factor for the deaths of the Père David's deer in captivity. However, there are currently no detailed studies on the intestinal microorganisms in Père David's deer.

The gut microbiota is a collective term for microorganisms that live in the digestive tracts of animals, and refers to the microbial diversity formed between intestinal microorganisms and their host and environment. This diversity is interdependent, interinhibitive, and relatively stable. Unlike single-stomach animals, ruminants have a large forestomach, and the microbes are closely related to the production and health of ruminants (Malmuthuge and Le, 2017). The digestion of ruminants depends on the complex microflora in the rumen. The rumen is a feed processing plant in ruminant, and 70 to 85% of the digestible and 50% of the crude fibers in the feed are digested in the rumen. The gut microbiota is closely related to the nutritional metabolism and immune system of their host species, and an important factor in the maintenance of its health. The gut microbiome helps maintain the immune and digestive systems of the host species. Once damaged, it could lead to various diseases (Buddington and Sangild, 2011; Chinen and Rudensky, 2015; Ding et al., 2017). Most studies on the gut microbiota are based on fecal samples. Fecal microbial data reflect the overall status of microbial communities in the intestinal tract (Li C. X. et al., 2007), and are easily collectible and non-destructive. Studying the structural characteristics of the gut microbiota in Père David's deer allows us to understand their health status and provide scientific data for their *ex situ* conservation.

Studies have shown that the interaction between animals and microorganisms inside them as well as their living environment determine the state of health or disease in the animal's body (Wei, 2008). The many differences in the hydrothermal conditions and food and nutritional statuses in Père David's deer breeding areas may lead to variances in the profile of their gut microbiota, which has an impact on their health. Therefore, analyzing and comparing the differences in the gut microbiota of Père David's deer in different breeding areas will increase our understanding of changes in the composition of gut microbiota in Père David's deer populations in *ex situ* conservation sites and provide a scientific reference for us to manually improve and evaluate the health of the Père David's deer in different areas.

Therefore, this study applied 16S rRNA high-throughput sequencing to analyze Père David's deer feces in the Beijing and Shishou areas. The 16S rRNA sequencing technology combines the advantages of high-throughput sequencing and bacterial identification based on 16S rRNA genes. This facilitates the integrated study of the structures and functions of mixed bacterial strains in complex samples, rendering it possible to compare the structural differences of the gut microbiota in different regions with higher accuracy. This was conducted to enable the comparison of changes and differences of gut microbiota between the *ex situ* conservation population of semi-wild Père David's deer in Shishou, Hubei and their counterparts in Beijing Milu Park, to understand the changes in gut microbiota of Père David's deer during *ex situ* conservation, and to provide scientific guidance for such conservation. The study has great significance for the understanding of the health status of Père David's deer in *ex situ* conservation reserves, the exploration of the most suitable environment for the deer's survival, and the improvement of the success rate of *ex situ* conservation of Père David's deer.

MATERIALS AND METHODS

Basic Facts of the Research Areas

Table below Information on Beijing Nanhaizi Père David's deer Park and Hubei Shishou Père David's deer National Nature Reserve (Liu et al., 2011).

	Beijing Nanhaizi Père David's deer Park	**Hubei Shishou Père David's deer National Nature Reserve**
Geographic coordinates	N 39°46′ E 116°26′	N 29°49′ E 112°33′
Annual average temperature	12.6°	16.5°
Relative humidity	65%	80%
Annual average precipitation	620 mm	1,200 mm
Altitude	31.5 m	32.9–38.4 m
Area	60 hm^2	1,567 hm^2
Vegetation	Aquatic vegetation, shrubs, arboreal forest	Poplar forest, reed swamps, grassland, Chinese willow (*Salix matsudana*), shrubs
Population	180	550

The geographical information of the Beijing Nanhaizi Milu Park (the Beijing area) and the Hubei Shishou Milu National Nature Reserve (the Shishou area) is outlined in the respective tables above. In 1993, 1994, and 2002, 30, 34, and 30 Père David's deer, respectively, were brought to the Shishou reserve from the Beijing Milu Park. Breeding populations were established and allowed to range freely in enclosures.

The Shishou area is situated in a sub-tropical monsoon climate zone, where summers are hot and winters are cold and dry. Precipitation is relatively heavy in spring, early summer and late autumn. The eight major vegetation populations are *Populus nigra var. italica*/*Phragmites communis*, *Salix matsudana*, *Phragmites communis* / *Miscanthus floridulus*, *Cynodon dactylon*, *Leonurus artemisia*, *Scirpus triqueter*, *Scirpus*

yagara, and *Eleocharis acicularis*. This study collected Père David's deer feces in the spring of 2017. In spring, the dominant species in the Père David's deer feeding areas in the Shishou area are *Phragmites communis*, *Roegneria kamoji*, *Cynodon dactylon*, *Hydrocotyle sibthorpioides*, *Leersi hexandra*, *Imperata cylindrical*, and *Carex argyi Levl. et Vant*.

The Beijing area is situated in a temperate and semi-humid monsoon zone, where the four seasons are distinct. Summers are hot and rainy, while winters are cold and dry. The dominant tree in the Père David's deer activity areas is *Salix matsudana*, while the dominant plant species include *Medicago sativa Linn.*, *Eleusine indica*, *Eragrostis cilianensis*, *Digitaria sanguinalis*, and *Setaria viridis*. Due to the limitations of climate conditions, manual feeding is conducted in Beijing Milu Park in spring. The major components of the concentrated feed is 50% corn, 26% soybean meal, 11% bran, 10% barley, 2% calcium hydrogen phosphate, and 1% sea salt. Corn and barley are common feed crops that are rich in nutrients such as sugars, protein, and fat. Soybean meal is a by-product of soybean oil extraction and one of the major protein feeds for animals. Bran is a by-product obtained from the processing of wheat to flour and contains a large amount of vitamins.

Animals and Sample Collection

Twelve healthy adult Père David's deer were selected from each of the Shishou and Beijing areas (Between the ages of 4 and 8, the body weight is 120–180 kg, six female elk and six male elk.), and ear tags were used to distinguish each individual deer. Sampling at the same time in mid-march 2017. Having cleaned the Père David's deer habitat the previous night, fresh fecal samples were collected in the early morning. Large, fresh, and relatively intact pieces of feces were collected. Disposable sterile gloves were worn when collecting samples to avoid human contamination. Samples were stored in sterile centrifuge tubes immediately after collection and sealed to avoid cross-contamination between samples. Immediately after sampling, all fresh fecal samples were stored in liquid nitrogen and returned to the laboratory, where they were stored at −80°C until DNA was extracted.

DNA Extraction and Purification

Total bacterial DNA was extracted with the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The integrity of the nucleic acids were determined visually by electrophoresis on a 1.0% agarose gel containing ethidium bromide. The concentration and purity of each DNA extract were determined using a Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, United States). The extracted total DNA was preserved at −80°C.

MetaVxTM Library Preparation and Illumina MiSeq Sequencing

Next generation sequencing library preparations and Illumina MiSeq sequencing were conducted at GENEWIZ, Inc. (Suzhou, China). DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, United States). The 40–60 ng DNA was used to generate amplicons using a MetaVxTM Library Preparation kit (GENEWIZ, Inc., South Plainfield, NJ, United States). V3, V4 hypervariable regions of microbial 16S rDNA and ITS1 regions of fungus were selected for generating amplicons and following taxonomy analysis. GENEWIZ designed a panel of proprietary primers aimed at relatively conserved regions bordering the V3, V4, and ITS1 hypervariable regions of the bacterial and archaeal 16S rRNA gene and fungus gene. (For samples containing eukaryotic DNA, only V3 and V4 regions will be amplified). The V3 and V4 regions were amplified using forward primers containing the sequence "ACTCCTACGGGAGGCAGCA" and reverse primers containing the sequence "GGACTACHVGGGTWTCTAAT." The ITS1 regions were amplified using forward primers containing the sequence "CTTGGTCATTTAGAGGAAGTAA" and reverse primers containing the sequence "GCTGCGTTCTTCATCGATGC." The first round PCR, respectively, amplified the V3–V4 and ITS1 regions to obtain the target fragment and part of the adapters sequence, and the second round PCR mixed the first round PCR amplification products. At the same time, indexed adapters were added to the ends of the 16S rDNA amplicons to generate indexed libraries ready for downstream NGS sequencing on Illumina Miseq. DNA libraries were validated by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States), and quantified by Qubit 2.0 Fluorometer. DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, United States). Sequencing was performed using a 2300/250 pairedend (PE) configuration; image analysis and base calling were conducted by the MiSeq Control Software (MCS) embedded in the MiSeq instrument.

Data Analysis

The QIIME data analysis package was used for 16S rRNA data analysis. The forward and reverse reads were joined and assigned to samples based on barcode and truncated by cutting off the barcode and primer sequence. Quality filtering on joined. sequences was performed and sequence which did not fulfill the following criteria were discarded: sequence length >200 bp, no ambiguous bases, mean quality score 20. Then the sequences were compared with the reference database (RDP Gold database) using UCHIME algorithm to detect chimeric sequence, and then the chimeric sequences were removed. The effective sequences were used in the final analysis. Sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH (1.9.6) against the Silva 119 database preclustered at 97% sequence identity. The Ribosomal Database Project (RDP) classifier was used to assign taxonomic category to all OTUs at confidence threshold of 0.8. The RDP classifier uses the Silva 119 database which has taxonomic categories predicted to the species level. Novel clusters (OTUs that did not match the reference database) were removed when performing analysis.

Sequences were rarefied prior to calculation of alpha and beta diversity statistics. Alpha diversity indexes were calculated using the Mothur software (Schloss et al., 2009) from rarefied samples using for richness and diversity indices of bacterial community (i.e., ACE, Chao1, Shannon, and Simpson). Principal coordinate

analysis (PCoA) performed using unweighted UniFrac. A oneway analysis of similarity (ANOSIM) was performed to determine the differences among groups (Clarke and Gorley, 2006). Here, the Bray–Curtis similarity index was used as a metric of similarity between the bacterial communities based on the abundance of OTUs between samples. The heatmap figures, Venn diagrams, and ANOSIM were produced using R1, and the cladogram was generated using the online LEfSe project2. Differences in phylum and genus relative abundances are presented as means ±SD. Student's *t*-test by SPSS 25 was used for data analysis. A P-value < 0.05 was considered statistically significant. The raw sequences obtained in this study were available through the NCBI Sequence Read Archive (accession number SRR5839043).

RESULTS

Analysis of 16S rRNA Sequencing Results

Illumina MiSeq sequencing technology was used to detect 16S rRNA gene sequences in the fecal microbiota of the semi-wild Père David's deer that were bred in the Beijing and Hubei areas. After a series of purification and filtration processes on the sequencing results, 59,837 valid sequences were obtained from each sample, and a total of 1,436,086 sequences were obtained (average length: 413.42 base pairs). The statistics of the filtered sequencing data of each sample are shown in **Table 1**. The reads sequences in the corresponding length range of each sample after quality control filtration were counted. The effective sequence length distribution is shown in **Figure 1**.

The number of sequences in each sample's OTU was obtained within the 97% sequence similarity threshold. By comparing the OTU representative sequences with a microbial reference database, we obtained classification information for each species corresponding to each OTU. The bacteria that could be detected were classified into 12 phyla, 22 classes, 27 orders, 47 families, and 94 genera. At each level (phylum, class, order, family, genus and species) the composition of each sample community was calculated. The number of each species at different levels are shown in **Table 2**, and the total number of OTUs covered by each sample in their subordinate levels are shown in **Table 3**. The dilution curves of the OTUs measured in this study indicated that the number of OTUs increased with the depth of sequencing. The final curve became stable, signifying that the amount of sequencing data is somewhat reasonable (**Figure 2**).

TABLE 1 | Statistics of sequencing data of each sample after filtration.

Sample	PE_reads	Effective tags	AvgLen(bp)	GC(%)	Effective(%)
B1	79,947	58,904	413	52.34	73.68
B2	80,039	58,496	412	52.43	73.08
B3	79,946	57,561	412	52.33	72.0
B4	80,105	58,334	413	52.21	72.82
B5	80,378	58,855	412	52.25	73.22
B6	80,014	58,720	412	52.37	73.39
B7	80,189	59,118	412	52.34	73.72
B8	80,189	58,047	412	52.27	72.32
B9	79,714	59,990	412	52.38	75.26
B10	79,878	59,408	412	52.35	74.37
B11	79,973	58,408	414	52.1	73.03
B12	79,795	59,307	413	52.2	74.32
S1	79,714	59,021	414	52.21	73.69
S2	79,726	59,137	415	52.01	74.18
S3	80,233	59,971	414	52.04	74.75
S4	79,836	59,971	414	52.13	73.23
S5	79,946	60,503	415	51.92	75.68
S6	79,783	59,661	415	52.13	74.78
S7	80,022	59,005	414	52.14	73.74
S8	79,961	60,124	414	52.25	75.19
S9	79,773	59,581	414	51.97	74.69
S10	79,896	59,695	414	52.15	74.72
S11	80,309	60,391	416	51.83	75.2
S12	80,250	60,440	414	52.11	75.31

Sample, name of sequencing sample; PE reads, paired-end reads number obtained by sequencing; Raw tags, number of original sequences obtained by splicing paired-ended reads; Clean tags, number of optimized sequences after filtering the original sequence; Effective tags, Clean tags Number of effective sequences after chimera filtered by clean tags; AvgLen (bp), Average sequencing length of the sample; GC (%), GC content of the sample, i.e., the percentage of G and C bases relative to the total number of bases; Effective (%): Proportion of effective tags among PE reads.

Comparison of Core Intestinal Microflora Between Père David's Deer in the Beijing and Shishou Areas

Venn diagrams were used to confirm the core intestinal microflora of the Père David's deer in the Beijing and Shishou areas. The bacterial populations common to all individuals in each group were considered the core microflora. As shown in **Figure 3**, the number of OTUs shared by all individual

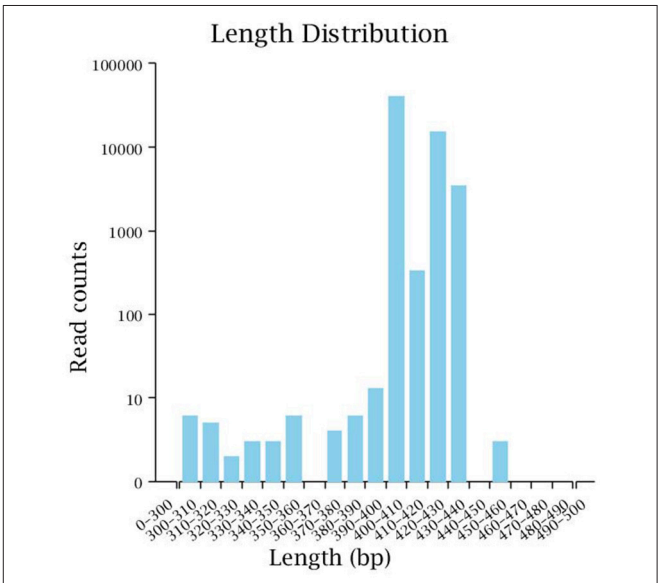

FIGURE 1 | Effective sequence length distribution. The x-coordinate is the sequence length range; the y-coordinate is the reads number.

TABLE 2 | Statistics of OTU species of samples on various levels.

Sample	Kindom	Phylum	Class	Order	Family	Genus	Species
B1	1	12	23	28	47	91	6
B2	1	12	21	26	44	87	5
B3	1	12	22	26	43	88	6
B4	1	12	21	25	43	89	6
B5	1	12	20	25	42	87	6
B6	1	12	20	25	43	88	6
B7	1	12	21	24	44	88	5
B8	1	12	20	24	44	88	6
B9	1	12	20	23	40	86	6
B10	1	12	22	26	45	90	6
B11	1	12	23	26	44	89	6
B12	1	12	22	25	43	88	6
S1	1	11	21	24	44	90	6
S2	1	12	23	25	43	91	6
S3	1	12	23	26	45	91	6
S4	1	12	23	25	45	90	5
S5	1	12	23	25	43	90	6
S6	1	11	21	23	42	89	6
S7	1	12	23	25	45	91	6
S8	1	11	19	21	42	90	6
S9	1	11	20	22	42	89	6
S10	1	11	21	23	42	88	6
S11	1	11	17	21	39	84	4
S12	1	12	22	23	43	91	6

B represents Père David's deer samples from Beijing Nanhaizi Milu Park and S represents Père David's deer samples from Shishou Milu Nature Reserve, Hubei Province.

TABLE 3 | Statistics of OTU clustering results of samples on various levels.

Sample	Kindom	Phylum	Class	Order	Family	Genus	Species
B1	33,145	33,104	33,104	33,103	32,962	32,169	31,746
B2	33,761	33,727	33,727	33,727	33,496	32,286	31,746
B3	32,277	32,163	32,163	32,161	32,054	31,280	30,894
B4	32,879	32,862	32,862	32,862	32,727	31,969	31,354
B5	33,708	33,691	33,691	33,691	33,546	32,572	32,097
B6	32,741	32,704	32,704	32,704	32,584	31,462	31,037
B7	33,915	33,907	33,907	33,905	33,756	32,711	31,928
B8	33,354	33,348	33,348	33,348	33,206	32,218	31,853
B9	34,619	34,616	34,616	34,616	34,468	33,518	32,688
B10	33,525	33,411	33,411	33,411	33,293	32,430	32,059
B11	32,866	32,790	32,790	32,789	32,678	31,715	31,112
B12	31,720	31,547	31,547	31,546	31,430	30,741	30,386
S1	33,535	33,532	33,532	33,525	33,344	32,208	31,015
S2	33,684	33,672	33,672	33,429	33,339	32,472	31,495
S3	33,079	33,040	33,040	33,036	32,922	31,967	31,241
S4	32,219	32,208	32,208	32,207	32,072	31,337	30,544
S5	34,816	34,791	34,791	34,776	34,656	33,925	32,927
S6	34,625	34,624	34,624	34,229	34,096	32,891	31,626
S7	32,699	32,683	32,683	32,680	32,544	31,822	31,039
S8	35,024	35,018	35,018	35,002	34,858	33,822	32,740
S9	35,245	35,234	35,234	35,202	35,089	33,950	33,077
S10	34,763	34,761	34,761	34,364	34,228	32,999	31,741
S11	35,525	35,520	35,520	35,520	35,458	33,917	33,592
S12	35,345	35,341	35,341	35,071	34,981	34,142	33,175

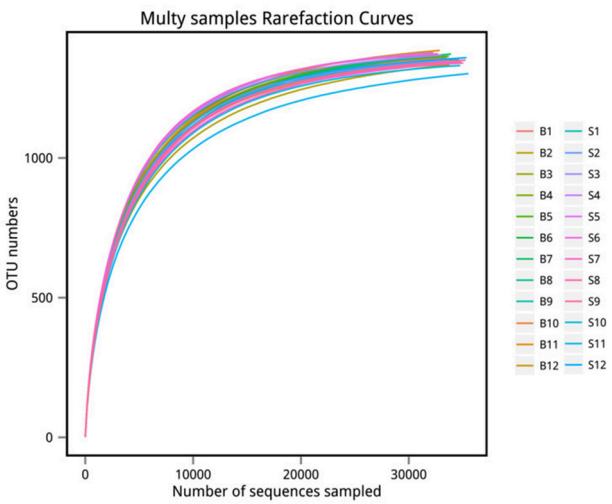

FIGURE 2 | Rarefaction curve. The x-coordinate is the number of sequences sampled and the y-coordinate is the number of observed OTUs. Each curve in the graph represents a sample, which is labeled with a different color. The number of OTUs increases with the sequencing depth. When the curve becomes stable, the number of detected OTUs does not increase with the expansion of extracted data, indicating a time when the amount of sequencing data is reasonable.

Père David's deer in the Beijing and Shishou areas was 1,438 (**Figure 3A**), while the number of OTUs was 1,059 for individual deer in the Beijing area (**Figure 3B**) and 1,058 for individual deer in the Shishou area (**Figure 3C**). The main bacterial phyla in the intestines of each group of Père David's deer are shown in **Figure 3D** (the Beijing area) and **Figure 3E** (the Shishou area). The two dominant phyla in these sequences were *Firmicutes* and *Bacteroidetes*.

A heatmap (**Figure 4**) is a graphical representation that uses a system of colored gradients to represent the size of values in a data matrix and cluster data based on species or the abundance similarity of samples. High-abundance and low-abundance species are clustered by color gradient and similarity to reflect the similarities and differences between multiple sample communities. A heatmap analysis was performed based on the species composition and relative abundance of each sample to extract the species at each taxonomic level. Mapping was achieved using R language tools, and a heatmap cluster analysis was performed at each of the phylum, class, order, family, genus, and species level.

Diversity Analysis of Microbial Communities in Père David's Deer in the Beijing and Shishou Areas

Alpha Diversity Analysis

Alpha diversity reflects the richness and diversity of a single sample species and has several indices for measurement, such as the Chao1, ACE, Shannon, and Simpson indices. The Chao1 and ACE indices measure the richness of species (i.e., the number of species), whereas the Shannon and Simpson indices measure the diversity of species and are affected by the richness and

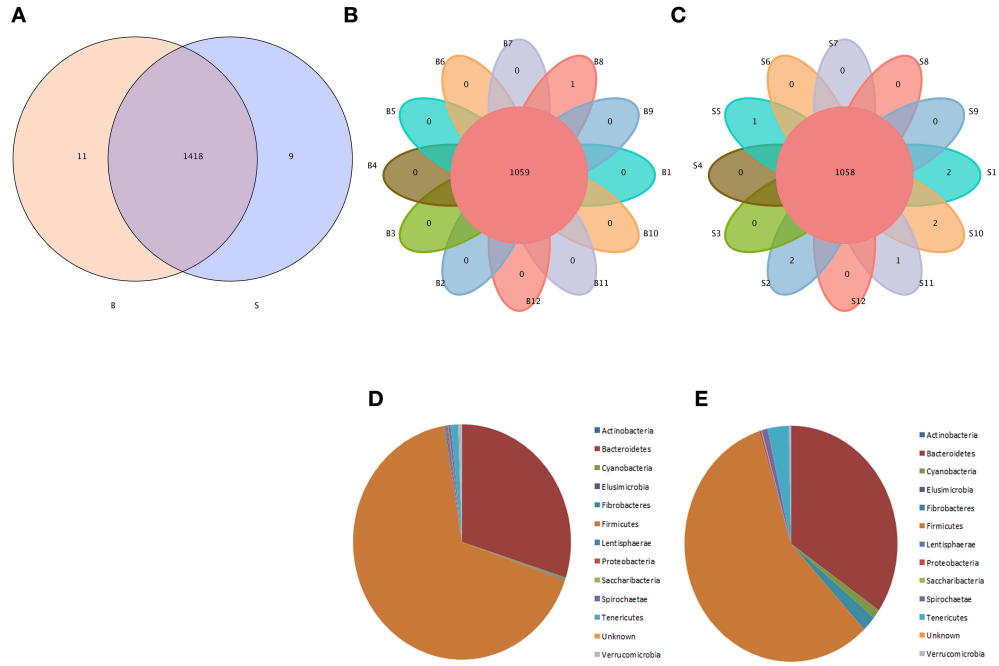

FIGURE 3 | Venn diagram and pie charts. The Venn diagrams show the numbers of OTUs (97% sequence identity) that were shared or not shared by B and S individuals, respectively, depending of overlaps. For this presentation, two individuals had to be combined (e.g., C1_2) thereby reflecting the number of OTUs shared by both individuals. **(A)** The number of OTUs shared by B and S. **(B)** The number of OTUs shared by B. **(C)** The number of OTUs shared by S. The pie diagram shows the 20 most abundant taxa (calculated over the combined dataset) in B and S. **(D)** B, **(E)** S.

community evenness of the sample community. In the case of equal richness, a higher community evenness among the species in the community is considered greater diversity, and the higher the Shannon index, the lower the Simpson index, which indicates higher diversity among the species in the sample (Wang and Wang, 2011). The completeness of the sequencing was tested by Good's coverage, which was close to 99% in this study, indicating that the majority of the bacterial species present in the sample had been detected.

We calculated the alpha diversity (ACE, Chao1, Shannon, Simpson, and Good's Coverage) for the gut microbiota in Père David's deer in the Beijing and Shishou areas, as shown in **Figure 5**. There was a significant difference in the ACE, Chao1, and Shannon indices between the Beijing and Shishou areas ($P < 0.05$), but no significant difference was found in the Simpson index of the two areas ($P > 0.05$). Therefore, the alpha diversity indices of the two areas are considered to be significantly different ($P < 0.05$).

Beta Diversity Analysis

Principal Coordinate Analysis (PCoA) (Li et al., 2005b) is an approach to sequencing using dimension reduction. It assumes that where there is data to measure the differences or distances between N number of samples, this method can be used to plot a rectangular coordinate system representing the N samples as N points, and the square of the Euclidean distance between the points is exactly equal to the original differential data. Thus, qualitative data is converted into quantitative data and the most important elements and structures are extracted from the multi-dimensional data. The classification of multiple samples can be achieved through PCoA, which further demonstrates the differences in diversity between samples. As observed in PC1 vs. PC2 shown in **Figure 6**, samples that are closer together in the graph indicate greater similarity.

Analysis of the Differences in Gut Microbiota Between Père David's Deer in the Beijing and Shishou Areas at the Phylum and Genus Levels

The cladogram (**Figure 7**) showed differences in 88 taxa between B and S. And **Figure 8** shows the differences in relative abundances at the phylum level of the top 5 bacterial communities and genus level of the top 10 bacterial communities in the Père David's deer samples from the Beijing and Shishou areas. In the Père David's deer from the Beijing area, the relative abundance of Firmicutes and Verrucomicrobia were significantly higher than that in the Père David's deer from the Shishou area ($P < 0.05$). In contrast, the relative abundances of the Bacteroidetes and Fibrobacteres phyla in the Shishou area were significantly lower than in the Beijing area ($P < 0.05$). Both groups did not show any significant differences in the relative abundances of Tenericutes ($P > 0.05$). At the genus level, the relative abundances of Christensenellaceae_R-7_group, Peptoclostridium, Lachnospiraceae_NK4A136_group, Ruminococcaceae_UCG-002, Ruminococcaceae_UCG-009 and

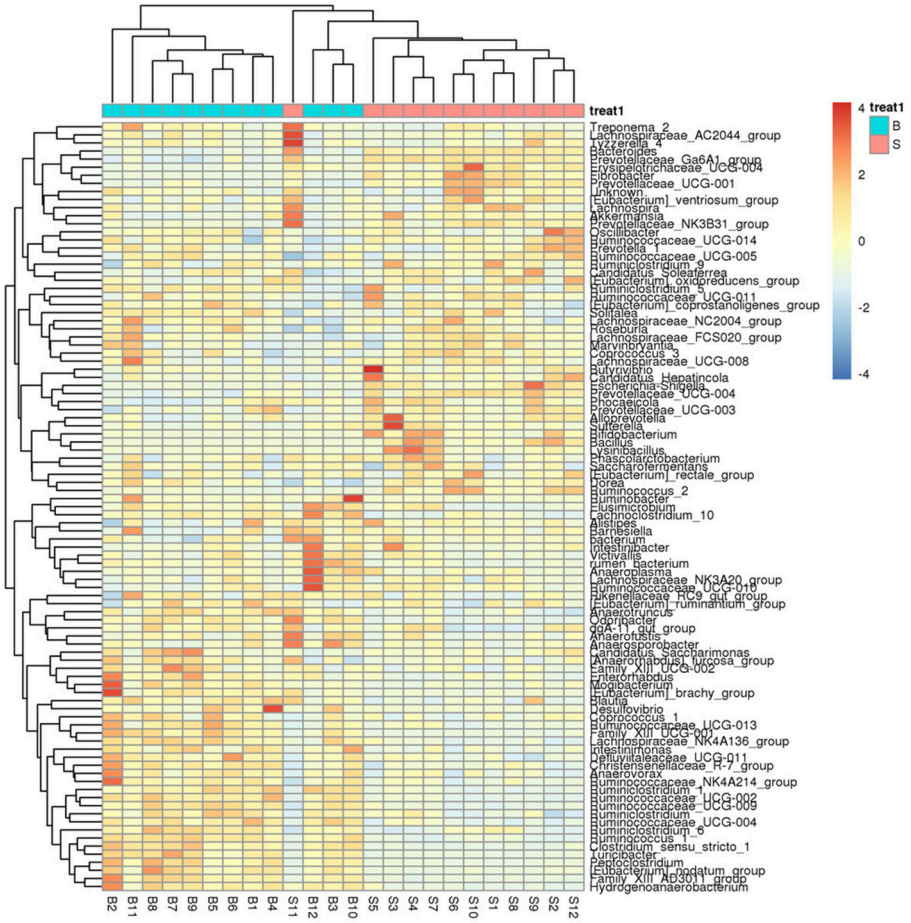

FIGURE 4 | Heatmap showing richness of species at each level. The corresponding values of the heatmap are the *Z* values obtained by normalizing the relative abundance of species on each row. The color gradient from blue to red indicates a low to high relative abundance. The vertical clustering indicates the similarity in the richness of different species among different samples. The closer the distance between two species, the shorter the branch length, indicating greater similarity in richness between the two species. Horizontal clustering indicates the similarity of species richness in different samples. Similarly, the closer the distance between two samples, the shorter the branch length, indicating greater similarity in richness of species between the two samples.

Ruminococcus_1 in the Beijing area were significantly higher compared with the Shishou area ($P < 0.05$), while the relative abundance of Bacteroides, Prevotellaceae_UCG-001, Prevotellaceae_UCG-004, and Fibrobacter was lower in the Beijing area than in the Shishou area ($P < 0.05$).

DISCUSSION

As research into the mammalian gut microbiota has deepened and high-throughput sequencing technology extensively applied, we have seen progress in the study of the relationship between the gastrointestinal micro-ecology and health of wildlife animals. The study of mammalian gut microbiota has currently gone deep into the gene level and has even begun to explore their mechanisms of action. Relevant research has extended from simple analyses on microbiota composition to function analyses, which involve multiple aspects, such as feeding patterns, nutrition, and habitats (Ding et al., 2017). As an important and rare wild animal in China, the Père David's deer plays an essential role in demonstrating how other endangered species can be protected through the *ex situ* conservation of some existing deer populations as well as the application of artificial management and reproduction to expand their populations. Since the Shishou Nature Reserve in Hubei Province re-introduced the Père David's deer from Beijing Milu Park in 1993, the population has grown to 550, three times the deer population of Beijing. However, due to technical limitations, there is relatively limited comparative analysis on the health status between the deer populations in the two areas, especially the analysis of the differences in their gut microbiota. This study applied 16S rRNA Illumina MiSeq high-throughput sequencing technology for the first time to compare the core gut microbiota between the two Père David's deer populations, analyzed the diversity of microbial communities, and conducted a differential analysis at the phylum and genus levels. Such analyses have expanded our understanding of the health status of deer populations in *ex situ* conservation sites and

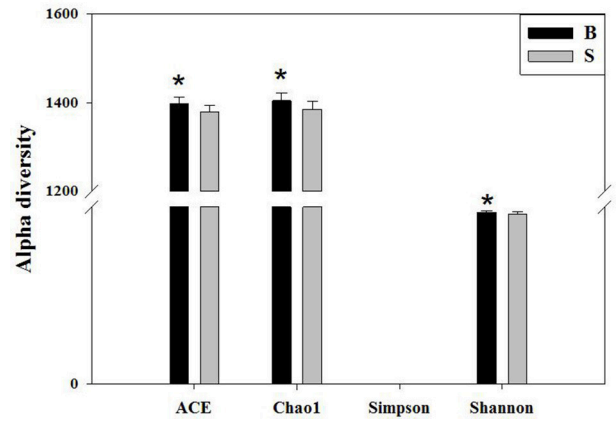

FIGURE 5 | Comparison of alpha diversity indices of gut microbiota in the Beijing and Shishou areas. ACE: an index used to estimate the number of OTUs in a community. It is one of the most commonly used indices for estimating species in ecology. Chao: an index that uses the Chao1 algorithm to estimate the number of OTUs included in a sample. Chao is commonly used in ecology to assess the total number of species. Shannon: an index often used to reflect alpha diversity and estimate microbial diversity in a sample. Simpson: a diversity index commonly used in ecology to quantitatively describe the biodiversity of a geographical area. *$P < 0.05$.

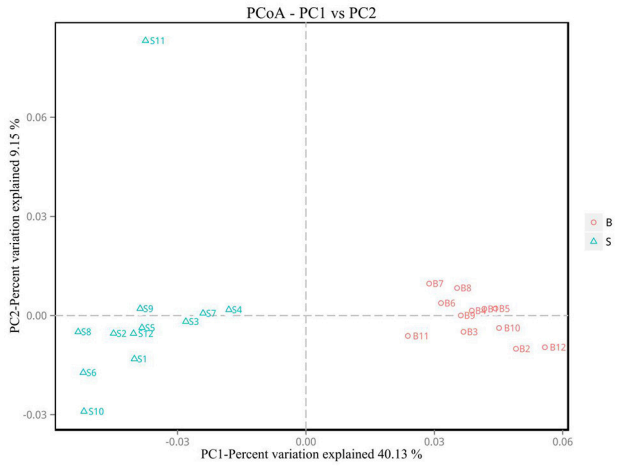

FIGURE 6 | PCoA plot. Principal coordinate analysis (PCoA) plot. Red dots represent Père David's deer samples from the Beijing area, and blue squares represent Père David's deer samples from the Shishou area. Samples in the same group are represented by the same color and shape. PC1 vs. PC2 is the PCoA plot obtained from the first and second main coordinates; the x-axis and y-axis represent the first and second main coordinates, respectively. The percentage of the main coordinates represent the relative contribution of this coordinate to sample differences, which is a measure of the amount of original information extracted by this main coordinate. The distances between the sample points represent the similarity of microbiota in the samples. A closer distance represents higher similarity and samples that cluster together are composed of similar microbiota.

provided a scientific basis for monitoring the health status of the Père David's deer.

The analysis results indicated that the amount of sequencing data in this study was reasonable (**Figures 1**, **2**). As seen in **Figure 3**, the gut microbiota in the Père David's deer populations in the Beijing and Shishou areas have a high degree of similarity (95.66%), with the common phyla being *Firmicutes* and *Bacteroidetes*. This finding was consistent with the majority of studies on the gut microbiota of ruminant animals (Sundset et al., 2007; Guan et al., 2017; Li et al., 2017). In terms of alpha diversity, the Chao1, ACE, and Shannon indices of the gut microbiota were significantly different between the Père David's deer populations of the Beijing and Shishou areas ($p < 0.05$) (**Figure 5**). Regarding beta diversity, the gut microbiota of both deer populations displayed two clear clusters (**Figure 6**). Thus, the richness and evenness of the intestinal microorganisms of the Père David's deer populations are still dissimilar. We observed from the analysis in **Figure 6** that there were greater differences between the deer populations in the Beijing and Shishou areas, while the differences in microbial communities within the same area were smaller.

The gut microbiome consisting of the host and the gut microbiota maintains the host's immune and digestive systems. The biodiversity and richness play an important role in maintaining the host's normal physiological functions, but is also affected by the host (Koboziev et al., 2014). The composition and functions of the gut microbiota is closely related to the health status of the host, and dysbiosis is the cause of many diseases. On the surface, dietary patterns are one of the key factors affecting the gut microbiota (Zheng et al., 2014), but differences between the gut microbiota in different geographical regions may also be due to other factors, such as host genotype, age, disease, probiotics, and drugs, all of which affect the structure and functions of the gut microbiota (Kovács et al., 1972). As the Père David's deer population in the Shishou Nature Reserve was originally transferred from the Beijing Milu Park and developed during the last century, the genotypes of individual deer from the two areas were not significantly different. In this study, we selected healthy adult Père David's deer of similar age and size as test subjects at approximately the same time, and the differences between the research areas and the samples were mainly the vegetation types caused by climatic conditions, which led to differences in the food sources of the deer populations. In spring, the deer in the Beijing area are manually fed, while in the Shishou area the feed on natural food sources. Therefore, by analyzing the differences in the food sources of the two areas could explain the biodiversity of the gut microbiota.

At the phylum level, the richness of *Firmicutes* in the intestines of the Père David's deer in the Beijing area was significantly higher than that in the Shishou area, whereas for *Bacteroidetes* it was exactly the opposite (**Figure 8A**). The main function of the *Firmicutes* (major food analysis) in the intestines is to hydrolyze carbohydrates and proteins, while *Bacteroidetes* (major food analysis) is responsible for the metabolism of steroids, polysaccharides, and bile acids, helping the host in the absorption of polysaccharides and the synthesis of proteins (Xu et al., 2003; Bäckhed et al., 2005). Other studies have found that a higher *Bacteroidetes* and *Firmicutes* content promotes animal fat deposition; this effect was more evident for *Firmicutes* than *Bacteroidetes* (Bäckhed et al., 2004; Turnbaugh et al., 2006).

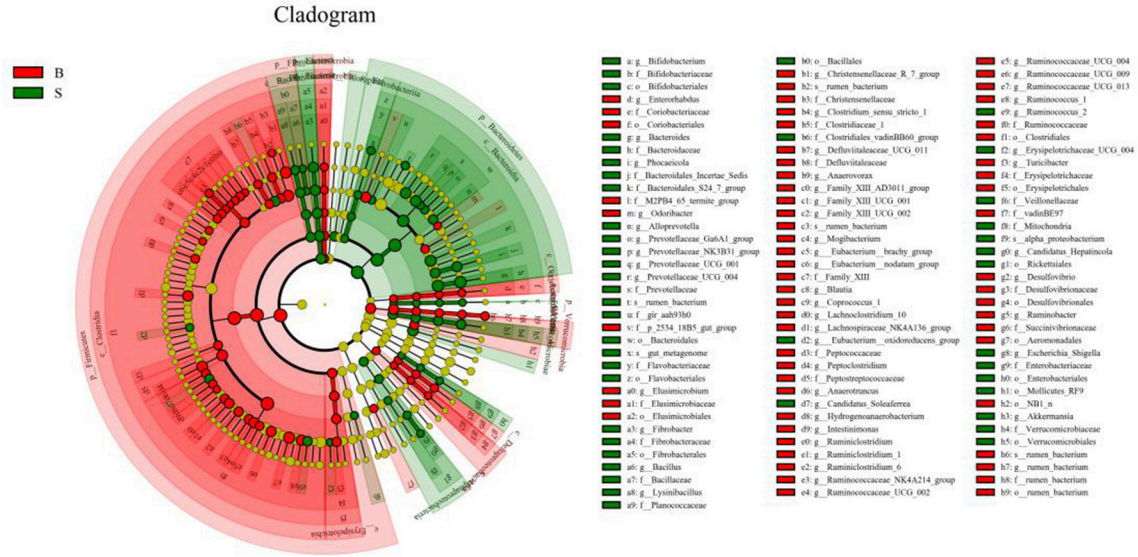

FIGURE 7 | LEfSe analysis of evolutionary tree. The circles from the inside to the outside of the evolutionary tree represent the classification from the phylum to the species level. Each small circle on a different classification level represents a classification below that level, and the diameter of the small circle is proportional to the relative abundance. Species with no significant differences are all represented by the color yellow, whereas the other significant different species are colored according to the group with the highest abundance to which the species belong. Different colors indicate different groups, and the nodes of different colors indicate the microorganisms that play an important role in the group represented by the color.

Moreover, it was found that the addition of soybean isoflavone aglycon in the diets of ruminantia, such as yellow cattle, enhances energy acquisition and stimulates the production of fat (Zhou, 2016). Hence we may infer that the significant difference in the amount of *Firmicutes* in the intestines of deer from the Beijing and Shishou areas may be because the deer diet in the Beijing area contains a certain proportion of soybean meal in springtime.

The results (**Figures 4**, **7**, **8B**) indicated that the relative abundance of *Bacteroides*, *Prevotellaceae*, and *Fibrobacter* of the Père David's deer in the Beijing area was significantly lower than those in the Shishou area on the family and genus levels ($p < 0.05$). The relative abundance of *Christensenellaceae*, *Peptoclostridium*, *Lachnospiraceae*, *Ruminococcaceae*, and *Ruminococcus* was the opposite. *Bacteroides* was also found to have other functions, such as promoting the improvement of the host's immune system and maintaining the balance of the gut microbiome (Hooper et al., 2001; Hooper, 2004; Sears, 2005; Jiang et al., 2014) studied changes in certain bacteria in the intestinal tracts of patients with ulcerative colitis, they found an increased number of *Bacteroides*, which has an inflammatory effect on patients with ulcerative colitis. Studies have also found that after adding soybean isoflavone aglycone to the diet of growing Jinjiang cattle, the richness of Bacteroides in the Bacteroidaceae family reduces, indicating that the aglycon lessens the inflammatory response in animals (Zhou, 2016). This study yielded similar results, as the abundance of *Bacteroidetes* and *Bacteroides* was significantly higher in the gut microbiota of Père David's deer in the Shishou area, compared to those in the Beijing area. The reason may be that the addition of soybean meal to the deer food in Beijing reduces the intestinal inflammation in the deer, whereas in the Shishou area the deer lack plants that contain soybean aglycon. Thus, more *Bacteroidetes* bacteria are needed to regulate the intestinal environment of the deer.

The experimental results indicated that the main types of intestinal microflora in Père David's deer are similar to those of humans (*Firmicutes* and *Bacteroidetes*) (Arumugam et al., 2011), due to the difference in food, People from different regions had significantly different in intestinal microbial communities. Therefore, dietary structure may have a significant impact on the microbial community in the intestines of deer. This finding has been verified in similar studies on other ruminants (Pitta et al., 2014). Moreover, a considerable number of studies have shown that differences in diet structure not only have an impact on the changes in the host's gut microbiota, but may also cause other physiological reactions or even diseases in animals. Studies have shown that different bacterial communities have different effects on the digestion of carbohydrates, protein, and cellulose in ruminant diets, not only affecting the host's nutrient utilization, but also possibly causing gastrointestinal diseases and obesity in the host (Bäckhed et al., 2005; Han et al., 2015). Hence, changes in the structure of the gut microbiota are mainly affected by dietary conditions, and the differences in feed composition are an inducing factor. For example, our results suggest that the differences in feed composition between the Beijing and Shishou areas explain the differences in the intestinal microorganisms of the Père David's deer. Nevertheless, whether the specific functions of different bacteria have an impact on the host's digestion and absorption and whether the change of gut microbiota after migration leads to diseases in the host and affects the health status of the deer population are issues yet to be investigated in future research.

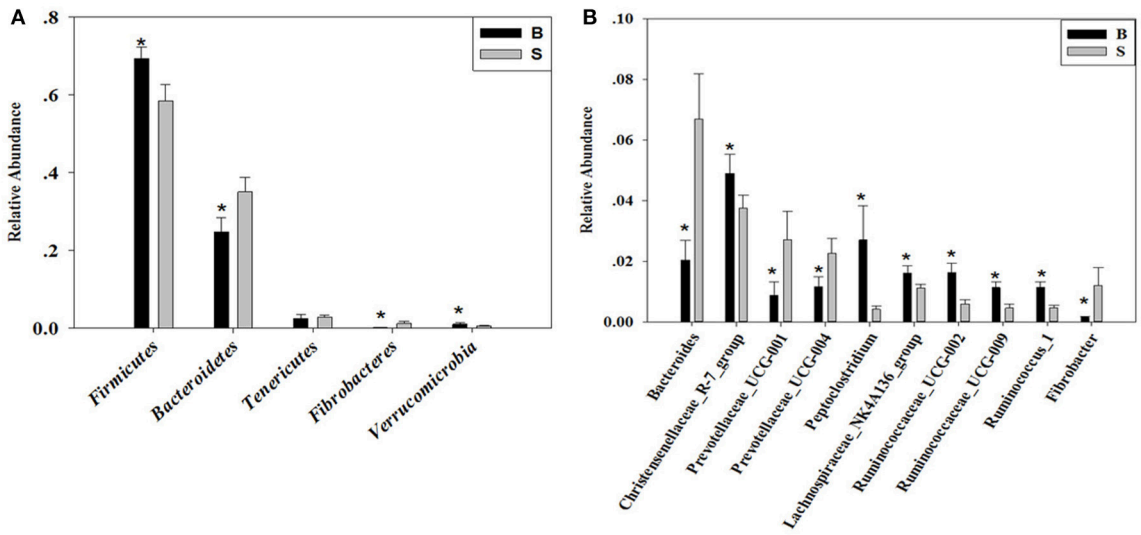

FIGURE 8 | Differences in relative abundance (mean % SD) of 5 major bacterial phyla and 10 major bacterial genus between the B and S groups. **(A)** Relative abundance (mean % SD) of five major bacterial phyla between the B and S groups. **(B)** Relative abundance (mean % SD) of 10 major bacterial genus between the B and S groups. The significance of Firmicutes, Bacteroidetes, Fibrobacteres, and Verrucomicrobia was determined using the independent-sample t-test, whereas the non-parametric Mann–Whitney U test was used to examine the significance of Proteobacteria. *represents $P < 0.05$.

In conclusion, our results showed that the gut microbiota of the Père David's deer population in the Shishou area is different from that of the Beijing area. This may be due to the fact that the deer population in Shishou has moved from Beijing to a new environment and has adapted to changes in their food and environment, leading to changes in their gut microbiota. Some researchers have analyzed the composition of the gut microbiota of tens of mammals and found that the diversity of the host's gut microbiota varies according to feeding patterns, gradually increasing from carnivorous, omnivorous, to herbivorous animals. Due to the differences in the digestive tract's anatomical structure and physiological metabolism, there is also a wide variation in the structures and functions of the gut microbiota (Ley et al., 2008). Therefore, after the Père David's deer migrates to a new environment, the composition and diversity of the gut microbiota are directly affected by changes in the natural environment and deer's feed, thus may impacting the adaptive changes of the digestive and immune health of the deer. To re-introduce the Père David's deer, we must ensure that there is reasonable feeding, planting of edible plants necessary for the deer's health, and scientific supervision over the deer. This study analyzed the gut microbiota of the Père David's deer in different geogrpahical areas and offered insights into the health status of the gut of the deer in China during the *ex situ* conservation process, providing an empirical reference and auxiliary measures for the scientific feeding and management of the Père David's deer.

AUTHOR CONTRIBUTIONS

MZ, MS, SZ, DH, SL conceived and designed the study. MZ, SX, YmL, TZ and MC performed the experiments. YL, XG, QC, and YpL processed the date. MZ, MS and MF wrote the paper. MF and MS reviewed and edited the manuscript. All authors read and approved the manuscripts.

ACKNOWLEDGMENTS

SL thank Beijing Milu Ecological Research Center (Grant No. 2017HXFWBHQ.LSQ.01) for support. The experimental facilities were provided by the innovation Laboratory of the College of Nature Conservation, Beijing Forestry University.

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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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 17 May 2018
doi: 10.3389/fmicb.2018.00896

The Growth and Protein Expression of Inflammatory Bowel Disease-Associated *Campylobacter concisus* Is Affected by the Derivatives of the Food Additive Fumaric Acid

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OPEN ACCESS

Edited by:
Zhaoping Li,
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Reviewed by:
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Ben Pascoe, University of Bath, United Kingdom

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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *13 December 2017*
Accepted: *18 April 2018*
Published: *17 May 2018*

Citation:
Ma R, Liu F, Yap SF, Lee H, Leong RW, Riordan SM, Grimm MC and Zhang L (2018) The Growth and Protein Expression of Inflammatory Bowel Disease-Associated Campylobacter concisus Is Affected by the Derivatives of the Food Additive Fumaric Acid. Front. Microbiol. 9:896. doi: 10.3389/fmicb.2018.00896

Inflammatory bowel diseases (IBD) are chronic inflammatory conditions of the gastrointestinal tract with multifactorial etiology. Both dietary factors and the microbe *Campylobacter concisus* have been found to be associated with the condition. The current study examined the effects of sodium fumarate, a neutralized product of the food additives fumaric acid and monosodium fumarate when in the intestinal environment, on the growth of *C. concisus* to determine the effects of these food additives on IBD-associated bacterial species. Through culture methods and quantification, it was found that neutralized fumaric acid, neutralized monosodium fumarate, and sodium fumarate increased the growth of *C. concisus*, with the greatest increase in growth at a concentration of 0.4%. Further examination of 50 *C. concisus* strains on media with added sodium fumarate showed that greatest growth was also achieved at a concentration of 0.4%. At a concentration of 2% sodium fumarate, all strains examined displayed less growth in comparison with those cultured on media without sodium fumarate. Using mass spectrometry, multiple *C. concisus* proteins showed significant differential expression when cultured on media with and without 0.4% sodium fumarate. The findings presented suggest that patients with IBD should consider avoiding excessive consumption of foods with fumaric acid or its sodium salts, and that the addition of 0.4% sodium fumarate alone to media may assist in the isolation of *C. concisus* from clinical samples.

Keywords: *Campylobacter concisus*, sodium fumarate, enteric bacteria, food additives, inflammatory bowel disease

INTRODUCTION

Inflammatory bowel disease (IBD) refers to the inflammatory disorders affecting the gastrointestinal tract, including Crohn's disease (CD) and ulcerative colitis (UC) (Cosnes et al., 2011). The symptoms of CD and UC are very similar, though the pathology of each differs, allowing for diagnosis. Inflammation in CD may occur in any part of the gastrointestinal tract with a

discontinuous pattern, while the inflammation in UC is continuous with diffuse and superficial lesions (Cosnes et al., 2011). Currently, the etiology of IBD is unknown, and several factors are suggested to contribute to its pathogenesis, such as host microbiota and diet (Sartor, 2006). *Campylobacter concisus* is an oral bacterium that has been linked to IBD. There was a significantly higher prevalence of *C. concisus* detected in intestinal biopsies of patients with IBD as opposed to healthy controls (Zhang et al., 2010; Mahendran et al., 2011; Mukhopadhya et al., 2011; Kirk et al., 2016). Studies have also shown that some oral *C. concisus* strains possess the ability to damage the intestinal epithelial barrier, suggesting that translocation of these strains to the lower gastrointestinal tract may increase the risk of the individual developing IBD (Nielsen et al., 2011; Ismail et al., 2012; Zhang, 2015; Mahendran et al., 2016). *C. concisus* strains are comprised of two genomospecies (Aabenhus et al., 2005; Engberg et al., 2005; Kalischuk and Inglis, 2011; Miller et al., 2012; On et al., 2013; Mahendran et al., 2015; Chung et al., 2016; Nielsen et al., 2016) and recently we have found that *C. concisus* genomospecies 2 strains were better adapted to the gastrointestinal environment (Wang et al., 2017).

Dietary factors have long been associated with a range of chronic illnesses. In IBD, there was a positive correlation of high intake of carbohydrates and fats to IBD incidence (Sakamoto et al., 2005). Non-Western countries historically have a low incidence and prevalence of IBD, but are showing an increasing incidence and prevalence of IBD coinciding with industrialization and westernization (Drewnowski and Popkin, 1997; Cosnes et al., 2011). The societal changes in these non-Western countries shift the diet toward high fat, high sugar, and processed foods, which are characteristic of Western diets, suggesting that dietary factors are likely to contribute to IBD incidence (Pingali, 2007). In murine models, it was shown that a high fat and high sugar diet changes the gut microbiota composition and promoted the colonization of IBD-associated adherent-invasive *Escherichia coli* (Martinez-Medina et al., 2014). With processed foods becoming increasingly consumed due to low cost and convenience achieved by food additives and industrial ingredients (Monteiro et al., 2013), their effects on IBD-related bacterial species require investigation.

Fumaric acid (E297) is a widely used acidity regulator (Smith and Hong-Shum, 2011), with its sodium salts, monosodium fumarate and sodium fumarate (E365) used less commonly for the same purposes. While the use of sodium fumarate as a food additive has been discontinued in Europe, it is allowed in Australia and New Zealand. There is no limit on the amount of fumaric acid used in both United States and Australia (CFR 172.350 in the United States, Standard 1.3.1 in Australia), while in the European Union a maximum of 4000 mg/L has been suggested with voluntary enforcement [Regulation (EC) no 1333/2008]. The neutralization of fumaric acid ($C_4H_4O_4$) via contact with the naturally occurring sodium bicarbonate in the intestinal tract can produce both monosodium fumarate and sodium fumarate. At less acidic pH, fumaric acid dissociates to form monosodium fumarate ($NaC_4H_3O_4$) and then sodium fumarate ($Na_2C_4H_2O_4$) at almost neutral pH (Roa Engel et al., 2013).

Previous studies utilized the combined supplementation of sodium formate and sodium fumarate into culture media to isolate *C. concisus* (Tanner et al., 1981; Macuch and Tanner, 2000). This suggests that sodium fumarate can increase *C. concisus* yield. Therefore, we hypothesize that sodium fumarate, the neutralized product of the food additive fumaric acid, affects the growth of *C. concisus* and protein expression. This hypothesis was examined in this study.

We found that sodium fumarate supplementation into culture media alone affected the growth of *C. concisus* and some other enteric bacterial species. In addition, sodium fumarate altered the protein expression in *C. concisus*.

MATERIALS AND METHODS

Bacterial Strains Used in This Study

A total of 50 *C. concisus* strains were examined in this study. Of these, 49 strains were isolated from previous studies with ethics approval granted by the Ethics Committees of the University of New South Wales and the South East Sydney Area Health Service, Australia [HREC09237/SESIAHS 09/078, HREC08335/SESIAHS(CHN)07/48, HREC06233/SESAHS(ES)06/164, HREC 09237/SESIAHS09/078, and HREC08335/SESIAHS(CHN)07/48] (Zhang et al., 2010; Mahendran et al., 2011). *C. concisus* strain 13826 was purchased from the American Type Culture Collection. The details of the *C. concisus* strains used in this study and genomospecies, if known (Mahendran et al., 2015; Chung et al., 2016; Wang et al., 2017), are shown in **Table 1**. Additionally, four other enteric species obtained from the American Type Culture collection were included in this study as representatives of other species in the enteric environment (Bachmann, 1972; Gregory et al., 1978; Moschetti et al., 1998): *Bacteroides fragilis* ATCC 25285, *Bacteroides vulgatus* ATCC 8482, *Enterococcus faecalis* ATCC 19433, and *E. coli*.

Examination of the Effects of Sodium Fumarate and Sodium Formate on *C. concisus* Growth

We first examined whether individual supplementation of fumarate is comparable to supplementation with both sodium fumarate (Sigma-Aldrich, St. Louis, MO, United States) and sodium formate (Sigma-Aldrich), five strains of *C. concisus* were randomly chosen. These strains were first cultured on horse blood agar (HBA, Oxoid, Hampshire, United Kingdom) plates consisting of 6% defibrinated horse blood (Oxoid) in anaerobic conditions with 5% hydrogen as previously described (Lee et al., 2014). Cultures with an optical density of 0.1 at a wavelength of 595 nm (OD_{595} 0.1) were prepared (Ma et al., 2015), and 5 μL was inoculated onto HBA, HBA with 0.2% sodium formate (HBA^{form}), $HBA^{fum0.4}$, and HBA plates with both 0.2% sodium formate and 0.4% sodium fumarate ($HBA^{fum+form}$) and streaked for single colonies. After incubation, all plates were examined for

TABLE 1 | Bacterial strains used in this study.

Strain ID	Health status	Source	Genomospecies
P10CDO-S1	CD	Saliva	1
P10CDO-S2	CD	Saliva	1
P11CDO-S1	CD	Saliva	1
P19CDO-S1	CD	Saliva	1
P20CDO-S4	CD	Saliva	1
P25CDO-S3	CD	Saliva	1
P27CDO-S2	CD	Saliva	1
P26UCO-S2	UC	Saliva	1
P3UCB1	UC	Intestinal biopsy	1
P3UCLW1	UC	Fecal sample	1
P3UCO1	UC	Saliva	1
H1O1	Healthy	Saliva	1
H10O-S1	Healthy	Saliva	1
H12O-S1	Healthy	Saliva	1
H15O-S1	Healthy	Saliva	1
H17O-S1	Healthy	Saliva	1
H21O-S3	Healthy	Saliva	1
H24O-S1	Healthy	Saliva	1
H25O-S1	Healthy	Saliva	1
H26O-S1	Healthy	Saliva	1
H26O-S1	Healthy	Saliva	1
H27O-S1	Healthy	Saliva	1
H28O-S1	Healthy	Saliva	1
P1CDO2	CD	Saliva	2
P1CDO3	CD	Saliva	2
P2CDO4	CD	Saliva	2
P6CDO1	CD	Saliva	2
P12CDO-S1	CD	Saliva	2
P18CDO-S1	CD	Saliva	2
P20CDO-S2	CD	Saliva	2
P20CDO-S3	CD	Saliva	2
P21CDO-S1	CD	Saliva	2
P21CDO-S2	CD	Saliva	2
P24CDO-S2	CD	Saliva	2
P24CDO-S3	CD	Saliva	2
P27CDO-S1	CD	Saliva	2
P26UCO-S1	UC	Saliva	2
P7UCO-S2	UC	Saliva	2
P8UCO1	UC	Saliva	2
P13UCO-S3	UC	Saliva	2
P16UCO-S1	UC	Saliva	2
P16UCO-S2	UC	Saliva	2
H3O1	Healthy	Saliva	2
H7O-S1	Healthy	Saliva	2
H9O-S2	Healthy	Saliva	2
H11O-S1	Healthy	Saliva	2
H14O-S1	Healthy	Saliva	2
H22O-S1	Healthy	Saliva	2
H29O-S1	Healthy	Saliva	2
13826	Gastroenteritis	Fecal sample	2

The 50 strains of C. concisus were previously isolated from our studies. The letters P and H under Strain ID indicate a patient with IBD or a healthy control, respectively, and the following number is unique for separate individuals. UC, CD, and H under Health status indicate whether the strain was isolated from a patient with ulcerative colitis, Crohn's disease, or a healthy control, respectively. Genomospecies status known from previous studies is listed (Mahendran et al., 2015; Chung et al., 2016; Liu et al., 2018).

colony size differences by visual inspection and plate photographs were obtained through a Samsung HZ30W digital camera attached to the stereomicroscope at a magnification of 20×.

The effects of sodium fumarate and sodium formate on the growth of *C. concisus* were also quantified through CFU determination of the randomly chosen *C. concisus* strain, P26UCO-S2. P26UCO-S2 was cultured first cultured on HBA as described above before inoculation onto HBA, $HBA^{fum0.4}$, HBA^{form}, and $HBA^{fum+form}$ in triplicates and incubated for 72 h. After incubation, cultures were collected and serially diluted for bacterial enumeration using the drop plate method as previously described (Ma et al., 2015). Experiments were repeated three times.

Quantitative Assessment of the Effect of Neutralized Fumaric Acid and Neutralized Monosodium Salt on *C. concisus*

The pH of H_2O solutions containing 0.05, 0.2, 0.4, 1, and 2% fumaric acid, monosodium fumarate, or disodium fumarate was measured with a pH meter (Eutech pH700, Eutech Instrument, Singapore). The solutions of fumaric acid and monosodium fumarate were acidic and it was previously shown that low pH abolishes the viability of *C. concisus* (Ma et al., 2015). However, the ingested fumaric acid and monosodium fumarate would be neutralized in the lower gastrointestinal tract. Therefore, to determine the possible effects of the neutralized ingested fumaric acid and sodium monofumarate, neutralized solutions of fumaric acid and monosodium fumarate were examined.

Solutions of 20% (w/v) fumaric acid (Sigma-Aldrich) and monosodium fumarate (Nippon Shokubai, Osaka, Japan) were neutralized with sodium bicarbonate (Sigma-Aldrich) and used as the stock solution. *C. concisus* strains P26UCO-S1 and P2CDO4 were chosen randomly to be examined. Cultures of OD_{595} 0.1 were prepared as described in the Section "Examination of the Effects of Sodium Fumarate and Sodium Formate on *C. concisus* Growth" and 5 μL was inoculated onto HBA and HBA supplemented with 0, 0.05, 0.2, and 0.4% fumaric acid adjusted to pH 7 ($HBA^{FA0.05}$, $HBA^{FA0.2}$, and $HBA^{FA0.4}$) or monosodium fumarate adjusted to pH 7 ($HBA^{MF0.05}$, $HBA^{MF0.2}$, and $HBA^{MF0.4}$) in triplicate and spread in a radial pattern with a sterile hockey stick. Plates were incubated for 72 h and colony-forming units (CFU) were quantified as described in the Section "Examination of the Effects of Sodium Fumarate and Sodium Formate on *C. concisus* Growth." Experiments were conducted three times.

C. concisus Cultivation for the Observation of Colony Size Differences in the Presence of Sodium Fumarate

As both neutralized monosodium fumarate and neutralized fumaric acid increased growth of *C. concisus*, further experiments were performed with the neutralized final product, sodium fumarate. All 50 *C. concisus* strains were cultured and suspensions of OD_{595} 0.1 were prepared as described in the Section "Examination of the Effects of Sodium Fumarate and

Sodium Formate on *C. concisus* Growth." From these bacterial suspensions, 5 μL was streaked for single colonies onto a HBA plate and HBA plates supplemented with 0.05% ($HBA^{fum0.05}$), 0.4% ($HBA^{fum0.4}$), 1% (HBA^{fum1}), and 2% (HBA^{fum2}) sodium fumarate in triplicates and the plates were incubated for 72 h. After incubation, plates were examined and photographed as described in the Section "Examination of the Effects of Sodium Fumarate and Sodium Formate on *C. concisus* Growth."

Quantitative Assessment of the Effects of Different Concentrations of Sodium Fumarate on the Growth of *C. concisus* and Other Enteric Bacterial

From the 50 strains of *C. concisus* examined for morphological colony changes above, 7 strains were randomly selected for quantitative assessment of growth in the presence of different concentrations of sodium fumarate, including P2CDO4, P11CDO-S1, and P20CDO-S3 from patients with CD, P26UCO-S1 from a patient with UC, H12O-S1, and H17O-S1 from healthy controls and *C. concisus* strain 13826. Furthermore, the growth of *B. fragilis*, *B. vulgatus*, *E. faecalis*, and *E. coli* were also quantified.

Four different concentrations of sodium fumarate were supplemented into HBA plates, including the $HBA^{fum0.05}$, $HBA^{fum0.4}$, HBA^{fum1}, and HBA^{fum2} used previously in this Section. *C. concisus* strains were first cultured on HBA plates as described in the Section "Examination of the Effects of Sodium Fumarate and Sodium Formate on *C. concisus* Growth." Bacterial cells were harvested, diluted to an OD_{595} of 0.1 and 5 μL of the suspension was spread plated onto HBA plates and $HBA^{fum0.05}$, $HBA^{fum0.4}$, HBA^{fum1}, and HBA^{fum2} plates in triplicate and incubated for 72 h. After incubation, cultures were collected and serially diluted for bacterial enumeration using the drop plate method as previously described (Ma et al., 2015). Experiments were repeated three times.

Bacteroides fragilis, *B. vulgatus*, *E. faecalis*, and *E. coli* were cultured as previously described (Liu et al., 2017). Bacterial pellets were then collected, diluted and cultured as described for all *C. concisus* strains above. Due to their faster growth rates, these species were incubated for 24 h instead of 48 h (Liu et al., 2017).

Quantitative Assessment of the Growth of *C. concisus* When Cultured With and Without Sodium Fumarate Over 96 h

As the previous experiment found that a concentration of 0.4% sodium fumarate was preferable for the growth of most strains, this concentration was then used to examine the growth of *C. concisus* over a period of 94 h to examine the effect of sodium fumarate on growth rate. Three strains were randomly chosen to observe their growth when cultured on media with and without sodium fumarate.

All three strains were cultured on HBA plates as previously described and bacteria were collected. To prevent bacterial oversaturation of the agar plates, a lower OD_{595} of bacteria were used. Each strain was diluted to an OD_{595} of 0.025 and 5 μL of each were spread plated onto HBA and $HBA^{fum0.4}$ in triplicates for enumeration at 0, 12, 24, 48, 72, and 96 h. After incubation for the respective amount of time, plates were removed from incubation and the bacteria were collected and quantified as previously described (Ma et al., 2015). Additionally, wet mounts of each strain were also prepared at each time point to observe the morphology of *C. concisus*.

Quantification of the Growth of 50 Strains of *C. Concisus* on Media With and Without Sodium Fumarate

Based on the results of the Section "Quantitative Assessment of the Growth of *C. concisus* When Cultured With and Without Sodium Fumarate Over 96 h," the biggest effect of 0.4% sodium fumarate on *C. concisus* could be observed at 24 h; consequently, this condition was utilized to quantify the growth of all *C. concisus* strains in this study. All strains of *C. concisus* were cultured as per the Section "Examination of the Effects of Sodium Fumarate and Sodium Formate on *C. concisus* Growth" and diluted to an OD_{595} of 0.025. From the bacterial suspensions, 5 μL were spread plated onto HBA and $HBA^{Fum0.4}$. Plates were incubated for 24 h before bacteria were harvest and serially diluted for enumeration as described previously in the Section "Examination of the Effects of Sodium Fumarate and Sodium Formate on *C. concisus* Growth."

Analysis of the Proteins of *C. concisus* 13826 When Cultured With and Without Sodium Fumarate

From the *C. concisus* strains examined, strain 13826 (accession number: NC_009802) was chosen as the representative strain for protein analysis to examine changes in protein profile when cultured with and without sodium fumarate as its genome is readily accessible from the National Center for Biotechnology Information. First, *C. concisus* strain 13826 was cultured on HBA for 48 h. The bacterial cells were then collected, prepared and cultured on HBA and $HBA^{fum0.4}$ as described in the Section "Examination of the Effects of Sodium Fumarate and Sodium Formate on *C. concisus* Growth."

After incubation, bacteria were harvested and washed according to the Section "Examination of the Effects of Sodium Fumarate and Sodium Formate on *C. concisus* Growth." Whole cell lysate proteins were prepared by three cycles of rapid freezing and thawing. Protein concentration was determined via the bicinchoninic acid method according to manufacturer's instructions (Pierce, Rockford, IL, United States) and 20 μg of proteins were subjected to SDS–PAGE. Mass spectrometry was performed on proteins as previously described (Ismail et al., 2012; Luu et al., 2017). Experiments were repeated three times.

Peak lists were generated using mascot daemon/extract_msn (Matrix Science, London, United Kingdom), with default parameters, and all MS/MS spectra were searched against the NCBIprot_3_11_15 database (68653660 entries, specificity selected for bacteria) using MASCOT (version 2.5.1, Matrix Science, London, United Kingdom). Data search parameters were as follows: precursor tolerance was 4 ppm, and product

ion tolerances were ± 0.4 Da; oxidation of methionine, carbamidomethyl alkylation of cysteine, and propionamide on cysteine was specified as a variable modification, enzyme specificity was trypsin and one missed cleavage was possible.

Scaffold Q+ (v.4.7.3, Proteome software, Portland, OR, United States) was used to validate peptide and protein identities (Searle, 2010). Peptide identifications were accepted if they could be established at greater than 95% probability by the Protein Prophetalgorithm (Nesvizhskii et al., 2003). Proteins were accepted if they could be established at greater than 99% probability and contained at least 2 identified peptides. Only proteins present in two or more biological replicates were included. Normalized spectral counts in Scaffold provided an estimation of relative protein quantification. Protein function was derived from the eggNOG database (Muller et al., 2010).

Statistical Analysis

The comparisons of CFU and total protein variation of *C. concisus* strains cultured on the varying media with the CFU on the control HBA plates were obtained through *t*-tests. To compare relative abundances of proteins, *t*-tests were also performed with multiple test correction using the Benjamini–Hochberg method (Benjamini and Hochberg, 1995). A *p*-value of less than 0.05 was considered significant.

RESULTS

Comparison of *C. concisus* Growth on HBA Plates Supplemented With Sodium Fumarate Alone With the Combined Supplementation of Sodium Fumarate and Sodium Formate

To investigate the effects of sodium fumarate and sodium formate on colony size, the growth of five *C. concisus* strains were examined. In all five strains, there was a visible decrease in colony size on HBA^{form} in comparison with HBA, while all *C. concisus* on $HBA^{fum0.4}$ showed an increased colony size. On $HBA^{fum+form}$, there was no visible difference in colony sizes compared to HBA plates.

To further investigate the effects of the supplements, the CFU of strain P26UCO-S2 was quantified. The CFU of P26UCO-S2 when supplemented with 0.4% sodium fumarate and 0.2% sodium formate was not significantly different to HBA. When cultured on $HBA^{fum0.4}$, the CFU increased significantly ($p < 0.05$) in comparison with HBA. On HBA^{form}, the CFU showed a significant decrease ($p < 0.05$ in comparison with HBA) (**Figure 1**).

Measurement of *C. concisus* Growth on HBA Plates Supplemented With Neutralized Fumaric Acid or Neutralized Monosodium Fumarate

The pH of different concentrations of fumaric acid, monosodium fumarate, and sodium fumarate were measured and are shown in **Table 2**. *C. concisus* strains P26UCO-S1 and P2CDO4 representing *C. concisus* genomospecies 1 and 2, respectively, were examined for growth on HBA plates supplemented with neutralized monosodium fumarate or neutralized fumaric acid. Both strains had significantly increased growth on $HBA^{MF0.05}$ compared with HBA ($p < 0.05$), with P26UCO-S1 showing a fold change of 2.5 and P2CDO4 showing a fold change of 1.3. On $HBA^{MF0.2}$, both P26UCO-S1 and P2CDO4 showed a further increase in fold change, with a fold change of 5.6 and 4, respectively. The greatest increase in fold change could be seen on $HBA^{MF0.4}$ for both strains, with P26UCO-S1 increasing to 20.1 times the CFU on HBA and P2CDO4 increasing 23.7 times (**Figure 2**).

TABLE 2 | The pH of fumaric acid and its sodium salts at different concentrations.

Concentration of solute	Fumaric acid	Monosodium fumarate	Sodium fumarate
0.05%	2.96	3.81	7.54
0.2%	2.76	3.67	7.22
0.4%	2.55	3.68	7.75
1%	2.5	3.65	7.76
2%	2.48	3.6	7.92

Fumaric acid, monosodium fumarate, and sodium fumarate solutions were made by suspension in distilled water at concentrations of 0.05, 0.2, 0.4, 1, and 2%.

When cultured on $HBA^{FA0.05}$, both *C. concisus* strains P26UCO-S1 and P2CDO4 showed a significant increase in CFU compared to on HBA plates, with fold changes of 3 and 9.1, respectively. As the concentration of neutralized fumaric acid increased, the CFU of P26UCO-S1 also increased with fold changes of 6.4 on $HBA^{FA0.2}$ and 10.4 on $HBA^{FA0.4}$. Similarly, for P2CDO4, the fold changes on $HBA^{FA0.2}$ and $HBA^{FA0.4}$ were significantly increased P26UCO-S1 and P2CDO4, at 11.7 and 43.3, respectively.

Changes in Colony Size on HBA Plates With and Without Sodium Fumarate

When cultured on $HBA^{fum0.05}$, 27 of the 50 *C. concisus* strains examined showed an increase in colony size compared to the same strain on HBA. The remaining 23 stains showed no visible change in colony size compared to the same strain on HBA. On $HBA^{fum0.4}$, all strains of *C. concisus* tested showed visibly larger colonies as compared to that of the same strain on HBA, although the colony size increments varied in different strains. When cultured on HBA^{fum1}, 45 of 50 strains examined noticeably decreased in colony size as compared to that of the same strain cultured on HBA. From the five remaining strains, four showed no obvious difference in colony size compared to the controls and the final strain was P11CDO-S1, which showed an increase in colony size compared to the control. When the concentration of sodium fumarate was increased to 2%, 6 of the examined strains showed complete inhibition of growth. All of the remaining strains had a clearly smaller colony size in comparison with HBA. Photos of the results of a randomly chosen representative strain, H14O-S1, are shown in **Figure 3**.

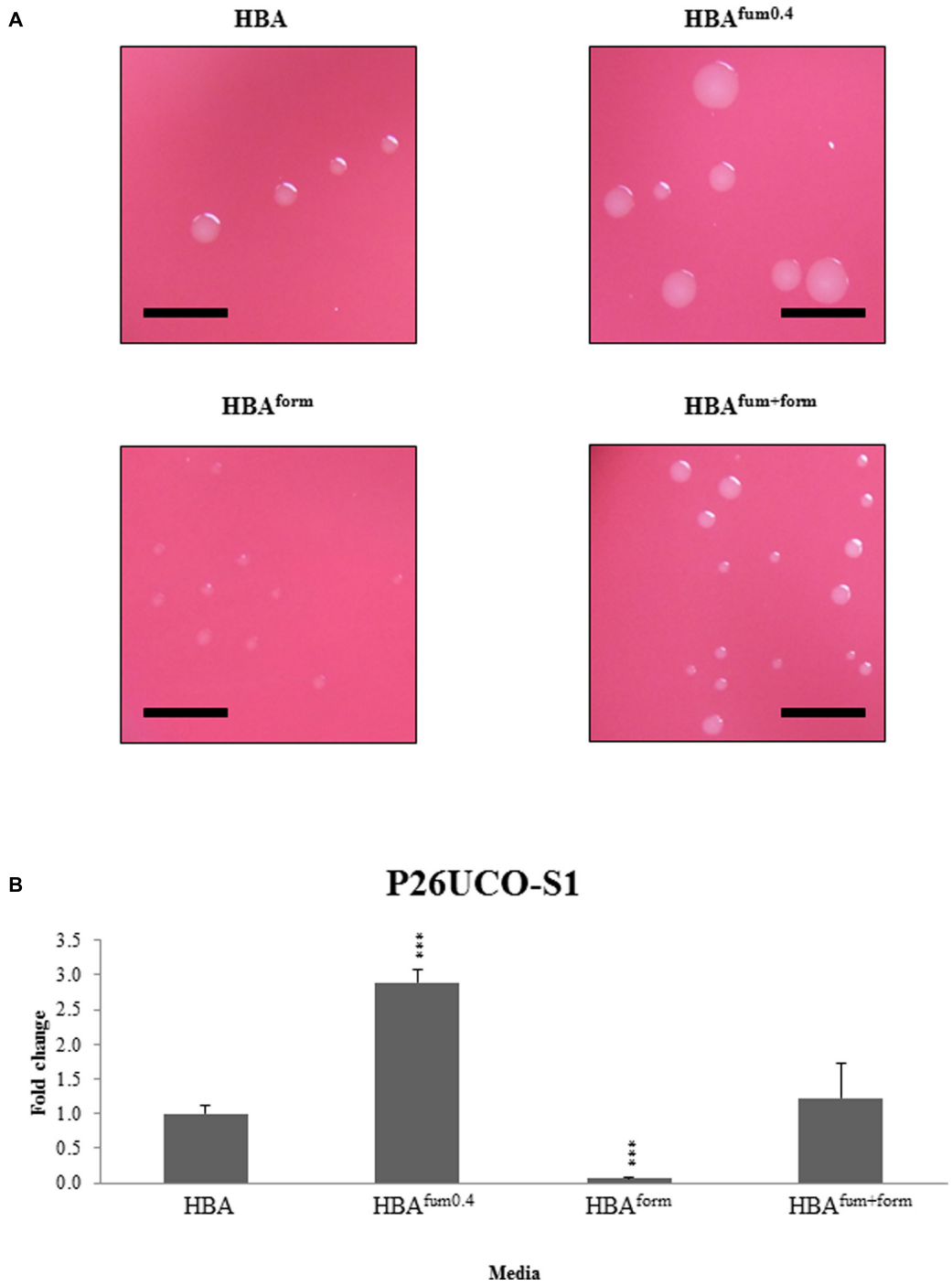

FIGURE 1 | Quantitative analysis of sodium fumarate and sodium formate on the growth of *C. concisus*. **(A)** Colonies of representative *C. concisus* strain P26UCO-S2. A total of five *C. concisus* strains were streaked onto HBA plates, HBA plates with 0.4% sodium fumarate ($HBA^{fum0.4}$), HBA with 0.2% sodium formate (HBA^{form}), and HBA with both sodium fumarate and sodium formate ($HBA^{fum+form}$). After incubation the colonies were observed under a stereomicroscope and photographs were captured at 20× magnification. Scale bars represent 5 mm. Of the five strains examined, all showed increased colony size when cultured on $HBA^{fum0.4}$ and decreased colony size on HBA^{form}. All strains showed no visible difference in colony size when cultured on $HBA^{fum+form}$. **(B)** The fold changes in CFU of *C. concisus* strain P26UCO-S2 cultured on HBA, $HBA^{fum0.4}$, HBA^{form}, and $HBA^{fum+form}$ is shown. Fold changes were calculated relative to the CFU of the same strain cultured on plates without fumarate or formate from the mean of quadruplicate counts on each media. *** indicates $p < 0.001$.

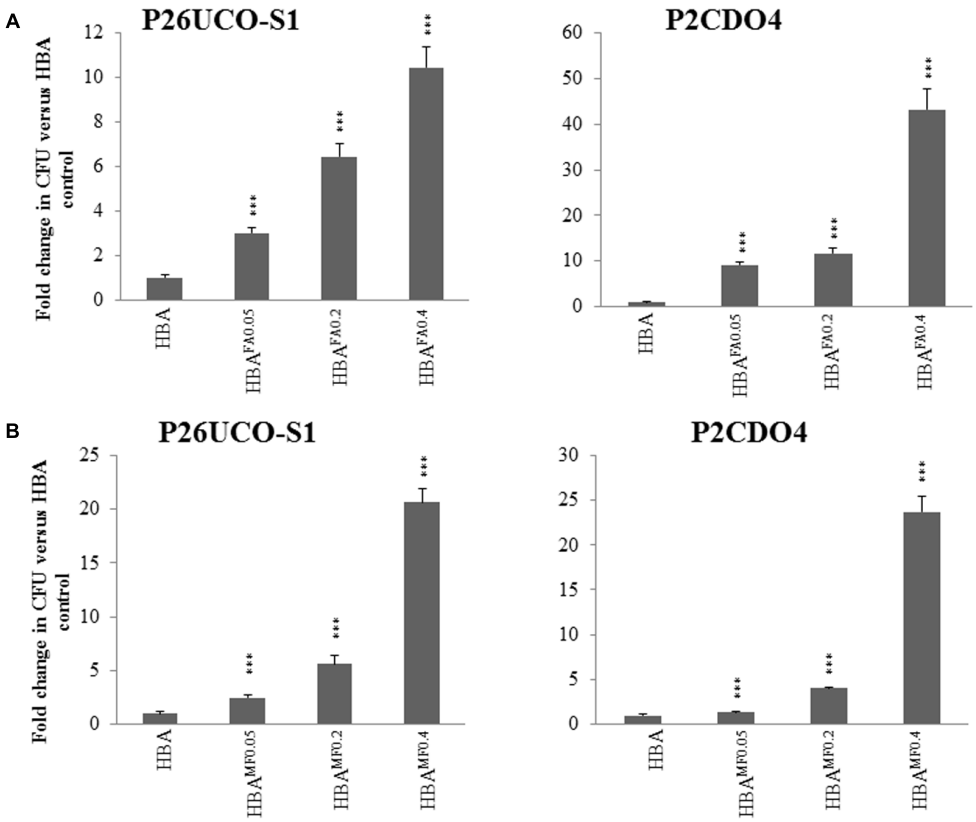

FIGURE 2 | Quantitative analysis of neutralized fumaric acid and neutralized monosodium fumarate on the growth of *C. concisus*. P26UCO-S1 and P2CDO4 are both *C. concisus* strains, the former isolated from a patient with UC and the latter from a patient with CD. Both strains were quantified after growth on HBA plates supplemented with varying concentrations of **(A)** neutralized fumaric acid and **(B)** neutralized monosodium fumarate. Fold changes were calculated relative to the CFU of the same strain cultured on plates without supplementation from the mean of quadruplicate counts on each media. *** indicates $p < 0.001$.

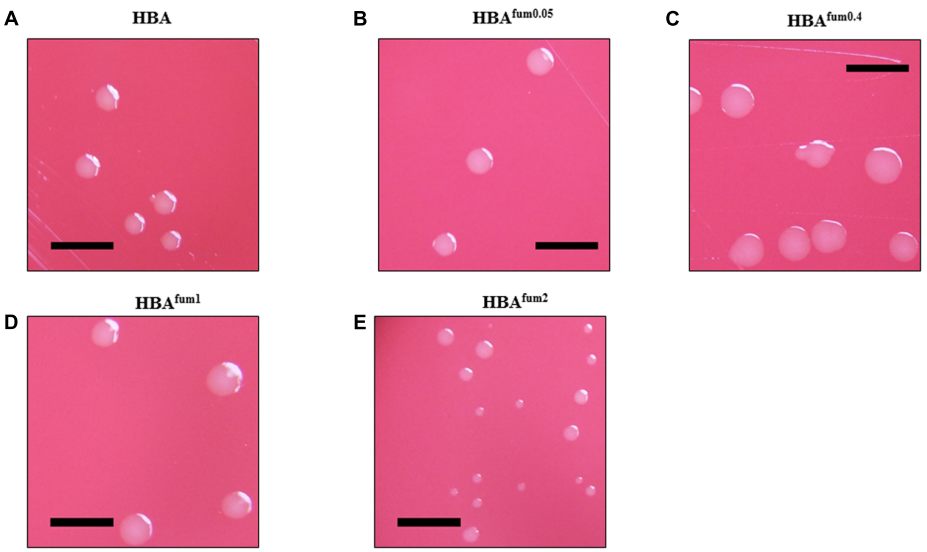

FIGURE 3 | The effects of sodium fumarate on the colony size of *C. concisus*. Colonies of representative *C. concisus* strain H14O-S1 on HBA plates **(A)** without supplementation and **(B–E)** with supplementation of sodium fumarate at 0.05, 0.4, 1, and 2%, respectively. Scale bars represent 5 mm. A total of 50 *C. concisus* strains were streaked onto HBA, $HBA^{fum0.05}$, $HBA^{fum0.4}$, HBA^{fum1}, and HBA^{fum2}. After incubation, colonies were observed under a stereomicroscope and photographs were captured at 20× magnification.

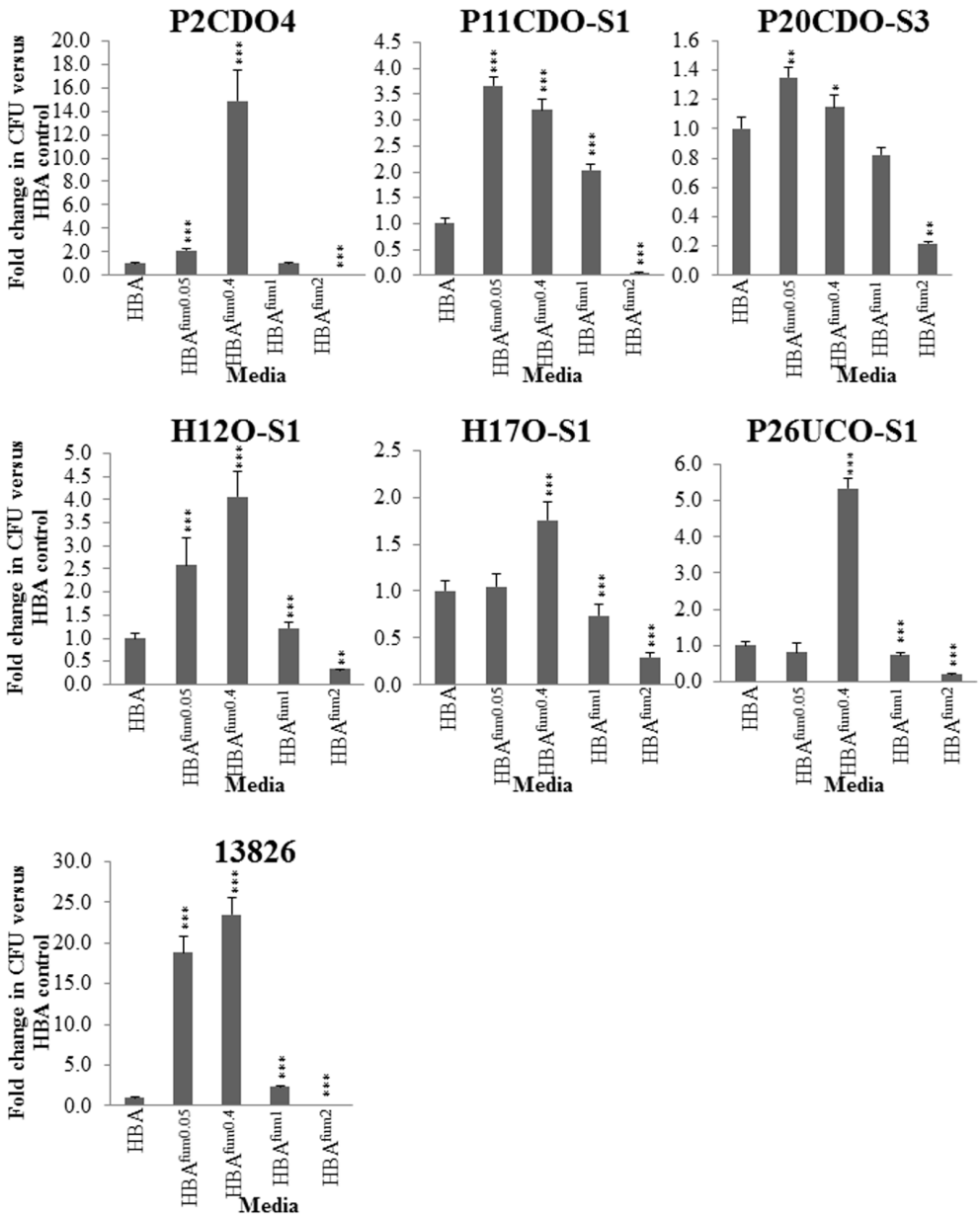

FIGURE 4 | Quantitative analysis of *C. concisus* cultured in the presence of varying concentrations of sodium fumarate. Seven randomly chosen strains of *C. concisus* were grown on HBA plates with varying concentrations of sodium fumarate; the number following Fum indicates the percentage of sodium fumarate in the HBA plate. Each strain was then collected and the CFU were enumerated. Fold changes were calculated relative to the CFU of the same strain cultured on HBA from quadruplicate counts. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

Quantitative Comparison of the Effects of Sodium Fumarate on *C. concisus* Growth

To further characterize the impact of sodium fumarate on the growth of *C. concisus*, the growth of seven strains of *C. concisus* were quantified after culturing on HBA supplemented with varying concentrations of sodium fumarate. When cultured on $HBA^{fum0.05}$, the CFU of five *C. concisus* strains (P2CDO4, P11CDO-S1, P20CDO-S3, H12O-S1, and 13826) were significantly increased compared to that on HBA (**Figure 4**). The CFU of *C. concisus* strain 13826 was increased significantly ($p < 0.05$), with an 18.8-fold change compared to its CFU on HBA plates. The remaining 4 strains had fold changes of between 1.3 and 2.7 on $HBA^{fum0.05}$ compared to the same strain on HBA. All strains of *C. concisus* examined showed a significant increase in CFU ($p < 0.05$) when cultured on $HBA^{fum0.4}$ as compared to HBA, with fold changes of 1.2- to 24.5-fold in CFU when cultured on $HBA^{fum0.4}$ as compared to HBA. When cultured on HBA^{fum1}, strains showed a differential

response with strains 13826, H12O-S1 and P11CDO-S1 showing an increase of 1.2- and 3.2-fold in CFU, respectively, P26UCO-S1 and H17O-S1 both showing 0.7-fold decrease in CFU, and P2CDO4 and P20CDO-S3 showing no change ($p > 0.05$) in CFU as compared to that on HBA (**Figure 4**). However, when cultured on HBA^{fum2}, all examined strains of *C. concisus* consistently showed a decrease in CFU as compared to the same strain cultured without sodium fumarate, with fold changes between 0.05 and 0.3.

Effects of Sodium Fumarate on the Growth of Other Enteric Species

For all the examined enteric species, there was no significant change between the CFU when cultured on $HBA^{fum0.05}$ as compared to HBA. *E. coli* and *E. faecalis* showed similar results on $HBA^{fum0.4}$ with growth significantly increased ($p < 0.05$) compared to HBA. *B. vulgatus* showed no change in CFU (**Figure 5**). When the concentration of sodium fumarate is increased to 1%, *E. coli*, *E. faecalis*, and *B. vulgatus* showed a significant increase ($p < 0.05$) in CFU. On HBA^{fum2}, *E. coli* and *B. vulgatus* displayed a significant decrease in CFU. *E. faecalis* showed a significantly increased ($p < 0.05$) CFU compared to HBA plates.

At all concentrations of sodium fumarate examined, *B. fragilis* showed no significant difference in CFU compared to the CFU on HBA plates ($p > 0.05$).

Growth Curve of *C. concisus* Cultured on HBA and $HBA^{Fum0.4}$

In the growth curves of all three strains of *C. concisus* examined, marked increase in CFU could be observed when cultured on $HBA^{fum0.4}$ as compared to HBA as early as after

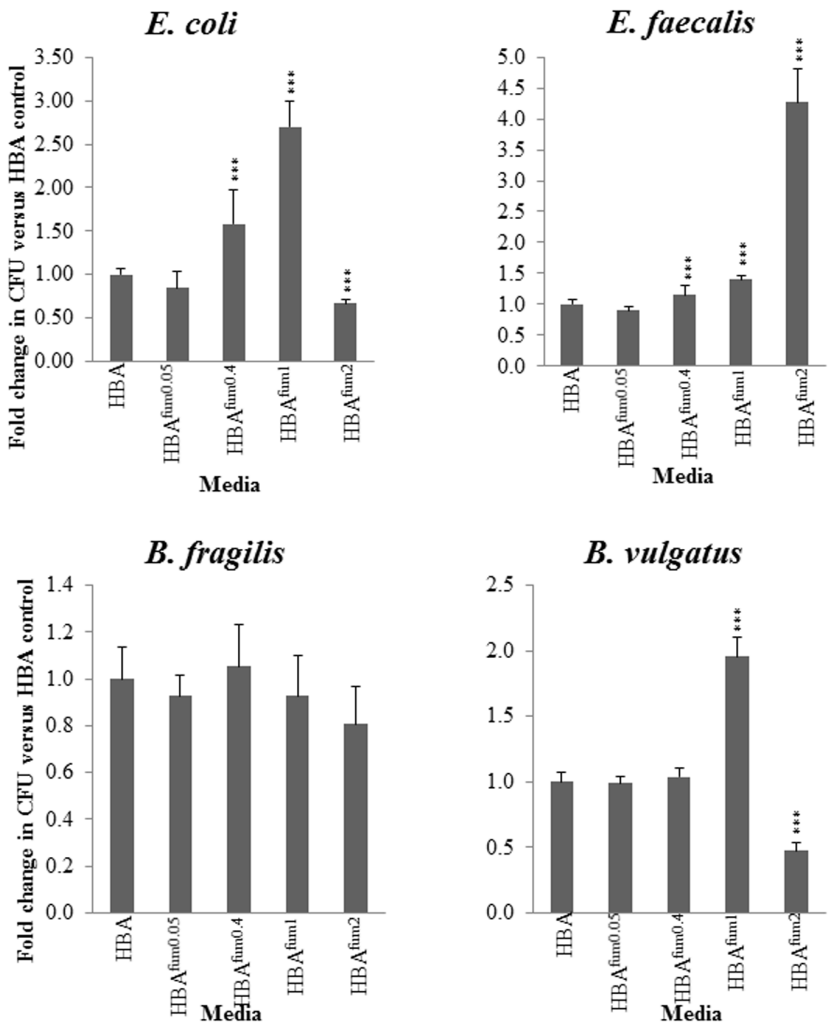

FIGURE 5 | Quantitative effects of varying concentrations of sodium fumarate on enteric bacterial species. *B. fragilis*, *B. vulgatus*, *E. faecalis*, and *E. coli* were chosen as representative bacteria of the enteric environment. Each species was cultured on HBA plates with varying concentrations of sodium fumarate; the number following Fum indicates the percentage of sodium fumarate in the HBA plate. Bacteria were then collected and the CFU were quantified. Fold changes were calculated relative to the CFU of the same species cultured on HBA from quadruplicate counts. *** indicates $p < 0.001$.

12 h of incubation (**Figure 6**). All three strains maintained a higher overall CFU when cultured on HBA$^{fum0.4}$ as compared to HBA at the same incubation time. While there was an increase in CFU on HBA$^{fum0.4}$, the trend of CFU increase and decrease followed the same pattern as that on HBA in all three strains. From the wet mount preparations, no difference in bacterial morphology could be seen in the same strains on HBA as compared to HBA$^{fum0.4}$ at the same time point.

Fold Change in Growth of 50 Strains of *C. concisus* When Cultured on Media With and Without Sodium Fumarate

All 50 strains of *C. concisus* examined displayed an increase in growth when cultured on HBA$^{fum0.4}$ as compared to HBA. The increase in CFU varied depending on the strain with the lowest fold change in comparison with HBA on HBA$^{fum0.4}$ being 1.1 ± 0.1 for P20CDO-S3 and the highest fold change being 28.8 ± 1.2 for P10CDO-S2 (**Table 3**). There was no significant difference in the average CFU fold changes from *C. concisus* strains isolated from patient groups (CD nor UC) versus those isolated from healthy controls ($p > 0.05$). No difference in average CFU fold changes were also observed between genomospecies 1 or 2 *C. concisus* ($p > 0.05$).

Comparison of Proteins Identified in the Whole Cell Lysates of *C. concisus* 13826 Cultured on HBA With and Without Sodium Fumarate

C. concisus strain 13826 was used to examine protein expression when cultured on HBA and HBA$^{fum0.4}$. In total, 391 proteins were

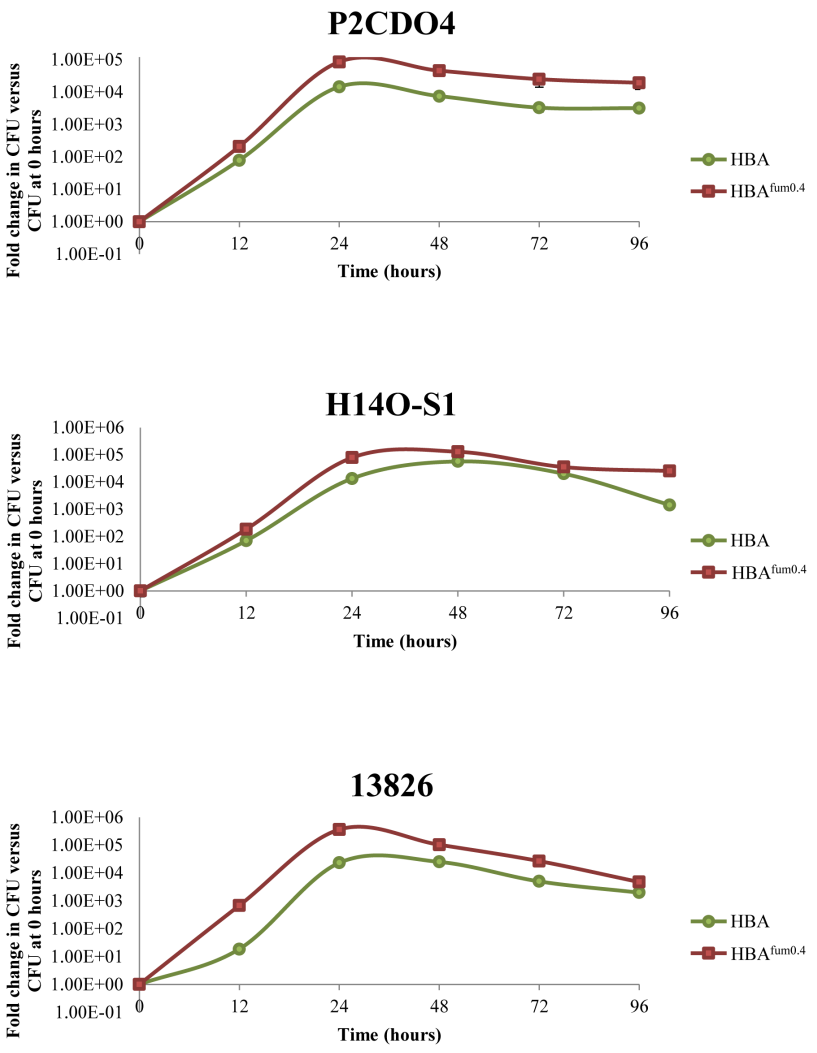

FIGURE 6 | Growth of *C. concisus* strains P2CDO4, H14O-S1, and 13826 cultured on HBA$^{fum0.4}$ and HBA over 96 h. A. Each strain was cultured on HBA and HBA with 0.4% sodium fumarate (HBA$^{fum0.4}$) and the total bacteria were enumerated at 0, 12, 24, 48, 72, and 96 h after incubation. Results are represented as a CFU fold change relative to the CFU at 0 h of incubation from quadruplicate counts.

TABLE 3 | CFU fold changes of all 50 strains of *C. concisus* cultured on HBA$^{fum0.4}$ compared to HBA plates.

Strain ID	Genomospecies	Fold change (mean ± SD)
P10CDO-S1	1	12.7 ± 1.3
P10CDO-S2	1	28.8 ± 1.2
P11CDO-S1	1	3.2 ± 0.3
P19CDO-S1	1	1.4 ± 0.0
P20CDO-S4	1	2.3 ± 0.3
P25CDO-S3	1	4.0 ± 0.6
P27CDO-S2	1	16.0 ± 1.9
P26UCO-S2	1	1.6 ± 0.2
P3UCB1	1	17.4 ± 1.9
P3UCLW1	1	3.4 ± 0.1
P3UCO1	1	17.1 ± 2.2
H1O1	1	2.2 ± 0.2
H10O-S1	1	6.5 ± 1.3
H12O-S1	1	1.8 ± 0.4
H15O-S1	1	6.5 ± 1.0
H17O-S1	1	1.3 ± 0.2
H21O-S3	1	20.8 ± 2.0
H24O-S1	1	5.5 ± 0.3
H25O-S1	1	2.1 ± 0.1
H26O-S1	1	9.4 ± 1.5
H26O-S1	1	14.5 ± 0.6
H27O-S1	1	13.6 ± 0.7
H28O-S1	1	22.7 ± 1.4
P1CDO2	2	18.2 ± 11.6
P1CDO3	2	8.5 ± 1.6
P2CDO4	2	5.9 ± 0.6
P6CDO1	2	11.9 ± 2.1
P12CDO-S1	2	10.2 ± 0.8
P18CDO-S1	2	2.0 ± 0.4
P20CDO-S2	2	5.2 ± 0.6
P20CDO-S3	2	1.1 ± 0.1
P21CDO-S1	2	2.8 ± 0.3
P21CDO-S2	2	4.3 ± 0.8
P24CDO-S2	2	7.1 ± 1.4
P24CDO-S3	2	1.4 ± 0.3
P27CDO-S1	2	3.1 ± 0.2
P26UCO-S1	2	14.3 ± 1.6
P7UCO-S2	2	5.8 ± 0.5
P8UCO1	2	1.2 ± 0.1
P13UCO-S3	2	15.8 ± 2.8
P16UCO-S1	2	11.4 ± 1.8
P16UCO-S2	2	10.1 ± 0.8
H3O1	2	6.9 ± 1.2
H7O-S1	2	2.4 ± 0.3
H9O-S2	2	3.6 ± 0.5
H11O-S1	2	18.7 ± 3.4
H14O-S1	2	2.0 ± 0.3
H22O-S1	2	2.2 ± 0.5
H29O-S1	2	10.5 ± 2.3
13826	2	15.5 ± 0.5

Fold changes were calculated from the CFU of the same strain on HBA$^{fum0.4}$ plates divided by the CFU on HBA plates. Results displayed are the mean fold changes ± the standard deviation (SD). The genomospecies which each strain belongs to is listed beside the Strain ID.

identified, with 355 being commonly expressed by *C. concisus* cultured on HBA and HBA$^{fum0.4}$. On HBA, there were 9 unique proteins that were not detected when cultured on HBA$^{fum0.4}$ (**Table 4**). When *C. concisus* was cultured on HBA$^{fum0.4}$, there were 27 unique proteins identified (**Table 5**).

TABLE 4 | Proteins only identified in *C. concisus* 13826 when cultured on HBA plates without fumarate.

Protein	Accession number	Functional category
Heavy metal translocating P-type ATPase	EAT98303.2	Inorganic ion transport and metabolism
Peptidylprolyl isomerase	WP_002940164.1	Post-translational modification, protein turnover, and chaperones
Peptidase	WP_012000970.1	Amino acid transport and metabolism
Flavocytochrome c	WP_012001109.1	Energy production and conversion
C4-dicarboxylate ABC transporter	WP_012140574.1	Carbohydrate transport and metabolism
Hypothetical protein	WP_012140660.1	Cell wall/membrane/ envelope biogenesis
LemA protein	WP_021084760.1	Function unknown
C4-dicarboxylate ABC transporter	WP_048809748.1	Energy production and conversion
UDP-N-acetylmuramate-alanine ligase	WP_048809780.1	Signal transduction mechanisms

The normalized total spectral count was used to quantitatively compare protein expression between *C. concisus* 13826 cultured on HBA and HBA$^{fum0.4}$. Of the 355 proteins detected in both conditions, 31 proteins displayed a significant difference in fold change ($p < 0.05$, **Table 6**). Of these, 14 proteins were detected to be significantly less abundant in *C. concisus* when cultured on HBA$^{fum0.4}$ as compared to the same *C. concisus* strain cultured on HBA, and the remaining 17 proteins were significantly more abundant (**Table 6**).

DISCUSSION

We investigated the effects of sodium fumarate on the growth of IBD associated bacterial species *C. concisus* and other enteric species. Additionally, protein expression differences when cultured on HBA and HBA$^{fum0.4}$ were also examined in *C. concisus*. We found that sodium fumarate supplementation into culture media alone affected the growth of *C. concisus* and some other enteric bacterial species. In addition, sodium fumarate altered the protein expression in *C. concisus*.

Fumaric acid is a widely used food additive, present in a variety of food products such as candy, jelly, juices, and flat breads. Both fumaric acid and monosodium fumarate are acidic (**Table 2**), which would inhibit the growth of *C. concisus* as shown by our previous study (Ma et al., 2015). However, the consumed fumaric acid will be neutralized in the intestinal tract. As the pH increases, the dissociation of fumaric acid causes a shift to monosodium fumarate and then ultimately to sodium fumarate at neutral pH. The human small intestine has

TABLE 5 | Proteins only identified in *C. concisus* 13826 when cultured on $HBA^{fum0.4}$.

Protein	Accession number	Functional category
Cell division protein FtsA	WP_002940409.1	Cell cycle control, cell division, chromosome partitioning
ATP-dependent protease ATP-binding subunit HslU	WP_002940444.1	Post-translational modification, protein turnover, and chaperones
Twitching motility protein PilT	WP_002941386.1	Cell motility, intracellular trafficking, secretion, and vesicular transport
3-isopropylmalate dehydratase small subunit	WP_002941843.1	Amino acid transport and metabolism
3-isopropylmalate dehydrogenase	WP_002941890.1	Amino acid transport and metabolism
Elongation factor P	WP_004317246.1	Translation, ribosomal structure and biogenesis
Cation ABC transporter substrate-binding protein	WP_004317362.1	Inorganic ion transport and metabolism
DNA-binding response regulator	WP_009294394.1	Signal transduction mechanisms
Amino acid ABC transporter substrate-binding protein	WP_012001169.1	Amino acid transport and metabolism
Competence/damage-inducible domain-containing protein	WP_012001188.1	Function unknown
Thioredoxin	WP_012001226.1	Post-translational modification, protein turnover, and chaperones
Hypothetical protein	WP_012001228.1	Function unknown
6,7-dimethyl-8-ribityllumazine synthase	WP_012001235.1	Coenzyme transport and metabolism
Iron ABC transporter ATP-binding protein	WP_012001386.1	Inorganic ion transport and metabolism
Hypothetical protein	WP_012001503.1	Function unknown
3,4-dihydroxy-2-butanone-4-phosphate synthase	WP_012139903.1	Coenzyme transport and metabolism
UDP-glucose 4-epimerase	WP_012140196.1	Cell wall/membrane/envelope biogenesis
Hypothetical protein	WP_012140308.1	Function unknown
Hypothetical protein	WP_012140384.1	Function unknown
Membrane protein	WP_012140437.1	Post-translational modification, protein turnover, and chaperones
ABC transporter substrate-binding protein	WP_012140548.1	Inorganic ion transport and metabolism
Superoxide dismutase	WP_012140576.1	Inorganic ion transport and metabolism
Transcription elongation factor GreA	WP_035142507.1	Transcription
Phosphoribosylformylglycinamidine synthase	WP_048809794.1	Nucleotide transport and metabolism
Hypothetical protein	WP_048809867.1	Function unknown
Endoribonuclease	WP_048809890.1	Translation, ribosomal structure and biogenesis
Protein-export membrane protein SecD	WP_054196430.1	Intracellular trafficking, secretion, and vesicular transport

a pH of 6.63 ± 0.53 (Evans et al., 1988); thus, the prevailing fumarate salt is likely to be sodium fumarate. The sodium fumarate in this study therefore represents the neutralized product of fumaric acid and monosodium fumarate in the intestinal tract.

Sodium fumarate at concentrations between 0.05 and 0.4% increased the growth of *C. concisus*. This finding suggests that fumaric acid from dietary sources may enhance the growth of *C. concisus* in the intestinal tract where it is neutralized. Sodium fumarate, although used less frequently as a food additive, may also enhance the growth of *C. concisus* in the gastrointestinal tract. As *C. concisus* was previously shown to be associated with IBD and that virulent strains could damage the intestinal epithelial barrier (Nielsen et al., 2011; Ismail et al., 2012), the data from our study suggests that patients with IBD should consider avoiding excessive consumption of foods containing fumaric acid or its sodium salts. *C. concisus* consists of two genomospecies and each genomospecies contains diverse strains (Chung et al., 2016). In our study, 0.4% sodium fumarate increased the growth of all *C. concisus* strains examined, with differences in the increments dependent on strain (**Table 3**).

At concentrations of 1% sodium fumarate, the growths of some *C. concisus* strains were inhibited, and at a concentration of 2%, the growth of all strains examined was inhibited. However, these concentrations of sodium fumarate are unlikely to be reach in the intestinal tract from dietary source.

Despite the association between *C. concisus* and IBD, the isolation rates of *C. concisus* from enteric samples are low (Mahendran et al., 2011; Mukhopadhya et al., 2011). Our finding that 0.4% sodium fumarate increases the growth of all *C. concisus* strains suggest that it can be used to enhance the isolation of *C. concisus* from enteric samples. We also found that 0.4% sodium fumarate affected *C. concisus* protein expression. Most of the proteins that decreased in expression when *C. concisus* was cultured on $HBA^{fum0.4}$ were involved in metabolism and most of the proteins with increased expression were involved in nutrient acquisition (**Tables 4**, **5**, **6**). These findings indicate that sodium fumarate has increased the growth most likely by affecting energy generation.

We also examined the effects of sodium fumarate on the growth of additional enteric bacterial species including *B. fragilis*, *B. vulgatus*, *E. faecalis*, and *E. coli*. In general, sodium fumarate showed less effect on the growth of these bacterial species as compared to *C. concisus*. For example, sodium fumarate did not affect the growth of *B. fragilis* at all concentrations tested, and a concentration of 0.05% sodium fumarate did not affect the growth of all 4 enteric species. Although sodium fumarate increased the growth of *E. coli* and *E. faecalis* at a concentration of 0.4%, the fold change was only 1.58 and

TABLE 6 | Proteins with a significant changed of expression profile when cultured on HBA plates and on $HBA^{fum0.4}$.

Protein	Accession number	*t*-Test ($p < 0.05$)	Fold change	Functional category
Flavocytochrome c	WP_012140366.1	0.0002	0.06	Energy production and conversion
Thiosulfate reductase	WP_012140134.1	0.0022	0.07	Energy production and conversion
Dehydrogenase	WP_012140508.1	0.0097	0.2	Energy production and conversion
Trimethylamine-N-oxide reductase	WP_012001034.1	0.0001	0.3	Energy production and conversion
Aryl-sulfate sulfotransferase	WP_012140335.1	0.0059	0.3	Function unknown
L-asparaginase	WP_012140623.1	0.0002	0.4	Amino acid transport and metabolism
Methyl-accepting chemotaxis protein	WP_012001460.1	0.0058	0.4	Cell motility, signal transduction mechanisms
Flavocytochrome c	WP_004317416.1	0.0025	0.5	Energy production and conversion
Peptidase M20	WP_012001703.1	0.0078	0.5	Amino acid transport and metabolism
Biotin attachment protein	WP_012001365.1	0.0380	0.5	Energy production and conversion
Flagellin	WP_012140492.1	0.0250	0.6	Cell motility
Hypothetical protein	WP_012001270.1	0.0160	0.7	Function unknown
DNA-binding protein	WP_012001719.1	0.0250	0.7	Replication, recombination and repair
Long-chain-fatty-acid-CoA ligase	WP_012001496.1	0.0260	0.8	Cell wall/membrane/envelope biogenesis
50S ribosomal protein L2	WP_002941522.1	0.0400	1.4	Translation, ribosomal structure, and biogenesis
30S ribosomal protein S2	WP_021091647.1	0.0015	1.5	Translation, ribosomal structure, and biogenesis
Hydrogenase accessory protein HypB	WP_002941014.1	0.0160	1.5	Transcription, post-translational modification, protein turnover, and chaperones
Hypothetical protein	WP_002941798.1	0.0260	1.5	Function unknown
50S ribosomal protein L15	WP_002941506.1	0.0250	1.6	Translation, ribosomal structure and biogenesis
Phenylalanine-tRNA ligase subunit beta	WP_048809769.1	0.0130	1.7	Translation, ribosomal structure and biogenesis
ABC transporter substrate-binding protein	WP_012001287.1	0.0150	1.7	Carbohydrate transport and metabolism
Glutamyl-tRNA amidotransferase	WP_048809879.1	0.0160	1.7	Translation, ribosomal structure and biogenesis
Peptide ABC transporter substrate-binding protein	WP_012001388.1	0.0072	1.9	Inorganic ion transport and metabolism
3-oxoacyl-[acyl-carrier-protein] reductase	WP_012140278.1	0.0160	1.9	Lipid transport and metabolism
Iron transporter	WP_009294901.1	0.0080	2.3	Inorganic ion transport and metabolism
Succinate dehydrogenase	WP_012001794.1	0.0120	2.5	Energy production and conversion
Heterodisulfide reductase subunit B	WP_012001792.1	0.0430	2.6	Energy production and conversion
Hypothetical protein	WP_012001093.1	0.0130	3.4	Function unknown
Molybdopterin molybdenumtransferase MoeA	WP_002942864.1	0.0160	5.5	Coenzyme transport and metabolism
2-Cys peroxiredoxin	WP_012140581.1	0.0058	7	Post-translational modification, protein turnover, and chaperones
(2Fe-2S)-binding protein	WP_012001793.1	0.0120	14	Energy production and conversion

The fold change reflects the spectral counts of a protein in C. concisus 13826 cultured on HBA plates divided by the spectral counts of the same protein in C. concisus 13826 cultured on $HBA^{fum0.4}$.

1.2, respectively (**Figure 5**). These results suggest that sodium fumarate from dietary source is more like to affect the growth of *C. concisus* in the intestinal tract rather than these enteric species.

As this study was performed under laboratory conditions, future studies should be conducted using animal models or in human volunteers to more accurately reflect the effects of food additives on the bacterial species in the gastrointestinal tract.

Clinical studies can also be conducted to examine whether the removal of fumaric acid and its salts from the diet of patients with IBD can help improve their condition.

In summary, the results from this study suggest that it may be prudent for patients with IBD to avoid the overconsumption of foods containing fumaric acid or its sodium salts. Additionally, $HBA^{fum0.4}$ plates can be used for the increased probability of isolating *C. concisus* from clinical samples. This was the first study showing that sodium fumarate, the neutralized product of the food additives fumaric acid and monosodium fumarate, affects the growth of IBD associated *C. concisus* in a dose- and strain-dependent manner.

ETHICS STATEMENT

No patient samples were collected in this study and all *C. concisus* strains used in this study were isolated in previous studies. Ethics approval was not required as per institutional and national guidelines.

AUTHOR CONTRIBUTIONS

RM, SY, HL, and FL performed the experiments and bioinformatics analyses. MG, SR, and RL provided an important feedback on clinical aspect. LZ and RM conceived the project. RM and LZ played a major role in writing the manuscript. All authors have read the manuscript and provided feedback. All authors have approved the final version of the manuscript.

FUNDING

This work is supported by Faculty Research Grant awarded to LZ from the University of New South Wales (Grant No: PS35329).

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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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 May 2018
doi: 10.3389/fmicb.2018.00948

Proteinaceous Secretory Metabolites of Probiotic Human Commensal *Enterococcus hirae* 20c, *E. faecium* 12a and L12b as Antiproliferative Agents Against Cancer Cell Lines

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 10 January 2018
Accepted: 23 April 2018
Published: 15 May 2018

Citation:
Sharma P, Kaur S, Kaur R, Kaur M and Kaur S (2018) Proteinaceous Secretory Metabolites of Probiotic Human Commensal Enterococcus hirae 20c, E. faecium 12a and L12b as Antiproliferative Agents Against Cancer Cell Lines. Front. Microbiol. 9:948. doi: 10.3389/fmicb.2018.00948

Cancer is the second leading cause of death worldwide and its incidence is expected to grow by almost 70% in the coming 2 decades. Recent microbiome studies in cancer mice models have shown that certain commensal bacteria play protective roles against cancer. Thus, the use of commensal microflora having anticancer activities for the treatment of cancer appears to be an attractive alternative therapeutic strategy. Lactic acid bacteria (LAB) form an integral component of commensal microflora in healthy individuals. As the vaginal ecosystem is enriched in LAB genera, we screened the vaginal LAB microflora of healthy women for their anti-proliferative abilities against various human cancer cell lines. The secreted metabolites of three enterococcal strains, *Enterococcus hirae* 20c, *Enterococcus faecium* 12a and L12b, out of 92 LAB isolates selectively inhibited the *in vitro* proliferation of various human cancer cell lines in a dose-dependent manner but had no activity against normal human peripheral blood monocytes. Further, proteinase K-treatment of the cell-free supernatant (CS) of all the three enterococci abrogated their anti-proliferative abilities, thereby showing the proteinaceous nature of the secreted metabolites in the CS. The microscopic examination of the cell lines showed that CS-treatment induced apoptosis-like morphological changes in the cancer cells. Further, the probiotic characters of the strains were studied, which showed that all the three strains had broad spectrum antimicrobial activities against various Gram-positive and Gram-negative pathogens, including *Mycobacterium smegmatis*. All the strains tolerated the gastric acidity and bile juice treatments, and had strong adhesive abilities to the colonic epithelial cell line HCT-15. Furthermore, none of the strains had any known secreted virulence factors or harbored virulence genes. This preliminary study highlights an important functional role of the commensal probiotic enterococcal strains *E. hirae* and *E. faecium* for the first time by demonstrating their anticancer properties that should be further tested in the *in vivo* mammalian models.

Keywords: probiotics, vaginal *Enterococcus*, anti-proliferative, anticancer, lactic acid bacteria

INTRODUCTION

Cancer has emerged as a leading cause of morbidity and mortality worldwide. In 2012, an estimated 14 million new cases of cancer were reported globally (Ferlay et al., 2015) and WHO (2017) predicted that in the coming 2 decades the number of cancer cases will rise by 70%. The number of cancer deaths stand at 8.8 million in 2015, making it the second leading cause of deaths worldwide. The rate of deaths in various types of cancer is in the order lung > liver > colorectal > stomach (WHO, 2017). Despite several therapeutic strategies available, the treatment of cancer still remains a formidable task. Chemotherapeutic drugs employed for the cancer treatment present considerable challenge due to their potential side-effects owing to their non-specificity toward normal cells. Moreover, in due course of treatment, cancer cells are known to develop resistance to the chemotherapeutic drugs. Therefore, there is an immense need for meticulous efforts to develop novel and safe anticancer agents. Recent progress in understanding the link between cancer and microbiome profiles of patients suggest that interventions that change the composition of the microbiome may affect the process of oncogenesis (Roy and Trinchieri, 2017; Zitvogel et al., 2017). Thus, the future anticancer therapies may combine the use of commensal-derived microorganisms or microbial products for the treatment of cancer (Zitvogel et al., 2017).

Probiotics are "live microorganisms which when administered in adequate amounts confer health benefits to the host" (FAO/WHO, 2002). Anticancer properties of few lactic acid bacteria (LAB) such as *Lactobacillus* (Murosaki et al., 2000; Gamallat et al., 2016) and *Pediococcus* (Dubey et al., 2016) are known. The various mechanisms through which probiotic LAB strains exert anticancer activities are by immunomodulation (Rafter, 2002), production of anticancer metabolites such as short chain fatty acids (Scheppach et al., 1995), bacteriocins (Kaur and Kaur, 2015), and the regulation of cell differentiation and apoptosis (Zhong et al., 2014). LAB probiotics are mostly shown to inhibit the growth of cancer cells indirectly through enhancing the adaptive immunity by inducing cytotoxic T cells (Lenoir et al., 2016), NK cells (Takagi et al., 2001), or cytokines (Seow et al., 2008). The direct effect of the secretory components of the LAB strains on inducing apoptosis of cancer cells is not much known. The probiotic strains that secrete anticancer metabolites may have additional advantage along with immunomodulatory properties and thus might be better putative anticancer agents.

Enterococcus is an important LAB genera that forms an integral part of healthy human microbiota of the gut (Noble, 1978), vagina (Brown et al., 2007), and oral cavity (Sedgley et al., 2004). Enterococci are among the earliest colonizers of the human gut (Houghteling and Walker, 2015) in the newborns. Several enterococcal strains have shown health benefits in the human clinical trials conducted for diseases such as diarrhea, irritable bowel syndrome, obesity, allergy, etc. (Franz et al., 2011). Few studies have shown the anticancer properties of enterococcal strains of food origin both *in vitro* and *in vivo* (Castro et al., 2010; Nami et al., 2015). However, not much is known about the anticancer activities of human-derived enterococcal strains. Human-derived microbial strains are known to have superior epithelial cell adhesion properties (Duary et al., 2011) and probiotic characters and therefore are successfully able to colonize the human intestinal tract (Dunne et al., 2001). Thus, the aim of the current study was to screen the cell-free supernatant (CS) of the probiotic LAB isolates from the vaginal tract of healthy women for their anti-proliferative potential against different cancer cell lines. Further, the nature and mode of action of the anti-proliferative agent in the secreted metabolites was also studied.

MATERIALS AND METHODS

Bacterial Isolates and Physico-Chemical Characterization

The LAB were isolated from the vaginal swab samples collected aseptically from the lateral vaginal wall of 40 healthy women visiting gynecology out-patient department of Lal hospital, Amritsar, India, after taking their written informed consents. The study was approved by the Institutional Human Ethics Committee. Vaginal swabs were immersed in tubes containing sterile thioglycollate broth (HiMedia Laboratories Private Limited, Mumbai) and transported to the laboratory. Broth was incubated at 37°C and 5% carbon dioxide (CO_2)-containing atmosphere in the CO_2 incubator (Astec Co. Ltd., Japan) under stationary conditions for 4 h and 10-fold serial dilutions of the samples were spread onto De Man, Rogosa, and Sharpe (MRS, HiMedia) agar-medium. The MRS plates were incubated at 37°C; 5% CO_2 atmosphere. Bacterial colonies with different morphologies were selected and preserved in 20% (v/v) glycerol-containing MRS broth at −80°C. The LAB were identified by their abilities to grow on the selective MRS media, Gram-positive staining, and catalase-negative phenotype as described in the second edition of Bergey's manual (Holt et al., 1994; Day et al., 2001). A total of 92 LAB strains were isolated, out of which 23 isolates were identified as lactobacilli and the rest were Gram-positive cocci. Further, to characterize the genera of selected cocci isolates, physico-chemical tests such as growth at temperatures 10°C and 45°C, in MRS containing 6.5% NaCl, production of gas and hydrolysis of bile esculin disks were done (Devriese et al., 2006). To obtain fresh cultures from the frozen stocks, strains were propagated twice in MRS medium at 37°C before the experiments.

Various pathogenic bacterial indicator strains used in this study were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The pathogens used were *Staphylococcus aureus* subsp. *aureus* MTCC 96, *Escherichia coli* MTCC 119, *Pseudomonas aeruginosa* MTCC 741, *Klebsiella pnueumoniae* subsp. *pneumoniae* MTCC 1704, *Shigella flexneri* MTCC 1457, *Vibrio cholerae* MTCC 3906, *Salmonella enterica* Typhimurium MTCC 733, *Listeria monocytogenes* MTCC 657, and *Mycobacterium smegmatis* MTCC 6. All the pathogenic bacterial strains, except *M. smegmatis* were grown and maintained on Brain Heart Infusion (BHI; HiMedia) medium at 37°C. *M. smegmatis* was grown aerobically at 37°C in 7H9 broth (HiMedia) supplemented with Middlebrook OADC growth supplement

(HiMedia) containing bovine serum albumin fraction V and Tween-80.

Lactobacilli isolates (Kaur et al., 2018) used as indicator cultures were isolated from stool samples of healthy children and cultured in MRS medium at 37°C in anaerobic jars.

Molecular Identification of the Enterococcal Isolates by 16S rDNA Sequencing

The genomic DNA of the three enterococcal strains was isolated according to the method described by Moore et al. (2004). Following DNA isolation, 16S rDNA was amplified by PCR using universal primers 27F Forward: 5′-AGA GTTGATCCTGGCTCAG-3′ and 1492P Reverse: 5′-TACGGCT ACCTTGTTACGACTT-3′. DNA amplification was carried out in 0.2 mL PCR tubes by using mastercycler personal (Eppendorf, Hamburg, Germany). The PCR reaction mixture (50 μL) consisted of 25 μL of 2X PCR Master Mix (3B BlackBio Biotech India Ltd., India), 1 μL of each primer (Bioserve Biotechnologies Pvt. Ltd., India), 5 μL of template DNA, and 18 μL of nuclease free water. For PCR reaction, the initial denaturation of DNA was carried for 4 min at 95°C followed by 32 cycles of amplification. The amplification cycle consisted of denaturation step at 95°C for 1 min, primer-annealing at 56°C for 1 min 30 s, and extension at 72°C for 1 min. Reactions were completed with 10 min elongation at 72°C followed by cooling to 4°C. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel at 100 V for 45 min against 100 bp step ladder. The bands were visualized with bioimaging system (GeneGenius Imaging System, Syngene Bioimaging Pvt., Ltd., India). The isolates were identified by using partial sequencing of 16S rDNA. The sequences were aligned using BLAST (NCBI) version 2 (Altschul et al., 1990). All 16S rDNA sequences were submitted to NCBI GenBank with submission numbers SUB2507370 for 12a, SUB2507390 for 20c, and SUB2507388 for L12b.

Antimicrobial Activity of CS of Enterococci

Antimicrobial potential of the CS of 92 vaginal LAB isolates was determined by agar-well diffusion method against various pathogenic indicator strains and *Lactobacillus* isolates. To prepare CS, LAB isolates were grown overnight at 37°C in MRS media in anaerobic jar. The overnight grown cultures were centrifuged at 800 × *g* for 10 min at 4°C to obtain CS, passed through 0.22-μm syringe filters and stored at 4°C till further use.

For performing agar-well diffusion assay, the protocol by Geis et al. (1983) was followed. Briefly, the indicator strains in the log phase were spread onto their respective agar media plates and the wells with 6 mm diameter were made in the inoculated plates by using a sterilized borer. Thereafter, 100 μL of CS of the LAB isolates were added to the wells and the plates were incubated at 4°C overnight to allow the CS to diffuse. The plates were then incubated at 37°C under aerobic conditions for the growth of pathogenic strains and in anaerobic jars for the growth of fecal lactobacilli strains. The zones of inhibition were measured in mm after 24 h. The strains having broad spectrum antimicrobial activity against both Gram-positive and Gram-negative pathogens were selected for further studies.

Probiotic Properties of Enterococcal Strains

Gastric and Bile Juice Tolerance

Cells from overnight culture of enterococcal isolates were harvested by centrifugation at 800 × *g* for 10 min at 4°C and washed three times with 0.1 M phosphate buffered saline (PBS; pH 7.2). The cell pellet was suspended in simulated gastric juice (SGJ) at the concentration of 1×10^8 CFU/mL and incubated at 37°C with 5% CO_2. SGJ was composed of 3.2 g/L pepsin and 2 g NaCl/L, and the pH was adjusted to 2.0 with sterile 5N HCl (Huang and Adams, 2004). Cells suspended in PBS were used as controls. Survival cell counts were determined at different time points (0, 2, and 4 h) by plating onto MRS agar plates and incubating at 37°C with 5% CO_2.

Bile salt tolerance of enterococcal isolates was determined by using method of Pereira and Gibson (2002) with some modifications. MRS broth with 0.3% (w/v) oxgall and without oxgall was inoculated with 1×10^8 CFU/mL of overnight grown cultures and incubated at 37°C; 5% CO_2. Survival cell counts were determined at different time points (0, 2, and 4 h) by plating onto MRS agar plates and incubating at 37°C with 5% CO_2. Both the experiments were performed three times in triplicates.

Biofilm Formation

Biofilm formation potential of the enterococcal isolates was estimated by crystal violet assay (Stepanović et al., 2000). The potential of enterococci to form biofilm was tested at 3 different pH values (4, 5, and 6) for different time points (24, 48, and 72 h). The optical density (OD) at wavelength 595 of overnight grown cultures was adjusted to 0.2 and 15 μL was added to the wells of 96-well plate containing 135 μL MRS broth. Plates were incubated at 37°C with 5% CO_2 for different time periods to allow the formation of biofilms. After incubation period, plates were washed thrice with 100 μL sterile PBS to remove non-adherent cells. The adhered biofilm was fixed with methanol and stained with 2% (w/v) crystal violet. The wells were washed thrice with PBS to remove excess stain. The stain was released from the biofilms with 160 μL of 33% (v/v) glacial acetic acid and absorbance of the wells was determined at wavelength 595 nm. Uninoculated MRS broth served as control. Three independent experiments were performed in triplicates. Based on the absorbance, the strains were categorized as non-biofilm producers if $OD \leq OD_C$, weak biofilm producers if $OD_C < OD \leq 2OD_C$, moderate biofilm producers = $2OD_C < OD \leq 4OD_C$, strong biofilm producers = $4OD_C < OD$, where OD = OD of inoculated well and OD_C = OD of control well.

Adhesion to Intestinal Cells

Adhesion of the enterococcal isolates was assayed on the monolayers of HCT-15 cells grown on cover slips in 60 mm petridishes. HCT-15 monolayers were washed twice with PBS and 3 mL of DMEM containing enterococcal cells in the ratio 1:100 was added and incubated for 1 h at 37°C; 5% CO_2. Following

incubation, all of the dishes were washed four times with PBS to release unbound bacteria. The cells were then fixed with methanol for 10 min and stained with Giemsa stain solution for 30 min. The dishes were washed with PBS until no color was observed, dried, and observed under bright field microscope. Each adhesion assay was performed in triplicates with cells from three successive passages (8–13 cell passages). The adherent enterococci in five random microscopic fields were counted for each test. Bacterial strains were scored as non-adhesive when fewer than 40 bacteria were present in 5 fields, adhesive when 41–100 bacteria were present in 5 fields, and strongly adhesive when more than 100 bacteria occurred in 5 fields (Fernández et al., 2003).

Safety Determinants

Hemolytic, Gelatinase, and Casein Hydrolase Activity

To evaluate hemolytic activity, enterococcal isolates were streaked on Columbia agar plates containing 5% (v/v) sheep blood (HiMedia) and incubated at 37°C for 24 h. Hemolytic activity was detected by appearance of halo around the colonies: green zone for α-hemolysis, clear zone for β-hemolysis and no halo indicated γ-hemolysis (Barbosa et al., 2010). To evaluate the proteolytic activity of the isolates against gelatin and casein, BHI media was supplemented with 1% gelatin (Kanemitsu et al., 2001) and 1.5% skim milk (Lisiecki, 2017), respectively. Cultures were streaked on the BHI plates and incubated at 37°C for 24 h. A halo around the colonies indicated a positive result for gelatinase and casein hydrolase production.

PCR Amplification of the Virulence Determinant Genes

Enterococcus isolates were tested for the presence of genes encoding various virulence factors by using PCR amplification method. The amplification conditions and primers used are mentioned in **Table 1**. Total chromosomal DNA from overnight grown cultures of the isolates was extracted according to the method by Moore et al. (2004). DNA was quantified by using 1% agarose gel stained with ethidium bromide. For the detection of virulence genes, PCR was performed in 50 μL reaction mixture containing 5 μL bacterial DNA template (50 ng), 25 μL of 2X PCR Mastermix, 1 μL of each primer (Bioserve Biotechnologies), and 18 μL of nuclease free water by using mastercycler personal (Eppendorf). The PCR reaction conditions used were DNA denaturation at 95°C for 4 min. followed by 32 cycles of amplification. The amplification cycle consisted of denaturation step at 95°C for 1 min, annealing temperature as mentioned in **Table 1** followed by extension at 72°C for 1 min. Reactions were completed with 10 min elongation at 72°C followed by cooling to 4°C. PCR products were analyzed in 1.5% agarose gel stained with ethidium bromide and visualized by using bioimaging system.

Antibiotic Susceptibility

Antibiotic susceptibility of the isolates was determined by Kirby Bauer disk diffusion method (Bauer et al., 1966) in MRS agar media. Isolates were grown overnight in MRS broth at 37°C. A total of 100 μL of the culture having 1×10^6 CFU/mL was spread plated onto MRS agar-containing plates. Antibiotic disks (HiMedia) were placed on the MRS agar plates and incubated at 37°C for 24 h at 5% CO_2. Diameters of the zone of inhibition around the disks were measured in mm. The results were reported as sensitive, intermediate or resistant to various antibiotics according to the breaking points recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012) guidelines for enterococci.

Assessment of Anti-proliferative Activity of CS

Cancer Cell Lines and Preparation of Peripheral Blood Mononuclear Cells (PBMC)

The various human cancer cell lines used in the study were cervical cell line, HeLa; lung carcinoma, A549 and colonic epithelial cell line, HCT-15. These cell lines were procured from National Cell Center, Pune, India. The cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, United States) supplemented with 10% fetal bovine serum (Sigma-Aldrich, United States) in tissue culture flasks at 5% CO_2-containing atmosphere and at 37°C. These cells were collected in the confluent phase by treating with Trypsin–Hank's Balanced Salt Solution. The cells suspension was centrifuged at $120 \times g$ for 15 min. The supernatant was removed and 5 mL of DMEM was added to the cell pellet. The cell number was counted using hemocytometer.

The PBMC were isolated from the whole blood by density gradient centrifugation. For this, 5 mL of peripheral blood was withdrawn from the antecubital vein and transferred to ethylenediaminetetraaceticacid-coated vial. The blood was then diluted with PBS in the ratio of 1:1 and 5 mL of HiSep (HiMedia) was added in a centrifuge tube. The diluted blood was poured onto HiSep solution along the wall in the ratio of 1:2 and centrifuged at $128 \times g$ for 20 min. The white ring of PBMC was harvested carefully, washed thrice with PBS, and suspended in DMEM. The cell number was counted using hemocytometer.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide] Assay

The MTT assay (Mosmann, 1983) was used to determine the anti-proliferative activities of the CS of the enterococcal isolates against cancer cell lines HeLa, HCT-15, and A549 and normal human cells, PBMC. CS was prepared by centrifuging overnight grown culture of the enterococcal isolates at $800 \times g$ for 10 min. The pH of CS was set at 7.2 with 1N NaOH and CS was lyophilized and diluted in DMEM media to obtain desired concentrations of 1.5–50 μg/mL. The solution was filtered sterilized through 0.22-μm syringe filters (Milipore, United States) and used for the MTT assay.

For setting up the MTT assay, 100 μL of the cell suspension containing 4×10^5 cells/mL was added to each well of the 96-well plate and the plate was kept in the CO_2 incubator with 5% CO_2, at 37°C for 24 h. After 24 h, 100 μL of the serially diluted CS or lyophilized MRS (as negative control) in DMEM was added to the wells. The plate was again maintained in the CO_2 incubator

for 24 h. The supernatant was discarded and 100 μL of MTT (5 mg/mL for PBMC and 0.5 mg/mL for cell lines) was added to each well and the plate was incubated in CO_2 incubator at 37°C for another 4 h. After incubation, medium containing MTT was discarded and 100 μL of dimethyl sulfoxide (HiMedia, India) was added to the wells to dissolve the blue formazan crystals. The OD was read at wavelength 570 nm on the microplate reader (Multiskan® EX by LabSystems, Finland). The proliferation of cells under treatment was assessed according to the following formula:

$$\text{Percentage of proliferation} = \frac{\text{Absorbance of CS-treated wells}}{\text{Absorbance of MRS-treated control wells}} \times 100$$

To determine the proteinaceous nature of the component of CS responsible for the anti-proliferative activity, the CS was subjected to treatment with proteinase K (1 mg/mL; HiMedia) for 2 h at 37°C, followed by deactivation of proteinase K by heating at 60°C for 15 min. The residual anti-proliferative activity of the CS was determined by using MTT assay as described previously. The untreated-CS was used as positive control and MRS was used as negative control.

Apoptosis Detection by Staining Techniques

Apoptosis of eukaryotic cells is associated with certain morphological changes in their cell membranes and DNA that could be studied microscopically (Elmore, 2007). To study the effect of CS on the morphology of HCT-15 cells, they were treated with 50 μg/mL of CS and then stained with dyes, Hoechst 33342, propidium iodide (PI), and Giemsa (Merck, Darmstadt, Germany). Sterile coverslips were placed in wells of 6-well tissue culture plate (CoStar, United States). HCT-15 cells were added to each well of the plate at a concentration of 1.2×10^6 cells/well in DMEM and incubated at 37°C in the CO_2 incubator. After 24 h, CS of enterococcal isolates and MRS as controls were added to the wells and the plate was again incubated at 37°C in the CO_2 incubator for 24 h. The cells were fixed with 4% paraformaldehyde for 5 min and washed thrice with PBS before staining with Hoechst 33342 (1 μg/mL) and PI (5 μg/mL) for 15 min, and with Giemsa (1:9) for 20 min. After staining, the coverslips were washed thrice with PBS to remove excess stain. Hoechst 33342 and PI-stained coverslips were viewed under fluorescent microscope and Giemsa-stained coverslips were viewed under bright field microscope (Nikon A1R, Japan).

Statistical Analysis

Data were analyzed by one-way ANOVA. Significant differences of means ($p < 0.05$) were compared through independent Student's *t*-test by using SPSS 17.0.

RESULTS

Antimicrobial Activity

Out of the 92 tested vaginal LAB isolates, 3 cocci, 12a, 20c, and L12b had broad spectrum antimicrobial activities in CS against both Gram-positive and Gram-negative pathogens. The CS of 12a and 20c inhibited the growth of *S. enterica* Typhimurium, *E. coli*, *S. flexneri*, *M. smegmatis*, *V. cholerae*, and *L. monocytogenes*, whereas, CS of L12b inhibited *S. enterica* Typhimurium, *E. coli*, *S. flexneri*, *M. smegmatis*, *L. monocytogenes*, and *S. aureus* (**Table 2**). None of the isolate inhibited the growth of human pathogens *P. aeruginosa* and *K. pneumoniae*. Further, the antimicrobial activities of CS were also screened against 7 Lactobacilli spp. isolated from human gut. None of the CS inhibited any *Lactobacillus* isolate (**Table 2**).

Further, the zones of inhibition of CS of all the three isolates against various susceptible strains remained same with and without pH neutralization (data not shown), thereby showing that the antimicrobial effect was not due to acidic conditions of CS.

Physico-Chemical and Genetic Characterization of the Enterococcal Isolates

Three LAB isolates 12a, L12b, and 20c selected for the study were characterized by both physico-chemical and genetic methods, and were further screened for their probiotic, virulence, and anti-proliferative properties. Physico-chemical tests showed that all the three isolates belonged to the genera *Enterococcus* as they all showed growth at temperatures 10°C and 45°C, in MRS-containing 6.5% NaCl and hydrolyzed bile esculin (data not shown). The isolates were further identified by using partial sequencing of 16S rDNA. The sequences were aligned using BLAST (NCBI) version 2. The isolates 12a and L12b with Genbank NCBI accession numbers KY785661 and KY785374,

TABLE 1 | PCR primers and reaction conditions used for the detection of genes implicated in virulence of *enterococcal* isolates.

Virulence determinant genes	Primer sequence (5′-3′)	Amplicon (bp)	Annealing temperature (°C)	Reference
agg (aggregation substance)	F:AAGAAAAAGAAGTAGACCAAC R:AAACGGCAAGACAAGTAAATA	1553	50	Eaton and Gasson, 2001
esp (Enterococcal surface protein)	F:AGATTTCATCTTTGATTCTTGG R:AATTGATTCTTTAGCATCTGG	510	48	Vankerckhoven et al., 2004
gel E (extracellular metallo-endopeptidase)	F:ACCCCGTATCATTGGTTT R: ACGCATTGCTTTTCCATC	419	51	Sabia et al., 2008
cyl (cytolysin)	F: ACTCGGGGATTGATAGGC R: GCTGCTAAAGCTGCGCTT	688	58	Vankerckhoven et al., 2004

TABLE 2 | Antimicrobial activity of the enterococcal isolates against various indicator microorganisms.

Indicator microorganism	Susceptibility profile		
	12a	L12b	20c
S. enterica Typhimurium	S	S	S
E. coli	S	S	S
S. flexneri	S	S	S
L. monocytogenes	S	S	S
V. cholera	S	R	S
M. smegmatis	S	S	S
P. aeruginosa	R	R	R
S. aureus	R	S	R
K. pneumonia	R	R	R
L. plantarum L14	R	R	R
L. fermentum L32	R	R	R
L. pentosus S45	R	R	R
Lactobacillus spp. L13	R	R	R
Lactobacillus spp. L12	R	R	R
Lactobacillus spp. L18	R	R	R
Lactobacillus spp. S49	R	R	R

The antimicrobial activities of the CS of enterococcal isolates were determined by using agar gel diffusion assay. The pH of CS was set to 6.5 by using 1 N NaOH. The experiment was performed in triplicates. S, diameter of the zone of inhibition > 10 mm. R, diameter of the zone of inhibition < 10 mm.

respectively, had 99% sequence similarities to *E. faecium*; while the isolate 20c (accession number KY785319) had 99% similarity to *E. hirae*.

Probiotic Properties

Gastric and Bile Juice Conditions

The oral administration of probiotics subjects them to the harsh acidic environment in the stomach and to bile juices in the small intestine. Therefore, to determine the ability of enterococcal isolates to survive in gastric juice, the survival of the isolates 12a, L12b and 20c were evaluated in SGJ having pH 2.0 and in the presence of bile salts. Both SGJ and bile treatment resulted in less than 0.5 log CFU change in the viabilities of all the three enterococcal isolates after 4 h (**Figure 1**). Isolate L12b appeared to be comparatively more resistant to the effects of both SGJ and bile treatment as compared to isolates 12a and 20c.

Biofilm Formation and Adherence of Enterococci to HCT-15 Cell Line

Biofilm formed by probiotic microorganisms cover epithelial cell receptors in the gut and inhibit colonization by undesirable microorganisms. Thus, biofilm formation is a desirable characteristic for probiotic cultures. In this study, we observed that the three enterococcal isolates possessed the ability to form well-structured biofilm. All the isolates formed strong biofilm at pH 6 and at 48 h. At pH 4 and 5, only isolate L12b showed strong biofilm formation at 48 h (**Table 3**).

Further, the adherence of the enterococcal isolates to the HCT-15 cell line was studied microscopically. All the three enterococcal isolates were strongly adhesive as 1 h incubation of bacterial cells

TABLE 3 | Biofilm-forming potential of the enterococcal isolates.

Enterococcal isolate	Biofilm forming potential at different pH and time periods								
	pH 4			pH 5			pH 6		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
12a	W	W	M	W	M	M	S	S	S
L12b	W	M	S	W	S	M	S	S	M
20c	W	W	W	W	M	W	M	S	S

S: strong biofilm producers, M: moderate biofilm producers, W: weak biofilm producers. The enterococcal biofilms were formed in MRS media of different pH in 96-well microtitre plates and analyzed at different time points by using crystral violet assay. Based on OD_{595} of the well, strains were classified as: weak biofilm producers ($OD_C < OD \leq 2OD_C$), moderate biofilm producers ($2OD_C < OD \leq 4OD_C$), and strong biofilm producers ($4OD_C < OD$), where OD = OD of inoculated well and OD_C = OD of uninoculated well. Experiments were performed three times in triplicates.

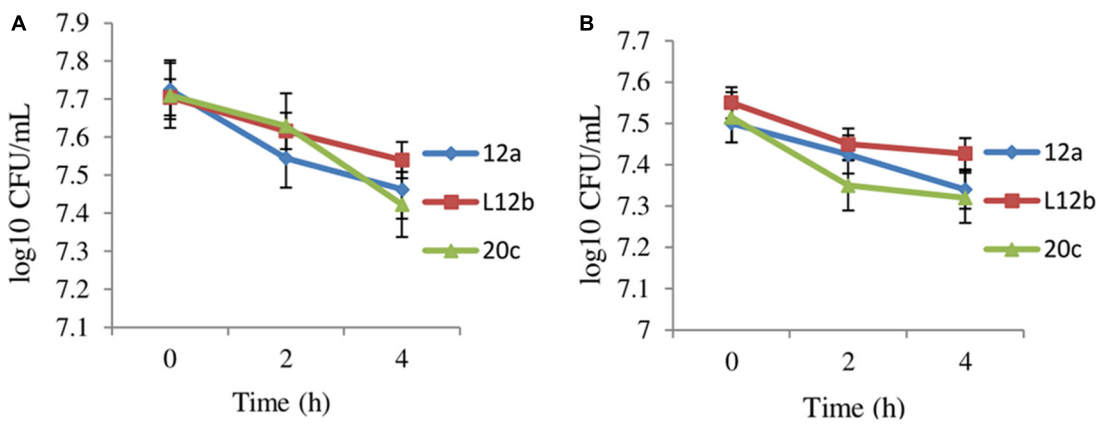

FIGURE 1 | Effect of **(A)** simulated gastric juice and **(B)** bile juice treatment on the viabilities of enterococcal isolates after 4 h. Error bars are representative of SD of the three independent experiments performed in triplicates.

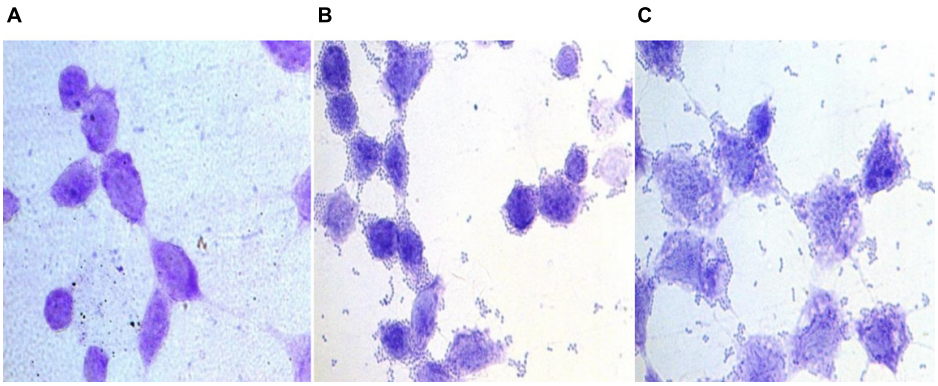

FIGURE 2 | Bright field microscopic images showing adhesion of enterococcal cells to colonic epithelial cell line HCT-15. Giemsa-stained HCT-15 cells **(A)** without enterococci, **(B)** with *E. faecium* 12a, and **(C)** with *E. hirae* 20c.

with the cell line resulted in more than 100 bacterial cells bound to the HCT-15 cell line in the 5 fields (**Figure 2**).

Virulence Factors

As some pathogenic strains of *Enterococcus* are known to possess virulence factors (Jett et al., 1994) that play important role in the pathogenesis of enterococcal infection, the secreted virulence factors were studied. The results showed that no gelatinase, casein hydrolase or hemolytic (γ-hemolysis) activities were observed in any of the three enterococcal cultures.

Further, the PCR amplification of virulent genes *agg*, *esp*, *gel E*, and *cyl* was negative in all the three enterococcal isolates thereby showing them as safe non-virulent strains.

Antibiotic Susceptibility

Probiotic candidate should be screened for antibiotic resistance as they can act as potential reservoirs of transmissible antibiotic resistance genes. Antibiotic susceptibility pattern of the isolates was evaluated against various antibiotics (**Table 4**). All the three enterococcal isolates were sensitive to all the three β-lactam antibiotics and teicoplanin. Among fluoroquinolones, L12b and 12a were sensitive to both moxifloxacin and gatifloxacin. Both 20c and L12b were susceptible to tetracycline. All the three isolates were resistant to ciprofloxacin, erythromycin, and azithromycin.

Anti-proliferative Assay

Evaluation of anti-proliferative activities of the CS of enterococcal isolates on the cancer cell lines HeLa, A549, and HCT-15 was done. CS of all the three enterococcal isolates had dose-dependent anti-proliferative activity against all the cancer cell lines tested. CS of isolate 12a, 20c, and L12b at the highest dose of 50 μg/mL reduced the viabilities of HeLa to 8.4, 9.7, and 16.4%, respectively (**Figure 3A**). The 50% inhibitory concentration (IC_{50}) values of the CS of 12a, 20c, and L12b for HeLa were calculated as 12.5, 7.7, and 15.9 μg/mL, respectively.

Similarly, the secretory metabolites all the three enterococcal isolates inhibited the growth of HCT-15 (**Figure 3B**) at the maximum dose of 50 μg/mL. The viabilities of HCT-15 were reduced to 13.7, 14.4, and 15.8% after treatment with the CS of 12a, 20c, and L12b, respectively. The IC_{50} values calculated for the CS of 12a, 20c, and L12b were 9.9, 14.4, and 15.3 μg/mL, respectively.

Further, 24 h treatment of the cell line A549 with the CS of the enterococcal isolates12a, 20c, and L12b at the concentration 50 μg/mL, reduced the viabilities to 17.5, 21.3, and 18.7%, respectively (**Figure 3C**). The IC_{50} values calculated for the CS of 12a, 20c, and L12b were 14.01, 21.3, and 11.7 μg/mL, respectively.

The antiproliferative effect of CS of all the three enterococcal isolates was also determined against normal human PBMCs.

TABLE 4 | Antibiotic susceptibility profile of vaginal enterococcal isolates.

Antibiotic	Sensitivity profile		
	20c	12a	L12b
β-lactams			
Penicillin	S	S	S
Ampicillin	S	S	S
Carbenicillin	S	S	S
Macrolides			
Azithromycin	R	R	R
Erythromycin	R	R	R
Fluoroquinolones			
Ciprofloxacin	R	R	R
Moxifloxacin	R	S	S
Gatifloxacin	I	S	S
Glycopeptides			
Vancomycin	I	S	S
Teicoplanin	S	S	S
Oxazolidinones			
Linezolid	I	R	S
Tetracyclines			
Tetracycline	S	I	S

Kirby Bauer disk diffusion method was performed, and zone of inhibition was measured in the presence of various antibiotics. Standard interpretation of antimicrobial susceptibility tests of enterococci with disk diffusion method in accordance to CLSI (2012) standards. S, susceptible; I, intermediate; R, resistant. Experiment was performed in triplicates.

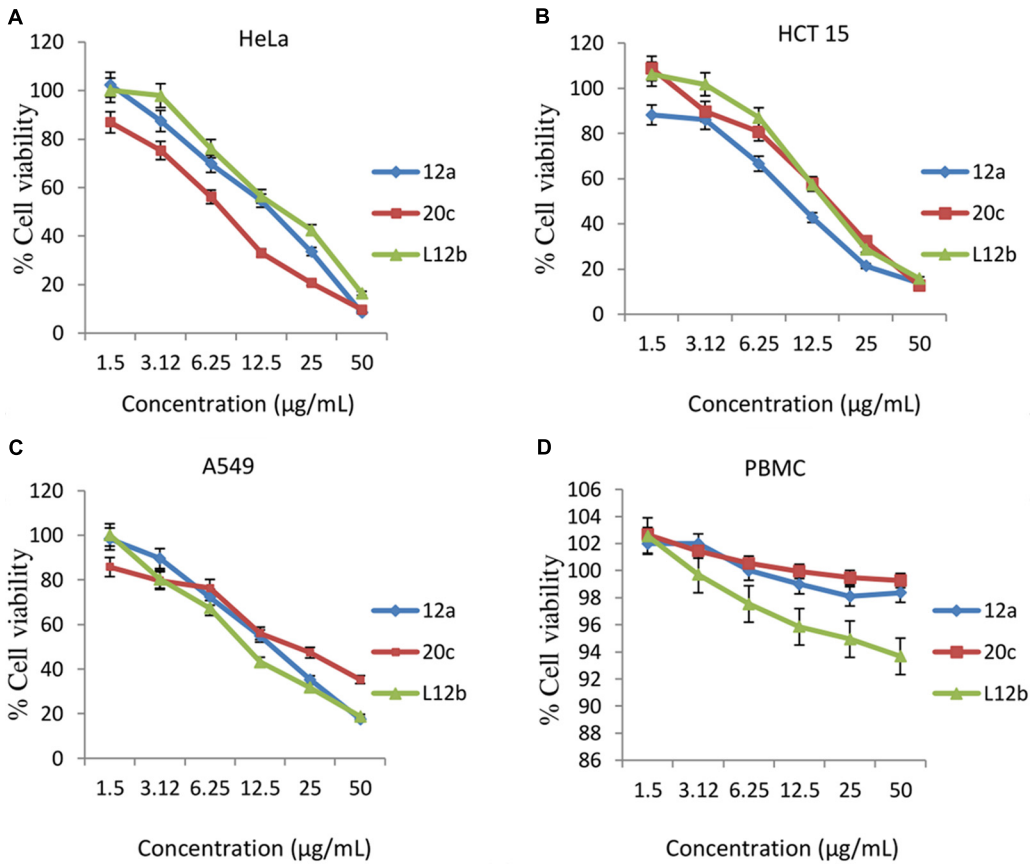

FIGURE 3 | Dose-dependent effect of the secreted metabolites of the CS of enterococcal isolates on the viabilities of **(A)** HeLa, **(B)** HCT 15, **(C)** A549, and **(D)** Human PBMCs after 24 h treatment. Error bars are representative of SD of the three independent experiments performed in triplicates.

The residual viabilities of PBMC after treatment with the CS of 12a, L12b, and 20c were 74.5, 76.5, and 81.5 %, respectively (**Figure 3D**).

Proteinase K treatment of CS of all the three enterococcal isolates resulted in abrogation of the anti-proliferative effect of CS (**Figure 4**). The viabilities of HCT-15 were reduced only by 18.7, 22.9, and 26.8% with proteinase K-treated CS of 12a, 20c, and L12b, respectively. Thus, the anti-proliferative activity of CS of all the isolates is due to some proteinaceous substance present in the CS.

Microscopic Detection of Morphological Changes in the CS-Treated Cell Lines

Further, to study the mechanism of the anti-proliferative activity of CS, the morphological changes of CS-treated cancer cell line HCT-15 cells were studied by bright field and fluorescent microscopy. Apoptosis also known as programmed cell death involves microscopically visible morphological changes such as chromatin condensation, margination, cell shrinkage, membrane blebbing, and formation of apoptotic bodies (Leite et al., 1999). Most of the cells in the control sample (treated with MRS) remained viable with normal cell morphology with clear outline of cell membrane and nucleus (**Figure 5**; Lanes A1–C1). On the other hand, the cells treated with the CS of enterococcal isolates 12a and 20c showed morphological changes typical of apoptosis (**Figure 5**). The CS-treated and Giemsa-stained cells (Lanes A2 and A3) showed cell shrinkage, chromatin condensation and nuclear fragmentation. Whereas CS-treated and fluorescent-stained (Lanes B2, B3, C2, and C3) cells showed nuclear condensation and fragmentation.

DISCUSSION

In this study, the selective anti-proliferative and apoptotic effects of the secreted proteinaceous metabolites of the vaginal *E. hirae* and *E. faecium* have been shown against various cell lines for the first time. Further, the strains exhibited good probiotic properties that make them promising candidates for use as prophylactic and therapeutic anticancer agents. The other two studies that reported the *in vitro* anti-proliferative effects of the secreted proteinaceous metabolites of LAB were that of *E. durans*, isolated from fermented food (Haghshenas et al., 2014) and *E. faecalis* isolated from human vagina (Nami et al., 2014b). In both the studies, the CS was shown to inhibit the human cancer cell lines HeLa, mammary cancer cell line MCF-7 and gut epithelial cell lines, AGS and HT-29. The effect of the CS of *Enterococcus* spp.

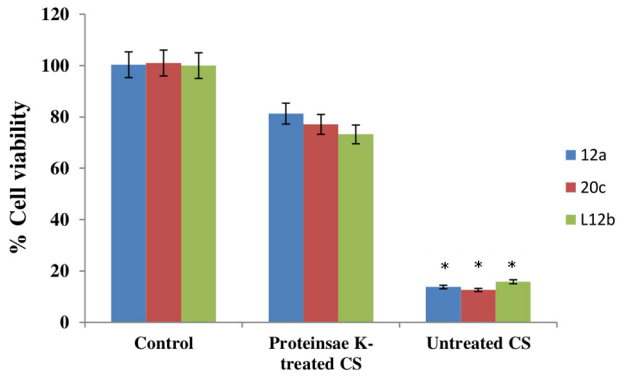

FIGURE 4 | Anti-proliferative effect of CS (50 μg/mL) of enterococcal isolates, with and without proteinase K (1 μg/mL) treatment on HCT-15 cell line. Untreated HCT-15 was used as control. Error bars are representative of SD of the three independent experiments performed in triplicates. Asterisk denotes statistically ($p < 0.001$) significant difference as compared to the respective controls.

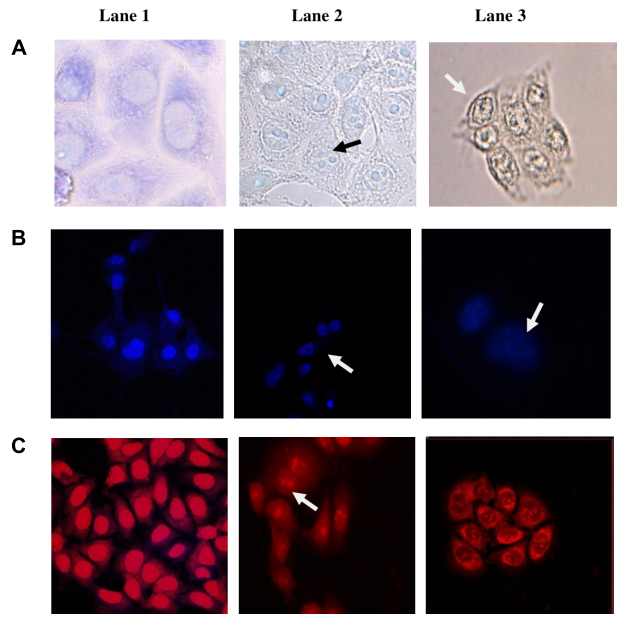

FIGURE 5 | Morphological changes induced in HCT-15 following 24 h treatment with CS of enterococcal isolates. **(A)** Giemsa-stained, **(B)** Hoechst 33342-stained, and **(C)** PI-stained HCT-15 cells. Lane 1: untreated HCT-15 cells; Lane 2: HCT-15 cells treated with CS of 12a; and Lane 3: HCT-15 cells treated with CS of 20c. Arrows marked in Giemsa-stained cells show cell shrinkage along with nuclear fragmentation. Arrows marked in PI and Hoechst 33342-stained cells show nuclear condensation and fragmentation.

on the lung carcinoma cell line A549 is reported for the first time in our study. Furthermore, the cell viability of HeLa in our study was reduced in the range from 82.6 to 91.6%; whereas in the previous studies the CS of *E. durans* induced 60% (Haghshenas et al., 2014) and *E. faecalis* induced 71.9% reduction (Nami et al., 2014b) in the viabilities of HeLa cells after 24 h at the same concentration.

The secreted metabolites from other LAB isolates such as *L. plantarum* 17C (Haghshenas et al., 2015), *L. plantarum* 5BL (Nami et al., 2014a), *Pediococcus pentosaceus* GS4 (Dubey et al., 2016), and *E. lactis* IW5 (Nami et al., 2015) were also shown to inhibit the proliferation of cancer cell lines, *in vitro*, but the type of the component in the CS responsible for the anti-proliferative activity is not known.

The proteinaceous nature of the component in the CS responsible for the anti-proliferative activities was confirmed by its proteinase K treatment, which significantly ($p < 0.05$) reduced the anti-proliferative activity against HCT-15 cell line. LAB are known to secrete cationic proteins or peptides known as bacteriocins in the CS that inhibit the growth of cancer cells (Kaur and Kaur, 2015; López-Cuellar et al., 2016). Bacteriocins are positively charged peptides that are known to selectively bind and destabilize the membranes of cancer cell lines owing to the enhanced negative charge, membrane fluidity and numbers of microvilli on the surface of cancer cells as compared to normal cells (Kaur and Kaur, 2015). Thus, the protein component of the CS that mediated anticancer activities may be a bacteriocin-like molecule or other proteins such as enzymes. The morphological changes observed microscopically suggested that the CS of the enterococcal isolates induced apoptosis in the cell line HCT-15. Other studies also reported apoptosis as the mode of action of CS on cancer cell lines (Haghshenas et al., 2014; Nami et al., 2014b). Further, as reported earlier our results also showed that CS selectively inhibited cancer cell lines and had no effect on the normal human cells (Haghshenas et al., 2014; Nami et al., 2014b).

Another indirect mechanism through which enterococci may have prophylactic action against cancer is by modulating the gut microbiota. All the three *enterococcal* isolates inhibited the growth of Gram-negative pathogens but not that of *Lactobacillus* spp. The Gram-negative microorganism-associated molecular patterns such as lipopolysaccharide have been implicated in various types of cancer (Schwabe and Jobin, 2013). Also Gram-negative bacteria such as *E. coli*, *S. enterica*, *Shigella* spp., etc., are known to produce genotoxins such as cytolethal distending toxin (Graillot et al., 2016) and colibactins (Arthur et al., 2012) that cause DNA damage and are known to promote the development of cancer. Thus, *enterococcal* isolates through inhibition of the growth of Gram-negative pathogens may protect against the development of cancer. The potential of enterococci to inhibit the *Mycobacterial* spp. has been shown for the first time in this study.

Further, the probiotic properties of enterococci were explored. Probiotics are administered orally and therefore the condition of the gastrointestinal tract is the first physiological challenge faced by them. Human stomach has a pH value ranging from 1.5 to 4.5 and it can take up to 3 h for food to get ingested (Jacobsen et al., 1999). The putative probiotic strain should be able to remain alive during transit through gastrointestinal tract at low pH and in the presence of bile salt. All the enterococcal isolates in this study were resistant to SGJ and their viabilities were not affected by more than 0.5 log CFU. These results are in concordance with the results of previous studies (Banwo et al., 2013; Nami et al., 2014b). Once inside the

intestine, probiotic microorganisms must adhere to intestinal mucosa to facilitate their colonization and to prevent their removal by peristalsis. The ability for biofilm formation is associated with cell adherence potential (Boris et al., 1997). All the isolates possessed the ability to form strong biofilms at pH 6 that is similar to the physiological intestinal pH. Furthermore, adhesion to intestinal epithelial cells is an important feature for colonization and competitive exclusion of pathogens by probiotics. *In vitro* studies performed to study the adherence abilities of the isolates to the colonic epithelial-like cell line HCT-15, showed that more than 100 enterococcal cells could be counted in each field having 3–5 HCT-15 cells, thereby showing strong adherence of all the isolates to the colonic cells.

The safety features of enterococci were evaluated in terms of antibiotic profile and virulence factors. FAO/WHO (2002) addressed the problem of transmissible antibiotic resistant genes in the probiotics and recommended the evaluation of its antibiotic profile. The antibiotic susceptibility profile showed that all the isolates were sensitive to β-lactam antibiotics, which are the first line of treatment for enterococci infections. Among fluoroquinolones, L12b and 12a showed sensitivity to moxifloxacin and gatifloxacin; however, all of them were resistant to ciprofloxacin. Similarly, L12b and 12a were sensitive to vancomycin, whereas L12b and 20c were susceptible to tetracycline. Thus, the molecular mechanisms of vancomycin resistance in 20c and tetracycline resistance in 12a should be further evaluated to study their transmissible nature. All the isolates were resistant to macrolides, erythromycin, and azithromycin. Thus, based on the antibiotic susceptibility profile, L12b and 12a appear to be more sensitive to various classes of antibiotics as compared to 20c.

Safety aspect of the strains was assessed as per European Food Safety Authority (2012) guidelines both by determining PCR amplification of virulence genes such as *agg*, *gel E*, *cyl*, and *esp*, and by using bioassays to screen for the secreted virulence factors. As reported for the *E. faecium* probiotic strains, such as SF68, none of the isolates showed the presence of virulence factors and therefore all can be considered as safe probiotic candidates (Kayser, 2003).

Thus, this preliminary study explored the functional properties of human commensal enterococci. The beneficial roles of gut microflora in inhibiting tumor growth (Zitvogel et al., 2017) and modulating the therapeutic effects of anticancer drugs (Viaud et al., 2013; Roy and Trinchieri, 2017) is increasingly being realized. For example, Sivan et al. (2015) showed that the gut microflora of mice that responded to anticancer immunotherapy had the abundance of commensal *Bifidobacterium* spp. as compared to those that did not respond. Hence, the commensal *Bifidobacteria* were isolated and orally administered to melanoma mouse model that resulted in reduced tumor growth. Studies in germ-free mice have shown that gut microflora, particularly Gram-positive bacteria are required for the anticancer activity of the anticancer drug, cyclophosphamide (Viaud et al., 2013). Cyclophosphamide-treatment of mice increased the gut permeability allowing gut commensal, *E. hirae* to translocate to lymph nodes, where it induced cytotoxic T cell response to the lung and ovarian cancer (Viaud et al., 2013). In line with these studies, our results further demonstrated that commensal *Enterococcus* spp. have the ability to directly inhibit the proliferation of cancer cell lines by secreting anticancer metabolites.

CONCLUSION

All the three probiotic enterococcal strains *E. hirae* 20c, *E. faecium* 12a, and L12b secreted potent proteinaceous anticancer components that selectively inhibited the human cancer cell lines but not the normal cells. Therefore, they seem to be the promising candidates that need further evaluation in *in vivo* studies for their therapeutic potential. Secondly, the protein component of the CS should be purified and characterized to determine its therapeutic efficacy both *in vitro* and *in vivo*.

AUTHOR CONTRIBUTIONS

SrK conceived the idea and supervised the experiments. PS and SrK designed the experiments. PS performed all of the experiments except MTT assay. SpK performed the microscopic studies, whereas RK and MK designed and performed the MTT assay. All authors discussed the results and contributed to the final manuscript.

FUNDING

This work was supported by research grant (Grant No. 42-478/2013 to SrK) sponsored by University Grants Commission (UGC), New Delhi, India.

ACKNOWLEDGMENTS

PS is thankful to University with Potential for Excellence scheme of UGC for the fellowship. SpK is thankful to UGC for the fellowship.

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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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 December 2017
doi: 10.3389/fmicb.2017.02406

EPSP of *L. casei* BL23 Protected against the Infection Caused by *Aeromonas veronii* via Enhancement of Immune Response in Zebrafish

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Aquaculture is the fastest-growing food production sector in the world, and it supplies nearly 50% of the global food fish supply. However, disease outbreaks have become a major problem in the fish farming industry. The beneficial contribution of probiotic bacteria to aquatic animals' health has been widely described, and they have been widely used in aquaculture for disease control and growth promotion. However, the action of probiotic bacterial components and mechanisms underlying protection against pathogens afforded by probiotic bacteria remain poorly understood. In the present study, we pre-colonized zebrafish larvae (before hatching) with 17 potential probiotic bacterial strains and screened for those possessing anti-infective effects against *Aeromonas veronii*. We found that *Lactobacillus casei* BL23 significantly increased the survival of zebrafish larvae upon *A. veronii* infection. Using a germ-free (GF) zebrafish model and gut microbiota transplant experiment, we showed that *L. casei* BL23 *per se* has anti-infective effects in zebrafish larvae, which does not involve microbiota. Furthermore, we identified an exopolysaccharide-protein complex (EPSP) extracted from *L. casei* BL23 cells, which consisted of a 40–45 KD size protein and an exopolysaccharide composed of α-Rha, α-Glc, β-GlcNAc, and β-GalNAc. EPSP significantly increased the survival rate of GF zebrafish at a dose of 10–20 μg/ml after *A. veronii* infection ($P < 0.01$). In addition, the EPSP induced a higher expression of TLR1 and TLR2, and modulated the expression profile of pro-inflammatory and anti-inflammatory cytokines in zebrafish liver (ZFL) cells. Our data indicated that the anti-infective effect of EPSP from *L. casei* BL23 was mediated by enhancement of immune responses in zebrafish, which might involve the TLR1/TLR2 signal pathway.

Keywords: probiotic, *L. casei* BL23, EPSP, *Aeromonas veronii*, immunomodulation

OPEN ACCESS

Edited by:
Haitao Lu,
Shanghai Jiao Tong University, China

Reviewed by:
Maria de los Angeles Serradell,
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *26 March 2017*
Accepted: *20 November 2017*
Published: *08 December 2017*

Citation:
Qin C, Zhang Z, Wang Y, Li S, Ran C, Hu J, Xie Y, Li W and Zhou Z (2017) EPSP of L. casei BL23 Protected against the Infection Caused by Aeromonas veronii via Enhancement of Immune Response in Zebrafish. Front. Microbiol. 8:2406. doi: 10.3389/fmicb.2017.02406

INTRODUCTION

Aquaculture is the fastest-growing food production sector in the world and a major contributor to global food production, contributing nearly 50% of the global food fish supply (Subasinghe et al., 2009). Aquaculture production of aquatic animals amounted to 73.8 million tons in 2014, with an estimated value of US $160.2 billion. It supplies 17% of animal protein in people's diets worldwide

and supports the livelihoods of about 12% of the world's population (FAO, 2016). However, disease outbreaks have become a major problem in the fish-farming industry due to the increasing intensification and commercialization of aquaculture practices (Bondad-Reantaso et al., 2005). Infectious diseases have been estimated to cost billions of dollars in the global aquaculture industry annually (Lafferty et al., 2015). For example, the outbreaks of motile *Aeromonas* septicemia (MAS) caused by *Aeromonas* spp. often have high mortality and cause severe economic losses in aquaculture worldwide (Cipriano et al., 1984; Beaz-Hidalgo and Figueras, 2013). During the past few decades, antibiotics have been the standard strategy for management of fish diseases and for improving their growth (Romero et al., 2012). Unfortunately, antibiotic resistance among bacterial pathogens and antibiotic residues in the animal products has piqued global interest in limiting the use of antibiotics in aquaculture (Chen et al., 2015; Huang et al., 2015; Pereira et al., 2015).

Probiotics are live microorganisms that have beneficial effects on the host when properly administered. Extensive studies have demonstrated that probiotics are a promising alternative to antibiotics in aquaculture, and that they have a variety of beneficial effects, including counteraction of dysbiosis, promotion of gut health and homeostasis, promotion of growth, enhancement of immune defenses, and protection of the host from pathogen infection (Newaj-Fyzul et al., 2014; Hai, 2015). Probiotics, especially *Lactobacillus*, have been widely used in aquaculture for disease control, notably against bacterial diseases (Newaj-Fyzul et al., 2014; Fečkaninová et al., 2017). In recent years, there has been increasing interest in determining the biological roles of each probiotic bacterial component. Several factors, i.e., metabolites, enzymes, surface or secreted proteins and cell surface polysaccharides, that influence the immune response of the host have been identified in lactobacilli (Kim et al., 2006, 2009). In particular, studies have indicated that the health benefits of lactic acid bacteria are associated with the production of exopolysaccharides (EPS), which showed antitumor, antiulcer, immunomodulating, and cholesterol-lowering activities (Ruasmadiedo et al., 2002; Welman and Maddox, 2003).

Zebrafish have become a popular model for studying host-bacteria interactions and bacterial pathogenicity (Sullivan and Kim, 2008; Allen and Neely, 2010; Kanther and Rawls, 2010). Zebrafish have an innate immune system and develop adaptive immunity by the age of 4 weeks (Trede et al., 2004; Kanther and Rawls, 2010). In addition, the availability of germ-free (GF) zebrafish larvae combined with available genetic tools make zebrafish particularly suitable for molecular analyses from both the host and bacterial perspectives (Phelps and Neely, 2005; Pham et al., 2008).

In this study, we developed a new experimental approach to direct analysis of bacterial factors involved in the protection of zebrafish larvae by exogenous probiotic bacteria against pathogens. We found *L. casei* BL23 able to robustly protect zebrafish larvae from *A. veronii* infection from 17 potential probiotic bacterial strains. Further, our data indicated that *L. casei* BL23 can enhance host immune responses that may involve the activity of EPSP from BL23 via TLR1/TLR2 pathways.

MATERIALS AND METHODS

Bacteria and Culture Condition

The probiotic strains are listed in **Table 1**. The bacteria were stationarily cultivated in MRS medium at 37°C for 24 h. After growing in MRS medium for 24 h, lactobacilli cells were collected by centrifugation (10 min, 4000 × *g*, 4°C). The pellet was washed by sterile water three times, and resuspended in sterile water at a final concentration of 1.0×10^9 CFU/ml. *A. veronii* was grown in Luria–Bertani (LB) broth for 18 h at 37°C with 200 rpm shaking.

Animals

Adult zebrafish and larvae (*Danio rerio*) (TU line) were reared in the lab. The adult animals were kept in tanks (length × width × height; 25.5 cm × 18.5 cm × 18.0 cm) in a recirculating aquaculture system under controlled conditions (28 ± 0.5°C, under a 14-h light, 10-h dark photoperiod). The inlet water flow was approximately 1 L/min. The fish were fed twice per day with freshly hatched brine shrimp (8:30 a.m. and 5:30 p.m.). Procedures involving animals were performed in accordance with Chinese legislation associated with animal experimentation and the studies were approved by the Ethics Committee of the Feed Institute, Chinese Academy of Agricultural Sciences (2016-ZZG-ZF-001).

Probiotics Screening

Probiotic strains were grown stationarily in MRS medium at 37°C for 24 h. Bacteria were then pelleted and washed twice in sterile water, and resuspended in water at a final concentration of 1.0×10^7 CFU/ml. At 3 days post fertilization (dpf), about

TABLE 1 | List of probiotic strains.

Probiotic strains
Lactobacillus acidophilus LABCC IMAUFB058
Lactobacillus casei LABCC IMAU10005
Lactobacillus casei LABCC IMAU10007
Lactobacillus casei LABCC IMAU10316
Lactobacillus casei LABCC IMAU10325
Lactobacillus casei LABCC IMAU10333
Lactobacillus casei LABCC IMAU10408
Lactobacillus casei BL23
Lactobacillus rhamnosus 20300
Lactobacillus rhamnosus LGG
Lactobacillus amylovorus JCM 1126
Lactobacillus johnsonii 466
Lactobacillus brevis CGMCC 1.2028
Lactobacillus plantarum LABCC IMAU10012
Lactobacillus plantarum LABCC IMAU10058
Lactobacillus plantarum LABCC IMAU10707
Lactobacillus plantarum LABCC IMAU10722

12 h before hatching, zebrafish eggs were put in contact with the probiotic strains by transferring them to probiotic-containing bottles (60 eggs per bottle). At 4 dpf, the water was exchanged, and fresh probiotic bacteria cells were added. At 7 dpf, fish were infected with virulent *A. veronii* at a dose of 2×10^7 CFU/ml after water renewal. The mortality was recorded for 5 days.

GF Zebrafish Husbandry and Gut Microbiota Transplantation

The protocol to generate and rear GF zebrafish was descripted by Pham et al. (2008) with slight modifications. Freshly fertilized zebrafish eggs were washed by sterilized water three times in a 90 mm sterilized dish, and then the eggs were separated into 50 ml Falcon tubes (100 eggs per tube). Eggs were treated with AB-GZM (gnotobiotic zebrafish medium with antibiotics, which contained 250 ng ml^{-1} of amphotericin B, 5 $\mu g\ ml^{-1}$ of kanamycin, 100 $\mu g\ ml^{-1}$ of ampicillin, and 10 $U \cdot mL^{-1}$ of penicillin and streptomycin) for 4.5 h at room temperature. Then the eggs were washed three times with AB-GZM, and treated with 0.05% of PVPI (polyvinyl pyrrolidone-iodine complex) for 35 s and washed three times with GZM. Next, they were bleached (0.002%) for 15 min. Eggs were washed again three times in GZM and transferred to Petri dishes to be distributed into 300 ml culture bottles with vented caps containing 150 mL of GZM (60 eggs/bottle). GF animals were monitored for sterility every day by spotting 100 μL from each flask on tryptic soy medium agar plates at 28°C under aerobic or anaerobic conditions. Before the gut microbiota transplantation, adult zebrafish were reared in the control or *L. casei* BL23-added water (1.0×10^6 CFU/ml) for two weeks. Then the fish of the control or *L. casei* BL23 treatment group were sacrificed and the intestinal contents of every five fish from each group were pooled. Three replicate bottles of GF zebrafish larvae (4 dpf, $n = 60$) were transplanted with gut microbiota of control or BL23-treated fish at a dose of 10^5 and 10^6 CFU/mL. After 3 days of colonization, fish were challenged with *A. veronii* at a dose of 2.0×10^7 CFU/mL after GZM was renewed. The zebrafish mortality was observed for 120 h after infection.

DNA Extraction and Sequencing

Total bacteria DNA was extracted from intestinal contents samples by using Power Fecal™ DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, United States) according to manufacturer's instruction. Sequencing was performed at the Novogene Bioinformatics Technology Co., Ltd. Briefly, DNA was amplified by using the 515F/806R primer set (341F: 5′-CCTAYGGGRBGCASCAG-3′ 806R: 5′-XXXXXX GGACTACHVGGG TWTCTAAT-3′), which targets the V-V43 region of the bacterial 16S rDNA, with the reverse primer containing a 6-bp error-correcting barcode unique to each sample. PCR reaction was performed using phusion high-fidelity PCR Mastermix (New England Biolabs LTD., Beijing, China) with the following condition: 94°C for 5 min (1 cycle), 94°C for 20 s/55°C for 20 s/72°C for 30 s (30 cycles), and a last step of 72°C for 10 min. PCR products were purified by using the QIAquick Gel Extraction Kit (QIAGEN, Dusseldorf, Germany). Pyrosequencing was conducted on an Illumina HiSeq 2500 platform according to protocols described by Caporaso et al. (2012). Paired-end reads were merged using FLASH[1] (V1.2.7) (Magoc and Salzberg, 2011). Sequences were then demultiplexed and quality filtered using the default parameters of the Quantitative Insights into Microbial Ecology (QIIME) software package (Caporaso et al., 2010). The operational taxonomic unit (OTU) clustering pipeline UPARSE was used to select OTUs at 97% similarity (Edgar, 2013). The representative sequence sets were aligned and given a taxonomic classification using Ribosomal Database Project (Wang et al., 2007). The similarity among microbial communities was determined using histograms, UniFrac principal coordinates analysis (PCoA), and the unweighted pair-group method with arithmetic mean (UPGMA).

L. casei BL23 Exposure

Lactobacillus casei BL23 cells were grown stationarily in MRS medium at 37°C for 24 h. Bacteria were then pelleted and washed twice in sterile water, and resuspended in sterile water at a final concentration of 1.0×10^9 CFU/ml. To obtain dead cells, the bacterial pellets were treated with 4% paraformaldehyde for 2 h, and washed three times in sterile water. After hatching (4 dpf), conventional or GF zebrafish were exposed with live or dead cells of *L. casei* BL23 at a dose of 1×10^5 CFU/ml, 1×10^6 CFU/ml and 1×10^7 CFU/ml, respectively for 3 days. After three days of probiotics exposure, fish were then infected with virulent *A. veronii* at a dose of 2×10^7 CFU/ml after GZM was renewed. The mortality was recorded for 5 days. At the infected time of 0, 24, and 48 h, thirty fish (GF and fish treated with live cells or dead cells of *L. casei* BL23 at the dose of 1×10^6 CFU/ml) from each culture bottle were sacrificed and the whole body was sampled. The samples were immediately frozen in liquid nitrogen and stored at −70°C for cytokine expression analysis.

EPSP Preparation and Characterization

The method to extract EPSP was previously described by Zhang et al. (2016) in our lab. *L. casei* BL23 cells were grown stationarily in MRS medium at 37°C for 48 h. Bacteria were then pelleted and washed twice in sterile water, and were resuspended in sterile water. Then bacteria were incubated in water bath incubation (70°C, 24 h). The extracts were precipitated by gradually adding cold ethanol to 75% (v/v), and the supernatant was removed after 24 h, followed by centrifugation at 12000 rpm for 20 min. The precipitated product was washed and dissolved in water obtained from an Alpha-Q reagent grade water purification system (Millipore Co., Milford, MA, United States). The aqueous solution of the extracts were further treated with sevage reagent (trichloromethane – *n*-butanol, 4:1, vol/vol) at a final concentration of 25% and incubated for 2 h under gentle agitation and then precipitated proteins were removed by centrifugation at 8000 *g* for 20 min (repeat this step for two times). After centrifugation, the solution

[1]http://ccb.jhu.edu/software/FLASH/

containing EPS was dialyzed (molecular weight cut-off: 3000 Da) against 5 l of distilled water for 2 days with water changes three times per day. The extract solution after dialysis was lyophilized.

The purity of the extract (5 mg/ml) was tested by SDS-PAGE electrophoresis and size-exclusion chromatography (SEC) on a column of Superdex75 (10/300 GE) (Pharmacia, Uppsala, Sweden), which fitted to an AKTA FPLC system (Pharmacia) and were eluted with 0.3 M NaCl buffer.

The monosaccharide composition was determined by TLC. Briefly, 20 mg EPSP was hydrolyzed with 2 ml sulfuric acid (1 mol/l) at 100°C for 4 h. The residual sulfuric acid was removed by neutralization with excessive $BaCO_3$ reaction for 12 h. This solution was adjusted to pH7 and diluted to 20 ml. The hydrolyzate was evaporated under reduced pressure, dissolved in 2 ml ultra-pure water. The resulting hydrolyzate was analyzed by TLC. TLC analysis was conducted according to the previous report (Tanaka et al., 1999). Migration was performed twice on a silica gel TLC plate (20 cm × 20 cm) using *n*-butanol–methanol–25% ammonia solution–water (5:4:2:1 [vol/vol/vol/vol]). Carbohydrates were visualized by heating the TLC plate after spraying with aniline-diphenylamine reagent (4 ml of aniline, 4 g of diphenylamine, 200 ml of acetone, and 30 ml of 85% phosphoric acid). Monosaccharides of α-Rha, α-Glc, β-GlcNAc, and β-GalNAc were used as standard and the plate was baked at 110°C for 5 min.

Cell Culture and Treatments

The cell line of ZFL was purchased from American Type Culture Collection (ATCC). The ZFL cells were cultured at 28°C in modified limit dilution factor (LDF) culture medium. The complete medium consisted of 50% Leibovitz's L-15 (L-15), 30 % Dulbecco's modified Eagle's (DMEM), and 20% Dulbecco's Modification of Eagle's Medium/Ham's F-12 medium (DMEM/F12) supplemented with 5% fetal bovine serum (FBS), 0.5% trout serum, 10 μg/ml bovine insulin, 50 ng/ml mouse Epidermal Growth Factor (EGF). Mediums were supplemented with 1% penicillin–streptomycin. All basal mediums and FBS were obtained from Corning (NY, United States). The cells were treated with EPSP (10 μg/ml) or equal volume of dd H_2O after the cells covered the plate, and cells were harvested at 24 h after treatment.

Real-Time PCR

Total RNA was isolated from zebrafish larvae and ZFL cells with TRIzol (Invitrogen) extraction. First-strand complementary DNA synthesis was performed using the Superscript First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR reaction were performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7500 (Applied Biosystems) with reaction volumes of 20 μl. The reaction mixtures were incubated for 5 min at 95°C, followed by 40 cycles of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C, and finally the melt curve was performed from 65to 95°C with a 0.5°C increment for 10 s. Two genes, including rpl13 and rps11 were used as references. The primer sequences are listed in **Table 2**.

Statistical Analysis

Animal survival rates were analyzed by Kaplan–Meier survival estimate with Bonferroni *post hoc* test with GraphPad Prism version 5.0 software. Other data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test with GraphPad Prism version 5.0 software. In addition, unpaired *t*-test was used to compare data from two groups when appropriate. Wherever applicable, *P*-values are reported, and a *P*-value of ≤ 0.05 is considered significant.

RESULTS

Identification of Probiotic Bacteria That Mitigate *A. veronii* Infection in Pre-colonized Zebrafish Larvae

In order to screen the probiotic strains that protect zebrafish larva from damage induced by *A. veronii* infection, we pre-colonized unhatched (3 dpf) conventional zebrafish larvae with 17 Gram-positive bacteria commonly often used as probiotics in aquaculture and elsewhere in the food industry (**Table 1**). These

TABLE 2 | Primers for RT-Qpcr.

Primer	Sequence (5′–3′)
rps11 F	ACAGAAATGCCCCTTCACTG
rps11 R	GCCTCTTCTCAAAACGGTTG
rpl13 F	TCTGGAGGACTGTAAGAGGTATGC
rpl13 R	TCAGACGCACAATCTTGAGAGCAG
TNF-α F	CAGAGTTGTATCCACCTGTTA
TNF-α R	TTCACGCTCCATAAGACCCA
IL-10 F	ATTTGTGGAGGGCTTTCCTT
IL-10 R	AGAGCTGTTGGCAGAATGGT
Saa F	CGCAGAGGCAATTCAGAT
Saa R	CAGGCCTTTAAGTCTGTATTTGTTG
IL-1β F	GAGACAGACGGTGCTGTTTA
IL-1β R	GTAAGACGGCACTGAATCCA
TLR-4a F	TGTCAAGATGCCACATCAGA
TLR-4a R	TCCACAAGAACAAGCCTTTG
TLR3 F	CTACGTGATAGCTCCGCCTC
TLR3 R	ACAAGCGTAGAACAAGGGCA
TLR5a F	CATTCTGGTGGTGCTTGTT
TLR5a R	CTGCTGCTTCAGGATTGTT
TLR2 F	ATACAAGCCAAACGGAAACCT
TLR2 R	CTTCTCACATTTCCGCATCAT
NF-κB F	GCAAGATGAGAACGGAGACAC
NF-kB R	CTACCAGCAATCGCAAACAA
TLR5b F	GTGAGGAGCCTGATCCTGATAG
TLR5b R	CATACTAAATGTATAATAAGTCTACCATG
Myd88 F	TCCACAGGGACTGACACCTGAGA
Myd88 R	GCTGAGTCTTCAGCACAGCAGAT
TLR1 F	CCCAAGCTTGAAGGCGACTGTG
TLR1 R	GTACTTTGAGGGAATGAGATACAG
IL-6 F	TCAACTTCTCCAGCGTGATG
IL-6 R	TCTTTCCCTCTTTTCCTCCTG

pre-colonized larvae were then infected at 7 dpf with *A. veronii* and their mortality rate was compared to that of the control larvae. The result showed that pre-incubation with *Lactobacillus casei* BL23 significantly increased the survival rate of larvae upon *A. veronii* infection (**Supplementary Figure S1**).

Assessment of *L. casei* BL23 Protection against *A. veronii* Infection in Zebrafish Larvae by Dose

To characterize the protective effect of *L. casei* BL23 in zebrafish larvae, we first determined whether the protective effect towards zebrafish larvae was dose-dependent. As shown in **Figures 1A,B**, zebrafish larvae pre-colonized with increasing dosage of *L. casei* BL23 correlated with increased larvae survival rate after *A. veronii* infection. No significant difference in survival rate was observed between the larvae pre-colonized with *L. casei* BL23 at the dose of 1.0×10^5 CFU/ml and control larvae after *A. veronii* infection (**Figures 1A,B**). However, the survival rate of larvae pre-colonized with BL23 at 1.0×10^6 CFU/ml or 1.0×10^7 CFU/ml was significantly higher than that of control larvae after *A. veronii* infection (**Figures 1A,B**, $P < 0.05$). These data indicated that the protective effect of *L. casei* BL23 in zebrafish was dose dependent.

The Protection of *L. casei* BL23 in Zebrafish Was Mediated by the Bacteria Itself and Did Not Involve the Microbiota

In order to determine whether the protective effect of *L. casei* BL23 on zebrafish is affected by the bacterium itself or via alterations in the gut microbiota, a GF zebrafish and gut microbe transplant model was established. Here, the intestinal microbiota associated with administration of control treatment and *L. casei* BL23 treated (*L. casei* BL23 administration for 2 weeks at a dose of 1.0×10^6 CFU/ml) zebrafish were transferred to freshly hatched GF zebrafish at 4 dpf. These gut microbiota recipient larvae were then infected with *A. veronii* at 7 dpf and their mortality was compared to that of the *A. veronii*-infected GF larvae. The results showed survival to be significantly higher in zebrafish colonized with microbiota from either the control or fish treated with *L. casei* BL23 at doses of 1.0×10^5 CFU/ml and 1.0×10^6 CFU/ml compared with the GF fish (**Figures 2A,B**, $P < 0.001$). No significant difference in survival rate was observed between larvae colonized with microbiota from control or BL23-treated zebrafish (**Figures 2A,B**).

High-throughput sequencing with the 16S *r*RNA gene was performed to characterize the gut microbiota of zebrafish treated with the control or *L. casei* BL23. The results showed that phyla Fusobacteria and Proteobacteria and genus of *Cetobacterium* and *Aeromonas* were dominant in the intestines of zebrafish (**Figures 2C,D**). The gut microbial community exhibited no statistical difference between the two groups at the level of phylum to genus (**Figures 2C,D** and **Supplementary Figures S2A–C**). These data indicated that administration of *L. casei* BL23 did not alter the gut microbiota in zebrafish, which is consistent with the results of the microbiota transfer experiment described above.

We then mono-colonized GF zebrafish larvae (4 dpf) with *L. casei* BL23, then the larvae were infected with *A. veronii* at 7 dpf. We found that the survival of BL23-treated GF larvae was significantly higher compared with the GF larvae after challenge with *A. veronii*. The protection mediated by BL23 was found to be dose-dependent (**Figures 2E,F**, $P < 0.001$). Among larvae treated with *L. casei* BL23, the transcription levels of several chemokines, specifically interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), interleukin-10 (IL-10), and serum amyloid A (Saa), increased 4–80 fold at 24 h after *A. veronii* infection, but it decreased to near basal levels at 48 h (**Figure 3**). In contrast, the transcription level of these cytokines in GF fish increased slowly and the increasing trend was maintained for 48 h post challenge (**Figure 3**). The mRNA levels of TNF-α, IL-1β, IL-10, and Saa were higher in *L. casei* BL23-treated larvae than in GF larvae at 24 h (**Figures 3A–D**, $P < 0.01$) and lower (except IL-10) at 48 h after challenge with *A. veronii* (**Figures 3A,B,D**, $P < 0.05$).

Collectively, these data indicated that the protection of *L. casei* BL23 against *A. veronii* infection in zebrafish larvae was mediated by baceterium itself and did not involve the microbiota.

Protective Effect of *L. casei* BL23 Irrespective of Cells' Viability

In order to determine whether protective effect of *L. casei* BL23 in zebrafish is mediated by cellular metabolites or cell structural components, we tested the effect of live and dead (4% paraformaldehyde fixed) cells of *L. casei* BL23 in both conventional and GF zebrafish. The results showed that, for both conventional and GF zebrafish, live and dead cells of *L. casei* BL23 both efficiently increased the survival rate of zebrafish larvae after infection with *A. veronii* (**Figures 4B–D**, 2E,F, $P < 0.05$). In addition, a similar difference in cytokine expression was observed between dead BL23-treated larvae and live BL23-treated larvae after *A. veronii* infection (**Figures 4E–H**). These data suggest that the immuno-regulation and anti-infectious activity of *L. casei* BL23 was mediated by certain cell components irrespective of cell viability.

EPS Extract from *L. casei* BL23 and *A. veronii* Infection

Using the data given above, we speculated that the protective effect of *L. casei* BL23 in zebrafish might involve the EPS of the bacteria. So, we extracted and tested the biological roles of the EPS from *L. casei* BL23. GF larvae (4 dpf) were treated with 2, 10, and 20 μg/ml EPS from *L. casei* BL23, respectively. Then the larvae were infected with *A. veronii* at 7 dpf, and the survival rates of these fish were compared to the *A. veronii*-infected GF fish. The survival rate of fish treated with the *L. casei* BL23 EPS at 10 and 20 μg/ml was significantly higher than those of GF fish after *A. veronii* infection (**Figures 5A,B**, $P < 0.01$).

Extraction and Characterization of the EPS Extract from *L. casei* BL23

The EPS extract was purified by SEC on a column of Superdex75 (10/300 GE). Interestingly, as shown in

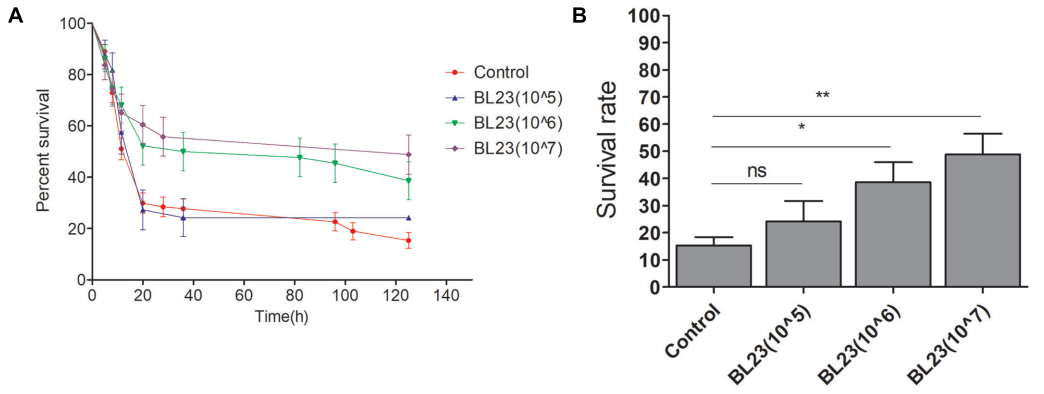

FIGURE 1 | Dose dependent of *Lactobacillus casei* BL23 protection against *Aeromonas veronii* infection in conventional zebrafish larvae. The survival rate **(A)** and final survival rate **(B)** of zebrafish larvae treated with *L. casei* BL23 at a dose of 10^5, 10^6, and 10^7 CFU/ml, respectively, after infected with *A. veronii*. Asterisks indicate significant difference compared with control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

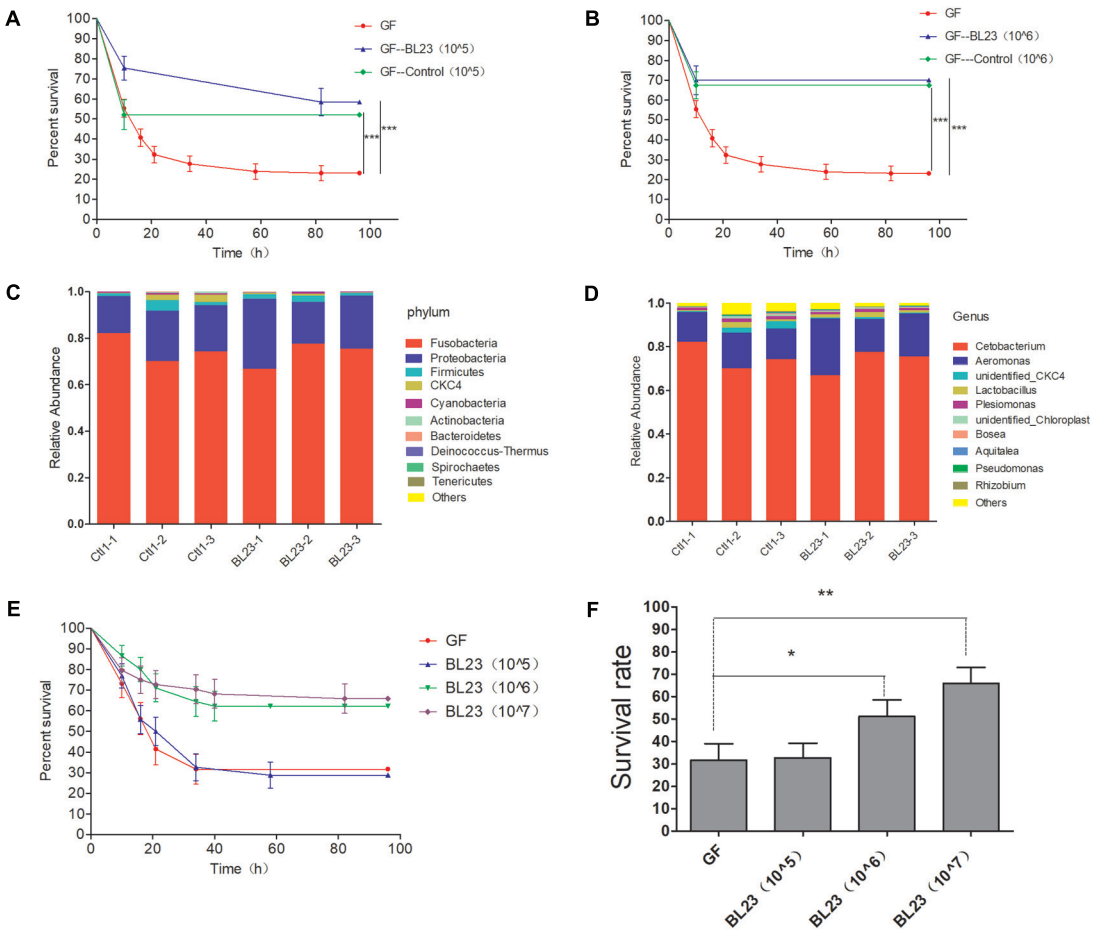

FIGURE 2 | The protection of *L. casei* BL23 was mediated by the bacteria itself and does not involve intestinal microbiota of host. The survival rate of GF zebrafish larvae colonized with gut microbiota from control or *L. casei* BL23 treated fish at a dose of 10^5 CFU/mL **(A)** and 10^6 CFU/mL **(B)** after infected with *A. veronii*. Gut microbiota of zebrafish from control or *L. casei* BL23 treated fish at phylum **(C)** and genus **(D)** level. The survival rate **(E)** and final survival rate **(F)** of GF zebrafish larvae treated with *L. casei* BL23 at a dose of 10^5, 10^6, and 10^7 CFU/Ml, respectively after infected with *A. veronii*. Asterisks indicate significant difference compared with control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

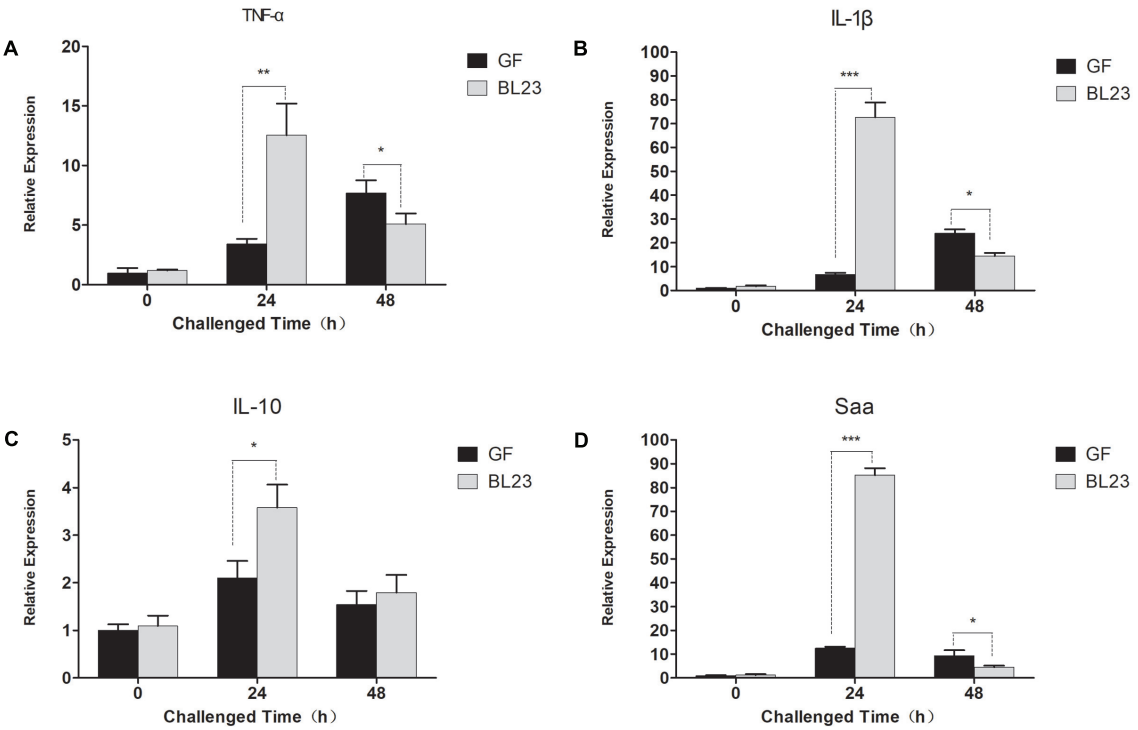

FIGURE 3 | *Lactobacillus casei* BL23 modulated immune response against *A. veronii* infection in zebrafish. The mRNA levels of TNF-α **(A)**, IL-1β **(B)**, IL-10 **(C)**, and Saa **(D)** from GF and *L. casei* BL23(10^6 CFU/mL) treated zebrafish at certain time points after infected with *A. veronii*. The results are given as mean ± SEM ($n = 3$). Asterisks indicate significant difference compared with control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Figure 6A, the EPS extract showed a single, symmetrical protein peak, indicating that the EPS extract contains a homogeneous protein. It was also confirmed by the SDS-PAGE analysis, which showed a single band with 40–45 KD in size (**Figure 6B**). In addition, qualitative carbohydrate analysis of the fractions (purified by SEC) with the phenol–sulfuric acid method showed that all the positive reactions were within the peak of the protein fractions. Furthermore, the monosaccharide composition of the EPS extract was determined by TLC after acid hydrolysis. TLC indicated that the EPS of the cell extract from *L. casei* BL23 was composed of α-Rha, α-Glc, β-GlcNAc, and β-GalNAc (**Figure 6C**). Together, these findings indicated that the EPS extract is an exopolysaccharide-protein complex (EPSP).

Immuno-regulation of EPSP in ZFL Cells

In order to characterize EPSP-mediated immuno-regulation in zebrafish, we tested the immune response of ZFL cells after EPSP treatment. The transcription level of toll-like receptors (TLRs) and cytokines were evaluated in ZFL cells at 24 h after EPSP treatment. The results showed that EPSP induced more expressionl of TLR1 and TLR2 compared with the control (**Figures 7A,B**, $P < 0.05$). In addition, the transcription level of IL-10 and TNF-α was significantly higher in ZFL cells treated with EPSP than in the control (**Figures 7C,D**, $P < 0.05$). Gene expression of IL-1β was less pronounced than in the control after EPSP treatment in ZFL cells (**Figure 7E**, $P < 0.05$). No significant difference between the EPSP-treated and control ZFL cells was observed in the transcription levels of TLR3, TLR4a, TLR5a, TLR5b, MyD88, NF-κB, or IL-6 (**Figure 7F** and **Supplementary Figures S3A–G**). These data suggested that the immuno-regulation of EPSP on zebrafish were might involve the TLR1/TLR2 signal pathway.

DISCUSSION

Infectious diseases remain the major problem in aquaculture production and food security (Leung et al., 2013; Stentiford et al., 2017). Industry-wide losses to aquatic animal diseases exceed 6 billion dollars per year (World Bank, 2014). In certain sectors (e.g., farming of shrimp and various fishes), disease outbreaks have particularly devastating economic and social impacts, with total losses exceeding 40% of the worldwide capacity (Israngkura and Haesae, 2002; Lafferty et al., 2015).

Probiotics have been widely used in aquaculture for disease control and immune improvement for decades (Newaj-Fyzul et al., 2014). However, several studies and meta-analyses of randomized probiotic trials have shown that application of probiotics has varying success rates (Sazawal et al., 2006; Kalliomaki et al., 2010; Li-Li, 2010). This is in part because

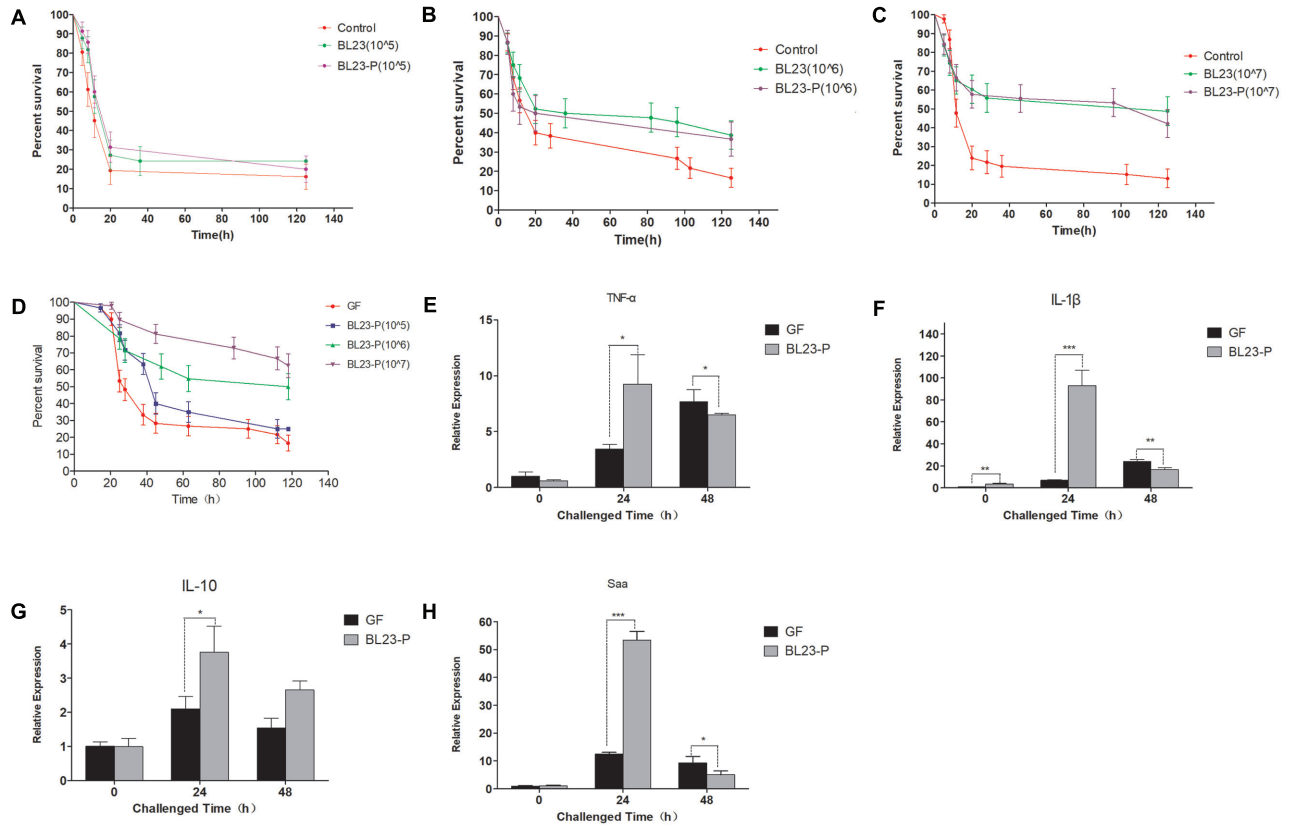

FIGURE 4 | Anti-infective activity of *L. casei* BL23 was mediated by some cellular structural components irrespective of cell viability. The survival rate **(A)** of conventional zebrafish larvae treated with live or dead cells of *L. casei* BL23 at a dose of 10^5 CFU/ml after infected with *A. veronii*. The survival rate **(B)** of conventional zebrafish larvae treated with live or dead cells of *L. casei* BL23 at a dose of 10^6 CFU/ml after infected with *A. veronii*. The survival rate **(C)** of conventional zebrafish larvae treated with live or dead cells of *L. casei* BL23 at a dose of 10^7CFU/ml after infected with *A. veronii*. The survival rate **(D)** of GF zebrafish larvae treated with dead cells of *L. casei* BL23 at a dose of 10^5, 10^6, and 10^7 CFU/Ml, respectively after infected with *A. veronii*. The mRNA levels of TNF-α **(E)**, IL-1β **(F)**, IL-10 **(G)**, and Saa **(H)** from GF and dead BL23 (10^6 CFU/mL) treated zebrafish at certain time points after infected with *A. veronii*. Asterisks indicate significant difference compared with control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

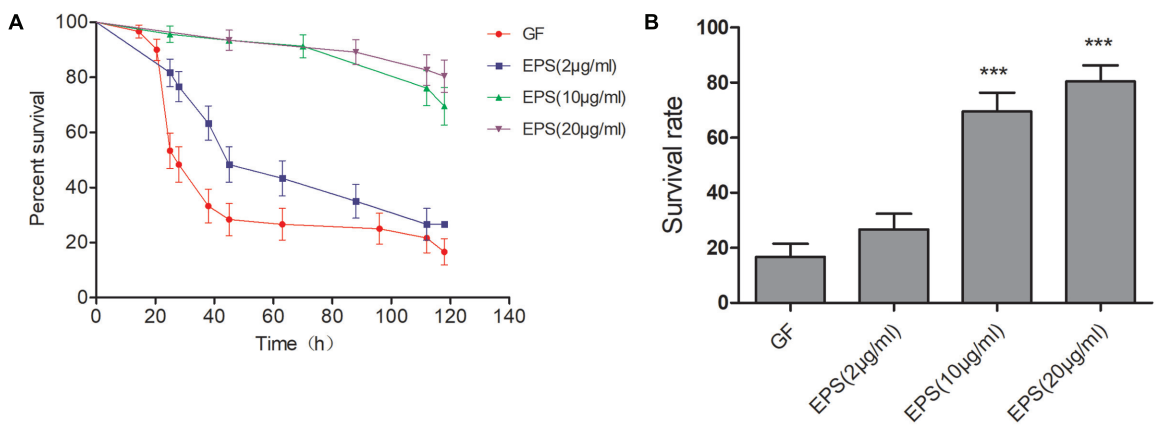

FIGURE 5 | Exopolysaccharides (EPS) extracted from *L. casei* BL23 protected against *A. veronii* infection in GF zebrafish larvae. The survival rate **(A)** and final survival rate **(B)** of GF zebrafish larvae treated with EPSP at a dose of 2, 10, and 20 μg/mL, respectively after infected with *A. veronii*. Asterisks indicate significant difference compared with control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

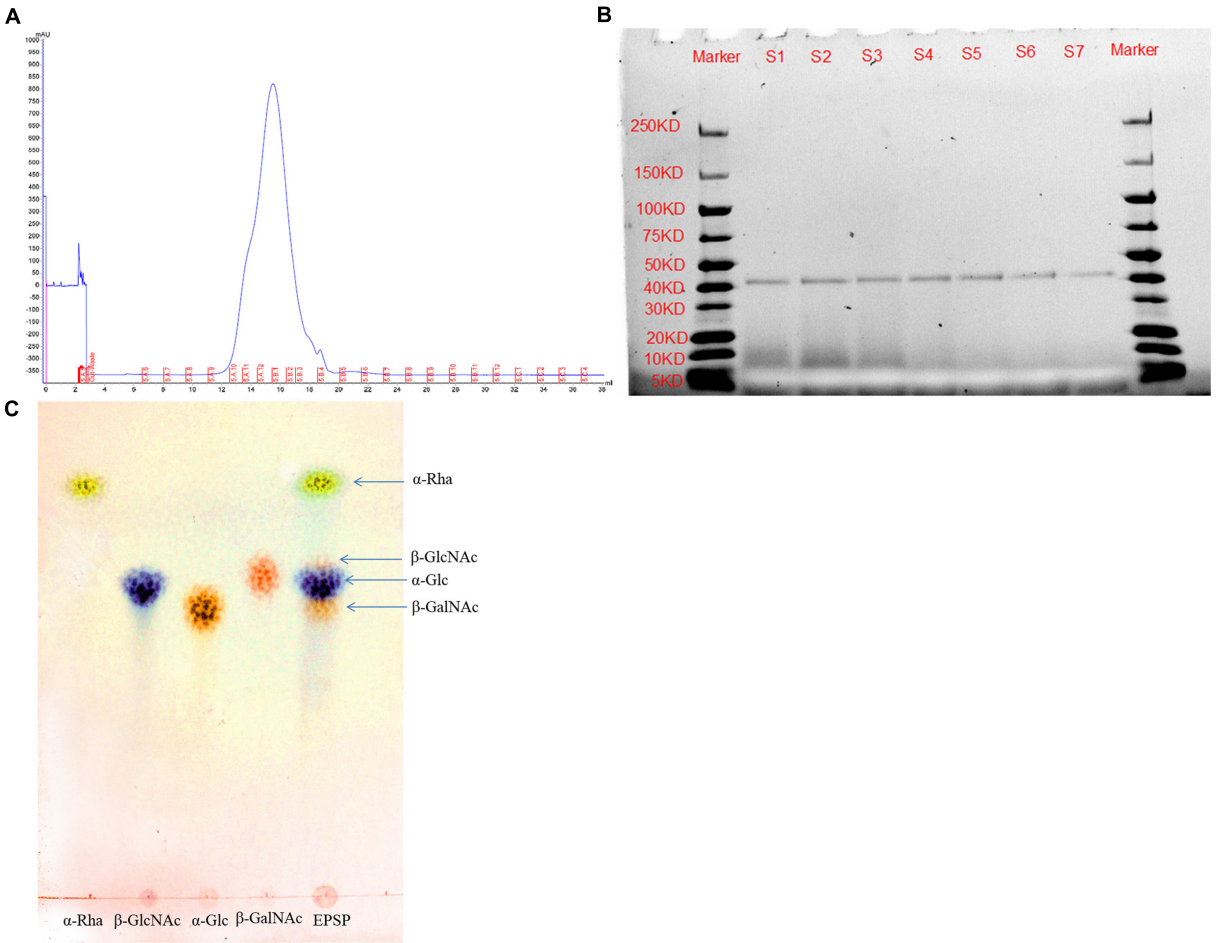

FIGURE 6 | Characterization of the EPSP from *L. casei* BL23. Purity of EPSP analyzed by size-exclusion chromatography (SEC) on a column of Superdex75 (10/300 GE) **(A)** and SDS-PAGE **(B)**. Monosaccharide composition of EPSP (α-Rha, α-Glc, β-GlcNAc, and β-GalNAc) analyzed by TLC **(C)**.

the efficacy of different probiotic strains against particular pathogens is often both host-specific and probiotic strain-specific. Moreover, knowledge concerning the precise molecular mechanisms underlying the action of specific probiotic strains is limited (Nayak, 2010). In this study, we showed that among 17 selected probiotic strains, only *L. casei* BL23 was able to protect zebrafish from *A. veronii* infection (**Figure 1**).

Possible benefits of probiotic treatment have already been suggested: competitive exclusion of pathogenic bacteria, production of inhibitory compounds, inhibition of the expression of virulence genes, disruption of quorum sensing of the pathogens, improvement in water quality, and enhancement of the immune response against pathogens (Pandiyan et al., 2013; Fuente Mde et al., 2015; Reddy, 2015; Selvaraju, 2015). Here, we developed a GF zebrafish and gut microbiota transplant model to characterize the mode of action of *L. casei* BL23 in protecting zebrafish from *A. veronii* infection. The results showed that the gut microbial community exhibited no statistically significant difference between *L. casei* BL23 administration group and control group at the level of phylum to genus (**Figures 2C,D** and **Supplementary Figures S2A–C**). This might be because *L. casei* BL23 does not colonize the intestine of zebrafish (Qin et al., 2014). In addition, we showed that both live and dead cells of *L. casei* BL23 significantly increased resistance against *A. veronii* infection in GF and conventional zebrafish (**Figures 2E,F**, **4**, $P < 0.05$). *L. casei* BL23 also modulated the expression of the pro-inflammatory cytokines IL-1β and TNF-α, the inflammation marker Saa, and the regulator cytokine IL-10 after *A. veronii* infection (**Figure 3**, $P < 0.05$). We speculated that the anti-infective effect of *L. casei* BL23 was mediated by the enhancement of immune responses against pathogens in zebrafish, which was induced by certain cellular structural components of BL23, but not mediated by metabolites. We also speculated that this anti-infective effect of *L. casei* BL23 did not involve alteration of the gut microbiota.

Bacterial cell components such as peptidoglycan (PGN) (Mackenzie, 2010), lipoteichoic acid (LTA) (Grangette et al.,

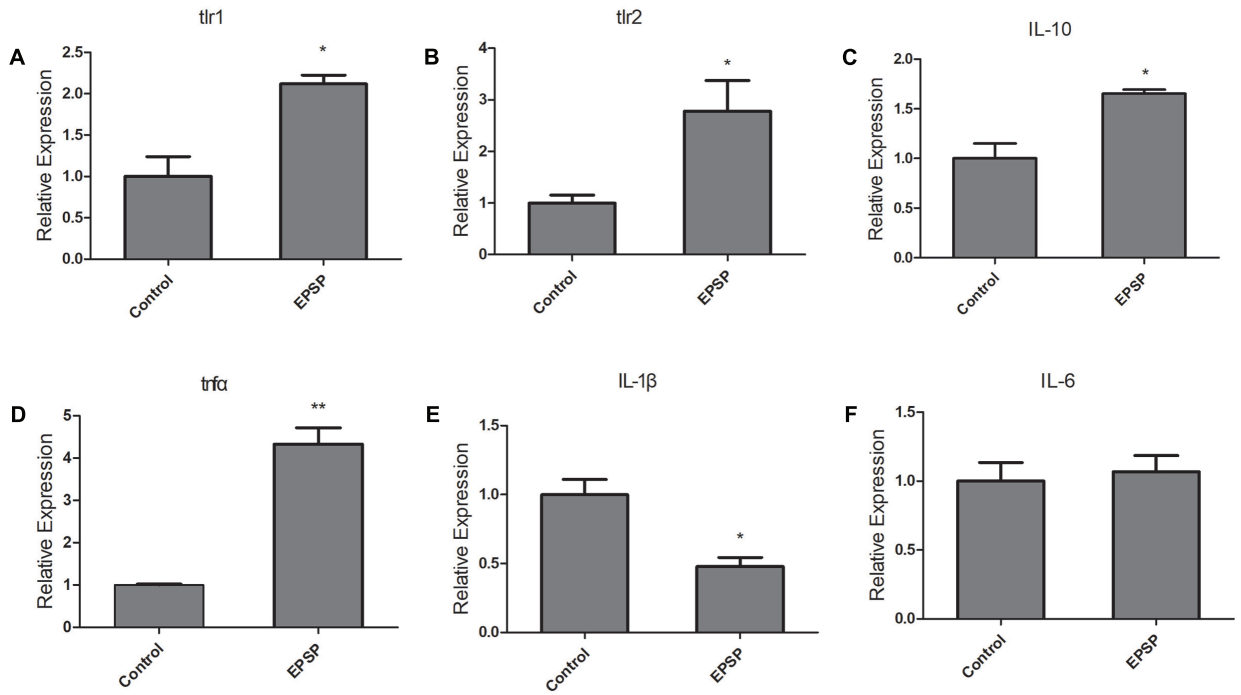

FIGURE 7 | Exopolysaccharide-protein complex (EPSP) modulated immune response in ZFL cells via TLR1 and TLR2. The mRNA levels of TLR1 **(A)**, TLR2 **(B)**, IL-10 **(C)**, TNFα **(D)**, IL-1β **(E)**, and IL-6 **(F)** in ZFL cells from control and EPSP treatment (10 μg/mL) groups after 24 h treatment. Asterisks indicate significant difference between the control and EPSP treatment group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

2005), EPS (Vinderola et al., 2006; Wu et al., 2010), outer membrane proteins (OMP), and extracellular proteins (ECP) (Abbass et al., 2010; Sharifuzzaman et al., 2011) from Gram-positive bacteria have been reported to act as potent immunostimulants for animals. These molecules possess conserved microbe-associated molecular patterns (MAMPs), which can be recognized by pattern recognition receptors (PRRs), e.g., TLRs, nucleotide oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs). They then modulate the immune response of the host (Lebeer et al., 2010; Bron et al., 2012).

Exopolysaccharides produced by some strains of LAB have been shown to possess beneficial health effects, such as blood cholesterol reduction (Nakajima et al., 2010), immunostimulatory capacities (Chabot et al., 2001a), and antitumor activity (Kitazawa et al., 1998). For example, the antihypertensive and anti-infective activities of strain *L. casei* YIT9018 were attributed to polysaccharide–glycopeptide and polysaccharide–peptidoglycan (PS-PG) complexes, respectively (Nagaoka et al., 1990; Sawada et al., 1990). Immunomodulating properties were also reported for the cell wall polysaccharide (WPS) of *L. casei* strain Shirota (YIT 9029) (Emi et al., 2008). Kefiran, an EPS produced by a number of strains of lactobacilli in the fermented milk drink kefir, may play a role in promoting intestinal homeostasis by increasing luminal IgA and both pro- and anti-inflammatory cytokines, such as IFN-c, TNFα, IL-6, and IL-10, as observed in the small and large intestine (Vinderola et al., 2006). Additionally, EPSs isolated from strains of lactobacilli and bifidobacteria have been found to augment the release of both pro- inflammatory and anti-inflammatory cytokines, such as TNF-α, IL-6, and IL-10 in murine macrophages (Chabot et al., 2001b; Bleau et al., 2010; Wu et al., 2010). Chabot et al. (2001a) suggested EPS could exert their action via the mannose receptor. Lin et al. (2011) reported that TA-1 (novel EPS) can stimulate the release of the pro-inflammatory cytokines TNF-α and IL-6 from murine macrophages via a TLR2 mediated pathway.

In the present study, EPSP extracted from *L. casei* BL23 was characterized and consists of EPS and a 40–45 KD ECP (**Figure 6B**). The EPS have been shown to consist of α-Rha, α-Glc, β-GlcNAc, and β-GalNAc (**Figure 6C**). Very recently, Vinogradov et al. (2016) showed that the structure of the EPS of *L. casei* BL23 consists of α-Rha, α-Glc, β-GlcNAc, and β-GalNAc, forming a branched heptasaccharide repeating unit (variant 1) with an additional partial substitution with α-Glc (variant 2) and a modified non-reducing octasaccharide end, corresponding to a terminal unit of the EPS (variant 3). We showed that the survival rate of zebrafish was significantly higher in fish treated with EPSP of BL23 at 10–20 μg/ml than in control fish after *A. veronii* infection (**Figure 5**, $P < 0.001$). In addition, the EPSP induced more expression of TLR1, TLR2, IL-10, and TNF-α and reduced the expression of IL-1β in ZFL cells (**Figure 7**, $P < 0.05$). These findings indicated that the EPSP enhanced zebrafish immune

response against *A. veronii* might involve the TLR1/TLR2 signal pathway.

CONCLUSION

Our results indicated that the *L. casei* BL23 showed high efficiency against *A. veronii* infection in zebrafish irrespective of cell viability. This protective effect of *L. casei* BL23 might involve membrane PRR signaling pathways induced by EPSP. Accordingly, *L. casei* BL23 may be suitable for disease control in aquaculture, especially for use in larval fish. However, gene knockout zebrafish lacking related factors are needed to further investigate the mechanisms underlying the action of EPSP.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by the Key Project of Chinese National Programs for Fundamental Research and Development (2015CB150605), the National Natural Science Foundation of China (31272672, 31572633), and National gm project (National gm project).

ACKNOWLEDGMENT

The authors thank LetPub (http://www.letpub.com) for providing linguistic assistance during the preparation of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02406/full#supplementary-material

FIGURE S1 | The anti-infective effect of potential protiotics against *Aeromonas veronii* in zebrafish larvae.

FIGURE S2 | Gut microbiota of zebrafish from control or *Lactobacillus casei* BL23 treated fish at class **(A)**, order **(B)**, and family **(C)** level.

FIGURE S3 | The mRNA levels of TLR3a **(A)**, TLR3b **(B)**, TLR4a **(C)**, TLR5a **(D)**, TLR5b **(E)**, MyD88 **(F)**, and NF-κB **(G)** in ZFL cells from control and Exopolysaccharide-protein complex (EPSP)-treated (10 μg/mL) groups after 24 h treatment.

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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.

frontiers in Immunology

GENERAL COMMENTARY
published: 27 July 2017
doi: 10.3389/fimmu.2017.00850

Commentary: Dietary Polyphenols Promote Growth of the Gut Bacterium *Akkermansia muciniphila* and Attenuate High-Fat Diet-Induced Metabolic Syndrome

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Keywords: gut microbiota, polyphenols, *Akkermansia*, diet, obesity

A commentary on

Dietary Polyphenols Promote Growth of the Gut Bacterium *Akkermansia muciniphila* and Attenuate High-Fat Diet-Induced Metabolic Syndrome
by Roopchand DE, Carmody RN, Kuhn P, Moskal K, Rojas-Silva P, Turnbaugh PJ, et al. Diabetes (2015) 64:2847–58. doi: 10.2337/db14-1916

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: *08 April 2017*
Accepted: *05 July 2017*
Published: *27 July 2017*

Citation:
Anonye BO (2017) Commentary: Dietary Polyphenols Promote Growth of the Gut Bacterium Akkermansia muciniphila and Attenuate High-Fat Diet-Induced Metabolic Syndrome. Front. Immunol. 8:850. doi: 10.3389/fimmu.2017.00850

Dietary polyphenols exert a range of beneficial outcomes on the intestinal microbiota, and in metabolic syndrome, such as anti-inflammatory, antioxidant, anticarcinogenic, and antidiabetic effects. Research by Roopchand and colleagues demonstrated that concord grape polyphenols (GP) led to changes in the gut microbiota and reduction in conditions associated with metabolic syndrome arising from high-fat diet (HFD) in mice (1). Mice were divided into three groups and fed with HFD only or HFD supplemented with 10% soy-protein isolate (SPI) or HFD supplemented with 10% GP-SPI, respectively, for 13 weeks. When compared to the other diet groups, mice on the GP-SPI diet had lower body weight and adiposity though the food intake was similar across the groups (1).

Also, mice on the GP-SPI diet reduced markers of systemic inflammation as IL-6 was undetectable, and low levels of TNF-α and bacterial lipopolysaccharide was detected in the serum in comparison to the SPI group. However, levels of cholesterol, triglycerides, and IL-1β in the serum were not significantly different from mice fed with the SPI diet (1).

So, how did the GP-based diet impact the gastrointestinal tract? Just as observed in the serum, lower expression of TNF-α and IL-6 was detected in the colon. Fasting-induced adipose factor, a circulating lipoprotein lipase inhibitor, was significantly increased in the ileum compared to the SPI diet (1). This suggests that the GPs may aid in the suppression of fatty acid storage thereby attenuating the effects of diet-based metabolic syndrome. Further evidence in the ileum was provided by increased gene expression of proglucagon, a precursor of proteins associated with production of insulin and maintenance of gut barrier integrity (1). However, it would have been nice to see how these evidences compared with the controls used in this study as the data were not shown. Glut2, a gene for glucose transport, was also significantly lower in the jejunum tissue when compared to the mice on the SPI-diet (1).

Grape polyphenol-based diet led to decreased ratio of Firmicutes to Bacteroidetes and significant increase in the relative abundance of *Akkermansia muciniphila* in the cecal and fecal microbiota (1). This decrease in the proportion of Firmicutes to Bacteroidetes has been reported in other

diet-induced obesity studies (2, 3). However, another study did not find a causal effect of Firmicutes to Bacteroidetes ratio in relation to obesity (4).

Similarly, the effects of dietary polyphenol from cranberry extract were evaluated in mice fed with high-fat/high-sucrose diet for 8 weeks (5). Cranberry extract prevented weight gain, enhanced insulin sensitivity, and reduced triglycerides in the jejunum. Cranberry extract-supplemented diet also led to a dramatic increase in *Akkermansia* (5). Furthermore, a reduction in body weight gain and insulin was observed in rats fed with a standard-chow diet supplemented with pterostilbene (6). Changes in the gut microbiota with increase in *Akkermansia* were also observed (6).

Of what importance is *Akkermansia* in the intestinal microbiota and how does it influence diet-induced obesity and metabolic disorders? *A. muciniphila* is a mucin degrading bacterium present in the mucus layer of the intestinal epithelium and may represent 3–5% of the gut microbiota in healthy adults (7). Several studies have shown an increase in *Akkermansia* in diet-induced obesity studies and correlates with the reduction of weight gain, adiposity, and improved glucose tolerance (1, 5, 8). Administration of live *A. muciniphila* reversed the symptoms of obesity and metabolic syndrome in HFD mice by reducing adiposity, inflammatory markers, insulin resistance, and improved gut barrier (7). Recently, it was shown that the introduction of capsaicin, a dietary polyphenol, led to an abundance of the genera *Akkermansia*, *Bacteroides*, and *Coprococcus* in mice fed with HFD and a decrease in weight gain (9). Potential mechanisms by which *Akkermansia* influences the host microbiota leading to these beneficial outcomes are depicted below (**Figure 1**).

These and other studies suggest that dietary polyphenols play a role in the modulation of the gut microbiota that may favor positive outcomes. Understanding the mechanism of action of dietary polyphenols is likely to be key in the development of new diet-based therapies. This is because two different polyphenols can give complementary and dissimilar effects on the gut microbiota as observed in black tea and red wine grape extracts (11).

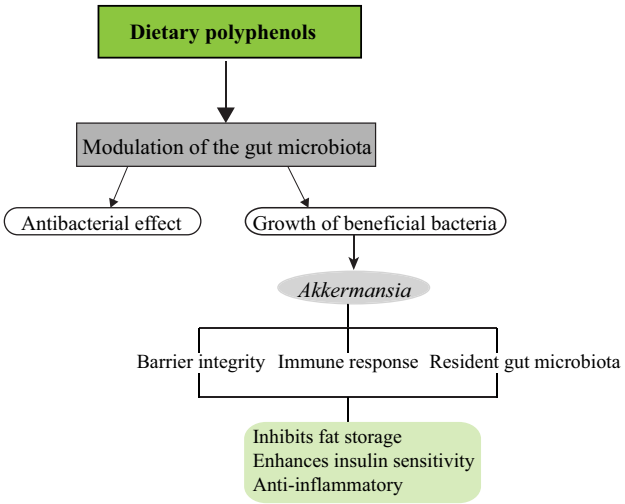

FIGURE 1 | Dietary polyphenols and impact on the gut microbiota. Different classes of dietary polyphenols modulate the gut microbiota by various means. This could be by exerting antibacterial effect as observed in flavonoids on six pure cultures of intestinal bacteria (10) and stimulating the growth of beneficial bacteria in the gut microbiota such as *Akkermansia*. *Akkermansia*, in turn, generates short-chain fatty acids from the breakdown of mucins, which stimulates the goblet cells to produce more mucus thereby preserving/replenishing the intestinal barrier integrity. Mucus secretion is associated with the activation of the immune system, by preventing increased interaction of microbe-associated molecular patterns with intestinal epithelial cells, and stimulating other immune responses. This helps to reduce intestinal inflammation. *Akkermansia* may also influence resident gut bacteria by acting as an oxygen scavenger thereby, creating a favorable environment for the growth of strict anaerobes, which could have a synergistic effect on the host.

As such, more studies are needed to unravel the bioactivity of this class of xenobiotics to fully understand their effects on the host.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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

frontiers in Physiology

ORIGINAL RESEARCH
published: 24 July 2017
doi: 10.3389/fphys.2017.00487

Gut Microbiome Response to Sucralose and Its Potential Role in Inducing Liver Inflammation in Mice

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Sucralose is the most widely used artificial sweetener, and its health effects have been highly debated over the years. In particular, previous studies have shown that sucralose consumption can alter the gut microbiota. The gut microbiome plays a key role in processes related to host health, such as food digestion and fermentation, immune cell development, and enteric nervous system regulation. Inflammation is one of the most common effects associated with gut microbiome dysbiosis, which has been linked to a series of human diseases, such as diabetes and obesity. The aim of this study was to investigate the structural and functional effects of sucralose on the gut microbiota and associated inflammation in the host. In this study, C57BL/6 male mice received sucralose in their drinking water for 6 months. The difference in gut microbiota composition and metabolites between control and sucralose-treated mice was determined using 16S rRNA gene sequencing, functional gene enrichment analysis and metabolomics. Inflammatory gene expression in tissues was analyzed by RT-PCR. Alterations in bacterial genera showed that sucralose affects the gut microbiota and its developmental dynamics. Enrichment of bacterial pro-inflammatory genes and disruption in fecal metabolites suggest that 6-month sucralose consumption at the human acceptable daily intake (ADI) may increase the risk of developing tissue inflammation by disrupting the gut microbiota, which is supported by elevated pro-inflammatory gene expression in the liver of sucralose-treated mice. Our results highlight the role of sucralose-gut microbiome interaction in regulating host health-related processes, particularly chronic inflammation.

Keywords: artificial sweetener, sucralose, gut microbiota, metabolomics, inflammation

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: *09 April 2017*
Accepted: *26 June 2017*
Published: *24 July 2017*

Citation:
Bian X, Chi L, Gao B, Tu P, Ru H and Lu K (2017) Gut Microbiome Response to Sucralose and Its Potential Role in Inducing Liver Inflammation in Mice. Front. Physiol. 8:487. doi: 10.3389/fphys.2017.00487

INTRODUCTION

Artificial sweeteners are commonly used food additives that have much higher sweetness intensities than table sugars (Gardner et al., 2012). Consumption of artificial sweeteners is increasing in the United States, and in 2008, the prevalence of consumption of beverages containing artificial sweeteners was 24.1% among adults. Sucralose, which is 600 times sweeter than sucrose, is one of the most commonly used artificial sweeteners in the market due to its extremely sugar-like taste, lack of a bitter aftertaste, stability at high temperatures, and long shelf-life (Grice and Goldsmith, 2000; Sylvetsky et al., 2012). The health effects of sucralose have been highly debated over the years. A number of previous studies concluded that sucralose is safe for its intended use as an artificial

sweetener and that the body acquires no calories from sucralose (Grotz and Munro, 2009; Sylvetsky et al., 2012). Most ingested sucralose is not absorbed or metabolized and moves through the gastrointestinal tract unchanged (Roberts et al., 2000). However, this does not prove that sucralose has no effect on the gut microbiota. One study showed that a product containing sucralose altered the rat gut microbiota and induce inflammatory lymphocyte infiltration (Abou-Donia et al., 2008), but the study was considered to be deficient in several aspects (Brusick et al., 2009), including the use of high doses and a sucralose mixture instead of pure sucralose. Another study that focused on the metabolic effects of sucralose on environmental bacteria showed that sucralose can inhibit the growth of certain bacterial species (Omran et al., 2013). Therefore, sucralose may inhibit intestinal bacteria and alter the gut microbiota, and these alterations could affect host health.

The mucosal surfaces of the human intestines are host to more than 100 trillion microbes (including bacteria, fungi, viruses, and parasites) from more than 1,000 species (Ley et al., 2006; Qin et al., 2010). Gut microbes interact with the host mucosa directly via the recognition of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide, flagellin, and bacterial DNA and RNA, by mucosal pattern recognition receptors (PRRs; Maloy and Powrie, 2011). Alternatively, the interaction can occur indirectly through secreted metabolites (Nicholson et al., 2012). These interactions are involved in maintaining symbiotic homeostasis. Increasing evidence indicates that this homeostasis is vital for human health (Holmes et al., 2011; Tremaroli and Backhed, 2012). Gut microbes can help maintain good host health by participating in digestion and fermentation of food, development of immune cells, regulation of the enteric nervous system, and prevention of colonization by pathogens (Holmes et al., 2011). The host, in turn, provides a habitat and nutrients and secretes antibodies to inhibit the aggressive expansion of microbes (Kamada et al., 2013). Being highly diverse, the gut microbiota can be shaped by various factors, including aging, diet, drugs, antibiotics, diseases, stress, exercise, and environmental pollutants (Ley et al., 2006; Nicholson et al., 2012), and if homeostasis is disrupted as a result of this shaping, many adverse outcomes may occur, such as cardiovascular disease, obesity, diabetes, allergies, and cancer (Ley et al., 2006). For example, an increased ratio of Firmicutes to Bacteroidetes was found in obese mice compared with their lean littermates (Turnbaugh et al., 2006), and obesity-related phenotypes were found to be transmissible in a study in which fecal microbes from obese and lean human twins were transferred to germ-free mice (Ridaura et al., 2013). Likewise, a remarkable increase in taurocholic acid and *Bilophila wadsworthia* induced by dietary fat promotes colitis in IL10-deficient mice (Devkota et al., 2012). Metabolites produced from dietary choline by gut microbes have been shown to be modulated in obesity, diabetes, and cardiovascular diseases (Kim et al., 2010; Wang et al., 2011).

Abbreviations: PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; IBD, inflammatory bowel disease; LPS, lipopolysaccharide; iNOS, inducible nitric-oxide synthase; MMP-2, matrix metalloproteinase 2; ROS, reactive oxygen species; GI, gastrointestinal.

Inflammation is one of the most common physical conditions associated with gut microbiota dysbiosis. For example, acute or chronic inflammation is the primary characteristic of inflammatory bowel diseases (IBDs; Xavier and Podolsky, 2007), of which a disrupted gut microbiota is one of the major triggers in addition to genetic factors and the host immune system, although the precise etiology remains unclear (Hill and Artis, 2010). Moreover, increasing evidence demonstrates that low-grade chronic inflammation induced by gut microbiota disruption is associated with metabolic diseases (Holmes et al., 2011). Obesity and diabetes are associated with low-grade inflammation not only in adipose tissues but also systemically. A study of bariatric surgery, a method of reducing body weight for obese individuals, showed that one gut microbe species, *Faecalibacterium prausnitzii*, is directly related to the reduction of low-grade inflammation in obesity and diabetes (Furet et al., 2010). Dyslipidemia induced by a high-fat diet results in increased levels of lipopolysaccharide (LPS), which is a pro-inflammatory mediator (Holmes et al., 2011). Moreover, chronic inflammation induced by gut microbes can drive the progression of colorectal cancer from adenoma to invasive carcinoma (Uronis et al., 2009). Thus, inflammation can be triggered and modulated by an altered gut microbiota, and exposure to compounds that can alter the gut microbiota may induce inflammation in the host.

In this study, we first used 16S rRNA gene sequencing to examine the effects of sucralose on the gut microbiome of C57BL6/J mice over a 6-month administration period. Next, we used metabolomics to profile fecal metabolome changes associated with a perturbed gut microbiome. Finally, we assessed several markers of inflammation to define the effects of sucralose consumption on host tissues. Our results show that sucralose altered the gut microbiome and associated metabolic profiles, which may contribute to inflammatory response in the mouse liver.

MATERIALS AND METHODS

Animals and Sucralose Exposure

Male C57BL/6J mice (∼8 weeks old) purchased from the Jackson Laboratory (Bar Harbor, ME) were used in this study. Twenty male mice were housed in the University of Georgia animal facility for a week before the study and then assigned to the control or treatment group (ten mice in each group), which received tap water or sucralose (Sigma-Aldrich, MO) in tap water, respectively, for 6 months. The concentration of sucralose was 0.1 mg/ml, which was equivalent to the FDA-approved acceptable daily intake (ADI) in humans (5 mg/kg/day). Fresh solutions were made every week, and the consumption of water was measured for both groups. Standard pelleted rodent diet and tap water were provided to the mice *ad libitum*, and the mice were housed in environmental conditions of 22°C, 40–70% humidity, and a 12:12 h light:dark cycle before and during the experiment. Body weight was measured before and after the treatment. Fecal pellets were collected at baseline and at three and 6 months of treatment. Mice were euthanized with carbon dioxide and necropsied after 6 months. All experiments were approved by the University of Georgia Institutional Animal Care and Use

Committee. The animals were treated humanely and with regard for alleviation of suffering.

16S rRNA Gene Sequencing of the Gut Microbiota

The gut microbiota was investigated using 16S rRNA gene sequencing in fecal samples at different time points. DNA was isolated from the feces of individual mice using a PowerSoil DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions, and the resultant DNA was quantified and stored at −80°C until further analysis. The V4 region in the 16S rRNA gene was targeted using the universal primers 515 (5′-GTGCCAGCMGCCGCGGTAA) and 806 (5′-GGACTACHVGGGTWTCTAAT). For each sample, 1 ng of the purified fecal DNA was used as a template for amplification and then barcoded with specific indexes. The amplified products were then normalized, pooled and sequenced by an Illumina MiSeq at the Georgia Genomics Facility. Paired-end 250 × 250 (PE250, v2 kit) reads were generated at a depth of at least 25,000 reads per sample. Geneious 8.1.5 (Biomatters, Auckland, New Zealand) was used to process the raw fastq files, and the mate-paired files were trimmed to dispose of bases with an error probability higher than 0.01 and then merged. The data were then analyzed using quantitative insights into microbial ecology (QIIME, version 1.9.1) (Caporaso et al., 2010), and UCLUST was used to match operational taxonomic units (OTUs) with 97% sequence similarity against Greengenes database 13.8. The matched sequences were assigned at five different levels: phylum, class, order, family and genus. The raw data of controls and treated-mice have been uploaded into the MG-RAST server (http://metagenomics.anl.gov/) with the following job IDs: 317595, 317584, 317588, 317586, 317583, 317589, 317596, 317592, 317585, 317598, 317590, 317599, 317582, 317594, 317591, 317581, 317597, 317580, 317593, and 317587.

Functional Gene Enrichment Analysis

An open-source R package, Tax4Fun, was first used to analyze the enrichment of functional genes of the microbiome of each group (Asshauer et al., 2015). The output from QIIME with a SILVA database extension (SILVA 119) was used for this analysis. Tax4Fun can survey the functional genes of bacterial communities based on the 16S rRNA sequencing data and provide a good approximation to the gene profiles obtained from metagenomic shotgun sequencing methods. The results from Tax4Fun were further analyzed using Statistical Analysis of Metagenomic Profiles (STAMP) (version 2.1.3) (Parks et al., 2014).

Fecal Metabolomics Analysis

Metabolites in fecal samples collected at 6 months were extracted using methanol and water as previously described (Lu et al., 2014). In brief, 20 mg of feces was disrupted in 1 ml of a methanol/water solution (1:1) with a TissueLyser at 50 Hz for 5 min, followed by centrifugation at 12,000 rpm for 10 min. The resultant upper phase was collected and dried using a SpeedVac, and the dried samples were re-suspended in 20% acetonitrile for MS analysis. Metabolomic profiling was conducted using a quadrupole-time-of-flight (Q-TOF) 6520 mass spectrometer (Agilent Technologies, Santa Clara, CA) with an electrospray ionization source interfaced with an Agilent 1200 HPLC system. The detailed method for metabolomics was published previously (Lu et al., 2012). The Q-TOF was calibrated with standard tuning solution (Agilent Technologies) daily to ensure a mass accuracy within 5 ppm. A YMC Hydrosphere C18 column was used to separate the metabolites, and all detectable molecular features in a mass range of 30–2,000 m/z were captured in the positive mode.

Metabolomic Data Processing and Metabolite Identification

The data obtained from the HPLC-Q-TOF system were processed and analyzed as previously described (Lu et al., 2012). Briefly, the raw.d data were converted to .mzdata format using MassHunter Workstation software (Agilent), and only signals with an intensity higher than 1,000 counts were included in the subsequent analysis. Peak alignment, intensity calculations, and comparisons between the control and treatment group were performed using the XCMS Online tools. Significantly changed molecular features were profiled and searched against the Human Metabolome Database (HMDB) (http://www.hmdb.ca) and METLIN (http://metlin.scripps.edu) with a 10-ppm mass accuracy threshold. The matched exact masses were stored and used for the generation of MS/MS data to tentatively identify the metabolites. The matched molecular features were fragmented using MS/MS in the Q-TOF 6520 mass spectrometer to obtain the product ions, and the spectra were compared with the HMDB and METLIN MS/MS database to identify significantly altered metabolites, with at least two matched fragments within a 200 ppm mass accuracy as the matching threshold.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Liver was first segmented into left lobe, media lobe, right lobe, and caudate lobe during necropsy. Each liver segment was put into a 2 ml tube, followed by immediate addition of 1 ml of RNAlater® solution (Thermo Fisher Scientific). The tubes were stored at +4°C overnight to allow the RNAlater® solution to inhibit the RNase before they were transferred to the −80°C freezer for storage. Liver samples (right lobe) treated with RNAlater® were used to isolate RNA with an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and the resultant RNA was digested with a DNA-free™ DNA Removal Kit (Thermo Fisher Scientific) to remove genomic DNA contamination. RNA integrity number (RIN) for each RNA sample was measured with an Agilent Bioanalyzer. The RIN typically was >9.0, indicating no RNA degradation in the samples and processing. Then, cDNA was synthesized from 1 μg of total RNA using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, CA), and the products were diluted to 1:5 before use in subsequent reactions. Quantitative real-time PCR was performed on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). The sequences of the primers used for quantitative PCR were

as follows: TNF-α, 5-CCCTCACACTCAGATCATCTTCT and 5-GCTACGACGTGGGCTACAG; IL-6, 5-TAGTCCTTCCTA CCCCAATTTCC and 5-TTGGTCCTTAGCCACTCCTTC; IL-1β, 5-GCAACTGTTCCTGAACTCAACT and 5-ATCTTT TGGGGTCCGTCAACT; iNOS, 5-GTTCTCAGCCCAACAATA CAAGA and 5-GTGGACGGGTCGATGTCAC; MMP-2, 5-CAG GGAATGAGTACTGGGTCTATT and 5-ACTCCAGTTAAA GGCAGCATCTAC; MMP-9, 5-ATCTCTTCTAGAGACTGG GAAGGAG and 5-AGCTGATTGACTAAAGTAGCTGGA; MMP-13, 5-GTGTGGAGTTATGATGATGT and 5-TGCGAT TACTCCAGATACTG; and β-actin, 5-CGTGCGTGACATCAA AGAGAA and 5-TGGATGCCACAGGATTCCAT. All results were normalized to the β-actin or GAPDH gene (endogenous control). The fold change in expression over control samples was calculated using the ΔΔCT method by CFX manager software (Bio-Rad). The qPCR conditions were 95°C for 10 min, 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, and a final melting curve analysis performed by raising the temperature from 65 to 95°C in 0.5°C increments for 0.05 s each. Potential genomic DNA contamination was controlled for by DNase digestion and the inclusion of a No-RT control, and technical contamination was controlled for by the inclusion of a No-template control.

Data Analysis

The difference in individual gut bacterial components between control and sucralose-treated mice at different time points was assessed with the Mothur software. A two-tailed Welch's *t*-test ($p < 0.05$) was used to compare the difference in metabolites between the control and sucralose-treated mice. Additionally, principle component analysis (PCA) was used to examine the intrinsic clusters and outliers. Partial least squares discriminant analysis (PLS-DA) and a hierarchical clustering heat map was used to visualize metabolomic difference in different groups. A two-tailed Student's *t*-test was used to determine the statistical significance of pro-inflammatory gene expression between the controls and treated mice.

RESULTS

Sucralose Altered the Developmental Dynamics of the Gut Microbiome

The gut microbiome is a dynamic system, and its bacterial composition shifts over time. Maintaining normal developmental trajectories of the gut microbiome is critical for its functions. Feces collected from both groups of mice at baseline and after three and 6 months of administration were employed to investigate the effects of sucralose on the gut microbiome. Using 16S rRNA gene sequencing, we found that 14 genera exhibited different patterns over time in sucralose-treated mice compared with control mice, as shown in **Figure 1**. These bacterial genera exhibited no significant difference in abundance at baseline but were significantly different after three and/or 6 months of treatment, indicating that sucralose disrupts the developmental dynamics of gut bacteria. The genera included *Turicibacteraceae Turicibacter*, *Lachnospiraceae Ruminococcus*, *Ruminococcaceae Ruminococcus*, *Verrucomicrobiaceae Akkermansia*, *Staphylococcaceae Staphylococcus*, *Streptococcaceae Streptococcus*, *Dehalobacteriaceae Dehalobacterium*, *Lachnospiraceae Anaerostipes*, *Lachnospiraceae Roseburia*, and unclassified members in Family *Clostridiaceae*, *Christensenellaceae*, *Peptostreptococcaceae*, *Erysipelotrichaceae* and Order *Bacillales*..

Sucralose Increased the Abundance of Bacterial Genes Related to Pro-inflammatory Mediators

We next examined whether the changes in gut microbiome composition were associated with functional perturbations of the gut bacteria. Indeed, a number of bacterial functional genes were enriched in sucralose-treated mice. For example, functional gene enrichment analysis of the gut microbiome showed that genes related to bacterial pro-inflammatory mediators were highly elevated in sucralose-treated mice, as shown in **Figure 2**. Specifically, genes related to LPS synthesis were significantly increased after 6 months of treatment. In addition, multiple genes related to flagella protein synthesis were increased in sucralose-treated mice. Likewise, genes involved in fimbriae synthesis increased in sucralose-treated mice. Numerous bacterial toxin genes, such as toxic shock syndrome toxin-1 and shiga toxin subunits, were also elevated in sucralose-treated mice.

Sucralose Changed the Fecal Metabolome

We next conducted metabolomic profiling to examine the functional impact of sucralose on the fecal metabolome. The combination in feces of a large quantity of gut bacteria and their metabolic products creates an ideal biological sample to assess functional changes in the gut microbiome. A total of 13,611 molecular features were detected in fecal samples, 1,764 of which were significantly different ($p < 0.05$ and fold change>1.5) between the sucralose-treated and control mice (**Figure 3A**), clearly indicating that sucralose perturbed the fecal metabolome. A PLS-DA plot (**Figure 3B**) showed a separation in the molecular patterns of the two groups, and the hierarchical clustering heat map (**Figure 3C**) was consistent with this result. Molecular features matched with the HMDB and METLIN database were used for metabolite identification via MS/MS. We tentatively identified 66 metabolites, including quorum sensing compounds, amino acids and derivatives, lipids, fatty acids, bile acids, and nucleic acids, among others (Supplementary Table 1).

Sucralose Altered Quorum Sensing Signals

Bacteria control multicellular behaviors, such as biofilm growth and development, horizontal gene transfer, host-microbe cross-talk, and microbe-microbe interactions, by the cell-cell signaling process known as quorum sensing. Four acyl homoserine lactones known to be quorum sensing signals (Bainton et al., 1992; Winson et al., 1995; Passador et al., 1996; Stankowska et al., 2008; Lade et al., 2014) were identified: N-butanoyl-l-homoserine lactone, N-(3-oxo-hexanoyl)-homoserine lactone, N-tetradecanoyl-L-homoserine lactone, and N-pentadecanoyl-L-homoserine lactone. The reduced abundance of these quorum sensing signals in sucralose-treated mice (**Figure 4**) indicates that sucralose disrupts quorum sensing signaling.

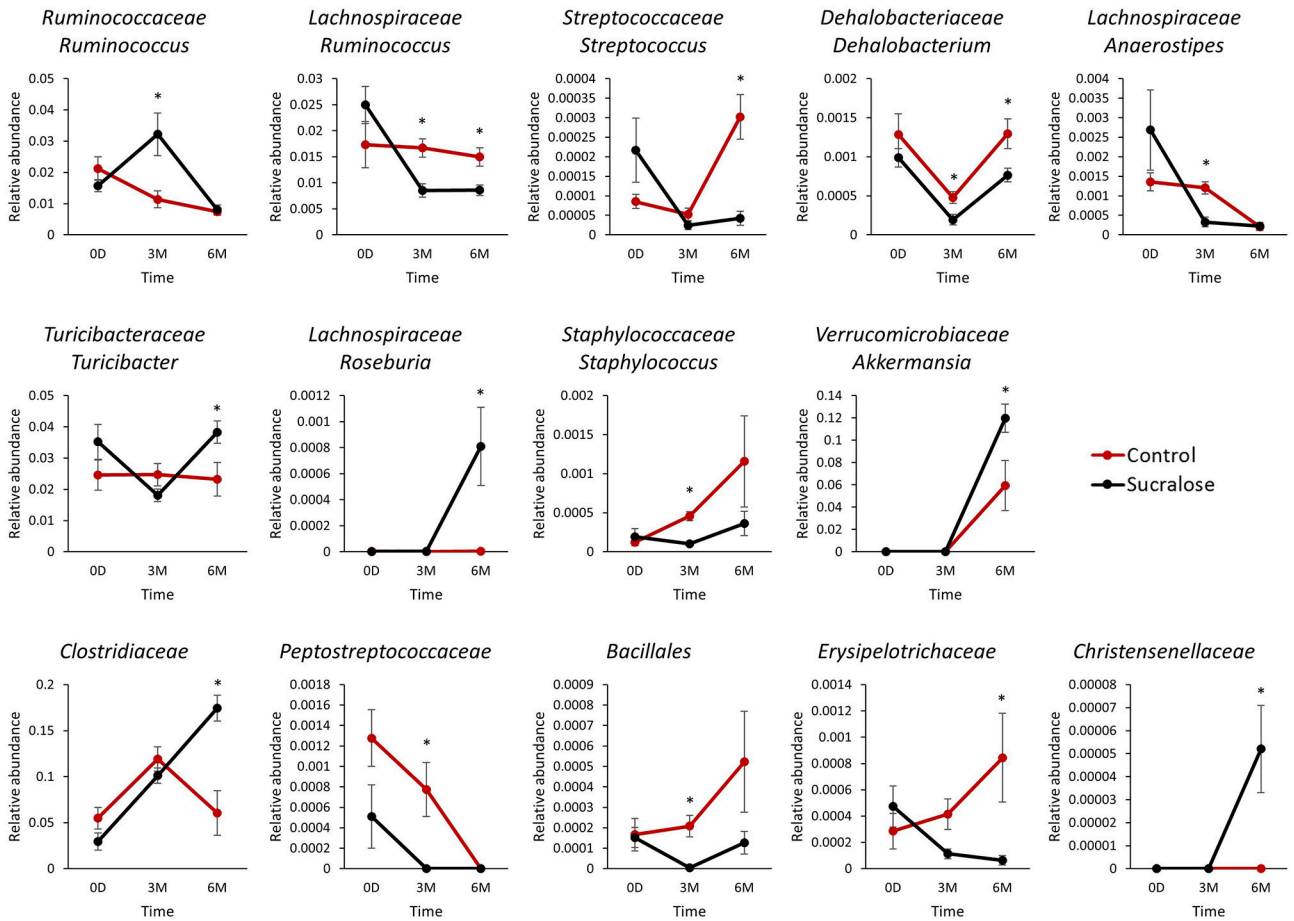

FIGURE 1 | Sucralose altered the dynamics of gut microbiome development in C57BL6/J mice. Bacterial genera exhibited different patterns over time between the control and sucralose-treated mice. *$p < 0.05$.

Sucralose Altered Amino Acids and Derivatives

The gut microbiome is highly involved in the synthesis and regulation of amino acids. Amino acids, such as L-tryptophan, L-tyrosine, L-leucine, and L-isoleucine, as well as their derivatives (Table S1) were affected by sucralose treatment. Four compounds involved in tryptophan metabolism were identified, including L-tryptophan (Trp), quinolinic acid, kynurenic acid, and 2-aminomuconic acid, as shown in **Figure 5A**. Compared with control mice, Trp, quinolinic acid, and 2-aminomuconic acid were increased by 1.71-, 5.45-, and 2.09-fold in sucralose-treated mice, while kynurenic acid was reduced by 2.45-fold in sucralose-treated mice. For tyrosine metabolism, though L-tyrosine increased (1.62-fold), two of its metabolites, p-hydroxyphenylacetic acid and cinnamic acid, decreased by 4.63- and 1.53-fold, respectively (**Figure 5B**).

Sucralose Altered Bile Acids

The gut microbiome can transform primary hydrophilic bile acids into secondary hydrophilic bile acids in the large intestine through deconjugation, dehydroxylation, and dehydrogenation. Bile acids not only facilitate fat and fat-soluble vitamin absorption and maintain cholesterol homeostasis but are also viewed as signaling molecules that bind to the nuclear receptor FXR and the G-protein-coupled receptor TGR5. Several bile acids were significantly different between the control and sucralose-treated animals (**Figure 6**). 3-Oxo-4,6-choladienoic acid was increased in sucralose-treated mice compared with control mice, while other bile acids were reduced, including 3β,7α-dihydroxy-5-cholestenoate, 3α,7β,12α-trihydroxyoxocholanyl-glycine and lithocholic acid/isoallolithocholic acid/allolithocholic acid/isolithocholic acid.

Sucralose Induced Elevated Pro-inflammatory Gene Expression in Liver

As described above, sucralose could increase the production of bacterial pro-inflammatory mediators, which may cause inflammatory responses in host tissues after being translocated into the host circulation. In fact, sucralose-treated mice exhibited elevated gene expression of pro-inflammatory markers in the liver (**Figure 7**), such as matrix metalloproteinase 2 (MMP-2) and inducible nitric-oxide synthase (iNOS).

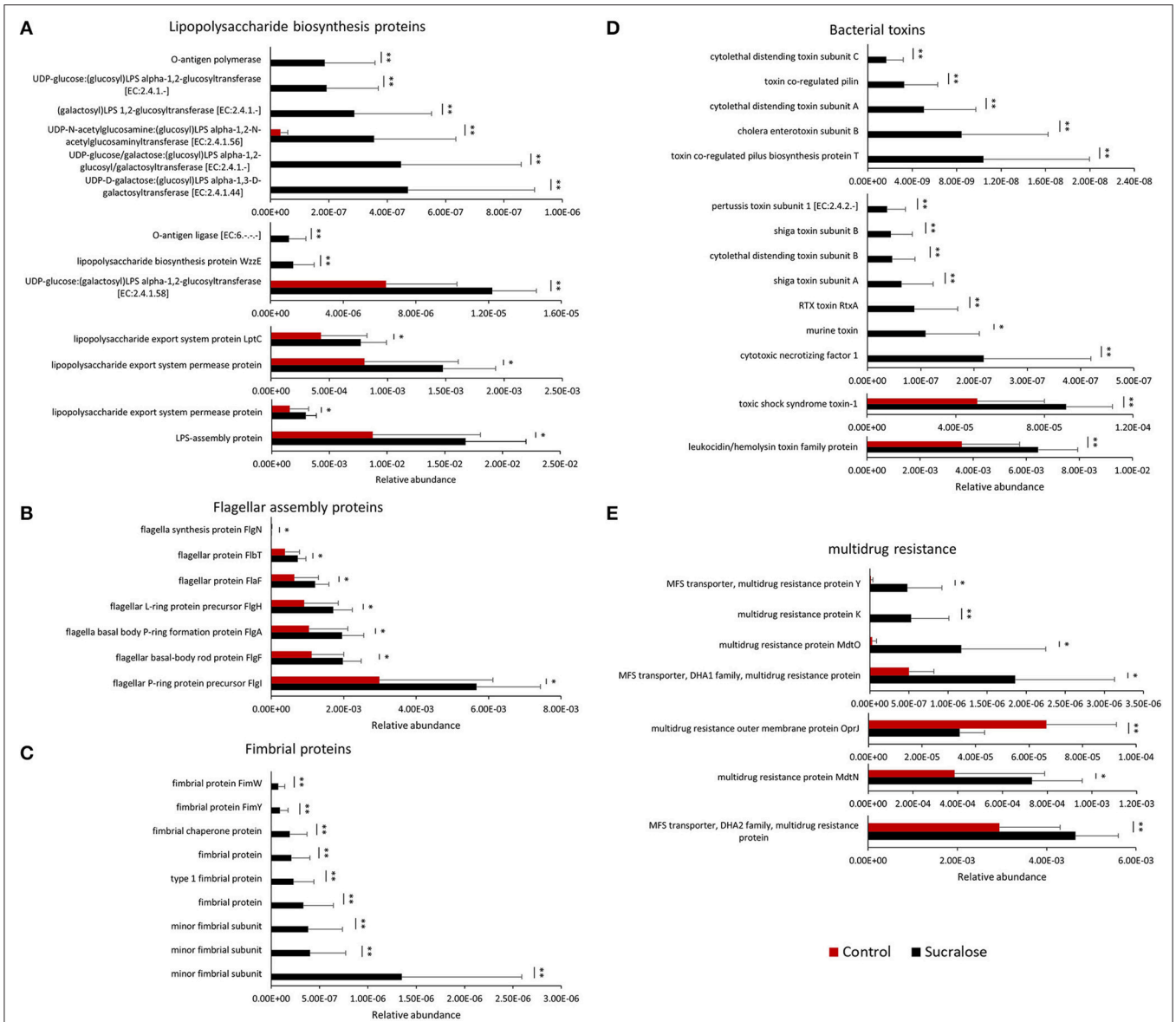

FIGURE 2 | Enrichment of bacterial pro-inflammatory genes after 6 months of sucralose treatment (*$p < 0.05$, **$p < 0.01$), including genes involved in LPS **(A)**, flagella **(B)**, and fimbriae synthesis **(C)** as well as toxins **(D)** and multidrug resistance genes **(E)**.

DISCUSSION

The gut microbiota is a dynamic system, and maintaining a healthy balance is vital for the host (Nicholson et al., 2012). Previous studies have demonstrated that changes to the gut microbiota affect numerous host processes, such as immune system development and energy metabolism and absorption, and can also impact diseases in and beyond the GI tract (Holmes et al., 2011). Xenobiotics in the food or environment can affect the gut microbiome and host health (Lu et al., 2014; Suez et al., 2014; Gao et al., 2017). One common argument used to support sucralose safety is that the majority of sucralose is not absorbed or metabolized in the body (Grice and Goldsmith, 2000). However, we demonstrate here that sucralose can affect the gut microbiome, its metabolic functions and the host even though it passes through the GI tract unchanged.

Specifically, we investigated the effect of sucralose consumption on the gut microbiota and host in mice using 16S rRNA gene sequencing, functional gene enrichment analysis, metabolomics and real-time PCR. Sucralose consumption for 6 months altered the gut microbiome composition, fecal metabolites, and pro-inflammatory gene expression in the liver. The alterations induced by sucralose consumption could affect the development of inflammation and further influence other physiological functions in the

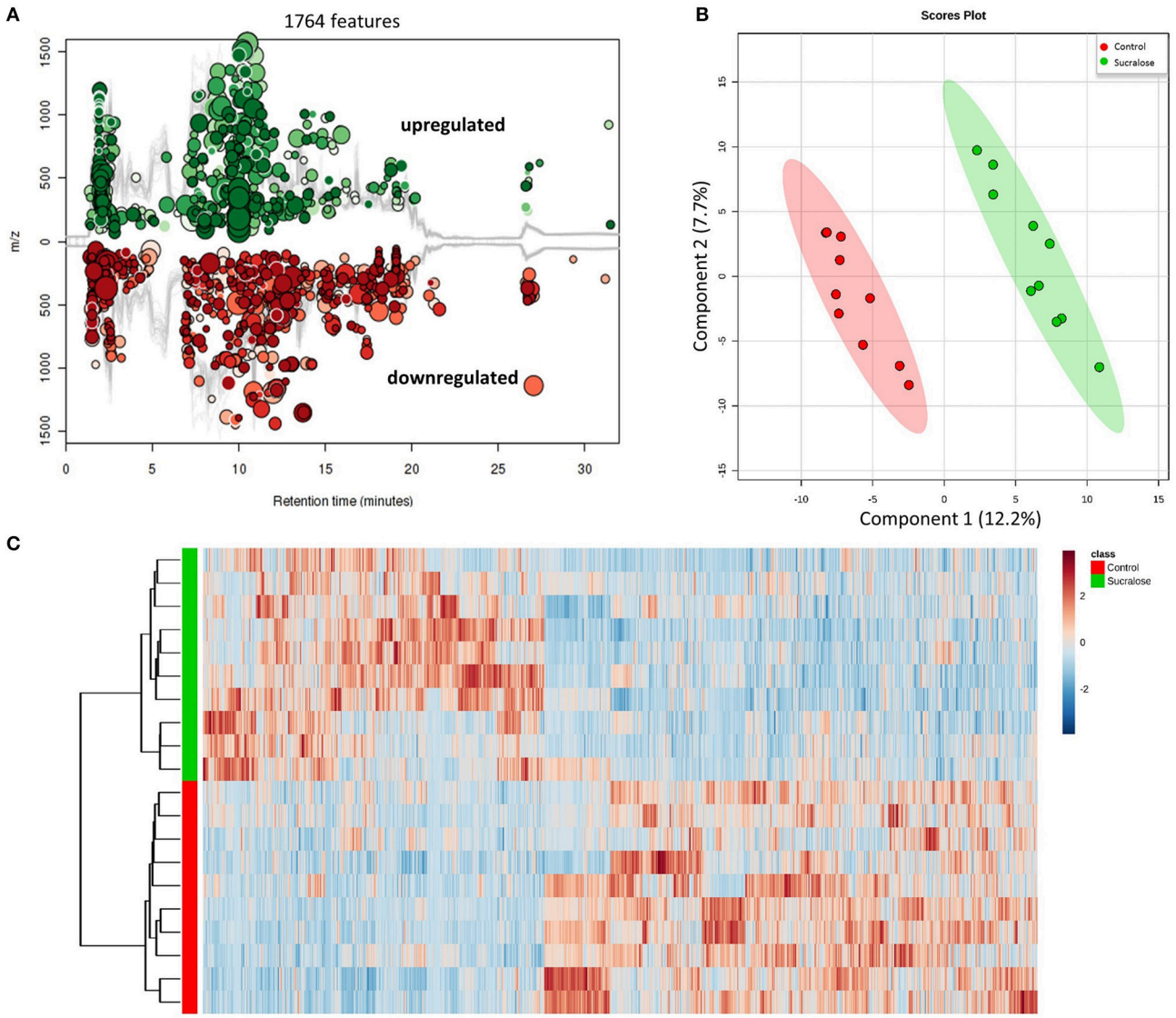

FIGURE 3 | Sucralose changed the fecal metabolome, as illustrated by the Cloud plot **(A)**, PLS-DA plot **(B)**, and heat map **(C)**. A total of 1,764 molecular features were significantly different ($p < 0.05$ and fold change>1.5) between sucralose-treated mice and controls.

body. This study provides a new understanding of the effect of artificial sweeteners on the gut microbiota and host health.

Sucralose has been shown to be safe using different endpoints in previous studies, but very few studies have reported its effects on the gut microbiome and, particularly, its functions (Grotz and Munro, 2009). In this study, we examined sucralose-induced gut microbiome functional perturbation, which may contribute to the development of systemic inflammation in the host. Altering the gut bacterial composition may confer an increased risk of developing inflammation in sucralose-treated mice. For example, among the 14 changed genera, several were found to be associated with host inflammation. *Ruminococcaceae Ruminococcus*, which were more abundant in sucralose-treated mice in this study, were shown to be more abundant in colonic Crohn's disease samples than in healthy samples in a previous study (Willing et al., 2010); *Streptococcaceae Streptococcus*, *Dehalobacteriaceae Dehalobacterium*, *Lachnospiraceae Anaerostipes*, and *Lachnospiraceae Ruminococcus*, which were reduced in sucralose-treated mice, were found to be negatively associated with inflammation in previous studies (Willing et al., 2010; Collins et al., 2014; Fernández et al., 2016; Munyaka et al., 2016). The functional impact of these altered gut bacteria remains to be further elucidated in the future. Nevertheless, alterations in gut microbiome composition may lead to differential functional bacterial metagenomes and metabolic capacities of the gut microbiome.

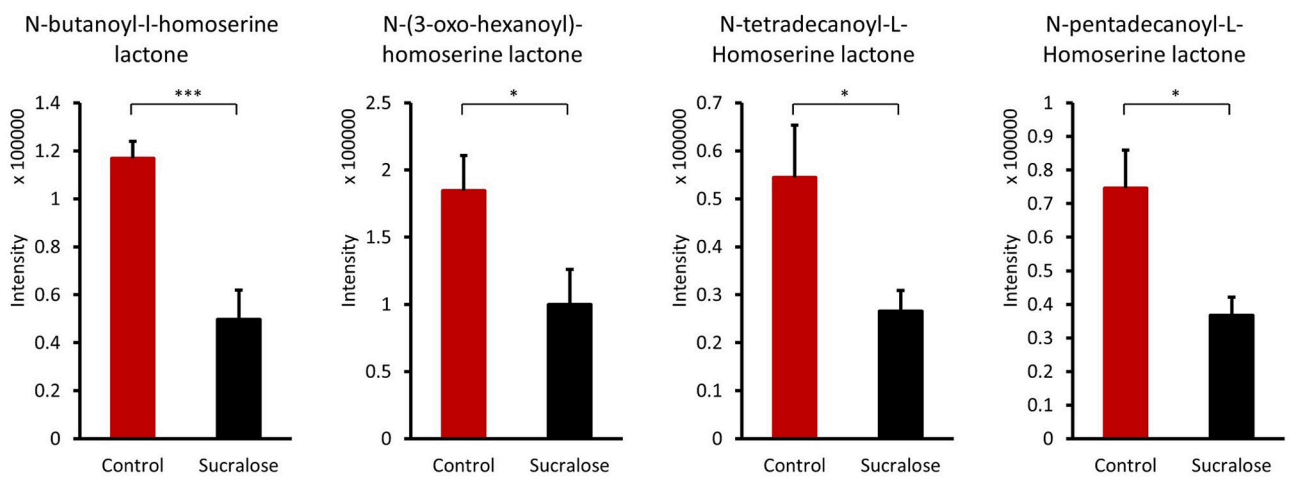

FIGURE 4 | Quorum sensing signals were altered by sucralose consumption. *$p < 0.05$, ***$p < 0.001$.

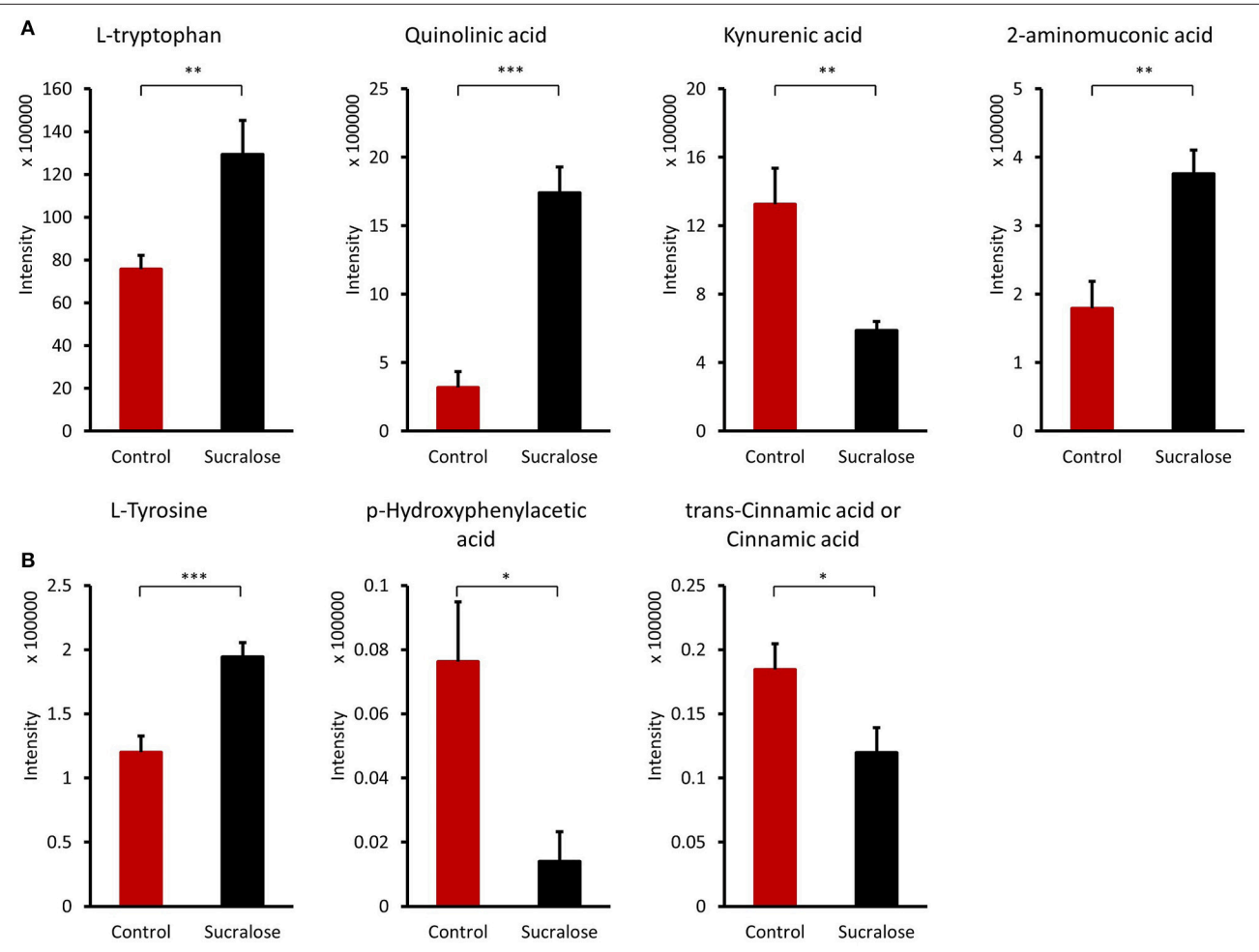

FIGURE 5 | Amino acids and derivatives altered by sucralose. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) **(A)** tryptophan metabolites; **(B)** tyrosine metabolites.

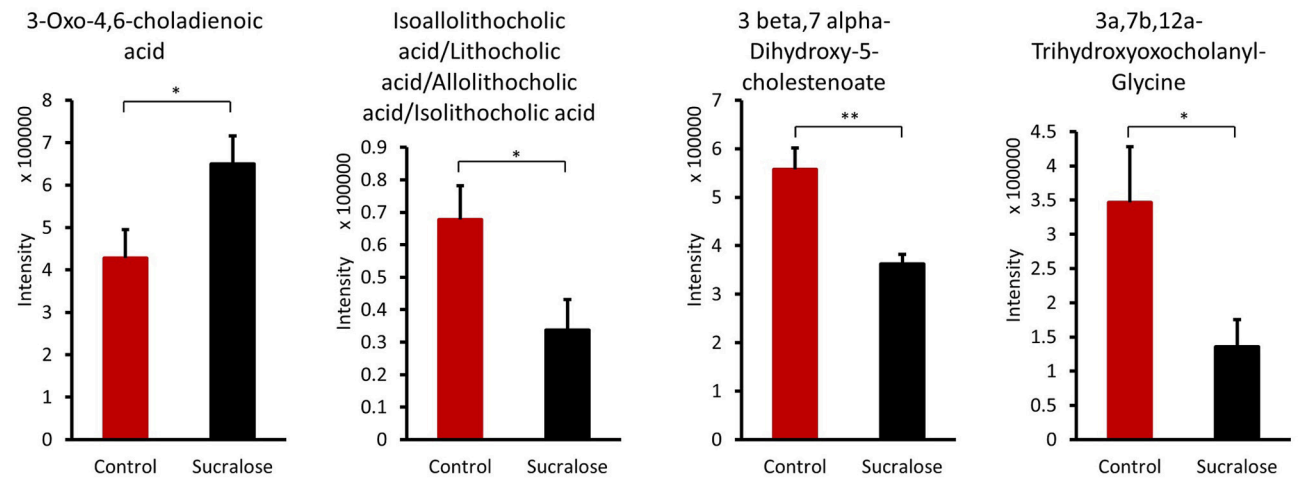

FIGURE 6 | Sucralose altered bile acids in the fecal samples of mice treated with sucralose for 6 months. *$p < 0.05$, **$p < 0.01$.

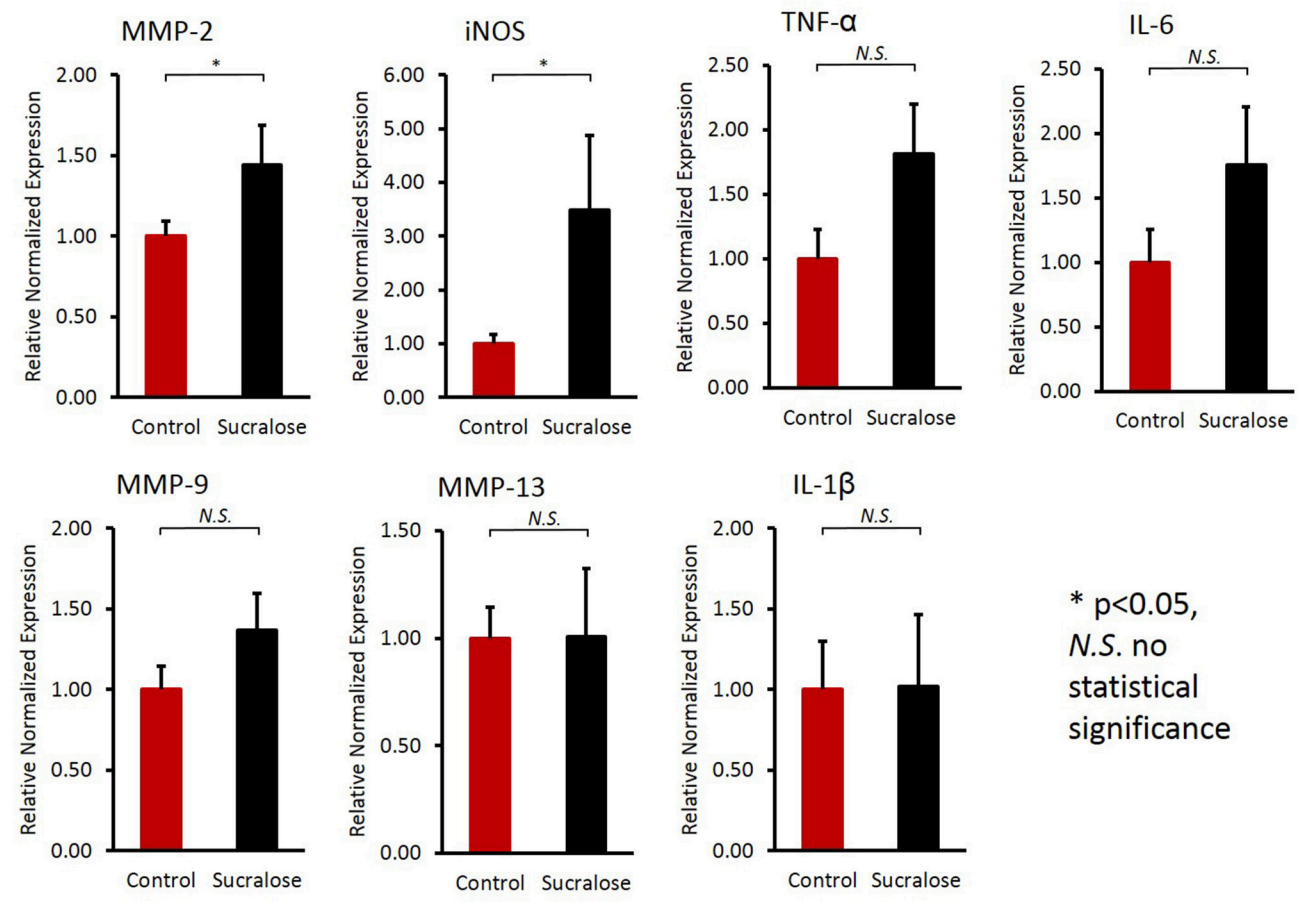

FIGURE 7 | Sucralose consumption increased the gene expression of inflammatory markers in the liver, as examined by qRT-PCR. The mRNA expression of matrix metalloproteinase 2 (MMP-2) and inducible nitric-oxide synthase (iNOS) was elevated in sucralose-treated mice. *$p < 0.05$, N.S., no statistical significance.

Previous studies have demonstrated that functional genes of the bacterial community are related to 16S rRNA marker genes, allowing the functional capacities of the gut microbiome to be surveyed using 16S rRNA gene sequencing (Asshauer et al., 2015). Using functional gene enrichment analysis, a number of genes related to bacterial pro-inflammatory mediators were shown to be significantly increased in the sucralose-treated gut microbiome, including genes involved in LPS synthesis, flagella protein synthesis, and fimbriae synthesis as well as bacterial toxins and drug resistance genes. LPS, flagella, and fimbriae are known PAMPs that can trigger pathological inflammation in the host, and various toxins produced by bacteria can induce toxicity in the host. LPS, a known endotoxin from the outer membrane of gram-negative bacteria, can initiate inflammatory events, such as the secretion of pro-inflammatory cytokines like interleukin-6 or tumor necrosis factor (TNF)-α (de La Serre et al., 2010). Flagella protein levels are low in a healthy gut, and high levels of flagella proteins have been shown to be associated with gut mucosal barrier breakdown and inflammation in previous studies (Cullender et al., 2013). Fimbriae play an important role in bacterial adhesion to and invasion of epithelial cells and are known virulence factors (Nakagawa, 2002). Additionally, multidrug resistance genes were increased in the sucralose-treated gut microbiome, and the increase in multidrug resistance genes and/or multidrug-resistant bacteria may lead to a more hostile gut environment (Marshall et al., 2009). These data indicate that 6 months of sucralose consumption increased the pro-inflammatory products of the gut microbiome and its ability to potentially induce systemic inflammation.

Likewise, the metabolites identified in fecal samples may be involved in regulating inflammation. For example, several amino acids were perturbed in sucralose-treated fecal metabolites in this study. In particular, we found that tryptophan metabolism was disrupted by sucralose, and this disruption was related to changes in the expression of functional genes of the gut microbiome. As shown in Supplementary Figure 1, several genes related to tryptophan metabolism were elevated, while the abundance of tryptophan and its metabolites were altered in the fecal samples. The four metabolites identified are involved in the kynurenine pathway, which is the most important tryptophan metabolism pathway, consuming 95% of the tryptophan in the body (Keszthelyi et al., 2009). The balance between two metabolites in this pathway, quinolinic acid, and kynurenic acid, plays an important role in mediating inflammation and the excitability of cells such as enteric neurons. Quinolinic acid is pro-inflammatory and excitotoxic, whereas kynurenic acid is anti-inflammatory and neuroprotective (Keszthelyi et al., 2009). Here, we found an elevated level of quinolinic acid and a decreased level of kynurenic acid. This indicates that sucralose may shift cells to a pro-inflammatory state. Likewise, tyrosine and two of its metabolites, p-hydroxyphenylacetic acid and cinnamic acid, have previously been shown to decrease the production of reactive oxygen species (ROS) in neutrophils (Beloborodova et al., 2012), and the reduced level of these compounds in our study indicated that ROS levels may be increased in sucralose-treated mice. Bacterial antioxidative enzyme genes, such as catalase and catalase-peroxidase, which respond to ROS, were also significantly enriched in sucralose-treated mice (Supplementary Figure 2). ROS can stimulate the release of pro-inflammatory cytokines (Chapple, 1997); therefore, the decrease in the two tyrosine metabolites may contribute to the development of a pro-inflammatory state. Additionally, secondary bile acids that have

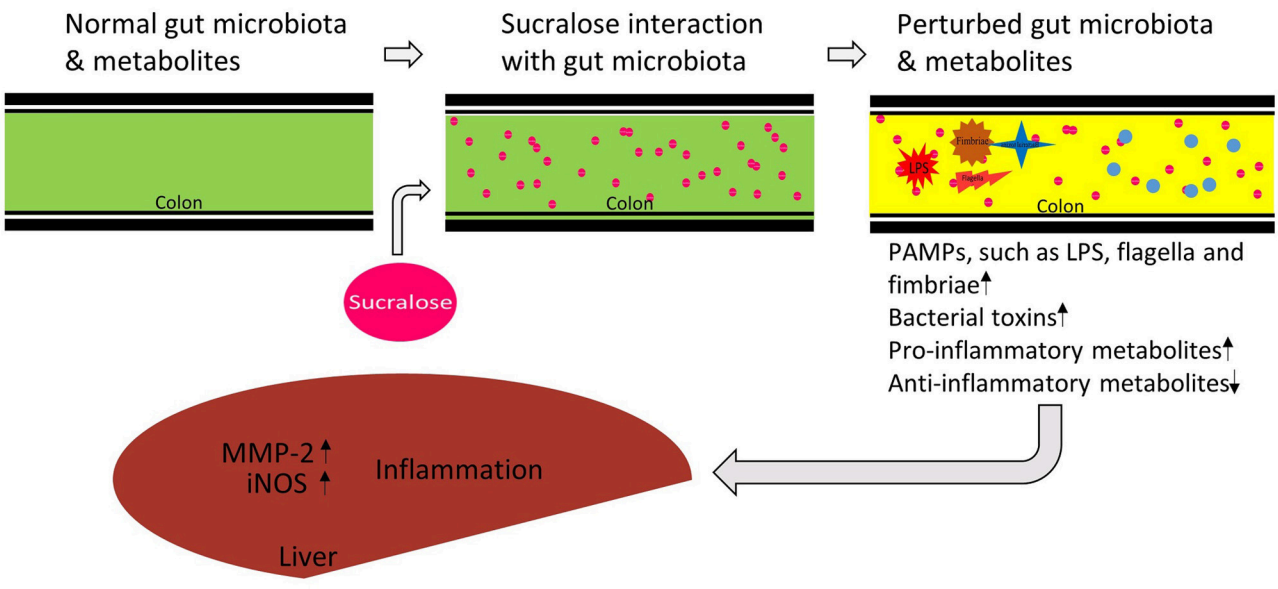

FIGURE 8 | Proposed functional link between sucralose-induced gut microbiota alterations and host inflammation. Sucralose perturbs the gut microbiome and its metabolic functions, inducing the enrichment of bacterial pro-inflammatory mediators, and disrupting metabolites involved in inflammation regulation. Together, these consequences may contribute to the induction of liver inflammation in the host.

antimicrobial effects were decreased, which may allow the growth of pathogens (Begley et al., 2005).

Pro-inflammatory mediators, such as LPS, and metabolites can translocate into host circulation and tissues, leading to systemic inflammatory response (de La Serre et al., 2010). In accordance with this expectation, real-time PCR results showed that MMP-2 and iNOS expression was elevated in the liver of sucralose-treated mice. MMP-2 is strongly associated with inflammatory responses, because it can cleave and activate TNF-α and IL-1β, which are both pro-inflammatory cytokines that contribute to the induction of inflammation (Medina and Radomski, 2006; Wang et al., 2006). Likewise, iNOS-derived NO regulates pro-inflammatory genes and significantly contributes to inflammatory liver injury. iNOS exerts numerous effects associated with the progression of inflammatory conditions in multiple liver diseases, such as increasing the liver inflammatory response, promoting the induction of liver tumors and contributing to liver fibrosis caused by a chronic viral infection (Sass et al., 2001; La Mura et al., 2014). The expression of both MMP-2 and iNOS was found to be increased in the liver of sucralose-treated mice compared with control mice, indicating that sucralose exposure increases the risk of developing inflammation in the liver. Notably, most of the sucralose consumed passes through the GI tract unabsorbed and unchanged (Roberts et al., 2000); therefore, the inflammatory response observed in the liver was unlikely to be stimulated by sucralose directly.

Taken together, as illustrated in **Figure 8**, our data show that 6-month sucralose consumption at human ADI alters the gut microbiome and its functions in mice. In particular, the enrichment of gut microbial pro-inflammatory genes and fecal metabolites suggests that sucralose alters the gut environment to release more pro-inflammatory mediators and alter functional metabolites, which may contribute to the increased expression of pro-inflammatory markers in the liver, such as iNOS and MMP-2. Notably, while the majority of ingested sucralose passes through the GI tract unabsorbed, it does disrupt the gut microbiota and its functions; therefore, our results highlight the role of sucralose-gut microbiome interaction in regulating host health-related processes, such as chronic inflammation.

There are a few limitations associated with this study. First, we only assessed inflammatory response in the liver of sucralose-treated mice by RT-PCR. Examination of host response using other endpoints and methods, such as circulating LPS and histological assessment, in related samples and tissues would be needed to better characterize the effects of sucralose in the body. Second, we conducted experiments using a single dose of sucralose at the human ADI, while human intake of sucralose is typically lower than this concentration. Our ongoing study using multiple human-relevant doses aims at better understanding time- and dose-dependent effects of sucralose on the gut microbiome and host. Third, the enrichment analysis of functional bacterial genes was performed based on the 16S rRNA sequencing data. Metagenomic shotgun sequencing and/or metatranscriptomics will further shed light on sucralose-induced functional perturbation of the gut microbiome. Finally, the identification of altered metabolites was based on the matching with the metabolite database. Future validation of key metabolites of interest with authentic compounds is warranted. Likewise, a more accurate quantitative analysis of altered metabolites using stable isotope labeled standards should be conducted.

AUTHOR CONTRIBUTIONS

XB, HR, and KL designed the study. XB, BG, LC, and PT acquired, analyzed and interpreted the data. XB, PT, HR, and KL drafted and critically revised the manuscript, approved the version to be published, and are accountable for all aspects of the work.

FUNDING

This research was partially supported by the University of Georgia, University of North Carolina at Chapel Hill and NIH/NIEHS (R01ES024950).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00487/full#supplementary-material

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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.

frontiers in Physiology

REVIEW
published: 26 January 2016
doi: 10.3389/fphys.2015.00363

Drug-Induced Liver Toxicity and Prevention by Herbal Antioxidants: An Overview

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The liver is the center for drug and xenobiotic metabolism, which is influenced most with medication/xenobiotic-mediated toxic activity. Drug-induced hepatotoxicity is common and its actual frequency is hard to determine due to underreporting, difficulties in detection or diagnosis, and incomplete observation of exposure. The death rate is high, up to about 10% for drug-induced liver damage. Endorsed medications represented >50% of instances of intense liver failure in a study from the Acute Liver Failure Study Group of the patients admitted in 17 US healing facilities. Albeit different studies are accessible uncovering the mechanistic aspects of medication prompted hepatotoxicity, we are in the dilemma about the virtual story. The expanding prevalence and effectiveness of Ayurveda and natural products in the treatment of various disorders led the investigators to look into their potential in countering drug-induced liver toxicity. Several natural products have been reported to date to mitigate the drug-induced toxicity. The dietary nature and less adverse reactions of the natural products provide them an extra edge over other candidates of supplementary medication. In this paper, we have discussed the mechanism involved in drug-induced liver toxicity and the potential of herbal antioxidants as supplementary medication.

Keywords: antioxidant, hepatoprotection, hepatotoxicity, herbal medicine

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: *02 September 2015*
Accepted: *16 November 2015*
Published: *26 January 2016*

Citation:
Singh D, Cho WC and Upadhyay G (2016) Drug-Induced Liver Toxicity and Prevention by Herbal Antioxidants: An Overview. Front. Physiol. 6:363. doi: 10.3389/fphys.2015.00363

INTRODUCTION

The leading cause of drug non-approval and drug withdrawal by the Food and Drug Administration (FDA) in the US is drug-induced hepatotoxicity (Ostapowicz et al., 2002; Pandit et al., 2012). More than a thousand medicines and chemicals have been reported to cause liver injury (Larrey, 2000; Biour et al., 2004; Upadhyay et al., 2010b; Porceddu et al., 2012). Drug-induced liver injury may account for approximately 10% of all cases of acute hepatitis, 5% of all hospital admissions, and 50% of all acute liver failures (Pandit et al., 2012). It is remarkable that more than 75% of cases of idiosyncratic drug reactions result in liver transplantation or death (Ostapowicz et al., 2002; Pandit et al., 2012). Drug-induced liver injury is a relatively common cause of acute liver disease and carries a mortality of around 10% (Lewis and Zimmerman, 1989; Shapiro and Lewis, 2007; Chalasani et al., 2008; Bell and Chalasani, 2009; Holt and Ju, 2010; Upadhyay et al., 2010b; Pandit et al., 2012; Björnsson et al., 2013). Inefficient drug metabolism, as is observed in the renal transplant (RT) recipients with the chronic liver disease, make them more prone to drug-induced hepatotoxicity (Contreras et al., 2007). Extensive research on drug-induced hepatic cell damage worldwide has been done in the past and is also an area of

major concern at present since hepatotoxicity due to these drugs, when it becomes terminal, results in malnutrition, organ dysfunction, and death (Björnsson, 2009; Porceddu et al., 2012).

The liver plays a major role in the metabolism and removal of drugs (Pandit et al., 2012). Detoxification of drugs and xenobiotics in the liver by drug metabolizing enzymes (DMEs) is an important phenomenon in the acquisition of homeostasis (Upadhyay et al., 2007, 2008). Alteration in homeostatic status leads to a shift in the dynamic equilibrium of metabolism toward the ROS generation thereby oxidative stress leading to organ malfunction. Liver insufficiency and damage resulted from exposure to environmental toxicants, particular combinations or dosages of pharmaceuticals, and microbial metabolites are major causes of disease and death worldwide (Upadhyay et al., 2007, 2008). Intake of drugs, their metabolism and removal make a condition of dynamic equilibrium to maintain homeostasis. Phase I and phase II enzymes play a crucial role in the metabolism and detoxification of various drugs and xenobiotics. For the acquisition of dynamic homeostasis, highly tuned metabolic control of drugs or xenobiotics by xenobiotic metabolizing enzymes is needed. Any imbalance in the activity of these enzymes ultimately leads to the shifting of equilibrium toward free radical generation that could finally bind to macromolecules such as DNA to cause mutation, lipid to cause membrane damage, or proteins to alter their activities (Upadhyay et al., 2007, 2008, 2010a,b). The generation of reactive intermediates is a common event in liver damage resulting from a variety of hepatotoxic drugs and solvents (Coleman et al., 2007). The hepatotoxic reactions caused by drugs may be summarized as acute reactions (which consist of hepatocellular necrosis), cholestasis (with or without inflammation), and miscellaneous reactions; however, some drugs can cause chronic damage and may even lead to tumor growth (**Table 1**). In this article, we have discussed the drug-induced hepatotoxicity, factors that may add to its toxicity, mechanism, and prevention.

TABLE 1 | Common hepatotoxic reactions.

Drugs	Hepatotoxic reactions
Acetaminophen	Acute, direct hepatocellular toxicity, chronic toxicity
Isoniazid	
Methyldopa	
Allopurinol	Miscellaneous acute reactions
Aspirin	
Quinidine	
Sulfonamides	
Valproate	
Amiodarone,	Chronic toxicity
Methotrexate	
Niacin	
Rifampicin	
Pyrogallol	
Vitamin A	
Chlorpropamide	Acute cholestasis, phenothiazine type
Erythromycin-estolate	
Phenylbutazone	
Diclofenac	Acute, idiosyncratic hepatocellular toxicity
Halothane-related anesthetics	
Indomethacin	
Phenytoin	
Propylthiouracil	
Hydrocarbons	Acute, direct hepatocellular toxicity
Tratcycline	
Methyltestisterone	Acute cholestasis, steroid type
Oral contraceptives	

FACTORS AFFECTING DRUG-INDUCED HEPATOTOXICITY

There are various factors that enhance a person's susceptibility to a potentially hepatotoxic drug (**Figure 1**). Advancing age, gender, lifestyle factors, obesity, nutritional status, genetic background, dose, and duration of drugs may affect the risk of drug-mediated hepatotoxic reactions. Persons suffering from other diseases, such as, human immunodeficiency virus, hepatitis C, rheumatoid arthritis, and systemic lupus erythematosus, are more prone to toxic drug reactions. Drug composition and drug-drug interaction may also be a reason for increased risk of drug-induced hepatotoxicity. For example, certain drugs containing a nitro-aromatic moiety or drugs interacting with nuclear receptors such as phenobarbital may cause organ-selective toxicity or may potentiate the toxicity of other drugs (Yamazaki et al., 2005; Boelsterli et al., 2006).

DRUG-INDUCED DAMAGE

A rising number of cases of acute liver failures are reported every year in the United States, and drugs contribute majority of them, e.g., acetaminophen and idiosyncratic reactions due to other medications (Pandit et al., 2012). Two to five percent of jaundice cases and over 10% cases of acute hepatitis are also contributed by drug-mediated side reactions (Pandit et al., 2012). Several drugs and chemicals have been withdrawn from the market worldwide due to their hepatotoxic reactions (Bakke et al., 1995; Shah, 1999; Lee, 2003; Mohapatra et al., 2005; Upadhyay et al., 2010a,b; Pandit et al., 2012), yet epidemiological studies are alarming and suggest that the drug trial process should be more rigorous. Some drugs have been extensively studied in humans and animals to elucidate the biochemical and molecular mechanism of drug-induced hepatotoxicity (Upadhyay et al., 2007, 2008, 2010a,b). Most of the drugs have a signature effect, and exhibit a pattern of liver injury. Nevertheless, certain drugs like rifampicin may produce all kind of symptoms associated with liver injury, ranging from cholestasis, hepatocellular injury, to even isolated hyper-bilirubinemia. Therefore, the knowledge of the most commonly used drugs/agents, such as, anti-tubercular drugs, acetaminophen, diclophenac, pyrogallol, statins

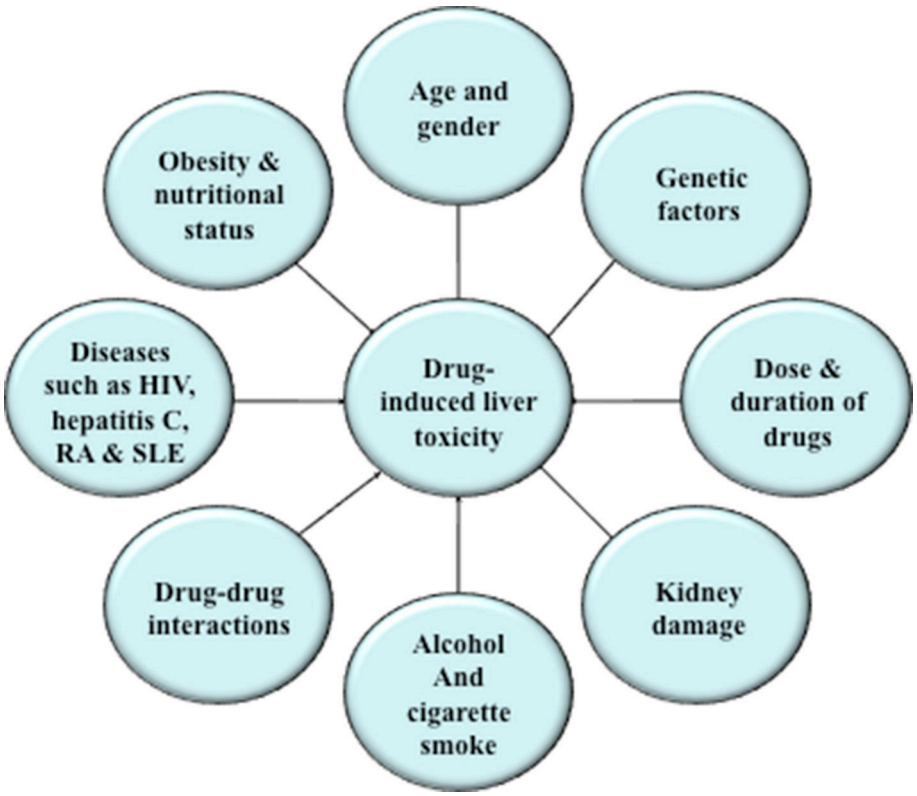

FIGURE 1 | Factors affecting drug-induced hepatic toxicity. Various factors, such as, advancing age, gender, lifestyle factors, obesity, nutritional status, genetic background, dose, and duration of drugs may increase the risk of drug-mediated hepatotoxic reactions.

and so forth, and a high index of suspicion are essential in diagnosis.

RIFAMPICIN

Hepatitis has been reported to occur 0.46% of individuals undergoing anti-tubercular therapy and receiving anti-tubercular drugs. The rate of hepatotoxic reaction was much higher in Indian patients as compared to that reported from developed countries (Ramachandran, 1980; Alexander et al., 1982; Mindie and Gabriel, 2002). It is readily absorbed from the gastrointestinal tract (90%) and most of it is bound to plasma proteins in circulation. The involvement of oxidative stress in rifampicin-induced hepatotoxicity has been demonstrated previously in experimental rats (Sodhi et al., 1998). Rifampicin is a potent inducer of cytochrome P450 action and enhances the covalent binding of reactive metabolites of acetyl hydrazine to the macromolecules of hepatocytes leading to hepatic cell damage (Powell-Jackson et al., 1982; Sinha, 1987). Additionally, desacetylrifampicin, another reactive metabolite of rifampicin, also contributes to some of its adverse effects. It has been reported to modulate the membrane permeability and cause membrane damage (Rana et al., 2006). Its prolonged exposure significantly decreases glucose-6-phosphatase activity, which could be a reason for the increased level of lipid peroxidation (Koster and Slee, 1980; Saraswathy and Shyamala Devi, 2001). Anti-tubercular drugs increase intracellular calcium concentration leading to the induction of phospholipase A2 which degrade membrane phospholipids (Karthikeyan, 2005; Tasduq et al., 2005). Additionally, CYP2E1 activation and fatty acid accumulation in the liver either due to excessive supply of lipids to the liver or interference with lipid deposition, has been documented in the anti-tubercular drug-induced liver disorders (Anundi et al., 1993; Farombi et al., 1999; Upadhyay et al., 2007). Anti-tubercular drugs also induce hypercholesterolemia that might be due to increased uptake of LDL from the blood, by the tissues (Kissler et al., 2005; Santhosh et al., 2006).

ISONIAZID

Isoniazid, an anti-tubercular drug, is used alone or in combination with other drugs to eliminate the active (growing) bacteria. Usually, the therapy with isoniazid is continued for a longer time (6–12 months) since the bacteria may exist in a resting state for a longer period of time. The studies have indicated severe and fatal hepatitis with isoniazid therapy (Huang et al., 2003; Saukkonen et al., 2006). The frequency of hepatotoxic reactions are higher in aged patients over 65 years. Additionally, daily alcohol consumption increases the risk of hepatitis. In the patients receiving isoniazid, the symptoms

of hepatic damage appear late, usually after 3 months of treatment.

In the liver, isoniazid is metabolized primarily by N-acetyl transferase 2 (NAT-2) to acetyl-isoniazid, which subsequently is converted to mono-acetyl hydrazine (MAH) and non-toxic diacetyl hydrazine, as well as other minor metabolites (Huang et al., 2002; Saukkonen et al., 2006). Studies have revealed that reactive metabolites of MAH are toxic to tissues due to ROS generation (Mitchell et al., 1975; Saukkonen et al., 2006). Isoniazid inhibits glutathione biosynthesis, activities of antioxidant glutathione peroxidase and catalase activity in rats (Sodhi et al., 1996; Attri et al., 2001; Saukkonen et al., 2006). Furthermore, acetyl-hydrazine, a metabolite of isoniazid, causes damage to hepatic cells by covalently binding to liver macromolecules (Mitchell et al., 1975; Saukkonen et al., 2006). In an epidemiological study, it has been reported that homozygous CYP2E1 c1/c1 host gene polymorphism, which results in enhanced CYP2E1 activity, causes higher risk of hepatotoxicity in patients (Huang et al., 2003; Saukkonen et al., 2006).

ACETAMINOPHEN

Acetaminophen (APAP) is a commonly used analgesic and antipyretic, which is relatively safe at recommended therapeutic doses (Blazer and Wu, 2009). However, its associated hepatotoxicity is a major concern and the leading cause of drug-induced liver failure in many countries when used at high doses (Larson et al., 2005; Mitka, 2014; Wang et al., 2015). Acetaminophen has been extensively studied in order to understand the mechanism of drug-induced hepatotoxicity. Its hepatotoxicity pattern is different in various age groups for example the hepatotoxic incidences are less in neonates than in older children and adults. It has been suggested that in neonates, the oxidative enzyme activity is limited therefore the incidences are less common (Jacqz-Aigrain and Anderson, 2006). The acetaminophen-induced liver injury leads to a functional suppression of the immune system as dictated by the hindrance of a deferred hypersensitivity reaction to dinitrochlorobenzene (Masson et al., 2007). Subsequent studies with adrenalectomized mice, recommended a role of corticosterone in the exhaustion of lymphocytes taking after APAP-instigated liver damage (Masson et al., 2007). Acetaminophen is metabolized to N-acetyl-p-benzoquinone imine by CYP enzymes, which is subsequently detoxified by reduced glutathione (GSH) to a threshold concentration, after which GSH depletion occur leading to the covalent binding of the metabolite to the macromolecules (Reid et al., 2005; Wolf et al., 2007; Olaleye and Rocha, 2008; Saito et al., 2010; McGill et al., 2012). APAP overdose has been reported to induce massive necrosis in the liver in animal models. It is remarkable that only those hepatocytes undergo necrosis in which acetaminophen-protein adducts formation occur (Hinson et al., 2004; Saito et al., 2010). It is also suggested that the APAP toxicity propagates through nitric oxide (NO), which scavenges superoxide to produce peroxynitrite, thereby causing protein nitration (3-ntrotyrosine) and tissue injury (Jaeschke et al., 2002; Hinson et al., 2004). Three-Nitrotyrosine is usually detoxified by conjugation with reduced GSH. Thus, acetaminophen toxicity occurs with increased oxygen/nitrogen stress (Hinson et al., 2004).

PYROGALLOL

Pyrogallol is an anti-psoriatic drug and has been reported to cause liver damage in mouse and rat models (Gupta et al., 2002; Upadhyay et al., 2007, 2008, 2010a,b). In the presence of metal ions under certain conditions *in vitro*, pyrogallol causes oxidative damage to macromolecules (Singh et al., 1994). Pro-oxidant action of pyrogallol is suggested to be the major cause of harmful effects such as mutagenesis, carcinogenesis and hepatotoxicity (Akagawa et al., 2003; Upadhyay et al., 2007). It is suggested that the auto-oxidation property of pyrogallol due to the attack by reactive oxygen species such as $\bullet OH$, O_2^-, and hydrogen peroxides contribute greatly to its pro-oxidant actions (Cao et al., 1997; Hayakawa et al., 1997; Mochizuki et al., 2002; Akagawa et al., 2003). Studies have now established the involvement of toxicant responsive genes for example CYP1A2, CYP2E1, glutathione-*S*-transferase, glutathione reductase, and glutathione peroxidase in pyrogallol-induced membrane damage and hepatotoxicity (Upadhyay et al., 2007, 2008, 2010a,b).

DICLOFENAC

Diclofenac-mediated liver toxicity is a prime example of idiosyncratic liver injury (Mitchell et al., 1973) and the increased hepatotoxicity markers have been noted in about 15% of patients receiving diclofenac regularly. Elderly females are more susceptible to diclofenac-induced liver injury. (Banks et al., 1995; Kaplowitz, 2001; Mitchell and Hilmer, 2010) Diclofenac predominantly exhibits hepatocellular pattern of liver injury; however, cholestatic pattern of liver injury and cases resembling autoimmune hepatitis have also been observed (Aithal, 2004). Metabolism of diclofenac by Cyp2C8/9 or by UDP-glucuronosyltransferase-2B7 results in unstable intermediate compounds that modify proteins covalently and increase the risk of hepatotoxicity. Additionally, covalent binding of reactive metabolites to "self" proteins results in the formation of neoantigens, which could be recognized by helper T cells leading to their activation and an effector-cell response. It is remarkable that hepatocytes express MHC I molecules on their surfaces and may present diclofenac adducts, making them prone for T-cell mediated liver injury. Alternatively, diclofenac adducts on the plasma membrane of hepatocytes may be recognized by B cells resulting in their maturation into plasmacytes, the secretion of antibodies and ultimately immunological destruction of hepatocytes (Aithal, 2011).

STATINS/HMG-CoA REDUCTASE INHIBITORS

Statins are the widely used drugs in the Western countries including United States. Statins inhibit the process of cholesterol biosynthesis by competitively inhibiting HMG-CoA reductase. Further, they also decrease the low-density lipoprotein levels

and increase the atherosclerotic plaques stability (Jacobson, 2006). The use of higher statin doses is shown to exhibit biochemical abnormalities of liver function as indicated by moderate elevations of hepatotoxicity markers in animal models (Horsmans et al., 1990). High doses of derivatives of statins, lovastatin and simvastatin, have been shown to cause significant hepatocellular necrosis in rabbits and guinea pigs respectively (Horsmans et al., 1990). Nevetheless, hepatocellular necrosis by statins is rarely observed in humans (Alonso et al., 1999).

OTHER DRUGS

There are some other commonly used drugs that cause liver damage, for example, valproic acid, an antiepileptic drug, may cause liver damage in approximately 20% patients; antibiotics namely ciprofloxacin, erythromycin, amoxicillin may produce symptoms ranging from jaundice, acute hepatotoxicity to liver malfunction; chlorpromazine is found to produce symptoms of jaundice; amiodarone causes acute hepatotoxicity in both animal and human studies; oral contraceptives may cause jaundice and cholestatic liver injury and so forth.

UNDERSTANDING THE MECHANISM OF HEPATOTOXICITY

Mechanism of drug-induced hepatotoxicity is variable and complex (**Figure 2**). Some drugs are directly toxic and begin to exhibit hepatotoxic reactions, which are dose related, within hours of exposure, whereas others may produce liver injury only in susceptible people and symptoms appear after few days or weeks. These reactions are rarely allergic or more accurately described as idiosyncratic. Drug-drug interaction, though the drugs are not hepatotoxic themselves, may also play a critical role in the propagation of toxicity (Yamazaki et al., 2005). The pathogenesis of drug-induced toxicity mediated by reactive metabolites has been a center of research enthusiasm since spearheading examinations in the 1950s uncovered the connection between these metabolites and chemical carcinogenesis (Park et al., 2005). A major cause of hepatotoxic reactions may be drug-induced intrahepatic cholestasis, which often occurs during the drug discovery and development process. The vital roles of ROS in the cellular damage are widely investigated and it has been suggested that the covalent binding of ROS as well as reactive intermediates to macromolecules could likely contribute to the severe harmful drug reactions (Racknagel et al., 1989; Masubuchi et al., 2007). There are several studies that suggest the generation of reactive metabolites and free radicals from hepatotoxic drugs (Racknagel et al., 1989; Park et al., 2005). Membrane lipid peroxidation is directly related to the depletion of tissue GSH (an intracellular antioxidant) leading to the altered functional integrity of these structures and if the damage is sever, it could be fatal (Ross, 1988; Guven and Gulmez, 2003). Membrane lipid peroxidation may lead to alteration in membrane fluidity and permeability, enhanced rates of protein degradation, and ultimately cell death (García et al., 1997). The assumption is upheld by the way that oxidative damage to erythrocytes causes loss of membrane capacity by enhancing lipid peroxidation (LPO) and modifying the erythrocyte antioxidant framework (Vajdovich et al., 1995). The concentration of intracellular GSH, therefore, is the key determinant of membrane integrity and the extent of toxicant-induced hepatic cell injury (Ross, 1988; Guven and Gulmez, 2003).

DRUG-METABOLIZING ENZYMES (DMEs)

DMEs and transporters are key factors that affect the disposition of xenobiotics (Cheng et al., 2005). These DMEs include mix function oxidases and phase II backup machinery that are actively involved in the detoxification process. Recently, the roles of transporters (Phase III enzymes) also have been implicated in the detoxification procedure.

PHASE I ENZYMES (MIXED FUNCTION OXIDASES)

Cytochrome P450 (CYP), a super gene family of heme-containing, mixed function oxidase enzymes, are involved in the metabolism of endogenous steroid hormones, vitamins, endobiotics (fatty acid derivatives), various drugs/xenobiotics, and are readily inducible by many exogenous and endogenous substances (Nelson et al., 1996; Ahmed and Pawar, 1997). The CYP-catalyzed oxidation of xenobiotics generate a highly electrophilic intermediates capable of forming covalent adducts with critical cell macromolecules, such as thiol-containing membrane proteins that direct Ca^{++} homeostasis (Bellomo and Orrenius, 1985; Dahm and Jones, 1996; Vessey, 1996). The activation of increased intracellular calcium may be the regular pathway promoting cell death. CYP-mediated reduction of halogenated hydrocarbons, for example, carbon tetrachloride or halothane, can likewise create free radical intermediates, which can straightforwardly harm cell layers through lipid peroxidation, or can target nucleophilic DNA deposits (Thor and Orrenius, 1980; De Groot and Noll, 1983; Racknagel et al., 1989; Dahm and Jones, 1996).

Phase I reactions, catalyzed by various drugs and xenobiotics, are mediated by various isoforms of CYPs and are regulated through various nuclear receptors. Phase I enzyme impelling by classes of microsomal inducers happens by means of enactment of transcription factors, for example, aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR) and peroxisome proliferator-actuated receptor α (PPARα) (Cheng et al., 2005). Various isoforms of CYP are reported to be involved in the metabolism and therefore bioactivation of drugs. CYP3A, CYP2D, and CYP2C sub-families are involved in the metabolic activation of 50%, 25%, and 20%, respectively, of the currently used clinical drugs (Rendic, 2002; Deng et al., 2008). CYP1A2, 2C7, 2C11, 2D2, 2E1, 2B1/2, and 3A1/2 are the major CYP isoforms involved in the drug metabolism in rats (Kobayashi et al., 2002; Deng et al., 2008). Involvement of CYP2E1 and 1A2 in the acetaminophen

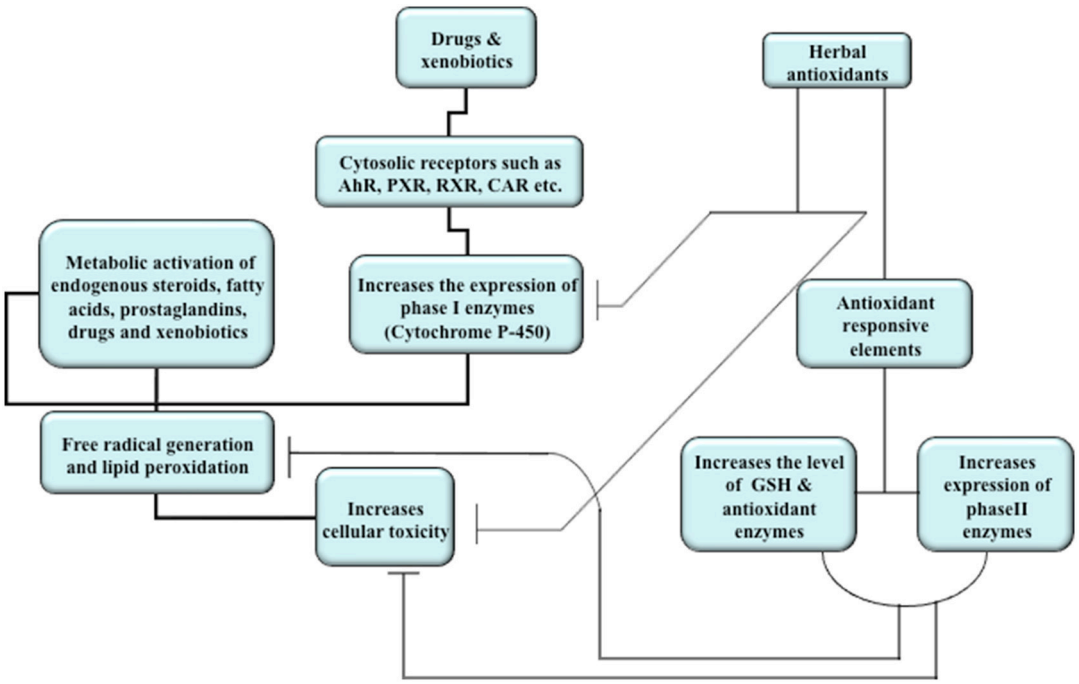

FIGURE 2 | Overview of mechanism of drug and xenobiotic metabolism and effects of herbal antioxidants. Induction of nuclear receptors by drugs and xenobiotics lead to translocation in the nucleus where they increase the expression of cytochrome P450 enzymes. The activity of these enzymes generates reactive metabolites and free radicals which in turn bind to macromolecules, cause membrane lipid peroxidation, and increase cellular toxicity. Natural products increase the expression of phase II enzymes, the level of intracellular antioxidant (GSH), and antioxidant enzymes. The natural products may also inhibit the activities of cytochrome P450 enzymes in order to attain dynamic homeostasis and reduce cellular toxicity.

and pyrogallol activation has been well established in animal models (Raucy et al., 1989; Snawder et al., 1994; Upadhyay et al., 2007). The activation of paracetamol to N-acetyl-para-benzoquinoneimine (NAPQI) is catalyzed by CYP2E1, 3A4, and 1A2 in humans (Raucy et al., 1989; Snawder et al., 1994). The involvement of CYP2E1 in rifampicin and ethanol-induced liver toxicity has also been reported (Jaeschke et al., 2002; Upadhyay et al., 2007).

REGULATION OF PHASE I ENZYMES: NUCLEAR RECEPTORS

Regulation of phase I enzymes, particularly CYPs is coupled with various transcription factors also called nuclear receptors (NR). These are the largest known family of transcription factors that modulates the tissue gene expression and are associated largely with metabolism, developmental function, and cell differentiation. There are 49 known members of the nuclear receptor superfamily and each share key structural features (Urquhart et al., 2007; Woods et al., 2007). These are the targets of approximately 10–15% pharmaceuticals and explore about $400 billion global pharmaceutical markets (Woods et al., 2007). The majority of the nuclear receptors target on various proteins included in xenobiotic metabolism, in particular, CYP enzymes (Waxman, 1999; Honkakoski and Negishi, 2000; Johnson and Klaassen, 2002). Several receptors require heterodimerization with retinoid X receptor (RXR) (Mangelsdorf and Evans, 1995). Activation of nuclear receptors, for example, aryl hydrocarbon receptors (AhR), RXR, pregane X receptors (PXR), and peroxisome proliferator-initiated receptors (PPAR) are crucial for some clinically imperative drug-drug interactions. Activation of phase I enzymes by agonists can be ascribed for activation of signal transduction pathways. Various microsomal protein inducers display their effects through prompting of diverse orphan nuclear receptors, therefore enhancing target gene expression (Denison and Nagy, 2003; Sonoda et al., 2003). For example, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) binds to the AhR, which releases the AhR from the cytosolic tethering protein HSP90, permitting AhR translocation to the nucleus, heterodimerization with aryl hydrocarbon receptor nuclear translocator (ARNT), and the binding to xenobiotic response elements on the CYP1A1 promoter. Also, CAR ligands affect CYP2B10, PXR ligands incite CYP3A11, and peroxisome proliferator-initiated receptor α (PPAR α) ligands activate CYP4A14 (Cheng et al., 2005). The pregnane X receptor (PXR, NR1I2), a member of the nuclear receptor superfamily, is activated by a lot of compounds and natural products. It is involved in the regulation of various CYP isoforms such as CYP 3A4, which is involved in the metabolism of about 60% of all prescription drugs (Lehmann et al., 1998; Guengerich, 1999; Kliewer, 2003). PXR sometimes mediates the protective effects also in case of some herbal remedies besides its undesirable effects in patients on combination therapy (Staudinger et al., 2006).

PHASE II ENZYMES

Detoxification by phase II enzymes eliminates the reactive intermediates from cellular environments and therefore decreases the burden of bio-molecular adducts on cellular homeostasis (Habig et al., 1974; Moon et al., 2006). Phase II enzymes are mainly involved in the conjugation of activated pro-toxicants with endogenous bio-molecules like GSH or glucuronic acid which reduces toxicity and increases water solubility (Habig et al., 1974; Moon et al., 2006). Glutathione-*S*-transferases (GST), a crucial Phase II enzyme, initiates the detoxification process by catalyzing the conjugation of –SH moiety of glutathione to xenobiotics, thereby neutralizing the electrophilic sites and rendering the products more water-soluble. Glutathione conjugates are thought to be metabolized further by cleavage of the glutamate and glycine residues, followed by acetylation of resultant free amino groups of the cysteinyl residue, to produce the final product, a mercapturic acid (Boyland and Nery, 1969; Wood and Woodcock, 1970).

REGULATION OF PHASE II ENZYMES

Phase II enzymes are regulated by regulatory elements called ARE (anti-oxidant response elements), which are situated upstream to promoter regions (Hayes and McMahon, 2001). Regulation is coupled with the activation of ARE through activation of a transcription factor, nuclear erythroid factor 2—related factor 2 (Nrf2; Lee and Surh, 2005). Nrf2 either upregulates or inhibits transcription of phase II enzymes through ARE by the heterodimerization with an array of leucine b-zip family members such as small maf proteins, fos, jun, and so forth (Jaiswal, 2000; Cheng et al., 2005). Nrf2 is tightly coupled with Keap1, which acts as a negative regulator of Nrf2 and as a sensor of xenobiotic and oxidative stresses (Motohashi and Yamamoto, 2004). Nrf2 activation leads to its release from Keap1 followed by translocation to the nucleus, heterodimerization to other leucine zipper proteins, and binding to ARE in order to transcriptionally activate the downstream targets (Motohashi and Yamamoto, 2004). Nrf2 activation is triggered by the ROS modulation in cells via the interaction of a various signaling molecules specified by cell types (Hayes and McMahon, 2001). Therefore, most chemopreventive operators are the modulators of cell ROS and subsequently activate Nrf2 pathway, which thusly incite phase II detoxifying response (Hayes and McMahon, 2001). In addition to the dynamic equilibrium of Phase I and Phase II enzymes, reduced GSH, glutathione peroxidase (GPx) and catalase (CAT) are also critical in acquisition of normal cellular physiology as these are involved in the elimination of lipid peroxides and toxic oxygen radicals formed during the cellular metabolism (Trackshel and Maines, 1988; Tanaka et al., 2004; Pal et al., 2006).

Another adoptive cellular response against drugs/toxicants is the induction of heme oxygenase-1 (HO-1), the rate-limiting enzyme in the breakdown of heme into carbon monoxide (CO), iron and bilirubin (Farombi and Surh, 2006). HO-1 is instigated by oxidative stress stimuli and its enactment is considered an adoptive survival response (Immenschuh and Ramadori, 2000). HO-1 impelling possibly provides security against oxidative damage, for example, in liver ischemia/reperfusion secondary to transplantation or hemorrhage/resuscitation. Induction of HO-1 controls the intracellular levels of "free" heme (a prooxidant), increases the delivery of biliverdin (an antioxidant), enhances nutritive perfusion, and cultivates the amalgamation of the Fe-binding protein ferritin to ensure the protection of cells against oxidative stress (Bauer and Bauer, 2002). CO and biliverdin/bilirubin, which are generated as a product of HO-1 activity, have been accounted for the protective effects in a few organs, including the liver (Farombi and Surh, 2006). Additionally, HO-1 is an intense defensive component for cytokine-and CD95-intervened apoptotic liver damages (Sass et al., 2003). Nevertheless, the heme oxygenase gives a defensive shield against oxidative stress to a certain level of expression and a large amounts of HO-1 may even sensitize the cells to oxidative damage by increasing the level of "free" reactive iron (Bauer and Bauer, 2002).

ROLE OF TRANSPORTERS

The reduction in expression of uptake transporters, and augmentation in expression of export transporters, as well as detoxification enzymes, may prevent the hepatocytes from accumulating potentially toxic products (Aleksunes et al., 2005). Therefore, the instances and propagation of liver injury appears to be a coordinated phenomenon involving both transport and detoxification genes (Aleksunes et al., 2005). The phenomenon of extraction and subsequent excretion of drugs and their toxic metabolites from portal blood is performed by basolateral and canalicular transporters in hepatocyte plasma membranes (Arrese and Accatino, 2002). Uptake carriers, such as organic anion-transporting polypeptides (Oatps) and the sodium/taurocholate-cotransporting polypeptide (Ntcp), and export transporters such as multidrug resistance proteins (Mdrs), multidrug resistance-associated proteins (Mrps), bile salt export pump (Bsep), and breast cancer resistance protein (Bcrp) are actively involved in the process of transportation of drugs/xenobiotics from absorption till excretion (Arrese and Accatino, 2002; Geier et al., 2002; Song et al., 2003). The transporters perform specialized functions, for example, canalicular transporters (Mrp2, Mdrs, Bcrp, and Bsep), are involved in the excretion of xenobiotics and their metabolites from hepatocytes into bile, and basolateral transporters (Mrp 1, 3–6) mediate the efflux of drugs and chemicals from hepatocytes into blood (Arrese and Accatino, 2002; Geier et al., 2002; Song et al., 2003). Various reports are available online implicating the involvement of these transporters in the xenobiotics metabolism and propagation of cell injury (Arrese and Accatino, 2002; Geier et al., 2002; Song et al., 2003; Ghanem et al., 2004; Heijne et al., 2004).

HERBAL MEDICATION

Herbal antioxidants have attracted the researchers due to its potential and efficacy against drug-induced liver toxicity. Natural products used in China and India as traditional medicine for the treatment of liver disorders are of great interest in these

days. These are the potential sources for new therapeutic agents that could be used in the prevention of hepatic injuries. Natural products rich in triterpenes, flavonoids or polyphenols, have been now established as powerful hepatoprotective agents in experimental liver-injury cell and animal models (**Table 2**; Gupta et al., 2002, 2004; King and Cousins, 2006; Upadhyay et al., 2007, 2008, 2010a,b). The basis behind the protection provided by the natural products is hypothesized to be their antioxidant property through which they remove the free radicals from the cellular environment and therefore provide protection against ROS mediated damage to membrane lipids and macromolecules (**Figure 2**; Gupta et al., 2002, 2004; Upadhyay et al., 2007, 2008, 2010a,b). Additionally, the protective potential of natural products is also contributed by its interaction with various CYP isoforms, its capability to increase GSH biosynthesis, level of Phase II/antioxidant enzymes and to inhibit the entry of toxins to the cells (**Figure 2**; Gupta et al., 2002, 2004; King and Cousins, 2006; Upadhyay et al., 2007, 2008, 2010a,b; Yarnell and Abascal, 2007). Some of the natural products containing polyphenols are considered as potential chemopreventive and hepatoprotective agents. In the last decade, several studies have been conducted to investigate the mechanism of action of natural products at biochemical, genomic and proteomic levels. The most extensively investigated natural products for hepatoprotection are silymarin, resveratrol, curcumin and gingko due to their high efficacies, low or no toxicity and easy availabilities.

SILYMARIN

Silymarin, isolated from the seeds of milk thistle (Silybum marianum), is an unique flavonoid-complex containing silybinin, silydianin, and silychristin and have been extensively studied (Flora et al., 1996; Madrigal-Santillán et al., 2013, 2014). Silibinin is major constituent of silymarin. Two main mechanisms of action of silymarin have been proposed based on its cell-regenerating function and cytoprotective effect. Cytoprotection is mediated by its antioxidant properties and direct interaction with cell membrane components (Muriel and Mourelle, 1990; Mira et al., 1994). Inhibition of lipid peroxidation, as reported in various *in vitro* studies using erythrocytes (Valenzuela et al., 1987), isolated and cultured hepatocytes (Joyeux et al., 1990; Miguez et al., 1994), and human mesangial cells, is accepted as one of silymarins major protective mechanisms. Silymarin has been also reported to have anti-inflammatory (De la Puerta et al., 1996), anti-fibrotic (Fuchs et al., 1997), and anti-proliferative effects (Scambia et al., 1996). Diverse biochemical reactions, such as, the incitement of the synthetic rate of ribosomal RNA (rRNA) species through the induction of polymerase I and rRNA transcription, shielding the cell from free radical-mediated injury and blockage of the uptake of toxins, also contribute to the protective potential of silymarin (Sonnenbichler and Zetl, 1986; Sonnenbichler et al., 1999; Wellington and Jarvis, 2001). Silymarin offers protection against enlarged liver by inhibiting 5-lipoxygenase, production of leukotrienes and generation of free radicals in Kupffer cells. Moreover, silybin, a major component of silymarin, protects against the membrane lipid peroxidation and cellular damage in the mouse hepatocytes (Yin et al., 2011; Bahmani et al., 2015).

Silymarin has been shown to be effective in combating against drug-induced hepatotoxicity in various animal models (Gupta et al., 2002, 2004; Upadhyay et al., 2007). Hepatoprotective potential of silymarin is suggested to be due to its antioxidant, cell regeneration and cytoprotection activity (Sonnenbichler et al., 1999; Gupta et al., 2002, 2004; Upadhyay et al., 2007; Ahmad et al., 2013; Yang et al., 2014b). It is equally efficient in animals as well as in humans. It increases the level of glutathione and glutathione peroxidase in the serum of patients and animals. Although silymarin has low oral absorption, oral dosages of 420 mg/day have shown some therapeutic potential, with good tolerability in alcoholic cirrhosis patients. Moreover, daily dose of silybin (20–48 mg/kg) is supposed to be an antidote for acute mushroom poisoning by Amanita phalloides. Some isoforms of Silybin have been shown to possess the strongest anti-NF-κB and anti-HCV activity (Polyak et al., 2007). It has been shown to offer protection against prostate cancer by the inactivation of erbB1-SHC (Src homology 2 domain containing) signaling pathway and induction of CDKIs, and a resultant G1 arrest (Zi et al., 1998; Zi and Agarwal, 1999). Additionally, it inhibits xanthine oxidase and mRNA expression of TNF-α, which plays critical role in mouse skin tumorigenesis (Zi et al., 1997; Sheu et al., 1998). At lower non-toxic concentrations, it inhibits transformation in cultured rat tracheal epithelial cells treated with benzo[a]pyrene, by which chemopreventive compounds that act at early stages of the carcinogenic process, could be identified (Steele et al., 1990; Kohno et al., 2002). Protective potential of silymarin against rifampicin and pyrogallol-induced hepatotoxicity has been found to be due to its modulatory effect on the augmented level of CYPs and attenuated level of phase II and antioxidant enzymes (Upadhyay et al., 2007). In a clinical trial with over 2000 patients with chronic liver diseases, administration of silymarin extract for 8 weeks resulted in a significant decrease in liver damage index in approximately 88% of the patients (Nasri et al., 2013). It was remarkable that some minor side effects were observed only in less than 1% of patients (Nasri et al., 2013).

RESVERATROL

Resveratrol (trans-3,4′,5-trihydroxy-trans-stilbene) is a natural polyphenol present in significant amounts in peanuts, the skin of grapes and red wine. Potential health benefits of resveratrol have been widely investigated in past few years. Its activity in chemoprevention, cardiovascular diseases and neurodegenerative disorders has been reported in preclinical studies (Surh, 1999; Frémont, 2000; Ignatowicz and Baer-Dubowska, 2001; Latruffe et al., 2002; Gescher and Steward, 2003; Srivastava et al., 2013). Mechanism based inactivation of CYP2E1 and CYP1A2 by resveratrol has been reported previously (Chang et al., 2001; Mikstacka et al., 2002; Upadhyay et al., 2008). Further, it has been demonstrated that the metabolic hydroxylation of resveratrol by CYP1B1, results in its conversion to piceatannol, a tyrosine kinase inhibitor and a compound of known anticancer activity (Geahlen and McLaughlin, 1989; Mikstacka et al., 2002; Potter et al., 2002). The substitution of the hydroxy with methoxy

TABLE 2 | Natural products, their active ingredients and effects.

Natural products	Major constituents	Effect	References
Barley grass	2-0-glycosylisovitexin (2-0-GlV), flavone-C-glycosides, saponarin and lutonarin contain natural SOD	Protect damage by inhibition of fat oxidation (lipid peroxidation) and by increasing antioxidant capacity	Duarte, 1995; Markham and Mitchell, 2003
Carrot	Xanthophyll, betacarotene, and other antioxidant carotenoids	Protects against damage due to powerful antioxidant capacity of xanthophyll, betacarotene	Duarte, 1995
Citrus fruit	Flavonoids and vitamin C	Provides protection against damage by its antioxidant capacity	King and Cousins, 2006
Eleuthero root (also known as Siberian ginseng)	Four shogaols contain antioxidants	Protects against damage due to its antioxidant and anti-lipid peroxidative activities	Bol'Shakova et al., 1997; DerMarderosian, 2001; Yu et al., 2003
Ginger root	About 40 different bioflavonoids, including proanthocyanidins and quercetin	Protects against damage due to its antioxidant and anti-lipid peroxidative activities	DerMarderosian, 2001; Halvorsen et al., 2002; Kim et al., 2002
Ginkgo leaf	About 40 different bioflavonoids, including proanthocyanidins and quercetin	Protects against damage due to its antioxidant and anti-lipid peroxidative activities; also inhibits nuclear factor-kappa B and activator protein 1 activation, and adhesion molecule expression in HAECs therefore effective against atherosclerosis disease	DerMarderosian, 2001; Chen et al., 2003; DeFeudis et al., 2003; King and Cousins, 2006
Grape seed/skin	A variety of antioxidant substances and is over 90% proanthocyanidins	Protects against damage by blocking free radical generation and lipid peroxidatin as well as by regulating bcl-X(L) gene, DNA damage and presumably by reducing oxidative stress	Bagchi et al., 2000; Young et al., 2000; DerMarderosian, 2001
Kudzu root	Isoflavone known as puerarin (its crude form is more efficient than puerarin)	Protects against damage due to its antioxidant capacity	Guerra et al., 2000; Allen et al., 2003; Rezvani et al., 2003
Milk thistle seed	Silymarin (major constituents silybinin)	Protects against damage due to its antilipid peroxidative capacity and antioxidant nature. It also inhibits CYP isoforms and increases antioxidant enzymes	Ben-Amotz et al., 1998; Shimizu, 2001; Kvasnicka et al., 2003; Upadhyay et al., 2007
Rosemary leaf	Flavonoids such as cirismarin, diosmin, hesperidin, homoplantiginin, and phegopolin	Protects against damage due to its antioxidant capacity and have analgesic property for myalgias and neuralgias	Duarte, 1995; Gruenwald et al., 2000
Schisandra fruit	At least 9 dibenzocyclooctene lignans	Protects against damage due to its antioxidant and anti-lipid peroxidative activities and decrease the extent of membrane fluidity of liver microsomes	Lu and Liu, 1992
Tomato	Lycopene and vitamin C	Protects against damage due to its antioxidant capacity	King and Cousins, 2006
Turmeric root	Curcuminoids	Protects against damage due to its antioxidant capacity and also inhibits different molecules involved in inflammation such as phospholipase, lipooxygenase, cyclooxygenase 2, leukotrienes, thromboxane, prostaglandins, nitric oxide, collagenase, elastase, hyaluronidase, monocyte chemoattractant protein-1, interferon-inducible protein, tumor necrosis factor, and interleukin-12	DerMarderosian, 2001

groups in resveratrol molecules may increase its lipophilicity and binding to the active sites of CYPs (Chun et al., 2001; Regev-Shoshani et al., 2004).

Recently it has been shown by Wang et al., that resveratrol prevents acetaminophen-mediated liver damage by inhibiting CYP-mediated bioactivation of acetaminophen and regulating SIRT1, p53, cyclin D1, and PCNA to facilitate liver regeneration (Wang et al., 2015). Zhang et al., found that pretreatment of resveratrol effectively reversed As_2O_3-induced liver toxicity indices and resulted in a significant improvement in hepatic function in cat models. Moreover, resveratrol also improved the glutathione levels, activities of antioxidant enzymes and attenuated As_2O_3-induced increases in reactive oxygen species and malondialdehyde production in liver tissues (Zhang et al., 2014). Resveratrol has also been shown to offer protection against methotrexate, sodium fluoride and azozymethane- induced hepatotoxicity in animal models (Dalaklioglu et al., 2013; Gurocak et al., 2013; Atmaca et al., 2014).

CURCUMIN

Curcumin is one of the most widely used herbal formulations to protect drug-induced toxicity. It is the major constituent of the spice turmeric extracted from the root of Curcumalonga Linn. It is metabolized to curcumin glucuronides, sulfates, tetrahydrocurcumin and hexacurcumin in the intestine and liver of human and rats and is reported to exhibit antioxidant, anti-inflammatory, choleretic, anti-carcinogenic, antiviral, and anti-infectious activities (Pan et al., 1999; Joe et al., 2004; Maheshwari et al., 2006). It inhibits the expression of the enzyme cyclooxygenase 2 via interference with activation of the transcription factor NF-κB and is a potential hepatoprotectant (Plummer et al., 1999; Duvoix et al., 2005; Aggarwal and Shishioda, 2006). Curcumin may be used in combination with conventional chemotherapeutic drugs to reverse multi drug resistance (MDR) in cancer cells and is the most effective MDR modulator among curcuminoids (Chearwae et al., 2004). Curcumin attenuates the proliferation of human lymphocytes and the production of several inflammatory mediators such as lipid mediators and cytokines and possess immunosuppressive and anti-rheumatic activity (Fahey et al., 2007; Gautam et al., 2007; Sandur et al., 2007). Curcuminoids have also been reported to inhibit pro-inflammatory induction by enhancing peroxisome proliferator-activated receptor-gamma (PPAR-gamma) activation (Jacob et al., 2007). Soliman et al., showed the curcumin-mediated protection against paracetamol-induced liver damage by restoring antioxidant activity of liver. They further showed that curcumin effectively restored the paracetamol-mediated increase in matrix metalloproteinase-8 (MMP-8), interleukin-1β (IL-1β), IL-8, tumor necrosis factor-α (TNF-α), and acute phase proteins and decrease in the expression of antioxidant genes (Soliman et al., 2014). Curcumin offers hepatoprotection against cisplatin, alcohol and heavy metals-induced liver damage in animals (Lee et al., 2013; García-Niño and Pedraza-Chaverrí, 2014; Waseem et al., 2014; Sankar et al., 2015; Soliman et al., 2015).

GINGKO

The Ginkgo biloba extract possess memory-enhancing, cognition-improving, immuno-modulating, apoptosis-inducing, and antiplatelet effects (Oken et al., 1998; Diamond et al., 2000; Wesnes et al., 2000; McKenna et al., 2001; Mahady, 2002; Andrieu et al., 2003; Ponto and Schultz, 2003; Yang et al., 2014a). The therapeutic benefits of this herbal medicine in neurological disorders, chronic refractory schizophrenia, hepatotoxicity, and in sleep disturbance of depressed patients have been reported (Hemmeter et al., 2001; Watanabe et al., 2001; De Smet, 2002; Yang et al., 2011). The major constituents of ginkgo include flavonoids (e.g., kaempferol), terpenoids (e.g., ginkgolides and bilobalaides), and organic acids (e.g., ginkgolic acids and alkylphenols) (Krieglstein et al., 1995; Jaggy and Koch, 1997; Tang et al., 2001; Lichtblau et al., 2002; Van Beek, 2002). As an adjuvant therapeutic drug, ginkgo appears to be promising in diabetics with respect to ischemic myocardium injury (Schneider et al., 2008). Intake of Ginkgo biloba extract may alter the hepatic metabolism by modulating hepatic drug metabolizing enzymes, altering the level of antioxidant enzymes and endogenous antioxidants such as GSH. The altered hepatic metabolism may result in the clearance of co-administered drugs, in particular, that have reduced renal and liver function (Deng et al., 2008).

OTHER HERBAL MEDICINES

Use of herbs in treatment of various liver disorders is common in China. The potential of these herbs against diseases with few and even no side effects continuously increases its popularity (**Table 3**). Now these medicines are being gradually accepted worldwide, particularly in Asia, Europe and North America. Nevertheless, the application strategy may differ in East and West due to variety of reasons, such as, philosophical viewpoint, concept of diseases, and treatment approaches.

In a review published in 2007 on Chinese medicine, the authors have summarized 274 species (in 216 genera among 92 families) of herbs possessing protective effect against liver toxicity (Wang et al., 2007). They further suggested a crude classification of these drugs in two groups; firstly, the main ingredients, for example silybin, osthole, cumarin, glycorrhizin, flavonoids and so on; and secondly, the supporting substances like sugars, amino acids, resins, tannins, and volatile oil.

LIVER INJURY ASSOCIATED WITH HERBAL PRODUCTS

The drug-induced liver injuries are debated since decades; the liver toxicity of certain herbal products has only recently been recognized. There are not many studies available on this alarming aspect of herbal products till date; however, the threat cannot be ignored as they may present with the same spectrum of liver pathologies as synthetic products (Stickel and Shouval, 2015). A large number of herbal and dietary products including Shou-Wu Pian (*Polygonum multiflorum*), *Breynia officinalis*, Germander (*Teucrium chamaedrys*), Chaparral (*Larrea tridentata*), *Actractylis gummifera*, Impila (*Callilepsis*

TABLE 3 | A list of herbal medicines and their effects.

Herbal medicines	Effects	References
Chai Hu Qing Gan Decoction	Ameliorates the central necrosis and fatty changes of the liver	Lin et al., 2007
Da Cheng Qi Decoction	Protects against gastrointestinal disorders, and liver diseases	Tseng et al., 2007
Xiao Cheng Qi Decoction	Protects against gastrointestinal disorders, and liver diseases	Tseng et al., 2007
Tiao Wei Cheng Qi Decoction	Protects against gastrointestinal disorders, and liver diseases	Tseng et al., 2007
Fenofibrate	Inhibits TNF-α and up-regulates PPAR-α and protects against NAFLD	Hong et al., 2007
Xuezhikang	Inhibits TNF-α and protects against NAFLD	Hong et al., 2007
Breviscapine extracted herb Erigeron breviscapus	Attenuating liver lipid accumulation and oxidative stress	Wu et al., 2007a,b
Inchinkoto	Increased hepatic levels of heme oxygenase-1 and GSH by a nuclear factor-E2-related factor (Nrf2)-dependent mechanism	Okada et al., 2007
Tanshinones	Decreases ALT and MDA levels, and increases ORAC, vitamin C and GSH levels in liver tissues	Xu et al., 2006
Root of Bupleurus spp.	Inhibitory capacity on superoxide anion formation and superoxide anion scavenging activity	Liu et al., 2006
BJ-JN (a traditional Chinese formulation)	ALT levels, hepatic NO and MDA content, and restores hepatic SOD activity and alleviates diminished (by toxin treatment) splenocyte proliferation	Zou et al., 2006
Piper betel leave	It has the biological capabilities of detoxication, anti-oxidation, and antimutation	Young et al., 2007
Cordyceps sinensis	Possesses the antitumor activity, antioxidant activity, and the capability of modulating the immune system	Chen et al., 2006
Baicalin	Decreases the leakages of LDH and ALT, and the formation of MDA; attenuates GSH depletion and oxidative stress. Histopathological evaluation of the rat livers revealed that baicalin reduced the incidence of liver lesions including hepatocyte swelling, leukocyte infiltration, and necrosis induced by toxin	Hwang et al., 2005
Crocetin	Protective action of crocetin operated via quenching of the superoxide anion and/or free radical	Tseng et al., 1995
Bidens bipinnata L.	Suppresses nitric oxide production and NF-κB activation, and possesses antioxidant property	Zhong et al., 2007
Mung bean, adzuki bean, black bean, and rice bean	Possess antioxidant property and suppresses LPO	Wu et al., 2001

laureola), Pennyroyal (*Mentha pulegium*), Greater celandine (*Chelidonium majus*), Kava (*Piper methysticum*), Black cohosh (*Cimifuga racemosa*), Noni juice (*Morinda citrifolia*), Gotu Kola (*Centella asiatica*), etc. are reported to have liver damaging effects (Stickel and Shouval, 2015). These products when consumed may cause symptoms ranging from acute, chronic, cholestatic, fulminant, and acute autoimmune-like hepatitis to acute liver failure, and liver cirrhosis. The mechanism of their toxicities are largely unknown; however, involvement of oxidative stress and apoptosis is also reported (Stickel and Shouval, 2015).

CONCLUSION

The nature and dose of a particular drug are not the only determining factors of cell injury. Other factors such as an individual's gene expression profile, antioxidant status and the capacity for regeneration are also crucial. Several mechanisms are involved in the initiation of liver cell damage and aggravate ongoing injury processes. Dysfunction of these vital cell organelles results in impairment of dynamic equilibrium in homeostatic condition, thus resulting in intracellular oxidative stress with excessive formation of reactive oxygen species. Major causes of the hepatotoxic reactions by drugs are elevated ROS generation, oxidative stress and suppressed immune responses. Hepatotoxicity remains a major cause of drug withdrawal from the market. Recent examples in the USA and Europe are ximelagatran, nefazodone, nimesulide, ebrotidine, trovafloxacin, troglitazone, bromfenac, and so forth.

Natural products have shown great promise in combating against the toxicity of several commonly used drugs, including acetaminophen and paracetamol. Additionally, some of these natural products, such as resveratrol and curcumin, are now widely accepted chemopreventive agents. Due to easy availability and dietary nature, it is time to promote the natural products as supplementary medication with drugs that also cause toxicity to cells. Although a majority of natural products investigated to date are non-toxic, some studies have shown liver toxicity by certain natural products. Therefore, the proper selection of the natural products is also necessary. It is envisioned that natural products will not only lower the risk of drug-induced liver damage, but also provide an alternative solution to remedy the drug-induced hepatotoxicity.

AUTHOR CONTRIBUTIONS

DS wrote the manuscript. WC and GU revised 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.

frontiers in Physiology

ORIGINAL RESEARCH
published: 07 August 2015
doi: 10.3389/fphys.2015.00216

Xylooligosaccharide supplementation alters gut bacteria in both healthy and prediabetic adults: a pilot study

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: 21 April 2015
Accepted: 17 July 2015
Published: 07 August 2015

Citation:
Yang J, Summanen PH, Henning SM, Hsu M, Lam H, Huang J, Tseng C-H, Dowd SE, Finegold SM, Heber D and Li Z (2015) Xylooligosaccharide supplementation alters gut bacteria in both healthy and prediabetic adults: a pilot study. Front. Physiol. 6:216. doi: 10.3389/fphys.2015.00216

Background: It has been suggested that gut microbiota is altered in Type 2 Diabetes Mellitus (T2DM) patients.

Objective: This study was to evaluate the effect of the prebiotic xylooligosaccharide (XOS) on the gut microbiota in both healthy and prediabetic (Pre-DM) subjects, as well as impaired glucose tolerance (IGT) in Pre-DM.

Subjects/Methods: Pre-DM ($n = 13$) or healthy ($n = 16$) subjects were randomized to receive 2 g/day XOS or placebo for 8-weeks. In Pre-DM subjects, body composition and oral glucose tolerance test (OGTT) was done at baseline and week 8. Stool from Pre-DM and healthy subjects at baseline and week 8 was analyzed for gut microbiota characterization using Illumina MiSeq sequencing.

Results: We identified 40 Pre-DM associated bacterial taxa. Among them, the abundance of the genera *Enterorhabdus, Howardella,* and *Slackia* was higher in Pre-DM. XOS significantly decreased or reversed the increase in abundance of *Howardella*, *Enterorhabdus,* and *Slackia* observed in healthy or Pre-DM subjects. Abundance of the species *Blautia hydrogenotrophica* was lower in pre-DM subjects, while XOS increased its abundance. In Pre-DM, XOS showed a tendency to reduce OGTT 2-h insulin levels ($P = 0.13$), but had no effect on body composition, HOMA-IR, serum glucose, triglyceride, satiety hormones, and TNFα.

Conclusion: This is the first clinical observation of modifications of the gut microbiota by XOS in both healthy and Pre-DM subjects in a pilot study. Prebiotic XOS may be beneficial in reversing changes in the gut microbiota during the development of diabetes.

Clinical trial registration: NCT01944904 (https://clinicaltrials.gov/ct2/show/NCT01944904).

Keywords: xylooligosaccharide, prediabetic, diabetes, gut, microbiota

Abbreviations: XOS, xylooligosaccharide; Pre-DM, prediabetic; BMI, body mass index; T2DM, Type 2 Diabetes Mellitus; FOS, fructooligosaccharides; GOS, galactooligosaccharides; OGTT, oral glucose tolerance test OGTT.

Introduction

Increasing evidence indicates that changes in gut microbiota composition might contribute to the development of metabolic disorders such as obesity and T2DM (Qin et al., 2012). Several mechanisms have been proposed regarding how gut bacteria could facilitate the pathogenesis of T2DM (Shen et al., 2013). Studies suggest that gut bacteria influence whole-body metabolism through regulation of the host's immune response, energy extraction and utilization, intestinal glucose absorption, and lipid metabolism (Musso et al., 2011). The central feature of obesity and T2DM is insulin resistance, potentially caused by low-grade inflammation resulting from nutrient excess and leading to endoplasmic reticulum (ER) stress. More and more research has shown that gut microbiota is another important factor for this low-grade inflammation (Chassaing and Gewirtz, 2014). The *Bacteroidetes*/*Firmicutes* ratio is associated with increased plasma glucose concentrations and a decrease in butyrate-producing bacteria in T2DM patients (Larsen et al., 2010; Shen et al., 2013). Recent studies in mice have demonstrated that an increase in the abundance of *Bifidobacteria* and *Akkermansia muciniphila* attenuated high fat diet-induced metabolic complications (Everard et al., 2013).

Pre-DM refers to the intermediate stage between normoglycemia and overt diabetes mellitus. Pre-DM is characterized by glucose dysregulation and a higher risk of developing T2DM and other associated complications (Portero McLellan et al., 2014). However, not all individuals with Pre-DM progress to overt T2DM. With changes in lifestyle and diet, the progression of Pre-DM to T2DM can be prevented or delayed (Portero McLellan et al., 2014). Given the significant involvement of certain gut bacteria in host metabolism (Shen et al., 2013), therapeutic manipulation of the gut microbiota has been proposed for both individuals with T2DM and in those at risk of developing the condition.

Prebiotics are highly effective and important for many applications in medicine. They are not digestible and do not contribute to human nourishment, but rather exert a profound effect on the human gut microbiota (International Scientific Association for Probiotics and Prebiotics, 2004). Prebiotic-induced modulation of gut microbiota has been developed and widely used (Everard et al., 2011). The principal effect of prebiotics on the human gut microbiota is to stimulate the growth of the *Bifidobacterium* and *Lactobacillus* genera (Marotti et al., 2012). Prebiotic treatment is known to modulate host gene expression and metabolism as well (Everard et al., 2011). Dietary intervention using prebiotic inulin or oligofructose (FOS) alters the gut microflora composition by promoting the growth of beneficial bacteria such as *Bifidobacterium, Lactobacillus,* and *A. muciniphila* (Rossi et al., 2005; Choi and Shin, 2006). However, the mechanisms associating prebiotics and its beneficial effects have yet to be fully understood. Further studies are needed to examine the precise physiological roles of prebiotics on human bowel flora and in host immune function.

Xylooligosaccharide (XOS) is a recent prebiotic that can be incorporated into many food products (Aachary and Prapulla, 2011). Our lab previously reported that XOS, at the dose of 2.8 g/day, was well tolerated, and modified the gut bacterial composition in healthy people (Finegold et al., 2014). A consideration of the gut microbiota in the context of health benefits of XOS in Pre-DM is especially relevant since recent research has indicated a critical role of gut microbiota in the development of T2DM (Qin et al., 2012). The present study was designed to determine the effect of XOS supplementation on the gut microbiota in healthy and pre-DM individuals. Since the ultimate objective of this research is to explore the potential effects of XOS in the prevention of progression of Pre-DM to T2DM, we also evaluated the effects of XOS in the management of IGT, body composition, and inflammatory marker in Pre-DM subjects.

Materials and Methods

Subjects

This was a double-blind, randomized, placebo-controlled study with 34 subjects who were recruited based on inclusion and exclusion criteria. The study population consisted of 16 healthy subjects (placebo: $n = 9$; XOS: $n = 7$) and 13 Pre-DM subjects (placebo: $n = 6$; XOS: $n = 7$).

Ethics

The study was carried out in accordance with the guidelines of the Office for Protection of Research Subjects of the University of California, Los Angeles and the Institutional Review Board of the VA Greater LA Health Center. All subjects provided written informed consent before the study began.

Study Design

The enrollment criteria for healthy participants was fasting plasma glucose of 65–100 mg/dl, Participants in the Pre-DM study were selected based on the American Diabetes Association criteria for impaired fasting glucose (fasting plasma glucose of 100–125 mg/dl) and/or HgbA1c (5.7–6.4%) (American Diabetes Association, 2014). Over the span of 8 weeks, both healthy and Pre-DM subjects were randomly assigned to take daily a capsule supplement containing either 2 g XOS (2.8 grams of 70% XOS) or placebo. The XOS and placebo were provided by Life Bridge International (Riverside, CA). The XOS was manufactured by Shandong Longlive Bio-Technology Co., Ltd., China. The placebo capsules contained maltodextrin. The study consisted of three phases: a 2-week run-in phase, and an 8-week intervention phase.

Stool Collection

A total of two stools were collected from each subject: at baseline and week 8 of the intervention periods. Each time the entire stool specimen was obtained. The specimen was placed in a large, zip-lock freezer bag and all air was pushed out of the bag as the zip lock was closed. The specimen was delivered on ice to the UCLA Center for Human Nutrition within 24 h of collection where it was immediately stored at −20°C.

Miseq Sequencing

DNA from stool was extracted using a commercial extraction system (QIAamp Stool DNA Extraction Kit, Qiagen, Valencia,

CA). The quality of the DNA samples was confirmed using a Bio-Rad Experion system (Bio-Rad Laboratories, CA, USA). The 16S rRNA gene V4 variable region PCR primers 515/806 with barcode on the forward primer were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. After amplification, PCR products are checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA). Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis et al., 2006).

Body Composition

Body composition was measured using the Tanita-BC418 bioelectrical impedance analyzer (Tanita Corp., Japan).

Glucose Tolerance Test

On the test days at baseline and week 8, Pre-DM subjects remained in a fasting state for 2.5 h prior to the beginning of OGTT. The 75 g glucose cola was administrated immediately after the basal blood draw at 0 min. Subsequent blood samples were taken 30, 60, and 120 min afterwards. Serum samples were kept at −80°C. The insulin resistance index assessed by the homeostasis model (HOMA-IR) was calculated as follows (Allard et al., 2003): (fasting blood glucose [mmol/l] × fasting plasma insulin [μU/ml])/22.5.

Blood Biochemical Analysis

Blood samples were collected and coded to protect patient confidentiality. Lipids, insulin, glucose, and satiety hormones were measured. Serum triglycerides were determined using a enzymatic method (Pointe Scientific, MI). Serum glucose was determined using Glucose Assay kit (Cayman Chemical Company, MI). Serum insulin, active GLP-1, leptin, pancreatic polypeptides (PP) and TNFα were determined using the MILLIPLEX map kit (EMD Millipore, Billerica, MA) and data were captured and processed using Luminext 200 with xPonent software.

Statistics

Demographic data at baseline were analyzed and presented as mean ± SD and two sample *t*-tests were used for comparisons between the placebo and XOS groups. For analysis of serum variables, such as insulin, glucose, values of mean ± SE at baseline and week 8 were presented for all groups, and two sample *t*-test was used to compare the two groups at baseline and 8 weeks. For DNA sequencing analyses, Wilcoxon rank sum test was utilized to evaluate the differences between study groups. All tests are two sided and all analyses were conducted using SAS 9.3 (Statistical Analysis System, Cary, NC, 2008) and R (www.r-project.org) software.

Results

Subjects

The study population consisted of 16 healthy participants (4 men and 12 women) aged between 21 and 49 years, and 13 Pre-DM participants (9 men and 4 women) aged between 30 and 63. Participants did not report any adverse effects or symptoms with the XOS intervention at 2 g/day. **Table 1** shows the baseline characteristics of the participants in the four groups. The composition of the fecal microbiome from all healthy participants and Pre-DM participants was analyzed by Miseq sequencing.

Gut Microbial Composition Changes Related to Pre-DM

Miseq sequencing was used to compare the gut microbial composition of baseline samples from 16 healthy and 13 Pre-DM subjects. The abundance (percentage of total sequences) of 1 phylum, 1 class, 3 families, 13 genera, and 22 species, was significantly different between healthy and Pre-DM (Supplementary Table 1). Composition of phyla in healthy and Pre-DM groups is displayed in **Figure 1A**. The abundance of infectious and T2DM related phylum *Synergistetes* (Baumgartner et al., 2012; Qin et al., 2012) was significantly higher in Pre-DM compared with healthy subjects ($P \leq 0.05$) (**Figure 1A**). In addition, 13 genera responded significantly in Pre-DM ($P \leq 0.05$) (**Figure 1B**). The abundances of *Allisonella*, *Cloacibacillus, Enterorhabdus*, *Howardella*, *Megamonas,* and *Slackia* were significantly higher, while *Adlercreutzia*, *Anaerococcus*, *Ethanoligenens*, *Gordonibacter*, *Lactococcus*, *Parasutterella,* and *Tissierella* were greatly reduced in Pre-DM compared with healthy subjects (**Figure 1B**).

TABLE 1 | Baseline characteristics of the study participants.

	Healthy placebo ($n = 9$)	Healthy XOS ($n = 7$)	Pre-DM placebo ($n = 6$)	Pre-DM XOS ($n = 7$)
Age, year	31.9 ± 6.9	31.7 ± 9.3	44.8 ± 11.2	55.0 ± 6.2
Weight, kg	65.4 ± 14.0	69.9 ± 13.0	94.9 ± 15.9	97.4 ± 22.7
BMI, kg/m^2	23.4 ± 3.2	25.6 ± 3.2	33.6 ± 7.2	32.2 ± 4.2

Values are presented as mean ± standard deviation (SD). For all characteristics, there were no significant differences between the placebo and XOS groups (all P > 0.05, based on independent sample t-tests). BMI, body mass index.

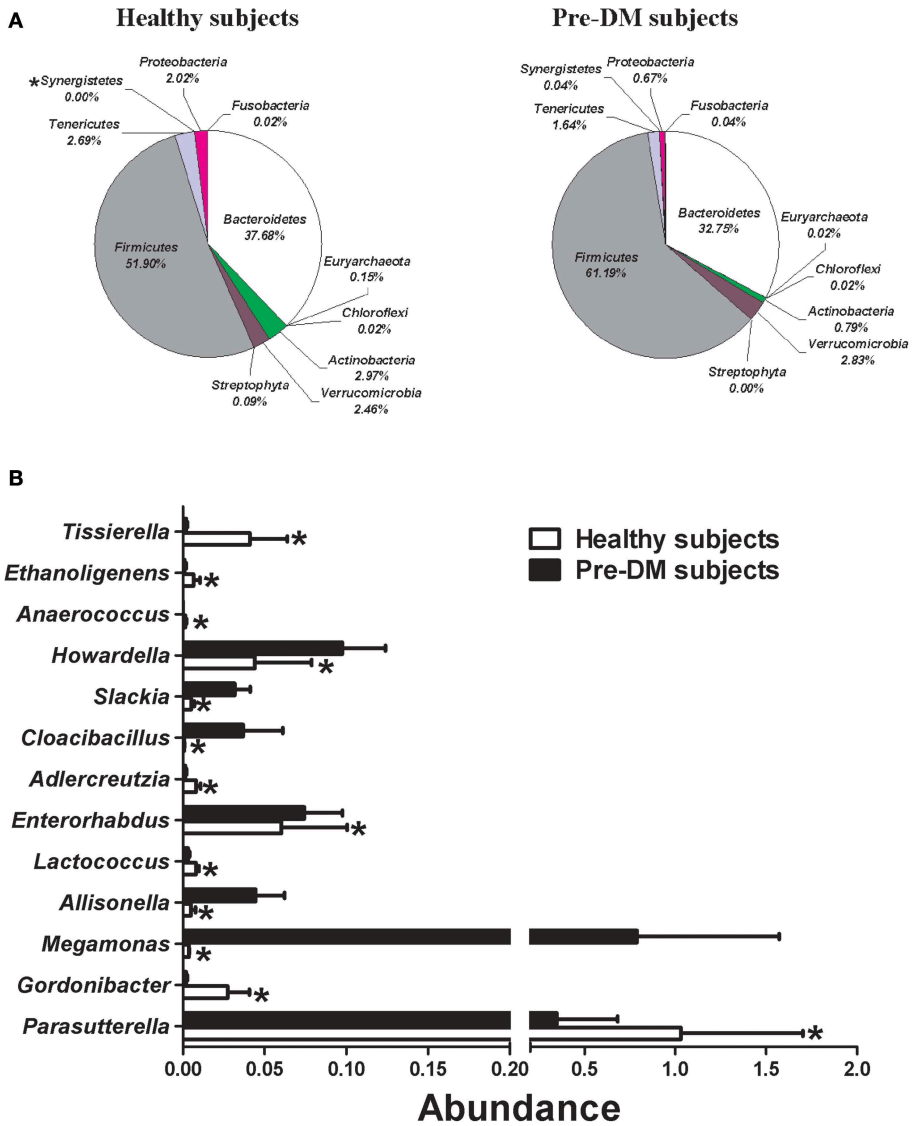

FIGURE 1 | Comparison of gut microbiota composition between healthy ($n = 16$) and Pre-DM ($n = 13$) subjects. (A) Pie charts depict mean abundance (% of total) of the indicated phyla. **(B)** Bar graph of genera shows significant differences in abundance between healthy and Pre-DM subjects. Values are presented as mean ± standard error (SE) $^*P \leq 0.05$.

Effects of XOS Supplementation on the Gut Microbiota in Healthy and Pre-DM Subjects

The overall changes of bacterial composition with 8-week XOS intervention were assessed at the phylum and genus levels (**Figures 2**, **3**). Significant XOS-induced changes were found for 1 phyla, 3 classes, 7 families, 23 genera, and 25 species in healthy subjects (**Figure 2** and Supplementary Table 2), and 3 classes, 1 families, 7 genera, and 17 species in Pre-DM subjects (**Figure 3** and Supplementary Table 3). The mean abundance of indicated phyla of healthy subjects in placebo and XOS groups at baseline and week 8 are displayed in **Figure 2A**. The phylum *Firmicutes* showed a 20% increase in abundance over 8 weeks in placebo groups of healthy subjects, while XOS intervention significantly reversed this increase ($P \leq 0.05$) (**Figure 2A**). The mean abundance of *Verrucomicrobia* increased in XOS groups of healthy subjects as well. Among genera with an average abundance >1% in at least one group, six were significantly regulated by XOS. An increase of infectious disease related *Streptococcus* and *Subdoligranulum* in placebo groups was largely inhibited by XOS in healthy subjects (**Figure 2B**). The 8-week XOS intervention did not induce significant changes of gut microbiota at the phylum level in Pre-DM subjects (**Figure 3A**). At the genus level, *Blautia*, *Anaerotruncus*, *Dialister*, and *Oscillospira* were four abundant genera identified with significant XOS-induced changes in Pre-DM subjects. XOS diminished or reversed the magnitude of population decline in all four genera (**Figure 3B**).

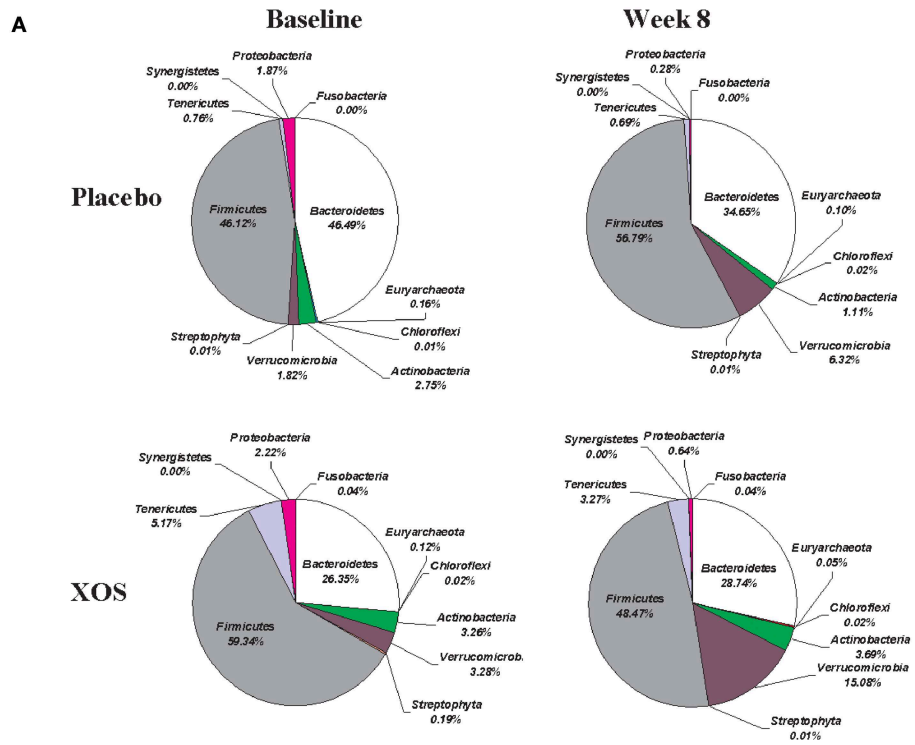

FIGURE 2 | Effects of XOS supplementation on gut microbiota in healthy subjects ($n = 16$). (A) Pie charts display the mean abundance of indicated phyla of healthy subjects receiving placebo ($n = 9$) and XOS ($n = 7$) at baseline and week 8. **(B)** Bar graph of genera shows significant difference in abundance between placebo and XOS groups. Values are presented as mean ± standard error (SE) *$P \leq 0.05$.

XOS Supplementation Reversed Gut Bacterial Alterations Associated with Pre-DM

Of the 40 Pre-DM associated bacterial taxa (Supplementary Table 1) identified in this study, the abundances of the *Enterorhabdus, Howardella,* and *Slackia* genera were elevated in Pre-DM. The 8-week XOS intervention significantly diminished or reversed the abundance increase of *Howardella* and *Slackia* observed in the placebo group of healthy subjects, as well as *Enterorhabdus* in Pre-DM subjects (**Figures 4A–C**). *B. hydrogenotrophica* was less abundant in Pre-DM subjects (Supplementary Table 1), but XOS intervention significantly reversed the decrease in *B. hydrogenotrophica* abundance observed in the placebo group of Pre-DM subjects ($P \leq 0.05$) (**Figure 4D**).

Effects of XOS on Body Composition, Metabolic, and Immunological Markers in Pre-DM Subjects

In Pre-DM subjects, body composition, blood tests, and oral glucose tolerance tests (OGTT) were done at baseline and after 8 weeks of XOS intervention. Body weight and indexes of overall adiposity such as BMI, % fat, and % trunk fat were not changed by 8-week XOS intervention (**Figure 5**). Despite significant inter-individual variations in insulin responses

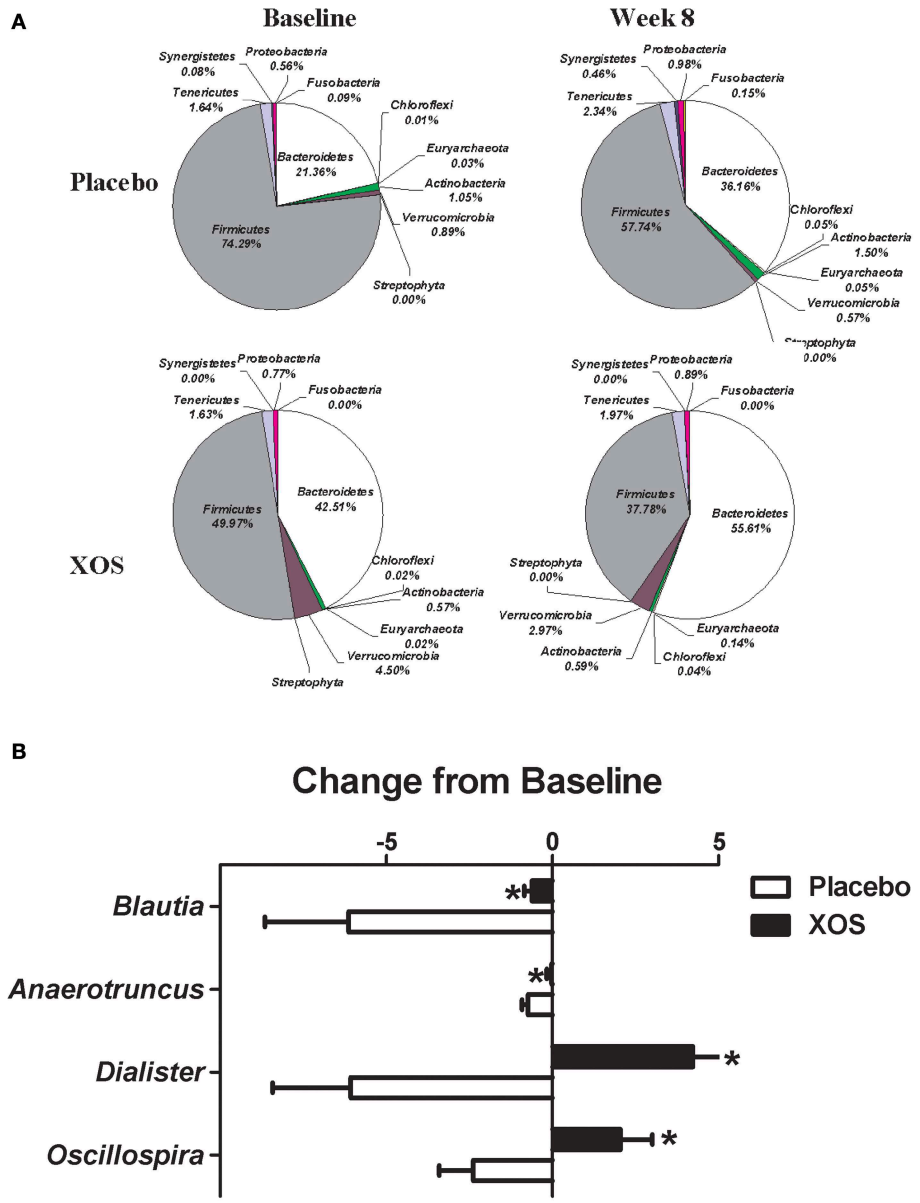

FIGURE 3 | Effects of XOS supplementation on gut microbiota in Pre-DM subjects ($n = 13$). (A) Pie charts display the mean abundance of indicated phyla of Pre-DM subjects receiving placebo ($n = 6$) and XOS ($n = 7$) at baseline and week 8. **(B)** Bar graph of genera shows significant difference in abundance between placebo and XOS groups. Values are presented as mean ± standard error (SE) $^*P \leq 0.05$.

among Pre-DM subjects, OGTT 2-h insulin response showed a tendency to decrease with XOS intervention in Pre-DM ($P = 0.13$) (**Figure 6A**). No significant XOS-related differences were observed in serum glucose, HOMA-IR, active GLP-1, triglycerides, leptin, PP, or the inflammatory marker TNFα (**Figures 6B–H**).

Discussion

Emerging evidence suggests that metabolic disorders including T2DM are associated with a pro-inflammatory state secondary to dysbiosis of gut bacterial flora (Larsen et al., 2010; Esteve et al., 2011; Musso et al., 2011). In addition, literature has documented the translocation of gut bacteria to blood and tissues in T2DM, and probiotic *Bifidobacterium* treatment prevents bacterial translocation and protects against T2DM (Cani et al., 2007; Amar et al., 2011). Together, these findings suggest that gut bacteria are an important modifier of T2DM.

The effects of oligosaccharides including XOS, FOS, and galactooligosaccharides (GOS) in the treatment of T2DM have gained interest. However, studies have shown inconsistent results. In T2DM patients, Yamashita et al. demonstrated that

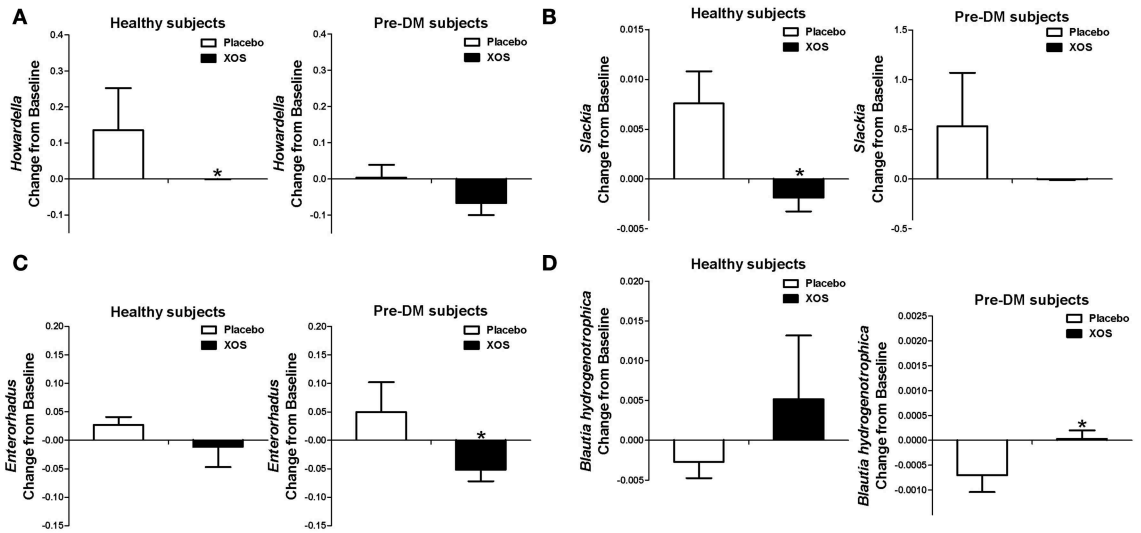

FIGURE 4 | XOS selectively regulated some of the Pre-DM associated bacterial taxa in healthy subjects ($n = 16$) or Pre-DM subjects ($n = 13$) during 8 weeks. The abundances of Pre-DM associated *Howardella* **(A)**, *Slackia* **(B),** and *Enterorhabdus* **(C)** were greatly reduced by XOS in healthy and Pre-DM subjects, respectively. **(D)** The abundance of healthy associated *Blautia hydrogenotrophica* was enhanced by XOS in healthy and Pre-DM subjects. Values are presented as mean ± standard error (SE) $^*P \leq 0.05$.

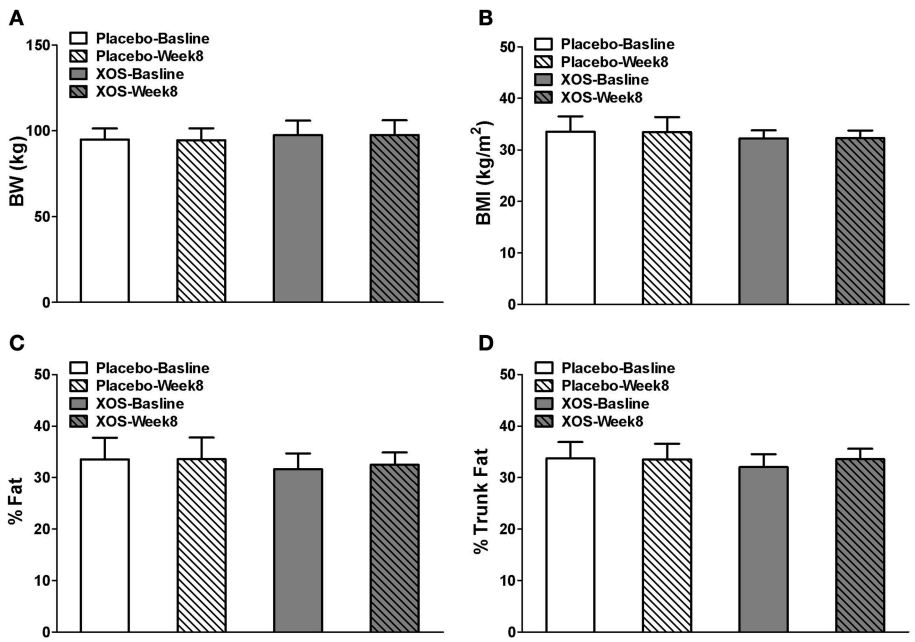

FIGURE 5 | Body weight (A), BMI (B), % Fat (C), and % Trunk fat (D) in Pre-DM subjects at baseline and after 8 weeks placebo ($n = 6$) or XOS ($n = 7$) treatment. Data are means ± standard errors (SE).

FOS at a dose of 8 g per day for 14 days resulted in a reduction of serum glucose, while Alles et al. showed that daily consumption of FOS at 15 g for 20 days had no effect on serum glucose level (Yamashita et al., 1984; Alles et al., 1999). Chan et al. showed that 4 g per day of XOS for 8 weeks was effective in reducing blood glucose and lipids in Taiwan T2DM patients (Sheu et al., 2008), and for 21 days benefited intestinal health and increased of *Bifidobacteria* in elderly subjects (Chung et al., 2007). Another study reported that both XOS and FOS dietary intervention reduced hyperglycaemia in diabetic rats (Gobinath et al., 2010). In addition, we found that 2 g per day of XOS for 8 weeks increased the *Bifidobacteria* abundance in healthy Americans without any gastrointestinal side effects (Finegold et al., 2014). Overall, a significant number of studies have shown oligosaccharides to be an effective option for lowering blood sugar in T2DM as well as improving intestinal health.

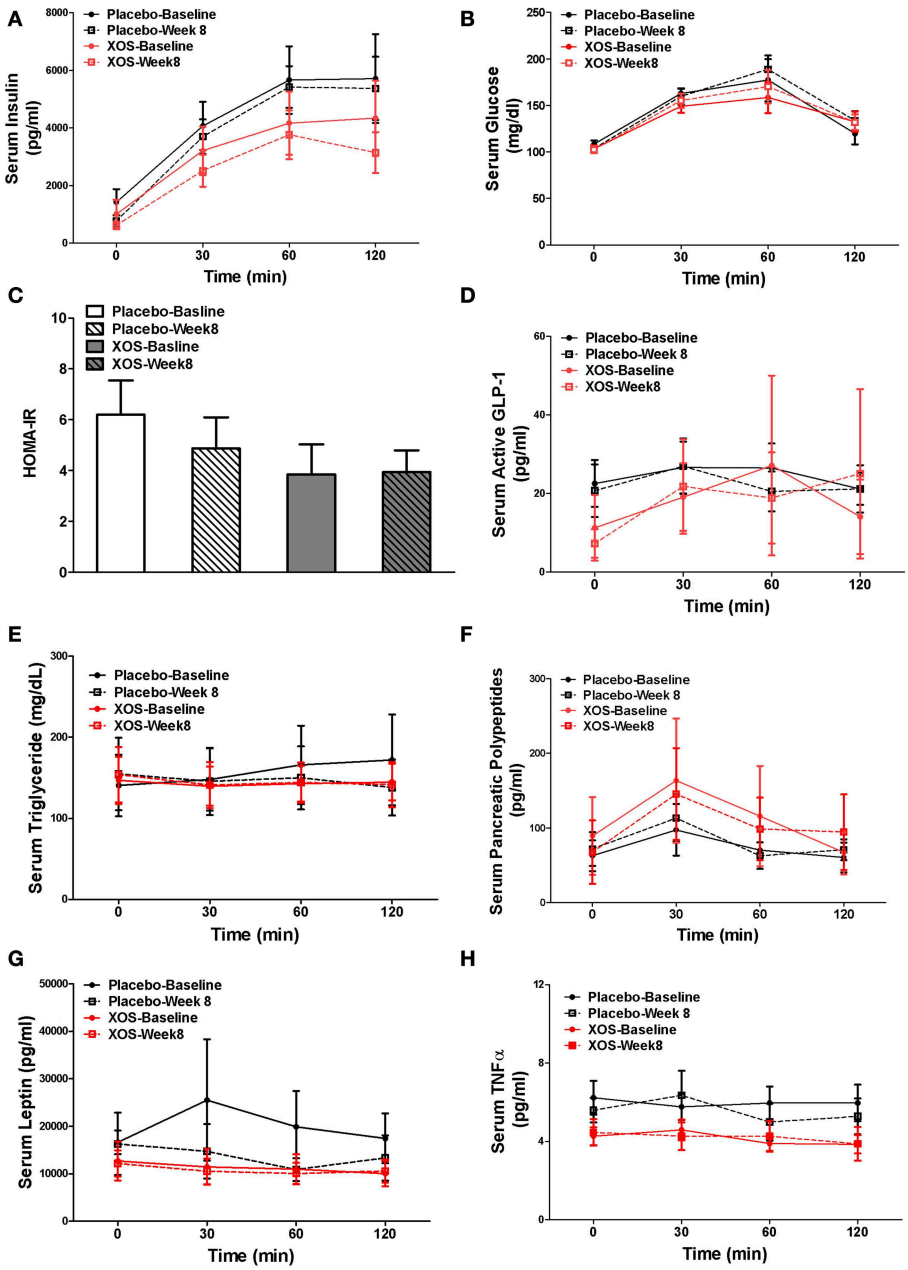

FIGURE 6 | Mean of parameters with SE at baseline and 8 weeks were compared between placebo- ($n = 6$) and XOS-treated ($n = 7$) group in Pre-DM subjects during the 120-min OGTT test. (A) Serum Insulin. **(B)** Serum glucose. **(C)** HOMA-IR. **(D)** Serum active GLP-1. **(E)** Serum triglyceride. **(F)** Serum pancreatic polypeptides. **(G)** Serum leptin. **(H)** Serum TNF α. Values are presented as mean ± standard error (SE).

In the present study, both healthy and Pre-DM subjects were given 2 g per day of XOS for 8 weeks. Miseq sequencing was used to evaluate the potential of XOS in preventing the dysbiosis of gut microbiota during the development of T2DM. We found that XOS had a clear impact on gut microbiota in both healthy and Pre-DM groups, and resulted in dramatic shifts of several bacterial taxa associated with Pre-DM. Among them, *Dialister spp.* and *Slackia* are pro-inflammatory (Rocas and Siqueira, 2006; Kim et al., 2010), and were greatly reduced by XOS. Additionally, T2DM associated lactic acid bacteria *Enterococcus*, *Streptococcus,* and *Lactobacillus* (Remely et al., 2013) were also greatly reduced by XOS. The inhibitory effect of XOS on other opportunistic pathogens, such as *Clostridia*, *Streptococcaceae*, and *Subdoligranulum*, further supports that XOS can potentially promote an optimal gut microbiota profile, and consequently reduce the risk of T2DM.

Miseq sequencing data also revealed that gut microbial composition of healthy subjects at different taxonomic levels was different from Pre-DM subjects. Some of our findings are consistent with previous T2DM studies of gut microflora

(Qin et al., 2012; Zhang et al., 2013; Finegold et al., 2014), substantiating the significant association of these changes with the progression of T2DM (Supplementary Table 1). Studies showed that the *Megamonas* OTU was most enriched in Pre-DM, compared to healthy or T2DM individuals (Qin et al., 2012; Zhang et al., 2013). The abundance of *Megamonas* in our study was 200-fold higher in Pre-DM than healthy subjects. We also found that the abundance of the phylum *Synergistetes* in Pre-DM was about 50 fold higher compared with healthy subjects. The *Synergistetes* appear to be more numerous in individuals with oral-related diseases as well as gut and soft tissue infections (Vartoukian et al., 2007). Another two infectious or metabolic disease related bacteria *Eubacteriaceae* (Plieskatt et al., 2013) and *Slackia* (Kim et al., 2010), were more abundant in Pre-DM individuals. The Pre-DM associated enrichment of infectious bacteria and the appearance of oral bacteria in the gut suggest that the host immune system may lose control over these opportunistic pathogens during the development of T2DM.

Both animal and clinical studies have shown that XOS supplementation greatly increases the *Bifidobacterium* population (Campbell et al., 1997; Chung et al., 2007; Finegold et al., 2014). We previously showed that an increase of *Bifidobacterium* abundance was detectable only with an *in vitro* culture method, and not pyrosequencing (Finegold et al., 2014). Using Miseq sequencing alone, the abundances of the *Bifidobacterium* genus, as well as the *Bifidobacterium longum*, *Bifidobacterium bifidum,* and *Bifidobacterium adolescentis* species were not significantly increased by XOS in healthy subjects (Supplementary Table 4). However, we found that XOS largely inhibited bacterial taxa related to infectious and metabolic disease, such as family *Streptococcaceae*, class *Clostridia*, and genera *Subdoligranulum*, *Gordonibacter,* and *Streptococcus* in healthy subjects (Supplementary Table 1). Furthermore, in healthy subjects, XOS greatly reduced the abundance of bacteria related to obesity or T2DM, including phylum *Firmicutes* and genera *Subdoligranulum* and *Bacilli* (Remely et al., 2013; Zhang et al., 2013). In Pre-DM subjects, XOS diminished or reversed the magnitude of population decline in about 70% bacterial taxa identified with a significant change from its baseline levels between treatment groups (Supplementary Table 2). The family *Veillonellaceae* and genera *Oscillospira* and *Dialister* exhibited population declines in the placebo group, but demonstrated large increases in abundance in the XOS group. Abnormally low levels of *Veillonellaceae* and *Dialister* have been described in autistic children (Kang et al., 2013) and patients of Crohn's disease (Joossens et al., 2011). Dietary whole grain intervention (Martinez et al., 2013) and corn fiber (Hooda et al., 2012) increased the *Dialister* and *Veillonellaceae* abundance. The genus *Oscillospira* has been associated with lean BMI (Tims et al., 2013). The inhibition of *Firmicutes* and increase of *Oscillospira* abundance suggest a potential role of XOS in weight control.

To the best of our knowledge, this is the first clinical study evaluating the effects of daily treatment with 2 g of XOS on glucose tolerance and insulin resistance in Pre-DM adults. In our experience, a dose of 2 g does not cause any gastrointestinal side effects (Finegold et al., 2014). Eight weeks of XOS supplementation tended to increase insulin sensitivity by lowering OGTT 2-h insulin response ($P = 0.11$), while no significant improvement of Pre-DM subjects' metabolic situation was observed, using the parameters of body composition, serum glucose, triglyceride, satiety hormones and inflammation marker TNFα. The TNFα levels (4.91 ± 1.85 pg/ml) of Pre-DM subjects in our study are normal, slightly lower than the reported TNFα (∼15–20 pg/ml) of eastern Indian Pre-DM population (Dutta et al., 2013) and much lower than T2DM (range from 87 to 112 pg/ml) (Goyal et al., 2012). Since we enrolled pre-DM subjects with impaired glucose tolerance (IGT) and without any other medical conditions we possibly did not observe elevated TNFα level. Pre-DM is a dynamic intermediate stage in the progression to T2DM, therefore it is very likely, subjects that met the selection criteria for Pre-DM are at different stages even though they are all classified as Pre-DM. Besides, studies also suggest a connection between TNFα gene polymorphism, its blood levels and the tendency to progression from Pre-DM to T2DM (Dutta et al., 2013). Therefore, more studies are needed to improve our understanding of the relationship between TNAα, Pre-DM staging, and T2DM progression. Our results do not agree with Chan et al. study in T2DM. However, this discrepancy could be explained by difference in XOS dose, study population and disease stages. It is possible that 2 g per day of XOS may have been too low to induce a difference in glucose tolerance. However, we observed a trend of increased insulin sensitivity by lowering OGTT 2-h insulin response at this dosage. Pre-DM is a strong risk factor for the development of T2DM, and the regulation of glucose metabolism and insulin sensitivity could be really dynamic during this stage. We think future clinical study with large sample size will be needed to confirm the benefits of XOS in Pre-DM.

In conclusion, XOS significantly modified gut microbiota in both healthy and Pre-DM subjects, and resulted in dramatic shifts of 4 bacterial taxa associated with Pre-DM. Future studies with larger sample size are needed to study the metabolic impact of XOS and understand the connection between XOS-mediated gut microbiota changes and the pathogenesis of T2DM.

Source of Support

Supported by departmental funds from the Center for Human Nutrition, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles.

Acknowledgments

ZL and DH designed research and had primary responsibility for final content. SH, JY, MH, HM, JH, PS, and G. Thames conducted research. SF, CT, and JY analyzed the data. JY wrote paper.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2015.00216

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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.

frontiers in Physiology

ORIGINAL RESEARCH
published: 22 May 2015
doi: 10.3389/fphys.2015.00129

Curcuminoids and ω-3 fatty acids with anti-oxidants potentiate cytotoxicity of natural killer cells against pancreatic ductal adenocarcinoma cells and inhibit interferon γ production

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OPEN ACCESS

Edited by:
Ravinder Abrol, Cedars-Sinai Medeical Center, USA

Reviewed by:
Christopher J. Scarlett, University of Newcastle, Australia
Edwin Charles Thrower, Yale University, USA

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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: *12 January 2015*
Accepted: *11 April 2015*
Published: *22 May 2015*

Citation:
Halder RC, Almasi A, Sagong B, Leung J, Jewett A and Fiala M (2015) Curcuminoids and ω – 3 fatty acids with anti-oxidants potentiate cytotoxicity of natural killer cells against pancreatic ductal adenocarcinoma cells and inhibit interferon γ production Front. Physiol. 6:129. doi: 10.3389/fphys.2015.00129

Pancreatic cancer has a poor prognosis attributed in part to immune suppression and deactivation of natural killer (NK) cells. Curcuminoids have a potential for improving the therapy of pancreatic cancer given promising results in cancer models and a clinical trial, but their oral absorption is limited. Our objective in this study is to show curcuminoid anti-oncogenic effects alone and together with human NK cells. We tested curcuminoids in an emulsion of ω-3 fatty acids and anti-oxidants ("Smartfish") regarding their direct cytocidal effect and enhancement of the cytocidal activity of NK cells in pancreatic ductal adenocarcinoma (PDAC) cells (Mia Paca 2 and L3.6). Curcuminoids (at ≥10 μM) with ω-3 fatty acids and anti-oxidants or with the lipidic mediator resolvin D1 (RvD1) (26 nM) induced high caspase-3 activity in PDAC cells. Importantly, curcuminoids with ω-3 fatty acids and anti-oxidants or with RvD1 significantly potentiated NK cell cytocidal function and protected them against degradation. In a co-culture of cancer cells with NK cells, interferon-γ (IFN-γ) production by NK cells was not altered by ω-3 fatty acids with anti-oxidants or by RvD1 but was inhibited by curcuminoids. The inhibition was not eliminated by ω-3 fatty acids or RvD1 but was relieved by removing curcuminoids after adding NK cells. In conclusion, curcuminoids with ω-3 fatty acids and anti-oxidants or with RvD1 have increased cytotoxic activity on PDAC cells alone and with NK cells. The effects of curcuminoids with ω-3 fatty acids and anti-oxidants on pancreatic cancer will be investigated in a mouse model with humanized immune system.

Keywords: pancreatic cancer, curcumin, ω-3 fatty acids, natural killer cells, interferon-γ

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer-related deaths in the United States with an overall survival of less than 1 year and based on current statistics is predicted

to become the 2nd leading cause of cancer-related deaths by 2020 (American Cancer Society, 2012). Chemotherapy has been less successful in PDAC in comparison to other cancers. The first line drug gemcitabine increased overall 1-year survival to 18% for gemcitabine-treated patients in comparison to 2% for 5-fluorouracil-treated patients (Burris et al., 1997).

The inflammatory milieu in pancreas may precede cancer onset due to chronic pancreatitis and be intimately involved with cancer progression. Although initially the immune cells tend to eliminate cancer cells, they ultimately fail and actually promote oncogenesis through IL-6 (Sideras et al., 2013). The immune system is modulated by cancer to inhibit the cytotoxic function of natural killer (NK) cells and CD8 T cells through activation of regulatory T cells, myeloid derived suppressor (MDSC) cells, tumor associated macrophages and fibroblasts. Indeed, the cytotoxicity of NK cells from tumors and peripheral blood of cancer patients is significantly reduced (Lai et al., 1996; Jewett et al., 2013). The immune therapies of pancreatic cancer using vaccination with cancer proteins and inactivated allogeneic cancer cells and adoptive cell therapy using cytotoxic T cells have not produced clear results in short-term trials (Sideras et al., 2013). Thus, new approaches to pancreatic cancer treatment are urgent.

Curcuminoids are under intensive study against cancer as they inhibit cell proliferation through suppression of NFκB and of a NFκB-dependent protein Akt, and suppress anti-apoptotic genes (Aggarwal et al., 2006). By activation of various transcription factors, curcuminoids target manifold proteins *in vivo* (Goel et al., 2008). In a rat model, curcuminoids attenuated the severity of pancreatitis (Gukovsky et al., 2003). Curcuminoids produced promising results in pancreatic cancer patients in combination with gemcitabine (Kanai et al., 2011). However, the plasma concentrations of curcuminoids achieved in that study were very low (<0.5 μg/ml). Thus, proper formulation of curcuminoids for increased absorption, stability and cellular activity is needed for clinical applications. In addition, curcuminoids block the production of the cytokine interferon-γ that has a key role in anti-oncogenic differentiation of cancer cells. Interferon-γ is a member of the type II interferon family with its own receptor, which signals through STAT-1 to transactivate IFN response factor 1 (IRF1) and downstream secondary response genes. (Pestka et al., 2004). Biological activities involve anti-proliferative, anti-angiogenic, and proapoptotic effects against cancer cells (Chawla-Sarkar et al., 2003). However, IFN-γ has also negative pro-tumorigenic effect in cancer (Maio et al., 1991; Zaidi and Merlino, 2011). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are ω-3 fatty acids with anti-cancer properties through production of toxic intermediates (Gleissman et al., 2010) and specialized proresolving mediators (SPMs) called resolvins, such as resolvin D1 (RvD1). Resolvins are lipidic mediators which resolve acute inflammation and may prevent chronic inflammation leading to cancer (Greene et al., 2011). The Western diet lacks DHA and EPA for production of SPMs. The low ratio of ω-3/ω-6 fatty acids in the Western diet promotes macrophage recruitment and activation of tumorigenic pathways. As changing the diet is difficult for many people, supplementation with ω-3 fatty acids may optimally increase the ω-3/ω-6 ratio in the diet.

Both curcumin and lipidic mediators from ω-3 block signaling by NFκB but their anti-oncogenic effects have not been

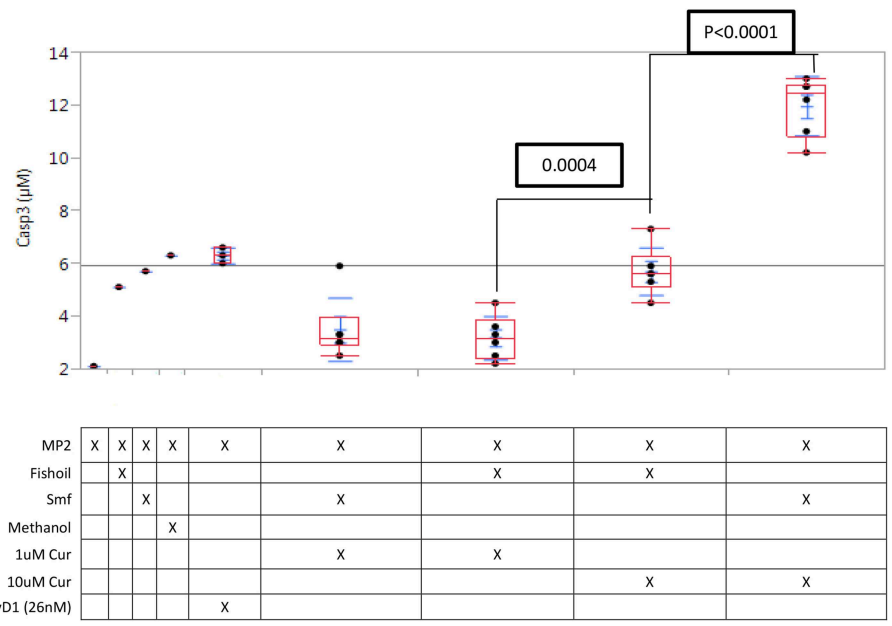


FIGURE 1 | Caspase-3 induction in MP2 cells is potentiated by curcuminoids with ω-3 and anti-oxidants. Replicate MP2 cell cultures ($N = 3$) in 24-well plates were treated 24 h with curcuminoids in Smartfish emulsion or fish oil, the cells were harvested and caspase-3 was assayed in the whole lysate. The experiment was repeated twice with comparable results. The maximal induction of caspase-3 reached by 10 μM curcuminoids in Smartfish was greater than any other treatment ($P < 0.0001$).

compared. Here we have examined whether the combination of curcuminoids with ω-3 fatty acids and anti-oxidants will increase cytotoxicity directly and/or synergistically with NK cells.

Materials and Methods

Cell Culture

Pancreatic Cancer

Pancreatic cancer cells called Mia Paca2 (MP2) and L3.6 PDAC cells were propagated in DMEM with 10% fetal calf serum and antibiotics.

Human Subjects

The human investigations were performed after approval by the UCLA institutional review board #11-000005 and in accordance with an assurance filed with and approved by the U.S. Department of Health and Human Services. Thirty healthy donors and a patient with disseminated prostate cancer were recruited into the study after signing an informed Consent.

NK cells

NK cells were isolated from the blood of 30 healthy subjects by negative selection using the NK cell isolation kit (Stem

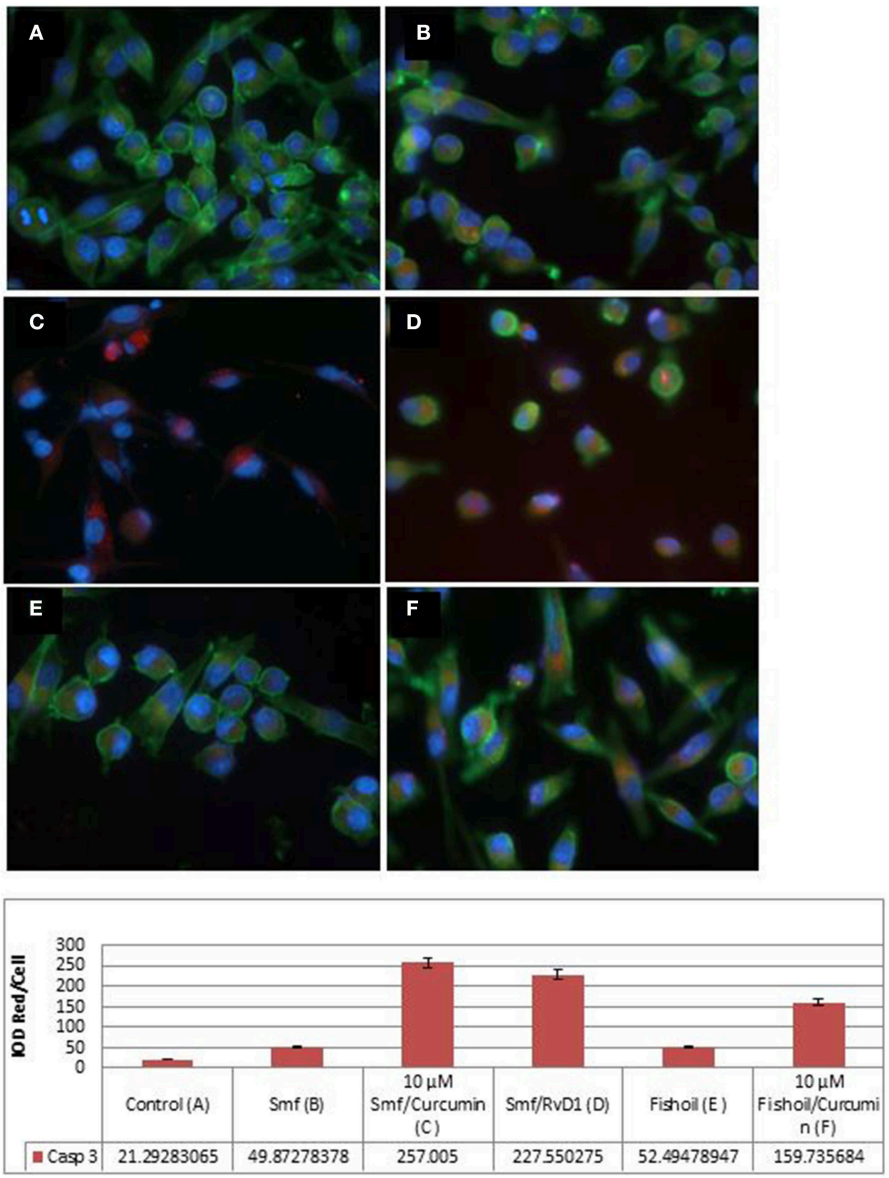
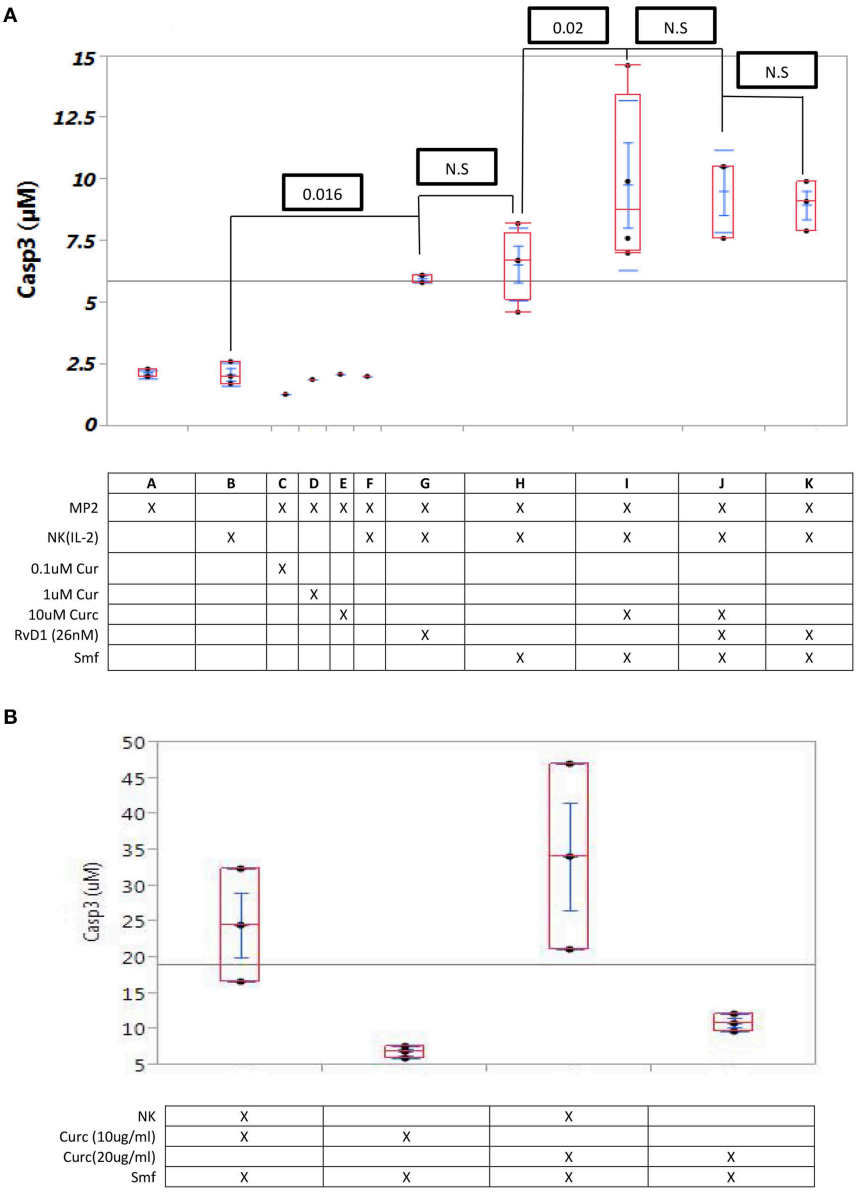


FIGURE 2 | Active caspase-3 expression in MP2 cancer cells is potentiated by curcuminoids and ω-3 or Resolvin D1. The MP2 cells were stained by phalloidin (green) and by rabbit antibody to active caspase-3/anti-rabbit ALEXA568 (red). **(A)** Untreated MP2 cells; **(B)** MP2 cells treated by ω-3 with anti-oxidants (Smartfish); **(C)** MP2 cells treated by curcuminoids with ω-3 and anti-oxidants (Smartfish); **(D)** MP2 cells treated by RvD1 and ω-3 with anti-oxidants(Smartfish); **(E)** MP2 cells treated by fish-oil; **(F)** MP-2 cells treated by curcuminoids in fish oil. Note the highest expression of caspase-3 in **(C,D)**.

cell technologies, Vancouver, Canada). NK cells were activated by treatment with IL-2 [termed NK(IL-2)] or IL-2 and CD16 antibody [termed NK(IL2CD16)] (BioLegend, San Diego, CA) as described (Tseng et al., 2010). NK (IL-2) cells are activated for cytotoxicity. NK (IL-2CD16) have decreased cytotoxicity but increased cytokine production ("split anergy").

Lipidic Emulsions

Smartfish^R (Smartfish Company, Oslo, Norway), a lipidic emulsion with DHA (1 gm/200 ml) and EPA (1 gm/200 ml) (from local cod and salmon) protected against oxidation by botanical anti-oxidants (polyphenols, pectin and whey proteins, tocopherols and rosemary extract, pomegranate, chookberry) and extremely low in environmental toxins; Smartfish/CUR^R containing a supplement of curcuminoids (760 mg /200 ml); control fish-oil emulsion (from imported sardines and anchovies not protected against oxidation). For use in cell culture, each emulsion was diluted 1:2 with fetal calf serum, sonicated for 30 s and then diluted 1:100 in DMEM with 10% fetal calf serum. For nutritional supplementation, the prostate cancer patient was taking one carton of the drink Smartfish/CUR^R daily.



	A	B	C	D	E	F	G	H	I	J	K
MP2	X		X	X	X	X	X	X	X	X	X
NK(IL-2)		X				X	X	X	X	X	X
0.1uM Cur			X								
1uM Cur				X							
10uM Curc					X				X	X	
RvD1 (26nM)							X			X	X
Smf								X	X	X	X

NK	X		X	
Curc (10ug/ml)	X	X		
Curc(20ug/ml)			X	X
Smf	X	X	X	X

FIGURE 3 | Curcuminoids or RvD1 potentiate caspase-3 induction in MP2 cells by NK cells. (A) Caspase -3 induction in MP2 cells: Quadruplicate MP2 cell cultures in 24-well plates were co cultured 24 h with NK cells (5 NK cells/1 cancer cell) with ω-3 emulsion and anti-oxidants and with or without curcuminoids or RvD1. (A–F) No caspase-3 induction by NK cells alone or curcuminoids alone; (G,H) low induction by NK cells with ω-3 or RvD1 (26 nM) ($P = 0.016$); (I–K) maximum induction by NK cells with ω-3or RvD1 ($P = 0.02$). **(B)** Comparison of caspase-3 induction by curcuminoids and smartfish with or without NK cells.

Nutritional Substances

Curcuminoids (prepared from Curcuma longa L root by extraction with acetone as granulated powder by Naturex, Avignone, France) were prepared by sonication as an emulsion in Smartfish^{R} by the Smartfish company. Resolvin D1 (RvD1) (Cayman Chemical company, Ann Arbor, MI) was emulsified in Smartfish^{R}.

Direct Cancer Cell Cytocidal Assay by Curcuminoids and ω-3

MP2 cells (10,000 cells) were plated in each well of a 24-well plate and were grown at 37 C 24 h before testing. Triplicate MP2 cell cultures were treated for 24 or 48 h with curcuminoids, RvD1, or other additives, harvested into the lysis buffer and tested by the enzymatic caspase-3 assay.

Combined Cancer Cell Cytocidal Assay by NK Cells together with Curcuminoids and ω-3

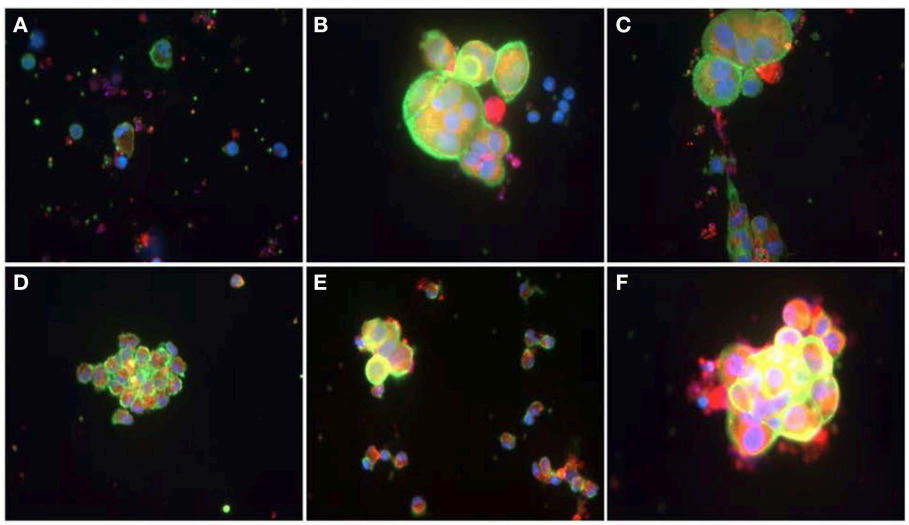
MP2 cell cultures were prepared as above. NK cells were added to MP2 cells at a ratio of 5 NK cell per 1 MP2 cancer cell. The co cultures were treated with various additives for 24 or 48 h and processed as in the direct assay.

Enzymatic Caspase-3 Assay

Enzymatic caspase-3 assay was performed by the CaspACE-3 assay G-7351 (Promega Corporation, Madison, WI) according to the manufacturer's instructions.

Caspase-3 Immunofluorescent Assay

MP2 cells were grown to partial confluence in 8-well chamber slides (Corning) in DMEM with 10% fetal calf serum and were treated 24 h as stated. Then they were fixed by 4% paraformaldehyde, permeabilized using 0.25 Triton, stained using the indirect technique with rabbit anti-caspase-3 (active) (Genetex, Irvine, CA) at 1:200 dilutions followed by ALEXA-fluor-donkey anti-rabbit 568 (InVitrogen, Carlsbad, CA) at 1:200 dilutions and FITC-phalloidin (Sigma, St. Louis, M0) at 1:500 dilution, mounted using Prolong Gold antifade with DAPI (In Vitrogen, Carlsbad, CA). The slides were examined using Olympus B-max fluorescence microscope at 20× and 40×



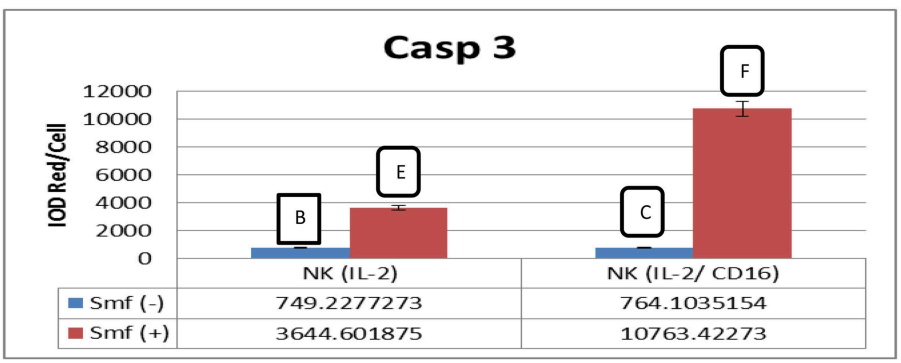


FIGURE 4 | Induction of active caspase-3 in L3.6 cancer cells by NK cells and potentiation by ω-3 and anti-oxidants. (A) NK(IL-2) cells alone; **(B)** NK(IL-2) cells and L3.6 cells; **(C)** NK (IL-2/CD16) cells and L3.6 cells, **(D)** NK cells with ω-3 and anti-oxidants, **(E)** NK(IL-2) cells and L3.6 cells with ω-3 and anti-oxidants; **(F)** NK(IL-16) and L3.6 cells with ω-3 and anti-oxidants. Phalloidin (green) and caspase-3 (red). NK cells are small green cells; cancer cells are large green cells. Caspase-3 is yellow or red. Red pixels were scanned by Image-Pro.

magnification with the Hamamatsu camera, and fluorescence was determined in four fields using Image Pro software and expressed as Integrated Optical Density (IOD) per macrophage.

Interferon-γ Assay

Supernatants were tested by interferon-γ ELISA assay (BioLegend, San Diego, CA).

Statistical Analysis

Means were compared using analysis of variance methods. Means and their approximate 95% confidence bounds (mean ± 2 standard errors of the mean) are reported. Computations were carried out using JMP software (SAS Inc, Cary, NC).

Results

Curcuminoids or RvD1 Have Increased Cytocidal Activity against Pancreatic Cancer Cells When Emulsified in ω-3 with Anti-oxidants

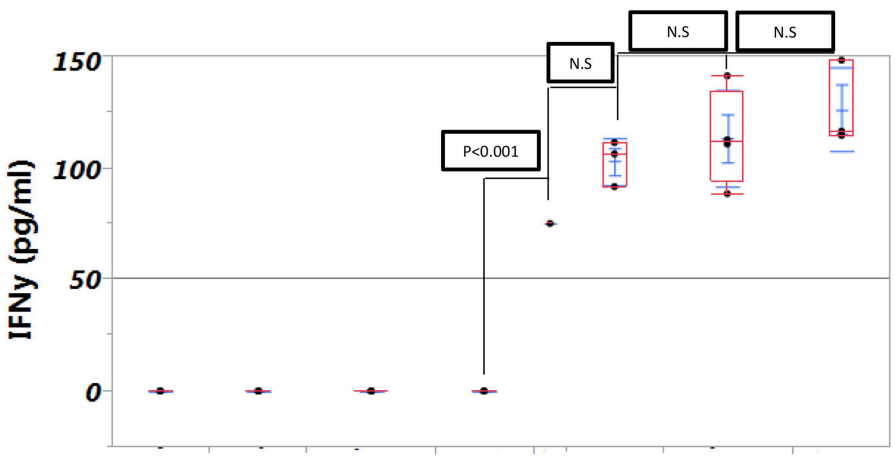
We tested apoptosis induction using the enzyme assay for the executioner caspase-3 expression. In MP2 cells alone, caspase-3 expression was low (1.2 μM) and was not significantly increased by curcuminoids (1 μM) alone or with ω-3 and anti-oxidants, or by fish oil; methanol is used as a control However, curcuminoids (10 μM) emulsified in ω-3 and anti-oxidants induced robust capase-3 expression, which was significantly higher ($P < 0.0001$) than that induced by curcuminoids (10 μM) in fish oil **(Figure 1)**.

We examined apoptosis by immunofluorescence microscopy of caspase-3 antibody stained cells (**Figure 2**). As shown in integrated optical density (IOD) of red per cell, caspase-3 expression was not observed in untreated MP2 cells (IOD Red/Cell = 21.29), but was seen at a low level in MP2 cells treated with ω-3 and anti-oxidants (IOD Red/Cell = 49.87), and at a high level in MP-2 cells treated with curcuminoids or RvD1 emulsified in ω-3 with anti-oxidants (IOD Red/Cell = 257.01 and 227.55, respectively). The cancer cells treated by ω-3 and anti-oxidants became rounded, detached and some displayed pyknotic nuclei confirming their apoptosis. The emulsification of curcuminoids in fish oil was clearly inferior in the production of apoptosis (IOD Red/Cell = 159.74). Comparable results were obtained in three experiments.

Curcuminoids with NK Cells Are More Cytocidal in Cancer Cells than Curcuminoids or NK Cells Alone

In MP2 cells alone, MP2cells with NK (IL-2) cells, and MP2 cells with curcuminoids (0.1–10 μM), caspase-3 expression was low (means 2.0–3.1 μM) (cf. A through F in **Figure 3A**). Caspase-3 expression was higher in the co culture of MP2 cells with NK (IL-2) cells in ω-3 and anti-oxidants or with RvD1 (means 6.0–6.6 μM) (cf. G and H in **Figure 3A**) and in a co culture of MP2 cells with NK (IL-2) cells and curcuminoids. However, the cytocidal activity of NK(IL-2) cells was significantly increased by curcuminoids ($P = 0.02$) in ω-3 and anti-oxidants (mean = 9.8 μM), in ω-3, anti-oxidants, and RvD1 (mean = 9.0 μM) or in ω-3 and RvD1 (mean = 9.5 μM) (cf. I through K in **Figure 3A**). Comparable results were obtained in four experiments with NK cells, curcuminoids, ω-3 and anti-oxidants.

A comparative study of cytotoxicity by curcuminoids alone (in ω-3 and anti-oxidants) vs. curcuminoids (in



MP2	X		X	X	X	X	X	X
NK (IL-2)		X	X	X	X	X	X	X
Smf/Cur (10uM)			X	X				
RvD1 (26nM)				X		X		X
Smf						X	X	

FIGURE 5 | Interferon-γ production by NK cells is not changed by ω-3 or RvD1 but is blocked by curcuminoids. Triplicate MP2 cell cultures in 24-well plates were cultured with NK cells with or without Smartfish emulsion, curcumin, or RvD. Note blockade of IFN-γ by curcumin.

ω-3 and anti-oxidants) and NK cells demonstrated that curcuminoid-enhanced NK cell cytotoxicity was greater than direct curcuminoid cytotoxicity (**Figure 3B**). The experiment was repeated three times with comparable results.

ω-3 Fatty Acids with Anti-oxidants Protect NK Cells and increase Apoptosis of Cancer Cells

We examined caspase-3 induction by immunofluorescence microscopy. Small NK cells are distinguished by immunofluorescence microscopy from large L3.6 cancer cells. Both cells are stained green in the cytoplasm and blue in the nuclei. In this experiment **(Figure 4)**, NK cells were incubated overnight in DMEM with ω-3 and anti-oxidants (Smartfish) either alone or with cancer cells. NK cells alone remained intact when incubated in DMEM with ω-3 and anti-oxidants in comparison to NK cells incubated in DMEM (cf. A–D and B–E in **Figure 4**). In a co culture of NK(IL-2) or NK(IL-2CD16) cells with L3.6 pancreatic cancer, cancer cells showed increased caspase-3 expression. Active caspase-3 was shown by increased red or yellow color in the cytoplasm of cancer cells stained with caspase-3 antibody (cf. B–E and C–F in **Figure 4**) when incubated in the presence of ω-3 and

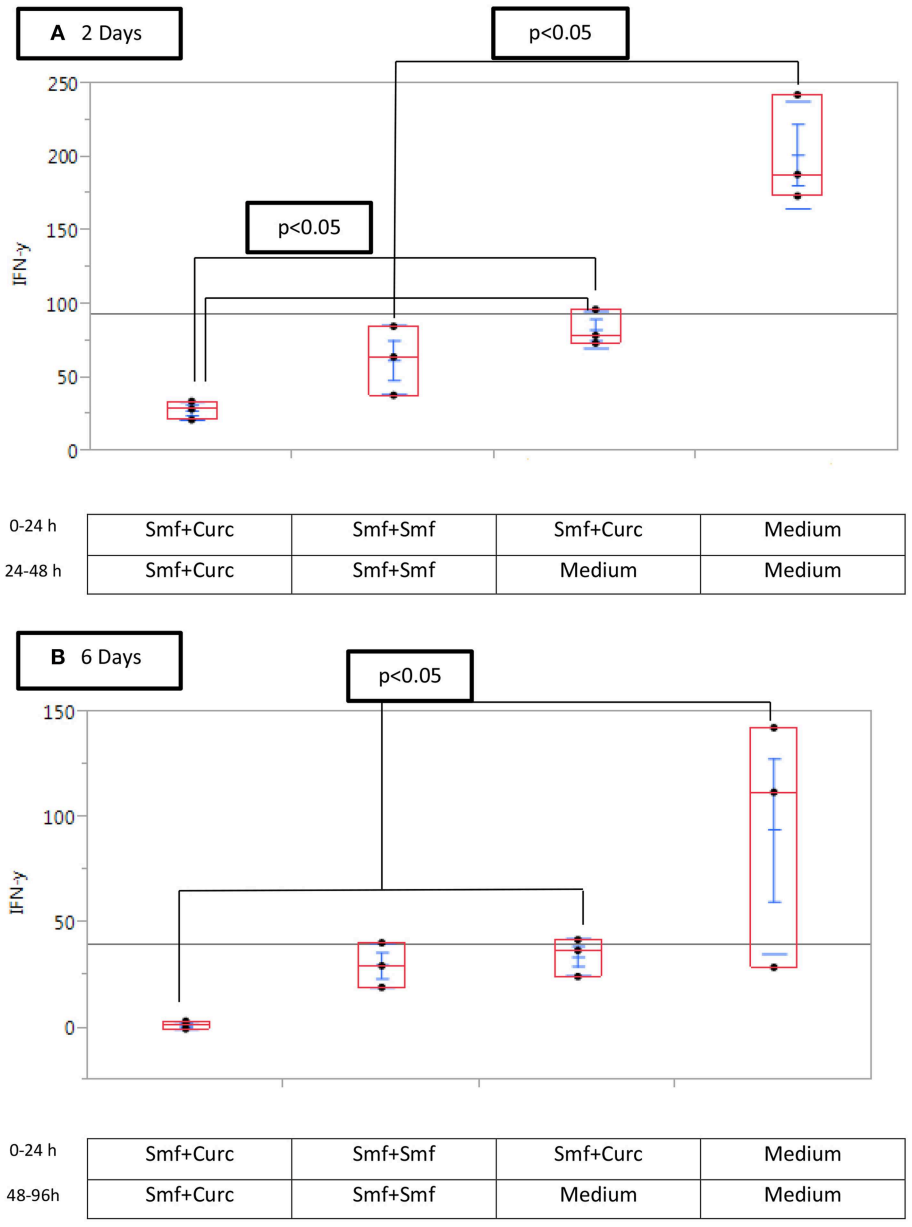


FIGURE 6 | Curcuminoid blockade of interferon-γ production is reduced by removal of curcumin (A) after 2 days, (B) after 6 days. Triplicate MP2 cell cultures in 24-well plates were cultured with or without Smartfish emulsion and curcuminoids or with DMEM, washed and replaced with the indicated medium and NK cells for 24 or 48 h. Note that the inhibition of IFN-γ by curcuminoids was reduced by washing MP2 cells.

anti-oxidants in comparison to medium as shown in integrated optical density (IOD) of red per cell. In case of NK(IL-2), IOD increased from 749.23 to 3644.60 in the presence of ω-3 and anti-oxidants, and in case of NK(IL-2/CD16), IOD increased from 764.10 to 10763.42 in the presence of ω-3 and anti-oxidants (**Figure 4**).

Curcuminoids Inhibit the Production of Interferon-γ by NK Cells during Co-incubation with MP2 Cancer Cells

No IFN-γ was produced by NK (IL-2) cells alone. High level of IFN-γ was produced by NK(IL-2) cells in a co culture with cancer cells with or without ω-3 and anti-oxidants or RvD1. Curcuminoids (10 μM) with or without ω-3 and anti-oxidants or RvD1 completely blocked production of IFN-γ **(Figure 5)**.

We tested the effect on IFN-γ production of incubation of the cells in a curcuminoid-free medium after addition of NK cells. The curcuminoid-free interval after addition of NK cells resulted in the following mean IFN-γ levels: (a) co culture of NK and cancer cells without curcuminoids for 48 h: 201 pg/ml; (b) co culture without curcuminoids for 24 h: 90 pg/ml; (c) co culture with constant presence of curcuminoids for 48 h: 27 pg/ml (**Figure 6A)**. When the experiment was increased to 96 h, the reduction in inhibition was still observed (**Figure 6B**).

Discussion

We evaluated the induction of the apoptosis executioner caspase-3 by curcuminoids and ω-3 or the lipid mediator RvD1 in PDAC cells as (a) direct effects, and (b) combined effects with NK cells. The analysis of direct effects showed that curcuminoids alone were poorly effective but had strong cytotoxic effects in dose-related fashion when administered in an emulsion of ω-3 with anti-oxidants in comparison to an emulsion in fish oil. The cytotoxic activity of NK cells was enhanced by ω-3 and anti-oxidants and was maximally enhanced by curcuminoids and ω-3 fatty acids with anti-oxidants. Thus, NK cells either with curcuminoids, ω-3 fatty acids and anti-oxidants or RvD1 (at 1000 lower concentration) induced maximal caspase-3. We also evaluated cytotoxicity by immunofluorescence microscopy in MP2 and L3.6 cancer cells. The results confirmed the increase of cidal effects from the culture in ω-3 and anti-oxidants to the culture in ω-3, anti-oxidants and curcuminoids (**Figure 2**) and from the culture with NK(IL-2) cells to NK(IL-2)cells, ω-3, anti-oxidants (**Figure 4**). Caspase-3 was activated in both cancer cells and NK cells, but the activation was much increased by ω-3 and anti-oxidants or RvD1 (**Figure 3A**). In addition, NK cells were protected against degradation after overnight incubation in DMEM with ω-3 and anti-oxidants in comparison to DMEM (**Figure 4**). Although previous studies showed potentiation of cytotoxic effects of NK cells against cancer cells by curcuminoids (Zhang et al., 2007), in our study these effects were further enhanced by the synergy between curcuminoids and ω-3 or RvD1. A previous study demonstrated an increase by curcumin of the caspase-8, which is upstream of caspase-3 in the execution-phase of apoptosis (Kim et al., 2005).

Curcuminoids inhibited the production of IFN-γ by NK cells in our study of pancreatic cancer cells and in a study of human melanoma cells (Bill et al., 2009). The latter group prepared a curcumin analog FLLL32 which does not block IFN-γ production by NK cells (Bill et al., 2010). The effect of curcuminoids on IFN-γ was reduced when curcuminoid treatment was reduced in time after the addition of NK cells (**Figure 6**).

In conclusion, our cell culture study showed dissociation between enhancing effects of curcuminoids on apoptosis and inhibiting effects on IFN-γ production by NK cells. In application to cancer therapy, it will be important to determine whether the inhibitory effect of curcuminoids on IFN-γ production could be modified by intermittent therapy. In the future work, we plan to study the cytocidal effects of curcuminoids, ω-3, and anti-oxidants *in vivo* in an animal model and ultimately in human patients. We are preparing such study in a model of pancreatic cancer with humanized immune system.

Acknowledgments

We thank Dr. Jeff Gornbein for advice regarding statistical analysis of data and Rachel Rubattino for assistance with manuscript preparation. This study was supported by Smartfish Inc., Oslo, Norway.

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