INTERPLAY OF INFECTION AND MICROBIOME

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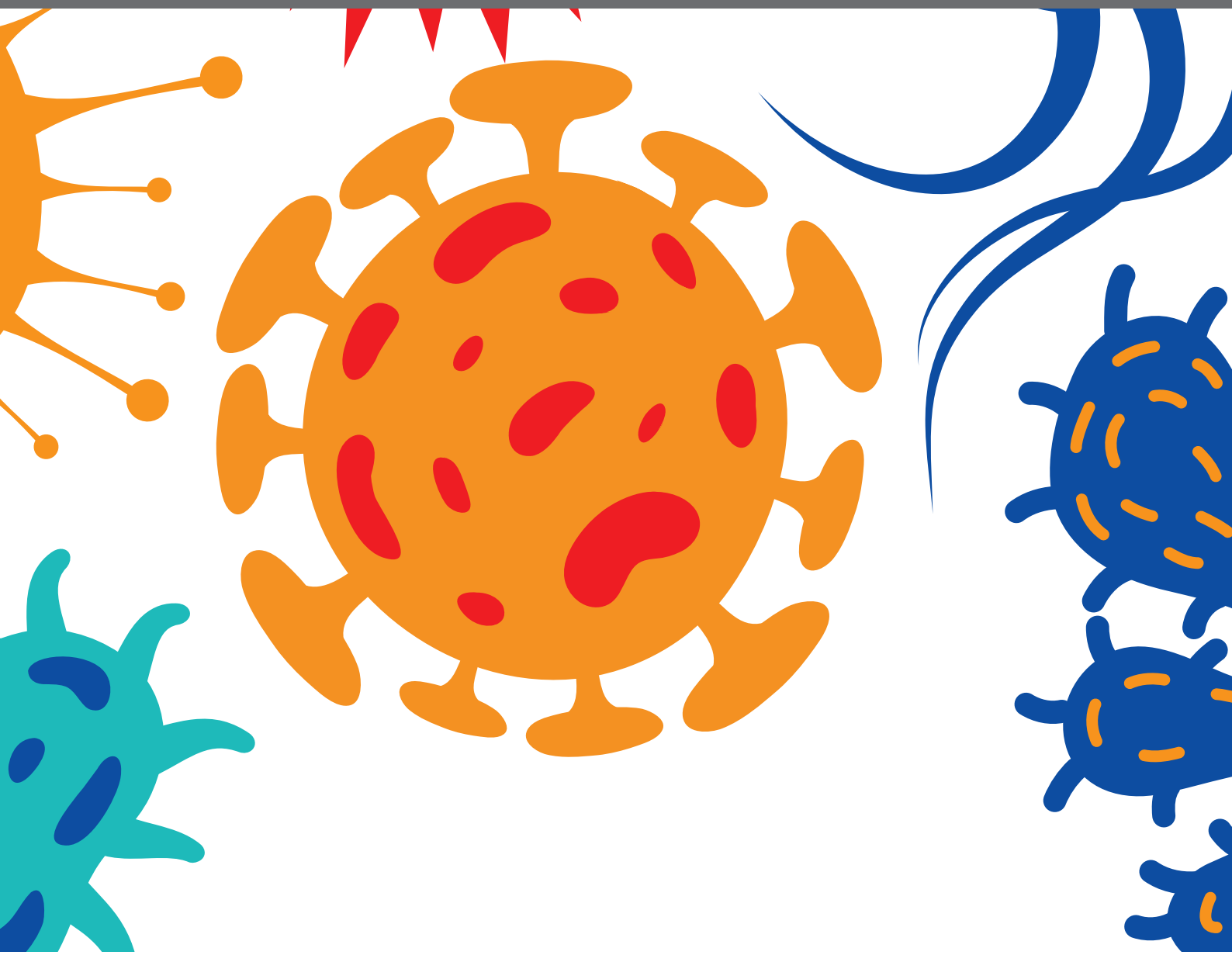

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INTERPLAY OF INFECTION AND MICROBIOME

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Table of Contents

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EDITORIAL
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Editorial: Interplay of Infection and Microbiome

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Editorial on the Research Topic

Interplay of Infection and Microbiome

The composition and function of the animal body is now widely acknowledged to be critically linked with the numerous microscopic organisms that have a habitat within the animal host. There are numerous lines of evidence that these tiny ecosystems can be critical for the local function of each tissue site, or can act as a pathogen defense system, or in some cases can be associated with disease. There is gathering evidence that the hosts diet, genotype, and lifestyle can impact on these microbial ecosystems throughout the body. The composition of the microbiota, or microbiome of the host, from humans to a myriad of animal hosts, has been the focus of recent research.

More and more it is becoming apparent that interactions between pathogenic infections and the local microbial community at the infection site is an important factor in the outcomes of diseases across humans and various animals.

The topics in this Research Topic issue span across the human and animal sphere and explore the infection and microbiota interplay through three major themes. The first theme focusses on the interactions between microbiome, metabolites and host responses; the second theme addresses microbiome compositions as predictive indicators or associated factors in cancers; and the final theme addresses the functional molecular and genomic knowledge that is now emerging on key microbiome players.

IS IT THE MICROBIOME COMPOSITION OR THE RESULTING METABOLOME THAT IS MOST INFLUENTIAL?

A priority to microbiome researchers and those considering probiotic prophylaxis is the concept of a pre-disposing immune environment due to the status of the local microbiome and how that may then interplay into the infection.

The role of L-tryptophan as a keystone metabolite that is critical for host nutritional and immune regulation, but also as a target for the gut microbiome manipulation to orchestrate the host immune status is comprehensively reviewed (Gao et al.). The review explores the molecular processes and metabolic compounds involved, identifies the knowns and unknowns for the major metabolites in the host and microbial pathways, outlines lifestyle and environmental factors that may influence these pathways, and outlines the diseases where this host and microbiome interplay around a keystone metabolite may be important.

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In the article by Delgado-Diaz et al. the influence of metabolic constituents associated with optimal and non-optimal vaginal microbiome compositions on cervicovaginal epithelial cellular responses was explored. The authors examined the inflammatory response profile of epithelial cells exposed to the metabolic profile associated with a bacterial vaginosis microbiome (non-optimal) compared to that of a lactobacilli dominated (optimal) vaginal microbiome. They found that short chain fatty acids, at levels observed in women with bacterial vaginosis, were associated with dysregulation of the immune response to inflammatory stimuli that may explain, in part, the pro-inflammatory cytokine and chemokine profile observed in women experiencing bacterial vaginosis.

Further to this insight into the disease burden of bacterial vaginosis, an approach to define a standardized ("universal") donor screening for a vaginal microbiome transplant has been outlined in the issue by DeLong et al.. Microbiome transplants, whilst limited success has been reported to date, are viewed as a likely more successful methodology to create a longer term change in a microbial ecosystem than administration of a probiotic, and particular in the case of this polymicrobial condition.

Finally, in this theme, the current knowledge of how the host gut microbiota interplays into the immune responses to nematodes and protozoa in mice and mosquito vectors is reviewed (Yordanova et al.), presenting a fascinating summary of the structural and physiological environments the parasites have to adapt in distinct life-cycle phases.

COULD THE MICROBIOME BE AN INDICATOR OR PREDICTIVE BIOMARKER FOR CANCER?

The role of microbiome signatures as a potential indicator of oral and oropharyngeal cancers was explored in a case-control study of 83 participants conducted by Lim et al.. In this study, which requires further validation, the findings indicated that profiling the oral rinse microbiome had 100% sensitivity and 90% specificity to predict these cancers.

Differences in the bacterial diversity profiles and also higher representations of certain bacterial genera were identified when microbial profiles of mid-stream urine samples of males with bladder cancer was compared to controls. This study (Wu et al.) of 60 men in total has provided preliminary evidence that there may be a microbiome profile consistent with the presence of bladder cancer.

A study presented in the issue comparing the profile of the mucosal microbiome from the gastrointestinal surfaces of patients with gastric antrum or duodenal ulcers identified that there was a higher proportion of Helicobacter in those with the gastric ulcers (Chen et al.). There were also a number of species that were over-represented in the specimens from the duodenal ulcers than the gastric sites, information which may have relevance in future management of this condition as the composition of the local microbiome could interplay into the pathology (in addition to the causation by *Helicobacter*).

INSIGHTS INTO KEY MICROBIOME PLAYERS

Intestinal helminth infections have been associated with alterations in the gut microbiome; however, whether this directly relates to helminths or is an indirect consequence of the immunological and metabolic response to the infection has not been certain. Given helminths have to compete with the gut microbiome to establish the infection, Midha et al.. have examined the antibacterial properties of the intestinal roundworm *Ascaris suum*, and identified excretory and secretory factors with broad spectrum antimicrobial factors. These findings demonstrate a specific functional impact on the microbiome as part of the pathogenic process for at least this helminth.

Campylobacter concisus, an organism known to colonize the oral cavity of humans, but has been identified as over-represented or dominant in a number of conditions (gastroenteritis, Barrett's Esophagus, and gingivitis or periodontal diseases), although as this comprehensive review points out many of the studies were underpowered or did not include appropriate controls meaning association with these conditions cannot yet be concluded. However, of particular interest is irritable bowel disease, where over-representation of *C. concisus* in the oral cavity has been reported in more than one study. The review also presents genomic analysis of the species, pathogenic mechanism, pathology and inflammatory associations, and anti-microbial resistance properties. The review (Liu et al.) also shares insights into other potentially important members of the genus.

The capacity of Lactobacilli strains (and their secretions) isolated from fecal samples from healthy children to prevent or disperse biofilms formed by *Vibrio cholera* and *Vibrio paraheamolyticus* was analyzed Kaur et al. to investigate their preventative or therapeutic potential against this diarrheal disease. The authors found that the low pH elicited by the strains was the main mechanism of antimicrobial activity against the Vibrio. However, in a strain specific manner, some culture supernatants where able to inhibit biofilm formation or disperse biofilms, even when the pH was neutralized.

CONCLUSIONS

The evidence is strong that the microbiome, metabolic profiles, and interplay with the host are determining factors in infection risk or potentially pathology associated with infection. However, the precision medicine applications that could emerge, and the therapeutic interventions remain in the distant future until greater understanding of the physiological and molecular features at play can be more rigorously and thoroughly profiled. This issue presents some up to date and promising data and reviews on this topic that point to the microbiome being a critical focus for on-going research into infection.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Impact of the Gut Microbiota on Intestinal Immunity Mediated by Tryptophan Metabolism

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The gut microbiota influences the health of the host, especially with regard to gut immune homeostasis and the intestinal immune response. In addition to serving as a nutrient enhancer, L-tryptophan (Trp) plays crucial roles in the balance between intestinal immune tolerance and gut microbiota maintenance. Recent discoveries have underscored that changes in the microbiota modulate the host immune system by modulating Trp metabolism. Moreover, Trp, endogenous Trp metabolites (kynurenines, serotonin, and melatonin), and bacterial Trp metabolites (indole, indolic acid, skatole, and tryptamine) have profound effects on gut microbial composition, microbial metabolism, the host's immune system, the host-microbiome interface, and host immune system–intestinal microbiota interactions. The aryl hydrocarbon receptor (AhR) mediates the regulation of intestinal immunity by Trp metabolites (as ligands of AhR), which is beneficial for immune homeostasis. Among Trp metabolites, AhR ligands consist of endogenous metabolites, including kynurenine, kynurenic acid, xanthurenic acid, and cinnabarinic acid, and bacterial metabolites, including indole, indole propionic acid, indole acetic acid, skatole, and tryptamine. Additional factors, such as aging, stress, probiotics, and diseases (spondyloarthritis, irritable bowel syndrome, inflammatory bowel disease, colorectal cancer), which are associated with variability in Trp metabolism, can influence Trp–microbiome–immune system interactions in the gut and also play roles in regulating gut immunity. This review clarifies how the gut microbiota regulates Trp metabolism and identifies the underlying molecular mechanisms of these interactions. Increased mechanistic insight into how the microbiota modulates the intestinal immune system through Trp metabolism may allow for the identification of innovative microbiota-based diagnostics, as well as appropriate nutritional supplementation of Trp to prevent or alleviate intestinal inflammation. Moreover, this review provides new insight regarding the influence of the gut microbiota on Trp metabolism. Additional comprehensive analyses of targeted Trp metabolites (including endogenous and bacterial metabolites) are essential for experimental preciseness, as the influence of the gut microbiota cannot be neglected, and may explain contradictory results in the literature.

Keywords: gut microbiota, Trp metabolism, intestinal immunity, intestinal inflammation, aryl hydrocarbon receptor

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INTRODUCTION

The gut microbiota and microbial metabolites are important for maintaining healthy bowels. Although the high complexity of the gut microbial composition and metabolites has presented significant research challenges, direct and indirect evidence of correlations between the gut microbiota, microbial metabolites, and intestinal immune function have been demonstrated, largely through the application of modern molecular biology techniques.

The gut microbiota can influence the scope and quality of the immune system response; in turn, the immune system participates in regulating the localization and composition of the gut microbiota. Recent studies have emphasized the profound effects of diet and nutrients on the localization and composition of the gut microbiota as well as on the connection between the gut microbiota and immunological pathways (Thorburn et al., 2014). As an essential nutrient in mammals, L-tryptophan (Trp), and its endogenous metabolites are involved in gut immune homeostasis and in several immune diseases.

Manipulating the gut microbial composition can modulate plasma concentrations of Trp and Trp metabolites (Clarke et al., 2014). In this review, we discuss studies that increase our understanding of how Trp metabolism interacts with the gut microbiota, how Trp functions in host–microbiota interactions and how Trp influences gut immune homeostasis. We provide a brief outline of studies that support an influence of dietary Trp on intestinal inflammation and other peripheral inflammation, outline the connection between Trp metabolism and the gut microbiota, and discuss which Trp metabolites intersect with the immune system. Understanding these interactions may provide novel targets for the treatment of various intestinal disorders that are associated with the microbiota and Trp metabolism.

GUT MICROBIOTA AND HOST IMMUNITY

The microbiome, consisting of microbes and their collective genomes, modulates the host metabolic phenotype and influences the host immune system (Gordon, 2012). Interactions between the gut microbiota and the host immune system begin at birth: the microbiota influences the development of the immune system; and the immune in turn system shapes the composition of the gut microbiota (Nicholson and Wilson, 2003). Later in life, the gut microbiota also influences immune cell recruitment and initiates inflammation. Crosstalk between the gut microbiota and enterocytes shapes the gut environment and profoundly influences intestinal immune homeostasis (Hold, 2016), which lasts a lifetime. Alterations in the gut microbiota, coupled to increased gut permeability (leaky gut), are widely recognized as relevant to the pathogenesis of several diseases, including autoimmune and neurodegenerative disorders (Anderson et al., 2016).

On the one hand, the host immune system is affected by the intestinal microbiome. The connection between microbes and the host immune system is mediated by a series of molecules (Anders et al., 2013) and signaling processes, which can impact the gut, liver, brain, and other organs. Complex host–microbe metabolic axes offer a lasting influence on metabolic reactions, the host immune system, and long-term health outcomes (Blumberg and Powrie, 2012; Cerf-Bensussan and Eberl, 2012; Hooper et al., 2012).

On the other hand, the intestinal immune system plays a crucial role in exposing bacteria to host tissues, alleviating the potential for pathologic outcomes and determining the stratification of intestinal bacteria on the luminal side of the epithelial barrier (Blumberg and Powrie, 2012). Moreover, the immune system controls the composition of the gut microbiota, and at the same time, resident microbes provide signals that foster normal immune system development and regulate ensuing immune responses. Disruption of these dynamic interactions may have far-reaching effects on host health (Hamard et al., 2007).

ENDOGENOUS AND BACTERIAL TRP METABOLISM

Endogenous Trp Metabolism

Trp absorption in the intestine is primarily mediated by B^0AT1 (SLC6A19). In addition to serving as a substrate for protein synthesis, Trp is primarily metabolized through two metabolic pathways: the kynurenine pathway (KP) and the serotonin pathway. Approximately 95% of the Trp ingested is degraded to kynurenine, kynurenic acid (KA), quinolinic acid, picolinic acid, and nicotinamide adenine dinucleotide (NAD) through KP, which is regulated by two rate-limited enzymes: tryptophan 2,3-dioxygenase (TDO) in the liver and indoleamine 2,3-dioxygenase (IDO) in extrahepatic tissues (Peters, 1991). Specifically, Trp is degraded to kynurenine, which is then largely metabolized to 3-hydroxykynurenine by kynurenine hydroxylase and marginally metabolized to anthranilic acid (AA) by kynureninase and KA by kynurenine aminotransferase. Furthermore, 3-hydroxykynurenine is mainly degraded to 3-hydroxyanthranilic acid by kynureninase and marginally degraded to xanthurenic acid (XA) by kynurenine aminotransferase. Through multi-stage enzymatic reactions, 3-hydroxyanthranilic acid is converted to quinolinic acid, pyridine carboxylic acids (such as picolinic acid, acetyl CoA), nicotinic acid, NAD^+, and other active molecules (Badawy, 2015).

Approximately 1–2% of ingested Trp is converted to serotonin (5-HT) and melatonin via the serotonin pathway. 5-HT is synthesized from Trp through two-stage enzymatic reactions involving Trp hydroxylase (TPH) and aromatic amino acid decarboxylase. In animals, serotonin is primarily found in the gastrointestinal tract (GI tract), blood platelets, and the central nervous system. Approximately 90% of total serotonin in humans is located in enterochromaffin cells in the GI tract, where it can promote intestinal peristalsis (Bai et al., 2017). Melatonin (N-acetyl-5-methoxytryptamine) is derived from 5-HT via two-step enzymatic conversion reactions (acetylation and methylation) mainly in the pineal gland but also in other tissues such as the retina, GI tract, skin, and leukocytes (Radogna et al., 2010). Although endogenous Trp metabolites play important roles in regulating gut immune homeostasis in mammals, the potential

contribution to intestinal immune function by Trp metabolites from resident microbiota should not be ignored.

Bacterial Trp Metabolism

The gut microbiota can directly utilize Trp, which partially limits Trp availability for the host. Approximately 4–6% of Trp is metabolized to indole, indican, tryptamine, and skatole as well as indole acid derivatives by the gut microbiota (**Figure 1**) (Yokoyama and Carlson, 1979). Intestinal microorganisms convert Trp to tryptamine (**Figure 1A**) and indole pyruvic acid and indole pyruvic acid to indole (**Figure 1C**), indole acetaldehyde (**Figure 1B**), and indole lactate (**Figure 1D**). Indole acetaldehyde can be converted to indole acetic acid and tryptophol, and the former can then be converted to skatole (**Figure 1B**). Indole lactate may be converted to indole acrylic acid and subsequently to indole propionic acid (**Figure 1D**) (Smith and Macfarlane, 1997). Although the conversion of these bacterial Trp metabolites are easily defined at the molecular level, it is in practice complicated to determine which type of metabolites are produced. Because different microbes possess different catalytic enzymes, mutual cooperation among more than two bacteria is needed to generate one metabolite from Trp. Unlike the relatively simple background of animal endogenous Trp metabolism, the intestinal environment is relatively complex with regard to bacterial Trp metabolism. Many strains that possess catalytic enzymes for Trp metabolism remain unknown, and research on the coordination of different species of bacteria in generating Trp metabolites is needed. Indeed, before designing strategies to manipulate Trp bacterial metabolism, differences in the gut microbiota and the intestinal environment among individuals should be evaluated.

Interplay between Endogenous and Bacterial Trp Metabolism

The microbiota can directly and indirectly modulate host endogenous Trp metabolism, and variations in Trp metabolism can negatively influence microbial proliferation and microbiota diversity. Reports have shown that the bacterial community can influence Trp metabolism and the serotonergic system. The balance between bacterial Trp metabolism and Trp synthesis determines local GI and circulating Trp availability for the host.

Circulating total Trp levels are increased in germ-free (GF) mice that lack gut microbiota (Wikoff et al., 2009; Clarke et al., 2012, 2013; El Aidy et al., 2012; Mardinoglu et al., 2015); KP metabolism and circulating 5-HT concentrations are also decreased (Wikoff et al., 2009; Clarke et al., 2013). This may be attributable to gut microbial metabolites such as short-chain fatty acids or Trp-derived indole metabolites, which may promote colonic 5-HT production to modulate circulating Trp levels (Reigstad et al., 2015; Yano et al., 2015).

In addition to being a rate-limited enzyme in KP, IDO1 also plays an essential role in maintaining microbial diversity (Le Floc'h et al., 2011). Host Trp depletion resulting from

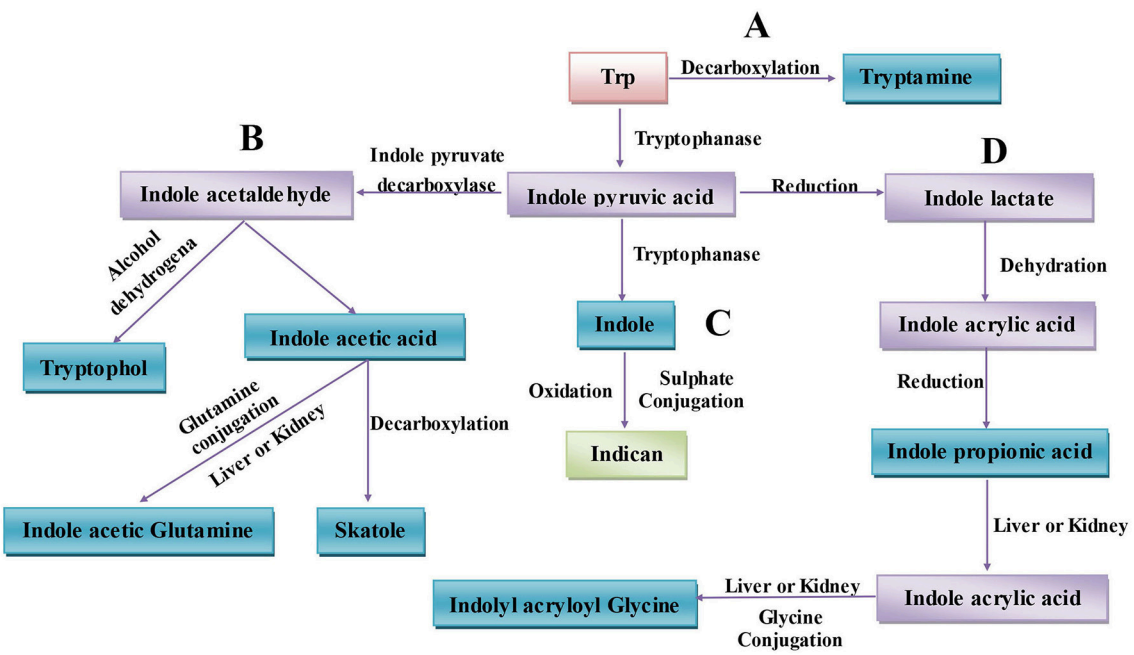

FIGURE 1 | Microbiota-associated tryptophan metabolism in the gut. **(A)** Trp is decarboxylated to tryptamine by the common gut Firmicutes *Clostridium sporogenes* and *Ruminococcus gnavus*. **(B)** Derivatization of indole pyruvic acid from Trp is catalyzed by tryptophanase, and then indole pyruvic acid is decarboxylated to indole acetaldehyde, which is the precursor of tryptophol and indole acetic acid. Indole acetic acid can be converted to skatole by *Lactobacillus*, *Clostridium*, *Bacteroides,* and others. **(C)** Indole pyruvic acid can be catalyzed to indole by tryptophanase; after absorption, indole is oxidized to indoxyl, conjugated with sulfate and excreted as urinary indican. **(D)** Indole pyruvic acid can also be converted to indole lactate, to indole acrylic acid, and to indole propionic acid by intestinal microorganisms. Indole propionic acid can be further converted to indole acrylic acid in the liver or kidney and combined with glycine to produce indolyl acryloyl glycine.

IDO1 activation can reduce microbial proliferation, and IDO1-induced depletion of Trp caused by host immune activation may lead to microbial amino acid deprivation and immune tolerance. For example, increased production of bacterial Trp metabolites was detected in IDO1-knockout mice (Zelante et al., 2013). In particular, dietary Trp insufficiency alters gut microbial composition and impairs intestinal immunity in mice (Hashimoto et al., 2012). Host Trp modulation in the microenvironment is presumed to involve arrest of microbial proliferation, providing a significant benefit for the host (Le Floc'h et al., 2011). Altering either the gut microbial composition during the host's lifespan or the trajectory of microbial colonization of the GI tract early in life can modulate Trp metabolism.

HOST IMMUNE-MICROBIOME INTERACTIONS ASSOCIATED WITH TRP METABOLISM

Trp

Trp has proven to exert anti-inflammatory effects in mammals, and Trp and its regulatory pathway act as important regulators of inflammatory responses (Marsland, 2016). Mice fed a low-Trp diet are more susceptible to chemically induced inflammation (Hashimoto et al., 2012). Conversely, mice or piglets fed a sufficient-Trp diet had reduced inflammation and decreased severity of dextran sodium sulfate (DSS)-induced colitis (Kim et al., 2010; Zelante et al., 2013; Etienne-Mesmin et al., 2017). Moreover, mice fed a Trp-depleted diet had more severe central nervous system inflammation compared with mice fed a Trp-rich diet, and this manifestation was ameliorated after feeding a diet supplemented with Trp. These effects of dietary Trp on mammalian immunity can be attributed to the production of Trp metabolites.

Aryl Hydrocarbon Receptor (AhR) Signaling

AhR, a cytosolic ligand-activated transcription factor that mediates xenobiotic metabolism, is a critical regulator of immunity and inflammation, involving fine-tuning of adaptive immunity and mucosal barrier function, maintenance of intestinal homeostasis, and carcinogenesis (Hubbard et al., 2015b; Korecka et al., 2016). The function of AhR signaling in the GI tract has been reported. In DSS-inducible intestinal injury models, AhR-null mice exhibit severe symptoms and mortality (Arsenescu et al., 2011; Benson and Shepherd, 2011), and in another study of intestinal disease models, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced AhR activation decreased lethality and symptom severity (Takamura et al., 2010). $Ahr^{-/-}$ mice are more susceptible to intestinal challenge, indicating the critical role of AhR in maintaining gut immune and barrier functions (Sutter et al., 2011). Similarly, in the absence of AhR, mice show high disease susceptibility when infected by *Listeria monocytogenes* and *Citrobacter rodentium* (Shi et al., 2007; Qiu et al., 2012). The promotional effect of AhR on immune homeostasis is usually ascribed to two mechanisms. First, an antimicrobial role for AhR due to AhR-dependent IL-22 transcription [AhR mediates activation of innate lymphoid cell 3 (ILC3) to produce IL-22 in the gut] has been reported (Lee et al., 2012; Qiu et al., 2012); in the gut, IL-22 can regulate the release of antimicrobial peptides and affect the homeostatic balance between immunity and the microbiota by regulating microbial composition (Zelante et al., 2013; Zenewicz et al., 2013; Behnsen et al., 2014). Second, there is evidence for an anti-inflammatory role for AhR mediated by its effects on regulating the development of intraepithelial lymphocytes and innate lymphoid cells (Zelante et al., 2014; Hubbard et al., 2015b). These cells play important roles in defending against infiltrating pathogenic microbes and facilitating gut homeostasis (Hubbard et al., 2015b).

As ligands of AhR, several microbial metabolites are vital to host immunity, especially in protecting the mucosa from inflammation (Rooks and Garrett, 2016). Excessive degradation of AhR ligands induces harmful effects on intestinal immunity, and these effects can be counterbalanced by increased supplementation of dietary AhR ligands (Schiering et al., 2017). Through exposure to AhR ligands, AhR can directly target and activate certain genes. During inflammation, targeted genes, including interleukin-6 (*IL-6*), interleukin-22 (*IL-22*), prostaglandin G/H synthase 2 (*PTGS2*), vascular endothelial growth factor A (*VEGFA*), and cytochrome P450 1A1 (*CYP1A1*), can be regulated by AhR activation (**Figure 2**). Moreover, consumption of AhR ligands can reverse AhR-mediated regulation of intestinal homeostasis (Hubbard et al., 2015b).

A Trp-rich diet can increase AhR mRNA expression and activate AhR and subsequently increase colonic *IL-22* mRNA expression. During acute colitis, Trp supplementation protects the epithelial layer and prevents the intestinal inflammation mediated by AhR signaling (Hashimoto et al., 2012). In a DSS-inducible intestinal injury murine model, dietary Trp alleviated colitis symptoms and severity through the activation of AhR (Islam et al., 2017). However, the effects of dietary Trp are mediated by its metabolites, which act as AhR ligands, and not by Trp itself (Opitz et al., 2011). Some endogenous and bacterial Trp metabolites have been proven to act as AhR ligands, and their binding activates AhR to regulate intestinal immunity (Zelante et al., 2013; Cheng et al., 2015). Endogenous Trp metabolites such as kynurenine, KA, XA, and cinnabarinic acid (CA) can function as direct AhR ligands, with the capacity to stimulate AhR-dependent gene expression (Romani et al., 2014) (**Figure 2**). After activation, AhR mediates transcription of IL-22 in human and murine $CD4^+$ T-cells (Lowe et al., 2014). Several bacterial Trp metabolites, including indole, indole propionic acid, indole acetic acid, skatole, and tryptamine, have also been proven to be AhR ligands (Bittinger et al., 2003; Chung and Gadupudi, 2011) (**Figure 2**).

AhR and IDO1 play crucial roles in connecting microbial Trp catabolism and host endogenous Trp metabolites with regulatory T-cell function, especially in AhR-dependent T-cell immune homeostasis at the mucosa. When induced by proinflammatory cytokines, IDO1 is activated, and kynurenines are produced. Acting as AhR ligands, kynurenines regulate immune homeostasis and induce the generation of regulatory

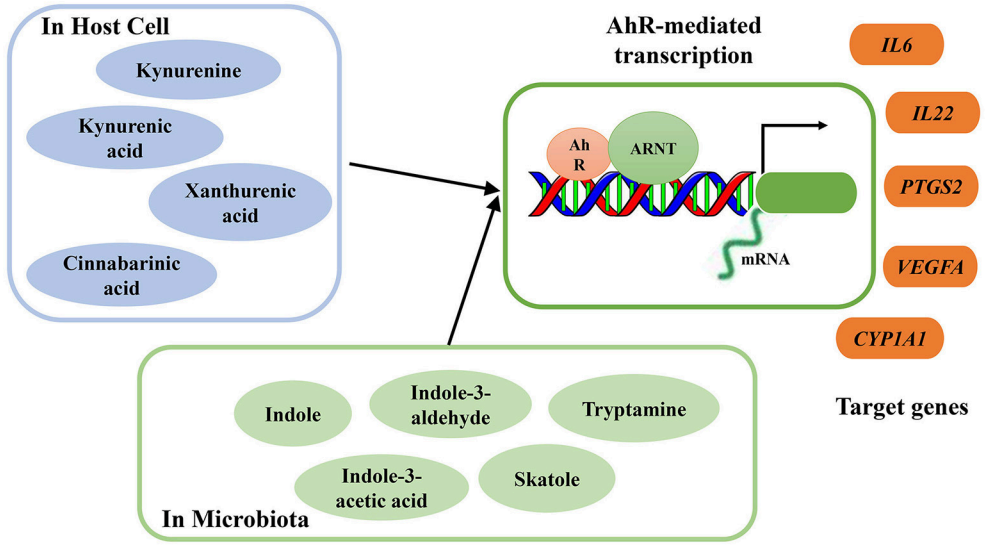

FIGURE 2 | Summary of endogenous and bacterial tryptophan metabolites as AhR ligands. AhR nuclear translocator protein (ARNT), AhR activation and transcription of target genes interleukin-6 (*IL-6*), interleukin-22 (*IL-22*), prostaglandin G/H synthase 2 (*PTGS2*), vascular endothelial growth factor A (*VEGFA*), cytochrome P450 1A1 (*CYP1A1*).

T-cells, which protect mice from hyper-inflammatory responses (Bessede et al., 2014). The coevolutionary commensalism between host and microbes may be relevant to the AhR ligands catabolized from Trp (Hubbard et al., 2015b). The absence of IDO1 induces upregulation of commensal-driven AhR/IL-22 activity, but AhR stimulation may in turn affect IDO1 (Bessede et al., 2014). The positive feed-forward loop between IDO1 and AhR is necessary for driving commensal fungi to coevolve with the mammalian immune system and microbiota, which is beneficial for host survival and fungal commensalism under strong inflammatory conditions and prevents dysregulated immunity (Romani et al., 2014).

The generation of AhR ligands and AhR activation is influenced by several factors such as diet, gut microbial composition, and endogenous enzymatic activity (Hubbard et al., 2015b). Through AhR, microbial Trp metabolites may provide important cues to the host for resisting colonization and defense against mucosal inflammation. *Lactobacilli* spp can metabolize Trp to produce AhR ligands, such as indole-3-aldehyde (IAld), which can active innate lymphoid cells (ILCs) (Zelante et al., 2013), after which mucosal resistance against the potential pathogen *Candida albicans* is increased. At the same time, ILC-induced IL-22 production can regulate release of antimicrobial peptides in the gut epithelium and enhance AMP expression, which reduces pathogen infectivity via sequestration of metal ions (Zelante et al., 2014; Rooks and Garrett, 2016).

Previous studies have highlighted the bidirectional interaction between AhR and the microbiome, and the microbiome-AhR axis can influence host metabolism (Korecka et al., 2016). Microbial metabolites such as short-chain fatty acids (SCFAs) and several microbial Trp metabolites can activate AhR and AhR target genes in the intestine or liver. In turn, AhR signaling can influence microbial composition in the small intestine. In addition, AhR acts an important regulator of host-microbiota communication, which can influence host metabolism and modulate the immune system.

Host Trp Metabolism

IDO1

As the rate-limiting enzyme of KP, IDO1 also plays an important role in regulating the adaptive immunity of vertebrate hosts (Zelante et al., 2013). When attempting to counterbalance tissue damage, high expression of IDO1 by intestinal mononuclear cells can mediate anti-inflammatory and immunosuppressive effects of IDO1 on the intestinal mucosa (Wolf et al., 2004) by regulating host immunomodulatory activity via kynurenine production, mucosal amino acid nutrition, mucosal immune reactivity, and gut microbial community metabolism (Dai and Zhu, 2010). Moreover, through the influence of IDO1 on T-cells, KP may also serve as the basis of the sensitive balance between pro-inflammatory (excitotoxic quinolinic acid) and anti-inflammatory (neuroprotective KA) states in the GI tract (Kaszaki et al., 2008). This balance can affect intestinal motor or sensory function through a profound influence on the excitability of enteric neurons. In turn, inflammatory mediators tightly regulate KP (Campbell et al., 2014). Specifically, inflammatory cytokines and interferon (IFN)-γ induce expression of IDO1 in the GI tract and other tissues. In addition, the severity of the inflammation induced is linked to the translocation of β-catenin from the cell membrane to the cytoplasm/nucleus (Cooper et al., 2000). Impairment of IDO1 activity, which is detected in inflamed or neoplastic intestinal epithelial cells, can reduce nuclear β-catenin and cell proliferation (Thaker et al., 2013).

In addition, IDO1 is an important activating enzyme in host-microbiota symbiotic relationships via regulation of Trp

metabolism (Niño-Castro et al., 2014; Romani et al., 2014). The gut microbiota may influence host Trp degradation and circulating Trp concentrations through KP (O'mahony et al., 2015). In GF animals, KP metabolism (kynurenine:Trp ratio) is decreased due to the microbiota deficiency (Clarke et al., 2013), and colonization by a normal microbiota in GF animals increases KP metabolism (kynurenine:Trp ratio) and reduces plasma Trp. In microbiota-deficient animals, KP cannot be detected in the central nervous system (CNS), but mice infected with Toxoplasma gondii have increased levels of kynurenine, KA, 3-hydroxykynurenine and quinolinic acid in brain tissue (Notarangelo et al., 2014). In contrast, colonization by *Bifidobacteria infantis* in rodents increases Trp levels, circulating KA and the KA:kynurenine ratio and decreases the kynurenine:Trp ratio, suggesting reduced activity of IDO and Trp metabolism through KP, with no effect on kynurenine concentrations (Desbonnet et al., 2008). This increase in KA and decrease in IDO activity appear to conflicting, and other factors need to be taken into account when considering the influence of *B. infantis* colonization on host Trp metabolism. Colonization by *Lactobacillus johnsonii* in rats also decreases ileum IDO mRNA levels and serum kynurenine concentrations, consistent with the effect of *L. johnsonii* culture cell-free supernatant in reducing IDO1 activity in HT-29 intestinal epithelial cells (47% reduction) (Freewan et al., 2013; Valladares et al., 2013). As an explanation, *L. johnsonii* feeding was proven to alter the distribution of ileum and colon IDO1 in rats, and increased ileum lumen H_2O_2 produced by *L. johnsonii* was found to be a strong inhibitor of IDO1 activity. The signaling molecule H_2O_2 possibly mediated host-microbiota symbiotic interactions. Moreover, the impact of altered IDO1 activity on the degradation of 5-HT is also important, as lower IDO activity leads to both decreased kynurenine and increased 5-HT concentrations.

There is also a correlation between IDO and inducible nitric oxide synthase (iNOS). On the one hand, the NO produced by iNOS inhibits IDO activity by direct interaction or by stimulating IDO degradation. On the other hand, 3-hydroxyanthranilic acid, a kynurenine metabolite, inhibits the expression and catalytic activity of iNOS. Other kynurenine metabolites, quinolinic, and picolinic acids, can also enhance IFN-γ-dependent iNOS expression (Xu et al., 2017). iNOS is involved in the immune response after gut microbiota exposure and helps to limit inflammation. During the inflammatory response, leukocyte recruitment, and adhesion are regulated by iNOS. iNOS-derived NO is maintained at high levels, which is considered a host-protective effect. Similarly, commensal bacterial exposure promotes iNOS expression, which further enhances IgA (a major class of immunoglobulin) secretion by intestinal B cells, which is beneficial for promoting the intestinal barrier function. Conversely, iNOS acts as a microbicidal mediator, reducing microbial growth, and indirect antimicrobial effects are suggested to be caused by local arginine depletion after induction of iNOS or NO-dependent induction of IFN-γ (Bogdan, 2001). In another report, increased iNOS stimulated by quinolinic and picolinic acids together with 3-hydroxykynurenine and 3-hydroxyanthranilic acids enhanced lipid peroxidation and activated an arachidonic acid cascade, resulting in the production of inflammatory factors such as prostaglandins and leukotrienes (Oxenkrug, 2010). Overall, the iNOS pathway acts as a mediator between the gut microbiota and host immune system, and it is also related to Trp metabolism. Nonetheless it remains unclear how the iNOS pathway and Trp metabolism simultaneously participate in this mutual interaction, and further elucidation is required.

The microbiota influences host IDO and Trp metabolism through KP, though there are conflicting results for the same or different experiments. For example, IDO and KP metabolism decrease due to the deficiency in the microbiota in GF animals, whereas GF animals colonized with a normal microbiota or *T. gondii* have increased IDO and KP metabolism. However, colonization of probiotics (*B. infantis* and *L. johnsonii*) in conventional rodents reduced IDO activity and KP metabolism. The reason for these differences may be variation in the original gut microbial background in the transplanted animals, with one being GF and others conventional. Another reason may be the different types of colonized microbes. In relation to both experimentation and therapy via IDO activity regulation, these factors should be further investigated.

Kynurenines

Kynurenines possess antimicrobial activities, which can directly impact proliferation of the gut microbiota (Niño-Castro et al., 2014). The influence of the gut microbiota on host Trp metabolism in KP is associated with the immune system. GF animals that lack a microbiota have an immature immune system, which is associated with reduced Trp metabolism in KP (Clarke et al., 2013). After intestinal microbiota colonization in GF animals, immune system function is reinstated, and aberrant KP metabolism is normalized (Clarke et al., 2013). GI Toll-like receptors (TLRs), which recognize microbial components in the GI tract, act as crucial junctions (Kawai and Akira, 2010; Wang et al., 2010). In the GF state, TLR expression is reduced, which is associated with increased KP metabolism that may be mediated by IFN-γ-dependent or -independent IDO1 induction (Clarke et al., 2012).

In KP, kynurenine, KA, CA, and XA act as direct ligands of AhR, stimulating AhR and AhR-dependent gene expression in a concentration-dependent manner, and simultaneously modulate intestinal homeostasis. Additionally, AhR itself plays a role in regulating levels of IDO1 and TDO1 expression (Bessede et al., 2014). The absence of AhR causes an increase in endogenous KA levels in mice (García-Lara et al., 2015). AhR may be an important mediator in the complex crosstalk between the gut microbiota, KP and the immune response.

In addition, transmembrane G protein-coupled receptors (GPCRs) sense metabolic intermediates to activate signaling pathways and play roles in regulating GI homeostasis and intestinal immunity. GPCRs, including GPR43, GPR109A, and GPR120, exert anti-inflammatory effects (Tilg and Moschen, 2015). For instance, GPR43 deficiency induces severe inflammatory reactions in the mouse intestine (Maslowski et al., 2009). GPR35 is predominantly expressed in immune cells and in the GI tract, suggesting it may play an important role in immunological regulation. Trp metabolites, such as

serotonin, melatonin, KA and niacin, are known GPCR ligands, and KA acts as a ligand for GPR35. Based on elevated KA levels, anti-inflammatory effects of KA during inflammation, and increased expression of GPR35 in immune cells, several studies have suggested that KA may have important functions in immunological regulation via GPR35 activation and subsequent signaling (Wang et al., 2006). Several bacteria can also catabolize Trp through KP (Genestet et al., 2014). Most strains of *P. aeruginosa* isolated from cystic fibrosis patients can produce a high level of kynurenine, which can promote bacterial survival and allow bacteria to circumvent the innate immune response by scavenging neutrophil reactive oxygen species (ROS) production (Genestet et al., 2014). Additionally, in *P. aeruginosa* KP, kynurenine acts as the main precursor of the *Pseudomonas* quinolone signal, which is another virulence factor of these bacteria (Genestet et al., 2014).

In summary, the interface for the gut microbiota, KP and immune response is tightly controlled and complex. Gut microbial composition plays an important role in regulating KP to subsequently influence host immunity, and variations in the composition of the gut microbiota can influence an individual's immunity and health through adjusted Trp metabolism in KP.

Serotonin

The serotonin pathway is one of the core signaling pathways in the gut (Gershon and Tack, 2007; Lesurtel et al., 2008). Serotonin plays a role in regulating the permeability of the intestine and mucosal inflammation. In the murine intestine, serotonin is associated with inflammation during chemically induced colitis. Suppressing the production of mucosal serotonin is beneficial for relieving inflammation (Margolis et al., 2013), and studies have found that GI-selective TPH (Trp hydroxylase) inhibitors may act as a cure for several GI diseases caused by serotonin pathway dysregulation (Shi et al., 2008). Changes in serotonin concentrations induced by the gut microbiota can regulate the host immune response and subsequently influence the coping strategy by which the host defends against pathogens or disease. Microbial SCFA metabolites can active GPCRs on intestinal epithelial cells and thus have a major role in regulating epithelial barrier integrity and intestinal immunity. At the same time, SCFAs promote *TPH1* transcription and colonic serotonin production from enterochromaffin cells and stimulate colonic transit, steps that are essential for serotonin homeostasis (Reigstad et al., 2015). Plasma serotonin levels were found to be decreased by 2.8-fold and levels of Trp increased in GF male Swiss Webster mice compared with conventional mice (Wikoff et al., 2009). At the same time, GF mice have decreased gut motility compared to normal animals, and decreased serotonin levels are one possible reason for this defect (Ridaura and Belkaid, 2015). In another experiment involving GF Swiss Webster mice, the concentration of hippocampal serotonin was significantly increased in GF and colonized GF animals compared with conventional mice (Clarke et al., 2013). The possible factors responsible for differences in serotonin levels in GF mice is worth further study. When discussing the effect of microbial colonization on host 5-HT levels, it is also important to consider the influence of altered IDO1 activity and KP metabolism induced by colonization, as lower IDO activity may result in decreased kynurenine and increased 5-HT concentrations.

Colonization of GF animals with the gut microbiota from humans or other mice can significantly increase gut motility, and this can be partially blocked by a pharmacologic antagonist of serotonin receptors (Kashyap et al., 2013). Indeed, serotonin can promote immunity and inflammation in various models of mucosal infections. Combining its effects on intestinal physiology and the gut microbiota, serotonin has been suggested to directly and indirectly influence GI motility and the immune system, which in turn shapes the composition and localization of the microbiota.

Melatonin

Melatonin acts as a powerful anti-inflammatory molecule, and the role of melatonin in the gut, especially its role in gut permeability, has recently been explored (Anderson and Maes, 2015). Melatonin release in the gut is 400-fold higher than that in the pineal gland (Bubenik et al., 1977), with a peak after food intake. Melatonin has positive impacts on gut disorders, including inflammatory bowel disease (IBD) (Eliasson, 2014) and GI cancer (Glenister et al., 2013).

The impact of melatonin on the gut microbiota has been examined. Specific gut bacteria determine the availability of Trp to the host and then regulate serotonin and subsequent melatonin synthesis (Wikoff et al., 2009). Moreover, melatonin can alleviate the increase in gut permeability and immune activation induced by *Escherichia coli* (Sun et al., 2013).

Inflammasomes, such as NOD-like receptor 3 (NLRP3) and pyrin domain-containing 6 (NLRP6), are known to be important effectors of gut permeability and interactions with gut bacteria. These inflammasomes are important for maintaining gut homeostasis (Zambetti and Mortellaro, 2014), including regulating gut permeability, and are crucial for IL-1b and IL-18 release. Activation of the alpha 7 nicotinic acetylcholine receptor (α7nAChr), an inflammasome activator in lipopolysaccharide (LPS) models (Kim et al., 2014), or melatonin (Galley et al., 2014) can reduce the incidence of sepsis by decreasing NLRP3 activation. Melatonin is a significant positive regulator of α7nAChr (Markus et al., 2010), suggesting that several regulatory effects of melatonin may be mediated by α7nAChr induction in the gut. For example, the protective effects of melatonin against induced gut permeability is mediated, at least in part, by α7nAChr (Sommansson et al., 2013). Several potential mediators that connect melatonin, gut bacteria, and gut immunity have been discovered, but the specific mechanisms of these connections remain to be determined.

Bacterial Trp Metabolites

Bacterial Trp metabolites, such as indole and indolic acid derivatives, are potent bioactive metabolites that affect intestinal barrier integrity and immune cells in mice by activating the pregnane X receptor (PXR) or aryl hydrocarbon receptor (AhR) (Zelante et al., 2013; Venkatesh et al., 2014; Lamas et al., 2016). The predominant Trp microbial metabolites in the intestine are indole, indole propionic acid, indole acetic acid, skatole, and tryptamine (**Figure 1**) (Yokoyama and Carlson, 1979).

The gut microbiota can directly influence the type and level of Trp microbiota-derived metabolites, which can target host AhR, and subsequently modulate the mucosal immune response (Levy et al., 2016) or regulate mucosal integrity through PXR. Moreover, via AhR, bacterial Trp metabolites can modulate the production of IL-22, which plays a key role in intestinal homeostasis.

In addition, AhR- and PXR-regulated pathways are relevant for expression of the mucin 2 (*Muc2*) gene in the intestine (Zelante et al., 2013; Venkatesh et al., 2014). PXR-deficient mice exhibit a leaky gut and reduced expression of *Muc2* in the small intestine (Venkatesh et al., 2014). Commensal bacteria can utilize mucins as an energy source, which is important for establishing a mucosal-associated niche for potential health-associated commensals (Wlodarska et al., 2017). The following sections will introduce the mechanisms underlying regulation by the main bacterial Trp metabolites of intestinal homeostasis and immune responses.

Host genes can also directly or indirectly modulate the production of microbial Trp metabolites and affect the composition and function of the gut microbiota. Lamas et al. found that the microbiota in caspase recruitment domain 9 (CARD9)-deficient mice lack Trp-catabolizing capacity and fail to metabolize Trp into metabolites that can act as AhR ligands (Lamas et al., 2016). Consistent with this, *Card9*$^{-/-}$ mice have decreased levels of bacteria with Trp-catabolizing functions, such as *Lactobacillus reuteri* (Zelante et al., 2013), and are more susceptible to intestinal inflammation. However, intestinal inflammation and the lack of Trp-catabolizing capacity in *Card9*$^{-/-}$ mice can be reversed by supplementation of *Lactobacillus* strains that are capable of metabolizing Trp (Lamas et al., 2016). Researchers have hypothesized that in *Card9*$^{-/-}$ mice, the altered immune response has an effect on the composition of the microbiota. In turn, the altered microbiota influence the production of Trp microbiota-derived metabolites, affecting the host's intestinal homeostasis and leading to the loss of intestinal homeostasis and intestinal inflammation.

Indole

Indole is a major bacterial Trp metabolite. The generation of indole is catabolized by tryptophanase, which can be induced by Trp or repressed by glucose in most bacteria (**Figure 1C**). Bacterial species including *E. coli*, *Proteus vulgaris*, *Paracolobactrum coliforme*, *Achromobacter liquefaciens,* and *Bacteroides* spp are capable of producing indole (Keszthelyi et al., 2009). Recently, indole has been recognized as a signaling molecule that can regulate bacterial motility, biofilm formation, antibiotic resistance, persister cell formation, and virulence (Li and Young, 2013) and that plays a role in affecting host cell invasion by other non-indole-producing species, such as *Salmonella enterica* and *P. aeruginosa* and even the yeast *C. albicans* (Li and Young, 2013). In the porcine gut with a low-non-starch polysaccharide diet, the maximum concentration of indole (∼0.12 mM) was found in the distal part of the cecum, where the majority of gut bacteria had settled, whereas the amount of indole in the hind intestine was lower in animals fed a high-non-starch polysaccharide diet (Knarreborg et al., 2002). An explanation is that easily fermented carbohydrates such as non-starch polysaccharides, but not protein, are preferentially fermented by the intestinal microbiota, decreasing the production of indole from Trp degradation. Indole can also be detected in human feces, between 0.25 and 1.1 mM (consistent with the levels that are readily produced by *E. coli* when cultured in a rich medium), which suggests that intestinal epithelial cells are exposed to indole when the fermented substrate is available for Trp degradation in gut (Bansal et al., 2010).

As a specific bacterial signal, indole is abundant in the healthy mammalian gut and positively influences intestinal health. Indole has also been recognized as a beneficial signal in intestinal epithelial cells that can ameliorate intestinal inflammation in mammals (Bansal et al., 2010). Moreover, compared to other bacterial Trp metabolites, indole is the most effective molecule (Davis, 2014). Indole administration can attenuate damage of the GI tract induced by non-steroidal anti-inflammatory drugs (NSAIDs), modulating inflammation mediated by innate immune responses and alterations in the gut microbiota composition (Whitfield-Cargile et al., 2016). At millimolar concentrations, indole can weaken the invasion and colonization capabilities of enteric bacteria by reducing expression of *Salmonella* Pathogenicity Island-1 (SPI-1) genes, which facilitate bacterial invasion into host cells. After exposure to indole, the expression level of genes associated with strengthening the mucosal barrier and mucin production is increased, which is usually positively correlated with improvement in the resistance of human enterocyte HCT-8 cells. Indole exposure can also reduce TNF-α-mediated activation of NF-κB, expression of the proinflammatory chemokine IL-8, and the adherence of pathogenic *E. coli* to HCT-8 cells, though it does increase production of the anti-inflammatory cytokine IL-10. Variations in NF-κB activation and cellular resistance are highly specific to indole, as exposure to other indole-like molecules does not induce a similar response (Bansal et al., 2010).

Indole has been shown to promote the epithelial barrier functions of intestinal cells by fortifying epithelial tight junctions between cells through the pregnane X receptor (PXR) (Bansal et al., 2010; Shimada et al., 2013; Thaiss et al., 2016), which might contribute to resistance to inflammation. Indole can also enhance secretion of glucagon-like peptide-1 (GLP-1), an incretin with profound influences on host metabolism (Chimerel et al., 2014; Thaiss et al., 2016). As an important Trp microbiota-derived metabolite, indole can activate AhR signaling and subsequently promote local IL-22 production, which is important for intestinal homeostasis and further drives the secretion of antimicrobial peptides and protects against pathogenic infection (Levy et al., 2016). Considering these *in vitro* and *in vivo* results, it can be concluded that indole has beneficial effect on intestinal health. For normal cells, indole exposure can strengthen the mucosal barrier and mucin production by inducing expression of associated genes, thereby increasing resistance to pathogen invasion; for inflammatory cells, indole exposure can suppress activation of NF-κB and proinflammatory chemokine production and simultaneously increase anti-inflammatory cytokine production, thus ameliorating inflammation and damage.

As Trp can induce the enzyme tryptophanase, exogenous Trp levels influence the production of indole in *E. coli* in the gut (Li and Young, 2013). High-protein diets have also been reported to induce bacterial tryptophanase activity, which can result in overproduction of indole in the colon. Because indole affects bacterial physiology in a concentration-dependent manner, the level of indole that can be produced by microbiota is noteworthy. For example, 0.5 mM indole affects the motility of bacteria, the formation of biofilm, and the secretion of several virulence factors. Higher indole concentrations (1–2 mM) influence expression of multidrug exporters and several virulence factors; even higher indole concentrations (3–5 mM) can inhibit cell division and affect plasmid stability (Li and Young, 2013). Regarding the relationship among bacterial physiology, intestinal homeostasis and intestinal inflammation, we suggest that several nutritional factors, such as a high-protein or high-Trp diet, may impact intestinal homeostasis and intestinal inflammation via indole as an intermediary. This may constitute an effective approach to increasing the production of indole to modulate intestinal immunity through nutritional regulation.

Indolic Acid Derivatives

In the gut, a portion of Trp can be catabolized to indolic acid derivatives by bacteria, including indole-3-acetic acid (IAA), indole-3-aldehyde (IAld), indole acryloyl glycine (IAcrGly), indole lactic acid, and indole acrylic acid (IAcrA) (**Figure 1**) (Keszthelyi et al., 2009). Several intestinal bacteria, such as *Bacteroides*, *Clostridia,* and *E. coli*, can catabolize Trp to tryptamine and indole pyruvic acid, which are then converted to indole-3-acetic acid, indole propionic acid, and indole lactic acid (Smith and Macfarlane, 1997). Indole-3-acetic acid can be further combined with glutamine to produce indolyl acetyl glutamine in the liver or oxidized to indole-3-aldehyde (IAld) through peroxidase-catalyzed aerobic oxidation (De Mello et al., 1980). Indolyl propionic acid can also be further converted to indolyl acrylic acid (IAcrA) and combined with glycine to yield indolyl acryloyl glycine (IAcrGly) in the liver or kidney (**Figure 1**) (Keszthelyi et al., 2009).

The effects of several indolic acid derivatives on the gut microbiota and intestinal homeostasis have been reported. Several *Peptostreptococcus* species are capable of producing IAcrA, which can suppress inflammation by promoting intestinal epithelial barrier function and mitigating inflammatory responses. After LPS stimulation, IAcrA enhances both IL-10 production and mucin gene expression. Mucins can be utilized as an energy source by commensal bacteria, and IL-10 acts as an anti-inflammatory cytokine (Hasnain et al., 2013). Therefore, IAcrA is suggested to have an important anti-inflammatory function in the intestine, and stimulating IAcrA production to promote anti-inflammatory responses has therapeutic benefits (Wlodarska et al., 2017). *Clostridium sporogenes* can metabolize Trp into indolyl propionic acid, which protects mice from DSS-induced colitis (Venkatesh et al., 2014). Indolyl propionic acid significantly enhances IL-10 production (an anti-inflammatory cytokine) after LPS stimulation and reduces TNF production (a proinflammatory cytokine). Indole-3-aldehyde (IAld), which is able to activate ILC3s to produce IL-22 via AhR, is abundantly produced by *L. reuteri* in the presence of Trp in the gut. Additionally, mutation of *L. reuteri* caused the loss of its capacity to produce IAld and induce IL-22 in the presence of Trp (Romani et al., 2014). Several indolic acid derivatives are toxic to the microbiota. For instance, certain indolic compounds are known to have bacteriostatic effects on gram-negative enterobacteria, especially the genera *Salmonella* and *Shigella*, Furthermore, indolyl acetic acid has been reported to inhibit the growth and survival of *Lactobacillus*, specifically *L. paracasei* (Nowak and Libudzisz, 2006). Indole-3-aldehyde (IAld), a metabolite produced from Trp by commensal lactobacilli, can act as a ligand of AhR and subsequently activate AhR-dependent *IL-22* transcription (Zelante et al., 2013). IL-22 mediates pivotal innate antifungal resistance in mice (De Luca et al., 2010) and humans (Puel et al., 2010) and provides colonization resistance against the fungus *C. albicans* and mucosal protection from inflammation. Several authors have suggested that indolyl acryloyl glycine (IAcrGly) can increase intestinal epithelial permeability, and it has been hypothesized that increased intestinal permeability is caused by membrane damage induced by the precursor of IAcrGly: indolyl acrylic acid. Such membrane damage results in increased permeability and permeation of compounds that can disrupt normal intestinal homeostasis (Keszthelyi et al., 2009).

What can influence the production of these derivatives? Increased and prolonged excretion of urinary indolic acid derivatives has been detected in a number of diseases, such as Hartnup disorder, celiac disease and other malabsorption syndromes (Haverback et al., 1960). However, the effects of increased indolic acid derivatives on the immune system, especially on intestinal immunity, have not been evaluated in these diseases, and doing so may be useful for interpreting pathology and improving available therapies. Additionally, excessive Trp overload in the colon and concomitant gut microbiota alteration has been hypothesized to increase the production of Trp microbiota-derived metabolites, though this is merely a hypothesis. The types of metabolites that can be accelerated and whether other factors can influence production need to be validated.

3-Methylindole (Skatole)

Skatole is another intestinal Trp microbiota-derived metabolite (Jensen et al., 1995), the precursor of which is indole-3-acetic acid (IAA) (**Figure 1B**) (Yokoyama and Carlson, 1979). Intestinal microbiota convert Trp to indole and IAA, and skatole is then synthesized from IAA via decarboxylation (**Figure 1B**). However, the level of skatole produced is usually low. Compared to other Trp bacterial-derived metabolites, skatole concentrations in the intestine are highly variable, which may be due to the two-step production process mediated by at least two different bacterial species; the concentration of the intermediate IAA may be another rate-limiting factor (Yokoyama and Carlson, 1979). *Lactobacillus*, *Clostridium,* and *Bacteroides* can convert IAA to skatole (Cook et al., 2007; Whitehead et al., 2008). The actual site of skatole production in the intestine is likely the small intestine and colon, and skatole can be efficiently absorbed by both. In pigs, the concentration of skatole in the

colon is in excess of 30 μg/g (Yoshihara and Maruta, 1977). After oral supplementation with L-Trp, the concentration of skatole in bovine ruminal fluid is ∼36 μg/ml. Skatole can influence the growth and reproduction of certain intestinal bacteria and has bacteriostatic effects on gram-negative enterobacteria. The genera *Salmonella* and *Shigella* are slightly more sensitive to the bacteriostatic effects of skatole than are *Escherichia* and *Aerobacter* species. In dilute solutions, skatole can also inhibit the growth and fermentation of *Lactobacillus acidophilus*. In this regard, skatole may determine the composition of intestinal microbes and the intestinal microbial ecosystem and protect the ecological niches of the bacteria that produce it (Yokoyama and Carlson, 1979). Regardless, the influence of skatole on host diseases through effects on intestinal microbes is not well-characterized.

The production of skatole is associated with both healthy and disease states. The fecal skatole concentration in humans varies considerably and may indicate different health statuses. Fecal skatole levels in healthy individuals are usually ∼5 μg/g feces, whereas fecal skatole levels may be as high as 80 to 100 μg/g feces in persons who suffer from disturbed intestinal digestion (Yokoyama and Carlson, 1979). Researchers have suggested that after absorption but before detoxification, skatole may have a damaging effect on the activity and function of intestinal epithelial mucosa (Yokoyama and Carlson, 1979). In cattle, intraruminal and intravenous administration of skatole induces clinical features and lung lesions similar to Trp-induced disease. Skatole has been recognized as a primary cause for Trp-induced disease, which manifests as acute pulmonary edema and emphysema, generally resulting in death (Yokoyama and Carlson, 1979).

With an efficacy equivalent to that of indole, skatole exhibits modest dose-dependent activation to stimulate murine and human AhR. Within the GI tract, abundant generation of skatole may underlie the establishment of an axis to regulate intestinal physiology, which may involve microbiota-indole-AhR-mediated maintenance of intestinal homeostasis throughout a longer lifespan (Hubbard et al., 2015a). In another report, skatole has been described as an inhibitory factor for *CYP11A1*, leading to decreased formation of pregnenolone, which is the precursor of mineralocorticoids, glucocorticoids, and sex steroids (Mosa et al., 2016). In the gut, synthesis of endogenous steroid hormones, such as the anti-inflammatory glucocorticoid cortisol, is critical for the maintenance of intestinal homeostasis (Bouguen et al., 2015). Along with reduced *CYP11A1* expression (Coste et al., 2007) and decreased glucocorticoid production (Huang et al., 2014), disorders of intestinal steroidogenesis have been associated with IBD. Skatole has also been suggested to play a role in the disturbance of intestinal homeostasis and in the development of IBD via inhibition of *CYP11A1* expression and glucocorticoid production.

Nutrients are important factors that influence the concentrations of skatole in the intestine and other tissues. As indicated above, oral supplementation with Trp can induce high concentrations of skatole in bovine ruminal fluid. Sugar concentrations in the intestine are another factor. Decarboxylase is a key enzyme in the second step of skatole production, and under induction-repression regulation, sugar concentrations in the intestine have a significant impact on decarboxylase activity and subsequently influence skatole production (Yokoyama and Carlson, 1979). Antibiotic use is another important factor influencing skatole levels. The most sensitive regulator of the microbial population and conversions mediated by bacteria in the gut is the application of antibiotics (Engberg et al., 2000). For instance, pigs fed zinc bacitracin have reduced skatole concentrations in the blood and backfat compared with non-supplemented control pigs (Hansen et al., 2000). In addition, significant gender differences in both indole and skatole concentrations in the blood and backfat were observed ($P \leq 0{\cdot}001$) (Hansen et al., 2000). As skatole stimulates physiological responses in a dose-dependent manner, maintaining appropriate concentrations in the gut is beneficial for maintaining the intestinal microbial ecosystem, intestinal homeostasis and intestinal health.

Tryptamine

Tryptamine is produced by the decarboxylation of Trp, which is common in the plant kingdom but rare in bacteria (**Figure 1A**) (Williams et al., 2014). The common gut Firmicutes *C. sporogenes* and *Ruminococcus gnavus* are capable of decarboxylating Trp to tryptamine (Williams et al., 2014). Although Trp decarboxylation is rare in bacteria, the Human Microbiome Project demonstrated that at least 10% of the human population possesses at least one bacterium encoding a Trp decarboxylase among the intestinal microbiota (Williams et al., 2014). Therefore, the generation and physiological role of tryptamine in the gut is non-negligible.

Tryptamine, a β-arylamine, is a neurotransmitter with documented effects on intestinal motility that acts on the enteric nervous system to modulate intestinal homeostasis (Wlodarska et al., 2015). However, tryptamine can induce ion secretion by intestinal epithelial cells. In an experiment using an Using chamber, 3 mM tryptamine induced a marked change in short-circuit currents, indicating that it can influence colonic ion secretion, which subsequently regulates GI motility. It is hypothesized that tryptamine-mediated signaling might affect the transit of food particles and bacteria through the gut lumen (Williams et al., 2014). Tryptamine can also reduce the invasion and colonization capabilities of enteric pathogens, such as *Salmonella enterica* serovar Typhimurium (Davis, 2014). In addition, tryptamine exerts inhibitory activity against IDO1, which then influences immune surveillance. Upregulation of IDO1 is reported to be associated with the escape of malignant cells from immune surveillance (Muller and Scherle, 2006; Katz et al., 2008), and inhibition of IDO1 activity is regarded as an important target in interventions related to immune escape (Whiteside, 2006). Therefore, effects of tryptamine on IDO1 inhibition may contribute to a more effective tumor-reactive response by immune cells, which is considered part of a viable strategy for anticancer therapies (Tourino et al., 2013). Similar to other Trp metabolites, tryptamine acts as a ligand for AhR and activates AhR to regulate intestinal immunity (Islam et al., 2017). In turn, AhR plays a role in regulating tryptamine production

when the intestinal balance is disturbed. For instance, in wild-type (WT) and *Ahr*-knockout (KO) mice, the concentrations of tryptamine in feces were similar in both Trp diet groups before DSS treatment. However, after DSS treatment, colonic tryptamine levels were markedly lower in *Ahr* KO mice compared to WT mice, which might be due to dysbacteriosis induction in the former (Islam et al., 2017). In addition, tryptamine is a ligand for trace amine-associated receptors (TAARs) and potentiates the inhibitory response of cells to serotonin (Zucchi et al., 2006). Tryptamine can also induce the release of serotonin (Takaki et al., 1985). Fluctuation in intestinal serotonin levels can modulate GI motility (Lundgren, 1998; Turvill et al., 2000) and is also involved in the pathology of IBD (Linden et al., 2003, 2005; Bischoff et al., 2009).

Alteration of tryptamine production is mediated by Trp-microbial metabolism. The concentration of tryptamine in feces increases ∼3-fold in conventional vs. GF mice (Marcobal et al., 2013). Although only a small fraction of Trp is converted to tryptamine by the intestinal microbiota, levels of tryptamine can be drastically increased after Trp supplementation (Vikström Bergander et al., 2012). Indeed, a Trp-supplemented diet can increase tryptamine levels compared with a control diet. For instance, the concentrations of tryptamine in the colon and serum were found to be increased in Trp-supplemented diet mice compared to control diet mice (Islam et al., 2017). On the basis of these studies, Trp can be absorbed from the diet by microbes and then converted to tryptamine; the type and distribution of Trp metabolites are altered and influence the normal physiological function of the host intestine.

FACTORS INFLUENCING TRP-MICROBIOME-HOST IMMUNITY INTERACTIONS IN THE GUT

Probiotics

Probiotics act as microbial food supplements that are beneficial to the host by improving the intestinal microbial balance. Studies indicate that probiotic supplements can modify the gut microflora and provide a practical means of enhancing gut and systemic immune function (**Figure 3**) (Nagata et al., 2016).

A particular focus has been the function of probiotics in Trp metabolism meditated by gut microbiota (**Figure 3**). Probiotics can selectively influence Trp metabolism and Trp concentrations. In bifidobacteria-treated rats, concentrations of plasma Trp (12.34 ± 0.87 vs. $8.03 \pm 0.81\,\mu g/ml$, $p < 0.005$) and kynurenic acid (19.5 ± 3.7 vs. $7.9 \pm 1.4\,ng/ml$, $p < 0.05$) were markedly increased compared with controls (Desbonnet et al., 2008). At the same time, bifidobacteria treatment significantly attenuated levels of proinflammatory cytokines, including IFN-γ, TNF-α, and IL-6, after mitogen stimulation in rats compared with untreated controls. These findings provide evidence that bifidobacteria treatment attenuates proinflammatory immune responses by elevating plasma Trp and kynurenic acid levels (Desbonnet et al., 2008). In an immunologically permissive environment, using the probiotic *L. reuteri* can effectively enhance the production of IAld through the Trp catabolism pathway when the substrate Trp can be accessed by the probiotic bacteria (Zelante et al., 2013; Marsland, 2016). As mentioned above, IAld, an AhR ligand, activates AhR in gut-resident T-cells and in ILCs, enhancing IL-22 production and protecting against inflammation in the colon. In addition, treatment with the probiotic *Lactobacillus fermentum* VRI-003 induced a certain increase in the IFN-γ response, a potent stimulus for IDO1 (Cox et al., 2010).

In what way might probiotic treatment affect Trp availability and Trp metabolism? First, probiotics can influence the gut microbiome composition, which may directly affect downstream metabolism and immunoregulatory pathways; the composition of gut bacteria can thus regulate Trp metabolism and the availability of Trp and Trp metabolites (Jenkins et al., 2016), influencing the immune response in the gut. As a therapeutic strategy in susceptible hosts, probiotic treatment can alter the intestinal microbiota and increase the generation of AhR ligands via Trp metabolism, which can protect the host from intestinal inflammation (Etienne-Mesmin et al., 2017). For example, in susceptible *Card9*$^{-/-}$ mice, administration of three commensal *Lactobacillus* strains with high Trp-metabolic activities restored intestinal IL-22 production and reversed susceptibility to colitis (Lamas et al., 2016). However, only a small portion of probiotics have been examined for their ability to regulate intestinal immune function by affecting Trp availability and metabolism; moreover, the key genes involved in the probiotics have not been verified. Thus, more types of probiotics should be tested. New probiotics carrying specific Trp-metabolism genes through genetic engineering may be an important direction. However, more studies will be necessary to address these possibilities.

Stress

The interaction between gut bacteria and regulation of the stress response is bidirectional (**Figure 3**). Physical and psychological stress can alter the composition of the GI microbiota in rodents and primates (**Figure 3**) (Bailey, 2014). In addition, manipulation of the intestinal microbiota has been shown to induce behavioral changes, including stress (Sudo et al., 2004), anxiety, and depression (Wilson et al., 2017). Stress responses of GF mice can be partially ameliorated by bacterial colonization with fecal matter from specific pathogen-free (SPF) mice to GF at early stages (Sudo et al., 2004).

Trp metabolism is closely related to stress-related challenges in animals. Stress, stress hormones and related neuropeptides have impacts on cerebral uptake of Trp, on serotonin synthesis, release and metabolism, and on other Trp-metabolism pathways. In detail, stress-induced increases in serotonin release in the brain is related to enhanced *Tph1* expression. Stress-related glucocorticoid hormones can induce TDO expression and activity at both the mRNA and protein levels (Ruddick et al., 2006), and immune stress induces IFN-γ release, which activates IDO expression and enhances NAD^+ synthesis in macrophages through quinolinic acid as a substrate (Grant et al., 1999; Ruddick et al., 2006).

In addition, altered IDO1 activity and Trp metabolism are involved in many stress-related disorders, and stress-induced cortisol increases IDO1 activity and gut permeability. Individuals

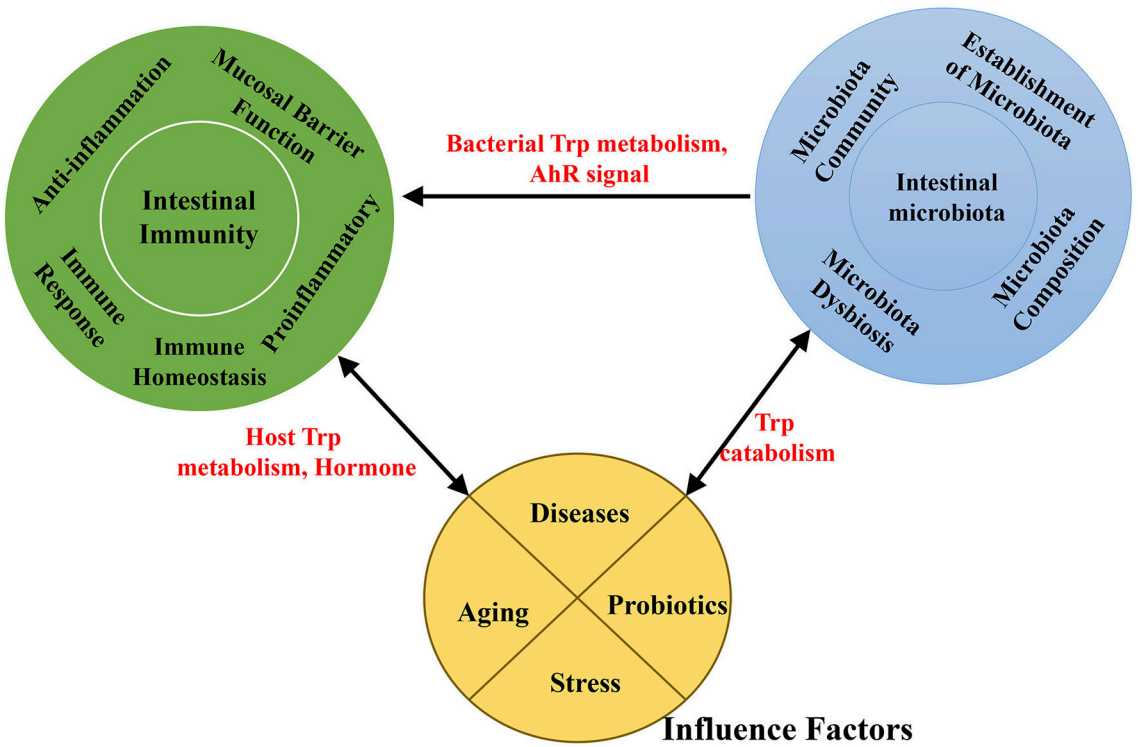

FIGURE 3 | A schematic representation of the effects of interaction among the intestinal microbiota, intestinal immunity and factors associated with Trp absorption and metabolism.

vulnerable to high levels of stress may benefit from Trp supplementation in the form of Trp-rich albumin or whey protein hydrolysates (Clarke et al., 2013). Nonetheless, little attention has been given to the possible connections among altered and affected Trp metabolites after stress inducement. Therefore, a rational strategy to alleviate stress-related disorders by adjusting and controlling Trp uptake and metabolism may be misguided.

Exhibiting a property of exaggerated stress reactivity, GF animals represent an effective model for studies to reveal the impact of the GI microbiota on Trp metabolism in response to stress (**Figure 3**). In GF animals, the kynurenine:Trp ratio is significantly decreased compared to conventionally colonized controls. This is however sex specific: the concentration of plasma Trp is increased in male GF animals (but not in females), and the concentrations of hippocampal 5-HT and 5-hydroxyindoleacetic acid are significantly elevated (again, not in females). After gut microbiota colonization in GF animals, the plasma Trp concentration was reduced and the kynurenine:Trp ratio increased compared with GF animals; plasma serotonin levels were also increased 2.8-fold (Wikoff et al., 2009). In addition, colonization by intestinal microbiota normalized stress in GF animals, displaying a more normal stress response. These results underline the ability of the microbiota to control Trp metabolism and the serotonergic system, which is particularly relevant to stress response and anxiety. Regardless, there are limited data pertaining to the relationship between plasma Trp concentrations and the kynurenine:Trp ratio, between plasma Trp and 5-HT concentrations, and between the same gut microbiota condition and the sex-specific serotonergic system.

The microbiota is suggested to play some role in regulating 5-HT synthesis, which is potentially mediated by IDO expression and functions in the stress response (Forsythe et al., 2010). Stress in the gut can alter the barrier function, enhance gut permeability and increase pro-inflammatory cytokines such as IL-1 and IL-6, which in turn alter IDO activity and Trp availability. Moreover, pro-inflammatory cytokines together with 5-HT influence the release of corticotropin-releasing hormone and vasopressin, which disorder the pressure response. Probiotic administration is shown to influence the stress response, and certain probiotic bacteria can alter the gut barrier function and 5-HT synthesis. Thus, probiotic administration is deemed a potential therapeutic for stress-related GI disorders such as irritable bowel syndrome (IBS). A more detailed understanding of the effects of stress on the induction of microbiome-gut-Trp alterations is needed, which may contribute to knowledge on the pathogenesis of several intestinal-related diseases.

Aging

Aging is related to changes in the gut microbiota, which is frequently linked to physiological changes in the GI tract, together with a decline in immune system function that may contribute to increased risk for infection, malnutrition, and other functional deficiencies (Salazar et al., 2016) (**Figure 3**). The

gut microbiota of elderly individuals is usually characterized by reduced bacterial diversity, altered dominant species, reduced beneficial microorganisms, and increased facultative anaerobic bacteria (Salazar et al., 2014), all of which indicate potential detrimental effects of microbial changes associated with aging. Changes in microbiota composition are connected to immunosenescence and inflammation in older individuals (Franceschi et al., 2000; Biagi et al., 2013; Cheng et al., 2013).

Trp metabolism is affected by aging (Rampelli et al., 2013). Trp plays crucial roles in the induction of immune tolerance and the maintenance of gut microbiota (**Figure 3**). Analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologous genes of intestinal core microbiomes in the elderly and young has shown that age-related genes with increased abundance are involved in the Trp metabolism pathway (ko00380) (Rampelli et al., 2013), which is consistent with the age-related reduction in Trp concentrations found in the serum of centenarians (Collino et al., 2013). Studies have suggested that a potential increase in Trp consumption by the gut microbiota may affect Trp bioavailability to the host (Rampelli et al., 2013). One recent study reported a relationship between reduced serum Trp levels and increased immune activation. For example, patients with inflammatory diseases show a significant depletion in serum Trp levels compared with healthy individuals (Gupta et al., 2012). It has also been speculated that a microbiota-dependent reduction in Trp enhances inflammation in centenarians (Rampelli et al., 2013).

In rats and mice, dietary Trp restriction is associated with a delayed aging process and prolonged lifespans (Van Beek et al., 2016). The accelerated aging *Ercc1*$^{-/\Delta 7}$ mouse, which exhibits characteristics of normal murine aging, has been used as a model to research the relationship among aging, the gut microbiota, and Trp metabolism (Gurkar and Niedernhofer, 2015). Compared with wild-type mice, *Ercc1*$^{-/\Delta 7}$ mice possess decreased microbial diversity, consistent with the reduced microbial diversity observed in aging humans (Biagi et al., 2012). Dietary Trp restriction can increase gut microbial diversity and cause the gut microbiota composition of older *Ercc1*$^{-/\Delta 7}$ mice to be more similar to that of young wild-type mice, which might provide a valuable nutritional intervention strategy to improve age-related decreases in gut microbial diversity. At the same time, dietary Trp restriction can arrest B cell development in the bone marrow of 16-wk-old wild-type and *Ercc1*$^{-/\Delta 7}$ mice. After dietary Trp restriction, decreased abundances of *Alistipes* and *Akkermansia* spp., which are both known to express tryptophanase, were positively correlated with decreased numbers of B cell precursors (Van Beek et al., 2016). In conclusion, dietary Trp restriction is a powerful intervention to modulate immunity, gut microbiota and aging. That is, aging is an important factor influencing host immunity, gut microbiota, and Trp metabolism. However, a beneficial interplay between dietary Trp, B cell development, and gut microbiota during aging can only be concluded, and there is no direct evidence for whether increased microbial diversity induces arrested B cell development or whether decreased B cell precursors cause changes in gut microbiota composition. In the gut microbiota, specific types of bacteria, *Alistipes* and *Akkermansia* spp., positively correlate with B cell precursors, though the key metabolites and the mode of action mediating this connection is unclear. Effects may include alleviating the harmful effects of aging or even slowing the aging process.

Diseases

The important role of the gut microbiota in host physiology and pathology has been extensively studied. A series of immune diseases, such as pediatric spondyloarthritis (SpA), IBS, IBD, and colon and GI cancer are associated with the gut microbiota (Uronis et al., 2009) (**Figure 4**). Trp metabolites also play important roles in regulating these immune diseases (Maes et al., 2007). As described above, the composition of the gut microbiota can influence Trp metabolite levels, and they both profoundly affect the immune status of the host, especially in the intestine.

Spondyloarthritis (SpA)

Spondyloarthritis (SpA) occurs in approximately one percent of the population in the United States (Lawrence et al., 2008). Half of SpA patients can have intestinal inflammation but not overt gastrointestinal symptoms (also called subclinical inflammation) (Vereecke and Elewaut, 2017). Based on the close relationship observed between SpA and intestinal inflammation, the microbiota has been suggested to be a potential factor in influencing immune responsiveness in SpA patients (Fantini, 2009; Scher et al., 2015). In pediatric or adult SpA patient feces, taxonomic differences in bacteria have been identified compared to healthy individuals (Costello et al., 2014; Stoll et al., 2014).

The influence of the gut microbiota in SpA has been studied (Lawrence et al., 2008; Costello et al., 2014; Stoll et al., 2014). With the decreased gut microbial diversity in pediatric SpA, relatively reduced Trp metabolism and lower levels of Trp metabolites in patients have been reported. The decrease in Trp metabolites in SpA patients is consistent with a similar finding in the synovial fluid of rheumatoid arthritis patients (Kang et al., 2015). Changes in fecal metabolomics and gut microbiota differences in Trp metabolism in pediatric SpA have been ascribed to alteration in the gut microbiota (Stoll et al., 2016). In the Trp metabolism pathway, 21 unique Trp metabolites were found to be reduced in the feces of SpA patients, as detected by charged ions, including the following: three bacterial Trp metabolites (indole-3-acetate, indole-3-acetaldehyde, and methyl indole-3-acetate); eight metabolites of kynurenine (such as 3-hydroxy-L-kynurenine, anthranilate, glutaryl-CoA, kynurenate); and six metabolites of 5-hydroxy-Trp. In addition, 16S sequence data of the fecal microbiome verify the relative decrease in Trp metabolism in SpA patients compared with controls (Stoll et al., 2016). Based on the above-described reports, the gut microbiota in enthesitis-related arthritis (one type of SpA) patients potentially acts as a proinflammatory factor due to altered Trp catabolism. As reported above, quite a lot of these Trp metabolites are associated with intestinal immune and intestinal inflammation, but it remains to be investigated which ones play dominant roles in causing subclinical inflammation in SpA. At the same time, which genes and gut microbes that target the specific lesser Trp metabolites should be further analyzed.

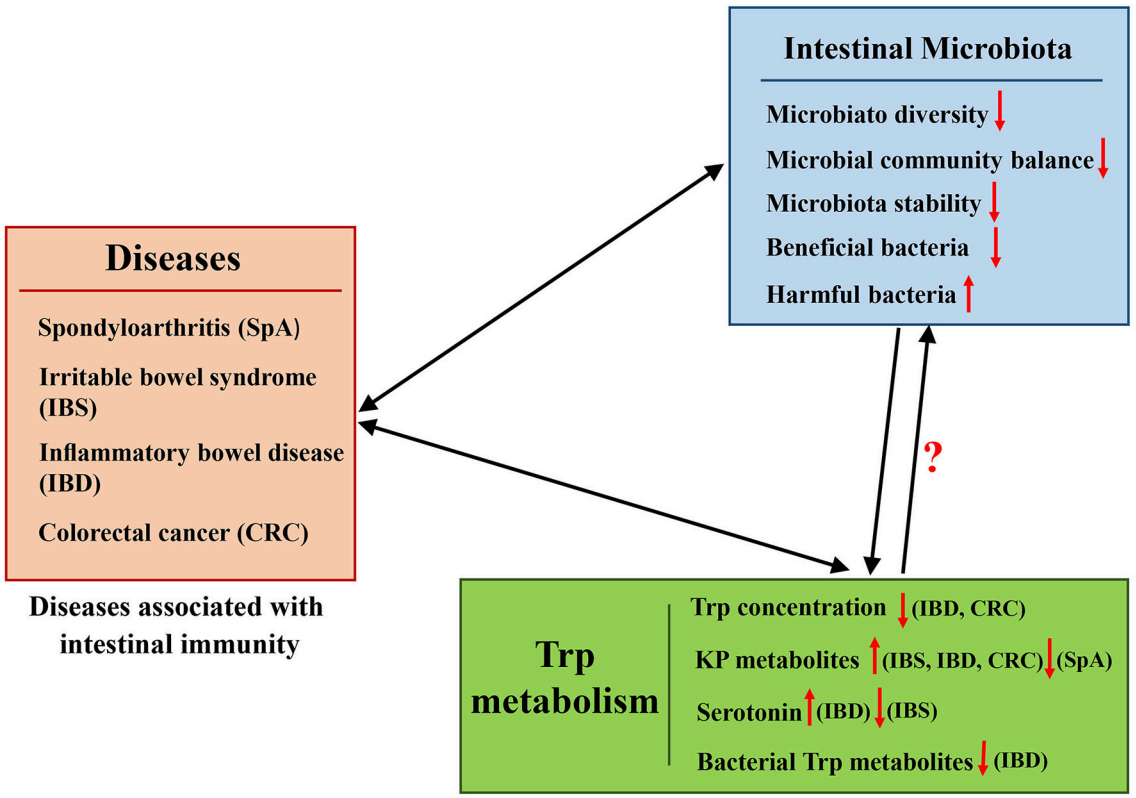

FIGURE 4 | Representation of the effects of interaction between the intestinal microbiota and Trp metabolism in diseases associated with intestinal immunity.

Irritable Bowel Syndrome (IBS)

IBS is one of the most commonly diagnosed chronic functional GI disorders, with a prevalence of ∼15% in western populations (Soares, 2014). The pathophysiology of IBS is related to intestinal epithelial cells, enteroendocrine cells, neuronal cells, and immune cells. Alterations in gut microbiota diversity and composition have been implicated in IBS. Focusing on the symptomatology of IBS, changes in the intestinal microflora balance are an important factor (Nakai et al., 2003; Spiller, 2008; Bhattarai et al., 2017). Increases in the *Firmicutes* to *Bacteroidetes* ratio (Clarke et al., 2009) and the abundance of *Streptococcus* and *Ruminococcus* species are observed in IBS patients (Hong and Rhee, 2014), whereas *Lactobacillus* and *Bifidobacterium* populations (Balsari et al., 1982) are decreased.

In addition, IBS is associated with increased Trp metabolism via KP (Jenkins et al., 2016). The kynurenine:Trp ratio is positively related to symptom severity in IBS (Fitzgerald et al., 2008), and IFN-γ activation and subsequent IDO1 oxidation of Trp may be a pathogenic mechanism of IBS (Fitzgerald et al., 2008). In addition, dysfunction of the serotonergic system is associated with the pathophysiology of IBS. Serotonergic modulation through acute Trp increase treatment in IBS patients induced more severe GI symptoms compared with acute Trp depletion treatment (Kilkens et al., 2004; Shufflebotham et al., 2006). In IBS, the severity of symptoms and alterations of both gut and brain serotonin concentrations are associated with changes in the microbiota balance (Jenkins et al., 2016). IBS patients have lower serotonin concentrations in the small intestine, but colonic serotonin production can be promoted by effects of bacterial products on enterochromaffin cells, such as SCFAs (Reigstad et al., 2015). At the genetic level, microbiota from humanized and conventional mice increased colonic Trp hydroxylase 1 (*Tph1*) (a rate-limiting enzyme for mucosal 5-HT synthesis) expression through the stimulatory activities of SCFAs. For instance, butyrate, a SCFA, can active *Tph1* expression in mice through the inducible zinc finger transcription factor ZBP-89 (Essien et al., 2013). In the intestinal tract, nearly all SCFAs are produced through bacterial fermentation. Concentrations of SCFAs in the cecum of GF mice were reported to be ∼1 mmol/kg, whereas average concentrations in the conventional mouse cecum are ∼125 mmol/kg. Moreover, the variety and concentrations of SCFAs in human feces vary widely among individuals, which may be attributable to the complex microbial community or dynamic diet composition (Høverstad and Midtvedt, 1986).

Microbial-origin stimuli and the gut serotonergic system may act as key factors influencing the symptomatology of IBS. To alleviate GI symptoms through serotonergic modulation in IBS patients, the *in vivo* effects of local concentrations and proportions of different colonic SCFAs on mucosal 5-HT homeostasis should first be investigated, as SCFAs affect *Tph1* expression in a concentration-dependent manner; how to

balance mucosal 5-HT homeostasis by manipulating the complex microbial community is challenging for this strategy. In addition, more data about the coadjustment between SCFAs and gut serotonergic system are required.

Inflammatory Bowel Disease (IBD)

IBD, defined as a chronic, remitting, and relapsing inflammatory disorder of the gut, includes Crohn's disease (CD), and ulcerative colitis (UC) (Matsuoka and Kanai, 2015; Lamas et al., 2017). Multifaceted factors, including dysregulation of the mucosal immune system, an unbalanced gut microbial community, and disruption of the mucosal barrier, are related to the pathogenesis of IBD (Flint et al., 2012; Pillai, 2013; Kostic et al., 2014; Matsuoka and Kanai, 2015; Lamas et al., 2017; Liu et al., 2017). Of these factors, the gut microbial community is gaining more attention due to its influence on intestinal health.

The pathogenesis of IBD is associated with alterations in the composition of the intestinal microbiome, but whether these alterations are causal or a result of inflammation in IBD is still under dispute (Gkouskou et al., 2014). In several reports, the gut microbiota has been considered to trigger inflammation in IBD (Sartor, 2008), as short-term treatment with antibiotics can markedly alleviate intestinal inflammation (Sartor, 2004; Kostic et al., 2014). In addition, an altered microbiome and altered interactions between intestinal microbes and mucosal immunity have been suggested to cause increased intestinal permeability and inflammation in IBD (Sartor, 2004; Hold et al., 2014).

Reductions in bacterial diversity have been reported in IBD patients (Martin-Subero et al., 2015), and these are accompanied by increased intestinal permeability and enhanced intestinal bacteria infiltration, which can induce immune responses and ultimately systemic inflammation (Xavier and Podolsky, 2007; Martin-Subero et al., 2015). IBD patients exhibit reduced intestinal mucosal barriers and decreased mucin glycosylation, and the disproportionate increase in several mucolytic microbes in IBD is suggested to be partly due to bacterial adaptation to the altered mucin glycosylation (Png et al., 2010). Several specific bacteria have been associated with IBD. For example, a decrease in *Bacteroidetes* (Frank et al., 2007) and *Bacteroides* (Nemoto et al., 2012) levels are reported in IBD patients, as were increases in *Desulfovibrio* and *Bilophila* levels (Rowan et al., 2010; Jia et al., 2012). A positive correlation between the invasive potential of *Fusobacterium* and the severity of IBD in the host was also found (Strauss et al., 2011), suggesting that invasive strains of *Fusobacterium* may influence IBD pathology (Kostic et al., 2014). In contrast, several specific species of gut bacteria may have protective effects against IBD (Kostic et al., 2014). For instance, species of *Bifidobacterium*, *Lactobacillus*, and *Faecalibacterium* may protect the host from mucosal inflammation by downregulating inflammatory cytokines or stimulating expression of IL-10, an anti-inflammatory cytokine (Sokol et al., 2008; Llopis et al., 2009).

IBD is also associated with altered host and gut bacterial Trp metabolites. Plasma levels of kynurenine and KA are increased (Forrest et al., 2003) and concentrations of plasma Trp are decreased in IBD patients, and increased IDO1 activity has been reported in the peripheral blood and colon cells of IBD patients (Munn and Mellor, 2007; Martin-Subero et al., 2015). In IBD, increased pro-inflammatory cytokines, including IFN-γ, IL-1, and IL-6, have been suggested to induce Trp catabolic pathways to reduce plasma Trp levels and increase Trp catabolite levels (Martin-Subero et al., 2015). In CD patients and IBD animal models, increased 5-HT levels are found in inflamed mucosa, suggesting the important role of 5-HT in driving intestinal inflammation in IBD (Levin and Van Den Brink, 2014). Several specific gut bacterial Trp metabolites are also involved in the pathophysiology of IBD (Matsuoka and Kanai, 2015). In dogs with IBD, bacterial Trp metabolites (indole acetate and indole propionate) that are suggested to have anti-inflammatory functions in the intestine are significantly decreased (Honneffer et al., 2015). In IBD patients, levels of IAA (anti-inflammatory function in the intestine) are reduced in feces, suggesting that reduced bacterial Trp metabolism may contribute to the etiology of IBD (Lamas et al., 2016). Furthermore, in IBD patients, the abundance of bacteria that can cleave terminal fucose residues from intestinal mucins by utilizing α-L-fucosidases is significantly reduced, correlating with the reduced production of indole acrylic acid and indole-3-propionic acid from Trp (Wlodarska et al., 2017). Studies have also suggested a strategy of increasing indole acrylic acid production by restoring bacterial Trp metabolism in the intestine to promote anti-inflammatory responses in IBD patients, which may have beneficial therapeutic effects.

Colorectal Cancer (CRC)

CRC is the third-most prevalent cancer that causes mortality. The effects of the gut microbiota on CRC progression have been examined. GF animals have lower tumor burdens compared to conventionally raised counterparts. Similarly, depletion of the gut microbiota through antibiotics has also been shown to reduce CRC occurrence (Grivennikov et al., 2012). Alterations in gut microbiota composition have been found in murine tumorigenicity studies. In CRC patients, the fecal microbiota demonstrate decreased temporal stability but increased diversity of *Clostridium leptum* and *Clostridium coccoides* subgroups compared with the control group (Grivennikov et al., 2012).

Trp metabolism via KP has proven to be a potent target for immunotherapy. KP is activated in multiple tumor types; IDO1 initiates KP and is expressed in the primary tumor and in infiltrating myeloid-derived cells in CRC (Uyttenhove et al., 2003; Ferdinande et al., 2012; Théate et al., 2015). IDO1 appears to be chronically activated in cancer patients (Huang et al., 2002; Weinlich et al., 2007). IDO1 is commonly overexpressed in CRC and often accompanied by reduced Trp levels and increased levels of KP metabolites (Liu et al., 2010; Walczak et al., 2011; Engin et al., 2015). As introduced in above, IDO1 plays an essential role in maintaining microbiota diversity, and IDO1 activation can reduce microbial proliferation. Hence, we suggest that overexpression of IDO1 in CRC may be the cause of alterations and decreased temporal stability of the gut microbiota. As depletion of the gut microbiota can reduce CRC occurrence, decreasing the microbial proliferation induced by IDO1 activation may constitute self-regulation as a defense against CRC. In fact, research has proven that blockage of IDO1

activity can directly enhance the immune capacity of tumor-bearing mice against the tumors (Uyttenhove et al., 2003; Muller et al., 2005). However, the influence of the blockage of IDO1 activity on the mouse gut microbiota was not discussed, and it remains unclear whether the mouse gut microbiota played a role in this IDO1-blockage treatment. Regardless, selective inhibition of IDO1 is proposed to upregulate cellular immunity, with therapeutic potential in cancer, including CRC, and the influence of IDO1 on the gut microbiota is a factor that must be taken into account.

IDO1 also functions as a mediator in host-microbe crosstalk, which has effects on the pathology of GI cancer. When mediating host-microbe interactions, expression of IDO1 can be induced by activation of several binding pattern recognition receptors (PRRs), especially TLR4 and TLR9 (Ciorba et al., 2010; Santhanam et al., 2016). For example, TLR4 can be activated by LPS, a primary bacterial toxin. The ability of LPS to activate PRRs and then IDO1, reducing the generation of LPS and other similar toxins by controlling gut microbes, may be important for reducing the risk of GI cancer. Furthermore, new antagonists for PRRs to reduce expression of IDO1 may be beneficial against tumors. Indoles, as Trp microbial metabolites, have particular relevance for Trp metabolism and CRC (Raman et al., 2013), and several indoles derived from dietary Trp can activate AhR, which has important roles in intestinal homeostasis and controlling GI cancer. The influence of the gut microbiota and Trp metabolism on CRC pathogenesis will guide the development of targets and new approaches for the prevention and treatment of CRC.

SUMMARY AND PERSPECTIVE

Our knowledge of interactions between Trp metabolism, gut microbiota, and host immunity has been greatly expanded over the past several years. Growing evidence shows that Trp, its endogenous host metabolites (kynurenines, serotonin, and melatonin) and its microbiome-modulated metabolites (indole, indolic acid, skatole, and tryptamine) have profound effects on gut microbial composition, microbial functions, the host-microbiome interface, and interactions between the host immune system and intestinal microbiota. Correspondingly, the gut microbiota affect host Trp absorption and metabolism, and directly or indirectly regulate subsequent host physiological and immune responses. In previous studies about Trp nutrition in conventional animals, some different or conflicting results have been reported. More attention was given to variation trends of endogenous host metabolites, whereas the effects of bacterial Trp metabolites were often overlooked. Thus, further comprehensive analyses of targeted Trp metabolites and associated genes are essential for experimental preciseness, which may explain the contradictory results in the literature.

Effects on host immunity by factors such as aging, stress, probiotic intake, and several diseases are partially associated with Trp-microbiome-immunity interactions. The influence of the gut microbiota on Trp metabolism should be assessed when studying Trp nutritional supplementation or Trp therapeutic applications. Furthermore, feasibly designed therapies that target Trp absorption, Trp metabolites, the gut microbiota, or Trp-microbiome-immunity interactions are promising approaches for treating intestinal or extra-intestinal inflammation. Moreover, the complex and variable gut microbiota may allow for identifying simple universal rules about bacterial Trp metabolites, and personalized gut microbiota analysis may be an effective approach for instituting clinical treatment. Similarly, further studies are required to determine which gut microbial species, the effective dose ranges of Trp metabolites, and which metabolite-targeted pathways may impact local and systemic inflammatory processes in the GI tract.

AUTHOR CONTRIBUTIONS

All the authors contributed extensively to the work presented in this manuscript. KX and JG mainly completed this review. KX, JG, and HL performed the literature search and wrote the manuscript. KX, MB, GL, CP, TL, and YY conceived the work and critically revised it. YY revised the manuscript.

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Anti-biofilm Properties of the Fecal Probiotic Lactobacilli Against *Vibrio* spp.

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Diarrheal disease caused by *Vibrio cholerae* is endemic in developing countries including India and is associated with high rate of mortality especially in children. *V. cholerae* is known to form biofilms on the gut epithelium, and the biofilms once formed are resistant to the action of antibiotics. Therefore agents that prevent the biofilm formation and disperse the preformed biofilms are associated with therapeutic benefits. The use of antibiotics for the treatment of cholera is associated with side effects such as gut dysbiosis due to depletion of gut microflora, and the increasing problem of antibiotic resistance. Thus search for safe alternative therapeutic agents is warranted. Herein, we screened the lactobacilli spp. isolated from the fecal samples of healthy children for their abilities to prevent biofilm formation and to disperse the preformed biofilms of *V. cholerae* and *V. parahaemolyticus* by using an *in vitro* assay. The results showed that the culture supernatant (CS) of all the seven isolates of *Lactobacillus* spp. used in the study inhibited the biofilm formation of *V. cholerae* by more than 90%. Neutralization of pH of CS completely abrogated their antimicrobial activities against *V. cholera*, but had negligible effects on their biofilm inhibitory potential. Further, CS of all the lactobacilli isolates caused the dispersion of preformed *V. cholerae* biofilms in the range 62–85%; however, pH neutralization of CS reduced the biofilm dispersal potential of the 4 out of 7 isolates by 19–57%. Furthermore, the studies showed that CS of none of the lactobacilii isolates had antimicrobial activity against *V. parahaemolyticus*, but 5 out of 7 isolates inhibited the formation of its biofilm in the range 62–82%. However, none of the CS dispersed the preformed biofilms of *V. parahaemolyticus*. The ability of CS to inhibit the adherence of *Vibrio* spp. to the epithelial cell line was also determined. Thus, we conclude that the biofilm dispersive action of CS of lactobacilli is strain-specific and pH-dependent. As *Vibrio* is known to form biofilms in the intestinal niche having physiological pH in the range 6–7, the probiotic strains that have dispersive action at high pH may have better therapeutic potential.

Keywords: *Vibrio*, anti-biofilm, biofilm-dispersion, lactobacilli, probiotic, antimicrobial

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INTRODUCTION

Cholera is an acute diarrheal infection caused by the ingestion of food or water contaminated with the cholera toxin-producing Gram-negative pathogen *Vibrio cholerae*. It has been the cause of 7 pandemics since 1,871, and still remains a major public health issue in more than one-third of the countries due to poor sanitation facilities and lack of safe drinking water (Morris and Acheson, 2003). The annual occurrence of cholera in 69 cholera-endemic countries is estimated to be 2.9 million that results in 95,000 deaths each year (Ali et al., 2015). It is an endemic disease in India and can be fatal if remained unmanaged. *V. parahaemolyticus* that secrets haemolysin is also known to cause gastroenteritis if consumed at high doses. The extensive use of antibiotics for the treatment of diarrhea has led to the emergence of drug resistance in *V. cholerae* (Kitaoka et al., 2011). Also the use of antibiotics can disrupt the homeostasis of the gut by killing the normal gut flora. Therefore, there is a need for safe alternative therapeutics to combat gut-bacterial infections. One of the safe alternative therapeutic agents is probiotics. Probiotics are live microorganisms which confer health benefits on the host when administered in adequate amounts (FAO/WHO, 2001). *Lactobacillus* spp. is the most widely accepted probiotic because of their GRAS (generally regarded as safe) status. Lactobacilli are rod-shaped Gram-positive, facultative anaerobes, comprising roughly 0.01% of the microbiome in the gastrointestinal tract (GIT) of humans (Harmsen et al., 2002) and range between 10^7-10^8 cells/gm of feces (Rinttilä et al., 2004). In GIT, they are known to possess health-promoting effects such as maintaining normal intestinal homeostasis by inhibiting the colonization of pathogens and modulating the immune responses (Lebeer et al., 2008; Kemgang et al., 2014). The use of probiotics for the treatment of gut-associated health disorders has yielded positive results as demonstrated by various clinical trials (Culligan et al., 2009). Meta-analysis of clinical trials showed the efficacy of lactobacilli probiotics for the treatment of antibiotic-induced diarrhea (D'Souza et al., 2002; McFarland, 2006), antibiotic-induced *Clostridium difficile* infection (McFarland, 2006), and reduction in the duration of rotavirus diarrhea (Huang et al., 2002; Ahmadi et al., 2015). However, the human clinical trial of probiotics against cholera was not successful (Mitra and Rabbani, 1990). *V. cholerae* is a biofilm-forming pathogen. It is known to form strong biofilms on the epithelial lining of gut in both mice (Millet et al., 2014) and humans (Yamamoto and Yokota, 1988). The biofilms play an important role in the pathogenesis of cholera (Fong et al., 2010; Almagro-Moreno et al., 2015). Also once the biofilms are formed, they resist the action of both immune defenses and antibiotics, and are also responsible for the recurrent nature of the infection. Therefore the probiotic strain having both antimicrobial and anti-biofilm properties may be expected to be therapeutically more effective. Some of the anti-biofilm agents effective against *Vibrio* spp. have been reported in the literature (Sambanthamoorthy et al., 2011; Sayem et al., 2011; Warner et al., 2015), but they do not have any antimicrobial properties against planktonic cells and thus are administered along with the conventional antibiotics. The antimicrobial probiotics having biofilm-dispersive properties can yield better clinical benefits for the treatment of diarrhea due to *Vibrio* spp as they may be used as stand-alone therapeutic agents. The *in vitro* antimicrobial activity of lactobacilli strains against different *Vibrio* spp. (Koga et al., 1998), *V. parahaemolyticus* (Shokryazdan et al., 2014; Chimchang et al., 2015) and *V. cholerae* (Petrova and Petrov, 2011) has been demonstrated by various workers. However the ability of lactobacilli in inhibiting the biofilm of *Vibrio* spp. has not been reported. With this in the background, we isolated lactobacilli from the fecal samples of healthy children and studied the antimicrobial and anti-biofilm activities of cell free culture supernatant (CS) of lactobacilli. The probiotic properties of the selected isolates were also studied with an idea to develop them as indigeneous probiotic strain.

MATERIALS AND METHODS

Microorganisms and Growth Conditions

For the isolation of lactobacilli, fecal samples were collected from 32 healthy children of age group ranging from 2 to 13 yrs after taking the written informed consent of their parents. The study was approved by the Institutional Human Ethics Committee. The stool sample weighing approximately 1 g was collected in thioglycollate broth (HiMedia laboratories, Mumbai, India) and incubated for 4 h at 37°C in anaerobic jars having 5% carbon dioxide (CO_2). Thereafter, 10-fold serial dilutions of the broth were plated onto De Man Rogosa and Sharpe (MRS; HiMedia) agar plates and incubated at 37°C under anaerobic conditions in anaerobic gas jars. Bacterial colonies with different morphologies were selected and preserved in 20% (v/v) glycerol (HiMedia)-containing MRS broth at −80°C. The lactobacilli were identified by Gram-positive staining and catalase-negative phenotype. For experimental purposes, the lactobacilli were cultured in MRS medium from the frozen stocks and propagated twice before use.

The various pathogenic indicator strains used in this study were *V. cholerae* strain 0139 MTCC 3906, *Salmonella enterica* Typhimurium MTCC 733, *Listeria monocytogenes* MTCC 657, *Escherichia coli* MTCC 119, *Shigella flexeri* MTCC 1457, *V. parahaemolyticus* MTCC 451, and *Staphylococcus aureus* MTCC 96. These strains were procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The fungal indicator strain *Candida* spp. was procured from the Department of Microbiology, Government Medical College, Amritsar. All the pathogenic bacteria were cultured in brain heart infusion (BHI) broth (HiMedia) and *Candida* spp. was cultured in Sabouroud dextrose broth (HiMedia) at 37°C under aerobic conditions. All the cultures were stored at −80°C in broth supplemented with 20% glycerol.

Characterization of the Lactobacilli Isolates

The lactobacilli isolates were characterized by using 16S rDNA sequencing and biochemical tests. For 16S rDNA sequencing, the genomic DNA of lactobacilli 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′-AGAGTTGATCCTGGCTCAG-3′ and 1492P Reverse: 5′-TACGGCTACCTTGTTACGACTT-3′. DNA amplification was carried out in 0.2 ml PCR tubes by using master cycle personal (Eppendorf Hamburg, Germany). The PCR reaction mixture (50 μl) consisted of 25 μl of 2X PCR master mix (3B Black Bio Biotech India, Ltd.,), 1 μl of each primer (Bioserve Biotechnologies Pvt. Ltd., India), 5 μl of template DNA, and 18 μl of nuclease free water. Initial denaturation of DNA was done at 95°C for 4 min, followed by 32 cycles of amplification comprising a denaturation step for 1 min at 95°C, 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 bio imaging system (Gene Genius gel imaging System, Syngene Bioimaging Private Ltd., India). The partial sequences of 16S rDNA were obtained and the isolate identified by aligning in the software BLAST version 2. The 16s rDNA sequences obtained have been submitted to NCBI and their accession no. were obtained (**Table 1**).

Determination of the Antimicrobial Activity of CS of Lactobacilli Isolates

The agar well diffusion assay was used to determine the antimicrobial activities of CS of lactobacilli isolates grown in MRS broth (Reinheimer et al., 1990; Gonzalez et al., 2007). To prepare the CS, lactobacilli were cultured in MRS broth for 16 h at 37°C and the broth was centrifuged at 9,000 g for 10 min at 4°C. The supernatant was then filter sterilized using syringe filters having pore size of 0.2 μm and stored at 4°C till further use.

For performing agar well diffusion assay, the indicator strains were grown at 37°C in the appropriate medium till an optical density (OD_{595}) of 0.2 is obtained. Following incubation, 100 μl of the indicator pathogen or commensal culture was spread onto suitable agar plates and 6 mm wells were cut into the agar plates by using sterile well-borer. An aliquot (100 μl) of CS was poured into wells and plates were placed at 4°C for 4 h to allow diffusion of CS into agar. Then the plates were incubated at 37°C for 24 h and the diameter of zone of inhibition around each well was measured in millimeters.

To nullify the effect of reduced pH on antimicrobial activity, the pH of CS was adjusted to 6.5 by using 1 N NaOH (HiMedia) and antimicrobial activity was similarly determined.

TABLE 1 | The GenBank accession numbers of fecal lactobacilli isolates characterized by using 16S rDNA sequencing.

Isolate	Genus species	Accession no.
L13	*Lactobacillus spp.*	KY780504
L14	*Lactobacillus plantarum*	KY582835
L18	*Lactobacillus spp.*	KY770976
L32	*Lactobacillus fermentum*	KY770983
S30	*Lactobacillus spp.*	KY780503
S45	*Lactobacillus pentosus*	KY780505
S49	*Lactobacillus spp.*	KY770966

Growth Curve

The growth curves of *V. cholerae* and *V. parahaemolyticus* were made in BHI medium supplemented with lyophilized MRS (as control) or pH-neutralized/non neutralized CS of lactobacilli isolates at the concentrations of 45 mg/ml. The overnight *Vibrio* cultures were grown in BHI and diluted to 0.1 OD_{595} before inoculating in BHI supplemented with MRS or CS at the concentration 45 mg/ml and incubated at 37°C for 48 h in the total volume of 220 μl in 96-well polystyrene plates in triplicates. Absorbance at the wavelength 595 was measured after every 4 h on the microplate reader (Biorad) till 48 h.

The Effect of CS on the Biofilm Formation by *Vibrio* spp.

The effect of pH non-neutralized CS of lactobacilli isolates was determined on the biofilm formation by both *V. cholerae* and *V. parahaemolyticus*, whereas, the effects of pH neutralized CS (pH set to 6.5 by using 1 N NaOH) was tested only on the biofilm formation by *V. cholerae*. The biofilm formation was determined by using modified crystal violet assay (Sharma et al., 2015) in a 96-well polystyrene microtiter plate (Tarsons Product Pvt. Ltd., Kolkata). To initiate biofilm formation, 100 μl of sterile BHI broth was added to each well along with 100 μl of CS or MRS broth (at concentration 45 mg/ml) and 20 μl of overnight grown *Vibrio* spp. having OD_{595} of 0.1. The microtiter plate was incubated at 37°C for 48 h to allow biofilm formation. Following incubation, the non adherent cells were removed by washing the wells gently 3 times with sterile distilled water. The adherent cells were fixed by using 200 μl of methanol (HiMedia) for 15 min and then the plate was emptied and air dried. The fixed biofilms were stained by using 200 μl of 2% crystal violet (HiMedia) in distilled water for 5 min. Excess stain was removed by washing under running tap water till color fades away. The stain was extracted from the adherent cells by using 160 μl of 33% glacial acetic acid (HiMedia) in distilled water and OD_{595} was measured using microplate reader. The experiment was conducted in triplicates. The percentage inhibition was calculated as,

Percentage inhibition = 100–[(OD_{595} of wells in the presence of CS X 100)/ OD_{595} of wells in the presence of MRS].

Effect of CS of Lactobacilli on the Dispersal of Biofilms of *Vibrio* spp.

The effect of pH non-neutralized CS was determined on the dispersion of preformed biofilm of both *V. cholerae* and *V. parahaemolyticus*; whereas the effect of pH-neutralized CS (pH set to 6.5 by using 1 N NaOH) was studied only on the biofilm of *V. cholera* (Wu et al., 2013). Biofilm of *Vibrio* spp. was developed in 96-well microtiter plate by adding 100 μl of autoclaved BHI broth along with 20 μl of overnight grown *Vibrio* culture having OD_{595} of 0.1. After 24 h incubation at 37°C, non adherent cells were removed by gentle pipetting without disrupting biofilm. CS of lactobacilli (at concentration 45 mg/ml) were added to each well along with 100 μl BHI broth. In the control wells instead of CS 100 μl of autoclaved MRS broth was added. The plates were incubated at 37°C

for 48 h. The experiment was conducted in triplicates. After specified incubation, quantification of biofilm formed was done as described previously.

Bacterial Adhesion Assays With HCT-15 Cell Line

The binding of *Vibrio* spp. to the intestinal cell line HCT-15 in the presence and absence of CS of lactobacilli isolates was determined. In separate set of experiments the binding of lactobacilli to HCT-15 was also determined. The intestinal cell line HCT-15 was cultured in Roswell Park Memorial Institute (RPMI)-1640 (Sigma-Aldrich) medium supplemented with 10% fetal calf serum (Biological Industries, USA) on the autoclaved glass coverslips kept in 60 mm petridishes and incubated at 37°C in 5% CO_2-containing atmosphere. After the cells formed 50% confluent monolayer, they were washed twice with PBS (pH 7.2) and the viable overnight grown cells of *Vibrio* spp. or lactobacilli suspended in 4 ml of RPMI were added to the petridishes at the multiplicity of infection 1:100. For determining the effect of CS, the cells of vibrio suspended in 2 ml of RPMI along with 2 ml of CS of lactobacilli were added to the cell line-containing petridishes. After incubation for 1 h at 37°C, the dishes were washed four times with PBS (pH 7.2) to remove the unbound bacteria. The cells were fixed with 3 ml of methanol for 5–10 min at room temperature. The cells were air dried and stained with 3 ml of Giemsa stain solution (HiMedia) by incubating at room temperature for 30 min. The dishes were washed until no color was observed in the washing solution, dried in an incubator at 37°C overnight, and examined microscopically under oil immersion. The adherent lactobacilli in 25 random microscopic fields were counted for each test. Bacterial strains were scored as non-adhesive when fewer than 40 bacteria were present in 25 fields, adhesive with 41 to 100 bacteria in 25 fields, and strongly adhesive with more than 100 bacteria in 25 fields. The adhesion assay was performed in duplicate.

Simultaneously the other set of HCT-15-containing petridishes were subjected to the treatment with lysis solution (0.05% trypsin-EDTA) for 30 min at 37°C in order to lyse and detach the cells, and thereafter plated onto BHI agar plates for CFU counting.

Estimation of Lactic Acid Production

The percentage of lactic acid produced by *Lactobacillus* isolates in CS was determined by titration method. Briefly, the overnight grown lactobacilli culture in MRS broth was centrifuged at 9,000 g for 10 min at 4°C in cooling centrifuge. One ml of 0.5% phenolphthalein indicator dissolved in 50% ethanol was added to 9 ml CS and then the solution was titrated using 1N NaOH (SRL) till the appearance of light pink color. The percentage lactic acid in the CS was equal to the percent NaOH used to neutralize the acidity.

Probiotic Potential of Lactobacilli

Gastric Juice Tolerance and Bile Tolerance

The overnight grown lactobacilli were pelleted down by centrifugation at 9,000 g at 4°C for 10 min. The pellet was resuspended in simulated gastric juice having 2 g/l NaCl (HiMedia), 3.2 g/l pepsin and pH adjusted to 2.5 with conc. HCl (HiMedia). The suspension was incubated at 37°C for 3 h. Cells suspended in 1X PBS buffer (pH-7.2) was used as control. The viabilities of bacterial cells were evaluated by spreading onto MRS agar plates.

To determine bile tolerance, the serially diluted cultures were spread onto MRS agar plates supplemented with 0.3% oxgall (HiMedia). MRS agar plates without oxgall were used as control. The plates were incubated at 37°C in anaerobic jars. The colonies were counted after 24 h.

Biofilm Formation by Lactobacilli

Biofilm-forming abilities of lactobacilli isolates was determined in 96-well microtiter plate in MRS broth set at two different pH−4 and 6 by using crystal violet assay. Briefly, 135 µl of the autoclaved MRS broth was added to each well along with 15 µl of overnight grown lactobacilli culture having OD_{595} of 0.1. The microtiter plates were incubated at 37°C for different time periods−24, 48, and 72 h and the non adherent bacteria were removed by gently washing 3 times with the autoclaved distilled water. The biofilms were then fixed and stained as described previously. The sterile MRS broth was used as control. The experiment was performed in triplicates.

Based on obtained OD, strains were classified as –

(1) Non-biofilm producers $OD \leq OD_c$.
(2) Weak biofilm producers $OD_c < OD \leq 2OD_c$.
(3) Moderate biofilm producers $2OD_c < OD \leq 4OD_c$.
(4) Strong biofilm producers $4OD_c < OD$.

OD: OD of experimental well having lactobacilli cells in MRS broth.

OD_c: OD of control well having only MRS broth.

Autoaggregation Assay

Overnight grown bacterial cells were centrifuged at 9,000 g for 10 min at 4°C. The CS was discarded and pellet was diluted in PBS buffer (pH-7.2) to give final OD_{595} of 1. The suspension of lactobacilli was incubated at 37°C for 4 and 8 h. After incubation period, 1 ml of the suspension from the top of the tube was removed and its absorbance was determined at 595 nm. Autoaggregation percentage was determined using equation: $(1–A_t/A_0) \times 100$; where A_t is absorbance of suspension at different time points and A_0 is absorbance at beginning of experiment (0 h). The experiment was performed in triplicates.

Antibiotic Susceptibility Test

The antibiotic susceptibility profiles of all the lactobacilli isolates was analyzed by using Kirby-Bauer diffusion test (Bauer et al., 1966). The antibiotic discs (HiMedia): tetracycline, streptomycin, ciprofloxacin, moxifloxacin, gentamycin, ampicillin, pencillin, vancomycin, clindamycin, kanamycin, and erythromycin were used in this study. The classification as "susceptible," "intermediate," or "resistant" was based on the European Food Safety Authority (EFSA)-recommended breakpoints for diameters of zone of inhibition (EFSA, 2012).

Effect of Commercially Available Drugs on the Growth of Lactobacilli

The agar well diffusion assay was used to study the effect of commercially available drugs on growth of lactobacilli isolates. The various tablet formulations used in this study were–paracetamol(500 mg/ml), diclofenac (10 mg/ml), nimugesic (20 mg/ml), ibuprofen (120 mg/ml), cetrizine HCl (2 mg/ml), and lansoprazole(4 mg/ml).

The lactobacilli were grown at 37°C for 18 h in MRS broth. Following the incubation period, 100 μl of culture was spread onto MRS agar plates (HiMedia). Using sterile well-borer, wells were cut onto agar plates. An aliquot (100 μl) of various drugs were poured in wells and plates were placed at 4°C for diffusion. After 4 h, plates were incubated at 37°C overnight. The diameter of zone of clearance was noted in millimeters.

Statistical Analysis

The results from at least three independent experiments represented as mean ± SD (standard deviation). Comparisons were performed by using unpaired Student's *t*-test in GraphPad Prism 5.04 software. In autoaggregation experiments, comparisons were performed by using paired Students's *t*-test.

RESULTS

Characterization of the Lactobacilli Isolates by 16S rDNA Sequencing

On the basis of antimicrobial activity, 7 lactobacilli isolates were selected and characterized by 16S rDNA sequencing. BLAST analysis showed that L14 was 99% similar to *L. plantarum*, L32 and S45 was 98% similar to *L. fermentum* and *L. pentosus* respectively. L13, L18, S30, and S49 had <97% sequence matching with any known species and thus appear to be novel strains (**Table 1**).

Determination of Antimicrobial Activity of Lactobacilli Isolates Against Test Organisms

The well diffusion assay was used to determine the antimicrobial activity of *Lactobacillus* isolates. Out of 55 isolates, 7 isolates had antimicrobial activities against *V. cholerae, E. coli* and *S. enterica*. Five of the isolates had antimicrobial activity against *L. monocytogenes*, 4 of them against *Sh. flexeri*, and only 3 isolates (S30, S45, and S49) inhibited *St. aureus* (**Table 2**). None of the CS had any antimicrobial activities against the pathogens *V. parahaemolyticus* and *C. albicans*. Also, the CS of all the isolates had no antimicrobial activity against the gut commensal lactobacilli isolates when they were tested against each other (**Table 2**).

The CS of all the 7 isolates after 16 h of growth in MRS broth had final pH in the range 3–4 (data not shown). Therefore in order to determine whether the antimicrobial property of CS is due to low pH, the pH of CS was set to 6.5 and its antimicrobial activity tested. The antimicrobial activities of all the CS against all the pathogens was completely abrogated after neutralizing the pH to 6.5 (data not shown). Further, *Lactobacillus* spp. is known to produce hydrogen peroxide that exhibits antimicrobial activity against various Gram-negative bacteria (Pridmore et al., 2008). Therefore, to negate the role of hydrogen-peroxide, we treated the CS of all the lactobacilli isolates with catalase before evaluating their antimicrobial activity. Our results showed that the catalase treatment had no effect on the antimicrobial activity of CS (data not shown).

TABLE 2 | The antagonistic activities of the cell-free culture supernatants of fecal lactobacilli against various pathogenic and commensal indicator strains.

Indicator strains	Zones of inhibition (mm) ± S.D.						
	L13	L14	L18	L32	S30	S45	S49
V. cholerae	20 ± 0.3	16 ± 0.1	24 ± 0.3	18 ± 0.2	26 ± 0.2	19 ± 0.2	18 ± 0.2
S. enterica	11 ± 0.1	11 ± 0.2	6 ± 0.2	12 ± 0.1	9 ± 0.1	8 ± 0.2	9 ± 0.1
E. coli	11 ± 0.2	12 ± 0.2	9 ± 0.1	14 ± 0.3	12 ± 0.2	6 ± 0.1	9 ± 0.2
St. aureus	–	–	–	–	20 ± 0.2	22 ± 0.2	19 ± 0.1
L. monocytogenes	24 ± 0.3	–	25 ± 0.2	–	18 ± 0.1	23 ± 0.3	17 ± 0.1
Sh. flexeri	10 ± 0.1	14 ± 0.2	0.8 ± 0.1	–	10 ± 0.2	–	–
V. parahaemolyticus	–	–	–	–	–	–	–
Candida spp.	–	–	–	–	–	–	–
Lactobacillus L13	ND	–	–	–	–	–	–
Lactobacillus L14	–	ND	–	–	–	–	–
Lactobacillus L18	–	–	ND	–	–	–	–
Lactobacillus L32	–	–	–	ND	–	–	–
Lactobacillus S30	–	–	–	–	ND	–	–
Lactobacillus S45	–	–	–	–	–	ND	–
Lactobacillus S49	–	–	–	–	–	–	ND

ND: Not Done, –: No zone of inhibition, The CS of lactobacilli isolates without pH neutralization were tested for the antimicrobial activities by using agar-gel diffusion assay. The experiment was performed three times in triplicate. The results are expressed as the means ± standard deviations.

Effect of CS on the Growth Kinetics of *V. Cholerae* and *V. Parahaemolyticus*

The growth kinetics of *V. cholerae* in BHI media supplemented with non-neutralized and pH neutralized CS was studied. The results showed that the supplementation of BHI growth medium with non-neutralized CS of all the 7 lactobacilli strains significantly ($p < 0.001$) inhibited the growth of *V. cholerae* till 12 h relative to that observed in BHI supplemented with MRS (**Figure 1A**). Beyond 12 h, no significant differences in the growth kinetics of the wells with and without CS was observed. On the other hand, the supplementation of BHI with pH-neutralized CS of all the seven lactobacilli strains had no significant ($p < 0.001$) effects on the growth kinetics of *V. cholerae* (**Figure 1B**) at all time points. Similarly the effect of non-neutralized CS of all the lactobacilli strains on the growth of *V. parahaemolyticus* was tested and the results showed that none of the CS inhibited its growth (**Figure 1C**).

Effect of CS of Lactobacilli on Biofilm Formation of *Vibrio Cholera*

Before determining the effect of CS of *Lactobacillus* spp. on the biofilm-forming abilities of both *V. cholerae* and *V. parahaemolyticus*. the biofilm-forming potential of *V. cholerae* and *V. parahaemolyticus* in microtiter plates was determined. As shown in **Figure 2**, *V. cholerae* formed 2.5 times stronger ($p < 0.001$) biofilms as compared to *V. parahaemolyticus* after 24 h of growth.

Next, the effect of CS of lactobacilli isolates on the formation of biofilm by *V. cholerae* was evaluated in an *in vitro* assay. The pH non-neutralized CS of all the seven isolates resulted in more than 90% inhibition (**Figure 3**) of the biofilm formation by *V. cholerae*. Maximum inhibition was observed in case of isolates L13 (96%), L14 (95%), and L32 (95.6%).

Further, as the pH neutralization of CS abrogated its antimicrobial activity, we evaluated the effect of pH neutralized-CS on the biofilm formation by *V. cholerae*. The results showed that the pH neutralized-CS of all the isolates except L32 and L18 resulted in similar inhibition of the biofilm formation by *V. cholerae*. (**Figure 3**). In case of L32 and L18, the pH neutralization of CS significantly ($p < 0.0001$) reduced their potential to inhibit biofilm formation by 22 and 9%, respectively.

Effect of CS of Lactobacilli on the Dispersion of Biofilm of *V. cholerae*

As probiotic treatment are prescribed after the infection has established, thus dispersive action of probiotics on *V. cholera*

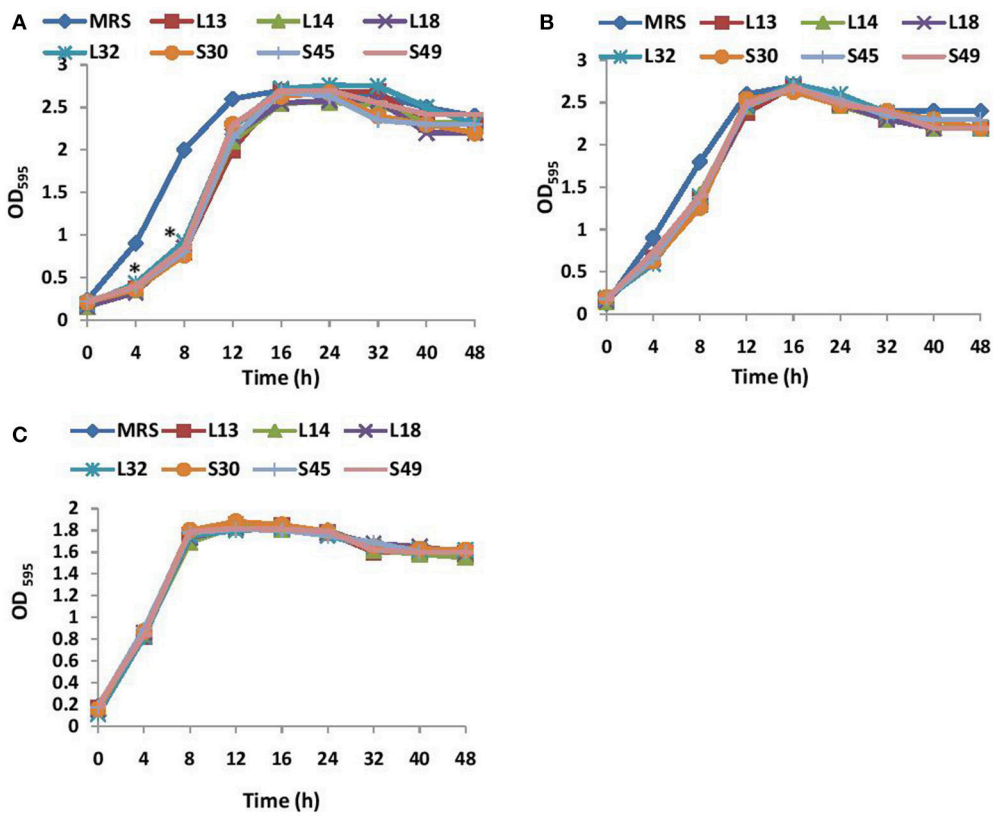

FIGURE 1 | (A) Growth curves of *V. cholera* in BHI supplemented with non-neutralized CS and **(B)** pH neutralized CS of lactobacilli strains. **(C)** Growth curve of *V. parahaemolyticus* in BHI supplemented with non-neutralized CS of seven different Lactobacilli strains. *Significance ($p < 0.001$) was measured by using an unpaired Student's *t*-test relative to the growth in BHI supplemented with MRS at 45 mg/ml.

biofilms may impart therapeutic benefits. Herein, the dispersion effect of CS at low pH (3.5) and after pH-neutralization was evaluated on the 24 h old preformed biofilms of *V. cholerae*. The results showed that CS of L14, S45, and S49 resulted in maximum dispersion of 85%, whereas the CS of the other lactobacilli isolates caused dispersion of *V. cholera* biofilms in the range 62–72% (**Figure 4**).

On adjusting the pH of CS to 6.5 the dispersive effect of the CS of the isolates S45, S49, and L18 was not affected; however, it was significantly ($p < 0.0001$) reduced in the case of four of the isolates viz. L13 (57% reduction), L14 (34%), L32 (19%), and S30 (19%). Thus, the dispersion effect of the CS is affected by pH neutralization in more than 50% of the isolates (**Figure 4**).

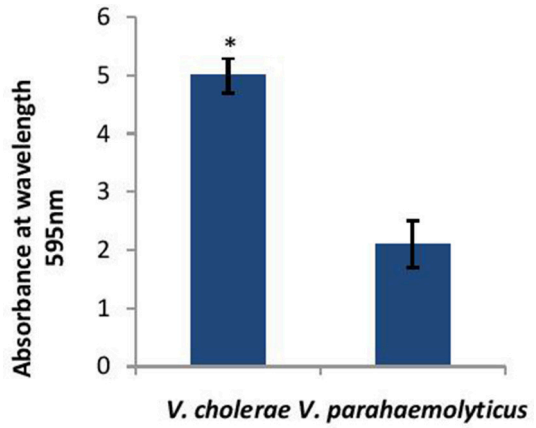

FIGURE 2 | Biofilm forming abilities of *V. cholerae* and *V. parahaemolyticus* evaluated by crystal violet assay performed in microtiter plate. Bars represent the mean and error bars represent standard deviation of three independent experiments. *Significance ($p < 0.001$) was calculated by using an unpaired Student's *t*-test.

Effect of CS of Lactobacilli on the Formation and Dispersal of Biofilm by *V. parahaemolyticus*

As the CS of lactobacilli had no antimicrobial activity against *V. parahaemolyticus,* therefore the inhibitory effects of only pH non-neutralized CS of lactobacilli on the biofilm formation by *V. parahaemolyticus* and its dispersion was determined (**Figure 5**). The CS of all the isolates, except S45, inhibited the biofilm formation by *V. parahaemolyticus* in the range 47–82%. Maximum inhibition of 82% was observed with the CS of the isolate S49 and L14, followed by S30 (72%), L32 (67%), L18 (62%), and L13 (47%). Interestingly, the CS of all the lactobacilli isolates had no or negligible biofilm-dispersing abilities.

Effect of CS of Lactobacilli on Adhesion of *Vibrio* to HCT-15 Cell Line

Adherence of *Vibrio* spp. to the intestinal epithelial cells is the primary step involved in the biofilm formation and pathogenesis of gut infection. Therefore, the effect of CS of lactobacilli on the initial adherence of pathogens-*V. cholerae* and *V. parahaemolyticus* with the intestinal cell line HCT-15 was evaluated by both plating technique and by using light microscopy. The binding of *V. cholera* to HCT-15 cell line was almost one log higher as compared to that of *V. parahaemolyticus*. Further, in the presence of CS of L18, there was significant ($p < 0.001$) reduction by approximately 2.5 $\log_{10}$ CFUs that adhered to HCT-15. On the other hand, the adherence of *V. parahaemolyticus* in the presence of the CS of L18 was not significantly reduced (**Figure 6**). The light microscopic images also showed similar effects (**Figure 7**). The CS of other lactobacilli isolates similarly reduced the adherence of *V. cholera* in the range 1.5–2.2 $\log_{10}$CFUs; whereas the adherence of *V. parahaemolyticus* was reduced in the range 0.6–0.9 $\log_{10}$CFUs (data not shown).

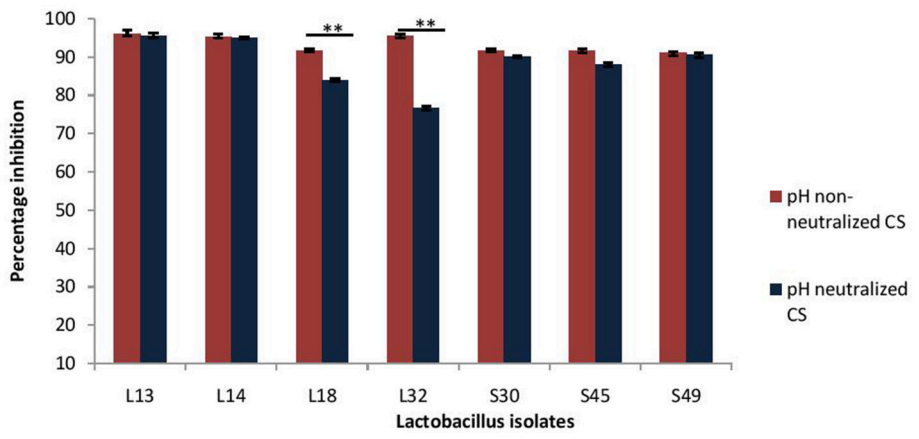

FIGURE 3 | Percentage inhibition of biofilm formation of *V. cholerae* by pH neutralized and non-neutralized CS of fecal *Lactobacillus* isolates evaluated by modified crystal violet assay performed in a microtiter plate. Bars are representative of the mean and error bars are representative of standard deviation of three independent experiments. **Significance ($p < 0.0001$) was measured by using an unpaired Student's *t*-test.

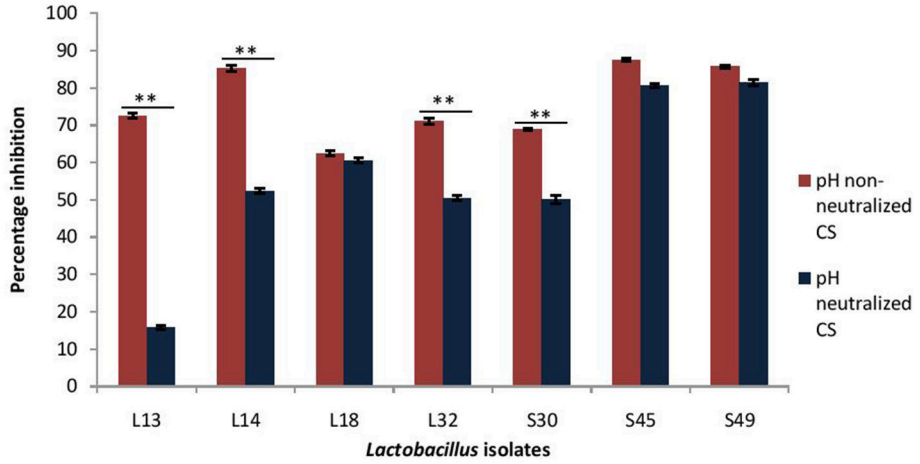

FIGURE 4 | Percentage dispersion of pre-formed biofilm of *V. cholerae* by pH neutralized and non-neutralized CS of fecal *Lactobacillus* isolates evaluated by modified crystal violet assay performed in a microtiter plate. Bars are representative of the mean and error bars are representative of the standard deviation of three independent experiments. **Significance ($p < 0.0001$) was measured by using Student's *t*-test.

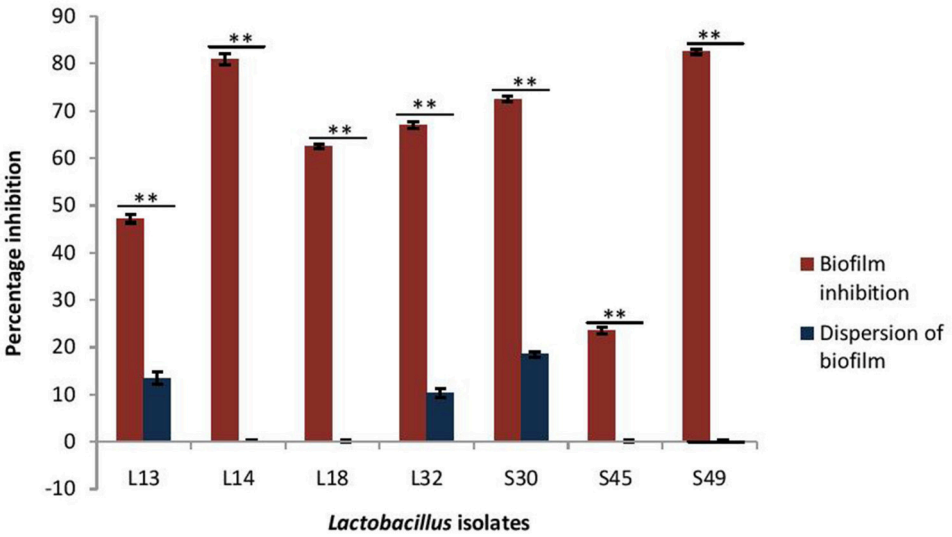

FIGURE 5 | Effect of CS of lactobacilli isolates on the biofilm formation and dispersion of 24 h old performed biofilms of *V. parahaemolyticus*. Bars are representative of the means and error bars represent standard deviation of three independent experiments. **Significance ($p < 0.0001$) was measured by using Student's *t*-test.

Lactic Acid Production Estimation

The lactic acid is the major organic acid produced by glucose fermentation by *Lactobacillus* spp. As biofilm-dispersive abilities in case of L13, L14, L32, and S30 is affected by pH neutralization, we evaluated the concentration of lactic acid in the CS. L13 produced highest amount of lactic acid followed by L32, S49, and L18 whereas S45 and L14 produced least amount of lactic acid (**Table 3**).

Probiotic Properties of Lactobacilli

The probiotic properties of lactobacilli is known to be strain specific, therefore we evaluated the probiotic potential of all the seven lactobacilli isolates. One of the most important criteria for the strain to be good probiotic is its ability to resist the action of bile salts and gastric juice during its passage in the GIT. As expected for the lactobacilli of fecal origin, all the isolates were able to survive after 3 h of incubation in the simulated gastric juice and also showed growth in the presence of bile salts (**Table 4**).

The ability of lactobacilli to form strong biofilms has been correlated with their abilities to persist *in vivo*. Therefore the abilities of the lactobacilli to form biofilms was evaluated at two different pH 4 and 6, and at different time intervals −24, 48, and 72 h. At pH 4 all the isolates except S45 and S49, formed strong biofilms at 48 and 72 h. Whereas, at pH 6, all the lactobacilli isolates formed strong biofilms after 48 h, but by 72 h biofilms

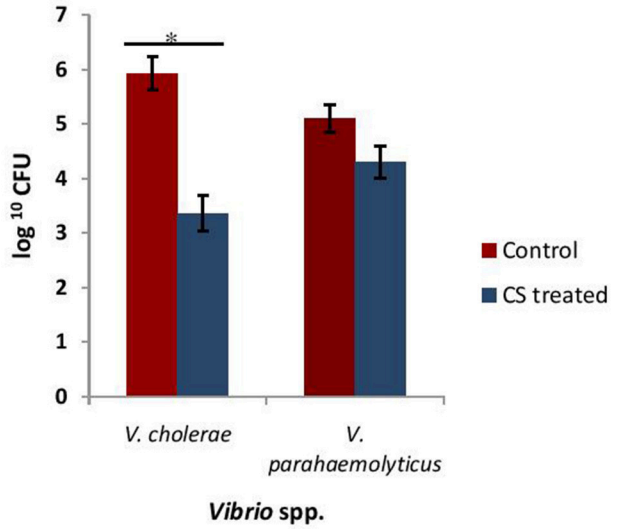

FIGURE 6 | The numbers of CFU of *Vibrio* spp. that adhered to HCT-15 cell line in the absence and presence of CS of L18. Bars are representative of the mean, whereas, error bars are representative of standard deviation of three independent experiments. *Significance ($p < 0.001$) was measured by using unpaired Student's *t*-test.

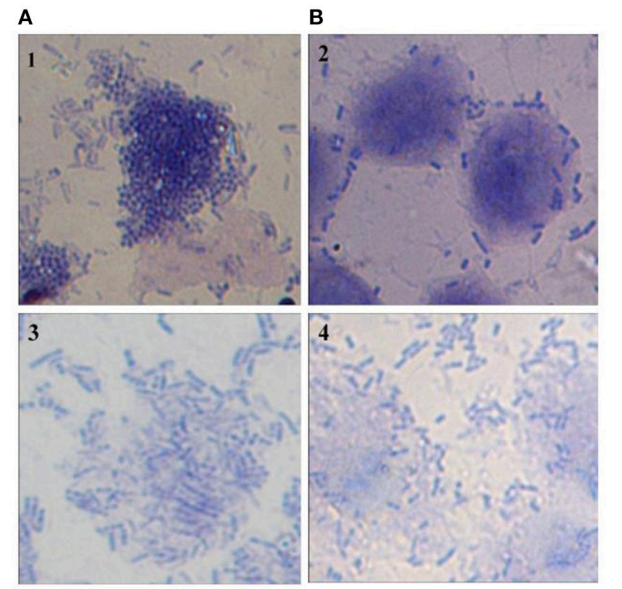

FIGURE 7 | Microscopic (1000X) image of Giemsa-stained HCT-15 cells showing adhered *V. Cholera* (1 and 2) and *V. parahaemolyticus* (3 and 4) in the absence **(A)** and presence **(B)** of CS of lactobacilli isolate L18.

TABLE 3 | Percentage of lactic acid produced by lactobacilli isolates.

Lactobacilli Isolates	% lactic acid produced
L13	1.60 ± 0.03
L14	1.18 ± 0.02
L18	1.31 ± 0.04
L32	1.42 ± 0.05
S30	1.27 ± 0.02
S45	1.17 ± 0.03
S49	1.40 ± 0.02

The lactic acid content of the CS was measured after 24 h of growth in MRS broth by titrating against NaOH. The experiment was performed three times in triplicates. The results are expressed as the means ± standard deviations.

TABLE 4 | Gastric juice and bile juice tolerance of lactobacilli isolates.

Lactobacilli Isolates	Gastric juice tolerance	Bile juice tolerance
L13	+++	+++
L14	++++	++++
L18	++++	++++
L32	+++	+++
S30	++++	+++
S45	++++	++++
S49	++++	+++

++++, no. of CFUs equal to that obtained in the control well.
+++, 0.2–0.6 log_{10} reduction in the CFUs as compared to control.
++, 0.6–2 log_{10} reduction in CFUs as compared to control.
+, more than 2 log_{10} reduction in CFUs.
–, no growth.

TABLE 5 | Biofilm formation by lactobacilli isolates at different pH and time points using crystal violet microtiter plate assay.

Lactobacilli Isolates	pH 4			pH 6		
	24 h	48 h	72 h	24 h	48 h	72 h
L13	S	S	S	S	S	S
L14	M	S	S	S	S	M
L18	S	S	S	S	S	S
L32	M	S	S	M	S	M
S30	S	S	S	M	S	M
S45	W	W	W	W	S	M
S49	W	W	W	S	S	M

S, Strong biofilm producers; M, Moderate biofilm producers; W, Weak biofilm producers. The lactobacilli biofilms were formed in MRS media of different pH in 96-well microtitre plates and analyzed at different time points by using crystal violet assay. Based on the OD_{595} of the wells, strains were classified as non-biofilm producers ($OD \leq OD_C$); weak ($OD_C < OD \leq 2 \times OD_C$); moderate ($2 \times OD_C < OD \leq 4 \times OD_C$) or strong biofilm producers ($4 \times OD_C < OD$); where OD_C is absorbance of control well having MRS broth with no cells and OD is absorbance of experimental well having MRS broth with lactobacilli cells. The experiment was performed three times in triplicates.

in all except L13 and L18 appeared to disperse and become moderate. L13 and L18 formed strong biofilms at all pH and time points (**Table 5**).

The maturation of biofilms depends on the autoaggregation properties of the lactobacilli. Thus, we assessed their abilities to autoaggregate. All the isolates showed significantly ($p < 0.0001$) higher auto aggregation (more than 90%) after 8 h of incubation that at 4 h (**Figure 8**).

Further, the ability of the lactobacilli isolates to adhere to the intestinal epithelial cell line HCT-15 was determined. Cell

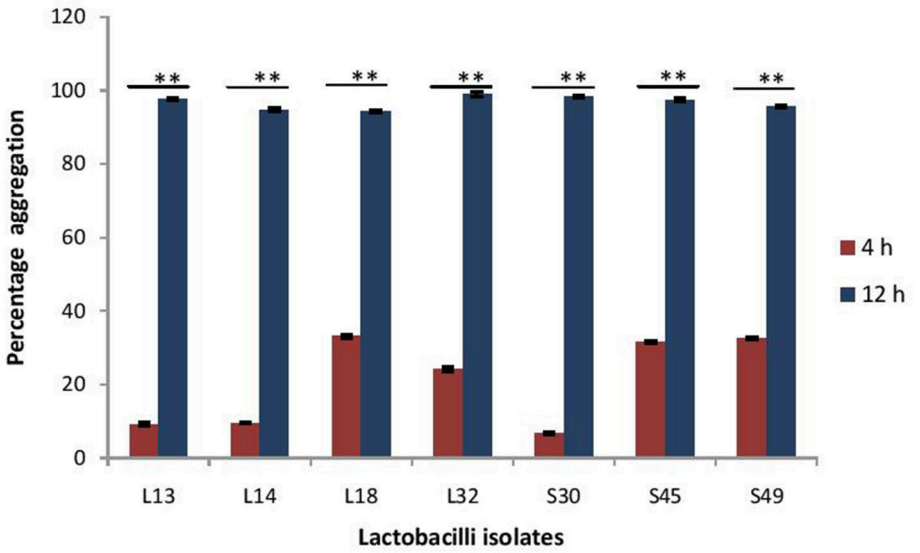

FIGURE 8 | Percentage auto-aggregation exhibited by lactobacilli isolates. Bars represent the mean, error bars represent standard deviation of three independent experiments and **significance ($p < 0.0001$) was measured by using paired Student's *t*-test.

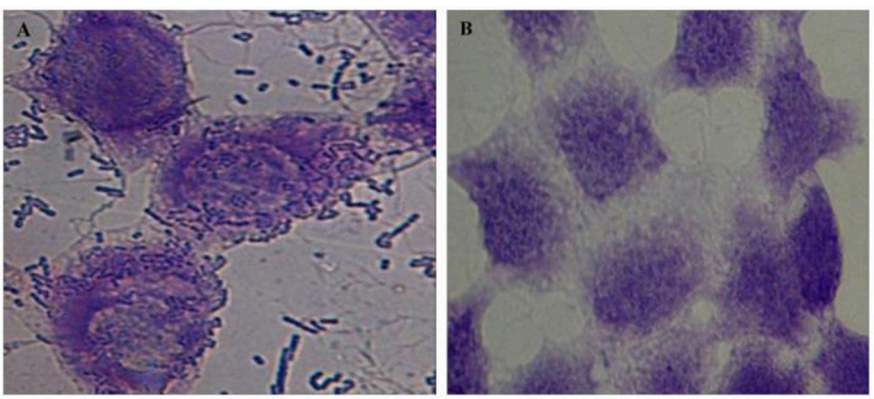

FIGURE 9 | Microscopic (1000X) image of Giemsa-stained intestinal cell line HCT-15 cells with **(A)** and without **(B)** adhered lactobacilli isolate L32.

adhesion assay demonstrated that all the selected lactobacilli isolates had strong adhesive ability to intestinal cell line – HCT-15 (**Figure 9**).

Antibiotic Susceptibility Test

Kirby Bauer disc diffusion method was used to analyze the susceptibility profiles of the lactobacilli isolates. Eleven antibiotics belonging to different classes: aminoglycosides (streptomycin, gentamycin, kanamycin), fluoroquinolones (ciprofloxacin, moxifloxacin), beta-lactams (ampicillin, penicillin), macrolides (erythromycin), glycopeptides (vancomycin), lincomycin (clindamycin), and tetracycline (tetracycline), were used in this study. The antibiotic profiles of all the isolates are summarized in **Table 6**. All of them were susceptible to tetracycline. All, except S45, were susceptible to penicillin and all, except S49, were susceptible to ampicillin, moxifloxacin, and erythromycin.

All the isolates were resistant to clindamycin. All except S45 were resistant to kanamycin and all except S45 and S49 were resistant to vancomycin, and ciprofloxacin. Similarly all, except L13 and S30 were resistant to streptomycin. In case of gentamycin, expect L13, L18, and L32, all were resistant.

Effect of Commercially Available Drugs on Growth of Lactobacilli

The effect of commercially available drugs on the growth of lactobacilli was evaluated. Paracetamol, nimugesic, cetrizine HCl, and lansoprazole had no antimicrobial activities against all the lactobacilli isolates. Ibuprofen inhibited the growth of L13, L14, and L18 whereas, diclofenac inhibited the growth of only L14 (**Table 7**).

TABLE 6 | Antibiotic susceptibility profile of fecal lactobacilli isolates.

Antibiotics (conc. in μg)	Lactobacilli Isolates						
	L13	L14	L18	L32	S30	S45	S49
TE (30)	S	S	S	S	S	S	S
S (10)	S	I	R	R	S	R	R
CIP(1)	R	R	R	R	R	S	S
MO (5)	S	S	S	S	S	S	I
GEN (10)	S	R	S	S	R	I	R
AMP (2)	S	S	S	S	S	S	R
P (2)	S	S	S	S	S	R	S
VA (30)	R	R	R	R	R	S	S
CD (2)	I	R	I	R	R	R	R
K (30)	R	R	R	R	R	S	R
E (15)	S	S	S	S	S	S	R

R: Resistant; I: Intermediate; S: Susceptible; TE: Tetracycline; S: Streptomycin; CIP: Ciprofloxacin; MO: Moxifloxacin; GEN: Gentamycin; AMP: Ampicillin; P: Penicillin; VA: Vancomycin; CD: Clindamycin; K: Kanamycin; E: Erythromycin.

The antibiotic susceptibilities of lactobacilli isolates were determined by Kirby Bauer method on MRS agar media and the classification as S, I and R was based on the recommended breakpoints for diameters of zone of inhibition as specified by EFSA (2012). The experiment was performed three times in triplicates.

TABLE 7 | Effect of commercially available drugs on growth of lactobacilli isolates.

Commercial drug (mg/ml)	Zone of inhibition (mm)						
	L13	L14	L18	L32	S30	S45	S49
Paracetamol (500)	–	–	–	–	–	–	–
Diclofenac (10)	–	15 ± 0.2	–	–	–	–	–
Nimugesic (20)	–	–	–	–	–	–	–
Ibuprofen (120)	15 ± 0.1	20 ± 0.2	12 ± 0.1	–	–	–	–
Cetrizine HCl (2)	–	–	–	–	–	–	–
Lansoprazole (4)	–	–	–	–	–	–	–

The antimicrobial effects of the commercial drugs were determined by agar well diffusion assay. The experiments were performed three times in triplicate. The results are expressed as means ± standard deviations.

DISCUSSION

Inhibition of biofilm formation by pathogens is an attractive target for therapeutic intervention (Bjarnsholt et al., 2013) that has received significant attention in recent years, leading to the discovery of biofilm inhibitors for many of the commonly encountered bacterial pathogens including *V. cholera* (Melander and Melander, 2015; Rabin et al., 2015). *V. cholerae* is well known for its ability to form strong biofilms *in vivo* on the intestinal mucosa in rabbit model (Jones and Freter, 1976; Nelson et al., 1976) and in humans (Yamamoto and Yokota, 1988). The experiments conducted in our laboratory also demonstrated strong *in vitro* biofilm-forming abilities of both *V. cholerae* and *V. parahaemolyticus* both in 96-well microtite plates and their abilities to bind to HCT-15 colonic epithelial cell line. The ability of *V. cholerae* to develop biofilms is critical to intestinal colonization (Silva and Benitez, 2016) and virulence (Xu et al., 2003; Fong et al., 2010). This is because, biofilm-derived cells could be more effective in competing for limiting nutrients in the small intestine, as suggested by elevated expression of the phosphate uptake system compared to planktonic cells (Mudrak and Tamayo, 2012). Also, biofilms of *V. cholerae* are reported to resist the action of acid inactivation in the gut (Zhu and Mekalanos, 2003). The biofilms once formed become resistant to the action of antibiotics as most of the conventional antibiotics are active only against planktonic *V. cholera* cells and have no biofilm-dispersive action (Warner et al., 2015). Thus, probiotics strains having both antimicrobial and anti-biofilm activities against *V. cholerae* are expected to be clinically superior.

We screened 55 fecal lactobacilli isolates for their antimicrobial potential against Gram-negative gut pathogens, out of which seven isolates having broad-spectrum antimicrobial activities against *V. cholerae*, *E. coli* and *S. enterica* were selected. Interestingly, the CS appears to inhibit specifically the gut-associated pathogens only and had no antimicrobial action against the commensal gut lactobacilli. However, when pH of the CS was neutralized, the antimicrobial activity was completely abrogated as shown by disappearance of zones of inhibition. This showed that low pH due to lactic acid or other organic acids secreted by the lactobacilli in the CS are responsible for the antimicrobial activities. Similar reports showing abrogation of antimicrobial activities of CS on pH neutralization has been reported earlier also (De Keersmaecker et al., 2006; Zhang et al., 2011). Lactic acid solution at concentration of 0.5% has been shown to effectively kill both Gram-negative (*Salmonella* and *E. coli*) and Gram-positive (*L. monocytogenes*) pathogens by causing cell membrane damage that resulted in leakage of proteins (Qiao et al., 2008; Wang et al., 2015). Thus, lactic acid in the CS (concentrations ranging from 1.17–1.8%) of all the lactobacilli isolates used in the study may be responsible for the antimicrobial activity. However the role of other organic acids for the antimicrobial activity cannot be negated. Also, the catalase enzyme treatment of CS did not altered the zones of inhibition, thereby showing that hydrogen peroxide is not responsible for the antimicrobial activity of CS. Next, the effect of non-neutralized CS on the growth kinetics of *V. cholerae* showed that it had bacteriostatic effects that significantly inhibited the growth till 12 h, beyond which the growth was similar to the wells with MRS. pH neutralized CS on the other hand had no significant effects on the growth kinetics of *V. cholerae*. Similarly, CS of all the lactobacilli strains had no effects on the growth kinetics of *V. parahaemolyticus*.

Further the effects of lactobacilli CS at low pH (3.5) and high pH (after pH neutralization to 6.5) was evaluated on the biofilm-forming ability of *V. cholera* because on pH neutralization the antimicrobial activity of CS is completely abrogated. Results demonstrated that CS at both low and high pH similarly inhibited the biofilm-formation by *V. cholera*. Further, in case of *V. parahaemolyticus* the CS of 5 out of 7 lactobacilli isolates inhibited 62–82% of the biofilm formation despite having no antimicrobial activity. The growth kinetics study also show that both the non-neutralized and the pH neutralized CS did not affect the growth of *Vibrio* at 48 h. Thus, the inhibition of

Vibrio spp. biofilm formation by lactobacilli CS was not due to its antimicrobial activity. The various components of CS of lactobacilli such as, exopolysaccharides (Kim et al., 2009) and biosurfactants (Walencka et al., 2008; Fracchia et al., 2010; Zakaria Gomaa, 2013) may inhibit the biofilm formation as reported against other pathogens. Purified EPS of *L. acidophilus* was shown to inhibit biofilm formation of a number of Gram-positive and Gram-negative pathogens. It was hypothesized that EPS interfered with the initial attachment of pathogen to small intestine cell line HT-29 (Kim et al., 2009). The physiological pH of mammalian stomach is acidic and that of intestine is toward neutral in the range 6–7. Thus, as the CS of all the lactobacilli isolates inhibited the biofilm formation at both low and high pH, these strains may have good prophylactic action against *Vibrio*-associated infection in both stomach and intestine. However, for the therapeutic action of lactobacilli spp., the dispersive action of CS against the preformed biofilms of *Vibrio* is important.

The CS at low pH dispersed the *V. cholerae* biofilm in the range 62–85%. Even on pH neutralization, the dispersal effect of CS of 6 isolates except L13 was in the range 50–75%; but it was reduced appreciably in the case of 4 isolates. However, none of the CS had any dispersal effects on the biofilm formation by *V. parahaemolyticus*. Thus, apparently the results showed that the biofilm dispersal effect of CS is dependent on its antimicrobial effect. But the role of CS components such as enzymes that caused the disintegration of *V. cholera* biofilm matrix but not that of *V. parahaemolyticus* cannot be negated. The *V. cholera* biofilm matrix is composed of exopolysaccharides containing glucose and galactose as the major components; whereas the biofilm matrix of *V. parahaemolyticus* is made up of capsular polysaccharides that contains many other sugar moieties apart from glucose and galactose (Yildiz and Visick, 2009). Thus, these differences may account for the differential dispersive effect. Further, CS may contain components that induced the secretion of quorum-sensing autoinducer and thereby caused dispersal of *V. cholerae* biofilms preferentially.

Further, we also assessed the abilities of CS of lactobacilli isolates to inhibit the adherence of both *V. cholerae* and *V. parahaemolyticus* to the intestinal epithelial cell line HCT-15. The CS of L18 was more effective at inhibiting the adherence of *V. cholerae* (2.5 $\log_{10}$ CFU reduction) to HCT-15 as compared to *V. parahaemolyticus* (0.7 $\log_{10}$ CFU reduction). Similar trend was observed with the CS of other isolates. The differences in the viable counts of *V. cholera* and *V. parahaemolyticus* on plating the lysed cell line can be due to the bactericidal action of CS against the *V. cholera* but not against *V. parahaemolyticus*. However, microscopic images of HCT-15 cells showed that L18 CS inhibited the binding of *V. cholera* more than *V. parahaemolyticus*.

Further, the probiotic potential of all the isolates were assessed. For an isolate to be a good oral probiotic candidate, it must have certain survival and adaptive characteristics such as resistance to the low pH, bile salts and various enzymes in the gut. All the *Lactobacillus* isolates showed more than 90% survival in the presence of bile salts and gastric juice. Probiotic bacteria having good biofilm-forming ability can prevent colonization of the gut epithelium by pathogenic bacteria (Jalilsood et al., 2015; Aoudia et al., 2016). Thus, the ability of probiotic bacteria to form biofilms and adhere to colonic cell line was evaluated. All the isolates showed strong adherence to the colonic cell line- HCT-15. Also all the isolates formed strong biofilms at pH-6 after 48 h; whereas, at pH 4 five of the isolates, except S45 and S49 formed strong biofilm. The maturation of biofilms is strongly dependent on the auto-aggregation properties of the probiotic bacteria as it helps the bacteria to form micro-colonies, which in turn secrete exopolysaccharides resulting in the maturation of biofilms. All the lactobacilli isolates used in this study exhibited more than 90% auto-aggregation after 8 h.

As probiotics are commonly used as adjuncts to antibiotic therapy, therefore the inherent resistance of lactobacilli to common antibiotics (Egervärn et al., 2009) was evaluated. Further the antibiotic susceptibility profiles to various classes of antibiotics could provide the hint to the presence of transferable resistance elements that can potentially be transmitted to the gut microbiota (Morelli et al., 1988). All the isolates were susceptible to tetracycline, and all except one were susceptible to erythromycin, ampicillin, penicillin, and moxifloxacin. High ciprofloxacin, gentamycin, streptomycin, and vancomycin resistance were observed among the isolates. The *Lactobacillus* species are known to be intrinsically resistant to vancomycin which is chromosomally-encoded and non-transmissible (Ruoff et al., 1988). Ciprofloxacin resistance in lactobacilli has been reported earlier among lactobacilli of fermented food (Kaktcham et al., 2012) and fecal origin (Shazali et al., 2014). Similar high resistance to aminoglycosides has been reported in probiotic lactobacilli (Zhou et al., 2005) and from fermented food (Kaktcham et al., 2012).

The probiotics are often prescribed along with various drugs such as, analgesic, anti-pyretic, non-steriodal anti-inflammatory drugs (NSAIDs), anti-allergic, and proton pump inhibitor. Therefore, the effect of these commonly used drugs on the growth of lactobacilli isolates need to be evaluated. The drugs - paracetamol, nimugesic, cetrizine hydrochloride and lanzoprazole had no inhibitory effects on the growth of isolates except ibuprofen which inhibited the growth of lactobacilli isolates–L13, L14, and L18 and diclofenac inhibited L14. Previous study also demonstrated the inhibitory effects of sodium diclofenac on the growth of *L. plantarum* ST8KF and ST341LD (Todorov and Dicks, 2008).

All the seven *Lactobacilli* isolates used in this study had broad-spectrum antimicrobial and biofilm-inhibitory activities. Thus, they may have good prophylactic properties to inhibit the gut-associated infectious diseases. The isolates S45, S49, and L18 seemed to possess the best biofilm dispersion abilities at both low and high pH, therefore their therapeutic potential to treat *V. cholera* infection in the mouse model should be further tested.

AUTHOR CONTRIBUTIONS

SukK conceived the idea and supervised the experiments. SumK and SukK designed the experiments and SumK performed almost all of the experiments, except those involving cell lines. PS, NK, and JS designed and performed the cell line studies. All authors discussed the results and contributed to the final manuscript.

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Comparisons Between Bacterial Communities in Mucosa in Patients With Gastric Antrum Ulcer and a Duodenal Ulcer

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Objective: To identify and compare the bacterial community profile of mucosal tissues from a gastric antrum ulcer and a duodenal ulcer in *Helicobacter pylori* (*Hp*) positive dyspeptic patients.

Methods: Genomic DNA was extracted from the mucosal tissues obtained from 18 patients diagnosed with gastric antrum or duodenal ulcers. A library was constructed using 16S rRNA gene amplification, and Miseq high-throughput sequencing was used to analyse the amplified products. Bioinformatics methods, including operational taxonomic units (OTUs), hierarchical clustering, and a diversity analysis, were performed to investigate and characterize the community composition.

Results: The proportion of *Helicobacter* in the mucosa of patients with a gastric antrum ulcer was significantly higher than that of patients with a duodenal ulcer. However, the diversity of the bacterial community in the gastric antrum ulcer mucosa was significantly lower compared with the mucosa of the duodenal ulcer. There were significant differences in microbial community structure between the gastric antrum ulcer and the duodenal ulcer. Notably, *Helicobacter*, *Prevotella*, *Neisseria,* and *Streptococcus* were also predominant genera in the bacterial community of the duodenal ulcer mucosa, and they outnumbered those species in gastric antrum ulcer mucosa.

Conclusion: The bacterial community composition and the corresponding abundance differ between the mucosal tissues of *Hp* positive gastric antrum ulcer and duodenal ulcer patients. Additionally, the bacterial community diversity in the mucosal tissues from gastric duodenal ulcer patients is higher than that from gastric antrum ulcer patients, and *Helicobacter* is not the absolutely predominant genus.

Keywords: microbial community structure, *Helicobacter*, gastric antrum ulcer, duodenal ulcer, 16S rRNA

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INTRODUCTION

Gastric ulcers and duodenal ulcers are two common peptic diseases seen in the clinic. A *Helicobacter pylori* (*H. pylori*) infection is an important causal factor (Graham and Shiotani, 2008) associated with more than 80% of peptic ulcers (Alam et al., 2014). Previous studies have reported that the occurrence of ulcers leads to alterations in the stomach microbiota (Li et al., 1995; Zhang et al., 1996), by aerobic bacteria, anaerobic bacteria, fungal isolation, and culture. However, this method has its limitations; it could not fully demonstrate the change of stomach microbiota in patients with peptic ulcers.

Recent years have witnessed the rapid development of high-throughput sequencing technology, especially the wide use of the bacterial 16S rRNA-encoding gene sequence analysis, which has made a significant improvement in the identification of bacterial lineages and their relative abundance in the gastric microbial community. For example, in non-*Hp* infected patients, Zilberstein et al. (2007) found predominantly acid resistant species—*Veillonella* sp. *Lactobacillus* sp. and *Clostridium* sp.—in the digestive tract microbiota, while in *H. pylori*-infected patients, some non- *Hp* bacterial genera such as *Streptococcus*, *Neisseria*, *Staphylococcus*, and *Roche* were also identified in gastric biopsy tissues (Hu et al., 2012). Nevertheless, *Hp* infection is a well-acknowledged causative agent of peptic ulcers (Groenen et al., 2009), and there have been few reports about the microbial structure of different ulcer types, such as gastric ulcers and duodenal ulcers.

The mechanisms of ulcer formation are diverse and include excessive gastric acid secretion and the use of non-steroidal anti-inflammatory drugs (NSAIDs), and these mechanisms are secondary to those in Crohn's disease and tuberculosis (Yuan and Tang, 2008). In recent years, the incidence of non- *Hp* non-NSAIDs-related ulcers has been on the rise (Hou and Zhang, 2015). From a microbial ecology viewpoint, the number of *Hp* that have colonized is not the only reason for a peptic ulcer. Other non- *Hp* bacteria may participate in the development of two common ulcers. Additionally, few studies have paid attention to the tissue structure of gastric and duodenal ulcers. The relevance of the *H. pylori* content and the proportion of other bacteria to ulcer formation and other issues also urgently require resolution.

In this study, to compare the bacterial community profile of the mucosa of a gastric antrum ulcer and a duodenal ulcer, we utilized the Miseq high-throughput sequencing technique and analysis to identify and compare the structural, biological diversity and abundance of the mucosal microbiota of 18 patients with *H. pylori*-positive gastric antrum and duodenal ulcers. The composition of the mucosal microbiota from the different ulcer types can provide a theoretical basis to understand the relevance of the mucosal flora to ulcer disease.

MATERIALS AND METHODS

Study Population

Gastric and duodenal biopsy samples were obtained between 2016 and 2017 from patients referred for an endoscopy examination at the First People's Hospital of Wenling. Biopsy samples were taken from the antrum and the body of the stomach for each patient. This study was approved by the First People's Hospital of Wenling Medical Ethics Committee, and written consent was obtained from the patients before they were included in the study.

All patients were outpatients admitted in the First People's Hospital of Wenling diagnosed with gastric and duodenal ulcers. Each patient received a ^{13}C urea breath test (^{13}C using a 75 mg dose) before endoscopy, and the test results were positive. All patients never had *Hp* eradication before gastroscopy. And we surveyed the patients' information, such as histopathological data, diet habits, economic status, and family history.

Specimen Collection and Processing

Each patient underwent endoscopic mucosal tissue sampling, and two mucosal tissue specimens were collected in parallel at the same biopsy site. Specifically, 3–5 cm of tissue around the gastric antrum ulcer surface was collected from gastric antrum ulcer patients, and tissue around the duodenal ulcer surface was collected for duodenal ulcer patients. For each patient, one of the collected mucosal tissue samples was placed in an *Hp* isolation culture tube containing a brain-heart infusion and the other was in a sample tube containing DNA preservation solution.

Bacterial Growth and Identification

After the sample was received, the tissue was first placed at room temperature, and the gastric mucosal tissue was sucked into a sander with a Pasteur pipette and then sufficiently ground to homogenize it; it was then inoculated on a 5% defibrinated sheep blood agar plate and evenly spread. The inoculated plate was placed in a 37°C three-gas incubator (5% oxygen, 10% carbon dioxide, 85% nitrogen), cultured for 3–11 days, and the growth was observed. Suspected colonies were smeared for microscopic observation of bacterial morphology consistent with *Hp*, and strains that were urease, oxidase, and catalase positive were classified as *Hp* positive, otherwise they were classified as *Hp* negative. Gastric antrum ulcer *H. pylori* positive patients were designated group A (antrum), and duodenal ulcer *Hp* positive patients were designated group B (duodenal).

DNA Isolation, Library Preparation and Sequencing

Genomic DNA from all the mucosal flora samples was extracted using an Invitrogen Purelink Genomic DNA kit (Life Technologies, Carlsbad, CA, USA). The operation was carried out strictly according to the kit instructions. The qualitative and quantitative detection of nucleic acids was accomplished by 1% agarose gel electrophoresis and a Qubit2.0 concentration analyser.

A specific primer with a "sequencing linker" was synthesized for the 16S rRNA V3 and V4 regions of mucosal flora samples. The first step PCR primer sequence was 356F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; 806R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3′. The PCR reaction system contained 2 × HiFi buffer 10 μL, forward primer 5 μmol/L, reverse primer 5 μmol/L, DNA 10 ng, plus ddH_2O to

the total volume of 20 μL. PCR reaction conditions: 95°C, 3 min, 25 cycles including 95°C 30 s, 60°C 30 s, 72°C 30 s; 72°C 5 min.

After PCR amplification, the PCR products were purified with Backman magnetic beads. Briefly, 20 μL of the PCR reaction product was mixed with 16 μL of magnetic beads to adsorb the DNA, and then washed with 80% ethanol twice and eluted with 40 μL of ddH_2O to obtain the purified PCR product. Then, the second step PCR reaction system contained 2 × HiFi buffer 10 μL, forward primer 10 μmol/L, reverse primer 10 μmol/L, first PCR Purify the product template 2 μL, plus ddH2O to a total volume of 20 μL. The PCR reaction conditions were 95°C, 3 min, 12 cycles including 95°C 30 s, 60°C 30 s, 72°C 30 s; 72°C 5 min. After completion of the PCR reaction, purification was accomplished according to the previously mentioned magnetic beads purification protocol, and finally, 30 μL of ddH_2O was eluted to obtain a DNA library of mucosal samples.

Quality control of all the libraries was conducted using an Agilent 2100 Bioanalyzer analyser and a Qubit 2.0 concentration analyser. The library that passed the qualification was sequenced using an Illumina MiSeq sequencer.

Analysis of 16S rRNA Gene Sequences

The original image data of the sequencing results were analyzed by the CASAVA software (v1.8.2), and the sequencing data were obtained after a preliminary quality analysis. Pandaseq (v2.7) was used to compare every two sequences and assemble them according to the end of the overlapping area. Using Trimmomatic (v0.30), the primers and linker sequences, and bases with a mass <20 at both ends as well as sequences with lengths <400 bp were also removed. Using usearch (v8.0), the remaining sequences were compared with those in the database, and the chimeric sequences were removed to obtain the final validated data. Operational Taxonomic Units (OTUs) are clustered sequences into bins, clustering was performed by the UCLUST method with the parameter similarity set at 97%, and an OTU list and OTU representative sequences were obtained. The sequences were randomized using the Qiime platform (v1.7), and dilution curves were constructed using the number of sample sequences and the number of OTU they could represent. According to the results of the OTU clustering, ACE, Chao1, Shannon, and Simpson were used to analyse the abundance and diversity of the mucosal samples. Using the Qiime platform (v1.7), a principal component analysis was performed based on the UniFrac distance.

Statistical Analysis

Statistical analyses were performed using SPSS19.0 software. The comparison of the continuity variables was analyzed using a *t*-test, and the classification of the data was analyzed using the chi-square test or Fisher's exact test. A P value ≤ 0.05 was considered as statistically significant.

RESULTS

Patient Clinical Data

In this study, 20 cases were enrolled in the study group, including 10 cases of gastric antrum ulcer and 10 cases of duodenal ulcer. The age range of the patients was 33–67 years, with an average age of 45.3 years and a male to female ratio of 1.857: 1. After *Hp* isolation and culture, one case of gastric antrum ulcer and one case of duodenal ulcer were determined to be *Hp* negative and removed. Thus, a total of 9 *Hp* positive cases remained for the gastric antrum ulcer group. The average age of the patients was 48.4 years, and the male to female ratio was 1.25: 1. For the duodenal ulcer *Hp* positive group of nine patients, the average age was 41 years and the male to female ratio was 2: 1. There was no significant difference between the two groups for age ($p > 0.05$, *T*-test) or sex ratio ($p > 0.05$, x^2 test). There was also no significant difference in histopathological data, diet habits, economic status, and so on between two groups (**Table 1**).

Diversity Analysis

A total of 1,564,734 primitive sequences were obtained by high-throughput sequencing of the 16S rRNA gene of the mucosal samples using a two-step PCR amplification method. Of these sequences, 1,520,613 sequences were effective, with a coverage index of 97.18%. The sequences were sorted by the 97% similarity of the OTUs, resulting in a total of 32513 OTUs. The number of OTUs was 21578 in the *Hp* positive gastric antrum ulcer patients (group A) and 24226 in the *Hp* positive duodenal ulcer patients (group B) (**Table 2**). As shown in the sample dilution curve in **Figure 1**, the number of OTU in the dilution curve increases with the number of sequences without reaching a plateau but tends to reach the platform stage. This indicates that sequencing quantity for each sample was sufficient to characterize the composition of the sample flora. The bacterial diversity in mucosa of duodenal ulcer patients was significantly higher than that of the gastric antrum ulcer patients (**Figure 1**).

Bacterial Community Composition in a *Hp* Positive Gastric Antrum Ulcer vs. a Duodenal Ulcer

As shown in **Figure 2A**, the predominant bacterial phyla in the two ulcer types were *Proteobacteria*, *Bacteroidetes,* and *Firmicutes*. It was noteworthy that *Proteobacteria* accounted for the major proportion in the *Hp* positive gastric antrum ulcers, whereas *Bacteroidetes* and *Firmicutes* accounted for the major proportion in the *Hp* positive duodenal ulcers, and the difference was statistically significant ($p < 0.05$, x^2 test).

The bacterial community composition at the genus level in the two ulcer types is shown in **Figure 2B**. Overall, the proportion of either the predominant or unknown bacteria was significantly higher for the *Hp* positive duodenal antrum ulcer than that for the *Hp* positive gastric ulcer. *Helicobacter* accounted for the major proportion in the *Hp* positive gastric antrum ulcer, with an over 40% ratio. However, *Helicobacter* was not the predominant bacterial genus in the *Hp* positive duodenal ulcer, only present in less than a 10% proportion in some samples. Notably, bacterial genera including *Prevotella* (9.83%), *Neisseria* (5.03%), *Streptococcus* (3.96%), *Veillonella* (1.92%), *Streptococcus* (3.96%), and *Porphyromonas* (2.62%) were also present in the *Hp* positive duodenal ulcer. Furthermore, the genera *Psychromonas*, *Frankia*, *Prevotella,* and *Porphyromonas* were identified and significantly

TABLE 1 | The patients' information.

Groups	Sex	Age	Diet habits				Economic status	Family history		Histopathological data		
			Wine	Salty	Vegan	Spicy		Stomach cancer	Hypertension	Ulcer size(cm)	Lymphoid hyperplasia	Activity
A1	male	42	U	S	No	Much	Middle	No	Yes	1.0*1.5	No	Yes
A2	male	67	U	S	No	Little	Middle	No	No	0.5*0.5	No	Yes
A3	male	51	S	U	No	Much	Middle	No	No	0.3*0.7	No	Yes
A4	male	40	No	No	No	Little	High	No	No	0.3*0.3	Yes	No
A5	female	60	S	No	No	Little	Middle	No	No	0.3*0.3	Yes	Yes
A6	female	36	S	S	No	Little	Middle	No	Yes	0.3*0.5	No	Yes
A7	male	54	No	S	No	Much	Middle	No	No	1.0*0.5	No	Yes
A8	female	46	No	S	No	No	High	No	No	0.3*0.4	No	Yes
A9	female	40	No	S	No	Little	Low	No	No	0.6*1.5	No	Yes
B1	male	35	No	S	No	Little	High	No	No	0.5*0.6	Yes	No
B2	female	39	S	U	No	Much	Low	No	Yes	0.3*0.4	No	No
B3	male	42	U	S	No	Little	Middle	No	No	0.3*0.5	No	No
B4	male	37	U	S	No	Little	High	No	Yes	0.5*0.4	No	Yes
B5	female	52	No	S	No	Little	Low	No	No	0.5*0.5	No	Yes
B6	male	42	S	S	No	Little	High	No	No	0.4*0.5	Yes	No
B7	female	33	U	No	No	Little	High	No	Yes	0.4*0.5	No	Yes
B8	male	46	No	U	No	Little	Middle	Yes	No	0.3*0.6	No	Yes
B9	male	43	U	U	No	Much	Low	No	Yes	0.6*1.2	No	No

"S" represents "sometimes", "U" represents "usually."

TABLE 2 | Bacterial community α-diversity indexes for *Hp* positive gastric ulcer types.

Group	OTUs	ACE	Chao1	Shannon	Simpson	Coverage
Antrum	21578	10224.67	9518.19	3.81	0.51	0.97
Duodenal	24226	10349.09	9561.53	7.61	0.91	0.96

OTUs, Operational Taxonomic Units.

distinguished the *Hp* positive duodenal ulcer samples ($p < 0.05$, x^2 test).

We then determined the proportion of colonization of *Hp* in the *Hp* positive gastric antrum and the duodenal ulcers. As shown in **Figure 2C**, the average proportion of *Hp* in the gastric antrum ulcer samples was 76.44%, and it was 24% in the duodenal ulcer samples. The difference was statistically significant ($p < 0.01$, x^2 test).

Bacterial Community Diversity in the *Hp* Positive Gastric Antrum and the Duodenal Ulcer

We investigated the bacterial community diversity of the two *Hp* positive ulcer types by first calculating the four α-diversity indexes, including the Chao1, Shannon, Simpson, and ACE indexes. For the *Hp* positive gastric antrum ulcers, the four indexes were 9518.19, 3.81, 0.51, and 10224.67 (**Table 2**). For the *Hp* positive gastric duodenal ulcer, the four indexes were 9561.53, 7.61, 0.91, and 10349.09 (**Table 2**). The ACE and Chao1 indexes represent the bacterial community richness in a single sample. Clearly, there were no significant differences in ACE and Chao 1 for the two ulcer types ($p > 0.05$, t-test, **Figures 2A,B**), indicating no difference in the total number of identified bacterial species for the two ulcer types. For the Shannon and Simpson indexes, a higher value indicates greater the bacterial community diversity.

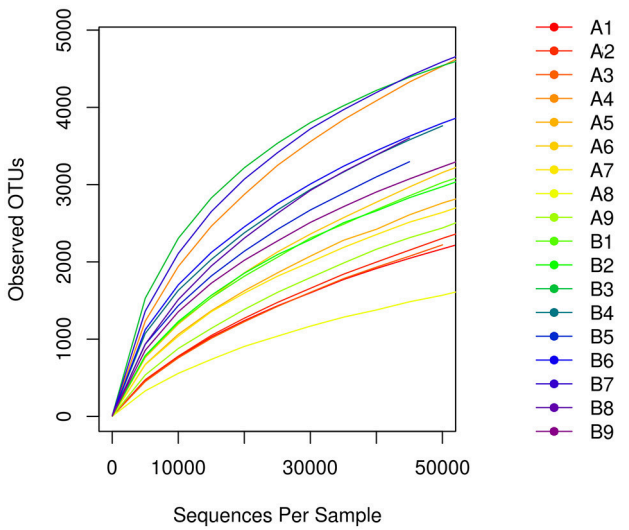

FIGURE 1 | The number of OTUs of the dilution curve increases with the number of sequences.

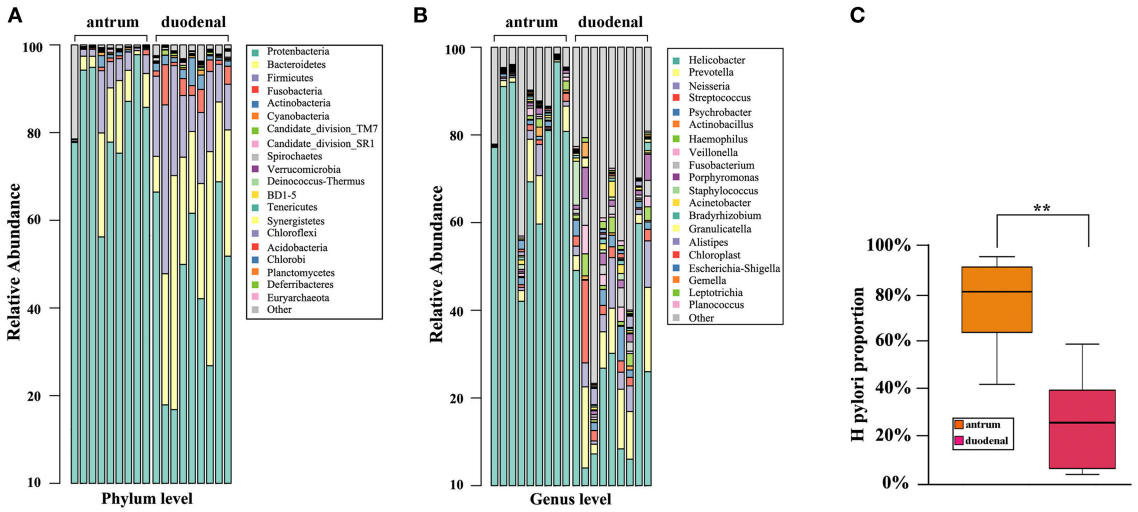

FIGURE 2 | Bacterial community composition for the *Hp* positive gastric antrum and duodenal ulcer types identified at the **(A)** phylum, **(B)** genus levels, and **(C)** groups. **Statistical significant difference in the two groups.

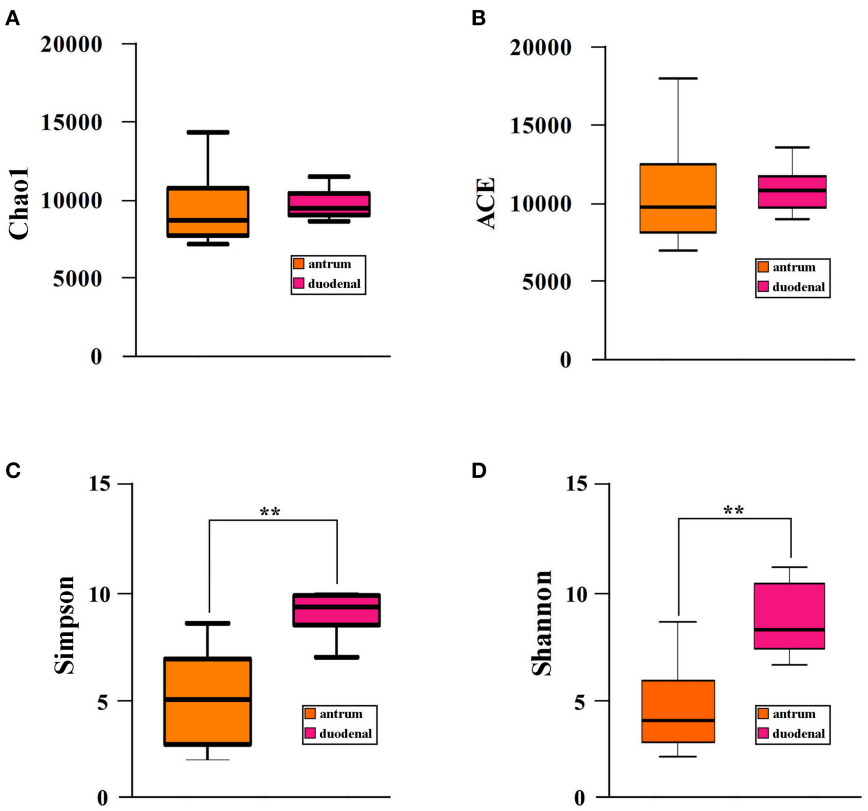

FIGURE 3 | Bacterial community α-diversity **(A)** Chao1, **(B)** ACE, **(C)** Simpson, and **(D)** Shannon, indexes for the *Hp* positive gastric antrum and duodenal ulcers. **Statistical significant difference in the two groups.

As shown in **Figures 3C,D**, the calculated value of the Shannon and Simpson indexes for the *Hp* positive gastric antrum ulcer was significantly lower than that of the *Hp* positive gastric duodenal ulcer ($p < 0.01$, *T*-test), indicating a lower bacterial community diversity.

We then performed clustering based on an unweighted distance matrix and a principal component analysis to characterize the β-diversity for both *Hp* positive gastric ulcer types, which compared the bacterial community difference between every two samples. As shown in **Figure 4**, the main

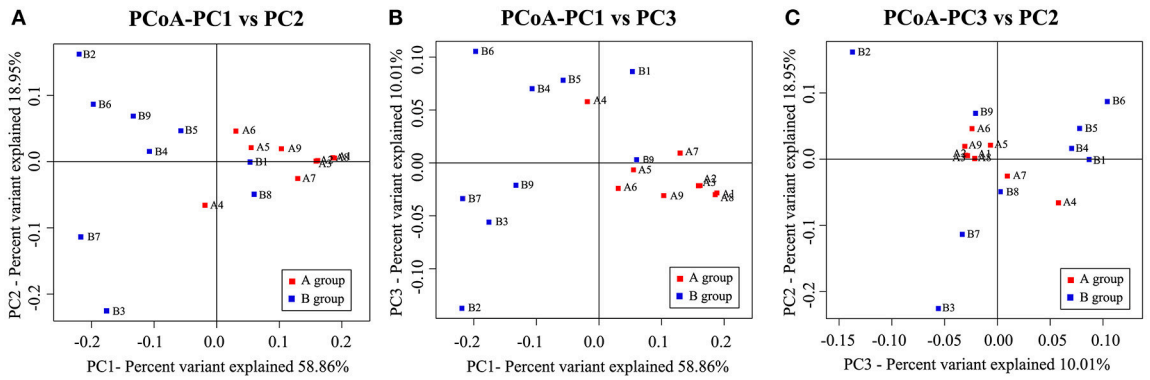

FIGURE 4 | Principal components analysis (PCoA) for bacterial community β-diversity in the *Hp* positive gastric antrum and duodenal ulcer samples. **(A)** PCoA using PC1 and PC2 as main axes; **(B)** PCoA using PC1 and PC3 as main axes; **(C)** PCoA using PC3 and PC2 as main axes.

axis 1 (PC1), 2 (PC2), and 3 (PC3) can explain 58.86, 18.95, and 10.01% of the variation, respectively. It is noteworthy that the samples of the *Hp* positive gastric antrum ulcer were mainly clustered in the positive-value quadrant, and their distribution was relatively concentrated. The samples of the *Hp* positive gastric duodenal ulcer were mainly clustered in the negative-value quadrant, and their distribution was relatively dispersed. This indicated that the intra-sample similarity is higher for the *Hp* positive gastric duodenal ulcer than for the *Hp* positive gastric antrum ulcer.

DISCUSSION

Previously, it has been well acknowledged that an acidic environment in the stomach and other antibacterial factors are not suitable for bacterial colonization; thus, the stomach should be in a relatively sterile state (1981). With the development of molecular biology and sequencing technology, *Hp* was first discovered in the stomach, and then other microorganisms were also identified (1981; Wang and Yang, 2014). The close relationship between an *Hp* infection and peptic ulcers has long been known; for example, in Japan, 94% of patients with gastric ulcer and 98% of patients with duodenal ulcer have an associated *Hp* infection (Alam et al., 2014). In Middle, duodenal ulcers occur in the duodenal bulb, and gastric ulcers occur in the antrum and other parts of the stomach. Even though *Hp* is the leading cause of ulcers, few studies found differences in the microorganism populations between the two ulcer types. In this study, we compared the mucosal flora structure in 18 cases of *Hp* positive gastric antral ulcer and duodenal ulcer.

Comparing bacteria at the phylum level, we found that Mycobacterium, Bacteroides, and *Firmicutes* were predominant in both ulcer types, which was consistent with the results reported by Bik et al. (Bik et al., 2006). In a genus level comparison, we also identified *Prevotella*, *Neisseria,* and *Streptococcus* in the *Hp* positive mucosal tissues in both ulcer types, despite the predominance of the bacterium *Helicobacter*. These bacteria are mainly found in the mouth and in food, and their presence has been confirmed in the stomach of the *Hp* negative patients in earlier studies (Jiang et al., 1990; Chen, 2004). Using a mass spectrometry biotyping analysis, Hu et al. (2012) found that *Streptococcus* and *Neisseria* were predominant bacterial genera in *Hp* positive gastric mucosal tissues. Using a 16S rDNA high-throughput sequencing analysis (Li et al., 2009; Ahn et al., 2013) also found that *Streptococcus* and *Neisseria* were predominant bacterial genera inout *Hp* negative gastric antral ulcer patients, and their colonization clearly affected that of *Neisseria* and *Streptococcus* as predominantly observed in the stomach of *Hp* negative patients.

However, few literature reports about the duodenal mucosal floral structure exist. Our study shows that in *Hp* positive duodenal ulcer patients, the proportion of *Helicobacter* is significantly lower than that of *Hp* positive gastric antrum ulcer patients (**Figure 1C**), whereas *Pseudomonas*, *Neisseria,* and *Streptococcus* accounted for a relatively larger proportion, so *Helicobacter* was not the only predominant genus. In addition, the proportion of *Bacteroides* and *Streptomyces* in patients with duodenal ulcers was significantly higher than that in patients with gastric antrum ulcers; this outcome was also the case for the bacterial genera *Halophilus*, *Frankesia*, *Prevobacteria,* and *Porphyromonas*. An explanation might be that the duodenum is part of the small intestine, which is closer to the jejunum and therefore will be affected by the diversity of the intestinal flora. It might also result from the fact that mucosal cells in the duodenum do not secrete gastric acid, and the relatively high pH value is more suitable for the growth of other flora.

With respect to biodiversity, the bacterial diversity in the mucosal tissues of patients with an *Hp* positive duodenal ulcer was significantly higher than that in patients with an *Hp* positive gastric antrum ulcer (**Figures 3A–D**, **4**). The clinical course of gastric ulcers is usually longer than that of duodenal ulcers and has a relatively higher probability of oncogenesis. The relationship between the lower diversity of gastric flora and oncogenesis has been reported (Ahn et al., 2013). Another advantage of floral diversity is that the metabolites are richer, especially the metabolites that are probiotics, which can inhibit the proliferation of the *Hp* to a certain extent Song (2012). Thus, the findings of our study highly suggest that gastric ulcer

treatment can be improved by eradicating the *Hp* infection together with probiotic adjuvant therapy to rebuild the diversity of gastric flora and maintain the stability of the stomach micro-ecological environment.

In conclusion, we compared the bacterial community composition and diversity in the mucosal tissues of *Hp* positive duodenal ulcer and gastric antrum ulcer patients using a 16S rRNA sequencing analysis and identified substantial differences. We believe that similar studies involving more patients that use other bacterial detection methods will further elucidate the relationship between the floral structure and different types of peptic ulcers, which will finally benefit clinical therapies.

AUTHOR CONTRIBUTIONS

XC and ZW conceived and designed the study; CX, QL, and LJ contributed to collected samples; LZ performed the laboratory tests; XC and ZW participated in analyzing data and writing the manuscript. All authors read and approved the final manuscript.

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Profiling the Urinary Microbiota in Male Patients With Bladder Cancer in China

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Mounting evidence indicates that microbiome plays an important role in the development and progression of cancer. The dogma that urine in healthy individuals must be sterile has been overturned. Dysbiosis of the urinary microbiome has been revealed responsible for various urological disorders, including prostate cancer. The link between chronic inflammation, microbiome and solid tumors has been established for various neoplastic diseases. However, a detailed and comprehensive analysis of urinary microenvironment of bladder cancer has not been yet reported. We performed this study to characterize the potential urinary microbial community possibly associated with bladder cancer. Mid-stream urine was collected from 31 male patients with bladder cancer and 18 non-neoplastic controls. DNA was extracted from urine pellet samples and processed for high throughput 16S rRNA amplicon sequencing of the V4 region using Illumina MiSeq. Sequencing reads were filtered using QIIME and clustered using UPARSE. We observed increased bacterial richness (Observed Species, Chao 1 and Ace indexes; cancer vs. control; 120.0 vs. 56.0; 134.5 vs. 68.3; and 139.6 vs. 72.9, respectively), enrichment of some bacterial genera (e.g., *Acinetobacter*, *Anaerococcus, and Sphingobacterium*) and decrease of some bacterial genera (e.g., *Serratia*, *Proteus,* and *Roseomonas*) in cancer group when compared to non-cancer group. Significant difference in beta diversity was found between cancer and non-cancer group, among different risk level, but not among different tumor grade. Enrichment of *Herbaspirillum*, *Porphyrobacter,* and *Bacteroides* was observed in cancer patients with high risk of recurrence and progression, which means these genera maybe potential biomarkers for risk stratification. The PICRUSt showed that various functional pathways were enriched in cancer group, including *Staphylococcus aureus* infection, glycerolipid metabolism and retinol metabolism. To our knowledge, we performed the most comprehensive study to date to characterize the urinary microbiome associated with bladder cancer. A better understanding of the role of microbiome in the development and progression of bladder cancer could pave a new way for exploring new therapeutic options and biomarkers.

Keywords: urinary bladder neoplasms, urinary tract, microbiota, extracellular matrix, inflammation

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INTRODUCTION

Bladder cancer is diagnosed in more than 430,000 patients worldwide every year, making it the ninth most common malignancy (Kamat et al., 2016). In addition, most of bladder cancer patients are male, five to six times more common than female in China (Li et al., 2015). In the past decades, bladder cancer has aroused scientists' attention for its high morbidity and mortality rates. Unfortunately, the etiology and pathophysiology of bladder cancer remain unknown. It may be caused by genetic mutations and external risk factors, including tobacco smoking, carcinogen exposure, the chlorination of drinking water and possibly cyclophosphamide (Babjuk et al., 2017).

Notably, all above factors are known to affect the composition of microbiota, the ensemble of symbiotic bacteria, fungi, parasites, and viruses that inhabit the epithelial barrier surfaces of our body (Costello et al., 2012). The microbiome affects human physiological functions, such as metabolism, immunity and haematopoiesis (Dzutsev et al., 2015). In addition, the microbiome also plays a role in the development of malignancies both at epithelial barriers and in tissues (Roy and Trinchieri, 2017). Studies suggest that microbial dysbiosis at various body sites may promote disease progression, such as periodontitis, inflammatory bowel disease, colorectal cancer and breast cancer (Darveau, 2010; Irrazábal et al., 2014; Chu et al., 2016; Urbaniak et al., 2016). Recently, emerging evidence overturns the dogma that urine in healthy individuals must be sterile (Whiteside et al., 2015). Furthermore, dysbiosis of the urinary microbiome has been revealed responsible for various urological disorders, such as urgency urinary incontinence, interstitial cystitis, overactive bladder and prostate cancer (Siddiqui et al., 2012; Pearce et al., 2014; Wu et al., 2017; Shrestha et al., 2018).

Accordingly, it is conceivable that alteration of urinary microbiome may be associated with bladder cancer. Although the link between specific pathogens and cancer is well established, such as *Helicobacter pylori* and gastric cancer (El-Omar et al., 1997), there is currently no hard evidence linking microbiome and bladder cancer. Intravesical instillation of *Mycobacterium bovis* bacillus Calmette–Guérin and oral administration of *Lactobacillus* after removal of the bladder tumor could reduce the probability of recurrence (Aso et al., 1995; Zitvogel et al., 2017). Dysbiosis caused by repeated antibiotic use can increase the incidence of cancers, including bladder cancer (Boursi et al., 2015). In addition, a case-control study showed that regular probiotic intake reduced the risk of bladder cancer in the healthy population (Ohashi et al., 2002). Taken together, these results strongly support the hypothesis that microbiome might be involved in bladder carcinogenesis, progression and relapse.

However, despite mounting researches on the human microbiome have yielded multiple insights into health and disease including cancers (Thomas et al., 2016), a detailed and comprehensive analysis of microbiota in urine of bladder cancer has not been yet reported. A recent study suggested that microbiome may be a factor in bladder cancer pathology and further studies on the urinary microbiota of bladder cancer would direct urologists to new therapeutic and prognostic options (Bucevic Popovic et al., 2017). Our primary purpose was to characterize urinary microbiota associated with bladder cancer in China and to explore the role of microbiome in bladder carcinogenesis.

MATERIALS AND METHODS

Subject Recruitment and Specimen Collection

Urine specimens were collected from male patients with bladder cancer and non-neoplastic patients admitted to Nanfang Hospital in China between March 2017 and September 2017. All cancer cases were histologically confirmed as urothelial carcinoma and male controls were cases for a wide spectrum of non-neoplastic conditions, such as renal cyst. Subjects with prior known sexually transmitted infection or a recent history of urinary tract infections or antibiotic usage for any indication (within 1 month) were excluded. All subjects were required to finish a structured questionnaire to collect information on socio-demographic characteristics. Data collection followed the principles outlined in the Declaration of Helsinki. All participants had signed a written informed consent to contribute their own anonymous information to this study. Our study was approved by the Medicine Institutional Review Board of Southern Medical University.

Mid-stream urine specimens were collected by the clean catch method under the guidance of urotherapy nurses, then centrifuged at 16,000 g for 10 min immediately and stored at −80°C until further processing.

DNA Isolation and 16S rRNA Gene Sequencing

To avoid contamination, DNA isolation was performed using the cultured cells protocol supplied with the DNeasy Blood and Tissue Kit (Qiagen, Germany) in a laminar flow hood. The concentration of extracted DNA was determined through a Nanodrop ND-1000 spectrophotometer (Thermo Electron Corporation, USA). The genomic DNA isolated from the clinical samples was amplified using primer sets specific for V4 regions (515F: GTGCCAGCMGCCGCGGTAA; and 806R:GGACTACHVGGGTWTCTAAT). In order to evaluate contribution of extraneous DNA from reagents, extraction negative controls (no urine) and PCR negative controls (no template) were included. The resultant PCR products were purified by Qiaquick PCR purification kit (Qiagen, Valencia, CA). Finally, purified samples were normalized to equal DNA concentration and sequenced using the Illumina Miseq sequencer (Illumina, Inc., USA). The 16S rRNA gene sequences have been submitted to the Short Read Archive (SRA) under accession number SUB3915640.

Bioinformatics Analysis

Raw data were filtered to eliminate reads with adapter pollution and low quality to obtain clean reads by using QIIME (Caporaso et al., 2010). Filtered sequences were clustered by 97% identity into operational taxonomic units (OTUs) using UPARSE (Edgar, 2013), and subsequently, a single representative sequence from each clustered OTU

was used to align to the SILVA database (Quast et al., 2013) and the Greengenes database (DeSantis et al., 2006) by Ribosomal Database Project Classifier (Wang et al., 2007).

QIIME was used to evaluate alpha diversity, which is composed of the Observed Species, Chao1, Shannon, Simpson and Ace indexes. Among them, the Observed Species, Chao1 and Ace indexes are indicators of species richness, while Shannon and Simpson indexes are indicators of species diversity. The difference of alpha diversity between groups was evaluated by Wilcoxon Rank-Sum Test (group number $= 2$) and Kruskal–Wallis test ($n > 2$) using SPSS (version 22).

To compare microbial composition between groups, beta diversity was evaluated by calculating the Bray Curtis, weighted UniFrac and unweighted UniFrac distances. Principal coordinate analysis (PCoA) was applied to generate three-dimensional plots in QIIME based on these distance matrices. The PERMANOVA was performed to test for statistical significance between groups using 999 permutations in QIIME.

Differential abundance analysis between groups was performed using Metastats and *P*-values were adjusted for multiple hypothesis testing using the False Discovery Rate based on the Benjamini-Hochberg (White et al., 2009). To identify significantly different bacteria between groups, taxa summaries were reformatted and input into Linear discriminant analysis effect size (LEfSe) via the Huttenhower Lab Galaxy Server (Segata et al., 2011). The Kruskal-Wallis rank sum test and Wilcoxon test were used to identify biomarkers, and linear discriminant analysis (LDA) was used to score them. Only taxa with logarithmic LDA score greater than 2 at a $P < 0.05$ were considered significantly enriched. To predict the functional pathways from microbiota composition data, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was performed for reconstruction of metagenome (Langille et al., 2013). Predicted functional genes were categorized into Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology and compared across patient groups using STAMP (version 2.1.3, Parks et al., 2014).

Statistical Analysis

Data are presented as median (first quartile to the third quartile) for continuous variables or number of cases (%) for counts data. The statistical significance of differences between groups were evaluated using Mann-Whitney U-test for continuous variables and Pearson's chi-square test or Fisher's Exact Test for count data through SPSS software (Version 22.0). All tests were two sided and $P < 0.05$ were considered statistically significant. PASS programme (PASS 11, NCSS, Kaysville UT, USA) provides estimates of power by simulation. Estimates were obtained 2000 simulations. We estimated that with 31 patients in cancer group and 18 subjects in control group, we would have 90% power to detect differences at the 0.05 significance level (alpha) using a two-sided Mann-Whitney Test.

RESULTS

Subjects and Samples Characteristics

A total of 60 samples were analyzed, including 35 from male cancer patients and 25 from male non-neoplastic controls, while 4 samples in cancer group and 7 samples in control group were excluded for samples with too little sequencing reads (Supplementary Table 1). Detailed information on clinical characteristics and pathological parameters can be found in **Table 1** and Supplementary Table 1. No significant difference was observed in the demographic characteristics between cancer and non-cancer group, except cigarette smoking (**Table 1**).

Cancer group was composed of 26 patients with non-muscle-invasive bladder cancer (NMIBC) and 5 patients with muscle-invasive bladder cancer (MIBC). The 2004 WHO grading system categorized cancer group as papillary urothelial neoplasm of low malignant potential (PUNLMP, $n = 5$) or low-grade papillary urothelial carcinoma group (LG, $n = 11$) or high-grade papillary urothelial carcinoma group (HG, $n = 15$) (Supplementary Table 2). Based on European Organization for Research and Treatment of Cancer (EORTC) scoring system (Lughezzani et al., 2011) (Supplementary Table 3), NMIBC group were stratified into lower risk of recurrence group (LER, recurrence score of EORTC ≤ 4, $n = 16$) and higher risk of recurrence group (HER, recurrence score of EORTC ≥ 5, $n = 10$), lower risk of progression group (LEP, progression score of EORTC ≤ 6, $n = 15$) and higher risk of progression group (HEP, progression score of EORTC ≥ 7, $n= 11$), respectively (Supplementary Table 2).

Sequencing Data, Alpha, and Beta Diversity

A total of 4,427,184 clean reads were obtained from the 49 samples. The median number of reads in cancer patients was 105,440, and in the non-cancer patients was 98,289 (**Table 1**, $P > 0.05$). The reads were classified into 1,653 OTUs that were used for downstream analysis. More OTUs were identified in urine from cancer patients, with an average of 120 OTUs in cancer group and 56 OTUs in control group ($P = 0.008$). Higher Observed Species index (richness, $P = 0.008$), Chao1 index (richness, $P = 0.008$), Ace index (richness, $P = 0.003$), Shannon index (diversity, $P > 0.05$) and lower Simpson index (diversity, $P > 0.05$) were presented in cancer group, which indicates that bacterial richness significantly increased in cancer patients while difference of species diversity was not significant (**Figures 1A–E**, **Table 1**).

Of interest was that we found that bacterial richness increased in HER group and HEP group, compared to LER group and LEP group, respectively (Supplementary Figures 1A–E, 2A–E; Supplementary Table 4), though no significant association was found between alpha diversity and tumor grade (Supplementary Figures 3A–E; Supplementary Table 5).

A comparison of urine from cancer patients and that from non-cancer controls showed significantly different bacterial profiles on unweighted UniFrac PCoA plots (**Figure 1F**). The PERMANOVA performed on the data set showed that the observed differences were statistically significant ($F = 1.97$, $P < 0.05$; $F = 2.53$, $P < 0.001$; and $F = 1.61$, $P < 0.05$, for weighted UniFrac, unweighted UniFrac and Bray

TABLE 1 | Comparisons of demographic characteristics and parameter of alpha diversity between cancer patients and non-cancer controls.

	Cancer (n = 31)	Non-cancer (n = 18)	P-value
DEMOGRAPHIC			
Age (y)	64.0 (49.0, 69.0)	55.5 (45.8, 64.8)	Ns
BMI	22.9 (20.8, 24.2)	22.1 (20.1, 23.4)	Ns
Smoking habit	24 (77.4)	7(38.9)	0.007
Smoking index	600 (50, 800)	150 (0, 300)	0.002
Drinking habit	8 (25.8)	8 (44.4)	Ns
Hypertension	10 (32.3)	4 (22.2)	Ns
Diabetes	6 (18.2)	1(5.6)	Ns
Hyperlipidemia	2 (6.5)	2 (11.1)	Ns
CHD	3 (9.7)	1 (5.6)	Ns
FHC	1 (3.0)	0 (0)	Ns
ALPHA DIVERSITY			
Number of reads	105440.0 (83300.0, 107694.0)	98289.0 (60820.0, 107601.0)	Ns
Observed species	120.0 (77.0, 147.0)	56.0 (49.0, 107.3)	0.008
Chao1	134.5 (82.5, 172.4)	68.3 (54.4, 116.8)	0.008
Ace	139.6 (41650.0, 53847.0)	72.9 (54.9, 111.9)	0.003
Shannon	2.4 (1.6, 2.9)	2.3 (1.8, 2.8)	Ns
Simpson	0.2 (0.1, 0.4)	0.2 (0.1, 0.3)	Ns

Data were presented as median (first quartile to the third quartile) for continuous variables or n (%) for counts. BMI, body mass index; CHD, coronary atherosclerotic heart disease; FHC, family history of cancer; Ns, not significant (based on P < 0.05).

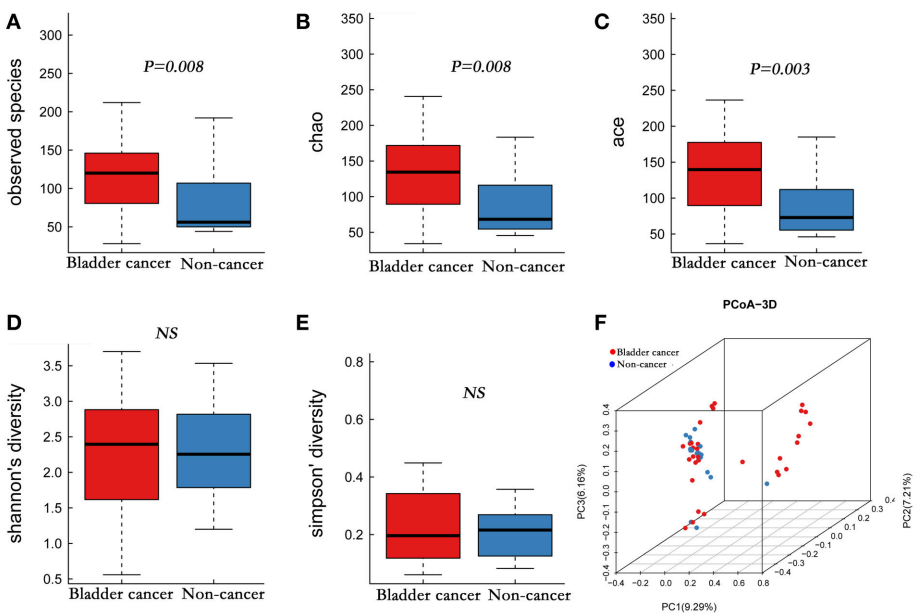

FIGURE 1 | Alpha and principal coordinate analysis (PCoA) for bladder-cancer samples and non-cancer samples. **(A–E)** Box-plot showing alpha diversity in samples using different metrics (**A,** observed species index; **B,** Chao1 index; **C,** Ace index; **D,** Shannon index; **E,** Simpson index). **(F)** PCoA plots of unweighed UniFrac distances in which samples were colored by clinical outcome. The PERMANOVA performed on the unweighted UniFrac distances showed that the observed differences were statistically significant (999 permutations; $F = 2.53$; $P < 0.001$).

Curtis distances, respectively). In addition, a clear hierarchical clustering of cancer samples was observed on dendrogram based on unweighted UniFrac distance metric (**Figure 2**). We next studied whether the microbial profile was different among different risk level and tumor grade. The results showed that the microbiota composition of patients with high risk of recurrence and progression was significantly different from that of patients with low risk of recurrence and progression (PERMANOA, $F =$

2.31, $P < 0.01$, LER vs. HER; $F = 2.40$, $P < 0.01$, LEP vs. HEP, for weighted UniFrac distances). However, no significant difference in the microbiota profiles of bladder cancer was observed for tumor grade (Supplementary Figures 1F, 2F, 3F, Supplementary Table 6).

Relative Abundance of Urinary Bacteria in Cancer and Control Samples

At phylum level, the urinary microbiota was dominated by *Proteobacteria* (39.7% cancer, 49.0% control) and *Firmicutes* (32.8% cancer, 28.1% control), followed by *Actinobacteria* (7.0% cancer, 6.2% control) and *Bacteroidetes*(3.9% cancer, 9.4% control) (**Figure 3A**, **Table 2**).The microbial composition of all samples at class, order and family level were demonstrated in **Figures 3B–D**. In addition, the genera compositions of all samples were demonstrated in **Figure 2**. Though bacterial relative abundance at phylum, class or order level differed between cancer and non-cancer group, no significant difference was found using Metastats algorithm after False Discovery Rate adjustment (**Table 2**). However, it is notable that *Sphingobacteriaceae* ($P = 0.047$) was significantly more abundant in patients and *Thermoactinomycetaceae* ($P = 0.005$) in control group at family level, while *Acinetobacter* ($P = 0.048$) were significantly more abundant in cancer patients and *Serratia* ($P = 0.003$), *Proteus* ($P = 0.003$), *Laceyella* ($P = 0.003$) in non-cancer group at genus level (**Table 2**).

Specific Genera Associated With Bladder Cancer

The LEfSe, which allows for identifying specific taxa associated with cancer, showed significantly higher compositional abundances of *Acinetobacter, Anaerococcus, Rubrobacter, Sphingobacterium, Atopostipes, Geobacillus* in cancer patients and *Serratia, Proteus, Roseomonas, Ruminiclostridium-6, and Eubacterium–xylanophilum* in control group at genus level (**Figures 4A,B**). Further analysis showed that *Acinetobacter* (cancer vs. control; 31 of 31 vs. 15 of 18, $P = 0.044$), *Anaerococcus* (cancer vs. control; 19 of 31 vs. 5 of 18, $P = 0.035$), *Rubrobacter* (cancer vs. control; 30 of 31 vs. 11 of 18, $P = 0.002$), *Sphingobacterium* (cancer vs. control; 13 of 31 vs. 2 of 18, $P = 0.024$), *Atopostipes* (13 of 31 vs. 2 of 18, $P = 0.024$) and *Geobacillus* (cancer vs. control; 12 of 31 vs. 1 of 18, $P = 0.017$) were detected in more cancer group samples than control group samples.

Since distinct difference of microbial profile was observed among groups with different risk of recurrence and progression, we next identified the specific taxa associated with high risk of recurrence and progression by using LEfSe analysis. The results showed that 6 genera were overrepresented in patients with high risk of recurrence and 4 genera in patients with high risk of progression, including *Herbaspirillum*, *Gemella*, *Bacteroides*, *Porphyrobacter*, *Faecalibacterium*, *Aeromonas* in HER group and *Herbaspirillum*, *Porphyrobacter*, *Bacteroides*, *Marmoricola* in HEP group (Supplementary Figures 4, 5).

TABLE 2 | Comparison of relative abundance of urinary microbiome between cancer group and control group at all taxonomic levels.

Taxa		Cancer	Non-cancer	*P*-value	FDR
Phylum	*Proteobacteria*	39.704	48.954	Ns	Ns
	Firmicutes	32.840	28.144	Ns	Ns
	Actinobacteria	7.073	6.183	Ns	Ns
	Bacteroidetes	3.932	9.439	Ns	Ns
Class	*Gammaproteobacteria*	31.218	39.250	Ns	Ns
	Bacilli	28.863	20.054	Ns	Ns
	Actinobacteria	6.143	5.771	Ns	Ns
	Betaproteobacteria	5.061	6.317	Ns	Ns
Order	*Enterobacteriales*	17.435	26.133	Ns	Ns
	Bacillales	17.112	12.756	Ns	Ns
	Lactobacillales	11.790	7.431	Ns	Ns
	Corynebacteriales	3.839	1.733	Ns	Ns
	Bacteroidales	3.049	8.619	Ns	Ns
Family	*Enterobacteriaceae*	17.470	26.173	Ns	Ns
	Staphylococcaceae	12.213	10.161	Ns	Ns
	Streptococcaceae	9.286	4.567	Ns	Ns
	Thermoactinomycetaceae	0.000	0.30185	0.001	0.005
	Sphingobacteriaceae	0.225	0.012	0.012	0.047
	Carnobacteriaceae	0.140	0.022	Ns	Ns
Genus	*Escherichia-Shigella*	15.373	24.460	Ns	Ns
	Staphylococcus	12.154	10.123	Ns	Ns
	Streptococcus	9.280	4.587	Ns	Ns
	Aeromonas	3.782	0.018	0.046	Ns
	Acinetobacter	3.252	1.092	0.018	0.048
	Bacteroides	0.064	1.584	0.028	Ns
	Lactobacillus	1.176	2.626	Ns	Ns
	Serratia	0.000	0.984	0.001	0.003
	Proteus	0.000	0.312	0.001	0.003
	Laceyella	0.000	0.304	0.001	0.003
	Fusobacterium	0.049	0.016	Ns	Ns

Data were reported as mean percentage; FDR, P-value after false discovery rate adjustment; Ns, not significant (based on P < 0.05).

Potential Functional Pathways Associated With Bladder Cancer

To infer the functional pathways based on the microbial community profiles we utilized PICRUSt. Overall, the microbial profiles present in patients with bladder cancer and non-cancer could not be distinguished clearly based on their functions (**Figure 5A**). The predicted KEGG pathways significantly enriched in bladder cancer included *Staphylococcus aureus* infection, glycerolipid metabolism, retinol metabolism, ethylbenzene degradation and carotenoid biosynthesis (**Figure 5B**).

DISCUSSION

In this study, we have characterized the urinary microbial profile of bladder cancer by using 16S rRNA gene sequencing and the results showed that bacterial richness significantly increased in

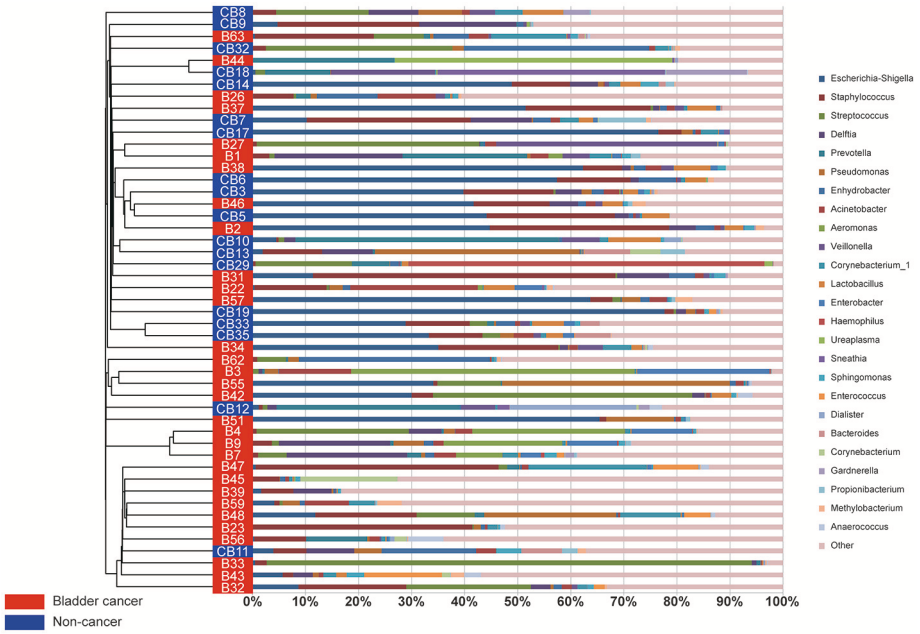

FIGURE 2 | The urinary microbiota profile of participants. A clear hierarchical clustering of cancer samples was observed in the dendrogram at genus level (**left**; based on the unweighted UniFrac distance metric). In the histogram **(right)**, each colored box represents a bacterial taxon and each bar, a subject. The height of a colored box represents the relative abundance of that organism within the sample. Bacterial genera with a relative abundance <0.5% and unclassified genera are grouped as "Other".

bladder cancer patients. In addition, higher bacterial richness was presented in urine samples with higher risk of recurrence and progression, which suggests that higher bacterial richness may be a potential indicator of high risk of recurrence and progression of NMIBC.

A reduction in microbial diversity has now been considered as a feature of gut disease, such as ulcerative colitis, Crohn's disease and colorectal cancer (Lepage et al., 2011; Ahn et al., 2013; Gevers et al., 2014). However, no consistent changes in microbial diversity were found among urinary tract disorders. Increased microbial diversity was observed in urgency urinary incontinence (Pearce et al., 2015), reduced diversity was found in interstitial cystitis (Siddiqui et al., 2012) and overactive bladder (Wu et al., 2017), while no significant difference in microbial diversity was found in prostate cancer (Shrestha et al., 2018).

Similar to microbiome inhabiting the gut, inter-individual microbial community heterogeneity of the urinary tract is influenced by lots of genetic and environmental factors, such as spatial distribution or lifestyle. However, despite the distinct inter-individual differences between samples, a common microbial feature appears to emerge, as shown in the PCoA analysis that clustered cancer group and control group separately (**Figure 1F**), indicating a possibility on common dysbiosis associated to bladder cancer. Studies suggest that changes in microbial composition and function may contribute to carcinogenesis, tumor progression and dissemination at these sites (Schwabe and Jobin, 2013). Alteration of urinary microbiome might be also involved in the development and progression of bladder cancer.

Bucevic Popovic et al. reported that no significant difference was observed in overall microbial profiles between bladder cancer ($n = 12$) and control group ($n = 11$) (Bucevic Popovic et al., 2017). Number of cases, ethnicity, gender and age may be reasons of different results between our study and Bucevic Popovic's study.

Acinetobacter and *Anaerococcus* were two genera found in higher abundances in bladder cancer patients than in non-cancer group. *Acinetobacter* spp. was reported one of the most abundant Gram-negative bacteria isolated from the urine of cattle affected by urothelial tumors of the urinary bladder (Roperto et al., 2012). *Acinetobacter* is a complex genus associated with nosocomial infections, including urinary tract infections. The virulence factors of *Acinetobacter baumannii*, a species of *Acinetobacter*, identified to date were confirmed to be involved in biofilm formation, adherence and invasion of epithelial cells, bacterial dissemination by degrading phospholipids present at mucosal barrier, and escape from the host immune response (McConnell et al., 2013). As for *Anaerococcus*, it was reported as a member of the Gram-positive anaerobic cocci, which was able to induce inflammation, remodeling of extracellular matrix (ECM) and re-epithelialization (Murphy and Frick, 2013). Based on above analysis, we raise an intriguing possibility that the interplay of ECM and microbiome and concomitant inflammation might play a role in bladder carcinogenesis.

The link between chronic inflammation, microbiome and the initiation and progression of solid tumors has been established for various neoplastic diseases, especially colorectal cancer (Irrazábal et al., 2014). Transient inflammation is considered as

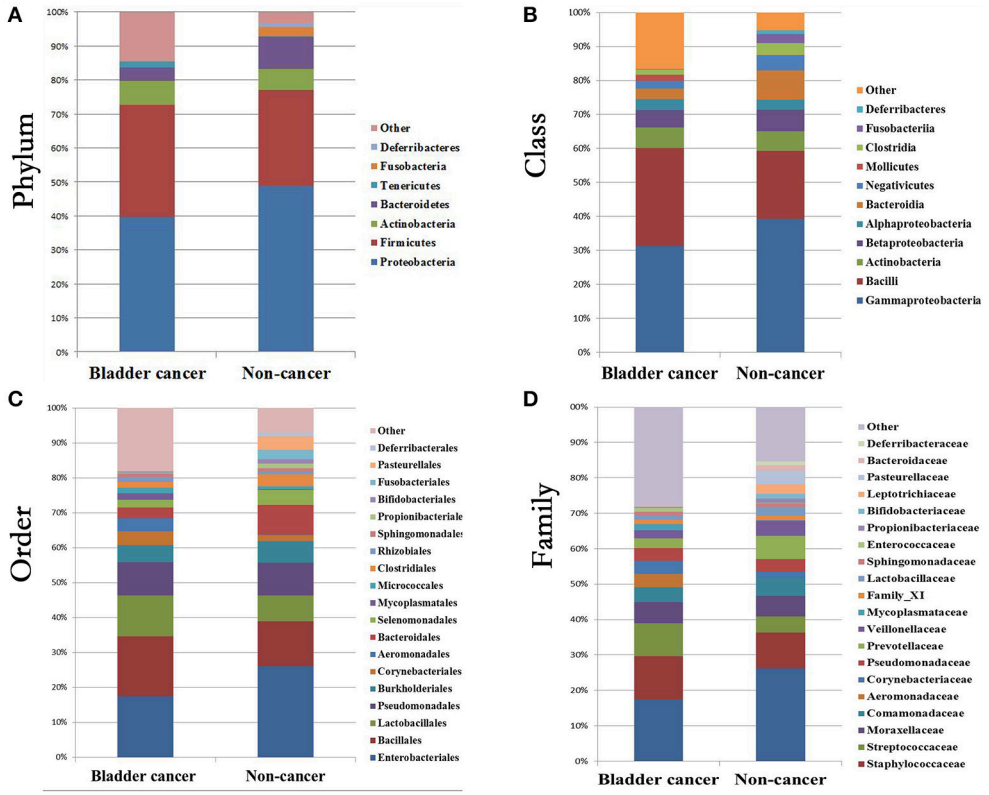

FIGURE 3 | Bacterial average relative abundance in bladder cancer and non-cancer samples. Average distribution of major taxa is represented by bar graphs. **(A)** phylum; **(B)** class; **(C)** order; **(D)** family. Each colored box represents a bacterial taxon and the height of a colored box represents the relative abundance of that organism within the sample. Bacterial genera with a relative abundance <1% and unclassified genera are grouped as "Other".

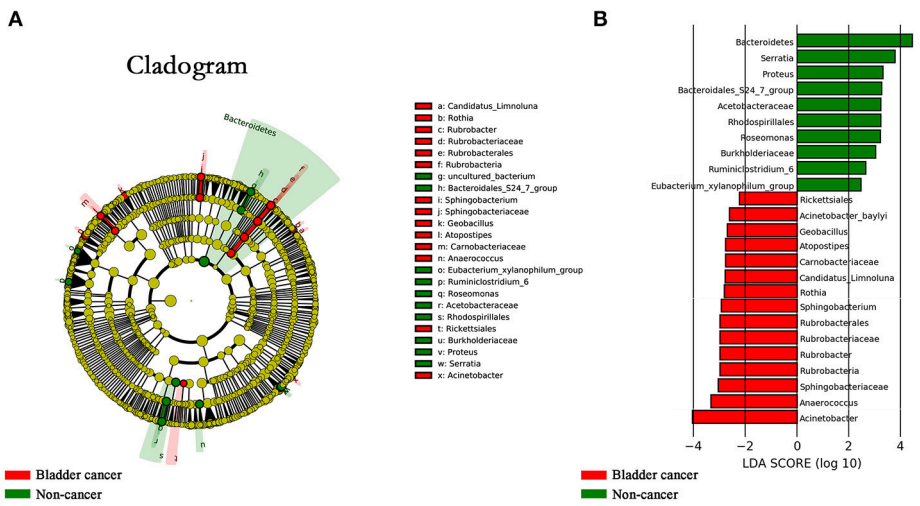

FIGURE 4 | Microbial taxa associated with bladder cancer. **(A)** Cladogram representation of the urinary microbial taxa associated with bladder cancer (red) and non-cancer (green). **(B)** Association of specific microbiota taxa with cancer group and non-cancer group by linear discriminant analysis effect size (LEfSe). Red indicates taxa enriched in cancer group and green indicates taxa enriched in non-cancer group.

part of body's immune defense against pathogen, but persistent inflammation could potentially contribute to the development of cancer (Ainsworth, 2017). One large epidemiological study reported that repeated, regular bouts of cystitis were associated with increased risk of bladder cancer (Vermeulen et al., 2015). Alfano et al. reported that bacteria produce proteases, acting as

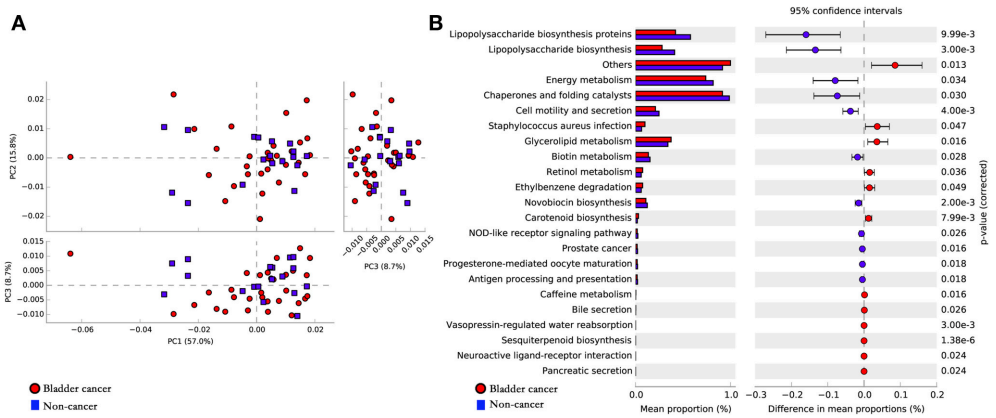

FIGURE 5 | Potential functional pathways associated with bladder cancer. **(A)** Principal component analysis (PCA) plot comparing the metagenome predictions (for KEGG orthology using PICRUSt) of the microbiota of patients with bladder cancer and non-cancer. **(B)** Microbial pathways that were significantly differentially enriched between the cases and controls. Red indicates taxa enriched in cancer group and blue indicates taxa enriched in non-cancer group.

virulence factors with crucial roles in host tissue degradation, as well as immune system evasion and destruction of host physical barriers, further promoting inflammation, remodeling of ECM and the generation of oxygen radicals, which results in mutagenesis that may promote the onset and progression of cancer (Alfano et al., 2016).

In our study, the LEfSe analysis showed that bacterial taxa along *Sphingobacteriaceae*-to-*Sphingobacterium* lineage (**Figures 4A,B**) were enriched in cancer patients. *Sphingobacterium* are recognized as etiological agents of cystic fibrosis and urinary tract infections. Besides, ceramides and sphingophospholipids of *S. spiritivorum*, one species of the genus *Sphingobacterium*, could induce DNA fragmentation, caspase-3 activation, changes in morphology and cell cycle shortening (Lambiase, 2014). The PICRUSt showed that in comparison with controls, *Staphylococcus aureus* infection was increased in patients with bladder cancer. *Staphylococcus aureus* could produce various enzymes such as alkaline protease, elastase and phospholipase C, which could degrade ECM components, break down elastin, disrupt tight junction, damage tissue, and cleaves various bonds in phospholipids. Above analysis also raises the possibility that microbiome-mediated modifications of ECM and concomitant inflammation might play an role in the initiation and development of bladder cancer. However, this study cannot answer the question whether changes in the microbiome contribute to cancer or vice versa. Numerous studies are required to determine whether the described profile is associated to, correlated with, or even responsible for bladder carcinogenesis and development.

Xu et al. reported enrichment of *Streptococcus spp.* in urine from urothelial carcinoma patients ($n = 8$) compared to healthy individuals ($n = 6$) (Xu et al., 2014). Bucevic Popovic et al. reported that genus *Fusobacterium* was significantly enriched in urine of bladder cancer patients. *Streptococcus* and *Fusobacterium* were also detected in our study. However, despite relative abundance of both genera was higher in bladder cancer patients, no significant difference was found between cancer and non-cancer group using Metastats algorithm after False Discovery Rate adjustment (**Table 2**).

Alfano et al. reported that specific pathogenic bacteria might promote the initiation and development of malignancies and tumor-associated microenvironments probably selectively facilitate the growth of specific bacteria in turn (Alfano et al., 2016). Enrichment of *Herbaspirillum*, *Porphyrobacter,* and *Bacteroides* was observed in bladder cancer patients with high risk of recurrence and progression (Supplementary Figures 4, 5), which suggests that these genera maybe potential biomarkers for risk stratification and to predict cancer prognosis. In addition, given the promising results of fecal microbiota transplantation, which was used to "re-establish the balance of nature" within the intestinal environment for the treatment of several refractory gastrointestinal disorders, strategies to restore normal bladder-associated microenvironment might be a potential option to reduce incidence or recurrence of bladder cancer.

Immune checkpoint targets have become the focus of investigation for the treatment of bladder cancer, including programmed death ligand-1 (PD-L1). Atezolizumab was the first PD-L1 inhibitor confirmed active in bladder cancer and is currently the only PD-L1 inhibitor approved by the FDA for patients with metastatic or locally advanced urothelial carcinoma based on promising overall response rates in clinical trials (Bellmunt et al., 2017). Though checkpoint blockade therapies have had remarkable results in management for bladder cancer, only small part of patients responds to PD-L1 blockers (Inman et al., 2017). It has become evident that microbiome plays a crucial role in modulating the response to cancer therapy, including immunotherapy (Roy and Trinchieri, 2017). Zitvogel *et al* reported that the microbiome could affect the therapeutic efficacy of PD-L1 blockade (Zitvogel et al., 2016). If the true association between microbial community and bladder

carcinogenesis is confirmed in the future, urinary microbiome might be a target for enhancing bladder cancer responses PD-L1 inhibitor.

Since gender can affect microbiome studies (Kim et al., 2017) and men are at considerably higher risk of developing bladder cancer, this study included only male patients (Dobruch et al., 2016). On one hand the restriction to male patients limited the generalizability of the data, on the other hand the restriction minimized confounding factor of gender. In addition, to avoid potential bias caused by hospital noise and environmental changes, we recruited non-neoplastic patients as controls. However, urinary microbiota may be influenced by various disorders, despite excluding confounding factors of neoplasms.

Our study is not devoid of limitations. Firstly, it is not possible to determine the cause-effect relationship between microbiome and bladder cancer for retrospective study and low number of cases. Thus, prospective follow-up studies with a larger sample number and animal experiment studies will be needed to clarify the role of microbiome in development and progression of bladder cancer. Secondly, despite the fact that potential harm brought by catheterization can be avoided, mid-stream urine sample collected by the clean catch method is potentially contaminated with microbiota surrounding the urethral orifice. Thirdly, bladder mucosa would provide the best samples to characterize the bladder-associated microbiome, which could rule out sample contamination with microorganisms present in the urethra. However, obtaining bladder biopsies or suprapubic aspirates in healthy individuals is unethical. Hence, profiling microbiome of tumor, peri-tumor and non-tumor tissues of bladder cancer patients would be crucial to ascertain the association between pathogenic effect of microbial dysbiosis and the onset, progression and relapse of cancer. Another limitation is that we did not have relevant data on urinary incontinence or retention profiles. The effect of profiles on urination may contribute to the results in the study. Last but not least, the 16S rRNA gene sequencing-based approach enabled us to detect bacteria that present even in low numbers on one hand, on the other hand this method cannot identify bacteria well at species level and detect non-bacterial microorganisms, such as viruses and fungi (Ainsworth, 2017).

In conclusion, we have profiled the urinary microbiome associated with bladder cancer in the most comprehensive study to date. Our study suggests that urinary microbiota may be associated with bladder cancer, but the cause-effect relationship remains unclear. When a true association between urinary microbiota and bladder cancer is determined in the future, a better understanding of the role of microbiome in the development and progression of bladder cancer could provide novel diagnostic and prognostic biomarkers, as well as more microbiome-targeted therapeutic options.

AUTHOR CONTRIBUTIONS

PW, GZ, JZ: conception and design; GZ, YC, JC: acquisition of data; GZ, YC, JZ: analysis and interpretation of data; PW, GZ, JZ: drafting of the manuscript; PW, WH, JRZ: critical revision of the manuscript for important intellectual content; PW, GZ, JZ, JLZ: statistical analysis; PW: obtaining funding; PW: supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00167/full#supplementary-material

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CORRECTION
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Corrigendum: Profiling the Urinary Microbiota in Male Patients With Bladder Cancer in China

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In the original article, there was an error. Readers were unable to access the 16S sequencing data submitted to the Short Read Archive (SRA) under the accession number SUB3915640.

A correction has been made to **MATERIALS AND METHODS, DNA Isolation and 16S rRNA Gene Sequencing**:

"To avoid contamination, DNA isolation was performed using the cultured cells protocol supplied with the DNeasy Blood and Tissue Kit (Qiagen, Germany) in a laminar flow hood. The concentration of extracted DNA was determined through a Nanodrop ND-1000 spectrophotometer (Thermo Electron Corporation, USA). The genomic DNA isolated from the clinical samples was amplified using primer sets specific for V4 regions (515F: GTGCCAGCMG CCGCGGTAA; and 806R:GGACTACHVGGGTWTCTAAT). In order to evaluate contribution of extraneous DNA from reagents, extraction negative controls (no urine) and PCR negative controls (no template) were included. The resultant PCR products were purified by Qiaquick PCR purification kit (Qiagen, Valencia, CA). Finally, purified samples were normalized to equal DNA concentration and sequenced using the Illumina Miseq sequencer (Illumina, Inc., USA). The 16S rRNA gene sequences have been submitted to the Short Read Archive (SRA) under accession number PRJNA486651".

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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REVIEW
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The Clinical Importance of *Campylobacter concisus* and Other Human Hosted *Campylobacter* Species

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Historically, Campylobacteriosis has been considered to be zoonotic; the *Campylobacter* species that cause human acute intestinal disease such as *Campylobacter jejuni* and *Campylobacter coli* originate from animals. Over the past decade, studies on human hosted *Campylobacter* species strongly suggest that *Campylobacter concisus* plays a role in the development of inflammatory bowel disease (IBD). *C. concisus* primarily colonizes the human oral cavity and some strains can be translocated to the intestinal tract. Genome analysis of *C. concisus* strains isolated from saliva samples has identified a bacterial marker that is associated with active Crohn's disease (one major form of IBD). In addition to *C. concisus*, humans are also colonized by a number of other *Campylobacter* species, most of which are in the oral cavity. Here we review the most recent advancements on *C. concisus* and other human hosted *Campylobacter* species including their clinical relevance, transmission, virulence factors, disease associated genes, interactions with the human immune system and pathogenic mechanisms.

Keywords: *Campylobacter concisus*, inflammatory bowel disease, human hosted *Campylobacter*, virulence factor, bacterial marker, transmission, oral *Campylobacter*, pathogenic mechanism

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INTRODUCTION

Campylobacter, along with *Arcobacter* and *Sulfurospirillum,* are the three genera that belong to the family, Campylobacteraceae. *Campylobacter* species are Gram-negative, curved or spiral shaped, and most of them are motile with a single polar flagellum present at one or both ends of the bacteria, allowing them to have a corkscrew-like motion during movement (Lastovica et al., 2014). *Campylobacter* species have low G + C content in their genome, and the median G + C content for most of the *Campylobacter* species ranges from 28 to 40% (Pruitt et al., 2007). There are few *Campylobacter* species which have G + C content of more than 40% in their genomes, including *Campylobacter curvus*, *Campylobacter rectus*, *Campylobacter showae* and *Campylobacter gracilis* (Pruitt et al., 2007). A majority of the *Campylobacter* species are microaerophiles, while some require anaerobic conditions for their growth (Debruyne et al., 2008).

Most *Campylobacter* species live as normal flora in the gastrointestinal tract of various animals (Lastovica et al., 2014). Some of these animal hosted *Campylobacter* species, such as *Campylobacter jejuni* and *Campylobacter coli*, can cause acute bacterial gastroenteritis in humans through consumption of contaminated food or water (Galanis, 2007). In addition to gastroenteritis, *C. jejuni* also causes Guillain-Barré syndrome, due to molecular mimicry between its sialylated lipooligosaccharides and the human nerve gangliosides (Takahashi et al., 2005).

As *C. jejuni* and *C. coli* are the main *Campylobacter* pathogens which cause human acute intestinal disease and they originate from animal sources, Campylobacteriosis has historically been considered to be zoonotic. Several *Campylobacter* species utilize humans as their natural host and accumulated evidence supports their role in chronic inflammatory diseases of the human intestinal tract. Here we review recent advancements on human hosted *Campylobacter* species, their clinical relevance, transmission, virulence factors, disease associated genes, interactions with human immune system and pathogenic mechanisms. Most of the studies on the human hosted *Campylobacter* species in the past decade were on *Campylobacter concisus*, this bacterium is therefore the focus of this review. In addition, other human hosted *Campylobacter* species were also reviewed.

THE NATURAL HOSTS OF *CAMPYLOBACTER* SPECIES AND HUMAN DISEASES ASSOCIATED WITH *CAMPYLOBACTER* SPECIES

The natural host of a bacterium refers to the host that the bacterium normally lives and reproduces (Haydon et al., 2002; Control and Prevention, 2006). Bacterial species are usually not harmful to their hosts, although there are exceptions. For example, *Helicobacter pylori* is a human hosted bacterial species, causing gastritis and gastric ulcers and being a risk factor for gastric cancer (Roesler et al., 2014).

To date, 40 *Campylobacter* species and subspecies have been isolated from a wide variety of animal or human sources (**Figure 1**). Many *Campylobacter* species are naturally hosted by domesticated animals raised as food such as chicken, cattle and pigs (Lastovica et al., 2014). They survive as commensal bacteria in their hosts, and some species, such as *C. jejuni* and *C. coli*, can cause human diseases. The main human disease caused by animal hosted pathogenic *Campylobacter* species is acute gastroenteritis and clinical disorders can also arise if bacterial species colonizing sterile sites of the body (**Table 1**). A number of *Campylobacter* species are also able to cause diseases in animals. For example, *Campylobacter fetus* is known to cause abortion in bovine and ovine, and *Campylobacter hepaticus* is the causative agent of spotty liver disease in chicken (Campero et al., 2005; Van et al., 2016).

Campylobacter species hosted by humans include *C. concisus, C. curvus, C. gracilis, Campylobacter hominis, C. rectus*, and *C. showae*; to date these six *Campylobacter* species have only been isolated from humans (Hariharan et al., 1994; Zhang, 2015). *Campylobacter ureolyticus* was isolated from human samples in most cases, with only one study isolating *C. ureolyticus* from the endometria of healthy horses (Hariharan et al., 1994). Therefore, in this review, *C. ureolyticus* is considered as a human hosted *Campylobacter* species. In contrast to animal hosted *Campylobacter* pathogens, the human hosted *Campylobacter* pathogens are more often involved in chronic inflammatory conditions of the gastrointestinal tract (**Table 2**).

Abbreviations: 5-ASA, 5-aminosalicylic acid; AZA, azathioprine; CD, Crohn's disease; CFU, colony forming unit; COX, cyclooxygenase; Erm, erythromycin ribosome methylase; GERD, gastroesophageal reflux disease; GS, genomospecies; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MP, mercaptopurine; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PCR, polymerase chain reaction; PLA_2, phospholipase A_2; TLR, toll-like receptor; TNF, tumor necrosis factor; UC, ulcerative colitis; Zot, zonula occludes toxin.

C. CONCISUS

C. concisus has a curved or spiral shape and a single polar flagellum, with the size being 0.5–1 by 4 μm (Tanner et al., 1981). Its colony appears as convex shaped, translucent and is ∼1 mm in diameter (Tanner et al., 1981). In early literature, *C. concisus* was described as a microaerophile, due to the growth of this bacterium under microaerophilic atmosphere enriched with hydrogen (H_2) (Lastovica, 2006). Later, Lee et al. demonstrated that *C. concisus* is not a microaerophile, as they found that none of the 57 *C. concisus* strains grew in the microaerophilic conditions generated using the Oxoid BR56A and CN25A gas-generation systems (Lee et al., 2014). These *C. concisus* strains were able to grow under anaerobic conditions, with tiny colonies observed after 3 days of culture under anaerobic conditions, showing that *C. concisus* is an anaerobic bacterium. The anaerobic condition used in the study was generated using AN25A gas-generation system which absorbs oxygen with simultaneous production of CO_2.

C. concisus is a chemolithotrophic bacterium, capable of using H_2 as a source of energy to markedly increases its growth (Lee et al., 2014). *C. concisus* is able to oxidize H_2 under both anaerobic and microaerobic conditions, although greater growth was observed under anaerobic conditions in the presence of 2.5–10% H_2 (Lee et al., 2014). *C. concisus* is catalase negative, contributing to its inability to grow under microaerobic conditions (Tanner et al., 1981).

Transmission of *C. concisus*

Currently, humans are the only known hosts of *C. concisus* with oral cavity being its natural colonization site (Zhang et al., 2010; Mahendran et al., 2013). *C. concisus* has been isolated from saliva samples of children as early as 3 years old, although the positive isolation rate was significantly lower than the other age groups (33 vs. 79–88%); and the highest *C. concisus* isolation rate was seen in the age group of 12–17 (Zhang et al., 2010) (**Figure 2**). When a PCR method was used for detection, the children from the 3 to 5 years old age bracket had a detection rate that was similar to other age groups (83 vs. 93–100%). These data show that although *C. concisus* colonize humans at the early stages of life, 3–5 year old children have lower bacterial loads of *C. concisus* in their saliva as compared to older children and the adults. Currently, no data are available regarding *C. concisus* colonization in children below 3 years old.

Given that *C. concisus* colonizes the oral cavity, transmission would occur through saliva. The stability of *C. concisus* in saliva samples is related to sample storage. We were able to isolate *C. concisus* from saliva samples stored at 4°C for 3–6 days. However, we were unable to isolate *C. concisus* from

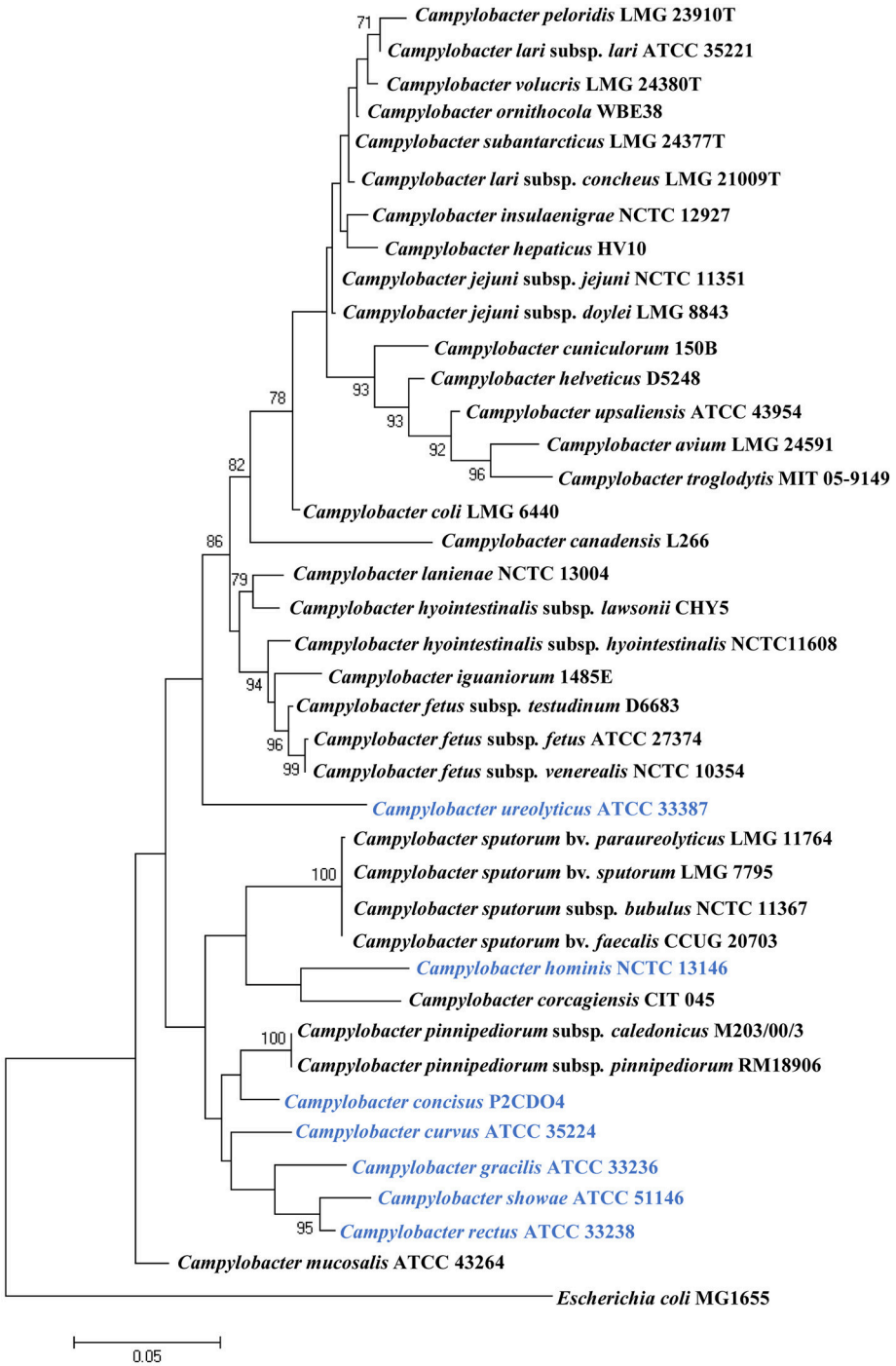

FIGURE 1 | Phylogenetic tree based on the 16S rRNA gene of *Campylobcter* species. The tree was generated using the maximum likelihood method implemented in MEGA7. *Bootstrap* values were generated from 1000 replicates. *Bootstrap* values of more than 70 were indicated. *Escherichia coli* MG1655 was included as an outgroup. Human hosted *Campylobacter* species are in blue. *Campylobacter geochelonis* was not included because its 16S rRNA sequence was not available.

the same saliva samples after storage at room temperature for 24 h. This suggests that in addition to direct contact, *C. concisus* contaminated food or drinks, particularly those stored in refrigerators, may also play a role in *C. concisus* transmission.

Multiple strains of *C. concisus* have been isolated from saliva samples and enteric samples of given individuals, suggesting a possible dynamic colonization of new *C. concicus* strains in the human gastrointestinal tract (Ismail et al., 2012).

Association of *C. concisus* With Human Diseases

Gingivitis and Periodontitis

Gingivitis is a common bacterial disease which affects 90% of the population (Coventry et al., 2000). The oral mucosa consists of stratified squamous epithelial cells (Lumerman et al., 1995). Periodontitis develops when gingivitis is not well-treated (Pihlstrom et al., 2005). As disease progresses, a loss of attachment between the gingivae and the teeth leads to the formation of a periodontal pocket, which then allows extensive colonization by anaerobic bacteria causing further inflammation of the mucosa (Highfield, 2009). In periodontitis patients, higher proportions of Gram-negative and anaerobic bacterial species are present in the oral cavity as compared to healthy controls (Newman and Socransky, 1977). *C. concisus* was initially isolated from the oral cavity of patients with gingivitis and periodontitis (Tanner et al., 1981). However, studies have not revealed a clear association between *C. concisus* and gingivitis and periodontitis, its role in human oral inflammatory diseases remains unclear.

Barrett's Esophagus

The esophagus is a muscular conduit with stratified squamous epithelial layers; its mucosa is colonized by microbes dominated by members of the genus *Streptococcus* (Di Pilato et al., 2016). Disturbances of the microbiota composition, such as the abnormal enrichment of some Gram-negative bacteria including *Campylobacter* spp. has been reported to be associated with gastroesophageal reflux disease (GERD), and is suggested to contribute to the development toward Barrett's esophagus and oesophageal adenocarcinoma (Di Pilato et al., 2016).

By analyzing the microbiome composition in biopsy samples of the distal esophagus collected from normal individuals and patients with esophagitis or Barrett's esophagus, Yang et al. found that type I microbiome (Gram-positive aerobic microbiome) was mainly associated with normal esophagus (11/12, 91.7%), while type II microbiome (Gram-negative anaerobic microbiome) was more closely associated with abnormal esophagus including esophagitis and Barrett's esophagus (13/22, 59.1%) (Yang et al., 2009). Furthermore, *Campylobacter* species was found to be one of the genera with increased abundance in type II microbiome (Yang et al., 2009).

By using bacterial cultivation methods, Macfarlane et al. found that high levels of *C. concisus* and *C. rectus* were present in oesophageal aspirate and mucosal samples of patients with Barrett's esophagus, but not in control subjects (Macfarlane et al., 2007). Similar findings were reported by another study, in which the *Campylobacter* genus dominated by *C. concisus* colonized patients with GERD and Barrett's esophagus with increased bacterial counts, accompanied by a significant decrease in bacterial counts for all other genera (Blackett et al., 2013). This relationship was not observed in patients with oesophageal adenocarcinoma. It is possible that *C. concisus* contributes to the inflammation associated with GERD and Barrett's esophagus.

Gastroenteritis

Gastroenteritis is an inflammatory condition of the gastrointestinal tract which is characterized by diarrhea, abdominal pain, fever and vomiting (Galanis, 2007). It is usually self-limiting within 2–5 days (Galanis, 2007). Gastroenteritis can be caused by bacteria, viruses, parasites and fungi; the most common causes are rotavirus and bacterial species such as *Escherichia coli*, *C. jejuni*, and *C. coli* (Galanis, 2007).

A number of studies have reported the isolation of *Campylobacter* species other than *C. jejuni* and *C. coli* in diarrheal stool samples. Lindblom et al. found that in stool samples from diarrheal patients, *Campylobacter upsaliensis*, *Campylobacter sputorum,* and *C. concisus* were the most common species in addition to *C. jejuni*, of which *C. concisus* was only isolated from children (Lindblom et al., 1995). Similarly, Lastovica et al. reported that in addition to *C. jejuni*, *C. concisus* was the second most frequently isolated *Campylobacter* species from diarrheic stools of pediatric patients (Lastovica and Roux, 2000). Nielsen et al. also found that *C. concisus* was more frequently found in diarrheal stool samples of young children and elderly (Nielsen et al., 2013). The same group later reported a clinical study comparing clinical manifestations between adult patients infected with *C. concisus* and *C. jejuni/C. coli*, and they showed that although *C. concisus* infection seems to induce a milder course of acute gastroenteritis in comparison to that induced by *C. jejuni/C. coli*, it is associated with prolonged diarrhea (Nielsen et al., 2012). Recently, Serichantalergs et al. reported a significantly higher detection rate of *C. concisus* in traveller's diarrhea cases as compared to that in asymptomatic controls in Nepal (Serichantalergs et al., 2017). Another recent study by Tilmanne et al. showed that *C. concisus* had a similar prevalence in children with acute gastroenteritis as compared with the control group (Tilmanne et al., 2018). Unfortunately, most of the studies that reported the isolation of *C. concisus* from diarrheal stool samples did not have control fecal samples from healthy individuals, making it difficult to judge the role of this bacterium in gastroenteritis; and for those studies with control groups included, the results were controversial. Thus, whether *C. concisus* plays a role in gastroenteritis remains to be investigated.

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract with Crohn's disease (CD) and ulcerative colitis (UC) being two of its major forms (De Souza and Fiocchi, 2016). The two forms of IBD mainly differ in pathology. CD is characterized by having discontinuous "skip lesions" of transmural inflammation and the abnormalities can be found throughout the gastrointestinal tract (Walsh et al., 2011). In contrast to CD, the inflammation involvement for UC is usually confined to the mucosa and submucosa without skip lesions and mostly occurs in the large intestine (Walsh et al., 2011).

The etiology of IBD is not fully understood. Accumulated evidence has suggested that the mucosal immune system in genetically predisposed individuals has mounted responses to intestinal commensal bacterial species, which is believed to contribute to the pathogenesis of IBD (Knights et al., 2013). Intestinal commensal bacterial species have co-evolved with the intestinal mucosal immune system thus the immune responses to intestinal commensal bacterial species would need an external

TABLE 1 | Clinical relevance of animal hosted *Campylobacter* species.

***Campylobacter* species**	**Isolation sources**	**Clinical relevance of human diseases**	**References**
Campylobacter coli	Gastroenteritis: feces Bacteraemia: blood Septic abortion: blood; maternal placenta; amniotic fluid Acute cholecystitis: gallbladder Retroperitoneal abscess Meningitis: CSF	Gastroenteritis* Abortion* Bacteraemia*	Skirrow, 1977; Kist et al., 1984; Blaser et al., 1986; Møller Nielsen et al., 1997; Lastovica and Roux, 2000; Galanis, 2007; Liu Y. H. et al., 2017;
Campylobacter fetus subsp. *fetus*	Bacteraemia: blood Gastroenteritis: feces Meningitis: feces; blood; CSF Chorioamnionitis: blood Cellulitis lesion: subcutaneous aspirate Cellulitis and bacteraemia: blood; feces Abortion: vagina; feces; blood; gastric aspirate; skin; liver; spleen; lung; spinal fluid Postsurgical abscess: groin abscess Post abortion infection: amniotic fluid Hemiparesis and aphasia: blood Cystic fibrosis: feces Surgical fever: blood Fever, chills, endocarditis: blood Immune deficiency disease: blood Sepsis, encephalitis, fever, myalgia: blood and CSF Cellulitis and diarrhea: ankle abscess Post-neurosurgery for metastatic esophagocardial carcinoma: brain abscess Chronic alcoholism: brain abscess Prematurity: brain abscess Heroin and barbiturate abuse: pulmonary abscess Alcoholism: gluteal abscess; blood Post-hemilaminectomy for disc herniation: epidural mass in pyogenic vertebral osteomyelitis Hyperthyroidism: thyroid gland abscess Health status unknown: Blood and synovial fluid	Bacteraemia* Abortion* Meningitis* Abscess* Gastroenteritis	Blaser et al., 1980a,b; Edmonds et al., 1985; Francioli et al., 1985; La Scolea, 1985; Klein et al., 1986; Simor et al., 1986; Morrison et al., 1990; Sauerwein et al., 1993; Kwon et al., 1994; Neuzil et al., 1994; Steinkraus and Wright, 1994; Morooka et al., 1996; Ichiyama et al., 1998; Lastovica and Roux, 2000; Viejo et al., 2001; Krause et al., 2002; Fujihara et al., 2006; De Vries et al., 2008; Liu Y. H. et al., 2017
Campylobacter fetus subsp. *testudinum*	Leukemia: blood Liver cancer (bloody diarrhea, pulmonary edema): pleural fluid Asthma: hematoma Lymphoma, hypertension, and heart disease (fever, chills, rigor, cough, and diarrhea): blood Diarrhea: bile Diabetes (cellulitis of leg): blood	Leukemia*	Tu et al., 2004; Patrick et al., 2013
Campylobacter fetus subsp. *venerealis*	Bacteraemia: blood Infective aneurysm: blood Vaginosis Health status unknown: blood		Garcia et al., 1995; Tu et al., 2001; Hagiya et al., 2015; Liu Y. H. et al., 2017

(Continued)

TABLE 1 | Continued

Campylobacter species	Isolation sources	Clinical relevance of human diseases	References
Campylobacter helveticus	Health status unknown: feces		Lawson et al., 1997
Campylobacter hyointestinalis (subsp. *hyointestinalis* and *lawsonii*)	Proctitis: rectum Gastroenteritis: feces	Gastroenteritis*	Fennell et al., 1986; Edmonds et al., 1987; Lastovica and Roux, 2000; Gorkiewicz et al., 2002
Campylobacter insulaenigrae	Gastroenteritis: blood Bacteraemia: blood	Gastroenteritis Bacteraemia*	Chua et al., 2007
Campylobacter jejuni (subsp. *jejuni* and *doylei*)	Bacteraemia: blood; feces Gastroenteritis: feces Sepsis: blood, feces, placenta Meningitis: CSF Appendicitis: appendix Myocarditis: feces Reactive arthritis: feces Guillain-Barré syndrome: feces Fisher syndromes: feces; gastric biopsy; CSF Recurrent colitis: blood; feces Acute cholecystitis: gallbladder Urinary tract infection: urine Chronic renal failure: peritoneal prodialysis fluid Ovarian cyst: peritoneal cyst fluid Thoracic wall abscess: thoracic wall Meningitis and hypogammaglobulinemia: CSF	Gastroenteritis* Bacteraemia* Guillain-Barré syndrome* Meningitis* Appendicitis Myocarditis Reactive arthritis	Skirrow, 1977; Thomas et al., 1980; Gilbert et al., 1981; Megraud et al., 1982; Chan et al., 1983; Blaser et al., 1986; Dhawan et al., 1986; Goossens et al., 1986; Klein et al., 1986; Kohler et al., 1988; Korman et al., 1997; Meyer et al., 1997; Møller Nielsen et al., 1997; Manfredi et al., 1999; Lastovica and Roux, 2000; Wolfs et al., 2001; Hannu et al., 2002, 2004; Cunningham and Lee, 2003; Takahashi et al., 2005; Lastovica, 2006; Galanis, 2007; Pena and Fishbein, 2007; Mortensen et al., 2009; Liu Y. H. et al., 2017
Campylobacter lanienae	Healthy: feces		Logan et al., 2000
Campylobacter lari (subsp. *concheus* and *lari*)	Urinary tract infection: urine Bacteraemia: blood Gastroenteritis: feces	Bacteraemia*	Bézian et al., 1990; Morris et al., 1998; Lastovica and Roux, 2000; Martinot et al., 2001; Krause et al., 2002; Werno et al., 2002
Campylobacter mucosalis	Gastroenteritis: feces		Figura et al., 1993
Campylobacter peloridis	Health status unknown: feces		Debruyne et al., 2009
Campylobacter sputorum (biovar faecalis, paraureolyticus, sputorum, and *bubulus)*	Gastroenteritis: feces Axillary abscess Leg abscess Pus from pressure sore Health status unknown: oral cavity; feces	Gastroenteritis Abscess*	Roop Ii et al., 1985; Steele et al., 1985; Lindblom et al., 1995; Tee et al., 1998; De Vries et al., 2008
Campylobacter troglodytis	Gastroenteritis: feces		Platts-Mills et al., 2014
Campylobacter upsaliensis	Abortion: blood and fetoplacental material Bacteraemia: blood Gastroenteritis: feces Breast abscess	Gastroenteritis Bacteraemia*	Lastovica et al., 1989; Gurgan and Diker, 1994; Jimenez et al., 1999; Lastovica and Roux, 2000; Lastovica and Le Roux, 2001; De Vries et al., 2008

CSF, cerebrospinal fluid. **Campylobacter species that have established associations with human diseases or have been isolated from a sterile site. Species which have not yet been isolated from humans were not included.*

TABLE 2 | Clinical relevance of human hosted *Campylobacter* species.

***Campylobacter* species**	**Isolation sites in human**	**Clinical relevance of human diseases**	**References**
Campylobacter concisus	Healthy: saliva; subgingival site; intestinal biopsy; feces IBD: saliva; intestinal biopsy Diarrhea: feces Barrett's esophagus: esophageal aspirate; distal esophageal biopsy Brain abscess: secondary to chronic frontal osteomyelitis Periodontal disease: subgingival site	Inflammatory bowel disease* Diarrheal diseases Barrett's esophagus* Periodontal disease	Tanner et al., 1981; Lindblom et al., 1995; Lastovica and Roux, 2000; Macuch and Tanner, 2000; Lastovica, 2006; Macfarlane et al., 2007; De Vries et al., 2008; Zhang et al., 2010; Kalischuk and Inglis, 2011; Mukhopadhya et al., 2011; Nielsen et al., 2011, 2013; Blackett et al., 2013; Mahendran et al., 2013; Zhang, 2015; Kirk et al., 2016
Campylobacter curvus	Healthy: subgingival site Periodontal disease: subgingival and periodontitis site Thoracic empyema: pleural effusion Premature birth: vaginal swabs Alveolar abscess Metastatic ovarian cancer: liver abscess Liver abscess: blood Lung cancer: bronchial abscess Guillain-Barré syndrome: feces Fisher's syndrome: feces Gastroenteritis: feces	Gastroenteritis Abscess	Koga et al., 1999; Lastovica and Roux, 2000; Macuch and Tanner, 2000; Abbott et al., 2005; Petersen et al., 2007; De Vries et al., 2008; Mendz et al., 2014; Horio et al., 2017
Campylobacter gracilis	Healthy: subgingival site Bacteraemia: blood Brain abscess (post-partum) Tubo-ovarian abscess Periodontal disease: subgingival and periodontitis site Visceral or head and neck infection	Periodontal disease*	Tanner et al., 1981; Johnson et al., 1985; Yu and Chen, 1997; Macuch and Tanner, 2000; De Vries et al., 2008; Shinha, 2015
Campylobacter hominis	Healthy: feces Septicaemia: blood CD: intestinal biopsy	Septicaemia	Lawson et al., 1998, 2001; Linscott et al., 2005; Zhang et al., 2009
Campylobacter rectus	Healthy: subgingival site	Periodontal diseases* IBD	Von Troil-Lindén et al., 1995; Lastovica and Roux, 2000; Macuch and Tanner, 2000; Han et al., 2005; Macfarlane et al., 2007; De Vries et al., 2008; Mahlen and Clarridge, 2009; Zhang et al., 2009; Man et al., 2010b; López et al., 2011; Mukhopadhya et al., 2011; Lee et al., 2012; Leo and Bolger, 2014; Noël et al., 2018

(Continued)

TABLE 2 | Continued

Campylobacter species	Isolation sites in human	Clinical relevance of human diseases	References
	Periodontal disease: subgingival and periodontitis site Barrett's esophagus: distal esophageal mucosal biopsy Fatal thoracic empyema: pleural liquid Septic cavernous sinus thrombosis: blood Gastroesophageal adenocarcinoma: palate abscess Breast abscess Vertebral abscess Gastroenteritis: feces		
Campylobacter showae	Healthy: subgingival site; gingival crevices Periodontal disease: subgingival and periodontitis site CD: intestinal biopsy Intraorbital abscess Bacteraemia: blood	IBD	Etoh et al., 1993; Macuch and Tanner, 2000; De Vries et al., 2008; Zhang et al., 2009; Man et al., 2010b; Suzuki et al., 2013
Campylobacter ureolyticus	Healthy: Male: urine Female: genital tract CD: intestinal biopsy Gastroenteritis: feces Periodontal disease: deep periodontal pockets Superficial soft tissue or bone infections Male: Non-gonococcal, non-chlamydial urethritis Non-gonococcal urethritis Superficial necrotic or gangrenous lesions Penile wound Female: Perineal, genital and peripheral ulcers Genital tract: excess vaginal discharge; lower genital tract symptoms Health status unavailable: Amniotic fluid; urine	IBD Gastroenteritis Genital tract diseae	Duerden et al., 1982, 1987, 1989; Johnson et al., 1985; Bennett et al., 1990, 1991; Petersen et al., 2007; Zhang et al., 2009; Bullman et al., 2011; Mukhopadhya et al., 2011; O'doherty et al., 2014

**Campylobacter species that have established associations with human diseases or have been isolated from a sterile site.*

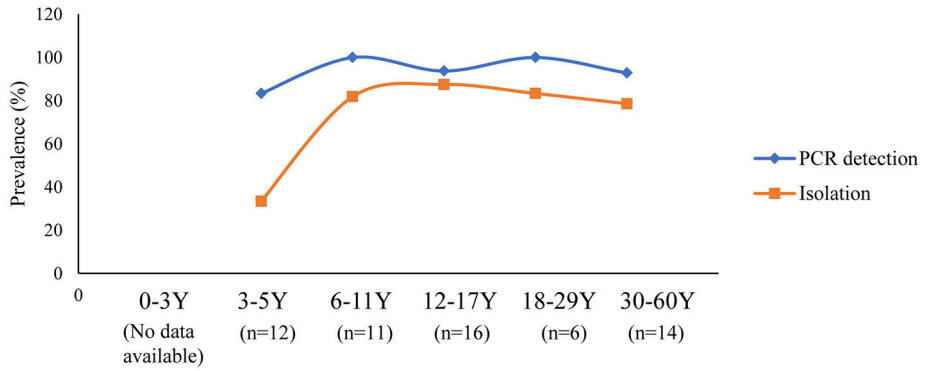

FIGURE 2 | Detection and isolation rates of *C. concisus* from saliva samples in different age groups. The PCR detection rate of *C. concisus* from children of different age groups varied between 80 and 100%. However, the isolation rate of *C. concisus* from children at 3–5 years was only 33% (4/12), which was significantly lower than that in other age groups, indicating younger children were colonized with lower numbers of bacteria. Data are from Zhang et al. (2010).

trigger. Microbes that have the ability to cause a prolonged primary intestinal barrier defect or alter the mucosal immune system are more likely to trigger IBD.

C. concisus is commonly present in the oral cavities of almost all individuals. However, patients with IBD have a significantly higher prevalence of *C. concisus* detected in their intestinal tissues as compared to healthy controls (Zhang et al., 2009, 2014; Man et al., 2010b; Mahendran et al., 2011; Mukhopadhya et al., 2011; Kirk et al., 2016). Comparison of the housekeeping genes and genomes of oral and enteric *C. concisus* strains suggests that enteric strains originate from oral *C. concisus* strains (Ismail et al., 2012; Chung et al., 2016).

C. concisus, *C. hominis*, *C. showae,* and *C. ureolyticus* have also been isolated from patients with CD. Additionally, *C. homonis*, *C. showae*, *C. rectus*, *C. gracilis,* and *C. ureolyticus* have been detected from fecal specimens of children with newly diagnosed CD (Zhang et al., 2009; Man et al., 2010b). However, no association has been found between the prevalence of these *Campylobacter* species in CD patients and healthy controls.

Genomospecies of *C. concisus*

C. concisus strains can be separated into two major genomospecies (GS), consistently defined by the analysis of core genomes and housekeeping genes (Istivan, 2005; Miller et al., 2012; Mahendran et al., 2015; Chung et al., 2016; Nielsen et al., 2016). *C. concisus* 23S rRNA gene has polymorphisms, which was also used to define the genomospecies by comparison of the entire 23S rRNA gene sequence or PCR amplification of 23S rRNA gene fragments (Engberg et al., 2005; Kalischuk and Inglis, 2011; On et al., 2013; Huq et al., 2017; Wang et al., 2017).

Quantitative PCR methods targeting the polymorphisms of the 23S rRNA gene revealed that there were more GS2 than GS1 *C. concisus* in samples collected from the upper and lower gastrointestinal tract of both patients with IBD and healthy controls, suggesting that GS2 *C. concisus* is better adapted to the human gastrointestinal tract (Wang et al., 2017). A meta-analysis of the composition of the isolated GS1 and GS2 *C. concisus* strains showed similar findings except that in healthy individuals, a significantly lower number of GS2 *C. concisus* strains than GS1 *C. concisus* were isolated from fecal samples. This suggests a potential difference in the *C. concisus* strains or the enteric environment between patients with gastrointestinal diseases and healthy controls (Wang et al., 2017).

The two GS of *C. concisus* do not differ in morphology but have GS-specific genes and may have different pathogenic potentials. By examining *C. concisus* strains isolated from diarrheal fecal samples, Engberg et al. found that bloody diarrhea was only present in individuals infected with GS2 *C. concisus* strains (Engberg et al., 2005). Furthermore, Kalischuk and Inglis demonstrated that GS2 *C. concisus* exhibited higher levels of epithelial invasion and translocation (Kalischuk and Inglis, 2011). Similarly, Ismail et al. reported that the oral *C. concisus* strains that were more invasive to intestinal epithelial cells were GS2 strains (Ismail et al., 2012).

Recently, comparative genomic analyses have identified two novel genomic islands CON_PiiA and CON_PiiB that carry proteins homologous to the type IV secretion system, LepB-like and CagA-like effectors (Chung et al., 2016). CON_PiiA and CON_PiiB were found in strains from both GS1 and GS2 (Chung et al., 2016). The effects of the proteins possessed by CON_PiiA and CON_PiiB on human cells require further examination.

Both GS1 and GS2 contain diverse *C. concisus* strains. The isolation sources of *C. concisus* strains do not seem to contribute to the phylogenetic relatedness. *C. concisus* strains isolated from saliva, intestinal biopsies and feces are found in both GS1 and GS2, as are the strains isolated from patients with enteric disease and healthy controls. A recent study examining the genomes of 104 *C. concisus* strains isolated from saliva, mucosal biopsies and fecal samples of patients with IBD, gastroenteritis and healthy individuals showed that sampling site rather than disease phenotype was associated with the particular GS (Kirk et al., 2018). In that study, authors reported that genes involved in cell membrane synthesis were common in oral strains, while those related to cell transport,

metabolism, and secretory pathways were more often found in enteric isolates. These results indicate that GS alone is unable to differentiate virulent *C. concisus* strains from commensal strains.

A recent study reported the identification of an exoprotein named *C. concisus* secreted protein 1 (Csep1) (Liu et al., 2018). The *csep1* gene was found to be localized in the bacterial chromosome and the pICON plasmid. The chromosomally encoded *csep1* gene was only found in GS2 *C. concisus* strains, while the pICON plasmid encoded *csep1* gene was found in both GS1 and GS2 strains. Some *csep1* genes contained a six-nucleotide insertion at the position 654–659 bp (*csep1-6bpi*). Importantly, the *csep1-6bpi* gene in oral *C. consisus* strains was found to be associated with active CD. So far, the *csep1-6bpi* gene is the only molecular marker in *C. concisus* reported to have an association with IBD.

Pathogenic Mechanisms of *C. concisus*

Motility, Adhesion, and Invasion

Campylobacter movement through the mucus layer is driven by its polar flagellum, which is crucial for approaching, attaching, and invading the intestinal epithelial cells (Young et al., 2007). In *C. jejuni*, the extracellular flagella filament is composed of multimers of flagellin proteins with a major flagellin protein FlaA and a minor flagellin protein FlaB. FlaA has been shown to be essential for invasion of INT 407 cells and optimal colonization in chicken gut (Wassenaar et al., 1991, 1993). The flagellum also serves as a type III secretion system for transportation of *Campylobacter* invasion antigens to the host cells (Buelow et al., 2011; Neal-Mckinney and Konkel, 2012; Samuelson et al., 2013).

Both *C. concisus* and *C. jejuni* are spiral shaped. Unlike *C. jejuni*. which can have either single or bi-polar flagella, *C. concisus* only has a single polar flagellum. Although the flagellum in *C. concisus* has not been comprehensively investigated, studies have shown that *C. concisus* flagellum might be a virulence factor that contributes to its pathogenicity. Man et al. have observed flagellum mediated attachment and invasion of *C. concisus* to Caco-2 cells (**Figure 3**) (Man et al., 2010a).

Several studies have examined the motility of *C. concisus* strains isolated from saliva, feces and intestinal biopsies of patients with IBD, gastroenteritis, and healthy individuals. The motility was shown to be strain dependent, and no statistically significant association was found between patients and healthy controls (Lavrencic et al., 2012; Ovesen et al., 2017). The authors also reported that the motility of *C. concisus* was lower than that of *C. jejuni* and *C. fetus* (Ovesen et al., 2017). Furthermore, comparative genomic analysis of *C. concisus* strains had identified proteins required for flagellin glycosylation pathway, which may affect bacterial flagellar filament assembly, autoagglutination, adhesion and invasion (Kaakoush et al., 2011).

Bacterial flagella are also involved in forming biofilm, a bacterial interaction that is important for its survival in the host (Reeser et al., 2007; Svensson et al., 2014). *C. concisus* was also shown to have the ability to form biofilms, and this ability did not differ between the strains examined (Lavrencic et al., 2012).

Damaging the Intestinal Epithelial Barrier

The intestinal epithelium is constructed by simple columnar epithelial cells (Clevers, 2013). Increased intestinal permeability is known to be associated with a number of chronic human diseases including IBD, being considered as a risk factor for its development (Hollander et al., 1986; Wyatt et al., 1993; Irvine and Marshall, 2000; D'Incà et al., 2006; Meddings, 2008). A compromised intestinal epithelial barrier may lead to loss of tolerance to the commensal enteric microbiota. Using *in vitro* cell culture models, several studies have indicated that *C. concisus* is able to increase the intestinal permeability. The intestinal epithelial permeability in Caco-2 cells was increased following *C. concisus* infection, and *C. concisus* also induced movement of tight junction proteins zonula occludens-1 and occludin from cell membrane into cytosol (**Figure 3**) (Man et al., 2010a). These changes in tight junction protein arrangement significantly increased the barrier permeability (Man et al., 2010a). These effects were also observed in HT-29/B6 intestinal epithelial cells, in which the cell monolayers infected with both oral and fecal *C. concisus* strains revealed epithelial barrier dysfunction (Nielsen et al., 2011).

Induction of Proinflammatory Cytokines

Inflammatory cytokines are produced in enteric infections (**Figure 3**). *C. concisus* strains were able to stimulate the productions of interleukin (IL)-8 and tumor necrosis factor (TNF)-α in THP-1 macrophages, and the productions of IL-8 and cyclooxygenase (COX)-2 in HT-29 cells (Man et al., 2010a; Ismail et al., 2013). COX-2 is an enzyme responsible for producing prostaglandins and other inflammatory mediators (Williams et al., 1999). Some *C. concisus* strains, mostly those isolated from patients with IBD, have been shown to upregulate surface expression of lipopolysaccharides (LPS) receptors including Toll-like receptor (TLR) 4 and myeloid differentiation factor 2 in HT-29 cells (Ismail et al., 2013). Activation of neutrophil adherence molecule CD11b and oxidative burst response have been shown in neutrophils following *C. concisus* infection (Sørensen et al., 2013). Additionally, by using transcriptomics analysis, assembly of IFI16 inflammasome has been observed in both intestinal epithelial cells and macrophages following *C. concisus* infection (Kaakoush et al., 2015; Deshpande et al., 2016). The IFI16 inflammasome plays important role in the innate immune responses, acting as a nuclear pathogen sensor that promotes caspase-1 activation (Xiao, 2015). Production of mature proinflammatory cytokines, IL-1β, and 1L-18, is caspase-1 dependent (Xiao, 2015). A recent study has found that *C. concisus* was able to elevate the mRNA expression of p53, TNF-α, and IL-18 in Barrett's cell lines, however these effects have not been demonstrated at protein level (Namin et al., 2015).

Flagellin from various Gram-negative and Gram-positive bacteria are capable of triggering nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signaling responses in intestinal epithelial cells, where it is recognized by TLR5 expressed on the surface of the host cells (Eaves-Pyles et al., 2001; Gewirtz et al., 2001). The conserved region on flagellin that is recognized by TLR5 has been studied in *Salmonella typhimurium* (Smith et al., 2003). *Campylobacter* flagellin shares

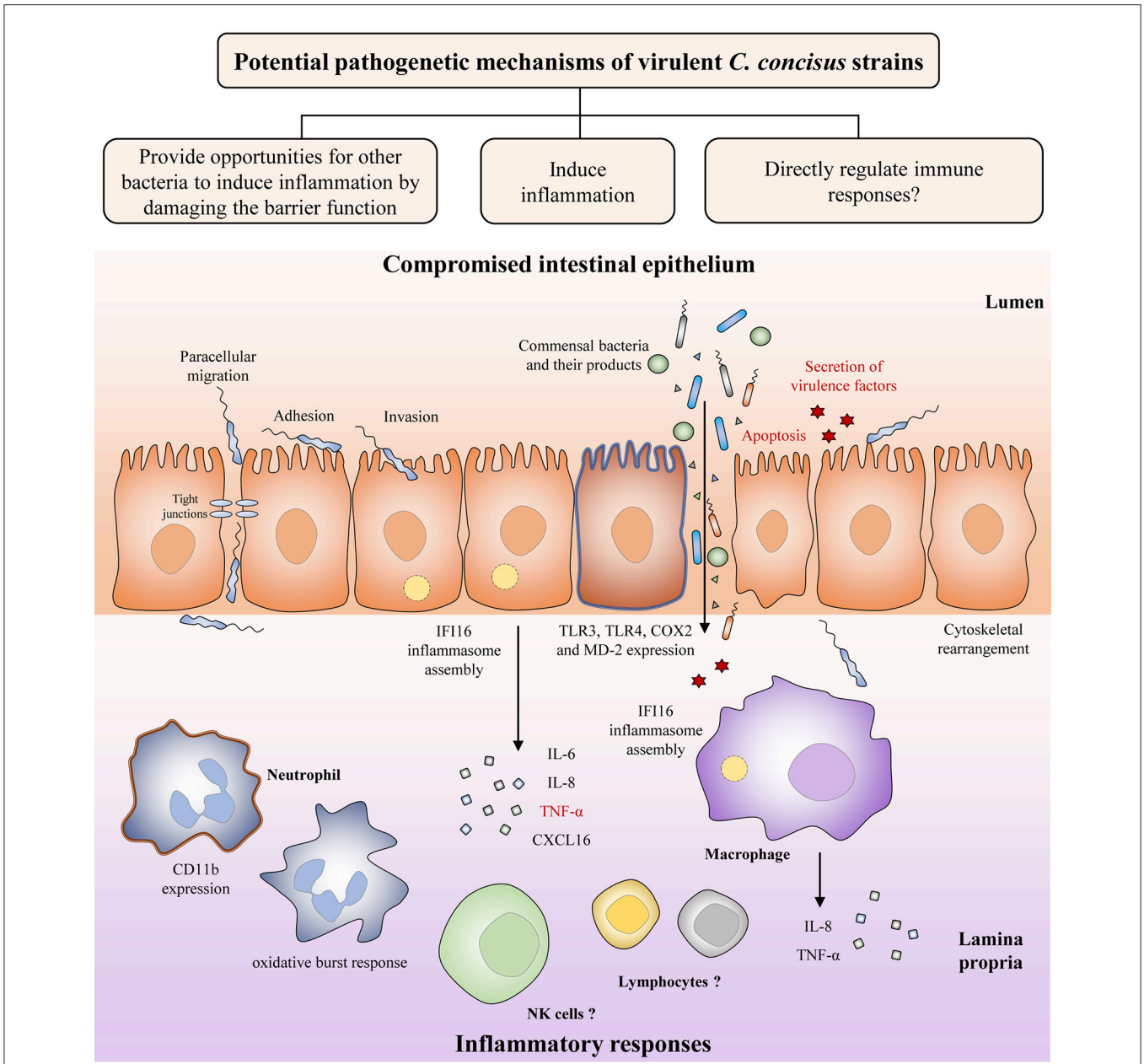

FIGURE 3 | Potential pathogenetic mechanisms of virulent *C. concisus* strains. Once it reaches the intestine, *C. concisus* adheres and invades the epithelium with the help of its flagellin and spiral shape. This is followed by a number of host responses such as cytoskeletal rearrangement, inflammasome assembly, expression of toll-like receptors, and the release of proinflammatory cytokines. Expression of virulence factors such as the zonula occludens toxin also results in proinflammatory cytokine production, as well as apoptosis (colored in red). The resulting compromised intestinal epithelium allows the increased translocation of commensal bacteria and their products from the lumen to the lamina propria. Proinflammatory cytokines and chemokines released by intestinal epithelial cells recruit immune cells to the site of infection and contributes to inflammatory response development.

limited sequence similarity with that of *S. typhimurium*, which explains why it is a poor stimulator of TLR5 (Watson and Galán, 2005). Studies have demonstrated that *C. jejuni*, *C. coli,* and *C. concisus* have limited potential to activate TLR5 (De Zoete et al., 2010; Ismail et al., 2013).

To date, only two virulence factors of *C. concisus* have been characterized, one of which is the zonula occludens toxin (Zot). Initially described in *Vibrio cholerae*, Zot is a known virulence factor that causes an increase in intestinal permeability. The *zot* genes in *Campylobacter* species are encoded by prophages and are divided into two clusters, which encode $Zot_{CampyType\_1}$ and $Zot_{CampyType\_2}$, respectively (Zhang et al., 2014; Liu et al., 2016). Although the two types of Zot toxins share common motifs, their overall sequence identities vary greatly, particularly

at the C-terminal compartment (Liu et al., 2016). Mahendran et al. have detected a similar prevalence of the cluster 1 *zot* gene in the oral *C. concisus* strains isolated from patients with IBD and healthy controls (Mahendran et al., 2013). Later on, Mahendran and colleagues found that the $Zot_{CampyType\_1}$ caused prolonged damage on Caco-2 monolayers (Mahendran et al., 2016). The damaging effect is different from that of *V. cholerae* Zot which induces transient and reversible damage to the intestinal epithelium (Fasano et al., 1991). This prolonged damaging effect induced by *C. concisus* Zot is at least partially due to the induction of cell apoptosis and/or intestinal epithelial cell production of proinflammatory cytokines such as TNF-α and IL-8 (Mahendran et al., 2016). Furthermore, pre-exposure to Zot causes increased phagocytosis of *E. coli* K12 by THP-1 macrophages, suggesting a possible role for *C. concisus* in enhancing responses of macrophages to other enteric bacterial species (Mahendran et al., 2016). Additionally, transcriptomics analysis showed that *C. concisus* Zot was able to upregulate the expression of TLR3, proinflammatory cytokines IL-6, IL-8, and chemokine CXCL16 (Deshpande et al., 2016). The *zot*-containing prophages are also found in a number of other human and animal hosted *Campylobacter* species including *C. ureolyticus*, *Campylobacter corcagiensis*, *C. gracilis*, *C. jejuni*, *Campylobacter hyointestinalis,* and *Campylobacter iguanorium*, however their pathogenic effects on human cells have not been examined (Liu et al., 2016).

In additional to Zot, another *C. concisus* virulence factor characterized is the membrane protein phospholipase A. Istivan et al. have examined the activity of haemolytic phospholipase A_2 (PLA_2) from *C. concisus* strains isolated from children with gastroenteritis and found that PLA_2 activity is detected in strains from both GS. Membrane extracts containing PLA_2 exhibited cytolytic effects on CHO cells, supporting the idea that *C. concisus* may have potential to cause tissue destruction related to intestinal inflammation (Istivan et al., 2004).

To date, only one study has examined the effects of *C. concisus* in animal model (Aabenhus et al., 2008). Mice displayed a significant loss of body weight between day 2 and day 5 following *C. concisus* inoculation via gastric route. Signs of inflammation in the gut were not consistently found, but micro abscesses were found in liver of infected animals. Additionally, infiltration of lymphocytes was also observed in the jejunum and colon of infected mice. However, the details of the *C. concisus* strains used were not provided.

Responses to Environmental Factors

Environmental factors present in the gastrointestinal tract such as bile and pH have been found to affect the growth of *C. concisus in vitro* (Ma et al., 2015). Reduction in *C. concisus* growth was observed with lower pH. The gastric pH values in patients with CD and UC range between 1.5–4.1 and 1.55–4.4, respectively, which are significantly higher than those in healthy individuals (0.95–2.6) (Press et al., 1998). Under laboratory conditions, *C. concisus* strains were unable to grow following exposure to pH 2 for 30 min, while only 20% of the strains were able to survive following exposure to pH 3.5 for 30 min, and exposure to pH 5 for 120 min had minor effects on *C. concisus* growth (Ma et al., 2015). The sensitivity of *C. concisus* toward low pH suggests that the acidic environment may prevent *C. concisus* from colonizing the stomach and intestinal tract. The less acidic gastric environment observed in patients with IBD may provide a better condition for *C. concisus* colonization (Press et al., 1998). Recently, Ma et al. reported that the derivatives of the food additive, fumaric acid, were able to enhance the growth of *C. concisus* strains (Ma et al., 2018). It was found that *C. concisus* strains showed the greatest increase in growth when cultured in media containing 0.4% of neutralized fumaric acid, neutralized monosodium fumarate and sodium fumarate. These results imply that removal of fumaric acid and its salts from diet of patients with IBD may help to improve their condition.

Campylobacter species have different ability in bile resistance. Some oral *C. concisus* strains are able to grow in the presence of 2% bile, suggesting they are able to survive in the enteric environment. Enteric *Campylobacter* species such as *C. jejuni* and *C. hominis* are bile resistant. Fox et al. have demonstrated that although significant growth inhibition was observed when *C. jejuni* was cultured in the presence of 2.5% bile, *C. jejuni* was still able to proliferate in cultures containing 5% bile (Fox et al., 2007). Lawson et al. showed that *C. hominis* strains isolated from stool samples were able to tolerate 2% bile (Lawson et al., 2001).

Antimicrobial Resistance of *C. concisus*

There has been an increase in antibiotic resistance in *Campylobacter* isolates from both humans and animals worldwide (Luangtongkum et al., 2009; Iovine, 2013). Among antimicrobial therapies for treatment of *Campylobacter* enteritis, fluoroquinolones, and macrolides are most commonly used, while tetracyclines are rarely used (Alfredson and Korolik, 2007). Intravenous aminoglycosides are sometimes used to treat serious bacteraemia and other *Campylobacter* induced systemic infections (Alfredson and Korolik, 2007). *Campylobacter* species resist antibiotics by several mechanisms such as modification or occupation of target sites thus preventing the binding of antibiotic compounds; efflux pump systems that reduce intracellular antibiotic concentrations; changes in bacterial membrane permeability that prevents entering of antibiotic compounds; and hydrolysis of antibiotic compounds (Alfredson and Korolik, 2007; Iovine, 2013). The resistance determinants in *C. jejuni* and *C. coli* and their mechanisms of action have been widely studied, however the resistance determinants in *C. concisus* have not been investigated in detail. It is thought that antibiotics resistance has not developed in *C. concisus* as it is susceptible to a variety of antibiotics, of which tetracycline, erythromycin, ciprofloxacin, and macrolides are most commonly reported (Johnson et al., 1986; Aabenhus et al., 2005; Vandenberg et al., 2006; Nielsen et al., 2013). Ciprofloxacin, along with other antibiotics, have been shown to be effective in treating certain phenotypes of CD (Sartor, 2004).

Antibiotics are used as primary or adjuvant treatment along with anti-inflammatory and immunosuppressive drugs for the treatment of IBD (Sartor, 2004). Antibiotics are used to selectively reduce tissue invasion, decrease luminal, and mucosal bacterial loads and their translocation (Sartor, 2004). Some patients with IBD are colonized with multiple *C. concisus* strains,

and a significantly higher prevalence of multiple oral *C. concisus* strains in patients with active IBD than healthy controls has been reported (Mahendran et al., 2013). Furthermore, for IBD patients who are in remission, those without antibiotic treatment usually have a higher prevalence of multiple oral *C. concisus* strains than those who were receiving antibiotics, indicating antibiotics were able to alleviate but not eradicate *C. concisus* growth in the intestinal tract (Mahendran et al., 2013).

In addition to antibiotics, the antimicrobial potentials of immunomodulating and anti-inflammatory drugs used for IBD treatments have not been widely tested. Immunosuppressive drugs such as azathioprine (AZA) and mercaptopurine (MP) used in the treatment of IBD have been reported to exhibit inhibitory effect on the growth of *C. concisus* strains, with the effect of AZA being more potent than MP (Liu F. et al., 2017). In their use as immunosuppressive drugs, both AZA and MP are eventually metabolized to purine analogs that interfere with DNA synthesis in immune cells (Nielsen et al., 2001). However, bioinformatics analysis has not identified all the enzymes required for AZA and MP metabolism in the *C. concisus* genome, indicating the inhibitory action of AZA and MP on *C. concisus* growth is not through the conventional pathway. AZA and MP can also reduce the growth of *Mycobacterium avium* subsp. *paratuberculosis*, another bacterium that is associated with human CD (Sanderson et al., 1992; Collins et al., 2000; Sieswerda and Bannatyne, 2006; Greenstein et al., 2007; Shin and Collins, 2008).

5-aminosalicylic acid (5-ASA) is an anti-inflammatory drug that is also commonly used to induce and maintain remission in IBD (Hanauer, 2006; Nikfar et al., 2009). Its effect on *C. concisus* growth varies between strains as it inhibits the growth of some *C. concisus* strains, while it promotes the growth of other strains, features which appears to be independent of *C. concisus* GS (Schwartz et al., 1982). Although 5-ASA is often used in the treatment of IBD, in some cases 5-ASA medications are found to cause exacerbations of colitis (Schwartz et al., 1982). The enhancement of *C. concisus* growth induced by 5-ASA may have implications in the deteriorated clinical conditions observed in patients with IBD following 5-ASA medications.

While antimicrobials may be effective in limiting the growth of *C. concisus* in the intestinal tract, continuous transportation of *C. concisus* from the oral cavity along with saliva and food makes eradication of this bacterium a challenge. Thus, antimicrobials targeting *C. concisus* in the oral cavity, particularly virulent strains, should be developed.

C. RECTUS

C. rectus (formerly named as *Wolinella recta*) is a small, straight, rod shaped, single polar flagellated bacterium with the size being 0.5 by 2–4 μm (Tanner et al., 1981). The colonies of *C. rectus* appear as convex shaped and spread or corrode blood agar plates (Tanner et al., 1981). *C. rectus* was initially identified using anaerobic condition consisting of 80% N_2, 10% CO_2, and 10% H_2, although the study indicated that some stains can grow in the presence of 5% oxygen (Tanner et al., 1981). A number of studies have used anaerobic conditions for the isolation of *C. rectus* from different clinical samples such as dental plaques, feces, and oesophageal mucosal biopsies (Rams et al., 1993; Gmur and Guggenheim, 1994; Von Troil-Lindén et al., 1995; Lastovica and Roux, 2000; Macuch and Tanner, 2000; Macfarlane et al., 2007). A study comparing different atmospheric conditions on the growth of *C. rectus* demonstrated that its growth could be observed at 30, 35, and 42°C under anaerobic conditions; in contrast it did not grow in a conventional microaerophilic atmosphere consisting 5% O_2, 10% CO_2, and 85% N_2 (Mahlen and Clarridge, 2009). Given all these findings, *C. rectus* is an anaerobic bacterium instead of microaerophilic bacterium.

C. rectus was first isolated from individuals with periodontal diseases (Tanner et al., 1981). It has since been isolated from various locations of the oral cavity including periodontal sulcus, tongue, cheek mucosa, and saliva (Könönen et al., 2007; Cortelli et al., 2008). *C. rectus* is predominantly localized in the middle and deep periodontal pocket zones, and tends to form clumps in tooth-attached and epithelium associated plaque areas (Noiri et al., 1997). A number of studies have reported the association between *C. rectus* and periodontal diseases. Macuch and Tanner detected *C. rectus* from 90% (42/47) of the subgingival sites of initial and established perondontitis individuals, which was signficianlty higher than that from gingivitis subjects (20%, 3/14) and healthy controls (10%, 2/18) (Macuch and Tanner, 2000). Dibart et al. also showed that *C. rectus* had a signfiicantly higher prevalence in supragingival plaque obtained from periodontally diseased subjects as comapred with healthy individuals (7/24 vs. 0/27) (Dibart et al., 1998). Furthermore, von Troil-Lindenl et al. reported that *C. rectus* was more frequently detected in saliva samples of subjects with advanced periondontitis (100%, 10/10) as comapred with individuals with initial or no periondontitis (40%, 4/10) (Von Troil-Lindén et al., 1995). Additionally, several studies have examined the prevalence of *C. rectus* in periondontitis patients with different disease status, however healthy subjects were not included. Several virulence factors of *C. rectus* including LPS, GroEL-like protein, and surface-layer protein have been characterized.

C. rectus acts as a stimulator of inflammation in the gingival tissue. It has been shown to enhance the production of IL-6 and IL-8 in human gingival fibroblasts (Dongari-Bagtzoglou and Ebersole, 1996). IL-6 is a multifunctional cytokine, as it can be both proinflammatory and anti-inflammatory, and its secretion is thought to drive the tissue damaging process in diseases such as periodontal disease (Irwin and Myrillas, 1998). *C. rectus* LPS is able to stimulate both plasmin activity and plasminogen activator activity in human gingival fibroblasts (Ogura et al., 1995). Plasmin is an enzyme that is converted from plasminogen via plasminogen activator catalysis (Martel-Pelletier et al., 1991). Plasmin is present in blood which degrades many blood plasma proteins such as fibrin clots (Martel-Pelletier et al., 1991). Significantly enhanced activities of plasmin and plasminogen activator activities have been observed in gingival fluid in periodontal disease (Hidaka et al., 1981; Talonpoika et al., 1990). Furthermore, *C. rectus* LPS also induces higher levels of IL-6 and prostaglandin E2 productions in aged human gingival fibroblasts as compared with those in younger cells, which may

explain the increased susceptibility of periodontal disease in aged individuals (Ogura et al., 1996; Takiguchi et al., 1996, 1997).

The *C. rectus* GroEL-like protein is a 64 kDa protein with antigenic properties (Hinode et al., 1998). It was found to cross-react with antibodies against human heat shock protein 60, *Helicobacter pylori* whole cells and the GroEL-like protein from *Actinobacillus actinomycetemcomitans*, a bacterial species that is associated with localized aggressive periodontitis (Hinode et al., 1998, 2002; Tanabe et al., 2003; Henderson et al., 2010). The *C. rectus* GroEL-like protein is also able to stimulate the production of IL-6 and IL-8 from the human gingival fibroblast monolayer (Hinode et al., 1998). The *C. rectus* GroEL-like protein is also found to possess immunodominant epitopes within both amino and carboxyl termini, and it may share same carboxyl epitopes with the *C. rectus* surface-layer (S-layer) protein (Hinode et al., 2002).

The S-layer is a monomolecular layer of a single secreted protein surrounding the entire surface which is found in almost all archaea and some Gram-positive and Gram-negative bacteria (Konstantinov et al., 2008). The S-layer protein (SLP) in *C. fetus* and *C. rectus* have been previously characterized, and the gene encoding SLP is also carried by *C. showae* (Tay et al., 2013). The *C. rectus* SLP has a molecular weight ranging between 130 and 166 kDa (Kobayashi et al., 1993; Nitta et al., 1997). *C. rectus* strains expressing SLP are resistant to complement and phagocytic mediated killing in the absence of specific antibodies (Okuda et al., 1997). However, *C. rectus* SLP does not play a major role in bacterial adhesion. *C. rectus* strains lacking the *crsA* gene which encodes the SLP protein are less effective at adhering to Hep-2 oral epithelial cells, with CrsA+ cells only 30 to 50% more adherent than the CrsA- cells (Wang et al., 2000). The CrsA- cells exhibit no difference in inducing the production of IL-6, IL-8, and TNF-α in Hep-2 cells as compared with CrsA+ cells. However, CrsA- *C. rectus* strains induce higher levels of these cytokines at early time points of the infection, suggesting that the S-layer in *C. rectus* may facilitate bacterial survival at the site of infection by delaying the cytokine responses (Wang et al., 2000).

Macrolides are a class of antibiotics used to treat a wide variety of infections (Hirsch et al., 2012). It inhibits protein synthesis by acting on the P site of the 50S ribosomal subunit, thus preventing peptide elongation (Payot et al., 2006). The rRNA methylase enzyme encoded by the erythromycin ribosome methylase (*erm*) gene methylates a single adenine in the 23S component of the 50S subunit, which sterically hinders the proper interaction between the macrolide and the 50S subunit, providing antibiotic resistance (Weisblum, 1995). *C. rectus* is the only *Campylobacter* species that has the *erm* determinants described. These determinants include Erm B, Erm C, Erm F, and Erm Q, though their clinical implications have not been examined (Roe et al., 1995).

In addition to its role in periodontal disease, *C. rectus* has been implicated in the association between maternal periodontitis and adverse pregnancy outcomes. Madianos et al. reported that a significantly elevated level of fetal IgM to *C. rectus* was observed among premature infants as compared to full-term neonates, indicating that *C. rectus*, as a maternal oral pathogen, may act as a primary infectious agent in fetus that leads to prematurity (Madianos et al., 2001). Later study by the same group demonstrated that *C. rectus* mediated growth restriction in a pregnant mice model, as higher numbers of growth-restricted fetuses were observed in the groups subcutaneously challenged by *C. rectus* as compared to the non-challenged groups (Yeo et al., 2005). The same group also showed that maternal *C. rectus* infection increased fetal brain expression of proinflammatory cytokines IFN-γ (Offenbacher et al., 2005). Furthermore, Arce and colleagues demonstrated that *C. rectus* was more invasive to human trophoblasts as compared to *C. jejuni*, paralleled with significantly upregulated mRNA and protein expressions of IL-6 and TNF-α in a dose-dependent manner (Arce et al., 2010). Moreover, the study also showed that *C. rectus* was able to translocate *in vivo* from a distant site of infection to the fetoplacental unit (Arce et al., 2010). Taking together, these studies suggest that as a pathogen in the oral cavity, *C. rectus* has the potential to contribute to the adverse pregnancy outcomes.

C. UREOLYTICUS

C. ureolyticus (formally known as *Bacteroides ureolyticus*) was first described in 1978 by Jackson et al., the strain was isolated from amniotic fluid using anaerobic condition (Jackson and Goodman, 1978). A later study has isolated *C. ureolyticus* from 103 superficial necrotic or gangrenous lesions. However, *C. ureolyticus* was rarely the sole bacterial species isolated from the site of infections, suggesting other microorganisms may be responsible for the pathogenesis of these diseases (Duerden et al., 1982).

C. ureolyticus has been frequently isolated from the genital tracts of both male and female. By examining the whole cell proteins of *C. ureolyticus* strains, Akhtar and Eley showed that strains isolated from urethra of men with and without non-gonococcal urethritis showed no difference in SDS-PAGE patterns (Akhtar and Eley, 1992). Later studies by Bennett et al. reported that *C. ureolyticus* was isolated from healthy individuals at a similar prevalence as compared with that from males and females presenting non-gonococcal urethritis (Bennett et al., 1990, 1991). These results indicate that *C. ureolyticus* is a part of the normal flora in the genital tract of both male and female.

In the past decades, *C. ureolyticus* has been considered as an emergent *Campylobacter* species in gastroenteritis as it is frequently isolated from fecal samples of patients presenting with diarrheal illness. By using a multiplex-PCR systems, Bullman et al. first reported the isolation of *C. ureolyticus* from feces of patients with gastroenteritis. Among all the *Campylobacter* positive samples being screened, 24% (83/349) was found to be positive for *C. ureolyticus*, of which 64% (53/83) had *C. ureolycitus* as the sole *Campylobacter* species detected (Bullman et al., 2011). By using PCR methods, another study by Bullman et al. also found that *C. ureolyticus* was the second most common non-*C. jejuni*/*C. coli* species in fecal samples collected from patients presenting diarrheal illness (Bullman et al., 2012). However, healthy controls were not included in these studies.

By combining the bacterial isolation and molecular detection methods, Collado et al. reported that *C. ureolyticus* is the

third most prevalent species of the Campylobacteria family in diarrheic stool samples in addition to *C. concisus* and *C. jejuni*, but no statistical difference was found between the diarrheic and healthy groups (Collado et al., 2013). A recent study has also reported the detection of *C. ureolyticus* from traveler's diarrhea cases (Serichantalergs et al., 2017). This study showed that *C. ureolyticus* was detected at a similar prevalence in patients presenting diarrhea and healthy individuals.

Despite being frequently isolated or detected from diarrheal stool samples, the association between *C. ureolyticus* and diarrheal diseases has not been established. However, the pathogenic potentials of *C. ureolyticus* have been examined by a number of *in vitro* studies. Through whole genome sequencing of *C. ureolyticus* strains, genes encoding known virulence factors have been identified. These factors were involved in bacterial adhesion, colonization, invasion, and toxin production (Bullman et al., 2013). By using an intestinal epithelial cell line model, *C. ureolyticus* was shown to be capable of adhering to Caco-2 cells but unable to invade, with the bacterial adhesion followed by cellular damage and microvillus degradation. Furthermore, secretome analysis had also detected release of putative virulence and colonization factors (Burgos-Portugal et al., 2012).

Hariharan et al. reported the isolation of *C. ureolyticus* from endometria of apparently normal mares under anaerobic condition (Hariharan et al., 1994). So far, this is the only study has reported isolation of *C. ureolyticus* from animal sources.

C. GRACILIS

C. gracilis (formally known as *Bacteroides gracilis*) was first isolated by Tanner et al. in 1981 from patients with gingivitis and periodontitis (Tanner et al., 1981).

C. gracilis has been isolated from patients with periodontal and endodontic infections, however its pathogenetic role in these diseases is still controversial. Macuch et al. reported that in addition to *C. rectus*, *C. gracilis* was the most dominant *Campylobacter* species isolated from subgingival sites among eight *Campylobacter* species being examined. However, similar levels of *C. gracilis* were detected in healthy, gingivitis and periodontitis sites, suggesting that its prevalence or quantity was unrelated to periodontal health or disease (Macuch and Tanner, 2000). A study by Siqueira and Rocas examining the prevalence of *C. gracilis* in patients with primary endodontic infections also found that it was not associated with clinical symptoms of the diseases (Siqueira and Rocas, 2003).

The first complete genome of *C. gracilis* was sequenced by Miller et al., which lead to identification of genes encoding for virulence factors including haemagglutinins, Zot, immunity proteins and other putative pathogenic factors (Miller and Yee, 2015). Nevertheless, supporting evidence for the virulence of *C. gracilis* is still lacking.

C. SHOWAE, C. HOMINIS, AND C. CURVUS

C. showae was first isolated by Etoh et al. in 1993 from dental plaque of gingival crevices of healthy adults (Etoh et al., 1993). A study later by Macuch et al. examining the prevalence of oral *Campylobacter* species from subgingival sites showed that in addition to *C. rectus*, *C. showae* was also found more frequently and in higher levels from patients with periodontitis and gingivitis than from healthy controls (Macuch and Tanner, 2000).

C. hominis was first described in 1998 by Lawson et al. through *Campylobacter* specific PCR assays. *C. hominis* was detected from stool samples of 50% of the 20 healthy individuals, while it was absent in all the saliva samples of the same individuals (Lawson et al., 1998). Later in a study by Lawson et al., *C. hominis* was isolated from the fecal samples of healthy individuals (Lawson et al., 2001).

By using PCR methods targeting the *Campylobacter* 16S rRNA gene, *C. showae* and *C. hominis* have been detected from intestinal biopsy samples collected from macroscopically inflamed and noninflamed areas. Furthermore, this was the first study reported the isolation of *C. concisus*, *C. showae*, *C ureolyticus,* and *C. hominis* from intestinal biopsies (Zhang et al., 2009). A later study by Man et al. also detected *C. showae* and *C. hominis* from stool specimens collected from children with CD (Man et al., 2010b). However, no association was found between the prevalence of *C. showae* and *C. hominis* in patients with CD and healthy controls.

C. curvus (previously named as *Wolinella curva*) was first described in 1984 by Tanner et al. (Tanner et al., 1984). *C. curvus* is a rarely encountered *Campylobacter* species in humans. Lastovica et al. reported an isolation rate of *C. curvus* as low as 0.05% (2/4122) from diarrheic stools of pediatric patients (Lastovica and Roux, 2000). A study by Abbott et al. also isolated *C curvus* from stool samples and showed that the prevalence of *C. curvus* was associated with sporadic and outbreak of bloody gastroenteritis and Brainerd's diarrhea in Northern California (Abbott et al., 2005). There was one study which reported isolation of *C. curvus* and *C. upsaliensis* from stools of patients with Guillain-Barré syndrome and Fisher's syndrome (Koga et al., 1999). However, serological examination of the patient from whom *C. curvus* has been isolated did not detect antibodies to this bacterium.

CONCLUSION

Accumulated evidence from the past decade supports the role of human hosted *C. concisus* in the development of IBD and possibly Barrett's esophagus and GERD. Recently, a CD-associated *C. concisus* molecular marker *csep1-6bpi* has been identified, strongly suggests that *csep1-6bpi* positive *C. concisus* strains is a potential causative agent of human CD. In addition to *C. concisus*, the association between human hosted *C. rectus* and periodontal diseases has also been established.

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FL played the major role in writing the review. LZ, RM, and YW provided critical feedback and helped in editing the manuscript.

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The Performance of an Oral Microbiome Biomarker Panel in Predicting Oral Cavity and Oropharyngeal Cancers

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The oral microbiome can play a role in the instigation and progression of oral diseases that can manifest into other systemic conditions. These associations encourage the exploration of oral dysbiosis leading to the pathogenesis of cancers. In this study, oral rinse was used to characterize the oral microbiome fluctuation associated with oral cavity cancer (OCC) and oropharyngeal cancers (OPC). The study cohort consists of normal healthy controls ($n = 10$, between 20 and 30 years of age; $n = 10$, above 50 years of age), high-risk individuals ($n = 11$, above 50 years of age with bad oral hygiene and/or oral diseases) and OCC and OPC patients ($n = 31$, HPV-positive; $n = 21$, HPV-negative). Oral rinse samples were analyzed using 16S rRNA gene amplicon sequencing on the MiSeq platform. Kruskal–Wallis rank test was used to identify genera associated with OCC and OPC. A logistic regression analysis was carried out to determine the performance of these genera as a biomarker panel to predict OCC and OPC. In addition, a two-fold cross-validation with a bootstrap procedure was carried out in R to investigate how well the panel would perform in an emulated clinical scenario. Our data indicate that the oral microbiome is able to predict the presence of OCC and OPC with sensitivity and specificity of 100 and 90%, respectively. With further validation, the panel could potentially be implemented into clinical diagnostic and prognostic workflows for OCC and OPC.

Keywords: oral cancer, oral rinse, saliva, biomarker, oral microbiome

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INTRODUCTION

Head and neck cancers (HNC) describe a broad category of aggressive heterogeneous tumor types arising from the upper aerodigestive tract (Lim et al., 2016b). Globally, HNC were responsible for 529,500 incident cases and 292,300 deaths in 2012, accounting for ∼3.8% of all cancer cases and 3.6% of cancer related deaths (Shield et al., 2017). Oral cavity cancers (OCC) and oropharyngeal cancers (OPC) constitute the majority of HNC with 300,000 men and 130,000 women being diagnosed annually (Adilbay et al., 2018). The number of newly diagnosed HNC patients is

predicted to increase by 62% by 2035, equating to 856,000 new cases due to demographic changes (Shield et al., 2017). While the risk factors for OCC in developing countries are mainly excessive tobacco and alcohol consumption, human papillomavirus (HPV) infection is a rising etiological factor for OPC in developed countries (Kulasinghe, 2015).

Early recognition of the symptoms and signs, as well as prompt diagnosis of OCC and OPC is vital for patient survival. However, due to the lack of early screening/diagnostic tools, these cancer types are often diagnosed at advanced stages, resulting in poor survival outcomes (5-year mortality rate of 40–50%) (Lam et al., 2007; Kulasinghe, 2015; Lim et al., 2016a). To improve patient survival, saliva diagnostics have played a central role in the discovery of biomarkers for OCC and OPC early detection. This is due to the fact that most biomarkers present in blood and urine can also be detected in a sample of saliva (Malamud, 2011). A recent study by Wang et al. demonstrated the feasibility of detecting tumor DNA in saliva from OCC and OPC patients with a sensitivity of 100% in contrast to 80% sensitivity when plasma samples were used from the same patients (Wang et al., 2015). These findings substantiate saliva as an ideal diagnostic medium to detect OCC and OPC. Nevertheless, none of these host biomarkers were able to successfully translate into the clinical setting due to the high amount of biological variation between individuals (Lim et al., 2016b, 2017a).

In recent years, the human oral microbiome has emerged as a new potential biomarker reservoir for OCC and OPC (Lim et al., 2017a,b). The oral microbiome, by definition, is the collective genomes of microorganisms that reside in the oral cavity. Bacteria are the primary cause of oral diseases that affect other systemic conditions such as cardiovascular diseases, adverse pregnancy outcomes, diabetes mellitus and respiratory diseases (Lim et al., 2017a). These associations encourage the exploration of oral dysbiosis and the pathogenesis of OCC and OPC due to their close proximity. For example, the association between chronic periodontitis and OCC and OPC could be measured objectively based on periodontitis history (Tezal et al., 2005). Furthermore, the periodontal pocket consists of stratified squamous epithelium and is continuously undergoing epithelial proliferation, migration, rete-ridge formation and ulcerations, providing an ideal site for HPV infection and persistence (Tezal et al., 2009). It is well-known that persistent HPV infection leads to the development of OPC (Chai et al., 2015). The oral cavity has the largest core of commonly shared microbes among unrelated individuals (Zaura et al., 2014). The incorporation of the oral microbiome with salivary tumor biomarkers may thus help overcome the challenge of molecular diagnosis for OCC and OPC due to human biological variation (Lim et al., 2017a).

Based on recent studies that describe changes in the oral microbiome between healthy individuals and OCC and/or OPC patients, it was concluded that prevalent bacterial groups in cancer are associated with tumor development and progression (Pushalkar et al., 2012; Schmidt et al., 2014; Guerrero-Preston et al., 2016; Wang et al., 2017; Wolf et al., 2017). In addition, these bacterial groups could also be used as potential biomarkers for cancer detection, prognosis, and monitoring. However, the use of these prevalent bacterial groups as OCC/OPC biomarkers remains to be demonstrated in a clinical setting. Furthermore, these data report on the abundance changes of specific bacterial species/phylo-types as individual biomarkers. The oral microbiome is an intricate entity, and a reflection of the complex interactions between all members of the community (Lim et al., 2017a). Due to the obligatory dependencies of oral bacteria for growth and survival, we feel that further analyses of these microbial fluctuations as a biomarker panel in a clinical setting is warranted.

We hypothesize that the differences between the oral microbiomes of OCC and OPC patients and healthy individuals are reflected in oral rinse samples, and detectable via 16S rRNA gene amplicon sequencing. These differences could then be associated to the pathogenesis of cancers, and used as a biomarker panel to predict OCC and OPC with high diagnostic accuracy in an emulated clinical setting. To that end, our study objectives were three-fold: (i) to characterize microbiome differences in oral rinse samples from normal healthy controls and OCC and OPC patients; (ii) to develop a robust oral microbiome biomarker panel to predict OCC and OPC based on microbial fluctuations and; (iii) to evaluate the oral microbiome biomarker panel in an emulated clinical setting.

MATERIALS AND METHODS

Study Cohort and Sample Collection

This study was approved by the Queensland University of Technology and University of Queensland Medical Ethical Institutional Boards (HREC no.: 1400000617 and HREC no.: 2017000662, respectively) and the Royal Brisbane and Women's Hospital (HREC no.: HREC/12/QPAH/381) Ethics Review Board. Written informed consent was obtained from all participants and all methods in this study were performed in accordance with the relevant guidelines and regulations. We have recruited normal healthy individuals between the ages of 20–30 years ($n = 10$) and above 50 years ($n = 10$) from the general population who self-reported to be in good general health. Individuals with bad oral hygiene and/or oral diseases such as gingivitis or periodontitis ($n = 10$) above the age of 50 years were also recruited in the same manner as high-risk population (full-mouth clinical examinations were performed by certified dentists). Newly diagnosed OCC and OPC patients were recruited upon diagnosis and were treatment naïve. Immunohistochemical detection of the $p16^{INK16a}$ protein was used as surrogate marker for HPV status. All participants were not on any local and/or systemic antibiotics prior to sample collection.

Participants were asked to refrain from eating and drinking for an hour prior to sample collection. Bottled water was provided for participants to rinse their mouth before sampling. Oral rinse samples were collected by asking participants to swish and gargle

Abbreviations: AUC, Area under curve; gDNA, Genomic DNA; HNC, Head and neck cancers; HPV, Human papillomavirus; OCC, Oral cavity cancer; OPC, Oropharyngeal cancer; OTU, Operational taxonomic unit; PLS-DA, Partial least squares–discriminant analysis; QIIME, Quantitative insights into microbial ecology; ROC, Receiver operating characteristic.

with 10 mL of 0.9% (w/v) saline solution (Baxter International Incorporate, Illinois, USA) for a minute and expectorate into a 50 mL sterile Falcon tube as previously published (Chai et al., 2016; Lim et al., 2017b; Sun et al., 2017). After collection, all samples were transported back to the laboratory on dry ice and stored at −80°C.

Bacterial Genomic (g)DNA Extraction

Total volume of 1 mL oral rinse samples were subjected to bacterial gDNA extraction using Maxwell® 16 LEV blood DNA kit (Promega Corporation, Wisconsin, USA) with an adapted protocol as published previously (Lim et al., 2017b).

16S rRNA Gene Amplicon Library Preparation and Sequencing

16S rRNA gene amplicons for sequencing by Illumina MiSeq system (Illumina Incorporate, California, USA) was prepared according to the manufacturers' protocol with gene-specific sequences targeting the V6–V8 hypervariable regions (primers 926F and 1392R) of the 16S rRNA gene (Shanahan et al., 2016). Q5® Hot Start High-Fidelity (New England Biolabs, Massachusetts, USA) polymerase enzyme was used for the amplicon and index PCR. The sequencing was performed at the Australian Centre for Ecogenomics (ACE, Brisbane, Australia).

Quantitative Insights Into Microbial Ecology (QIIME)

Illumina sequenced raw reads were analyzed using QIIME version 1.9.1 (Caporaso et al., 2010b). Chimeric sequences were identified and removed via USEARCH 6.1 to avoid perceived diversity (Edgar, 2010; Haas et al., 2011). Data sequences were clustered into operational taxonomic units (OTUs) by PyNAST with a 97% sequence identity threshold against Greengenes (chimera-checked 16S rRNA gene) core set database version 13.8 (DeSantis et al., 2006; Caporaso et al., 2010a; McDonald et al., 2012). Low abundance OTUs (≤0.1% of total sequences) and any sequences that were not of bacterial or archaeal origin were removed from the analysis. Based on the sample with the lowest OTU counts, random sampling (without replacement) was used to account for different sequencing depths that may occur for each individual sample.

Statistical Analysis

Rarefaction curve of the observed OTUs against sequences per sample and Shannon index was calculated using QIIME. On Calypso (version 5.4), bubble plot, partial least squares regression-discriminant analysis (PLS-DA, non-supervised) and redundancy analysis (supervised) was used to visualize the microbial communities (genus-level), based on OTU frequencies within respective categories in the metadata (Zakrzewski et al., 2017). In addition, the Kruskal–Wallis rank test was used to identify genera associated with OCC and OPC. Carstensen's multivariate receiver operating characteristic (ROC) curve with "Epi" package was used in R to evaluate the diagnostic potential of the biomarker panel. In brief, cancer status and microbial abundance (genus-level) was used as outcome and explanatory variable, respectively in a multivariable logistic regression statistical model. A predicted score was then generated for each sample using the estimated regression model and different cut-off values of this predicted score were used to classify samples into patients or controls. The performance of the biomarker panel was also tested in an emulated clinical setting by performing two-fold cross-validation test. In this case, a single subsample (randomly selected) was retained as the validation data for the testing model, and the remaining samples were used as training data. A bootstrap procedure was also implemented to repeat the process for 20 times to include all possible combinations of predictive model available (Devijver, 1982; Geisser, 1993; Kohavi, 1995).

RESULTS

Population Characteristics

Extracted bacterial gDNA from oral rinse samples were subjected to 16S rRNA gene amplicon sequencing. Based on our data, the average length of reads is 500 bp and the OTUs were subsampled to 1,044 counts. After processing, 770,061 high quality sequences were obtained in this study, with an average of 9,278 sequences per sample. From these sequences, 6 known phyla and 28 genera were identified, and a total of 108 OTUs were detected at the 97% sequence identify threshold.

The mean age for young normal healthy controls (between 20 and 30 years of age) was 26 years, and consisted of 8 males and 2 females. The mean age for elderly normal healthy controls (above 50 years of age) was 61 years and consisted of 4 males and 6 females. The mean age for high-risk controls (individuals with bad oral hygiene and/or oral diseases above 50 years of age) was 59 years and consisted of 8 males and 3 females (**Supplementary Table 1**). The patient cohort consists of mostly males with OCC or OPC and mean age of 65 years. A summary of the demographic and clinical characteristics of our patient cohort is presented in **Table 1**.

Oral Microbial Profiles From Normal Healthy Controls and Cancer Patients

A rarefaction curve of observed OTUs against sequence per sample was plotted for normal healthy controls, high-risk individuals and OCC and OPC patients to determine the efficiency of the sequencing process. The Shannon index was also calculated for each cohort and compared using rank test, with the patient cohort having significantly lower species diversity compared with the normal healthy controls and high-risk individuals (**Figures 1A,B**).

The taxonomic profiles of normal healthy controls, high-risk individuals and OCC and OPC patients were examined based on the proportion of bacterial sequences determined at genus-level (**Figure 2A**). The PLS-DA and redundancy plots show a clear distinct structure between normal healthy controls between the ages of 20–30 years and other categories (normal healthy controls above 50 years of age, high-risk individuals and OCC and OPC patients). While normal healthy controls above 50 years of age and high-risk individuals share similar features on

both the 2-D plots, they are well separated from the cancer cohort (**Figures 2B,C**). Our results demonstrate that age and cancers may play a role in the shift of oral microbiome and are consistent with previous findings (Kang et al., 2006). Due to the association of age and oral microbiome, normal healthy controls between the ages of 20 and 30 years were removed from subsequent analysis to avoid inherent bias. According to Kruskal-Wallis rank test, *Rothia* ($P < 0.0001$), *Haemophilus* ($P < 0.005$), *Corynebacterium* ($P < 0.01$), *Paludibacter* ($P < 0.01$), *Porphyromonas* ($P < 0.01$), and *Capnocytophaga* ($P < 0.05$) are found in significantly lower abundance in oral rinse samples from OCC and OPC patients while *Oribacterium* ($P < 0.05$) is significantly higher. While *Actinomyces* ($P < 0.05$), *Parvimonas* ($P < 0.05$), *Selenomonas* ($P < 0.05$), and *Prevotella* ($P < 0.05$) have a significantly higher abundance in OCC compared with OPC; HPV has a positive correlation on the abundance of *Haemophilus* ($P < 0.05$) and *Gemella* ($P = 0.06$). Pathogenic and/or opportunistic bacteria such as *Actinomyces* ($P < 0.01$), *Actinobacillus* ($P < 0.05$), *Lautropia* ($P < 0.05$), *Fusobacterium* ($P < 0.05$) and *Aggregatibacter* ($P < 0.05$) are significantly more abundant in high-risk individuals (**Supplementary Table 2**).

TABLE 1 | The demographic characteristics of the patient cohort ($n = 52$).

Explanatory variables	Patients	
	HPV-negative ($n = 21$)	HPV-positive ($n = 31$)
DEMOGRAPHICS		
Gender		
Male	16	30
Female	5	1
Age		
<50	1	0
>50	20	31
Race and ethnicity		
Caucasian	21	31
Other	0	0
Smoking		
Non-smoker	2	8
Ex-smoker	16	20
Smoker	3	3
Alcohol		
Non-drinker	7	19
Drinker	14	12
TUMOR CHARACTERISTICS		
AJCC TNM stage		
Stage I	3	1
Stage II	3	1
Stage III	6	4
Stage IV	9	25
Tumor anatomic site		
Oral cavity	10	5
Oropharyngeal	11	26

Oral Microbiome Biomarker Panel and Clinical Significance

Genera associated with OCC and OPC were selected as potential candidates to be included in the prediction panel. Hence, Carstensen's ROC curve for *Rothia*, *Haemophilus*, *Corynebacterium*, *Paludibacter*, *Porphyromonas*, *Oribacterium,* and *Capnocytophaga* were generated to investigate the panel's optimum performance based on the original samples that were used in building the model (**Figure 3**). With this approach, the panel has an area under curve (AUC) of 0.98 and sensitivity and specificity of 100 and 90%, respectively. The data were then processed using two-fold cross-validation and bootstrap to determine the performance of this panel in a "most likely scenario" with the intention of clinical translation. With the new enforced probability, the panel has an AUC of 0.82 and sensitivity and specificity of 90 and 61%, respectively (**Supplementary Table 3**). While the performance of the panel decreases, the AUC shows a prediction accuracy of 82%.

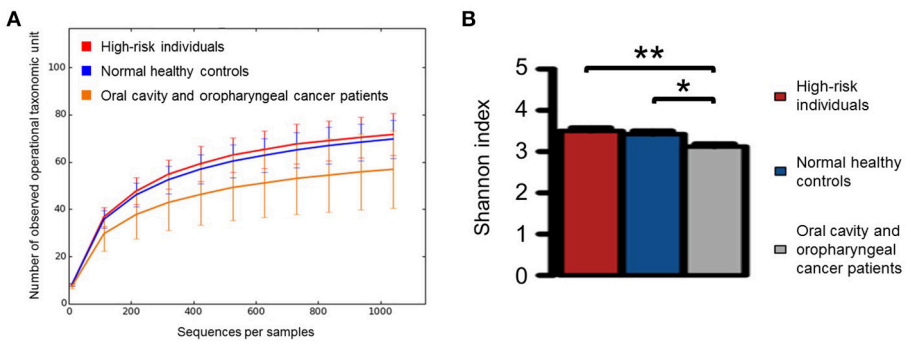

FIGURE 1 | (A) Rarefaction curve of observed operational taxonomic unites against sequences per sample for normal healthy controls ($n = 20$), high-risk individuals ($n = 11$), and oral cavity and oropharyngeal cancer patients ($n = 52$) as well as **(B)** the comparison of Shannon index for each category using rank test. Significant differences are denoted with $^*P < 0.05$ and $^{**}P < 0.01$, respectively.

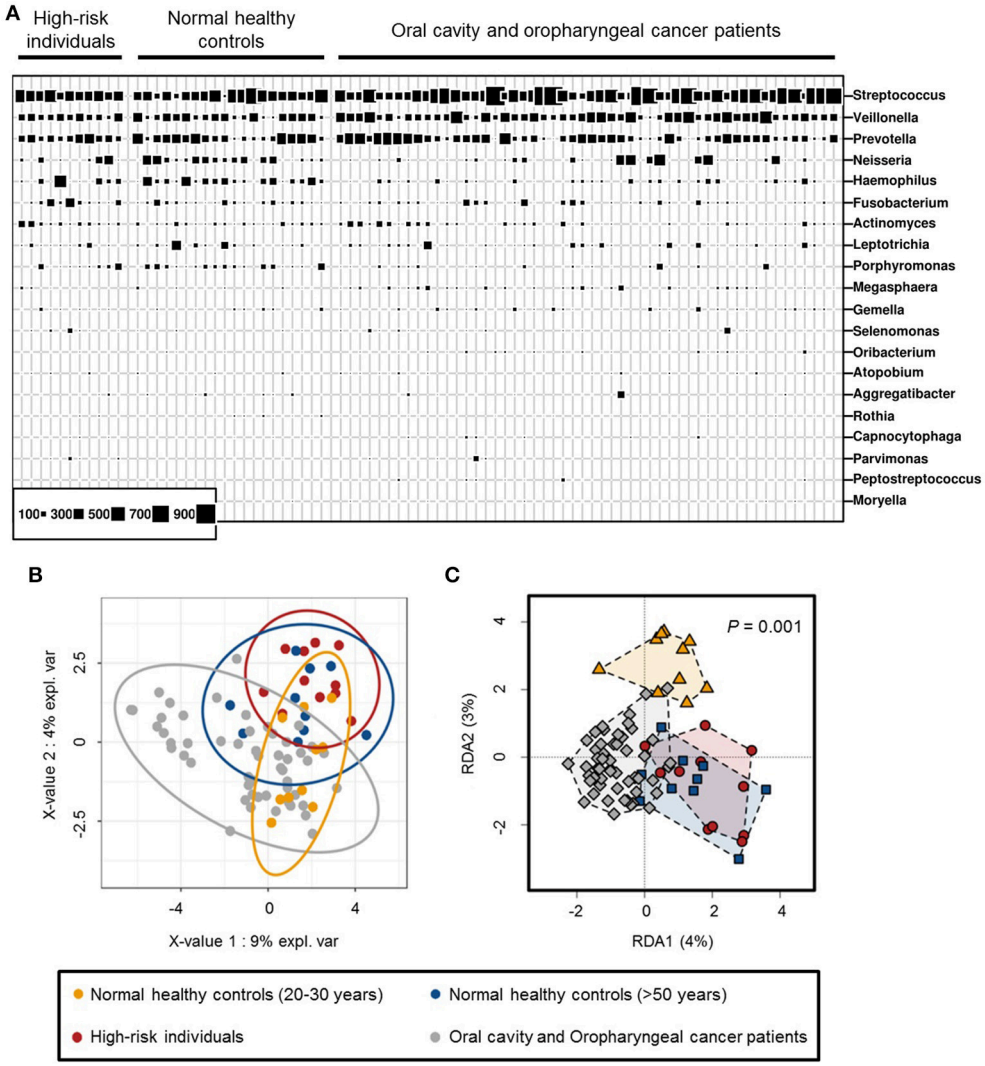

FIGURE 2 | (A) Bubble plot of 20 most abundant bacterial genera detected in oral rinse samples of normal healthy controls (between 20 and 30 years of age, $n = 10$ and above 50 years of age, $n = 10$), high-risk individuals ($n = 11$) and oral cavity and oropharyngeal cancer patients ($n = 52$). **(B)** Partial least squares regression-discriminant analysis (PLS-DA) and **(C)** redundancy analysis of microbial communities (genus-level) in oral rinse samples, based on OTU frequencies within respective categories.

DISCUSSION

Oral dysbiosis is a frequent event in oral and oropharyngeal carcinogenesis (Tezal et al., 2005; Galvão-Moreira and da Cruz, 2016; Laprise et al., 2016; Moraes et al., 2016; Furquim et al., 2017). In this study, we utilized oral rinse as a platform for oral microbiome characterisation and described an oral microbiome panel (*Rothia*, *Haemophilus*, *Corynebacterium*, *Paludibacter*, *Porphyromonas*, *Oribacterium,* and *Capnocytophaga*) that can discriminate OCC and OPC patients from age-matched normal healthy individuals. Our findings concur with previous findings, in which the differences in microbial abundance and diversity may have a role in cancer initiation and progression (Lim et al., 2017a). To our knowledge, this is the first study that evaluates the performance of an oral microbiome panel to predict OCC and OPC in an emulated clinical setting.

Based on our data, age is a contributing factor for microbial differentiation in the oral environment. Due to the fact that OCC and OPC generally manifest in the sixth decade of life in addition to the mean age from our patient cohort (∼65 years), individuals between the ages of 20 and 30 years were removed from the study to avoid the incorporation of any non-causal elements into the biomarker panel (Kang et al., 2006). While the microbial richness is generally consistent between all samples, OCC and OPC patients have a significantly lower diversity (Pushalkar et al., 2012; Schmidt et al., 2014; Guerrero-Preston et al., 2016). This may be due to the Warburg effect triggered by tumorigenesis, favoring the growth conditions of

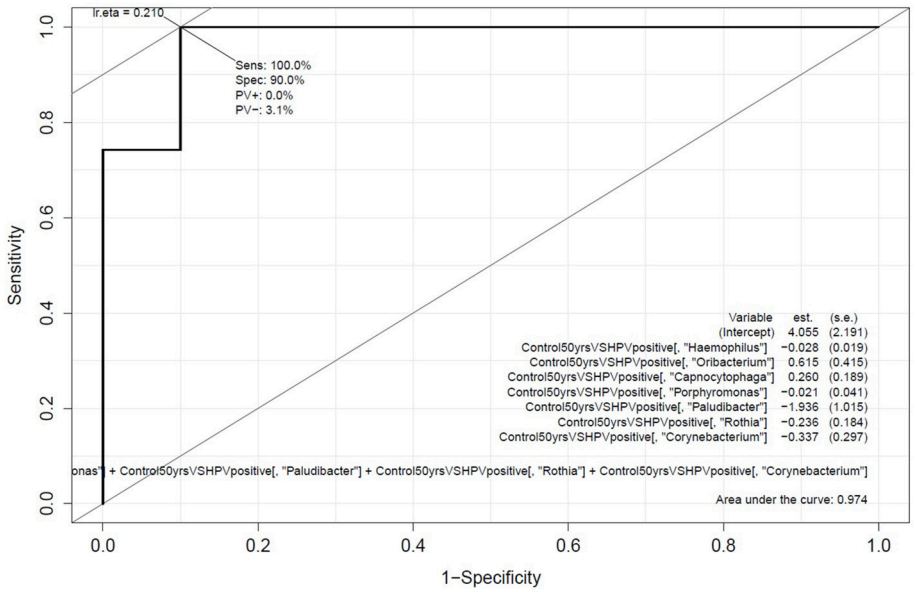

FIGURE 3 | Performance of the oral microbiome panel in predicting oral cavity and oropharyngeal cancers. Carstensen's multivariate receiver-operating characteristics curve based on the abundance of *Rothia*, *Haemophilus*, *Corynebacterium*, *Paludibacter*, *Porphyromonas*, *Oribacterium,* and *Capnocytophaga*; comparing normal healthy controls (above 50 years of age, $n = 10$) with oral cavity and oropharyngeal cancer patients ($n = 52$).

a subset of microbes (Cummins and Tangney, 2013). It is noted that while the majority of the genera have a lower abundance in cancers, *Oribacterium* was found to be significantly higher in abundance compared with normal healthy controls and high-risk individuals. In a recent study, Guerrero-Preston et al. also reported a higher *Oribacterium* abundance in OCC (Guerrero-Preston et al., 2016). This indicates that *Oribacterium* may play an important role in the pathogenesis of OCC and OPC.

HPV-positive OCC and OPC comprise a distinct molecular, clinical and pathologic disease entity that is likely causally associated with HPV infection (Gillison et al., 2000). In this study, we demonstrate that *Haemophilus* and *Gemella* have a positive correlation with HPV infection. A previous report described the increased abundance of *Leuconostoc* and *Gemella* in HPV-positive cancer samples (Guerrero-Preston et al., 2016). This differences may be due to the sample size variation as well as the demographics of the control cohort (in the previous study, individuals emitted to the same hospital with the cancer cohort for other diseases were used as controls provided that they have no history of cancer) (Guerrero-Preston et al., 2016). Nevertheless, these findings agree with previous studies on the impact of HPV on microbiome in cervical cancers (Mitra et al., 2015, 2016). It was also noted that certain genera were more abundant in OCC (*Actinomyces*, *Parvimonas*, *Selenomonas,* and *Prevotella*) compared with OPC. According to our previous study, there were no significant fluctuations in the microbiota composition between the oral and oropharyngeal regions, despite the distinct cellular, morphological and functional characteristics of these two regions (Lim et al., 2017b). Hence, we speculate that the microbiome differences may be directly influenced by the oral cavity tumor characteristics and/or secretomes.

Previous studies described oral diseases as a potential risk-factor for HNC (Tezal et al., 2005; Hasan and Palmer, 2014). In this study, we characterize the oral microbiome from individuals with gingivitis or periodontitis above the age of 50 years to determine if the oral microbiome could be used as a high-risk screening marker for OCC and OPC. Based on our data, the microbial composition from high-risk individuals bares a closer resemblance to normal healthy controls above the age of 50 years compared with OCC and OPC patients. This indicates that the oral microbiome differences detected in OCC and OPC patients may be cancer-specific, highlighting oral microbiome as an ideal biomarker candidate for cancer detection. However, due to the lack of dental records for the patient cohort, direct comparisons of oral health status could not be made. Future studies are warranted to compare the oral microbiome between high-risk individuals and cancer patients based on dental records to draw a more definite conclusion.

In previous studies, *Actinobacillus, Actinomyces, Aggregatibacter, Capnocytophaga, Fusobacterium, Oribacterium, Rothia, Haemophilus, Leptotrichia, Neisseria, Porphyromonas* and *Veillonella* were commonly found to be potential individual biomarker candidate for HNC due to the significant shift of abundance in cancer samples (Nagy et al., 1998; Mager et al., 2005; Schmidt et al., 2014; Guerrero-Preston et al., 2016; Wang et al., 2017; Wolf et al., 2017). Our investigation concurs with these findings. However, the compositions of *Aggregatibacter* and *Fusobacterium* between normal healthy controls and cancer patients are equivalent in our study. In addition to the genera mentioned, we also discovered a significant loss of abundance

with *Paludibacter* and *Corynebacterium* in cancer patients that has not been reported previously. The oral microbiome panel was conceptualized by using genera that have a strong correlation with OCC and OPC via an inclusion-exclusion principle. The panel is able to predict OCC and OPC with a high sensitivity and specificity. Although the performance of the panel decreases when placed against a more realistic scenario, the panel's prediction accuracy was not significantly affected. This is a good clinical endpoint as the panel enables non-invasive OCC and OPC detection with a simple genera quantification test on oral rinse samples.

One of the limitations of this study is the modest sample size and the lack of validation system. However, despite the fact that inherent value often obtained with greater numbers, we were able to detect and report statistically significant differences between our cohorts of interest. In addition, our findings correlate with previously established studies that consist of similar or larger sample cohort (Schmidt et al., 2014; Guerrero-Preston et al., 2016; Wang et al., 2017; Wolf et al., 2017). These results provide support in conducting larger cohort validation in the future to substantiate the diagnostic value of the oral microbiome panel in OCC and OPC. In addition, to best of our knowledge, the oral microbiome association between active smokers and OCC and OPC patients has not been investigated in the past. In our experience, this is due to the difficulty in recruiting individuals above the age of 50 years who actively smoke while having no oral hygiene problems and underlying conditions (oral, lung, and cardiovascular diseases) that could potentially influence the oral microbiome (Weidlich et al., 2008; Aho et al., 2015; Menon et al., 2017; Yamashita and Takeshita, 2017). Studies have shown that smoking affects the oral microbiome; the oral microbiome panel will have to be tested on age-matched active smoking individuals in the future to avoid overrepresentation (Wu et al., 2016). A longitudinal study using the oral microbiome panel should also be carried out to monitor the oral microbiome changes before and after treatment with multiple follow-up studies. The rate of oral microbiome recovery measured using the panel may lead to personalized medicine.

Overall, the results of this study have shown that the oral microbiome panel of *Rothia*, *Haemophilus*, *Corynebacterium*, *Paludibacter*, *Porphyromonas*, *Oribacterium,* and *Capnocytophaga* were able to discriminate age-matched normal healthy controls from OCC and OPC patients with high accuracy. Furthermore, two advanced statistical models were used to demonstrate the clinical relevance of the panel. While previous studies focus on the oral microbiome differences between controls and cases, we were able to establish that the information is clinically useful. We hope the findings presented here will contribute to the translation of microbial profiling techniques into technologies that improve the diagnosis and treatment of OCC and OPC.

AUTHOR CONTRIBUTIONS

YL collected samples, compiled all epidemiological data on all subjects, conducted all experiments, data interpretation, and wrote the manuscript. NF provided support on QIIME and Calypso and revised the manuscript critically for content. MT and MM contributed to the experimental design, laboratory facilities and technical support, data interpretation, and revised the manuscript critically for content. LK identified areas of tumor cellularity for all patients and conducted HPV assays on selected patients. CP participated in study concept and design, study coordination and revised the manuscript critically for content. All authors have read and approved the content of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00267/full#supplementary-material

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The Intestinal Roundworm *Ascaris suum* Releases Antimicrobial Factors Which Interfere With Bacterial Growth and Biofilm Formation

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Ascariasis is a widespread soil-transmitted helminth infection caused by the intestinal roundworm *Ascaris lumbricoides* in humans, and the closely related *Ascaris suum* in pigs. Progress has been made in understanding interactions between helminths and host immune cells, but less is known concerning the interactions of parasitic nematodes and the host microbiota. As the host microbiota represents the direct environment for intestinal helminths and thus a considerable challenge, we studied nematode products, including excretory-secretory products (ESP) and body fluid (BF), of *A. suum* to determine their antimicrobial activities. Antimicrobial activities against gram-positive and gram-negative bacterial strains were assessed by the radial diffusion assay, while effects on biofilm formation were assessed using the crystal violet static biofilm and macrocolony assays. In addition, bacterial neutralizing activity was studied by an agglutination assay. ESP from different *A. suum* life stages (*in vitro*-hatched L3, lung-stage L3, L4, and adult) as well as BF from adult males were analyzed by mass spectrometry. Several proteins and peptides with known and predicted roles in nematode immune defense were detected in ESP and BF samples, including members of *A. suum* antibacterial factors (ASABF) and cecropin antimicrobial peptide families, glycosyl hydrolase enzymes such as lysozyme, as well as c-type lectin domain-containing proteins. Native, unconcentrated nematode products from intestine-dwelling L4-stage larvae and adults displayed broad-spectrum antibacterial activity. Additionally, adult *A. suum* ESP interfered with biofilm formation by *Escherichia coli*, and caused bacterial agglutination. These results indicate that *A. suum* uses a variety of factors with broad-spectrum antibacterial activity to affirm itself within its microbe-rich environment in the gut.

Keywords: intestinal nematode, ascariasis, helminth, microbiota, antimicrobial peptides, biofilm, lectin

INTRODUCTION

Soil-transmitted helminth infections infect approximately 1.5 billion people worldwide (World Health Organization, 2017) as well as most companion, livestock, and wild animals (Eijck and Borgsteede, 2005; Nganga et al., 2008). The most prevalent helminth infection in people, Ascariasis, is caused by the intestinal roundworm *Ascaris lumbricoides* which infects approximately 800 million people (Brooker and Pullan, 2013) while the closely related *Ascaris suum* is commonly found in pigs raised for pork consumption (Dold and Holland, 2011; Thamsborg et al., 2013; Kreinoecker et al., 2017). The porcine host serves as a valuable infection model for humans for many diseases (Meurens et al., 2012), but particularly for Ascariasis, given the similarities between the human and pig intestinal tract and microbiota in comparison to that of mice (Heinritz et al., 2013) as well as the life cycles, genetic, and proteomic similarities of both *Ascaris* species (Leles et al., 2012; Xu et al., 2013; Shao et al., 2014). Infection begins with the ingestion of embryonated eggs containing L3-stage larvae which hatch in the small intestine before penetrating the intestinal wall of the cecum and colon to start their tissue migratory phase (Murrell et al., 1997). These L3-stage larvae then migrate through the liver before reaching the lungs by 6–8 days post-infection (Roepstorff et al., 1997). From the lungs, the larvae are coughed up and swallowed again, thereby reaching the small intestine where the nematodes will further develop into the L4 and adult stages and remain for approximately 1 year (Dold and Holland, 2011).

The small intestine hosts a microbiota, albeit at a lower density of microbes than that of the colon (Zoetendal et al., 2008; Isaacson and Kim, 2012; Sender et al., 2016). *A. suum* larvae invade host tissues in the distal small intestine, cecum, and proximal colon while adult worms reside in the small intestine; therefore, *A. suum* inhabits a microbial environment. Many studies have explored interactions between intestinal parasites and their hosts (Varyani et al., 2017), as well as hosts and their intestinal microbiota (Hooper et al., 2012); however, our understanding of how intestinal nematodes interact with the host microbiota is very limited. Recently, studies have linked various helminth infections to alterations in the host-intestinal microbiota (Zaiss and Harris, 2016). While host-immune factors and local metabolic factors have been implicated in shaping the microbiota, helminth components involved in the interaction with the microbial environment remain unexplored.

Studies in the free-living model nematode *Caenorhabditis elegans* suggest that these worms acquire an intestinal microbiota, distinct from their environments (Berg et al., 2016; Dirksen et al., 2016; Zhang et al., 2017). Though derived from environmental sources, the composition of the *C. elegans* microbiota was found to be selectively enriched and conserved across diverse sampling origins (Zhang et al., 2017). Additionally, certain microbes have been shown to support nematode growth and proliferation, while others pose infectious threats (Félix and Duveau, 2012; Samuel et al., 2016). Many laboratory-based studies have established *C. elegans* infection model systems with various bacterial pathogens (Couillault and Ewbank, 2002). Furthermore, other studies have also shown differential effects of biofilm-associated bacteria on *C. elegans* physiology (Tan and Darby, 2004; Begun et al., 2007; Smolentseva et al., 2017), demonstrating the diversity and importance of nematode-microbe interactions. Using these models, numerous details of the *C. elegans* antimicrobial defense response have identified detection mechanisms, transcription factors, and inducible effector molecules that form the nematode's innate immune system (Kim and Ewbank, 2015). In contrast in parasitic nematodes not much is known. Previous studies in *A. suum* have described induced transcription of members of two families of antimicrobial peptides (AMPs), *A. suum* antibacterial factors (ASABFs) and cecropins, in response to injection with heat-killed *Escherichia coli* (Pillai et al., 2003, 2005). In these studies, transcripts of some AMPs were also detected in the absence of an overt infectious challenge, suggesting that some defense molecules are produced constitutively. Homologs of ASABFs, called antibacterial factors, have also been described in *C. elegans* (Kato et al., 2002), as well as several other proteins and peptides involved in defense (Tarr, 2012).

Given the importance of interactions with bacteria for *C. elegans* physiology and longevity, as well as the absence of severe systemic inflammation of the host during Ascariasis despite migration of larvae originating in the intestine, we hypothesized a direct interaction of components of the intestinal parasitic nematode *A. suum* with the host gut-microbiota. Understanding the strategies that parasitic nematodes have evolved to control their microbial environments can provide insights into how the microbiota may be intentionally modified for therapeutic purposes, especially since nematodes do this without apparent detriment to their hosts. Herein we aimed to determine if *A. suum* nematodes release antimicrobial proteins and peptides in their excreted and secreted products (ESP) and whether or not these nematode products possess detectable antimicrobial activities.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted in accordance with the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and ethical approval was obtained from the Landesamt für Gesundheit und Soziales Berlin, Germany (approval numbers H0288/15 and H0005/18).

Parasite Material

Adult *A. suum* worms were obtained from infected pigs at a local slaughterhouse. Upon retrieval, worms were separated by sex and washed several times in a balanced salt solution (BSS), recipe modified from Locke's solution (Chehayeb et al., 2014), containing antibiotics and used as culture media for adult worms (127 mM NaCl, 7.5 mM $NaHCO_3$, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 200 U/mL penicillin, 200 μg/mL streptomycin, 50 μg/mL gentamicin, 2.5 μg/mL amphotericin B), then kept at 37°C with 5% CO_2. Three to five adult worms were kept together in 300 mL of BSS. Media changes were completed daily by transferring worms to fresh bottles containing fresh BSS. To generate ESP for use in our experiments, worms were cultured in antibiotic-free

BSS for several days with daily media changes. Spent media from the first 48 h were not used in microbiological assays. ESP were sterile filtered through a 0.22 μM vacuum-driven filter system and stored at −20°C until further use. For body fluid collection, adult worms were cultured in the absence of antibiotics as described for antibiotic-free ESP collection and body fluid was collected using the method of Chehayeb et al. (2014), sterile filtered using a 0.22 μM syringe-driven filter system and stored at −20°C until further use.

Third stage larvae were generated as previously described (Urban et al., 1981). Unembryonated *A. suum* eggs were collected from cultures of adult female worms, washed multiple times in water and placed in 0.1 N H_2SO_4 for 6–8 weeks at room temperature. Embryonation rates were assessed visually by light microscopy. Embryonated eggs were hatched using 5.25% hypochlorite treatment and incubation with slowly moving glass beads. Hatched third-stage larvae (L3) were cultured at a density of approximately 30,000 larvae/well of a 12-well tissue culture plate, in 1 mL of larval media [RPMI-1640 media (PAN Biotech, Aidenbach, Germany), 50 mM glucose, 200 U/mL penicillin, 200 μg/mL streptomycin, 50 μg/mL gentamicin, 2.5 μg/mL amphotericin B]. After 2 days in culture, worms were washed extensively with antibiotic-free media and then maintained in antibiotic-free larval media with media changes every 24 h for the first 2 days. Spent media from the first 48 h were discarded. Thereafter, supernatants were harvested every 48 h for 10–14 days, sterile filtered through a 0.22 μM syringe-driven filter system, and stored at −20°C until further use.

For tissue migrating larval stages, German Landrace piglets aged 8 weeks were orally infected with 12,000–15,000 embryonated *A. suum* eggs/pig. Pigs were sacrificed at 8 days post-infection for lung-stage larvae, and 16 days post-infection for L4-stage larvae. Lung-stage L3 larvae were retrieved as previously described with minor modifications (Slotved et al., 1997; Saeed et al., 2001). Briefly, harvested organs were ground using a hand-operated meat grinder. Ground organs were mixed with 0.9% NaCl to 300 mL and subsequently mixed with 300 mL of 2% agar solution which had been autoclaved and held at 45°C until use. The tissue-agar mixture was then poured into large glass petri dishes lined with plastic wrap and allowed to solidify, forming tissue gels. Tissue gels were wrapped in 200 μm woven synthetic mesh (Sefar, Edling, Germany), transferred to beakers with 0.9% NaCl, and incubated at 37°C for 3 h to allow worms to migrate into the saline solution. After 3 h, gels were removed and the remaining suspension transferred to Baermann funnels and allowed to sediment for 0.5–1 h. Worms were then collected and washed several times with larval media. Worms were cultured at 37°C with 5% CO_2 with media changes every 24 h. Unfortunately, we were unable to retrieve antibiotic-free lung-stage larvae, so this material was excluded from microbiological assays.

For L4-stage larvae, pigs were sacrificed at 16 days post-infection and the distal small intestine and proximal cecum were removed. Intestinal contents were incubated in pre-warmed NaCl at 37°C for 3 h to allow larval migration away from host tissue and ingesta. This mixture was then poured over a Baermann funnel and allowed to sediment, then collected and washed extensively, and the worms cultured as described for L3-stage larvae, except with approximately 100 larvae per well of a 12-well tissue culture plate in 1 mL of larval media.

For use in the agglutination assay, adult *A. suum* ESP were concentrated using Vivaspin centrifugal concentrators with a 5 kDa molecular weight cut off (Sartorius, Göttingen, Germany) to a final protein concentration of 1 mg/mL. For LC-MS/MS analysis, ESP and BF samples were prepared as previously described (Eberle et al., 2015), with modifications. Oasis HLB Plus cartridges (Waters 186000132, Milford, USA) were rinsed with 2 mL of pure methanol, equilibrated with 3 mL of 0.2% formic acid, and loaded with either 5 mL of *A. suum* ESP or 3 mL of BF. Samples were washed with 5 mL of 0.2% formic acid then eluted with 1 mL of 30% acetonitrile/0.2% formic acid, then 1 mL of 60% acetonitrile/0.2% formic acid, and finally with 1 mL of 80% acetonitrile/0.2% formic acid. Eluates were pooled and dried in a centrifugal evaporator.

Bacterial Strains

The strains used to evaluate antibacterial activities of *A. suum* products in the radial diffusion assay included: *Escherichia coli* IMT19224, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) ATCC 14028, and *Staphylococcus aureus* IMT29828, all obtained from the strain collection of the Institute of Microbiology and Epizoonotics, Freie Universität Berlin. The strains used to assess the effects of *A. suum* ESP on biofilm formation included the biofilm forming *E. coli* K-12 strains AR3110 and AR115. *E. coli* IMT19224, AR3110, and AR115 were used to assess agglutinating activity of *A. suum* ESP. Strains were selected to include representative gram-negative and gram-positive bacterial strains which may model *A. suum*-microbe interactions or to elucidate anti-biofilm activities of *A. suum* ESP. *E. coli* IMT19224 is a sequence type 131 (ST131) strain; ST131 isolates are commonly multidrug resistant, producing extended-spectrum β-lactamases and resistant to fluoroquinolones (Nicolas-Chanoine et al., 2014). *E. coli* AR3110, derived from *E. coli* K-12 strain W3110 by correcting a single nucleotide polymorphism in the *bcs* operon, produces phosphoethanolamine-modified cellulose and amyloid curli fibers as predominant extracellular matrix components in macrocolony biofilms (Serra et al., 2013; Thongsomboon et al., 2018). *E. coli* AR115 was derived from AR3110 by deleting *wcaE*, a gene involved in colanic acid synthesis (Miajlovic et al., 2014).

Radial Diffusion Assay

Antibacterial activities of ESP were assessed using the radial diffusion assay (Takemura et al., 1996). Overnight cultures were diluted 1:100 in Mueller-Hinton Broth (Carl Roth, Karlsruhe, Germany) and incubated at 37°C with shaking at 250 rpm until reaching an optical density of 0.3–0.4 at 600 nm. The bacteria were centrifuged at 880 x *g* for 10 min at 4°C, washed once with cold sodium phosphate buffer (100 mM, pH 7.4), and resuspended in cold sodium phosphate buffer. Bacteria were suspended in previously autoclaved, warm (50°C) underlay agar (10 mM sodium phosphate buffer, 1% (v/v) Mueller-Hinton broth, 1.5% (w/v) agar), at 4×10^5 colony forming units per mL. 15 mL of underlay agar was poured into 120 mm square petri dishes and allowed to solidify. Using the blunt ends of P10 pipet tips, evenly spaced 5 mm wells were punched into the agar

into which 5 μL of treatments and controls were added. Adult and larval growth media were included as negative controls. The *A. suum* AMP Cecropin P1 (Sigma-Aldrich, St. Louis, USA) was also included in the analysis. Plates were then incubated at 37°C for 3 h and then overlaid with 15 mL of overlay agar (4.2% (w/v) Mueller-Hinton broth, 1.5% (w/v) agar). The plates were incubated for 18 h at 37°C and the growth inhibition zones around each of the wells were measured. Antibacterial activity is herein represented as the diameter of the inhibition zone (mm) beyond the well.

Crystal Violet Assay

The influence of *Ascaris* ESP on biofilm formation was assessed using the microtiter dish biofilm formation assay (O'Toole, 2011). The biofilm forming *E. coli* K-12 strains AR3110 and AR115 were grown overnight in liquid salt-free Luria-Bertani (LB) medium at 37°C. The overnight culture was diluted in 2X LB medium (9×10^8 colony forming units per mL) for use in the biofilm assay. Hundred microliter of this bacterial suspension was used per well of a 96-well tissue-culture plate (Corning, New York, NY, USA) in replicates of four. The final volume per well was 200 μL with the remaining volume made up of controls and treatments at the concentrations indicated in the text. The plates were incubated for 24 h at 37°C. After incubation, cell suspensions were removed and the wells washed twice with phosphate buffered saline (pH 7.2) and stained for 15 min at room temperature with 0.1% (w/v) crystal violet solution (Sigma-Aldrich). The wells were then washed twice with distilled water and air-dried. For quantification, 125 μL of 30% acetic acid were added to each well and the plate incubated at room temperature for 15 min. The solubilized stain was transferred to a fresh flat-bottom 96-well plate and the absorbance read at 550 nm. Statistical analyses were performed using GraphPad Prism 7.0a to conduct 2-way ANOVA followed by Tukey's multiple comparison tests. *P*-values less than 0.05 were considered significant.

Macrocolony Biofilm Assay

The influence of *Ascaris* ESP on the morphology of biofilms was assessed using the macrocolony biofilm model (Serra and Hengge, 2017). Experiments were carried out using the same strains as for the crystal violet biofilm formation assay. Cells were grown overnight in salt-free LB medium at 37°C. 5 μL of the overnight culture was spotted on salt-free LB agar plates containing Congo red 40 μg/mL and Coomassie brilliant blue 20 μg/mL. 35 mm petri dishes (Sarstedt, Nümbrecht, Germany) were used to grow one colony per plate. After autoclaving and cooling to 42°C, agar was prepared with controls and treatments at the indicated final concentrations. Colonies were incubated at 28°C for up to 5 days. Macrocolonies were visualized at 10X magnification with a Stemi 2000-C stereomicroscope (Zeiss, Oberkochen, Germany) and photographed with an AxioCamICC3 digital camera (Zeiss).

Agglutination Assay

Agglutination activity of nematode products was assessed as previously described (Gasmi et al., 2017), using *E. coli* IMT19224. Bacteria were collected at mid-logarithmic phase by centrifugation at 880 × g for 5 min, washed then resuspended in BSS at approximately 10^9 cells/mL. 20 μL of bacteria were mixed with 20 μL of treatments in the presence and absence of 10 mM $CaCl_2$ and incubated for 1 h at room temperature on a glass slide. Concanavalin A from *Canavalia ensiformis* (Con A) and Lectin from *Triticum vulgaris* (Wheat germ agglutinin; WGA, both from Sigma-Aldrich) were included as positive controls. Samples were then visualized and photographed at 40X magnification on a Leica DM750 microscope equipped with an ICC50HD digital camera (Leica Microsystems, Wetzlar, Germany).

In-solution Tryptic Digestion and LC-MS/MS Analysis

Dried protein samples were resuspended in 50 μL of 50 mM ammonium bicarbonate in 5:95 (v/v) acetonitrile/water (digestion buffer) and reduced with 8 μL of 45 mM dithiothreitol in digestion buffer at 60°C for 30 min. After cooling to room temperature 8 μL iodoacetamide solution (100 mM in digestion buffer) were applied and the sample was kept in the dark for 30 min. Subsequently the samples were diluted with 190 μL digestion buffer and digested with 0.15 μg trypsin at 37°C for 4 h. The reaction was stopped with 2.5 μL of 10% (v/v) trifluoroacetic acid in water. The samples were concentrated to approximately 50 μL and desalted with μC18-ZipTips (Millipore, Darmstadt, Germany), dried and reconstituted in 0.1% (v/v) trifluoroacetic acid in 2:98 (v/v) acetonitrile/water. LC-MS/MS analyses of peptides were performed on an Ultimate 3000 RSLCnano system online coupled to an Orbitrap Q Excative Plus mass spectrometer (Thermo Fisher Scientific). The system comprised a 75 μm i.d. × 250 mm nano LC column (Acclaim PepMap C18, 2 μm; 100 Å; Thermo Fisher Scientific). Mobile phase (A) was 0.1% formic acid in 2:98 (v/v) acetonitrile/water and (B) 0.1% formic acid in 80:20 (v/v) acetonitrile/water. The gradient was 3–40% B in 85 min. Full MS spectra (350–1,600 m/z) have been acquired at a resolution of 70.000 (FWHM) followed by a data-dependent MS/MS fragmentation of the top 10 precursor ions (resolution 17.500; 1+ charge state excluded, isolation window of 1.6 m/z, normalized collision energy of 27%). The maximum ion injection time for MS scans has been set to 50 ms and for MS/MS scans to 80 ms.

Database Searching and Sequence Analysis

Protein identifications were performed with Mascot software version 2.6.1 (Matrix Science Ltd., London, UK). Data were searched against an *A. suum* protein database from nematode.net (http://nematode.net/NN3_frontpage.cgi?navbar_selection=speciestable&subnav_selection=Ascaris_suum), 17,843 sequences, 2017_05), *A. suum* proteins from Uniprot (9,213 sequences, 2017_05), the antimicrobial peptide database (http://aps.unmc.edu/AP/main.php, 2,338 sequences; 2017_05), SwissProt (555,100 sequences, 2017_07) and a contaminant database (247 sequences). The following parameters were set: enzyme: trypsin/P with one missed cleavage, static modification: carbamidomethylation (C), variable modifications: oxidation (M) and pyro-glu (Q), mass tolerances for MS and MSMS: 5 ppm and 0.02 Da. Proteins were accepted as identified if at

least two unique peptides with $p < 0.01$ were detected. Proteins identified only by one peptide were verified by comparison of their peptide fragment pattern with those of synthetic analogs. These reference peptides were synthesized in-house using Fmoc solid phase chemistry as previously described (Venken et al., 2011). In case of the common peptide (ISEGIAIAIQGGPR) of cecropin P1 and P2 an identification threshold of $p < 0.00001$ was set. Protein sequences were analyzed for the presence of classically secreted proteins containing signal peptides using SignalP 4.1 (Petersen et al., 2011) and for non-classically secreted proteins using SecretomeP 2.0 (Bendtsen et al., 2004).

RESULTS

Ascaris suum ESP Possess Antibacterial Activity

As intestinal parasitic nematodes inhabit a microbe rich environment, they are likely to experience microbial challenges while dwelling in the intestine. These challenges would need to be managed in order for the parasite to establish itself and survive during a long-term infection. We used the radial diffusion assay to test the antibacterial activity of native, unconcentrated secreted products (ESP) of different *A. suum* life stages and body fluid (BF) of adult male worms. The activities of nematode products against *E. coli* ST131 IMT19224, *S. aureus* IMT29828, and *S. typhimurium* ATCC 14028 were assessed. Adult ESP were obtained from 3 to 5 adult worms kept in 300 mL of culture medium (BSS), L3-stage material was harvested from the pooled supernatants of 30,000 larvae/well of a 12-well plate in 1 mL of larval media, while L4-stage material was harvested from the pooled supernatants of 100 larvae/well of a 12-well plate. BF was pooled from 5 adult males per batch. Treatments of ESP and BF were applied to proliferating bacteria and the resulting growth inhibition zones measured in comparison to BSS and larval culture media as controls (**Figure 1**). *Ascaris* ESP from *in vitro*-hatched L3-stage larvae resulted in no observable antibacterial activity. In contrast, ESP harvested from L4-stage larvae were very active, resulting in growth inhibition zones comparable to synthetic cecropin P1 against *E. coli*, and considerably more active than cecropin P1 against *S. typhimurium*. Interestingly, cecropin P1 had no detectable activity against *S. aureus*. Adult ESP were active against all strains tested and no considerable difference was detected between male and female ESP, thus they were considered together as "Adult ESP." BF from adult males demonstrated activity comparable to that of L4-stage larval ESP. Thus, these results show that native parasite material harvested directly from *A. suum,* including ESP and BF, possess considerable antibacterial activity. ESP from the intestinal L4 and adult life stages were most active, whereas ESP from *in vitro*-hatched L3 larvae did not show antibacterial activity.

Ascaris suum ESP Impair Bacterial Biofilm Formation

Many species of bacteria live in communities known as biofilms in which cells are embedded in an extracellular matrix of self-produced polymers. In addition to representing the preferred lifestyle in nature for many bacteria, biofilms are often of medical relevance for infectious diseases (Hall-Stoodley et al., 2004; Flemming et al., 2016). In the case of free-living *C. elegans* nematodes, biofilms have been shown to be harmful, contributing to the pathogenicity of *Staphylococcus epidermidis* against the worm (Begun et al., 2007), whereas biofilm formation by *Bacillus subtilis* enhances nematode stress resistance (Smolentseva et al., 2017). Therefore, as biofilms may also be of importance to intestinal nematodes, we evaluated the effects of *A. suum* ESP on biofilm formation using the submerged biofilm model (O'Toole, 2011) and the macrocolony biofilm model (Serra et al., 2013). We used the biofilm-forming *E. coli* K12 strain AR3110, which is a W3110 derivative with restored capacity to produce phosphoethanolamine-modified cellulose (pEtN-cellulose). AR3110 produce pEtN-cellulose along with amyloid curli fibers as key components of the extracellular matrix in biofilms (Serra et al., 2013; Thongsomboon et al., 2018). PEtN-cellulose production has been restored by repairing a single nucleotide polymorphism that resulted in a stop codon in the *bcs* operon (Serra et al., 2013). As adult worms can survive for approximately 1 year in the intestine, growing between 15 and 25 cm in length (Dold and Holland, 2011), they may present surfaces on which biofilms can form in the small intestine. Hence, we used adult material to study the impact of *A. suum* ESP on biofilm formation. In the submerged biofilm assay, bacterial suspensions were mixed with *A. suum* ESP in a volume-dependent manner as indicated and inoculated into the wells of flat-bottom 96-well tissue culture plates and grown for 18 h at 37°C. The same concentrations of adult culture media, BSS, were used as media controls. Biofilm formation was assessed by crystal violet staining of the biomass that had formed on the submerged wall and bottom of the wells, thereby staining bacterial cells as well as extracellular matrix components. *A. suum* adult ESP demonstrate a dose-dependent inhibition of bacterial biofilm formation for both strains tested, in comparison to control (**Figure 2**).

In the macrocolony biofilm assay, a dose-dependent disruption of colony growth was observed (**Figure 3**). With the AR3110 strain, the overall size of the resultant colony was decreased in the presence of *A. suum* adult ESP. Importantly, with 25% of ESP, *E. coli* responded to the treatment by producing large amounts of a white viscous substance (white shiny colony sectors; **Figure 3**). Since this substance was not formed in the *wcaE* mutant AR155, it can be ascribed to colanic acid, a mucoid exopolysaccharide that is typically produced in response to cell envelope stress and can confer resistance to antimicrobial insults and desiccation (Detweiler et al., 2003; Laubacher and Ades, 2008). This indicates that at least some of the ESP constituents act on the *E. coli* cell envelope, causing stress. However, also for the colanic acid-free mutant AR155, growth was not completely abolished suggesting that bacteria still resist the treatment by alternative mechanisms other than the production of colanic acid. Thus, these results show that the bacteria are able to adapt and survive to *A. suum* adult ESP, albeit while displaying signs of considerable stress. Notably, treatment with *A. suum* adult ESP did not interfere with curli and pEtN-cellulose production, since colonies of reduced size were still wrinkled as is particularly

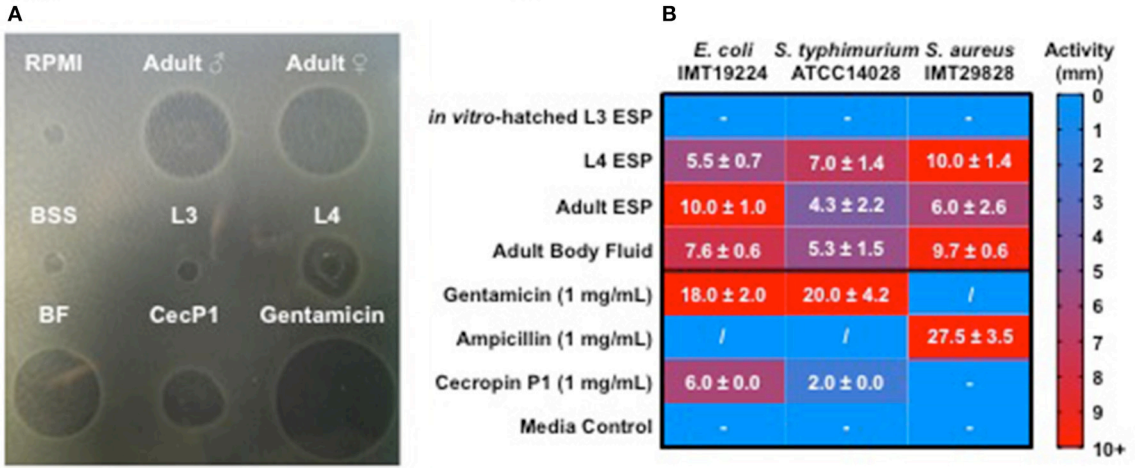

FIGURE 1 | *Ascaris suum* excretory/secretory products and body fluid possess antimicrobial activity. Five microliter of nematode products were applied to agar plates with proliferating bacteria for 18 h at 37°C and growth inhibition zones measured in millimeters. *Ascaris* products tested include native excreted and secreted products (ESP) from adult worms kept in culture for 24 h, body fluid (BF) from adult males, native ESP from approximately 30,000 L3-stage larvae hatched *in vitro*/mL media, native ESP from approximately 100 L4-stage larvae/mL media, and a synthetic form of the *A. suum* antimicrobial peptide, cecropin P1. Larval (RPMI) and adult worm media (BSS) were included as controls. **(A)** Representative agar plate of a radial diffusion assay, with nematode products tested against *E. coli*. **(B)** Activity shown as diameter (mm) of inhibition zones on agar plates. Results are expressed as means ± standard deviations obtained from 2 to 3 independent experiments with multiple batches of *A. suum* products (L3 $n = 3$, L4 $n = 2$, adult ESP and body fluid n = 3). "–" represents no detected activity. "/" = not tested.

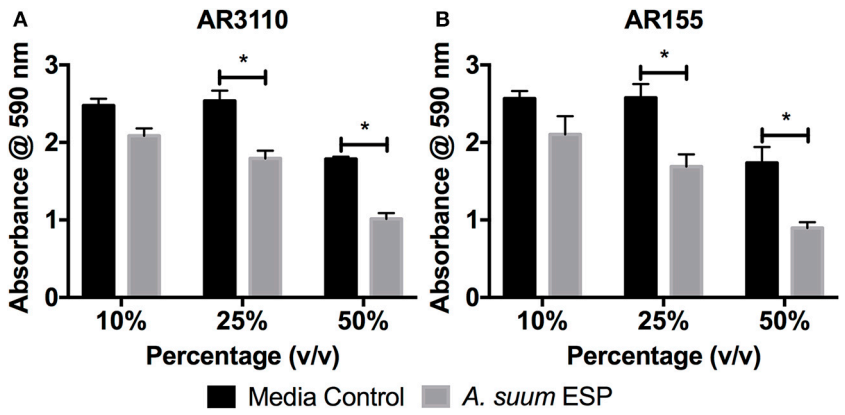

FIGURE 2 | *Ascaris suum* excretory/secretory products decrease biomass of submerged biofilms. Biofilm forming *E. coli* K-12 strains **(A)** AR3110 and **(B)** AR115 (a *wcaE* derivative of AR3110) were grown in 96-well cell culture plates in salt-free LB medium for 18 h at 37°C in the presence of adult *A. suum* excreted/secreted products (ESP) or adult worm media (BSS) as a control. Treatment doses were added as a percentage (v/v) of final culture volume (total = 200 μL per well). Results represented as the mean of three independent experiments ± SEM. Significance determined by 2-way ANOVA with Tukey's multiple comparison tests, $^*p < 0.05$.

visible in the absence of the large amount of viscous colanic acid with strain AR155 (**Figure 3**).

Ascaris suum ESP Possess Agglutinating Activity

Having demonstrated growth-inhibiting and biofilm-disrupting capabilities of *A. suum* ESP, we sought to determine if the nematodes could defend themselves against microbial threats without overtly killing bacteria. In addition to the inhibition of bacterial growth in the radial diffusion and macrocolony assays, we observed that also in our submerged biofilm assays some bacteria were still able to survive the treatment and reasoned that there may be non-lethal defense mechanisms employed by the worms such as neutralization via agglutination. In order to test the agglutinating activity of *A. suum* ESP, we treated *E. coli* ST131 IMT19224 with adult *A. suum* ESP (1 mg/mL) in the presence and absence of $CaCl_2$ (10 mM) and observed calcium-dependent agglutinating activity (**Figure 4**). The calcium-dependence implies the activity of C-type lectin domain-containing (CTLD) proteins which require calcium in order to exert their agglutinating and glycan-binding activities (Mayer et al., 2017). Similar results were obtained for the biofilm-forming *E. coli* K12 AR3110 and AR115 strains (**Supplementary Figures 1**, **2**).Thus, in addition to inhibiting

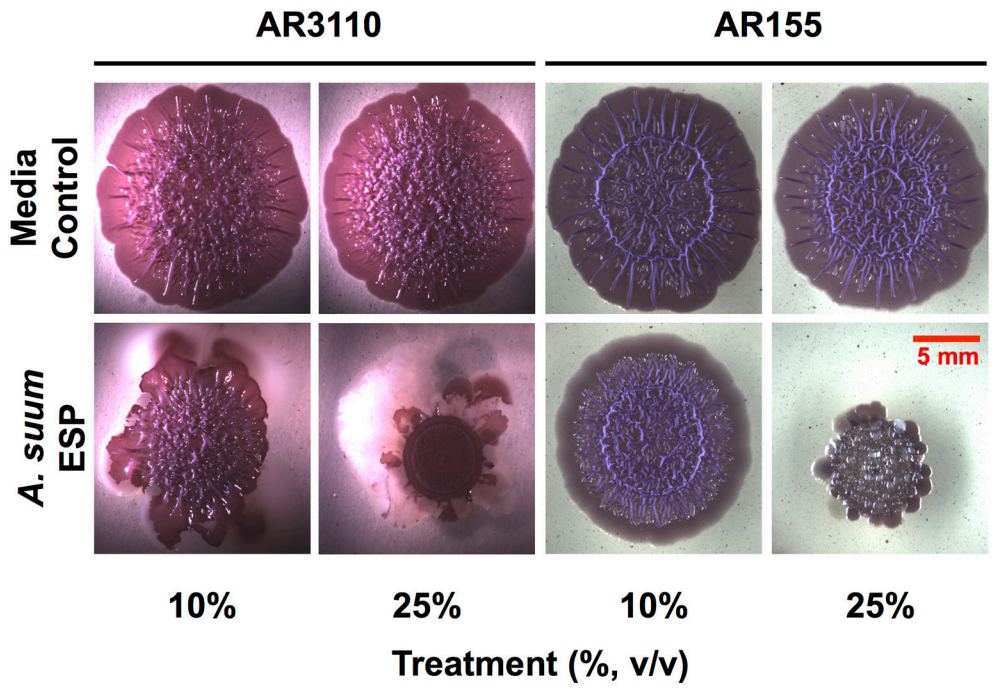

FIGURE 3 | *Ascaris suum* excretory/secretory products impair macrocolony biofilm formation. Five microliter of bacterial suspensions grown overnight were spotted on salt-free LB agar plates supplemented with Congo red, a dye that acs as an extracellular matrix indicator staining both pEtN-cellulose and curli fibers, as well as Coomassie brilliant blue and infused with either adult worm media (media control) or adult *A. suum* excreted/secreted products (ESP). Inoculated plates were then grown for up to 5 days at 28°C. Images shown here correspond to 5-days-old macrocolony biofilms of *E. coli* AR3110 and AR155 strains treated or untreated with *A. suum* ESP at two different concentrations.

bacterial growth and disrupting bacterial biofilm formation, *A. suum* adult ESP are also capable of neutralizing infectious threats by agglutinating bacteria.

Ascaris suum ESP and Body Fluid Contain Proteins and Peptides With Known and Predicted Antimicrobial Activities

In order to characterize ESP and body fluid of *A. suum* with respect to defense strategies that the nematode may employ in its microbial environment, we used native nematode material and omitted ultrafiltration-based concentration and trichloroacetic acid-mediated precipitation steps during our sample preparation which would have removed key antimicrobial components such as antimicrobial peptides from the final sample. By LC-MS/MS analysis, we assessed the protein and peptide constituents of ESP from different larval stages, including *in vitro*-hatched L3, lung-stage L3, intestinal-stage L4, and from adults, as well as BF obtained from adult males. The analysis revealed the presence of several proteins and peptides with known and predicted roles in nematode defense (**Table 1**), including galectins, C-type lectin domain-containing (CTLD) proteins, AMPs, a lysozyme (GH family 25 lysozyme 2), and a cysteine protease inhibitor (cystatin). Adult male and female ESP did not seem to differ in antimicrobial contents and are therefore shown together. Interestingly, we detected all of the aforementioned antimicrobial proteins and peptides in the ESP of adult nematodes, whereas we detected none of the proteins of interest in the ESP of lung-stage L3 larvae. ASABF-alpha, -beta, and –epsilon were detected only in adult ESP. In contrast, members of the cecropin family were detected in adult ESP and body fluid as well as in L4-larval ESP as well as cecropin P1 or P2 *in vitro*-hatched L3 ESP. While a significant and distinct peptide was detected and attributed to cecropin P1 in adult, L4, and *in vitro*-hatched L3 ESP as well as in BF, the same peptide could be attributed to cecropin P2; therefore, it is unclear if only cecropin P2 was detected or both cecropin P1 and P2. Cecropin P3 was detected in adult ESP and BF, as well as L4-stage ESP while cecropin P4 was detected only in adult ESP and BF, but not in larval material. The aforementioned lysozyme was detected only in adult ESP. Lectins, including CTLD proteins and galectins, were detected only in adult ESP but not in adult body fluid or in larval material. We detected seven unique CTLD proteins (including the three uncharacterized proteins, all of which contain CTLDs), though two of the seven did not contain signal peptides. Similarly, both galectins detected also did not contain signal peptides and were not predicted to be non-classically secreted using SecretomeP. Cystatin was detected in adult and *in vitro*-hatched L3 ESP. Cystatins from chickens and humans possess antibacterial activity (Blankenvoorde et al., 1998; Wesierska et al., 2005; Ganeshnarayan et al., 2012) whereas helminth cystatins, including from *Ascaris*, have a well-established role in modulating host immunity (Hartmann and Lucius, 2003; Mei et al., 2014; Coronado et al., 2017). Whether nematodes use cystatins to modulate the gut microbiota in

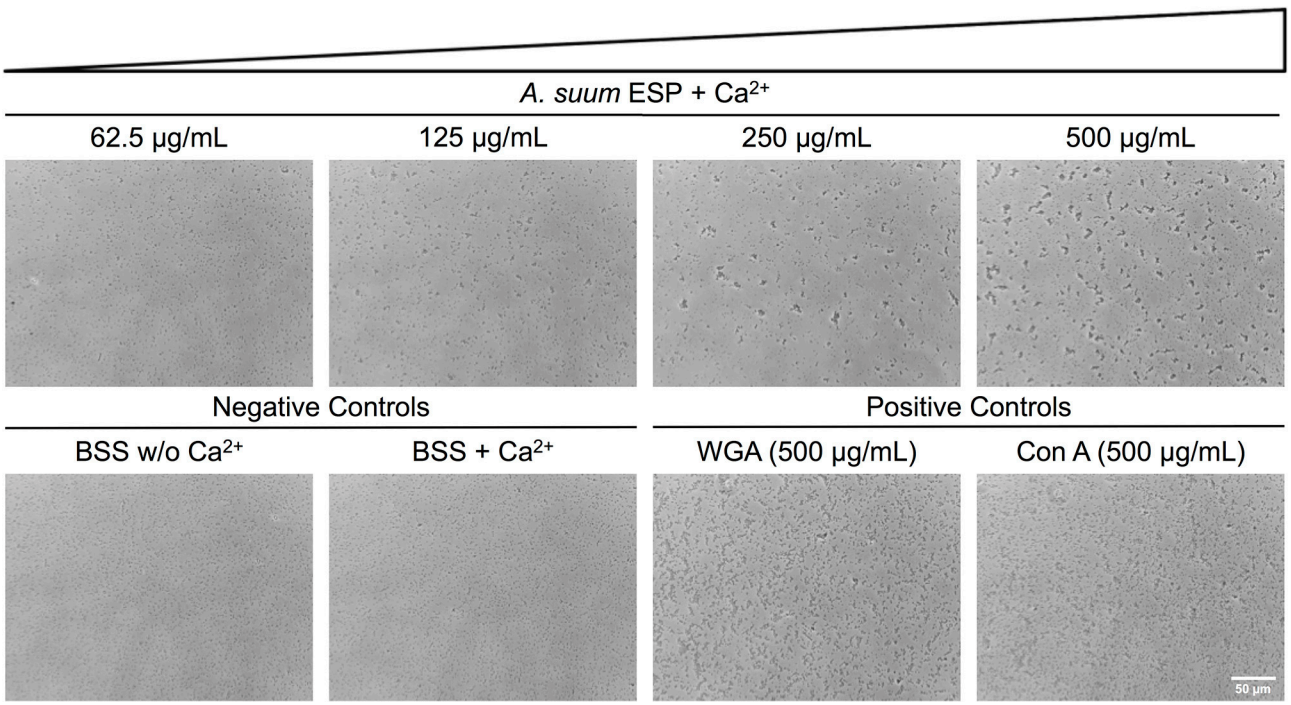

FIGURE 4 | *Ascaris suum* excretory/secretory products cause bacterial agglutination. Bacterial agglutination in the presence of adult *A. suum* ESP and 10 mM $CaCl_2$. Representative images of agglutination of *E. coli* IMT19224 with serial dilutions (1/2 factor) of *A. suum* ESP. Controls of agglutination include adult worm media (BSS) with and without $CaCl_2$ as well as the C-type lectins wheat germ agglutinin (WGA) and concanavalin A (Con A). Bacteria visualized at 400X magnification.

addition to host immune cells requires further study. The results of the LC-MS/MS analysis demonstrate that *A. suum* secretes diverse antimicrobial proteins and peptides which explain the various antibacterial activities we have observed. Furthermore, these factors likely act together to shape the nematode's microbial environment within the intestine of its host.

DISCUSSION

Intestinal parasites inhabit a microbe-rich environment. Diverse interactions between environmental microbes and free-living nematodes have been described, and similarly, the microbes in the host-gut may present benefits and risks for parasitic nematodes as they establish themselves in the niche of the intestine and migrate through the host tissue without eliciting overt inflammation. How *A. suum* survives in the small intestine of its porcine host has thus far been studied with a focus on host-pathogen interactions, whereas the interactions between *Ascaris* and the host-gut microbiota remain largely unexplored. Secreted products of helminths play various roles during the establishment of nematode infections, including invasion, migration, immune avoidance and immune modulation (Coakley et al., 2016). Hence, examining the role of secreted nematode products in nematode-microbe interactions is necessary to gain insights into the intricate trilateral interplay between the parasite, the host and the intestinal microbes during *A. suum* infection.

In this study, we demonstrated that *A. suum* ESP from different life stages possess antimicrobial activity against gram-negative and gram-positive bacteria (**Figure 1**). Interestingly, detectable antibacterial activity was limited to samples obtained from intestine-dwelling life stages, namely ESP from fourth larval-stage and adult worms, as well as body fluid from adult worms. Several proteins and peptides with known and predicted roles in antimicrobial defense were detected in these *A. suum* ESPs. In the nematode secreted products and BF samples, we detected members of the ASABF and cecropin AMP families (**Table 1**), previously shown to possess broad-spectrum antimicrobial activity (Pillai et al., 2003, 2005), accounting for observed antibacterial activities. Adult ESP also contained the highest diversity of potential antimicrobial components, including lectins, cystatin, and a lysozyme, GH family 25 lysozyme 2. To our knowledge, antibacterial activities of *Ascaris* lectins, cystatin, and lysozyme have not been reported previously; however, adult female BF has been reported to possess lysozyme-like and agglutinating activities, though specific factors were not identified (Kato, 1995). We were unable to detect antibacterial activity of ESP from *in vitro*-hatched L3 larvae and from lung-stage L3 larvae. Third-stage larvae hatch from infectious eggs protected by the L2 cuticle before migrating through host tissues (Douvres et al., 1969). As the liver is continuously exposed to microbial antigens from the gut, hepatic immune cells are particularly primed to deal with incoming threats (McNamara and Cockburn, 2016). L3-stage larvae may therefore be protected from microbial threats by cuticle barriers for the few hours in the intestine before entering the host, and by the host-antimicrobial immune system responding to any microbes that may be

TABLE 1 | Proteins and peptides with known and predicted antimicrobial activities detected in excreted/secreted products and body fluid of *A. suum*[a].

Protein name uniprot[b]	Protein mass (Da[b])	Signal peptide[c]	Accession number uniprot
C-type lectin domain-containing protein 160	41,886	+	F1L7R9
C-type lectin domain-containing protein 160	47,612	+	F1L4K4
C-type lectin domain-containing protein 160	43,174		F1L8I9
C-type lectin protein 160	60,173	–	F1L0R7
32 kDa beta-galactoside-binding lectin	32,483	–	F1L893
32 kDa beta-galactoside-binding lectin	31,791	–	F1LAD2
GH family 25 lysozyme 2	24,644	+	F1LE63
GH family 25 lysozyme 2	21,687	–	F1LEA7
Cystatin	13,961	+	F1LHQ3
ASABF-alpha	9,843	+	P90683
ASABF-beta	9,219	+	Q8MMG8
ASABF-epsilon	7,037	+	Q8IAC9
Cecropin-P1	7,876	+	P14661
Cecropin-P2	9,760	+	Q5H7N6
Cecropin-P3	8,381	+	Q5H7N5
Cecropin-P4	8,424	+	Q5H7N4
ADULT MALE BODY FLUID			
Cecropin-P1 and/or Cecropin-P2	7,876/9,760	+	P14661/Q5H7N6
Cecropin-P3	8,381	+	Q5H7N5
Cecropin-P4	8,424	+	Q5H7N4
L4-STAGE LARVAE			
Cecropin P1	7,876	+	P14661
Cecropin-P2	9,760	+	F1LBL1
Cecropin-P3	8,381	+	Q5H7N5
***IN VITRO*-HATCHED L3 LARVAE**			
Cecropin-P1 or Cecropin-P2	7,876	+	P14661/Q5H7N6
Cystatin	13,961	+	F1LHQ3

[a] *Extended version of table available in* ***Supplementary Material****.*
[b] *Protein name and mass from Uniprot database (https://www.uniprot.org).*
[c] *Identified proteins predicted to contain secretory signal peptide (+) or not (–) using SignalP.*

carried with the larvae as they penetrate through the intestinal tissue to the liver. While microbial threats are abundant in the intestine, tissue migration presents other unique challenges for the nematode larvae. Cystatin likely plays an important role in the interaction between migratory *A. suum* larvae and host immune cells; if it possesses antimicrobial activity as shown for cystatins from chickens and humans (Blankenvoorde et al., 1998; Wesierska et al., 2005; Ganeshnarayan et al., 2012) remains to be determined. Thus, our data indicate tissue migratory third-stage larvae may not produce high quantities of antimicrobials. In contrast, we detected considerable antibacterial activity in cecropin-containing ESP from L4-stage larvae which have undergone further development after re-entering the intestine and thereby facing the presence of the intestinal microbiota. Furthermore, material harvested from adult nematodes, which have to contend with the host microbiota for the majority of the worm's lifespan, also showed considerable antibacterial activity. To counteract a diversity of potential threats originating from the microbiota, *Ascaris* is armed with several antimicrobial factors resulting in broad-spectrum antibacterial activity.

Studies in *C. elegans* have demonstrated the importance of biofilms in bacterial-nematode interactions. Within biofilms, bacteria are bound together within an extracellular matrix composed of exopolysaccharides, proteins, and nucleic acids (Hall-Stoodley et al., 2004) which provides support and protection, allowing bacteria to withstand higher concentrations of antibiotics (Dufour et al., 2010). Biofilm exopolysaccharides have been shown to enhance virulence of *S. epidermidis* during colonization of the *C. elegans* intestine in addition to enhancing bacterial resistance to nematode antimicrobial factors (Begun et al., 2007). Interestingly, biofilm forming *B. subtilis* promote oxidative stress resistance, thermotolerance, and upregulated expression of a lysozyme leading to enhanced resistance to worm killing by the pathogenic *Pseudomonas aeruginosa* (Smolentseva et al., 2017). Though the experimental settings differ, these studies highlight the importance of the biofilm lifestyle to nematode health. Thus, as biofilms might also influence parasitic nematode physiology, we studied the impact of *Ascaris* ESP on biofilm formation by *E. coli* K-12 strain AR3110. ESP from adult worms clearly resulted in a dose-dependent reduction in biomass accumulation in the submerged biofilm model (**Figure 2**). *E. coli* AR3110 also form macrocolony biofilms with pEtN-cellulose and amyloid curli fibers as key components of the extracellular matrix (Serra et al., 2013; Thongsomboon et al., 2018). In the presence of adult *A. suum* ESP, macrocolony formation was considerably disrupted and was accompanied

by the production of the complex exopolysaccharide colanic acid, while the production of pEtN-cellulose and curli fibers (reflected by colony wrinkling) was not affected (**Figure 3**). Colanic acid production, which is under the control of the RcsC/RcsB phosphorelay cascade (Majdalani et al., 2005) and is induced in response to cell envelope stress (Laubacher and Ades, 2008), confers resistance to antimicrobial peptides (Detweiler et al., 2003). Hence, nematode antimicrobial factors present in the *A. suum* ESP, especially AMPs, represent extracytoplasmic stress and, by inducing production of colanic acid, modify bacterial biofilm formation. While the inability of *E. coli* to produce colanic acid increased the growth inhibitory effects of *A. suum* ESP, a portion of the bacterial population was still able to survive the treatment (**Figure 3**). Similarly, resistance to AMPs allows *S. typhimurium* to persist in the intestine of *C. elegans* (Alegado and Tan, 2008); while nematodes release factors to defend themselves against bacterial threats, some can withstand these assaults. While bacteria are able to colonize the intestine of *Ascaris*, as determined by culture-based methods (Nalin and McLaughlin, 1976; Hsu et al., 1986; Shahkolahi and Donahue, 1993), the role of biofilms in microbial colonization of *A. suum* and interplay with the host microbiota during ascariasis require further study.

In addition to bactericidal factors such as ASABFs and Cecropins, we also detected lectins, including C-type lectin domain-containing (CTLD) proteins and galectins (**Table 1**). CTLD proteins recognize and bind to carbohydrate ligands and are critical in immunity (Brown et al., 2018). CTLD proteins can be transmembrane proteins, functioning as cell surface receptors, or can be secreted. A previous study isolated three CTLD proteins from the murine intestinal nematodes *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* (Harcus et al., 2009). The authors reported that these lectins are primarily expressed in the intestine-dwelling adult stages; however, bacterial binding functions were not assessed in their study. *C. elegans* possesses an estimated 283 CTLD (*clec*) genes, the majority of which are thought to be secreted (Pees et al., 2016). Previous studies have demonstrated that during infection with *S. marcescens*, *clec-39,−49,* and *-50* are upregulated and worms deficient in *clec-39* are more susceptible to infection with *S. marcescens* (Mallo et al., 2002; Engelmann et al., 2011; Miltsch et al., 2014). Additionally, recombinant CLEC-39 and−49 were shown to bind *S. marcescens* without killing the bacteria (Miltsch et al., 2014). We demonstrated calcium-dependent agglutinating activity of adult *A. suum* ESP (**Figure 4**), likely due to the CTLD proteins we detected. Both mammalian and non-mammalian hosts use lectins to shape the intestinal microbiota (Pang et al., 2016), an effect which could be compounded by secreted *A. suum* CTLD proteins. Galectins are β-galactoside-binding proteins also thought to function in host defense (Vasta, 2009). *C. elegans* deficient in the galectin LEC-8 were more susceptible to infection with *Bacillus thuringiensis* (Ideo et al., 2009). Interestingly, galectins do not typically contain secretory signal peptides but many localize extracellularly and are thought to be non-classically secreted (Barondes et al., 1994; Hughes, 1999). The galectins reported in our study were not predicted to contain signal peptides or to be secreted through non-classical pathways. However, though their presence in the *A. suum* ESP may contribute to agglutinating activity, their roles in nematode defense and in shaping the porcine intestinal microbiota need further investigation.

In this study we described diverse impacts of *A. suum* ESP on bacterial species from direct antimicrobial activity, disrupted biofilm formation, and neutralization by agglutination. These observations correlated with proteins and peptides detected in the ESP by mass spectrometry analysis and suggest that intestinal nematodes employ multiple strategies in their interactions with bacteria. Studies in infection models of *C. elegans* reveal pathogen and tissue-specific gene expression changes (Engelmann et al., 2011) along with differentially synthesized proteins in response to different microbial pathogens (Bogaerts et al., 2010a,b). These studies identified a diversity of upregulated factors including antimicrobial peptides, lectins, and lysozymes, all of which we detected in *A. suum* ESP. These multiple factors would then act in concert with one another to endow nematodes with a broad-spectrum defense system to allow survival in a microbial environment, as faced by *A. suum* in the porcine intestine. While we focused on the protein components of *Ascaris* ESP, it is important to note that helminth ESP also contain RNAs (Buck et al., 2014) and metabolites such as short-chain fatty acids (Zaiss et al., 2015) which in addition to modulating host immunity, may also impact the microbiota. Further study is required to determine the role of non-protein contents in shaping the microbiota; however, antibacterial activity described in our study due to combination effects of the various constituents of *A. suum* ESP have been accounted for by our use of native material.

In summary, our findings suggest that intestine-dwelling life stages of *A. suum* employ diverse antimicrobial strategies to establish themselves amongst the host microbiota. Our results provide a first indication of the direct impact of an intestinal nematode on its immediate microbial environment. Furthermore, our results suggest that the antimicrobial potential of nematode products differ depending on the parasite life-stage and corresponding host-environments. While metabolic and host immune factors would also contribute to an altered microbiome during helminth infection, we propose that nematodes themselves also have a direct role in shaping the microbiota as they establish themselves in the host gut, involving the secreted products and antimicrobial activities described herein. These changes would be more pronounced with a high worm burden as the local concentration of nematode antimicrobials would likely be higher. The defense strategies discussed in this study involve killing and non-killing mechanisms exerted by several different secreted factors acting in combination, as exemplified by the constitution and diverse activities of *A. suum* ESP. Together, these factors allow nematodes to carve out a niche to survive within a microbial environment and while doing so, may be partially responsible for changes to the intestinal microbiome during helminth infection.

AUTHOR CONTRIBUTIONS

All authors gave final approval for manuscript publication. Project designed by AM, SH, and JS. Microbiological experiments

designed by AM, SG, RH, and DS. Mass spectrometry analysis performed by KJ and AN. Peptides for mass spectrometry analysis synthesized by PH. AM performed all experiments. All authors interpreted data. Manuscript was written by AM and SH with input from the other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00271/full#supplementary-material

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Corrigendum: The Intestinal Roundworm *Ascaris suum* Releases Antimicrobial Factors Which Interfere With Bacterial Growth and Biofilm Formation

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The correct affiliations appear in the author list above. The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

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REVIEW
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Micromanaging Immunity in the Murine Host vs. the Mosquito Vector: Microbiota-Dependent Immune Responses to Intestinal Parasites

Ivet A. Yordanova[1†], Suzana Zakovic[2†], Sebastian Rausch[1], Giulia Costa[2], Elena Levashina[2] and Susanne Hartmann[1]*

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The digestive tract plays a central role in nutrient acquisition and harbors a vast and intricate community of bacteria, fungi, viruses and parasites, collectively known as the microbiota. In recent years, there has been increasing recognition of the complex and highly contextual involvement of this microbiota in the induction and education of host innate and adaptive immune responses under homeostasis, during infection and inflammation. The gut passage and colonization by unicellular and multicellular parasite species present an immense challenge to the host immune system and to the microbial communities that provide vital support for its proper functioning. In mammals, parasitic nematodes induce distinct shifts in the intestinal microbial composition. Vice versa, the commensal microbiota has been shown to serve as a molecular adjuvant and immunomodulator during intestinal parasite infections. Moreover, similar interactions occur within insect vectors of deadly human pathogens. The gut microbiota has emerged as a crucial factor affecting vector competence in *Anopheles* mosquitoes, where it modulates outcomes of infections with malaria parasites. In this review, we discuss currently known involvements of the host microbiota in the instruction, support or suppression of host immune responses to gastrointestinal nematodes and protozoan parasites in mice, as well as in the malaria mosquito vector. A deeper understanding of the mechanisms underlying microbiota-dependent modulation of host and vector immunity against parasites in mammals and mosquitoes is key to a better understanding of the host-parasite relationships and the identification of more efficient approaches for intervention and treatment of parasite infections of both clinical and veterinary importance.

Keywords: microbiota, gastrointestinal parasite, immune response, mammalian host, mosquito vector, probiotics, *Plasmodium*

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INTRODUCTION

As early as 1885, Louis Pasteur postulated the preconceived idea that life under microorganism-free conditions would not be possible (Glimstedt, 1953). However, it was not until the 1950s that initial reports highlighted the importance of the symbiotic relationship between multicellular organisms and microorganisms, due to the generation of germ-free mammalian and insect animal models as novel research tools (Lancet, 1953). The complex communities of commensal bacteria, fungi, viruses and metazoans are collectively known as the microbiota. Importantly, the resident intestinal bacterial communities have been recognized as pivotal contributors to host development and metabolism, and for the induction and education of host immunity under homeostasis, during infection or inflammation (Grenham et al., 2011). Millions of years of evolution separate insects and mammals, which differ dramatically in many aspects, including the organization of their digestive and immune systems (**Figure 1**). However, they are often similarly exposed to the same microbes. Here we discuss how some of these commensal microbes affect immune responses of mice and mosquitoes to a number of parasites colonizing the intestinal tracts of their respective hosts.

In the context of immunity to gastrointestinal parasites, the microbiota of mammals is an essential functional player in the induction and maintenance of the mammalian immune system (Grainger et al., 2013). Removal of the resident gut microbiota inhibits the maturation of gut-associated lymphoid tissues, resulting in smaller and fewer Peyer's patches and lymphoid follicles (Cebra et al., 1998). From the initial colonization of the gut in neonates, the newly established commensal microbiota undergoes constant shifts in composition and diversity, reflective of the host's physiological development, diet, exposure to stress and gastrointestinal infections among other factors (Pickard et al., 2017). Mounting evidence has supported the notion that infection with intestinal parasites in mammals contributes to alterations in the diversity and abundance of commensal bacteria both locally and globally. On the other hand, insect vectors of medical importance like mosquitoes acquire and harbor a number of major human protozoan and nematode species in their own digestive tract. In recent years, stringent efforts have been placed to identify the factors that determine the mosquito vectorial capacity and to harvest that knowledge for the development of novel vector control strategies. Whereas, the midgut microbiota has emerged as a key factor shaping mosquito resistance to *Plasmodium*, the effector mechanisms driving these tripartite interactions remain largely unknown.

Although specific aspects of host-parasite-microbiota interactions have been recently reviewed separately for mice and mosquito vectors (Ippolito et al., 2018; Romoli and Gendrin, 2018; Stensvold and van der Giezen, 2018), in this review we contrast our current understanding of the relevance of microbiota-immunity interplay during gut infections in a comparative manner. We summarize and critically evaluate the role of the host intestinal microbiota in the instruction, support or suppression of mouse immunity to the non-invasive extracellular parasite *Giardia lamblia* and the invasive intracellular parasite *Toxoplasma gondii*, in comparison to the small intestinal nematode *Heligmosomoides polygyrus* and the large intestinal nematode *Trichuris muris*. We further discuss the most common insect effector mechanisms that control commensal and pathogenic bacteria, with a particular emphasis on their impact on *Plasmodium* infections in *Anopheles* mosquito vectors. Our review further highlights functional similarities and differences in microbiota-dependent induction and education of host immunity in such diverse hosts as mice and mosquitoes.

COMMENSAL MICROBIOTA COMPOSITION OF MICE AND MOSQUITOES

In the first year of life of most mammals, the microbial composition of their gastrointestinal tract develops from a sterile environment to one harboring a considerable density of commensal microorganisms, which over time develop a broad similarity to the microbiota composition of adults (Palmer et al., 2007). However, the infant microbiota presents considerably higher compositional variability and instability than the microbiota of adults, and factors like mode of delivery and breast feeding have been suggested as potential key factors in shaping the intestinal microbiota of mammals during the early stages of their life (Palmer et al., 2007; Milani et al., 2017; Pickard et al., 2017). Under homeostasis, the adult human intestinal tract harbors bacteria predominantly belonging to the *Bacteroides, Eubacterium, Ruminococcus*, and *Clostridium* genera, while the murine intestinal tract harbors predominantly *Clostridiales* and *Bacteroidales*, key in the enzymatic breakdown of complex polysaccharides (Palmer et al., 2007; Pickard et al., 2017). The high availability of mono and disaccharides in the gut, on the other hand, also allows for the proliferation of *Proteobacteria* and *Lactobacilli*–two other prominent members of the mammalian intestinal microbiota (Pickard et al., 2017). Notably, an abundance of evidence in recent years has highlighted that the normal composition of the adult intestinal microbiota suffers significant alterations following infections with gastrointestinal parasites, with significant implications to the regulation and maintenance of host metabolism and immunity.

Similar to mammals, the microbiota of *Anopheles* mosquitoes experiences dynamic changes in abundance and composition during the insect life cycle (Moll et al., 2001; Linenberg et al., 2016). The mosquito life cycle comprises four developmental stages: aquatic eggs, larvae, pupae and terrestrial adults. Larvae feed on environmental microorganisms, some of which establish residence in the larval guts and constitute the midgut microbiota. During pupariation, the resident microbiota is expelled together with the food bolus and the peritrophic matrix, resulting in dramatic losses of bacterial communities. Nevertheless, some larval bacteria can still be transmitted to pupae (Moll et al., 2001). In contrast, a stringent process of gut remodeling and sterilization during pupa-to-adult transition leads to complete loss of the pupal microbiome upon adult emergence (Moll et al., 2001). However, young adults re-establish microbial

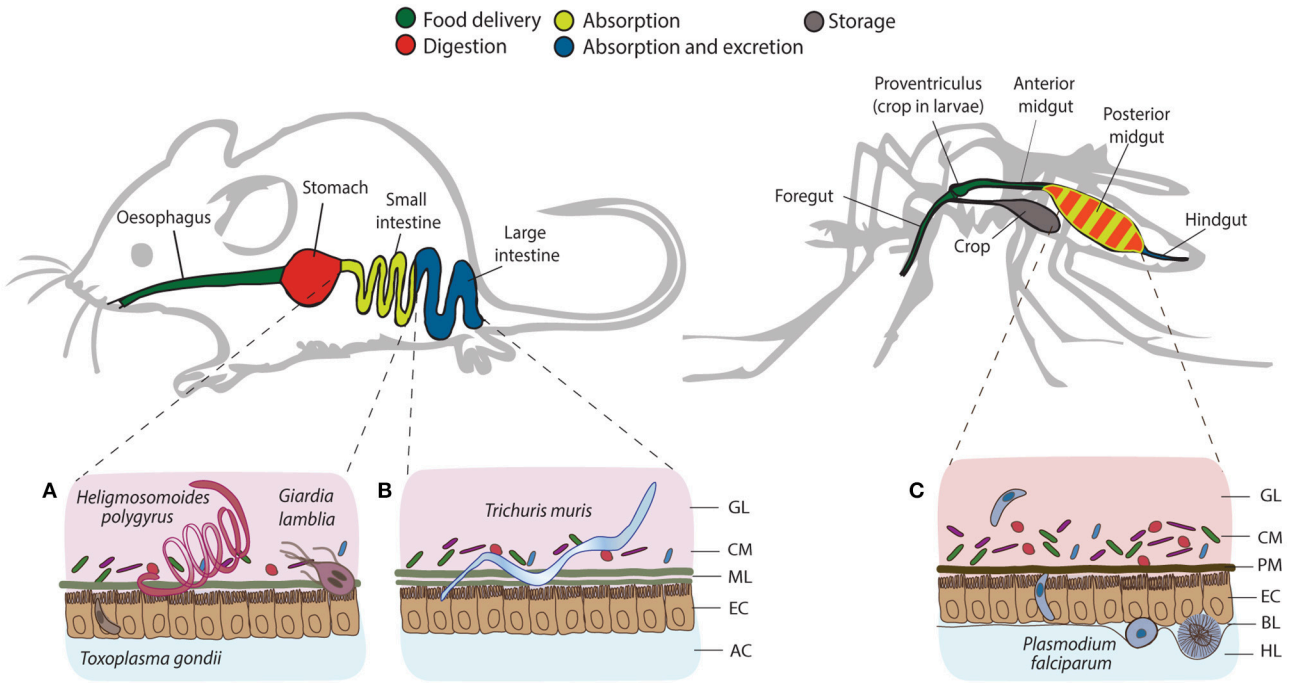

FIGURE 1 | Structural and functional differences of the gastrointestinal tracts of mice and mosquitoes, and the localization of their intestinal parasites. The gastrointestinal tract of mice consists of esophagus, stomach, small and large intestine, and colon, whereas in mosquitoes, it comprises the foregut, diverticulum (crop), midgut (anterior and posterior) and hindgut. One of the differences between gut organization is the separation of the food digestive and nutrient absorption functions taking place in the stomach and the small intestine of mice. In mosquitoes, digestion and absorption occur in the same organ, the midgut. The tracts secrete a protective layer that serves as a mechanical barrier against the acidic gastric environment and diverse pathogens. In mammals, epithelial goblet cells dispersed throughout the epithelium produce a viscous mucus layer that covers the entire tract, except the large intestine, which is characterized by a double mucus layer. Similarly to mucus, mosquitoes secrete the peritrophic matrix (PM), a semi-permeable extracellular structure composed of chitin and glycoproteins. In larvae, the PM is secreted continuously by a specialized organ cardia located in the anterior midgut, whereas in adult females, it is believed to be secreted by the midgut epithelial cells only after blood feeding. Small **(A)** and large **(B)** intestines of mice harbor a series of gastrointestinal parasites: *Toxoplasma* exploits the small intestine for the establishment of the infection; *Giardia* and adult *Heligmosomoides polygyrus* worms thrive in the lumen of the small intestine, while adults of *Trichuris muris* anchor to the epithelial cells of the large intestine. In mosquitoes, *Plasmodium* invades the posterior midgut to establish extracellular infection at the basal side of the epithelial cells **(C)**. GL, gut lumen; CM, complex microbiota; ML, mucus layer; EC, epithelial cells; AC, abdominal cavity; PM, peritrophic matrix; BL, basal lamina; HL, hemolymph.

communities from bacteria-rich breeding water, whereby re-acquiring the microbial fingerprint of their larval environment (Lindh et al., 2008). Indeed, the diversity of the adult microbiota resembles the microbial composition of the aquatic larval habitats (Boissière et al., 2012; Gimonneau et al., 2014; Dickson et al., 2017). Larval diet shapes the microbial communities, impacts mosquito development and female susceptibility to infection with the human malaria parasite *Plasmodium* (Linenberg et al., 2016). In *Anopheles*, only female adults feed on blood to initiate their reproductive cycle. The change of diet from carbohydrate-rich nectar to protein- and lipid-rich blood induces massive bacterial proliferation and changes in microbial composition in the gut (Dong et al., 2009; Tchioffo et al., 2016). Despite the vast variability in the microbiota of individual mosquitoes from diverse geographical locations, γ-*Proteobacteria* dominate mosquito microbial communities and in particular, *Enterobacter, Serratia, Pantoea, Asaia, Aeromonas, Pseudomonas,* and *Bacillus* (Straif et al., 1998; Lindh et al., 2005; Rani et al., 2009; Boissière et al., 2012; Osei-Poku et al., 2012; Ngo et al., 2015).

INTESTINAL PARASITE INFECTIONS AND ASSOCIATED CHANGES IN HOST MICROBIOTA

Microbial Changes Induced by Protozoan Parasite Infections

Giardia lamblia is an extracellular gastrointestinal parasite with a wide global distribution and still remains a common cause of food and waterborne-associated diarrhoeal disease (**Figure 1A**; Halliez and Buret, 2013). ***Giardia*** trophozoites attach to the epithelial lining of the small intestine and therefore they remain in intimate contact with the resident commensal microbiota of the host (Adam, 2001). *Giardia* infection in mice leads to localized shifts in the commensal microbial communities of the host small intestine. However, few studies have characterized the dynamics of *Giardia*-microbiota interactions in greater detail. During the early stages of infection, increased numbers of adherent and mucus-associated bacteria, as well as epithelial cell

damage-related translocation of commensal bacteria from the intestine to the spleen and liver have been demonstrated (Chen et al., 2013; Halliez et al., 2016).

Acute infection with *G. lamblia* in mice has been shown to cause a significant expansion of β- and γ-*Proteobacteria* in the small intestine, cecum and colon, whereas the relative abundance of *Clostridia* and the diversity of *Melainabacteria* is decreased (**Figure 2A**; Barash and Maloney, 2017). Importantly, additional perturbations of the microbiota following antibiotic administration did not prevent the observed shifts in gut microbiota during murine giardiasis (Barash and Maloney, 2017). Investigations of the impact of diet on *Giardia*-induced changes in microbial composition have further revealed that while a low protein diet resulted in an increased *Firmicutes/Bacteroidetes* ratio, *Giardia* infection further enhanced the compositional shift in favor of *Firmicutes,* predominantly of *Clostridiales*, *Turicibacter*, and *Enterococcus* (Bartelt et al., 2017). In addition, *Giardia* infection increases excretion of bile acid derivatives, phosphatidylcholine, and taurine metabolites, as well as the availability of byproducts of glucose metabolism, indicating *Giardia*-induced alterations of host metabolism as a potential contributor to commensal microbiota alterations observed during infection (Barash and Maloney, 2017).

In contrast, the intracellular apicomplexan parasite *Toxoplasma gondii* initially infects the small intestine, where it induces significant immunopathological damage before quick systemic dissemination in the host (**Figure 1A**; Wilhelm and Yarovinsky, 2014). Despite the direct damage to host epithelial cells inflicted during intracellular parasite development, compositional changes in host intestinal microbiota have been established as a key factor driving intestinal immunopathology of toxoplasmosis in wild-type and humanized mice (Heimesaat et al., 2006; Bereswill et al., 2014; Von Klitzing et al., 2017). *T. gondii* infection causes a notable expansion of *Enterobacteriaceae, Bacteroides*, and *Enterococcus* (Heimesaat et al., 2006, 2014) and a decrease in *Lactobacillus*, *Bifidobacterium*, *Clostridia*, and *Bacteroidetes* species (**Figure 2B**; Heimesaat et al., 2006; Molloy et al., 2013). The expansion of predominantly Gram-negative commensal bacteria elevates proinflammatory cytokine production in the gut and the immunopathology of toxoplasmosis (Heimesaat et al., 2006). Similar to *Giardia*, *T. gondii* infection induces strong compositional changes in the gut microbiota, that contribute to the intestinal immunopathology of toxoplasmosis (**Figure 2B**).

Microbial Changes by Intestinal Nematode Infections in Mice

Infection with the murine small intestinal nematode *H. polygyrus* leads to profound changes in the host commensal microbiota along the entire gastrointestinal tract (Walk et al., 2010; Rausch et al., 2013; Reynolds et al., 2014). In particular, *Lachnospiraceae*, *Ruminoccoccaceae*, γ-*Proteobacteria/Enterobacteria*, and *Bacteroides* dominate the cecum, while a greater abundance of *Porphyromonadaceae, Lactobacillaceae*, and *Clostridiaceae* was detected in the ileum (**Figure 2C**; Walk et al., 2010; Rausch et al., 2013). Importantly, Reynolds et al. (2014) have correlated host susceptibility to infection with a significant increase in *Lactobacilli*. Overall, infections with *H. polygyrus* appear to change the balance between *Bacteroidetes* and *Firmicutes* in favor of the latter (**Figure 2C**).

The murine whipworm *T.* muris, on the other hand, develops in the large intestine, where the adult parasites protrude through the epithelium into the lumen (Holm et al., 2015). *Trichuris* adults, therefore, reside in close proximity the resident commensal microbiota of the host. Several studies have previously demonstrated that murine infection with *T. muris* results in shifts in the microbiota composition in the caecum and colon (**Figure 2D**; Holm et al., 2015; Houlden et al., 2015). During the early stages of infection, an increase in the abundance of *Bifidobacteria, Lactobacilli, Alistipes, Parabacteroides*, and *Odirobacter* has been observed. Later chronic stages increased the abundance of *Oscillibacter, Butyricicoccus, Parasutterella, Sphyngomonas*, and *Halomonas,* paralleled by a decrease in *Roseburia, Allobaculum*, and *Barnesiella* genera (**Figure 2D**; Holm et al., 2015). A significant reduction of *Bacteroides* correlated with a shift in the metabolite composition of the gut, affecting the biosynthesis of fatty acids, amino acids and phospholipids (Houlden et al., 2015). Interestingly, type 1 fimbriae adhesins, structural components of the commensal bacterium *E. coli,* were demonstrated to induce *T. muris* egg hatching *in vitro* (Hayes et al., 2010). Consistently, depletion of the host microbiota inhibits the establishment of infection *in vivo* (Hayes et al., 2010). Interestingly, changes in host microbiota caused by primary *T. muris* infection have been shown to inhibit egg hatching of secondary infections with *T. muris*, suggesting that some microbial products facilitate establishment of *T. muris* during primary, but not secondary infections (White et al., 2018). Overall, intestinal nematodes appear to change the microbiota composition of their hosts and alterations of the balance in abundances of *Firmicutes* and *Bacteroides* have been highlighted as a shared feature in these infection models.

Microbial Changes Associated With *Plasmodium* Infections in Mosquitoes

In contrast to mammalian intestinal parasites residing primarily in the intestinal lumen and allow for continuous interaction between the parasites and the host microbiota, *Plasmodium* swiftly transits through the mosquito gut without establishing long-term residence in the lumen. The time that the parasite remains in contact with the luminal bacteria following a blood intake is limited to 24–30 h, by which time the majority of the parasites have already traversed the gut epithelium to escape the dangerous environment (Meis et al., 1989). Therefore, the impact of *Plasmodium* on the gut microbiota at this stage would be limited. Sporogonic parasite development at the basal side of the gut takes approximately 2 weeks. During this time, potential indirect parasite interactions with midgut bacteria could take place. Tchioffo et al. (2016) observed significant differences between mosquito microbial

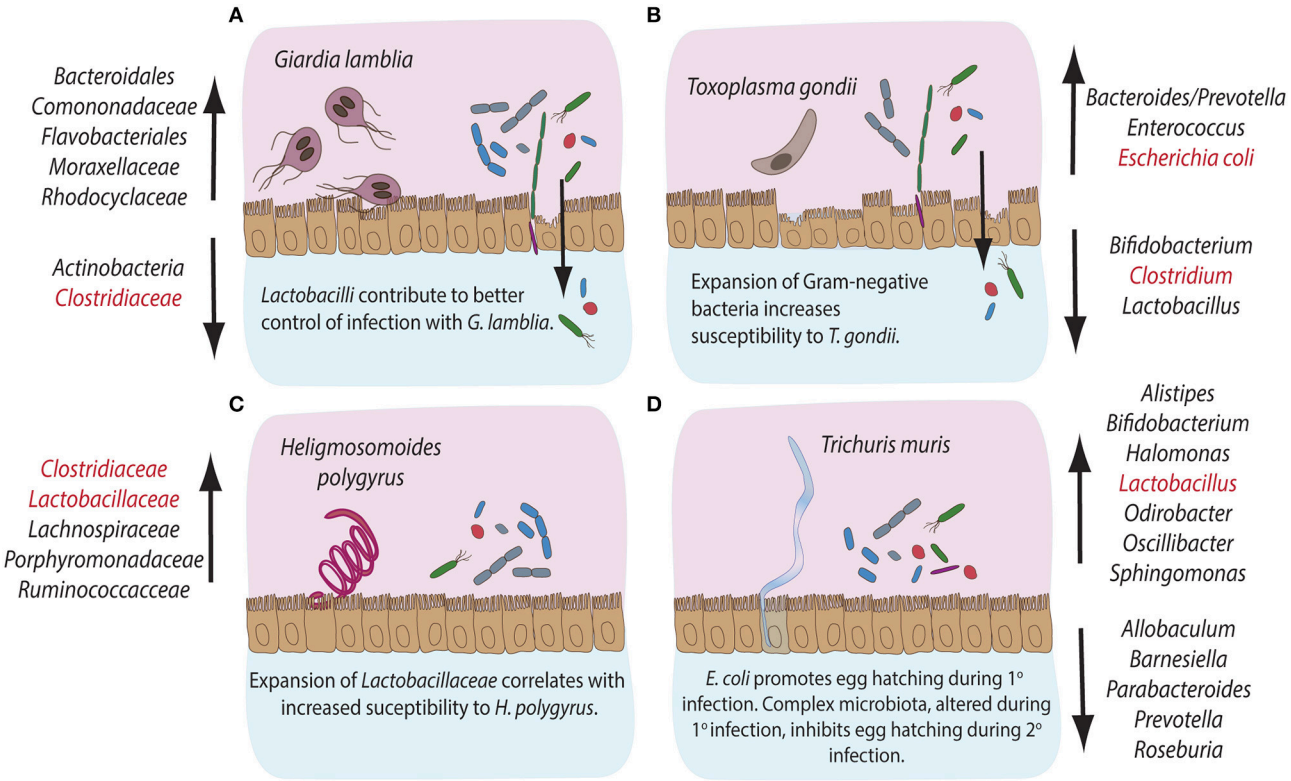

FIGURE 2 | Compositional alterations and functional significances of commensal bacterial groups during intestinal parasite infections in mice. The gastrointestinal tract of mice is home to diverse communities of commensal bacterial species. Infections with intestinal pathogens are known to lead to notable alterations in the commensal microbiota composition of the host. **(A)** Infection with the extracellular protozoan parasite *Giardia lamblia* leads to a significant expansion of bacterial groups like *Bacteroidales, Comononadaceae, Flavobacteriales, Moraxellaceae* and *Rhodocyclaceae*, while *Actinobacteria* and *Clostridiaceae* display reduced abundances. On the other hand, *Lactobacilli* are known to contribute to an overall better control of *Giardia* infection. **(B)** The intracellular protozoan parasite *Toxoplasma gondii* leads to expansion in *Bacteroides/Prevotella, Enterococcus* and *Escherichia coli*, while the abundance of *Bifidobacteria, Clostridiales*, and *Lactobacilli* decreases during infection. An overall expansion of Gram-negative bacteria has been shown to contribute to immunopathology during murine infection with *T. gondii*. **(C)** Infection with the small intestinal parasitic nematode *Heligmosomoides polygyrus* leads to a marked expansion of commensal *Clostridiaceae, Lachnospiraceae, Porphyromonadaceae*, and *Ruminococcacce* and *Lactobacilli*, the latter known to correlate with increased susceptibility to infection with *H. polygyrus*. **(D)** Infection with the large intestinal nematode *Trichuris muris* leads to the expansion of *Alistipes, Bifidobacteria, Halomonas, Lactobacilli*, *Odirobacter, Oscillibacter*, and *Sphingomonas*, while *Allobaculum, Barnesiella*, *Parabacteroides, Prevotella*, and *Roseburia* abundances decrease. Notably, commensal *E. coli* is known to promote egg hatching during primary infection, while complex microbiota alterations occurring during primary infection appear to inhibit egg hatching during secondary infection with *T. muris*. The bacterial groups highlighted in red have been demonstrated to take part in induction, maintenance or suppression of host immune responses during the corresponding parasitic infections.

communities in the gut, ovaries and salivary glands at 1 and 8 days after *P. falciparum* infection. However, it is unclear whether immune or metabolic factors mediate these changes. Mosquito immune responses target predominantly the midgut-traversing ookinetes, whereas exposure of early oocysts to immune attacks is limited, questioning the role of anti-parasitic immunity in the observed microbiota changes (Garver et al., 2012). As *Plasmodium* oocysts scavenge mosquito lipids, and possibly other nutrients essential for their proliferation and virulence (Costa et al., 2017), the parasite may directly impact mosquito metabolism. However, it remains to be investigated whether *Plasmodium* competes with bacteria during its gut development for the same resources, or directly impacts mosquito metabolism.

MICROMANAGING IMMUNITY

Microbiota-Associated Immune Responses to Protozoan Parasites

Protective immunity to *Giardia* predominantly relies on the secretion of intestinal IgA and the induction of pro-inflammatory Th17 responses, supporting neutrophil recruitment and secretion of antimicrobial peptides (Dann et al., 2015; Saghaug et al., 2015). However, our current understanding of the potential immunomodulatory roles of the commensal microbiota in the induction and maintenance of host immunity against *Giardia* remains very limited. Although microbiota-independent $CD4^+$ T-cell responses appear crucial for host protection, $CD8^+$ T cell activation has been shown to be ablated in the gut of

infected animals treated with antibiotics, suggesting a potential involvement of the intestinal microbiota in the activation of $CD8^+$ T cell responses during giardiasis (Keselman et al., 2016). The mechanisms of $CD8^+$ T-cell activation and their potential contribution to immune control during infection with *Giardia*, however, remain to be established (Keselman et al., 2016). Susceptibility to *G. lamblia* infection has previously been shown to vary in mice with an identical genetic background, but originating from independent commercial suppliers, further implicating host microbiota composition in shaping the course of *Giardia* infection (Singer and Nash, 2000). The microbiota of resistant mouse strains was later shown to contain Segmented Filamentous Bacteria (SFB), members of the family *Clostridiales*, while susceptible mice lacked this group (Ivanov et al., 2008). Importantly, SFB play a central role in the induction of intestinal Th17 responses (Ivanov et al., 2009), suggesting that microbiota-driven support for Th17 responses potentially facilitates immune control of *Giardia* infections. However, a direct link between the immunostimulatory properties of SFB and susceptibility to infection with *Giardia* remains to be established (**Figure 3A**).

T. gondii infection in mice, on the other hand, leads to potent Th1 immune responses and importantly, resident intestinal bacteria are known to play an important role in localized intestinal Th1 response polarization by providing molecular adjuvant signals in a TLR/MyD88-dependent manner, thus triggering IL-12 and IFN-γ production (**Figure 3B**; Benson et al., 2009). Studies have demonstrated the formation of distinct structured accumulations of host cells along the ileum of *T. gondii*-infected mice, named "intracellular casts," which contained elevated levels of γ-*Proteobacteria* and were enriched for highly activated neutrophils and ROS-producing inflammatory monocytes (Grainger et al., 2013; Molloy et al., 2013). Further analysis revealed that the presence and expansion of γ-*Proteobacteria* positively influenced neutrophil infiltration into the lumen and, hence, played a role in the induction of intraluminal casts formation, suggesting that commensal bacteria potentially contribute to bacterial overgrowth and support the control of overt pathology during acute murine toxoplasmosis (Molloy et al., 2013). Additionally, inflammatory monocytes from small intestinal lamina propria were shown to adopt a mixed proinflammatory/regulatory phenotype during acute infection with *T. gondii*. The parallel secretion of IL-10, TNF-α, and PGE2 by these monocytes was demonstrated to depend on a range of commensal bacteria-derived ligands and has been correlated with potential PGE2-dependent suppression of commensal-driven neutrophil activation (Grainger et al., 2013). Moreover, toxoplasmosis is associated with the differentiation of $CD4^+$ T cells specific for commensal microbes marked by a Th1 phenotype in a manner similar to parasite-specific Th1 cells (**Figure 3B**; Hand et al., 2012).

Microbiota-Associated Immune Responses to Parasitic Nematodes

Efficient immune control of intestinal nematodes largely depends on the development of protective type 2 immune responses driven by $CD4^+$ Th2 cells in concert with type 2 innate lymphoid cells. Both cell types produce the Th2 cytokines IL-4, −5, and −13 leading to concerted immune effector mechanisms. These mechanisms fortify intestinal barriers via enhanced mucus production, increased epithelial cell turnover, intestinal fluid influx and hypercontraction of smooth muscle cells (Sorobetea et al., 2018). Nematode infections additionally support the activation and expansion of regulatory T cells (Tregs) (Maizels et al., 2012), a phenomenon linked to the anti-inflammatory effect of nematode infections in several models of autoimmunity (McSorley and Maizels, 2012).

Whether the microbiota affects the development of immune effector mechanisms during nematode infections has only recently received attention. One study has reported that low dose antibiotic treatment during *H. polygyrus* infection increased the abundance of members of the *Lactobacillaceae* and *Enterobacteriaceae* bacterial families (Reynolds et al., 2014). *Lactobacilli* abundance correlated positively with worm burdens, and, importantly, with higher numbers of $Foxp3^+$ Tregs in gut-associated lymphoid tissue, suggesting *Lactobacilli*-mediated induction of Treg responses (**Figure 3C**). However, increased *Lactobacilli* abundance was observed only in C57BL/6 mice permissive for long-lasting infections with high worm burdens, while more resistant BALB/c mice did not display this phenotype. Interestingly, administration of *Lactobacilli* to more resistant mice increased their susceptibility to infection (Reynolds et al., 2014). In addition, *H. polygyrus*-dependent changes in intestinal microbiota composition have been correlated to the suppression of allergic airway inflammation. High concentrations of short-chain fatty acids (SCFA) resulted from the increased abundance of *Clostridiales* bacteria in *H. polygyrus* infected mice (**Figure 3C**; Zaiss et al., 2015). The SCFA increase supports mucosal Treg responses associated with the reduced susceptibility of mice to allergic airway inflammation (Zaiss et al., 2015). Furthermore, Ohnmacht et al. (2015) found that the microbiota is needed for the induction of highly activated $ROR\gamma T^+$ $Foxp3^+$ Tregs, which could be induced via the introduction of a cocktail of *Clostridia* species in germfree mice, most likely via the provision of the SCFA butyrate (**Figure 3C**). Importantly, the conditional removal of the $ROR\gamma T^+$ Treg subset rendered *H. polygyrus*-infected mice more resistant to infection due to the development of a more robust Th2 response. Together, these observations suggest that the microbiota can indirectly control intestinal Th2 responses via the induction and maintenance of suppressive Treg subsets in the context of intestinal nematode infections (**Figure 3C**; Ohnmacht et al., 2015).

T. muris infection, on the other hand, leads to a notable decrease in *Bacteroides* producing SCFA, and a concomitant decrease in $Foxp3^+$ Tregs in the lamina propria during chronic infection (Houlden et al., 2015). Moreover, Tregs in *T. muris* infection appeared less prone to release the anti-inflammatory cytokine IL-10. Therefore, chronically infected mice were more susceptible to intestinal inflammation and displayed poor worm expulsion due to the development of Th1 responses counteracting Th2 immunity (**Figure 3D**; Holm

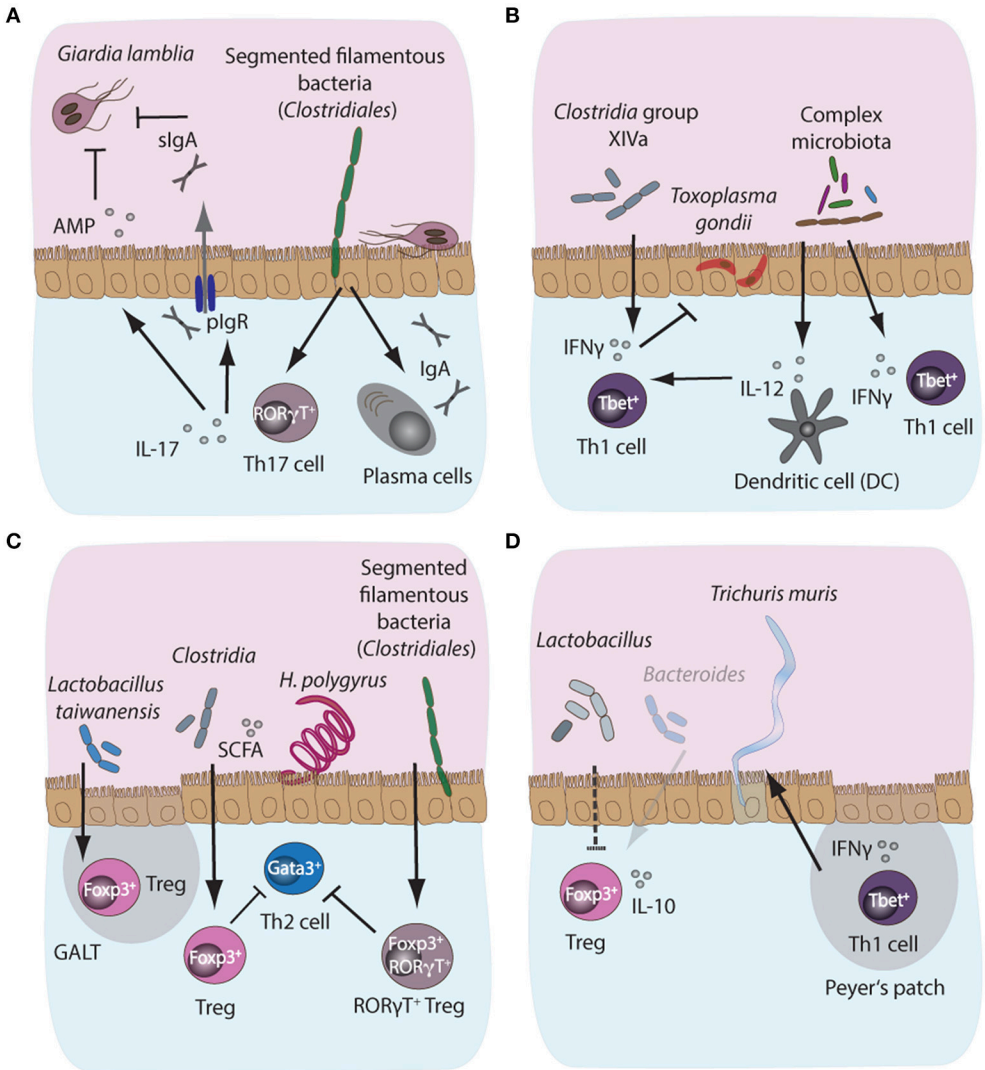

FIGURE 3 | Microbiota-dependent host immune responses during intestinal parasite infections in mice. **(A)** Segmented filamentous bacteria (SFB), members of the *Clostridiales* bacterial group colonizing the small intestinal tract of mammals, are potent inducers of intestinal IgA and Th17 responses in mice. Importantly, both IgA and Th17 responses are key host protective immune mechanisms against the protozoan parasite *Giardia lamblia*. IgA can directly target trophozoites, while IL-17A supports polymeric Ig receptor expression and hence IgA transport into the lumen, in addition to promoting antimicrobial peptide (AMP) secretion by epithelial cells. Resistance phenotypes to giardiasis have previously been linked to the presence or absence of SFB, suggesting a potential correlation between microbiota-induced intestinal IgA and Th17 responses and control of infection with *Giardia*. **(B)** During acute murine infection with *Toxoplasma gondii*, the presence of complex intestinal microbiota leads to MyD88-dependent signaling, triggering protective IL-12 responses in intestinal dendritic cells. **(C)** During infection with the small intestinal nematode *Heligmosomoides polygyrus*, the increased abundance of *Lactobacilli* and *Clostridiales* producing short chain fatty acids (SCFA)have been shown to induce the expansion of Foxp3$^+$ regulatory T cells (Treg) in the gut, while SFB additionally induce the development of a highly potent immunosuppressory subset of Foxp3$^+$RORγT$^+$ Treg, thus indirectly suppressing Th2 cell responses during infection. **(D)** A decrease in SCFA-producing *Bacteroides* and elevated abundances of *Lactobacilli* during chronic *T. muris* infection correlate with decreased intestinal Treg numbers and importantly, these Tregs appeared less prone to secrete IL-10 during chronicity.

et al., 2015). Thus, in nematode infection, the commensal microbiota and, importantly, specific bacterial groups, appear essential for both the induction and suppression of host immune responses. Contextually, this can contribute to resistance or susceptibility to infection, depending on the bacterial community and the invading parasite species.

Microbiota-Dependent Immune Responses in Mosquitoes

Multiple reports in the past have highlighted the ability of bacteria to inhibit the establishment of *Plasmodium* infection in the mosquito host (Gonzalez-Ceron et al., 2003; Dong et al., 2009; Cirimotich et al., 2011; Tchioffo et al., 2013; Bahia et al., 2014). Consistently, some antibiotic treatments promote parasite

development (Beier et al., 1994; Dong et al., 2009; Gendrin et al., 2015, 2016). Plasmodicidal properties have been reported for diverse microbes and must rely on some general rather than species-specific mechanisms (Lowenberger et al., 1999; Gonzalez-Ceron et al., 2003; Dong et al., 2009; Cirimotich et al., 2011; Tchioffo et al., 2013).

Female mosquitoes alternate between sugar and blood feeding. Such diverse diets expose the gut to strong physiological and oxidative challenges (reviewed in Sterkel et al., 2017). Uptake of proteins and lipids from iron and heme-rich blood induces massive metabolic and transcriptional changes in the midgut (**Figure 4**). Nutrient abundance sets off bacterial proliferation which, in turn, induces expression of immune genes (Dong et al., 2009). Blood intake elevates hydrogen-peroxide levels in the mosquito hemolymph and activates reactive oxygen species (ROS) detoxification responses (Kumar et al., 2003; Molina-Cruz et al., 2008; de Almeida Oliveira et al., 2012). However, how blood-feeding affects ROS levels in the midgut, and the underlying mechanisms remain unknown (**Figure 4B**). What is clear is that ROS inhibition after blood feeding causes lethal systemic bacterial infections, suggesting a crucial link between oxidative stress and immune activation (Molina-Cruz et al., 2008).

The peritrophic matrix (PM) provides the first line of defense against blood meal-induced oxidative stress and bacterial infections (**Figures 1**, **4B**). The cardia cells of mosquito larvae continuously secrete sleve-shaped PM (type II), whereas adult epithelial midgut cells synthesize type I PM only after blood feeding (Wigglesworth, 1930; Waterhouse, 1953). Feeding on complex organic matter continuously exposes larvae to bacteria. In contrast, blood feeding induces massive bacterial proliferation in the gut and renders adult females particularly vulnerable to microbial infections (Dong et al., 2009; Linenberg et al., 2016). Such differences in mosquito exposure to microbes at these developmental stages likely contribute to constant PM secretion in larvae vs. inducible PM secretion in adults. Interestingly, PM synthesis in adults requires midgut microbiota. Bacterial proliferation after blood feeding upregulates expression of hundreds of genes, including the genes encoding PM proteins such as glucosamine-fructose-6-phosphate aminotransferase (GFAT) and chitin synthase enzyme 1 (CHS1; Rodgers et al., 2017). Conversely, antibiotic treatment before blood feeding compromises the integrity of PM by inhibiting expression of *GFAT* and *CHS1*, but also the genes encoding peritrophic proteins 1 and 14, whereas it upregulates expression of the PM-degradation chitinase genes A and B (Rodgers et al., 2017; Song et al., 2018). The PM of mosquitoes, therefore, serves as an inducible protective mechanical barrier against bacteria.

The immune-deficiency (IMD) pathway, initially identified in *Drosophila*, is the major immune pathway that control bacterial infections by coordinated expression of antimicrobial peptide (AMPs) genes (Buchon et al., 2009; Broderick et al., 2014). In *Anopheles*, the pathway is activated by the recognition of DAP-type and lysine-type peptidoglycans by the peptidoglycan recognition protein LC (PGRP-LC; Meister et al., *2009).* Although relatively understudied in the mosquito gut, several lines of evidence suggest that IMD is functional in this tissue. *A. gambiae* mosquitoes with intact microbiota display higher basal expression levels of immune genes compared to antibiotic-treated controls (**Figure 4A**; Dong et al., 2009). These genes encode a range of AMPs, signal-transducing serine proteases, IMD pathway components and immune genes such as fibrinogen-related and thioester-containing proteins among others (Dong et al., 2009). Importantly, silencing of a transcriptional activator of the NF-kB family, *REL2* and of *PGRP-LC* receptor, promotes the proliferation of gut microbiota and increases mosquito susceptibility to *Plasmodium* infection (Dong et al., 2009; Meister et al., 2009). The proliferation of bacteria is believed to activate the IMD pathway via the PGRP-LC receptor. Since bacterial proliferation 24 h after blood feeding coincides with ookinete transversal of the midgut epithelium, it may also lead to the IMD-mediated killing of *Plasmodium* parasites (**Figure 4B**; Dong et al., 2009; Meister et al., 2009; Linenberg et al., 2016). Indeed, clearance of the mosquito microbiota by antibiotics before infection increases mosquito susceptibility to *Plasmodium*, whereas bacteria inoculation by feeding decreases it in a PGRP-LC-dependent manner (Meister et al., 2009).

Wolbachia, intracellular maternally transmitted bacteria, confer protection to their arthropod hosts against a range of pathogens (Hedges et al., 2008; Teixeira et al., 2008; Kambris et al., 2009; Moreira et al., 2009; Hughes et al., 2011). *Wolbachia* has been identified in natural *Anopheles* populations in Burkina Faso and Mali (Baldini et al., 2014; Gomes et al., 2017). Importantly, *Wolbachia* infections (experimental transovarian of the Asian malaria vector *A. stephensi*, natural infections of *A. coluzzii* and somatic infections in *A. gambiae*) significantly decrease the prevalence of *P. falciparum*-infected mosquitoes (Hughes et al., 2011; Bian et al., 2013; Shaw et al., 2016; Gomes et al., 2017). Although the mechanisms that cause mosquito resistance to *Plasmodium* remain to be elucidated, experimental *Wolbachia* infections induce expression of a series of immune effectors and ROS which could potentiate parasite killing (Kambris et al., 2009; Bian et al., 2013).

In addition to inducing the immunity-mediated *Plasmodium* killing, some bacteria show direct plasmodicidal activity. *Enterobacter* isolates from Zambian *Anopheles* kill *P. falciparum* in the midgut when administrated with the infectious blood meal (Cirimotich et al., 2011). This has been attributed to direct inhibition of ookinete development by the bacteria-produced ROS (Cirimotich et al., 2011). Soluble factors released by *Serratia marcescens* also inhibit *P. falciparum* ookinete development in the mosquito (Bahia et al., 2014). Inhibition of *P. berghei* ookinetes *in vivo* was linked to the length of the flagella and the motility of *S. marcescens* (Bando et al., 2013). Currently, it is unclear whether this inhibition is direct or immunity-mediated. Furthermore, oral administration of *Chromobacterium* sp. isolated from *A. aegypti* in Panama, induces high mortality in mosquito larva and adults, whereas the surviving mosquitoes exhibit low *Plasmodium* infection loads (Ramirez et al., 2014). Biochemical analyses identified romidepsin, the histone deacetylase inhibitor, as the plasmodicidal factor, uncovering an essential role of histone modifications in the mosquito stages of

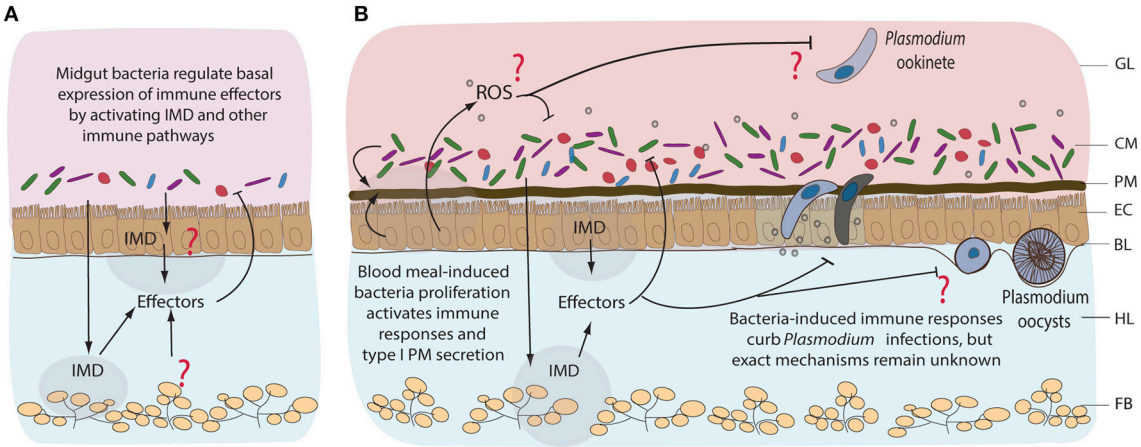

FIGURE 4 | Immune responses of mosquitoes to midgut bacteria and to *Plasmodium* parasites. Immune responses to resident bacteria before **(A)** and after blood feeding **(B)**. Proliferation of the midgut bacteria is regulated mainly by IMD pathway, however the tissue specificity of its expression remains unknown. Immune responses could be coming from the gut, hemocytes (mosquito blood cells), or fat body (nutrient storage and immune tissue). Involvement of other immune pathways, including reactive oxygen species (ROS), in response to midgut bacteria is not well-characterized. Blood feeding-induced bacteria proliferation activates immune responses that also curb the invasion of *Plasmodium,* mainly at the ookinete stage of development. GL, gut lumen; CM, complex microbiota; PM, peritrophic matrix; EC, epithelial cells; BL, basal lamina; HL, hemolymph; FB, fat body.

Plasmodium. Surprisingly, administration of romidepsin did not affect mosquito survival, suggesting that another bacterial factor mediates the *Chromobacterium*-induced mortality (Saraiva et al., 2018).

How plasmodicidal properties of mosquito microbiota correlate with dynamics of *Plasmodium* transmission in the field remains unknown. In laboratory conditions, *Serratia sp.* and *Enterobacteriaceae* curb *Plasmodium* infection (Bando et al., 2013; Tchioffo et al., 2013; Bahia et al., 2014). In contrast, two semi-field studies in Cameroon associated the same bacteria with higher *P. falciparum* loads (Boissière et al., 2012; Tchioffo et al., 2016). Most of the experimental studies examined the role of microbes in *Plasmodium* infections using microbiota-free mosquitoes, and the results of the few field studies stress the importance of studying the tripartite interactions between mosquitoes, microbes and parasites in natural conditions.

BACTERIAL FEEDING VS. BACTERIAL CLEARANCE

Manipulating Host Microbiota as a Treatment Strategy Against Protozoan Parasites

Treatment of giardiasis primarily relies on the administration of antibiotics like metronidazole. However, due to rising levels of resistance, frequent reports of side effects and clinical failures, there is a growing need for the development of novel alternative treatment strategies (**Table 1**; Ansell et al., 2015). In the past, it has been shown that the use of *Lactobacilli* as probiotics in the treatment of giardiasis holds promise. In *G. duodenalis*-infected gerbils, administration of *L. johnsonii La1* leads to reduced infection rates, lack of pathological damage to the epithelial cell layer and absence of immune cell infiltration and inflammation in mice receiving the probiotic compared to the placebo group (Humen et al., 2005). One proposed mechanism of the observed anti-giardial activities of *L. johnsonii La1* has been attributed to the metabolic generation of unconjugated bile salts (Pérez et al., 2001; Travers et al., 2016). Very recently, Allain et al. tested *in vitro* and *in vivo* the anti-giardial activity of three bile salt hydrolases (BSH), enzymes naturally produced by *L. johnsonii La1* (Allain et al., 2018). *In vitro* treatment of *G. duodenalis* with increasing concentrations of recombinant BSH47 and BSH56 enzymes revealed a dose-dependent giardicidal activity of both enzymes in the presence of bile. Importantly, the group further demonstrated the anti-giardial activity of rBSH47 after administration to *G. duodenalis*-infected suckling mice, supporting the view of *L. johnsonii La1* as a probiotic candidate for the treatment of *Giardia* infections.

Along the same line, *Enterococcus faecalis* SF68, a lactic acid bacterium indigenous to the mammalian commensal microbiota, has also been suggested as a potential probiotic treatment for *Giardia*. Oral administration of this strain prior to *G. lamblia* infection led to elevated levels of total IgA levels in small intestine during the acute stage of infection, as well as higher levels of specific anti-*Giardia* IgA and systemic IgG compared to control animals. Nevertheless, parasitological data were less conclusive and the use of *E. faecalis* SF68 as a probiotic supporting anti-*Giardia* immunity merits further investigations (Benyacoub et al., 2005). Overall, it appears that administration of probiotic *Lactobacilli* in *Giardia*-infected mice reduces epithelial damage, attenuates overt inflammation and fosters enzymatic giardicidal activity.

Treatment of toxoplasmosis, on the other hand, relies primarily on the administration of pyrimethamine and sulfonamide drugs (Alday and Doggett, 2017). Recently, the administration of *Bifidobacterium animalis* subsp. *lactis* to mice

TABLE 1 | Administration modes, outcomes and proposed mechanisms of action of probiotic bacteria as potential treatment strategies against intestinal parasitic infections in murine hosts and mosquito vectors.

Parasite species	Host species	Commensal bacterial group	Treatment	Outcome	Proposed mechanism of action	References
Giardia lamblia	*Meriones unguiculatus* *Mus musculus*	*Lactobacillus johnsonii* La1 (NC533)	*Ad libidum* administration of 10^8 CFU per animal 7 days prior to infection	Reduced infection rates, lack of epithelial cell layer damage, no immune cell infiltration and lower inflammation rates (compared to placebo-treated control animals)	Metabolic generation of bile salts with direct anti-giardicidal properties	Pérez et al., 2001; Humen et al., 2005; Travers et al., 2016; Allain et al., 2018;
	Mus musculus	*Enterococcus faecalis* SF68	*Ad libidum* administration of 5×10^8-1×10^9 CFU continuously during trial, starting 7 days prior to infection	Elevated total IgA levels in intestine, as well as higher titers of intestinal *Giardia*-specific IgA and serum IgG, increased proportion of $CD4^+$ T-cells in spleen and Peyer's patches	Induction of naturally polyreactive intestinal IgA secretion	Benyacoub et al., 2005
Toxoplasma gondii	*Mus musculus*	*Bifidobacterium animalis* subsp. *lactis*	*Ad libidum* administration of 1.6×10^7 CFU suspended in 0.1 mL milk continuously during trial, starting 15 days prior to infection	Elevated *Toxoplasma*-specific IgG in serum, elevated numbers of $CD19^+$ B-cells, reduced brain cyst numbers and lower intestinal villi inflammation	Induction of protective humoral immune responses during infection	Ribeiro et al., 2016
Trichuris muris	*Mus musculus*	*Lactobacillus casei* (ATCC7469)	*Ad libidum* administration of live or dead 1.8×10^9 CFU 7 days prior to infection	Suppression of localized IFN-γ and TNF-α responses in secondary lymphoid organs, elevated worm burdens	Unknown	Dea-Ayuela et al., 2008
	Mus musculus	*Lactobacillus rhamnosus* JB-1	*Ad libidum* administration of 1×10^9 CFU for 15 days or 36 days, starting 1 day prior to infection	Elevated tissue levels of IL-10, higher numbers of goblet cells and accelerated worm expulsion	Induction of protective IL-10 production by epithelial cells and enhanced mucus production during infection	McClemens et al., 2013
Plasmodium falciparum	*A. gambiae* *A. stephensi*	*Wolbachia*	Embryonic microinjection	Somatic infection, reduced prevalence and intensity of infection in *A. gambiae* Somatic and ovarian infection, invasion of *A. stephensi* populations, reduced infection rates	Unknown. Potentially through activation of immune defenses	Hughes et al., 2011; Bian et al., 2013
	A. gambiae	*Enterobacter*	Administration of 10^3–10^5 CFUs with the infectious blood meal	Inhibition of *Plasmodium* ookinete stage, reduced infection rates	Bacteria-produced ROS	Cirimotich et al., 2011
	A. gambiae	*Serratia marcescens*	10^3–10^7 bacteria/μl introduced to aseptic mosquitoes with the blood meal	Reduced infection rates and reduced survival after blood feeding	Unknown. Potentially through inhibition of *Plasmodium* ookinetes by *Serratia* soluble factors or bacterial flagellum (both demonstrated with *P. berghei*)	Bando et al., 2013; Bahia et al., 2014
	A. gambiae	Engineered *Serratia marcescens* expressing anti-malarial effector proteins	10^7 bacteria/ml introduced via sugar meal	Colonized midgut and reproductive organs, transmitted in three successive generations. Reduced infection rates	Through secretion of anti-*Plasmodium* effector molecules	Wang et al., 2017

chronically infected with *T. gondii* demonstrated enhanced serum levels of anti-*T. gondii* IgG and higher numbers of $CD19^+$ B-lymphocytes, a reduction in brain cysts, and attenuated intestinal villi inflammation in the probiotic-treated group (Ribeiro et al., 2016). Conversely, previous work in gnotobiotic mice colonized by probiotic *E. coli* strains revealed that these microbes contribute significantly to intestinal inflammation during *T. gondii*-infection (Bereswill et al., 2013). These results indicate that the use of probiotic bacterial strains in the treatment of intestinal parasite infections is highly contextual and reports on the potential benefits and risks for host health should be considered in the future design of novel probiotics.

Probiotic Administration and Microbiota Manipulation During Nematode Infections

In intestinal helminth infections, few *in vivo* or *in vitro* studies have demonstrated mechanisms of action of probiotic bacteria favoring either infection resistance or susceptibility. Treatment of *T. muris*-infected mice with viable or dead probiotic *Lactobacillus casei* led to the maintainance of high worm burdens in the chronic phase of infection and concurrently suppressed cellular and Th1/Th2 cytokine responses in secondary lymphoid organs (Dea-Ayuela et al., 2008). On the other hand, administration of live *Lactobacillus rhamnosus* JB-1 to *T. muris*-infected mice resulted in accelerated worm expulsion and elevated gut tissue concentrations of IL-10, but not Th2 or Th1 cytokines. Administration of *L. rhamnosus* enhanced worm expulsion in mice deficient in mucin 2 ($Muc2^{-/-}$), a key component of the intestinal mucus layer led to elevated goblet cell frequencies compared to medium-treated $Muc2^{-/-}$ mice, suggestive of probiotic-dependent induction of additional mucins involved in worm expulsion (McClemens et al., 2013).

The administration of probiotic bacteria against gastrointestinal protozoan parasite infections has gained attention as a novel and effective treatment strategy (**Table 1**). Numerous studies have highlighted a range of beneficial aspects of probiotic bacteria including support for intestinal antibody responses, reduction in immunopathology and elevated anti-inflammatory cytokine responses, as well as potential competition for nutrients with the invading intestinal parasite species. On the other hand, the introduction of certain probiotic bacteria during intestinal parasite infections could also have a number of detrimental effects, including increasing the numbers of intestinal Tregs and thus potentially facilitating nematode survival or providing support for damaging inflammatory reactions during infection.

Microbiota Manipulations in Mosquitoes as a Vector Control Strategy

The environmental bias of the mosquito microbiome poses a significant challenge for its manipulation for vector control purposes. However, several approaches have been explored for introducing into natural mosquito populations bacteria with anti-*Plasmodium* properties (**Table 1**). *Wolbachia* has been successfully exploited in *A. aegypti* mosquitoes to interrupt the transmission of dengue virus (Hoffmann et al., 2011, 2014). Efficient *Wolbachia* application for malaria control requires rapid bacterial spread in the mosquito populations. This spread relies on *Wolbachia* ability to induce cytoplasmic incompatibility (CI) in the host, which is manifested by the sterility of individuals with different *Wolbachia* infection status (Bordenstein and Werren, 2007). CI has not been observed in *Wolbachia* naturally occurring in *A. gambiae*. Instead, the bacteria appear to induce a modest acceleration of egg-laying rates (Shaw et al., 2016). For experimentally-introduced *Wolbachia*, successful maternal transmission and CI has been reported in *A. stephensi* (Bian et al., 2013). Another study has shown inhibition of vertical *Wolbachia* transmission in *A. gambiae* and *A. stephensi* by resident microbiota (Hughes et al., 2014), while other attempts to stably introduce *Wolbachia* in *A. gambiae* have been unsuccessful (Kambris et al., 2009; Hughes et al., 2011).

Recently, mosquito colonization with genetically engineered *Serratia* has been proposed as an alternative transmission-blocking strategy (Wang et al., 2017). In addition to the plasmodicidal activity of *S. marcescence* described above, the engineered *Serratia* strains express one or multiple anti-malarial effector proteins and successfully decrease *P. falciparum* infections in *A. stephensi* (Wang et al., 2017). *Serratia* naturally colonizes the mosquito midgut, ovaries and male accessory glands, and is expected to spread throughout mosquito populations. However, the persistence of genetically engineered *Serratia* under laboratory conditions was only demonstrated for three successive generations with a substantial drop in bacterial loads already in the second generation (Wang et al., 2017).

A surprising link between immune activation and changes in microbiota composition of the mosquito reproductive organs has been reported recently. Transgenic mosquitoes that expressed an active form of the REL2 transcriptional factor in the midgut after a blood meal were reported to inhibit bacterial proliferation and *Plasmodium* invasion (Dong et al., 2011). Unexpectedly, activation of the IMD pathway modified the mosquito mating behavior leading to the preferential mating of transgenic males with wild-type females (Pike et al., 2017). Although the exact mechanism is unclear, the authors proposed that transgene expression inhibits bacterial proliferation in the male reproductive organs and facilitates the spread of the transgene (Pike et al., 2017). Surprisingly, female mating preference was not affected by the transgene expression. Whether such behavioral manipulation was caused by a particular bacterial species or the overall bacterial loads and whether transgenesis-induced changes were male-specific remains to be investigated.

Another interesting approach to mosquito control exploits the entomopathogenic bacteria *Chromobacterium* sp. and the fungus *Beauveria bassiana* that exhibit plasmodicidal activities. As discussed above, *Chromobacterium* interferes with *Plasmodium* through histone modification processes and induces mortality by an as yet unknown mechanism (Saraiva et al., 2018). *B. bassiana*, on the other hand, downregulates IMD-mediated immunity and expression of dual oxidase DUOX, causing midgut dysbiosis and systemic infections by opportunistic bacteria that kill mosquitoes (Wei et al., 2017). Therefore, disrupting or manipulating the mosquito microbiome often has fatal consequences for the vector

TABLE 2 | Comparison of intestinal immunity, microbial diversity and microbiota-related interactions within the murine hosts and the mosquito vectors during intestinal parasitic infections.

Comparison	Mouse	Mosquito
Mechanical barriers	Mucus layer, enriched in mucin glycoproteins, permanently lining the intestinal epithelial layer as a shield from direct exposure to external stimuli and invading microorganisms. Commensal microbiota supports mucus secretion and maintenance.	Semipermeable peritrophic matrix (PM) composed of chitin and glycoproteins, lining the entire larval gut. In adult females, PM is secreted in the midgut upon blood feeding, which induces significant microbial proliferation in the midgut. Commensal microbiota stimulates peritrophic matrix synthesis.
Intestinal microbial diversity	Higher complexity and species diversity. Compositional changes occur subject to environmental and dietary changes like breastfeeding or gastrointestinal infections. Exhibits overall less drastic compositional shifts during lifespan of host. Dominant bacterial groups include *Enterobacter, Lactobacillus, Bifidobacteria, Bacteroides, Clostridia, Ruminococcus.*	Lower complexity and species diversity. High fluctuation in diversity and species abundance observed throughout the development and after blood meal uptake. Exhibits drastic compositional shifts during vector development. Dominant bacterial groups include *Enterobacter, Serratia, Pantoea, Asaia, Aeromonas, Pseudomonas, Bacillus.*
Microbial fingerprint during parasitic infections	Numerous studies have demonstrated significant alterations of intestinal microbial communities during infections with protozoan parasites (eg. *Giardia lamblia* and *Toxoplasma gondii*), as well as during nematode infections with *Heligmosomoides polygyrus* or *Trichuris muris*.	Not enough evidence of *Plasmodium* influence on midgut microbial communities, possibly due to limited time spent by the parasite in the midgut lumen.
Microbial priming of immune defenses against parasitic infections	Segmented filamentous bacteria (family *Clostridiales*) are known potent inducers of intestinal Th17 responses, key for host protection against *Giardia* infection.	Midgut microbiota is a potent inducer of IMD/NF-κB pathway. Basal activity of IMD is regulated by the midgut microbiome, and bacterial proliferation after blood feeding further enhances pathway activation. The microbiota-dependent activation of the IMD pathway impacts within-mosquito development of *Plasmodium falciparum*.
	Lactobacilli induce intestinal Treg responses during infection with *H. polygyrus*, but not during *T. muris* infection, and correlate with higher worm burdens	*Enterobacter* isolates have direct plasmodicidal activity. They produce ROS that kills *Plasmodium* ookinetes. Soluble factors of *Serratia marcescens* and motility of some *Serratia* strains impact development of *P. berghei* ookinetes.
	Clostridia can induce suppressive Treg responses via short-chain fatty acid production during *H. polygyrus* infection	Although understudied, immune activation after blood feeding is linked to oxidative stress. Blood feeding increases hydrogen peroxide levels and ROS detoxification responses. Moreover, inhibition of ROS leads to lethal systemic bacterial infections.
	γ-*Proteobacteria,* among the most abundant commensal bacteria in mice, contribute to intestinal immunopathology during *T. gondii* infection via activation of neutrophils and ROS-producing inflammatory monocytes	γ-*Proteobacteria* is the most abundant class of bacteria in mosquitoes. It limits mosquito vector competence for *Plasmodium* transmission by inducing the IMD pathway and probably other as yet unknown mechanisms.

that may directly or indirectly contribute to the inhibition of *Plasmodium* development.

CONCLUSIONS AND FUTURE PERSPECTIVES

Along the entire length of the mammalian intestinal tract from the small intestine infected by *Giardia, T. gondii*, and *H. polygyrus*, to the large intestine where *T. muris* thrives, there is evidence of a critical and vastly complex relationship between the host immune system, the resident microbiota and the invading pathogens. Alterations of the host microbiota can both affect the integrity and efficiency of protective immune responses, as well as the development of the parasite and its infection efficacy. However, we still lack a more comprehensive understanding of the causality vs. correlation between specific compositional shifts of the commensal bacterial communities in the gut and the induction of host immune mechanisms during intestinal parasite infections. More specifically, the majority of current studies are performed in laboratory conditions, often with microbiota-free animals. Despite their limited sample size, field and semi-field studies provide valuable insights into the microbiome complexity of natural *Anopheles* populations and the effects of natural microbial communities on *Anopheles* vectorial capacity and anti-parasitic responses in mice deserve further investigation.

The intestinal microbiota of mice and mosquitoes naturally faces vastly contrasting environmental and biochemical challenges due to major differences in host development, physiology and diet among many other factors (**Table 2**). The mouse microbiota displays higher complexity and species diversity than the resident microbial community in the mosquito, but at the same time experiences less drastic variations and

expansion compared to those seen in freshly blood-fed mosquito females. While the significant expansion of γ-*Proteobacteria* documented during acute *T. gondii* infection in mice contributes to immunopathology due to the activation of neutrophils and ROS-producing inflammatory monocytes, γ-*Proteobacteria* are important players in limiting vector competence of mosquitoes for *Plasmodium*. More importantly, the generation of ROS is a crucial immune response mechanism in insects, induced in response to a range of microorganisms. Considering the importance of murine resident γ-*Proteobacteria* in the induction of ROS-producing inflammatory monocytes against *T. gondii*, it merits further investigations whether a similar bacteria-mediated process is limiting vector capacity of mosquitos after uptake of *Plasmodium* with the blood meal.

Following each blood meal, the mosquito midgut synthesizes a semipermeable peritrophic matrix that shields the gut epithelium from direct exposure to potentially harmful external stimuli and invading microorganisms. Similarly, the murine gastrointestinal tract contains a viscous layer of mucus enriched in mucin glycoproteins protecting the intestinal epithelial layer (**Table 2**). Microbes enhance mucus secretion in the mammalian gut similarly to what has been suggested for PM deposition in the mosquito. Further studies are required to see if distinct microbes support the peritrophic membrane and thereby limit *Plasmodium* infection.

Changes in microbiota induced during intestinal parasite infections may provide valuable information on new intervention strategies against these pathogens. A better mechanistic understanding of how the microbial status and infection-induced microbiome alterations of an individual affects immune responses to parasites and of how the microbiome could be targeted to reduce infection success of parasites and arthropod vectorial capacity necessitates further studies. So far, the diversity in study designs using a gnotobiotic, mono-colonized, antibiotic-treated or standard-pathogen-free mouse and vector models offers invaluable tools for the elucidation of effector mechanisms responsible for the induction and maintenance of immune responses and to what extent specific members of the commensal microbiota are involved. Nevertheless, variations in study design like the colonization status of the host/vector, host age, sex, commercial provider, parasite strain and infection dosage, microbiota culturing and sampling techniques, as well as differences in data analysis at the phylum, order, family, or genus level present considerable challenges to microbiota research. Caution must also be taken in the interpretation of studies on microbiota-dependent immunity and the translation of their findings outside the laboratory, as inbred lab-reared mice/vectors experienced a considerable change in bacterial diversity/composition compared to wild animals, which provide a more representable animal model. Future studies should consider parallel investigations of wild rodent models and vectors from natural populations for a more comprehensive understanding of the tripartite interactions between host, microbiota and invading intestinal parasites. The administration of cocktails of probiotic bacteria with varying stimulatory effector functions, rather than single bacterial groups would represent successful future probiotic treatment strategies. As such, further studies focusing on how commensal and probiotic bacterial communities communicate and regulate each other in the context of gastrointestinal infections would be required in humans, laboratory and free-living mice and mosquito species alike.

AUTHOR CONTRIBUTIONS

All authors contributed to the work presented in this manuscript. EL and SH conceived the original framework of this paper. IY and SZ performed the literature search and wrote the manuscript. EL, SR, GC, and SH edited and critically revised the manuscript.

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Conceptual Design of a Universal Donor Screening Approach for Vaginal Microbiota Transplant

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The success of fecal microbiota transplant (FMT) in treating recurrent *Clostridioides difficile* infection has led to growing excitement about the potential of using transplanted human material as a therapy for a wide range of diseases and conditions related to microbial dysbiosis. We anticipate that the next frontier of microbiota transplantation will be vaginal microbiota transplant (VMT). The composition of the vaginal microbiota has broad impact on sexual and reproductive health. The vaginal microbiota in the "optimal" state are one of the simplest communities, dominated by one of only a few species of Lactobacillus. Diversity in the microbiota and the concomitant depletion of lactobacilli, a condition referred to as bacterial vaginosis (BV), is associated with a wide range of deleterious effects, including increased risk of acquiring sexually transmitted infections and increased likelihood of having a preterm birth. However, we have very few treatment options available, and none of them curative or restorative, for "resetting" the vaginal microbiota to a more protective state. In order to test the hypothesis that VMT may be a more effective treatment option, we must first determine how to screen donors to find those with minimal risk of pathogen transmission and "optimal" vaginal microbiota for transplant. Here, we describe a universal donor screening approach that was implemented in a small pilot study of 20 women. We further characterized key physicochemical properties of donor cervicovaginal secretions (CVS) and the corresponding composition of the vaginal microbiota to delineate criteria for inclusion/exclusion. We anticipate that the framework described here will help accelerate clinical studies of VMT.

Keywords: microbiota, fecal microbiota transplant (FMT), bacterial vaginosis (BV), *Lactobacillus*, urinary tract infection (UTI), cervicovaginal secretions (CVS), sexually transmitted infections

INTRODUCTION

Evidence continues to accumulate that demonstrates the critical role that bacteria play in human health and disease. It is often cited that the commensal bacteria colonizing our epithelial surfaces, glands, and fluids outnumber the cells that make up our bodies (Abbott, 2016; Sender et al., 2016). It has even been suggested that the gut microbiota should be considered an additional organ (Baquero and Nombela, 2012; Liu, 2016). The gut microbiota is perhaps the most extensively characterized, and has been shown to influence a wide range of diseases and disorders affecting the gut and beyond (Clemente et al., 2012; Valdes et al., 2018). Indeed, the premise of microbiota transplantation originated in the gut with the development of fecal microbiota transplantation (FMT) as a strategy for treating recurrent *Clostridioides difficile* (*C. difficile*) infection. The tremendous clinical success of FMT has led to explorations in using FMT for treating inflammatory bowel disease, obesity, liver disease, depression, food allergies, antibiotic resistance, malnutrition, multiple sclerosis, and more (Borody et al., 2013). Further, FMT has motivated the study of other forms of microbiota transfer, including skin microbiota transplant (Myles et al., 2018; Perin et al., 2018) and vaginal microbiota transfer from mother to babies born by Cesarean section (Dominguez-Bello et al., 2016).

Despite being one of the simpler commensal bacteria communities, cervicovaginal microbiota play a key role in sexual and reproductive tract health. The earliest attempts to characterize the cervicovaginal microbiota were conducted in the mid 1800s, a time when puerperal sepsis killed at least 15% of women giving birth in Europe and America (Hallett, 2005). In 1879, Louis Pasteur reported observation of streptococci in the blood of women with puerperal fever (Dunn, 2005). He believed this may have resulted from mechanical trauma that allowed bacteria in the vagina to enter the bloodstream. Later in 1892, Doderlein reported that women who had Gram-positive, lactic acid-producing bacilli in their vagina at the time of childbirth were less likely to develop puerperal sepsis after delivery (Doderlein, 1892; Thomas, 1928). Although there has been increasing awareness of the broad spectrum of "normal" (Smith and Ravel, 2017; Anahtar et al., 2018), it is generally considered that the "optimal" vaginal microbiota communities are dominated by one of only a handful of species of *Lactobacillus* (Linhares et al., 2011; Petrova et al., 2015). Diversity in the vaginal microbiota and lack of dominance by *Lactobacillus* species can be described clinically as bacterial vaginosis (BV), a condition that has been linked to increased risk of sexually transmitted infection acquisition and transmission (Cherpes et al., 2003; Wiesenfeld et al., 2003; Allsworth et al., 2008; Atashili et al., 2008; Cohen et al., 2012), urinary tract infections (Sumati and Saritha, 2009; Stapleton, 2016), and fertility and pregnancy outcomes (Hyman et al., 2014; Petricevic et al., 2014; García-Velasco et al., 2017; Stout et al., 2017). Further, rates of BV relapse after standard antibiotic treatment can be as high as 70% within 3 months (Larsson and Forsum, 2005). Beyond BV, vaginal microbiota have also been implicated in recurrent yeast infections (Zhou et al., 2009; Liu et al., 2013), colonization with group B streptococcus (Rosen et al., 2017; van de Wijgert, 2017), and potentially reproductive tract cancers (Xu et al., 2014; Kyrgiou et al., 2017). Many have drawn attention to the limitations of currently available treatments for BV and the need for innovation in approaches for modifying the vaginal microbiota (Bradshaw and Sobel, 2016; Martin and Marrazzo, 2016).

Vaginal microbiota transplant (VMT) has the potential to revolutionize the way we view and treat conditions affecting the female reproductive tract. Unfortunately, study of vaginal microbiota in preclinical animal models is severely limited by the fact that dominance of the vaginal microbiota and acidification by *Lactobacillus* species is a uniquely human phenomenon (Miller et al., 2016; Witkin and Linhares, 2017). Even our primate cousins have low vaginal colonization by *Lactobacillus* species, and it has been suggested that the normal rhesus macaque vaginal microbiota is a good model for human BV (Spear et al., 2010; Mirmonsef et al., 2012; Yildirim et al., 2014). Unlike FMT, there is no previous history of anecdotal clinical implementation of VMT. However, there is significant epidemiological evidence of vaginal microbiota transfer between women who have sex with women (WSW) (Marrazzo et al., 2002, 2009; Vodstrcil et al., 2015). Thus, a logical next step is to determine whether cervicovaginal secretions (CVS) can be used to transplant vaginal microbiota from a donor to a recipient in a clinical setting. Undoubtedly, ensuring safety and tolerability are top priorities. Here, we describe the development of a universal donor screening protocol intended for maximal risk reduction to mitigate potential transmission of infectious pathogens, as well as collection, characterization, and testing procedures for donor CVS.

MATERIALS AND METHODS

Ethics Statement

The sample collection and testing procedures described here were approved by the Johns Hopkins University Institutional Review Boards as a part of study IRB00131437. Informed consent was obtained from all human subjects prior to participation.

Screening Questionnaire

We have developed a questionnaire for pre-screening potential VMT donors. The questionnaire includes all questions listed in the FDA Guidance for Industry for Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps) (Section IV, Donor screening, §1271.75, section E). In addition, we included screening questions that are consistent with what has been observed to impact vaginal microbiota and stability of vaginal microbiota communities, including sexual history, sexual behavior, and vaginal product usage. We also included questions about medical history, and travel history (e.g., potential exposure to Zika or Ebola) that could have an impact on risk of incident sexually transmitted infections. For the pilot donor screening study described herein, we used an abbreviated questionnaire with the primary goal of correlating testing outcomes with self-reported sexual behavior, vaginal symptoms, vaginal product usage, and self-reported history of reproductive tract and sexually transmitted infections.

Participant demographics and questionnaire data can be found in **Table 1**.

Test List

The list of clinical tests and laboratory characterizations with their submeasures and readouts with normal ranges, if applicable, can be found in **Supplementary Tables 1** and **2**, respectively. We followed the FDA Guidance for Industry for Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps) (Section VI, Donor Testing, §1271.85, sections A and B) for testing of leukocyte-rich cells or tissues, which also required the use of a FDA-licensed, cleared, or approved donor screening test where such a test is available (Memorial Blood Centers, St. Paul, MN). Additional testing was conducted by Johns Hopkins Medical Institutions Medical Laboratories, including tests for herpes viruses, hepatitis A, *Toxoplasma gondii*, Epstein-barr virus (EBV), rubella virus, general immunocompetence, pregnancy, and bacterial and fungal cultures. Several tests were subsequently sent to Quest Diagnostics, as indicated by test codes beginning in "Q" in **Supplementary Table 1**. It was noted after the first four participants that many did not recall their hepatitis A vaccination status while testing positive for hepatitis A IgG, so a test for hepatitis A IgM was added. Additional laboratory characterizations for sexually transmitted infections, included nucleic-acid amplification tests (NAAT) for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, and *Mycoplasma genitalium*, and Human papilloma viruses (HPV), as well as quantitative polymerase chain reaction (qPCR) and 16S rDNA sequencing to determine composition of vaginal microbiota communities. As 16S rDNA sequencing runs typically require batching of samples into a 96 well plate format, we sought to develop a qPCR approach that was predictive of the relative abundance of *Lactobacillus* species and *Gardnerella vaginalis* and could be readily performed on individual samples on the same day for rapid screening and eligibility determination.

Sample Collection

Potential study participants were recruited from a list of participants from the investigator's prior studies that (i) had agreed to be contacted for potential participation in future studies, and (ii) had provided *Lactobacillus*-dominated CVS samples as part of these prior studies. To be included in the study, all participants identified as female and were pre-menopausal between the ages of 18–45 yrs. The potential participants were informed that at the time of sample collection, they must not be currently menstruating or within 3 days of their last menstrual period, they must be currently healthy and free of vaginal symptoms, and must not have used vaginal products or had vaginal intercourse in the prior 3 days. Participants were advised to drink extra water in the time leading up to their appointment to ensure proper hydration for blood collection. Participants were consented using an interactive question and answer approach that explained the purpose of the study, the associated risks, and the testing information that was to be obtained. The participants signed a consent form for HIV testing in the state of Maryland. The participants then filled out the abbreviated questionnaire and

TABLE 1 | Participant demographics and questionnaire data*.

Age	Median (range)
	26.5 (23–35)
Ethnicity	Number (%)
Hispanic or Latino	2 (10)
Not Hispanic or Latino	18 (90)
Race	Number (%)
White	12 (60)
Asian	3 (15)
White/Asian	2 (10)
Native Hawaiian or other Pacific Islander	1 (5)
Other	2 (10)
Type of Birth Control	Number (%)
None	4 (20)
Condoms	2 (10)
Oral Contraceptive	5 (25)
IUD	9 (45)
Copper IUD	3 (15)
Progestin IUD	6 (30)
Reported Symptoms	Number (%)
None	15 (75)
Staining of underwear	2 (10)
Vaginal Odor	1 (5)
Vaginal Discharge	2 (10)
Vaginal Itch	1 (5)
Previous Conditions	Number (%)
Yeast Infection	12 (60)
HPV	1 (5)
Bacterial Vaginosis	2 (10)
Vaginal Irritation	1 (5)
Vaginal Itch (Persistent)	1 (5)
UTI	1 (5)
Abdominal or Pelvic Pain	1 (5)
Chlamydia	3 (15)
Herpes	1 (5)
Products Used	Number (%)
None	16 (80)
Vaginal Douche	1 (5)
Feminine towelettes	2 (10)
Boric Acid	1 (5)
Number of sexual partners (lifetime)	Median (range)
Men	6.5 (0–29)
Women	0 (0–2)
Number of sexual partners in the last month	Median (range)
Men	0 (0–1)
Women	0 (0)
Is the current male partner circumcised?	Number (%)
Yes	15 (75)
No	3 (15)
No current male partner	2 (10)
Tobacco use	Number (%)
No	20 (100)
Have you ever given birth to a baby	Number (%)
No	20 (100)

**The following symptoms "you currently have" were not selected by any participant: pain during intercourse; abdominal or pelvic pain; vaginal irritation; pain during urination. The following options for conditions that "you have ever been diagnosed with" were not selected by any participant: trichomoniasis, gonorrhea, syphilis, pelvic inflammatory disease, other please specify. The following options for product use within the past 6 months were not selected by any participant: feminine hygiene spray, feminine hygiene powder, norforms, vaginal acid gel.*

asked questions for clarification as needed. The participants were then given a bag containing 5 unwrapped vaginal swabs [2 × BD Eswabs (Becton Dickinson), 1 × Mini-tip flocked swabs with viral transport media (Becton Dickinson), 1 × Digene HC2 DNA collection brushes (Qiagen), 1 × Aptima vaginal swab (Hologic)], a Softdisc menstrual fluid collection device (The Flex Company), a sterile wrapped urine specimen cup, and a 50 mL conical tube (Corning Falcon). The participants were shown diagrams to instruct them on how to use each swab and how to insert the Softdisc to collect CVS using a previously described method (Boskey et al., 2003). It was emphasized that the participants take their time collecting each specimen carefully, to ensure that none of the materials touched unintended surfaces, and to return for replacement swabs if any were dropped or contacted a surface other than the vaginal wall. The participant then went to a self-locked single stall restroom for sample self-collection. Upon their return to the clinical area, the participant was prepared for blood collection. The total volume of blood collected across various tubes to perform all tests was ∼50 mL. Specimens were then grouped for immediate overnight shipping to Memorial Blood Centers, transport to the JHMI Medical Laboratories, and transport on ice to the respective laboratory facilities for testing and characterization. Three participants had menstrual blood in their CVS, either trace amounts still present despite reporting 3 days post the end of their menstrual period, or because of an unanticipated early start to their period. They returned within 2–20 days to provide another CVS sample and vaginal Eswab for 16S rDNA sequencing, which were the samples used for the data reported herein.

Laboratory Characterizations

Panther System

Aptima (Hologic) swabs were transported on ice and stored at 4°C for <30 days until testing by the Aptima Combo 2 for chlamydia and gonorrhea, by Aptima TV for trichomonas, and by Aptima MG for *Mycoplasma genitalium*.

Human Papilloma Virus Assay

The Digene HC2 vaginal brushes were transported on ice and stored at 4°C for <60 days until testing. The Roche Linear Array HPV Genotyping Test is a qualitative test that detects 37 human papillomavirus genotypes including 17 high risk types, 15 low-risk types, and 5 unknown-risk/probable-high-risk (pHR) types (Muñoz et al., 2003; de Villiers et al., 2004). The tests were performed according to the manufacturer's protocol (Woo et al., 2007), with broad-spectrum amplification and reverse line blot hybridization for genotype discrimination (Gravitt et al., 1998; Low et al., 2015).

CVS Characterization

Specimens were transported from the clinical location to the laboratory in a cooler on ice. Immediately upon return to the laboratory, the 50 mL conical tube containing the Softdisc was centrifuged at 1,000 RCF for 2 min to collect the CVS. The CVS was transferred to a 1.5 mL Eppendorf tube using a 50 μL Wiretrol (Drummond Scientific). The approximate sample volume was noted (average 0.25 ± 0.14 mL, range 0.1–0.5 mL). We were able to characterize 3 out of 4 Amsel's criteria for BV diagnosis, including: (i) pH >4.5, (ii) basic amine "fishy" odor upon mixing with 10% KOH (positive whiff test), and (iii) presence of clue cells in the wet mount. A sample had to meet all 3 criteria to be categorized as BV based on Amsel's criteria. CVS sample pH was measured using a Mettler Toledo EL20 pH meter with a micro-combination pH electrode MI-411 (Microelectrodes, Inc.). Slides were prepared for wet mount by rolling a swab coated in CVS on a standard microscope slide followed by the addition of 10 μL of normal saline and covered with a glass coverslip. The wet mount slide was observed for the presence of clue cells using differential interference contrast (DIC) microscopy. The whiff test was performed by dipping a cotton swab in the CVS, pipetting 100 μl of 10% KOH onto the swab, and using a gloved hand to waft air over to determine whether a fishy odor was produced. Another swab covered in CVS was rolled onto a second standard microscope slide and left to air dry for gram staining and Nugent scoring for diagnosis of BV (Nugent et al., 1991). For lactic acid measurements, ∼10 μL of CVS was transferred to a preweighed Eppendorf tube to obtain the sample mass (6–20 mg). The CVS was diluted with 490 μL of normal saline and frozen at −20°C until performing the assay. The diluted samples were thawed and centrifuged at 1000 RCF for 5 min to pellet mucus solids. The supernatant was processed per manufacturer's instruction in a 96 well plate format using a D/L-lactic acid kit (R-Biopharm).

Multiple Particle Tracking to Assess HIV-1 Virion Mobility

Fluorescently labeled HIV-1 pseudoviruses were prepared as previously described (Nunn et al., 2015; Hoang et al., submitted). HIV virions (0.3 μL) were pipetted into 20 μL of undiluted CVS in a custom-made glass slide with a circular sample well, gently mixed, and immediately sealed with a glass coverslip. Twenty second videos of viral motion were recorded at room temperature using a Zeiss Axio Observer inverted epifluorescence microscope equipped with a 100x/1.46 NA oil-immersion objective and an EM-CCD camera (Evolve 512; Photometrics). The image resolution was 25 nm/pixel and sequential images were captured at a frame rate of 15 Hz. A minimum of 5 videos were collected per CVS sample. Virion trajectories were analyzed using automated MATLAB-based particle tracking software with a minimum of 16 frames (∼1 s) of consecutive tracking as previously published (Suh et al., 2005; Lai et al., 2007; Schuster et al., 2015).

16S Sequencing and Analysis

DNA Extraction

BD Eswab fluid (150 μL) or 10 mg of CVS were resuspended in 180 μL of lysis buffer (1% Triton X-100, 20 mM Tris-HCl pH 8.0, 2 mM EDTA) with 20 mg/mL of lysozyme and incubated for 1 h at 37°C. Following lysozyme treatment, DNA was extracted using the DNeasy® Blood and Tissue kit (QIAGEN®, Hilden, Germany), including the addition of 20 μL of proteinase K and incubation for 40 min at 56°C for pretreatment of gram-positive bacteria. Following DNA extraction, DNA concentrations were measured by NanoDrop.

DNA Amplification/Library Preparation

Library preparation was performed by the JHMI Deep Sequencing and Microarray Core at the Johns Hopkins Medical Institute according to the Illumina 16S metagenomic library preparation protocol (Illumina, San Diego, CA) with primers that amplified the V4 region of the 16S gene. Paired-end sequencing of the pooled library was performed with the MiSeq system (Illumina, San Diego, CA), generating 2 × 250 reads. Adaptor and barcode trimming was also performed by the JHMI Deep Sequencing and Microarray Core at the Johns Hopkins Medical Institute.

Sequence Processing

QIIME 1.9.1 was used to join paired reads, demultiplex samples, identify and filter out chimeras using USEARCH 6.1 (Edgar, 2010), perform open-reference OTU picking and make taxonomic assignments (Caporaso et al., 2010). Joining required a minimum of 15 base calls of overlap and <20% dissimilarity between matching sequences. Quality control during demultiplexing required 60% of base calls in a read to have a quality score above 17 with no more than 9 consecutive base calls below 17. The reference database used for OTU picking and taxonomic assignments is a custom reference database that combined an existing vaginal microbiome 16S database (Srinivasan et al., 2012) with the V4 regions of additional species represented in the VaHMP V1-V3 database (Fettweis et al., 2012).

Sequencing Data Analysis

Reads were rarefied in R (R 2017, R version 3.4.3) and k-means clustering was performed to assign samples to CSTs. To compare vaginal swab and CVS samples, Inverse Simpson Indices were calculated in R using the vegan (2.5–4) diversity command and a Bray-Curtis dissimilarity matrix, and NMDS analysis was performed using the vegan metaMDS command and a Bray-Curtis dissimilarity matrix. Stress of the NMDS analysis was 0.1725. PERMANOVA statistics were calculated using the vegan adonis command.

Bacterial Culturing

Lactobacillus crispatus (BEI Resources strain EX533959VC06), *Lactobacillus jensenii* (ATCC strain 25258), *Lactobacillus iners* (BEI Resources strain UPII 60-B), *Lactobacillus gasseri* (ATCC strain 33323), and *Gardnerella vaginalis* (BEI Resources strain JCP7275) were all grown in either MRS (*L. crispatus, L. jensenii, L. gasseri*) or NYC III (*L. iners*, *G. vaginalis*) liquid broth. Bacteria grown in MRS were plated on MRS agar plates while bacteria grown in NYC III were plated on BBL™ Brucella Agar plates with 5% Sheep Blood with Hemin and Vitamin K1 (Becton Dickinson). Plates were incubated in anaerobic jars with GasPak™ EZ anaerobe container system (Becton Dickinson) at 37°C for 2–3 days before CFUs were counted (O'Hanlon et al., 2011).

Quantitative PCR and Standard Curve Generation

Quantitative PCR

qPCR of vaginal samples and cultured bacteria was performed on Applied Biosystems, QuantStudio 3. Bacterial species-specific primers (Integrated DNA Technologies) for the 16S rRNA gene were used at a concentration of 100 nM (**Supplementary Table 3**) (Jespers et al., 2012). To standardize the qPCR, a fixed volume (2 μL) of DNA was used for cultured standards, CVS, and swab samples. The program started with an initial incubation at 95°C for 20 s, followed by 40 cycles with denaturing at 95°C for 1 s followed by anneal/extend at 60°C for 20 s. Data is shown for qPCR of CVS; the correlation coefficients for Ct values between CVS and swab was 0.97 for *L. crispatus*, 0.96 for *L. iners*, 0.96 for *L. jensenii*, 0.96 for *L. gasseri*, and 0.85 for *G. vaginalis*.

Species-Specific qPCR Standard Curve Generation

Bacteria were cultured overnight in liquid media (see above) then diluted 1:10 into fresh media and incubated for an additional 3–12 h to minimize the amount of dead bacteria. The subcultures were serially diluted 10-fold 5 times in triplicate. Serial dilutions were then plated as described above and used for DNA extraction. Following qPCR, the cycle thresholds (Ct) of the dilutions were plotted against the log of the CFU concentration for each species. Linear fitting provided an equation that was used to predict the CFU concentration in CVS based on the Ct for each species of bacteria.

Calculation of Lactobacilli Fraction

To calculate the fraction of a particular species of *Lactobacillus* using 16S sequencing data, the number of reads assigned to that species was divided by the total number of reads assigned to all *Lactobacilli*. To calculate the predicted fraction of a particular species of *Lactobacillus* using qPCR data, the predicted concentration (CFU/mL) for a particular species was divided by the total predicted CFU/mL for all *Lactobacilli* species.

Statistical Analysis

PERMANOVA statistics were calculated on the Bray-Curtis dissimilarity command using the adonis command of the vegan package in R. Inverse Simpson indices were compared using the student's *t*-test. Correlation analysis and significance for qPCR standard curves and comparisons between specific *Lactobacillus* species representation from 16S rDNA sequencing and qPCR was performed in GraphPad Prism 8.1.0.

RESULTS

Participant Demographics

Participant demographics are shown in **Table 1**. The median age of the participants was 26.5 yrs with a range of ages from 23 to 35 yrs. Two of the 20 (10%) of participants were Hispanic or Latino. The majority of participants were White (12/10, 60%) (**Table 1**).

Notable Findings From Self-Reported Questionnaires

The participants answered questions about their sexual history, history of sexually transmitted infections, vaginal symptoms, and vaginal product use. A summary of key questionnaire data is provided in **Table 1**. Birth control usage was high, with participants reporting None (4/20, 20%), Condoms (2/20, 10%), Oral contraceptives (5/20, 25%), and copper or progestin intrauterine device (IUD) (9/20, 45%). Two participants (10%) reported BV in the past, one of which appeared to have active BV by Amsel's criteria (also noted below) and one who had the appearance of mixed bacteria on wet mount and gram staining. Prior human papilloma virus (HPV) infection was reported by one participant (5%). Prior yeast infection was reported by 12/20 participants (60%). One participant reported prior UTI. Prior chlamydia infection or treatment for potential exposure was reported by 4/20 participants (25%). Prior herpes infection was reported by 1 participant who was also on viral suppressive therapy (also noted below). Self-reporting of vaginal symptoms was rare and did not correlate well with other test results that were criteria for exclusion. For example, vaginal discharge (2/20, 10%) was reported by two participants with *Lactobacillus*-dominated microbiota, and not by the one participant that apparently had active BV. Vaginal product use was also rarely reported and did not correlate with other negative findings. Interestingly, the 1 participant that reported use of boric acid did not report prior incidence of BV, which is the situation where boric acid is most likely to be used. There was a wide range of reported total numbers of sexual partners (0–29). The number of new sexual partners during the past month was low (0–1), and the percentage of participants reporting that their current male partner was circumcised was high (15/20, 75%). All participants in the study reported no tobacco use and no prior pregnancies resulting in birth of a baby. Many of the participants also had some extent of medical records that were accessible to supplement the self-reported questionnaire answers. It was noted that one participant was on testosterone therapy, presumably as part of gender affirming therapy.

Notable Findings From Testing

Blood Tests

As expected, a significant proportion (50%, 10/20) of the participants were CMV positive. Thus, while stool donors are typically not tested for CMV (Woodworth et al., 2017), we intend to ensure that CVS from CMV positive donors is used only in CMV positive recipients. Additionally, we found that 5/20 (25%) were positive for HSV-1 IgG, and two of these participants noted having oral cold sores in the past on follow-up. Out of an abundance of caution, we suggest that this be an exclusion criterion for potential VMT donors. Two participants out of 20 (10%) were HSV-2 IgG positive, where one was unaware of any past symptoms of infection and tested negative on the swab-based herpes NAT, and the other was on viral suppressive therapy. As expected, all participants (20/20, 100%) had rubella IgG and varicella zoster virus (VZV) IgG present due to past vaccination or exposure. Further, all participants tested were negative for rubella and VZV IgM, indicating there was no active infection. Likewise, 16/20 (80%) of participants were reactive for hepatitis A (HAV) IgG, but all participants tested were negative for HAV IgM. All participants (20/20) tested negative for current hepatitis B (HBV) and C (HCV) infections. One participant's bloodwork was indicative of microcytic anemia, which was consistent with a family history of thalassemia reported to the study team doctor on follow-up. While thassalemia does not have known impact on immunity or vaginal microbiota, for early investigations of VMT, a participant with thassalemia may be excluded under investigator discretion.

Swab Tests

We observed that there was not necessarily concordance between self-reporting of past yeast infection (12/20, 60%) and the presence of culturable yeast from a vaginal swab (6/20, 30%).

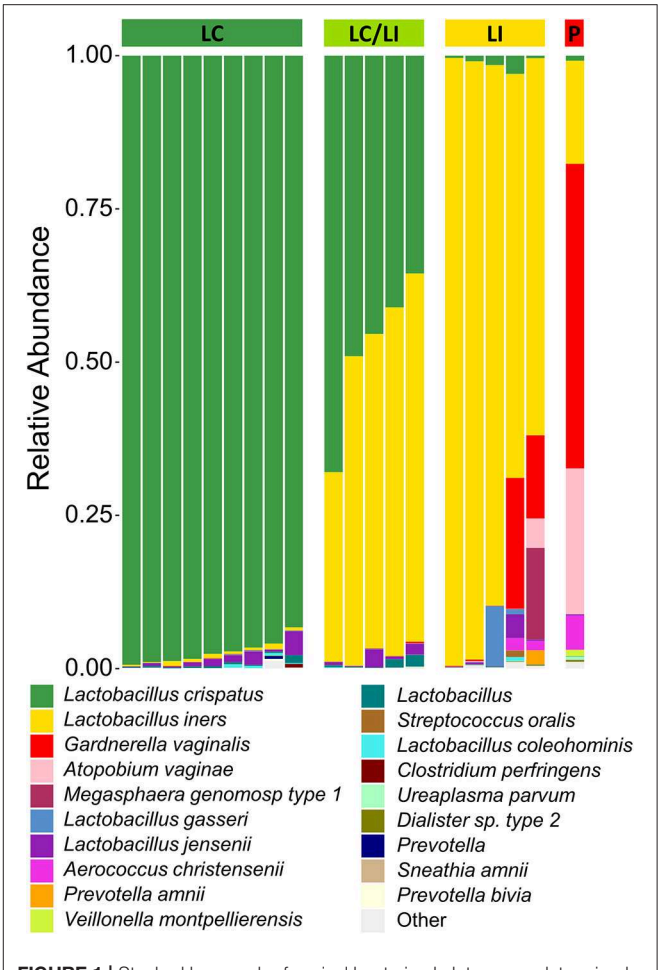

FIGURE 1 | Stacked bar graph of vaginal bacteria phylotypes as determined by 16S rDNA sequencing of CVS. Samples are organized according to community state types (CSTs) as indicated by the colored bar on top of the graph. From left to right, green = *L. crispatus* (LC); yellow-green = *L. iners*/*L. crispatus* mix (LI/LC); yellow = *L. iners* (LI); red = polymicrobial (P). Each column represents an individual sample ($n = 20$ total). The height of each color indicates the relative abundance of a specific bacterial phylotype, as indicated in the legend. The top 19 abundant phylotypes are included, with all additional phylotypes summed and labeled as "Other".

Three of the participants that had culturable yeast did not report prior yeast infection, and 9 of the participants that reported past yeast infection did not have yeast reported after fungal culture. Regardless, all six participants with cultivable yeast (typically *Candida albicans*) did not have any symptoms of a yeast infection on follow-up. Bacteria species not considered to part of the normal urogenital flora were detected in 4/20 (20%) of participants. Two of these participants had culturable *Staphylococcus* bacteria but reported no symptoms of active infection on follow-up. One of the participants had light growth of numerous bacteria species including *Escherichia coli* and *Bacteroides* species, but reported that she dropped the swab on the floor when the study team doctor contacted for follow-up. One of the participants had moderate growth of *Corynebacterium* species on culture, which are typically innocuous. However, this participant was also the only participant (1/20, 5%) to test positive for *Mycoplasma genitalium*, and was instructed to see her physician on follow-up. All participants (20/20) tested negative for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*. One 1/20 participants (5%) tested negative for all strains of HPV, though another 6/20 (30%) had only "weak" or "very weak" positives that may warrant additional confirmatory testing. All participants (20/20, 100%) were negative for HPV16 and 18, the strains responsible for most HPV-related cancers. Given the intermittent nature of viral shedding, we suggest that vaginal swabs be collected with every donor CVS sample to test for HPV and herpes viruses.

Urine Tests

All participants tested negative for pregnancy, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae*.

CVS

One participant did not report any history of BV, but the CVS sample characteristics were indicative of BV by fulfilling the 3 Amsel's criteria tested (positive whiff test, pH 4.62, visible clue cells/biofilm in wet mount). The presence of a polymicrobial bacteria community was confirmed by 16S rDNA sequencing (see below) and Nugent score (8–10). One participant had no notable exclusion criteria based on the questionnaire and screening tests, but the CVS itself appeared ovulatory (egg-white appearance, spinnbarkeit).

Microbiota Communities Were Consistent Between Swabs and CVS

There have been differing reports as to whether the sampling location and sampling method affects the composition of the vaginal microbiota (Kim et al., 2009; Virtanen et al., 2017). The Softdisc method described here collects material from the entirety of the cervicovaginal canal, which is then pooled together during centrifugation. Thus, we used 16S rDNA sequencing to characterize the vaginal microbiota communities for paired CVS and vaginal swab samples to determine whether the results would be similar. As shown in **Figure 1**, we found that 19/20 (95%) of the participants had CVS dominated by *Lactobacillus* spp., which was consistent with testing negative for BV based on the three Amsel's criteria characterized. CVS samples clustered into four distinct community state types (CSTs), three of which were predominated by *Lactobacillus spp.* (*L. crispatus*, *L. iners*, or a *L. crispatus*/*L. iners* mix). When comparing sequencing of DNA extracted from CVS samples and vaginal swabs, paired CVS samples and vaginal swabs were not discernible from each other using NMDS analysis (**Figure 2A**) and applying PERMANOVA did not reveal a significant difference between the distribution of the CVS samples or vaginal swabs ($p = 0.986$). The paired samples from each participant clustered into concordant CSTs and did not show a significant difference in diversity, as measured by the inverse Simpson Index ($p = 0.7138$) (**Figure 2B**).

Standardized qPCR Correlates With the Relative Abundance of Specific Bacterial Species

Because 16S rDNA gene sequencing is a high-throughput approach, samples are typically batched together in large numbers. For the purpose of screening CVS samples to rapidly determine individual donor and sample eligibility, we sought to determine whether qPCR could be used to provide rapid compositional information that was reflective of sequencing. Using species-specific primers for *L. crispatus*, *L. jensenii*, *L. iners*, *L. gasseri,* and *G. vaginalis*, we performed 5 qPCR reactions on DNA extracted from each CVS sample. By comparing the qPCR results with the relative abundance obtained from the 16S sequencing, we determined that in the case of *G. vaginalis*, a Ct cutoff of 20 readily distinguished three samples that had >10% relative abundance (red bars in the last three columns in **Figure 1**) from samples with low relative abundance of *G. vaginalis* (**Figure 3A**). Furthermore, Ct < 20 reliably predicted dominance by Lactobacillus species (**Figure 3A**, **Supplementary Figure 1A**), though not necessarily the relative abundance of each species in mixed samples. Therefore, to allow rapid predictive CST classification before 16S rDNA sequencing, we generated qPCR standard curves from laboratory strains of *L. crispatus*, *L. jensenii, L. iners*, and *L. gasseri* to relate Ct values with the concentration of colony forming units (CFU) per volume across four logs in concentration in serial dilutions. Strong linearity was observed in all four species over four logs in bacteria concentration (insets in **Figure 3B**, **Supplementary Figure 1B**). The comparison of the predicted fractional representation based on qPCR and the fractional representation based on the 16S rDNA sequencing is shown for the dominant species (*L. crispatus*, *L. iners*) in **Figure 3B** and for the minor species (*L. jensenii*, *L. gasseri*) in **Supplementary Figure 1B**. In all four cases, we observed strong correlations between the predicted relative abundance based on qPCR and the relative abundance based on 16S rDNA sequencing.

CVS Physicochemical Properties Correlate With the Presence of Lactobacillus

The protective role of the lactic acid and low pH of the vagina appears to be important for maintaining vaginal health (Olmsted et al., 2005; Aldunate et al., 2013; Hearps et al., 2017; Tachedjian et al., 2017), suggesting that the physicochemical characteristics of the CVS may be more important than the particular species

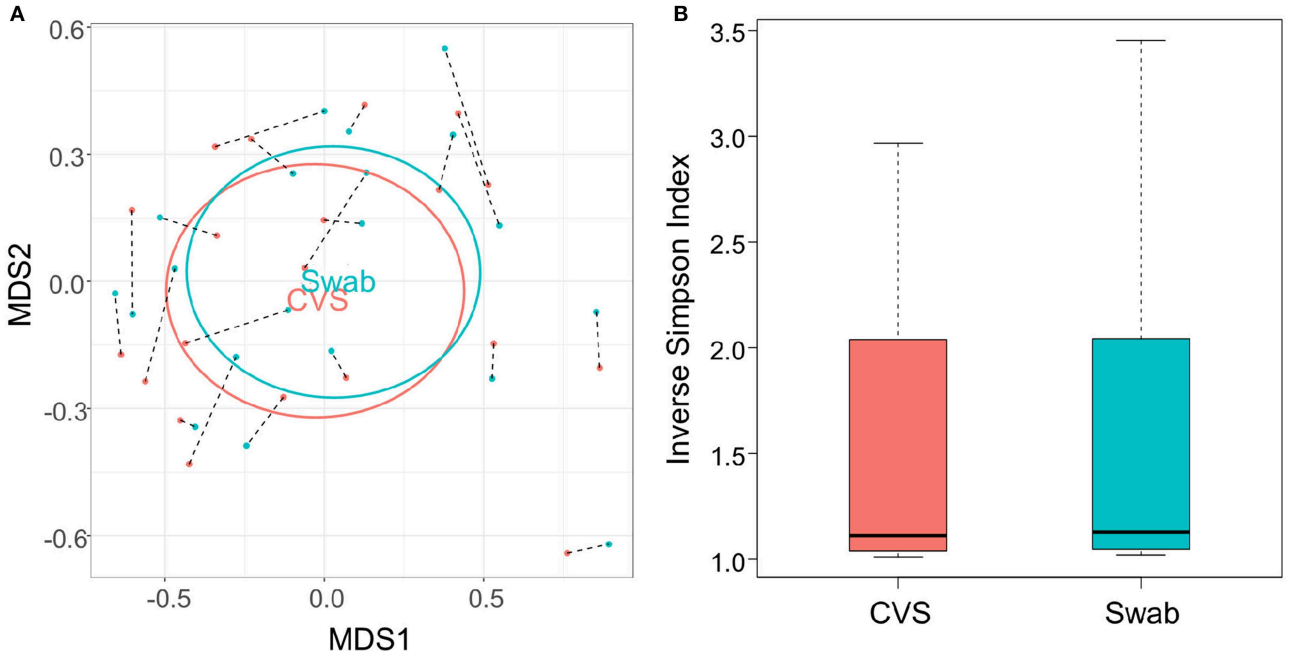

FIGURE 2 | (A) NMDS plot of the Bray-Curtis dissimilarity matrix of CVS (pink) and swab (blue) samples. Black dashed lines connect each CVS sample with the swab obtained from the same donor. "CVS" and "Swab" are positioned on the centroids of the CVS and swab points, respectively, with standard deviations indicated by the ellipses (not significantly different, $p = 0.986$). **(B)** Boxplot of Inverse Simpson Indices calculated for CVS and swab samples (not significantly different, $p = 0.7138$).

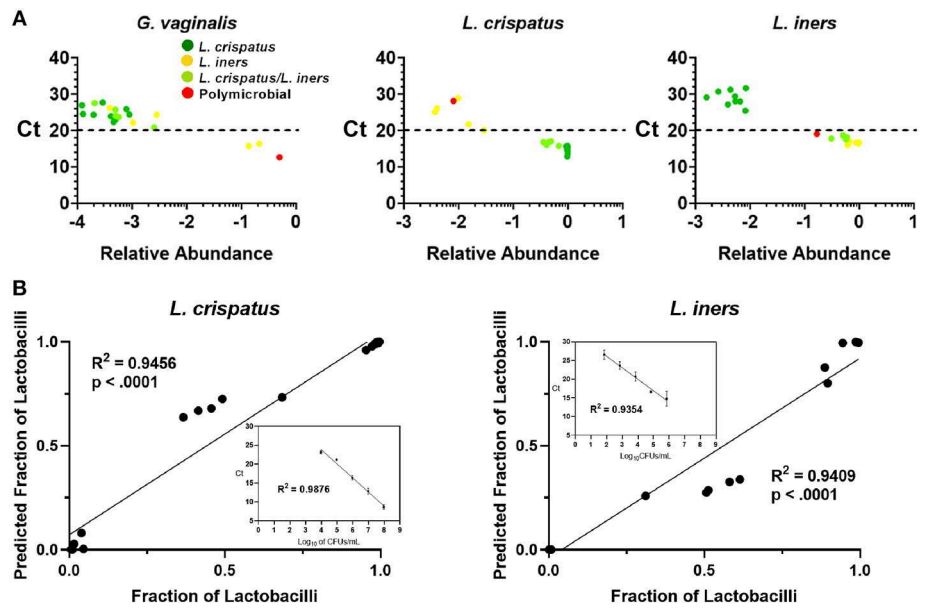

FIGURE 3 | (A) Individual CVS samples according to their relative species abundance, as obtained from 16S rDNA sequencing, and their Ct determined by qPCR using species specific primers for *G. vaginalis*, *L. crispatus*, and *L. iners*. Individual data points are color coded for each group based on sequencing. Dashed lines indicate Ct = 20, our suggested threshold. **(B)** Individual CVS samples according to the predicted fraction of *L. crispatus* or *L. iners* relative to the combined lactobacilli concentrations determined by qPCR compared to that obtained from the 16S rDNA sequencing. Insets show the standard curve used to estimate the concentration of the indicated species and calculate the predicted fraction by qPCR.

present. As shown in **Figure 4A**, dominance by *L. crispatus* is generally associated with lower pH. In comparison, samples dominated by *L. iners* or a mixture of *L. crispatus* and *L. iners* had a slightly higher average pH, though still below the clinical cutoff for BV (>4.5, gray dotted line). Indeed, only the one sample that was polymicrobial had a pH above 4.5 (**Figure 4A**). We also

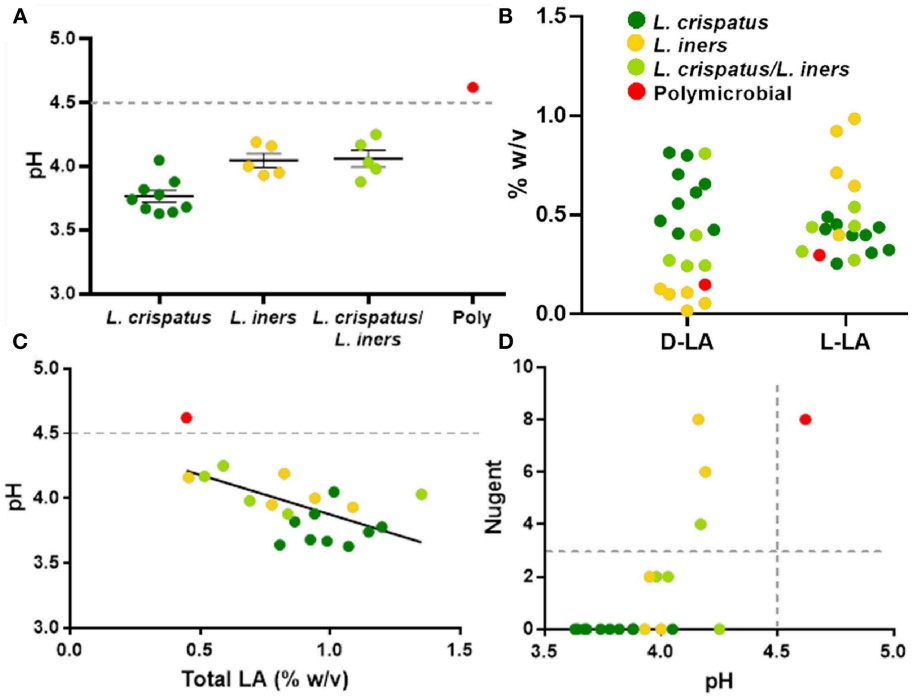

FIGURE 4 | (A) Individual CVS sample pH grouped based on 16S rDNA sequencing. The group mean ± SEM is shown. The gray dotted line corresponds to pH 4.5, which is the clinical cutoff for BV according to Amsel's criteria. **(B)** Concentrations of D- and L-isomers of lactic acid (LA) in CVS. Individual data points are color coded for each group based on sequencing. **(C)** CVS pH as a function of total (D + L) LA content. The gray dotted line corresponds to pH 4.5, which is the clinical cutoff for BV according to Amsel's criteria. Individual data points are color coded for each group based on sequencing. Linear regression line shown, $r^2 = 0.37$, $p = 0.004$. **(D)** Nugent score as a function of pH. Individual data points are color coded for each group based on sequencing. The gray dotted lines correspond to the threshold for Nugent score considered negative for BV (≤3) and the clinical cutoff for BV according to Amsel's criteria (pH 4.5).

found the relative concentrations of the D- and L- isomers of lactic acid (D-LA, L-LA) were reflective of the bacteria present in the CVS sample. Namely, there were relatively higher levels of D-LA compared to L-LA in CVS samples dominated by *L. crispatus*, where the opposite was true in samples dominated by *L. iners* (**Figure 4B**). For the samples that were a mixture of *L. iners* and *L. crispatus*, the concentrations for D-LA and L-LA were more similar, with the exception of one sample that had higher D-LA concentrations (**Figure 4B**), which may reflect the metabolic contributions of both species. The total concentration of lactic acid (D + L) generally increased with decreasing pH, meaning that *L. crispatus*-dominated samples typically had higher total LA concentrations, though there also were *L. iners*-dominated samples and *L. crispatus*/*L. iners* mix samples within the cluster (**Figure 4C**). We further found that the ability of the CVS to adhesively trap fluorescently labeled HIV-1 virions correlated with the *Lactobacillus* spp. present. *L. crispatus*-dominated CVS consistently trapped virions (**Supplementary Figure 2A**), supporting the mucosal barrier function to pathogens. However, virion mobility was more variable in *L. iners* and *L. iners*/*L. crispatus* mix CVS samples, where some samples trapped virions and other samples allowed for permissive diffusion of virions (**Supplementary Figure 2A**). Generally, virion mobility increased with increasing CVS pH, with the transition being relatively sharp around pH 4.2 (**Supplementary Figure 2B**). Thus, as pH is relatively easy to measure in real time and is an indicator of dominance by *Lactobacillus* spp. and the amount of lactic acid produced, we suggest a pH cutoff of ≤4.2 for use in VMT. Similarly, we found that a cutoff pH of ≤4.2 also correlated well with scoring negative for BV by Nugent (≤3) (**Figure 4D**). A few samples that were *Lactobacillus*-dominated by sequencing and pH < 4.5 had high Nugent scores in the intermediate (4–6) or BV (7–8) range. Two of these samples were dominated by *L. iners* but had significant amounts of *Gardnerella vaginalis* (Nugent scores 6 and 8). Based on these data, we suggest that Nugent score ≤2 is sufficient to identify *Lactobacillus*-dominated samples with high lactic acid content and low pH suitable for VMT.

Potential Eligibility Findings

The full lists of clinical and laboratory tests performed can be found in **Supplementary Tables 1, 2**, respectively. FDA testing requirements indicate that a potential donor of leukocyte-rich cells or tissues that is not sexually intimate with the recipient must be tested for HIV (types 1 and 2), HBV, HCV, *Treponema pallidum*; HTLV (types 1 and 2), CMV, *Chlamydia trachomatis*, and *Neisseria gonorrhea*. We propose that for VMT, a potential donor candidate should also be tested for HAV, HSV-1/2, VZV, EBV, rubella virus, *Toxoplasma gondii,* HPV, *Trichomonas vaginalis*, and *Mycoplasma genitalium*, cultivable yeast/fungi, and cultivable bacteria. The test list employed here included

redundant serological testing and vaginal swab-based testing of herpes viruses (HSV-1/2, VZV). All active infections should be excluded. Evidence of past exposure to CMV, VZV, and rubella (including vaccination) should be matched between donors and recipients. We suggest that the evidence of past exposure to HSV-1 or HSV-2 should be an exclusion criterion. The presence of cultivable yeast in the absence of symptoms of an active yeast infection and/or evidence of yeast growth in the wet mount preparation need not be an exclusion criterion unless the participant indicates having had a history of yeast infections (>1 in the past). We suggest similar guidelines that only UTI occurring on more than 1 occasion be grounds for exclusion. The HCT/P guidelines stipulate chlamydia infection is only an exclusion if it has occurred in the prior 12 months, though we suggest that infections occurring >12 months ago should be considered along with other history and could potentially be grounds for exclusion at investigator discretion upon review. The presence of cultivable bacteria that are not considered "normal" urogenital flora (terminology used by the pathology laboratory) would result in exclusion. We suggest that self-reported product use need not be an exclusion criteria alone, but that donor participants should be instructed to avoid insertion of any vaginal products (including tampons) during the entire CVS collection period. Our results suggest that there is not a clear rationale for setting a maximum limit for number of sexual partners for inclusion as a CVS donor. However, we suggest that donor participants should be required to avoid participating in vaginal, rectal, or oral intercourse, as well as use of sex toys, digital penetration, etc. during the entire CVS collection period. As the effects of exogenous hormones on the vaginal microbiota are not well-studied, we determined that this would be an exclusion criteria for participating as a VMT donor. Further, while the procedures for participation as a donor do not likely pose any risk to pregnant women, we propose that the potential impacts of hormones and other factors on the vaginal microbiome during pregnancy be grounds for exclusion. Ovulatory CVS or CVS collected at the time of menses would not be ideal for transplant due to low bacteria density and elevated pH, but would not exclude a donor from providing additional CVS samples in the future.

For the purposes of screening a potential donor's vaginal microbiota composition, our results suggest that qPCR can be used to perform rapid characterization of the relative abundances of the four common *Lactobacillus* species that is predictive of the relative abundance by 16S rDNA sequencing. We prefer the use of CVS isolated with the menstrual cup collection approach to a vaginal swab for this purpose, because with isolated CVS, sample mass can be standardized. In contrast, the amount of CVS collected with a vaginal swab is not known or easily standardized, which would affect the threshold Ct value between samples. Of course, use of this qPCR approach would require individual labs to generate standard curves using reference bacteria and standardization using individual instrumentation and reagents. Using our qPCR instrument and a standard mass of 10 mg CVS, we found that Ct < 20 (>10% relative abundance by 16S sequencing) for *G. vaginalis* should be an exclusion criterion. Similarly, dominance by *Lactobacillus* species was predicted by Ct < 20. Together with Nugent scoring, pH measurement, and lactic acid measurements, dominance by *Lactobacillus* species can be quickly confirmed in individual CVS samples. Further, all samples should still be characterized by confirmatory 16S rDNA sequencing, which is standard in the field. Also, we would suggest that a standard plating method and counting of CFUs should be used to determine the overall *Lactobacillus* "dose." While the HIV mobility assay is informative and correlative with other CVS physicochemical properties, the equipment and materials required are specialized and need not be considered a standard screening approach.

Based on the test results and exclusion criteria discussed above, we found that 7/20 (35%) of our participants may be eligible for CVS donation as part of a future VMT study. However, we anticipate that the actual success rate for participation as a CVS donor in a clinical trial will be much lower than 35% for several reasons. This small pilot study used an abbreviated screening questionnaire, as described in the Materials and Methods. The participants were selected from a pool of participants from the study team's prior clinical studies, increasing the likelihood that they would have *Lactobacillus*-dominated vaginal microbiota and fulfill the donor criteria. Of note, the majority of the participants were White or Asian, which is consistent with the observation that White and Asian women in the U.S. are less likely to have BV (Allsworth and Peipert, 2007; Fettweis et al., 2014; Peebles et al., 2019). However, efforts should be made to recruit a racially/ethnically diverse donor pool, as the potential impact of race/ethnicity on the success of VMT is unknown. Additionally, the more stringent cut-off values suggested based on the participants in this study (Nugent ≤ 2, pH ≤ 4.2) may need to be adjusted for a larger and more racially/ethically and/or geographically diverse donor pool. Further, the "universal" donor approach would typically consist of an initial full blood, swab, and urine screening like that described here, followed by a 30–60 day period of sequential CVS sample collections (excluding menses and ovulation if not using hormonal contraceptives). Out of an abundance of caution, we propose that donors be willing to abstain from vaginal intercourse and vaginal product use during the full duration of CVS collection, which was not an eligibility criterion for this small pilot study. Although not performed here, we also suggest that every donor CVS sample be tested for the presence of semen with a test like the ABAcard P30 test for the forensic identification of semen. We further propose that due to the intermittent nature of viral shedding (Wald et al., 1995; Phipps et al., 2011; Gravitt and Winer, 2017), vaginal swabs be collected to test for herpes viruses and HPV at the time of every CVS sample. After collection of the last CVS sample, the donor should go through the full blood, swab, and urine testing procedure again after a quarantine period of ∼30 days. It is likely that this series of additional testing will result in additional screening failures.

DISCUSSION

Although the first modern literature report of fecal microbiota transplant (FMT) was documented in 1958 (Eiseman et al., 1958), it was not until 2013 that the first randomized clinical

trial for treating recurrent *C. difficile* infection with FMT was reported (van Nood et al., 2013). Although the study did not involve blinding, it was considered to unequivocally represent the potential of FMT as a safe and effective treatment (Sampath et al., 2013). The prior year, the FMT process was greatly streamlined by demonstrating that equivalent efficacy could be achieved with (i) stool from standard volunteer donors rather than donors identified by the patient, and (ii) stool frozen prior to use (Hamilton et al., 2012). These findings paved the way for various stool banks around the country that screen potential donors and collect, process, and freeze stool for distribution to clinical providers. OpenBiome, a non-profit that reportedly shipped over 43,000 treatments since starting its service in 2013, was an early innovator in developing thorough screening and quarantining procedures for frozen, ready-to-use stool preparations to increase patient access and safety[1]. The development of the FMT field is an obvious source of inspiration for initiating study of other forms of microbiota transplantation, such as VMT. Thus, our goal here was to conceptualize a universal VMT donor screening process that is uniquely suited to what we understand about vaginal microbiota and sexually transmitted infections. For example, unique considerations for VMT donor participants include the number of sexual partners, practice of certain sexual behaviors and frequency, and use of vaginal products. Further, any history of sexually transmitted infections should be grounds for exclusion. The stringency of the universal donor screening process inherently limits the availability of eligible donor participants, which is why OpenBiome reports only 3% of their participants screened are deemed eligible for stool donation (Dubois et al., 2015)[1]. It is possible that if VMT were to be successful, a similar model for donor screening with CVS banking and distribution could be implemented. Further, the development of a rationally designed biotherapeutic product using clinical outcome data from VMT studies would be a logical next step, both from a safety perspective as well as the much larger potential patient population. The trajectory of the FMT field is similarly informative in this regard, and will aid in accelerating the development of other microbiota-based therapeutic strategies.

Much of what we understand about the dynamics of vaginal microbiota was learned in studies that employed vaginal swabs, self-collected or physician collected, as the means for isolating bacteria (Aagaard et al., 2012; Gajer et al., 2012; DiGiulio et al., 2015). Other studies have reported the physicochemical properties of undiluted CVS or cervicovaginal lavage fluids, which gives us added indirect measures of bacteria composition and some aspects of metabolic function (Lai et al., 2009a,b, 2010; O'Hanlon et al., 2011; Chappell et al., 2014; Nunn et al., 2015). However, we and others have begun characterizing both the CVS and the composition of the vaginal microbiota to begin to correlate community structure and function (Nunn et al., 2015; Hoang et al., submitted). Here, we further demonstrate that the community structure is strikingly similar whether using a vaginal swab or an aliquot of the CVS as the biological matrix for DNA extraction, which supports the use of vaginal swabs as a reliable and valid representation of the native mucosal environment. Similar to what was described for *Lactobacillus* bacteria in culture, we found that the relative concentrations of D- and L-lactic acid were reflective of the *Lactobacillus* spp. present in the CVS (Witkin et al., 2013). We also found that *L. crispatus*-dominated CVS tended to have higher total lactic acid content, lower pH, and more effective immobilization of fluorescently-labeled HIV virions, which is consistent with prior observations made by our group and others (Nunn et al., 2015; O'Hanlon et al., 2019). Our data shown here also suggested that using a CVS pH cut-off of $\leq$4.2 and a Nugent score cut-off of $\leq$2 would be most suitable for VMT, which is slightly more stringent than the criteria defined for categorizing samples as negative for BV (Amsel et al., 1983; Nugent et al., 1991).

The procedures and data shown herein are not intended to be wholly inclusive of every consideration for screening potential donors for VMT. Further, to operate under an investigational new drug application (IND), there are additional considerations for Chemistry, Manufacturing, and Controls (CMC). In the case of stool transplants, which were known to be efficacious for treating recurrent *C. difficile* prior to regulation by the FDA, we have yet to identify relevant measures of potency. In contrast to a small molecule drug or biologic, we do not know the key "active" component in stool, and thus cannot quantify the concentration or activity of that component. Further, the clinical success of using processed stool with 10% glycerol added prior to freezing (a standard approach for freezing bacteria stocks) was reported without characterization of the stool before and after freezing (Hamilton et al., 2012; Satokari et al., 2015). Many studies since have looked at the effect of processing and storage on the stability of particular bacteria or the overall bacteria community (Costello et al., 2015; Fouhy et al., 2015), though there is still not a general consensus on what is important for a successful clinical outcome. In the case of VMT, donor CVS is largely considered a monoculture of one *Lactobacillus* species, and thus, lends itself to performing characterization of the colony forming units (CFU) per unit volume before and after freezing. We can also determine the effect of cryoprotectants and other media on the *Lactobacillus* viability, which is an area of active study in our group. Of note, however, although we can directly characterize the "potency" (concentration in CFU/mg) of the *Lactobacillus*, it is also possible that the lactic acid, mucins, or other components of the host environment could play a role in the potential success of the transplantation process. While vaginal microbiota can be considered relatively monomicrobial, there is also evidence here and described by others that more than one species of *Lactobacillus*, such as *L. crispatus* and *L. iners*, can cohabitate (Ravel et al., 2011; Gajer et al., 2012). Further, although other species may be in the minority, it has yet to be determined whether the overall community structure with major and minor players is important. We anticipate that the trajectory of VMT will likely follow that of FMT, wherein there are many parallel efforts in academia and industry to determine what minimum cultivable components can be manufactured to produce uniform, standardized products with similar therapeutic efficacy as stool (Kelly et al., 2015; Hoffmann et al., 2017; Ott et al., 2017).

[1]OpenBiome (2019). Available online at: https://www.openbiome.org/impact (accessed March 09, 2019).

In addition to intensive and thorough screening of potential donor participants for VMT, it is also important to consider testing of potential recipient participants. An inherent risk of the VMT procedure is transmission of a sexually transmitted pathogen. For various reasons associated with participant safety and for determining the overall safety of the VMT procedure, the baseline infection status of the potential recipient should be known. Thus, potential recipients should be screened similarly to potential donor participants, but the inclusion and exclusion criteria would differ. In addition to the obvious consideration that BV would be an exclusion for donors but not for recipients, it is also feasible that a controlled herpes infection or HPV would not necessarily be an exclusion criterion for potential recipient participants. Because of the transient and intermittent nature of viral shedding, potential recipient participants would ideally be screened at multiple time points prior to undergoing the VMT procedure to maximize detection of asymptomatic viral shedding of HSV-1, HSV-2, and HPV. Further, samples for testing should be taken immediately prior to the VMT procedure, because the results will help interpretation of any notable findings that may occur during recipient follow-up, including whether they may be attributable to the donor CVS. As the field of VMT is in its infancy, ensuring safety and tolerability of the procedure is of paramount importance in the first clinical studies. Various standardized adverse event grading systems are available, such as the *Division of AIDS (DAIDS) Female Genital Grading Table for Use in Microbicide Studies* for local vaginal/reproductive tract adverse events. Further, follow-up testing of recipient participants can also determine whether a potential shift in vaginal microbiota composition impacts future risk of acquiring sexually transmitted infections. It is also conceivable that as VMT may be explored for other indications, additional follow-up considerations may be timing of childbirth and birth outcomes, or recurrence of conditions such as urinary tract infection or yeast infection.

Another important aspect to consider is the VMT procedure itself. As we describe, the donor CVS was collected using a non-absorbent polymer-based cup, allowing for collection of undiluted material by centrifugation. However, similar to FMT, dilution with fluid such as sterile saline would ease loading into a device for application and administration to the recipient. Thus, we anticipate that similar to FMT, dosing would be determined by the ratio of sample mass to diluent fluid volume. This is in contrast to the dosing approach for probiotic products, where the concentration of CFU/dose can be standardized during the manufacturing procedure—with CVS donation, the mucus itself may be as important as the bacterial colony count. However, in the case of CVS, we observe a wide range of CFU/mg between participants, which is also reflective of the functional differences of the dominant bacteria. As such, when working with CVS from individual donors, it is not straightforward to standardize the dose based on the concentration of CFU. This is perhaps particularly true for *Lactobacillus* species found in CVS, because when they are the predominant species, they are present in lower numbers of CFU than the total number of CFU when the community is polymicrobial (Hill, 1993). Indeed, the ability of lactobacilli to colonize and compete out other bacterial species in CVS does not appear to be directly dependent on their concentration.

In the discussion of how microbiota transplantation must be regulated differently than standard drugs and biologics, it is worth noting that VMT and vaginal microbiota transfer from mother to babies born by Cesarean section, or "vaginal seeding," uses the same "drug." However, the nature of the screening procedures and the handling of the CVS are quite different, and thus, VMT has many more parallels to FMT than vaginal seeding. For example, the notion of universal donors cannot apply to vaginal seeding, as the donor must be the mother. Thus, screening failure rules out the possibility of conducting the procedure at all. Further, because of the relevant time constraints, the vaginal seeding procedure also rules out the possibility of performing thorough testing of the CVS at the time of collection (1 h before Cesarean section) and quarantining in the frozen state until test results are known. The CVS collection procedure for vaginal seeding is also quite different and requires no subsequent processing; absorbent gauze or swabs are used to "soak up" the CVS for direct transfer to the baby's skin after birth. Thus, the definition of the "dose" is also different, in that a single dose would just be the amount of CVS that is collected in the gauze while it is left in the mother's vagina. Finally, the use of the term "transfer" rather than "transplant" reflects the fact that, by definition, transplanted material is placed in the same area of the body from which it was obtained. In the case of vaginal seeding, the CVS is swabbed onto the baby's mouth, nose, and skin in order to mimic the process of passing through the vaginal canal during birth. In contrast, VMT is a true transplant of CVS from the vagina of the donor to the vagina of the recipient.

CONCLUSION

This project represents a multidisciplinary effort to establish a protocol for comprehensive screening and characterization of donor CVS for the purpose of VMT, including a parallel rigorous screening process for potential recipients of VMT. The limitations of this pilot study include the small sample size and selection of participants from past study participant pools known to the study team to have optimal microbiota, which would not be generalizable to larger populations. It is possible that the requirement for sexual abstinence throughout the CVS sample collection period, or the requirement for providing multiple sequential self-collected swabs and CVS samples intensively over a month-long period, may constrain enrollment in future studies. The stringency of our exclusion criteria for donors, motivated by the ethical and clinical imperative to avoid potential transmission of pathogens, is both necessary and challenging from the perspective of recruitment. However, once a donor has been identified, using this protocol, as a safe donor, she could ideally donate CVS on multiple appropriately screened occasions; the idea of a "super-donor" with no identified past or current infections and with favorable *Lactobacillus*-dominated microbiota is one that should be explored and is of potential high impact to the project

and the field. It is certain that as the field of microbiota transplant expands, unique regulatory questions and dynamic scientific, ethical, and clinical considerations will continue to arise (Hoffmann et al., 2017). These should be met with equally dynamic innovative approaches that incorporate optimized human safety considerations while recognizing the potential value of microbiota transplantation in addressing BV and its varied adverse impacts on human health.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher. As of submission, the raw sequencing data is not publicly available, but public links or the data itself will be supplied on request.

AUTHOR CONTRIBUTIONS

LE, EW, CG, PG, EF, CH, RC, and JC contributed conception and design of the study. LE wrote the first draft of the manuscript and KD, SB, and EW wrote sections of the manuscript. KD, SB, FZ, HZ, TH, LE, CG, and PG conducted experiments, oversaw staff testing of clinical specimens, and/or collected clinical specimens. EW, EF, and CH oversaw the clinical study and coordinated clinical activities. KD, SB, FZ, HZ, TH, PG, EW, AA, and LE performed data analysis and/or data interpretation. All authors contributed to the manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00306/full#supplementary-material

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Distinct Immune Responses Elicited From Cervicovaginal Epithelial Cells by Lactic Acid and Short Chain Fatty Acids Associated With Optimal and Non-optimal Vaginal Microbiota

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Non-optimal vaginal microbiota, as observed in bacterial vaginosis (BV), is typically characterized by a depletion of beneficial lactobacilli and an abundance of numerous anaerobes. These non-optimal conditions are associated with subclinical cervicovaginal inflammation and an increased risk of HIV infection compared to women colonized with optimal vaginal microbiota dominated by lactobacilli. Lactic acid (LA) is a major organic acid metabolite produced by vaginal lactobacilli that elicits anti-inflammatory effects from cervicovaginal epithelial cells and is dramatically depleted during BV. However, it is unclear if LA retains its anti-inflammatory activity in the presence of vaginal microbiota metabolites comprising short chain fatty acids (SCFAs) and succinic acid, which are also produced by an optimal vaginal microbiota. Furthermore, the immunomodulatory effect of SCFAs and succinic acid on cervicovaginal epithelial cells at higher concentrations present during BV is unknown. Here we report that in the presence of physiologically relevant concentrations of SCFAs and succinic acid at pH 3.9 (as found in women with lactobacillus-dominated microbiota) LA induced an anti-inflammatory state in cervicovaginal epithelial cells and inhibited inflammation elicited by the toll-like receptor (TLR) agonists polyinosinic:polycytidylic acid and Pam3CSK4. When cervicovaginal epithelial cells were treated with a vaginal microbiota metabolite mixture representative of BV, containing a lower concentration of LA but higher concentrations of SCFA/succinic acid at pH 7, no anti-inflammatory was observed. Rather, the vaginal microbiota metabolite mixture representative of BV dysregulated the immune response of cervicovaginal epithelial cells during prolonged and sustained treatments. This was evidenced by increased basal and TLR-induced production of pro-inflammatory cytokines including tumor necrosis factor-α, but decreased basal production of chemokines including RANTES and IP-10. Further characterization of individual components of the BV vaginal microbiota mixture suggested that acetic acid is an important vaginal microbiota metabolite capable of eliciting diverse

immunomodulatory effects on a range of cervicovaginal epithelial cell targets. These findings indicate that elevated levels of SCFAs are a potential source of cervicovaginal inflammation in women experiencing BV, and support the unique anti-inflammatory properties of LA on cervicovaginal epithelial cells as well as a role for LA or LA-producing lactobacilli to reverse genital inflammation associated with increased HIV risk.

Keywords: vaginal microbiota metabolites, lactic acid, bacterial vaginosis, inflammation, short chain fatty acids, succinic acid, cervicovaginal epithelium

INTRODUCTION

The lower female reproductive tract epithelium plays important roles as a physical and immunological barrier against infection (Anderson et al., 2014); however its function is influenced by many factors including the effects of commensal vaginal microbiota and their metabolic products (O'Hanlon et al., 2011, 2013; Ravel et al., 2011; Aldunate et al., 2013; Anderson et al., 2014; Hearps et al., 2017; Tachedjian et al., 2017). The lower female reproductive tract microbiota is dynamic (Gajer et al., 2012) and can vary considerably between women of different ethnicities and geographical locations (Ravel et al., 2011; Gajer et al., 2012; Gosmann et al., 2017), although a predominance of beneficial lactobacilli is considered an optimal microbiota that is associated with eubiosis and favorable reproductive and sexual health outcomes (Aldunate et al., 2015; Fettweis et al., 2019; McKinnon et al., 2019).

Lactic acid (LA) is an organic acid metabolite predominately produced by lactobacilli (Boskey et al., 2001) and is present in the lower female reproductive tract at a concentration of ∼110 mM (∼1.0% ± 0.2% w/v) and a pH < 4.5 in women with lactobacillus-dominated microbiota (Boskey et al., 2001; O'Hanlon et al., 2013). There are two isoforms of LA, L-LA, and D-LA, which are produced in different ratios within the lower female reproductive tract by various *Lactobacillus* spp. (Witkin et al., 2013). At a vaginal pH below its pK_a (3.86), LA is predominantly present in its protonated form (Aldunate et al., 2013) which elicits an anti-inflammatory response from cervicovaginal epithelial cells *in vitro* (Hearps et al., 2017). LA has antimicrobial activities against human immunodeficiency virus (HIV) (Aldunate et al., 2013), herpes simplex virus (Conti et al., 2009; Isaacs and Xu, 2013), *Neisseria gonorrhoeae* (Graver and Wade, 2011), *Chlamydia trachomatis* (Gong et al., 2014), and bacteria associated with bacterial vaginosis (BV) (O'Hanlon et al., 2011), and inhibits the infection of epithelial cells with *Chlamydia trachomatis in vitro* (Edwards et al., 2019).

In contrast, the lower female reproductive tract of women colonized with a dysbiotic or non-optimal microbiota, such as in BV, is dominated by anaerobes, a dramatic depletion of beneficial lactobacilli and an elevated vaginal pH > 4.5. This altered microbiota is associated with distinct vaginal concentrations of organic acid metabolites including short chain fatty acids (SCFA) and succinic acid produced by commensal bacteria (reviewed in Aldunate et al., 2015). In addition to LA, which is the predominant metabolite of a lactobacillus-dominated microbiota, acetic and succinic acid, and to a lesser extent propionic and butyric acid, are major microbiota metabolites found within the lower female reproductive tract, particularly during BV where their concentration increases dramatically, up to ∼120 and 20 mM for acetic and succinic acid, respectively (Al-Mushrif et al., 2000; Chaudry et al., 2004; Mirmonsef et al., 2011, 2012b; Gajer et al., 2012).

BV is also associated with subclinical genital inflammation (Eschenbach et al., 1988) and an increased risk of transmitting and contracting HIV (Eschenbach et al., 1988; Atashili et al., 2008; Cohen et al., 2012). Women diagnosed with Nugent-BV, as determined by the Nugent score that enumerates bacterial morphotypes using a Gram stain (McKinnon et al., 2019), are at a 1.53-fold (95% CI = 1.24–1.89) higher risk of acquiring HIV than women without BV (Low et al., 2011), while young South African women colonized with a pro-inflammatory, diverse vaginal microbiota, as determined by next generation sequencing i.e., Molecular-BV (McKinnon et al., 2019), are at a 4.4-fold (95% CI = 1.17–16.61) higher risk of acquiring HIV than women colonized by *L. crispatus*-dominated microbiota (Gosmann et al., 2017). Another study in African women showed that bacterial diversity and specific BV-associated taxa (e.g., *Eggerthella* species type I, *Parvimonas* species types I and 2 and *Megasphaera* spp.) were associated with increased risk of HIV acquisition (McClelland et al., 2018). The pro-inflammatory female reproductive tract milieu associated with non-optimal vaginal microbiota is thought to increase HIV susceptibility by recruiting activated HIV target cells to the genital mucosa, decreasing epithelial integrity and changing the barrier properties of cervicovaginal mucus (Lewis et al., 2013; Borgdorff et al., 2016; Zevin et al., 2016; Gosmann et al., 2017).

Although the mechanisms contributing to the pro-inflammatory cervicovaginal milieu due to non-optimal vaginal microbiota (e.g., BV) have not been completely elucidated, overgrowth of vaginal commensal anaerobic bacteria such as *Gardenerella vaginalis, Prevotella* spp., *Atopobium vaginae,* and *Molibuncus* spp. may expose vaginal epithelial cells to pathogen-associated molecular patterns, stimulating pathogen recognition receptors such as toll-like receptors (TLRs) and eliciting an inflammatory response (Lavelle et al., 2010; Byrne et al., 2015; Gosmann et al., 2017). Organic acid metabolic products from BV-associated bacteria such as SCFAs and succinic acid at concentrations observed during BV (Al-Mushrif et al., 2000; Chaudry et al., 2004; Mirmonsef et al., 2011) elicit a pro-inflammatory response from peripheral blood cells and enhance TLR-associated inflammation (Mirmonsef et al., 2012a); however

the impact of SCFAs on cervicovaginal epithelial cells has not been reported.

We have previously shown that protonated LA at low pH (3.9), present during vaginal eubiosis (O'Hanlon et al., 2013; Tyssen et al., 2018), elicits an anti-inflammatory response and dampens TLR-induced pro-inflammatory responses from cervicovaginal epithelial cells (Hearps et al., 2017). However, it is unknown whether other vaginal microbial metabolic products present in the lower female reproductive tract such as SCFAs and succinic acid modulate this effect, or if they have immunomodulatory effects of their own. Accordingly, we investigated whether L-LA in combination with other vaginal microbiota metabolites, and at a pH relevant to vaginal eubiosis, maintains its anti-inflammatory effects. In addition, we investigated if individual or combinations of SCFAs and succinic acid, at conditions typically observed in BV, modulate the production of cytokines and chemokines relevant to increased HIV risk from cervicovaginal epithelial cells.

MATERIALS AND METHODS

Cervicovaginal Epithelial Cell Culture and Stimulations

Ectocervical Ect1/E6E7 (Ect) and vaginal VK2/E6E7 (VK2) epithelial cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in serum-free keratinocyte medium, supplemented with 0.1 ng /ml human recombinant epithelial growth factor, 0.05 mg/ml bovine pituitary extract (all from Life Technologies, Carlsbad, CA), 0.4 mM $CaCl_2$, 50 U/ml penicillin and 50 μg/ml streptomycin. Primary human cervicovaginal epithelial cells derived from discarded surgical tissue (Ayehunie et al., 2006) were obtained from MatTek (Ashland, MA) and were maintained in media as above but with 10 ng/ml human recombinant epithelial growth factor.

Cervicovaginal epithelial cells (100,000 per well) were seeded into either 6.5 mm diameter polycarbonate transwell inserts containing 0.4 μm pores (Corning, Corning, NY) in 24-well tissue culture plates or into 96-well tissue culture plates and cultured for 7 days to obtain a confluent cell monolayer. Epithelial monolayers were treated with combinations of vaginal microbiota metabolites, comprising L-LA, butyric acid, succinic acid (all from Sigma Aldrich, St. Louis, MO), acetic acid (Merck, Kenilworth, NJ), and propionic acid (MP Biomedicals, Santa Ana, CA). To mimic a lactobacillus-dominant microbiota (defined as eubiosis), cells were treated with media containing the predominate metabolites found during eubiosis and at concentrations and pH representative of this state. We assessed L-LA at 33 mM given our previous work showing that this concentration had significant immunomodulatory effects on cervicovaginal cells but minimal impacts on cell viability (Hearps et al., 2017). Acetic, succinic, propionic, and butyric acids are typically present at low to undetectable levels in vaginal fluid from women with eubiosis (Al-Mushrif et al., 2000; Mirmonsef et al., 2011; O'Hanlon et al., 2013) but can reach levels up to ∼4.6 mM for acetic acid (O'Hanlon et al., 2013) and 0.6 mM for succinic acid (Al-Mushrif et al., 2000) in women with lactobacillus-dominated vaginal microbiota, thus concentrations of 4 mM for acetic and 1 mM for succinic, butyric and propionic acid were assessed as part of the eubiosis mixture. Eubiosis treatment media was adjusted to pH 3.9 to mimic the physiological pH of women with lactobacillus-dominated microbiota (O'Hanlon et al., 2013). During BV, vaginal concentrations of acetic, succinic, propionic and butyric acid, as well as vaginal pH, are substantially increased whilst LA levels are decreased (Spiegel et al., 1983; Al-Mushrif et al., 2000; Mirmonsef et al., 2011; Gajer et al., 2012). To mimic these conditions, cells were treated with media containing L-LA 6 mM (reduced to ∼20% of that in the eubiosis mixture to reflect the approximate 5-fold reduction in LA concentration observed in women with BV), acetic acid 100 mM, succinic acid 20 mM, propionic acid 4 mM, and butyric acid 4 mM at pH 7. These concentrations of SCFAs and succinic acid were selected to reflect those reported in women experiencing BV, where levels of acetic acid can reach up to ∼120 mM (Chaudry et al., 2004; Mirmonsef et al., 2011), succinic acid up to ∼22 mM (Al-Mushrif et al., 2000) and propionic and butyric acids up to ∼4 mM (Al-Mushrif et al., 2000). This BV metabolite mixture was adjusted to pH 7 before adding to cells, to reflect the upper limit of vaginal pH commonly found in women with BV (Brabin et al., 2005; Sánchez-Hernández et al., 2012). To assess the effect of individual vaginal microbiota metabolites in the context of BV, cells were treated with each metabolite alone at the same concentration as in the BV mix. Vaginal microbiota metabolites were added in the absence or presence of the TLR1/2 agonist Pam3CSK4 (Pam, 1 μg/ml) or the TLR3 agonist high-molecular weight polyinosinic:polycytidylic (PIC, 20 μg/ml) (both from InvivoGen, San Diego, CA) to simulate pathogen challenge. The pH stated for each treatment media reflects that of the media added to the apical chamber for experiments conducted in transwells, and of the entire well for experiments conducted in culture plates.

Cells in transwell inserts were stimulated apically with vaginal microbiota metabolite-containing media for 1 h, washed with PBS, fresh media (containing no stimulant) was then added and cells were incubated for a further 18 h. Previous optimization identified the pH of treatment media in the apical chamber was maintained during this 1 h stimulation period (Hearps et al., 2017). For experiments involving prolonged stimulation with BV-relevant SCFA, transwells were not able to maintain the desired conditions for these extended periods due to diffusion between apical and basal compartments, thus cells were treated in tissue culture plates. For these experiments, cells cultured in 96-well plates were stimulated continuously with treatment media for 24 h. Supernatants were collected from the wells or the apical chamber of the transwell inserts and stored at −80°C for subsequent cytokine analysis. Cell viability was assessed following stimulation and supernatant collection using the CellTiter 96 Aqueous One Solution Cell proliferation assay (Promega, Madison, WI) as published (Hearps et al., 2017). Cell viability for all treatments remained above 70% of untreated cells (data not shown and as previously reported for LA) (Hearps et al., 2017) indicating minimal effects on cell viability under the conditions tested.

Cytokine and Chemokine Quantitation

The production of cytokines interleukin (IL)-1 receptor antagonist (IL-1RA), IL-6, tumor necrosis factor α (TNFα), IL-1β, and chemokines IL-8, C-C motif chemokine ligand 5 (RANTES), interferon gamma-induced protein 10 (IP-10), and macrophage inflammatory protein 3 alpha (MIP-3α) in culture supernatant was quantified using ProcartaPlex Multiplex Immunoassays (Affymetrix, Santa Clara, CA). Samples were analyzed on a Luminex 200 (Bio-Rad, Hercules, CA), or MAGPIX system (Merck Millipore, Burlington, MA), and analysis was performed using manufacturer-provided software. For some treatments, values for IP-10 were above the limit of quantification of the assay and were assigned the maximum value of the standard curve.

RESULTS

The Anti-inflammatory Effect of L-LA on Cervicovaginal Epithelial Cells Persists in the Presence of Other Organic Acid Metabolites Relevant to Vaginal Eubiosis

Our previous studies examined the immunomodulatory effect of LA alone on cervicovaginal epithelial cells (Hearps et al., 2017); however LA exists in combination with other organic acid metabolites from female reproductive tract-resident bacteria in the vaginal lumen (Aldunate et al., 2015). Our previous study demonstrated that L- and D-LA elicit similar responses from cervicovaginal epithelial cells with regard to cytokines and chemokines associated with increased risk of HIV transmission in women (Masson et al., 2015; Hearps et al., 2017). Accordingly, we performed experiments using one of the LA isomers (L-LA). To determine if L-LA maintains its anti-inflammatory effect when combined with other organic acid metabolites, cervicovaginal epithelial cells were treated in transwell inserts with a combination of vaginal microbiota metabolites relevant to eubiosis with and without TLR stimulation for 1 h, shown previously to be sufficient for L-LA to elicit its anti-inflammatory effects (Hearps et al., 2017), and cytokine and chemokine production were analyzed 18 h post-treatment. The eubiotic condition tested represents the maximum level of SCFAs and succinic acid measured in women carrying an optimal vaginal microbiota (Al-Mushrif et al., 2000; Mirmonsef et al., 2011, 2012b; O'Hanlon et al., 2013). Prior to addition to the apical compartment, the pH of treatment media was adjusted to 3.9 to mimic the physiological acidity in women with an optimal, lactobacillus-dominated vaginal microbiota (Aldunate et al., 2013).

Ectocervical (Ect) and primary ectocervical cells, treated with L-LA in combination with the eubiotic vaginal microbiota metabolite mixture elicited an 8.5-fold ($p = 0.01$) and 2.6-fold ($p = 0.02$) increase in the production of the anti-inflammatory cytokine IL-1RA as compared to untreated Ect and primary cells, respectively, which was similar to the effect elicited by L-LA alone (**Figure 1A** and **Supplementary Figure 1A**). A heightened production of IL-1RA was also observed from vaginal VK2 cells treated with eubiotic vaginal microbiota metabolite mixture containing L-LA compared to untreated cells, although this did not reach statistical significance (**Supplementary Figure 2A**). Furthermore, the production of IL-1RA by eubiotic and L-LA treatments was also observed in the presence of TLR1/2 (Pam) and TLR3 (PIC) agonists, which mimic bacterial and viral challenge, respectively (**Figure 1A** and **Supplementary Figures 1A**, **2A**).

Basal production of IL-6 and MIP-3α from Ect cells was significantly decreased by both the eubiotic treatment and L-LA ($p = 0.01$ for both, **Figures 1B,F**) consistent with our previous findings (Hearps et al., 2017), and a similar trend for IL-6 was also seen in VK2 cells (**Supplementary Figure 2B**). Similar to L-LA alone, the eubiotic treatment elicited a minimal 2-fold increase in the production of IL-1β from all cell types (**Supplementary Figures 1F, 2F, 3A**), which physiologically is likely to be mitigated by the substantially higher production of the antagonist IL-1RA elicited by L-LA (7420-, 5812-, and 10166-fold excess of IL-1RA to IL-1β in Ect, VK2 and primary cells, respectively (**Figure 1** and **Supplementary Figures 1, 2**). Taken together, these data indicate that the anti-inflammatory effect of L-LA on unstimulated cervicovaginal epithelial cells persists within a complex mixture of vaginal microbiota organic acids representing a eubiotic vaginal environment.

A Eubiotic Vaginal Microbiota Metabolite Mixture Containing L-LA Inhibits Pro-inflammatory Responses From TLR Stimulated Cervicovaginal Epithelial Cells

Genital inflammation, driven by a highly diverse vaginal microbiota, and increased cervicovaginal levels of pro-inflammatory cytokines and chemokines are associated with higher levels of HIV transmission (Masson et al., 2015; Gosmann et al., 2017). Beneficial vaginal lactobacilli (e.g., *L. crispatus*) dampen inflammatory and inhibit pro-inflammatory responses mediated by TLR agonists (Rose et al., 2012; Doerflinger et al., 2014) and vaginal pathogens (Santos et al., 2018), and we have demonstrated that this effect is mediated, at least in part, by LA (Hearps et al., 2017). Here, we asked whether this effect is maintained in the presence of physiological concentrations of other vaginal organic acid metabolites.

Apical stimulation of Ect cells with PIC and Pam, elicited an increased production of pro-inflammatory immune mediators including IL-6, IL-8, IP-10, RANTES, and MIP-3α, with PIC eliciting a greater response than Pam stimulation except for IL-8 and MIP-3α (**Figures 1B–F**). Similar to the effect of L-LA alone, in the context of PIC stimulation the eubiotic treatment significantly dampened the production of IL-6, IL-8, IP-10, MIP-3α ($p < 0.01$ for all) and RANTES ($p = 0.02$) from Ect cells (**Figures 1B–F**). A similar inhibitory effect was observed for Pam stimulated Ect cells, which was statistically significant for IL-6, IP-10, and MIP-3α production ($p = 0.03$ for all). TNFα production from Ect cells was largely below the limit of quantification, except for PIC-stimulated cells, with this response being completely inhibited by L-LA (**Supplementary Figure 3B**).

We next confirmed this result in VK2 and primary cervicovaginal cells and observed a similar ability of the eubiotic

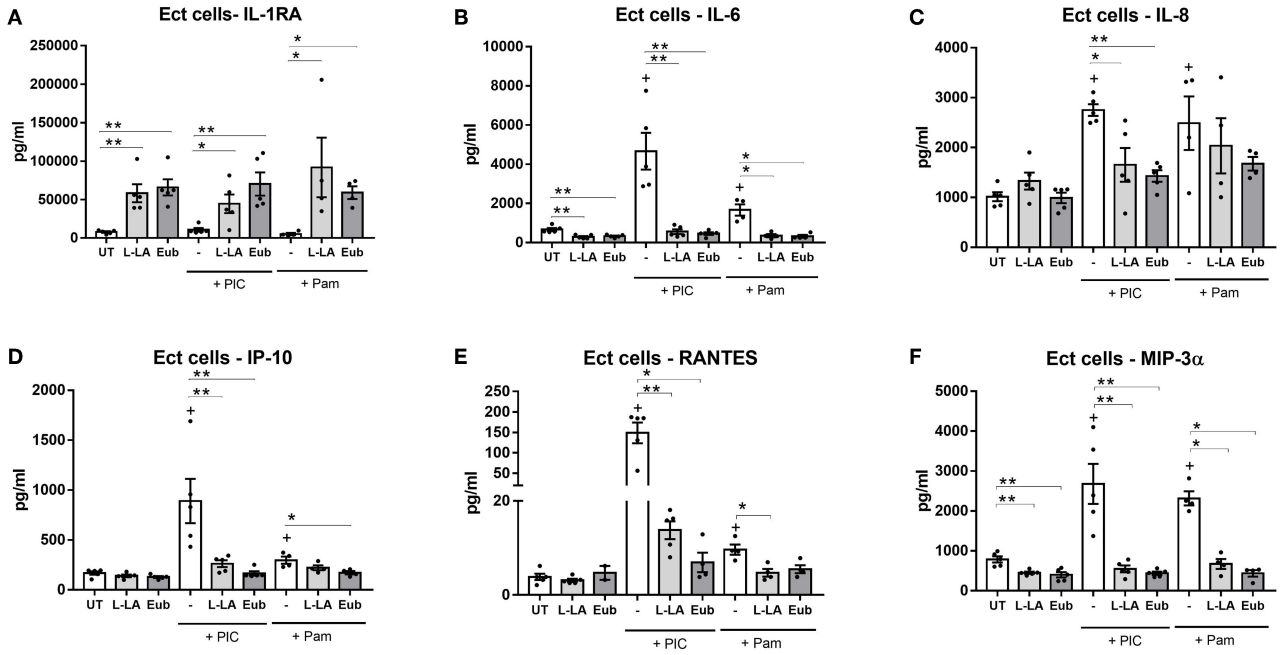

FIGURE 1 | Eubiotic vaginal microbiota metabolite mixture containing L-LA elicits a similar anti-inflammatory effect on Ect cells compared to L-LA alone. Ect cells seeded in transwells were apically treated with a mixture of vaginal microbiota metabolites at pH 3.9 recapitulating vaginal eubiosis (Eub) and L-LA alone in the absence or presence of the TLR agonists PIC (TLR3) and Pam (TLR1/2) as indicated. Cells were stimulated for 1 h and supernatant collected from the apical compartment after an additional 18 h of culture. Production of IL-1RA **(A)**, IL-6 **(B)**, IL-8 **(C)**, IP-10 **(D)**, RANTES **(E)**, and MIP-3α **(F)** was quantified using a luminex multiplex assay. Graphs show mean and standard error of the mean from $n = 4$ independent assays. Mann-Whitney tests were used to estimate statistical significance between treatment groups. + denotes a significant difference ($p < 0.05$) as compared to untreated (UT) cells. $P \leq 0.05$ and ≤ 0.01 are denoted as * and **, respectively. L-LA, L-isomer of lactic acid; TLR, toll-like receptor; PIC, polyinosinic:polycytidylic acid; Pam, Pam3CSK4.

treatment to inhibit TLR-mediated inflammation, similar to that observed with L-LA alone (**Supplementary Figures 1, 2**). These data demonstrate that the ability of L-LA to mitigate the pro-inflammatory response of cervicovaginal epithelial cells to TLR agonists mimicking bacterial and viral PAMPs is retained in the presence of physiological concentrations of vaginal microbiota organic acid metabolites found in women with a lactobacillus-dominated microbiota.

Short Exposure of Cervicovaginal Epithelial Cells to Vaginal Microbiota Metabolites Associated With BV Do Not Elicit Immunomodulatory Effects

BV is associated with vaginal inflammation, but whether this is mediated by an effect of vaginal microbiota metabolites produced by BV-associated bacteria on the production of inflammatory mediators by epithelial cells is not known. To determine if SCFAs and succinic acid, which are found at higher concentrations in the vagina of women with BV (Al-Mushrif et al., 2000; Chaudry et al., 2004; Mirmonsef et al., 2011; Gajer et al., 2012), have immunomodulatory effects on cervicovaginal epithelial cells, a combination of vaginal microbiota metabolites representing BV conditions was established and added apically to cervicovaginal epithelial cells seeded in transwells for 1 h. This mixture contained vaginal microbial metabolites at concentrations which represented the upper limits of what is reported *in vivo* for women with BV (acetic acid 100 mM, succinic acid 20 mM, propionic acid 4 mM, and butyric acid 4 mM) and at pH 7, to reflect the higher pH found in this state ranging from pH 4.5 to 8 (Brabin et al., 2005). In contrast to the eubiotic vaginal microbiota metabolite combination, a mixture of vaginal microbiota metabolites representing BV conditions did not significantly change the production of the anti-inflammatory cytokine IL-1RA compared to untreated cells, nor did it significantly alter the production of any other immune mediators tested from either unstimulated or TLR-stimulated Ect cells (**Figure 2**). A similar lack of immunomodulatory effect was also observed in VK2 and primary cells (data not shown). These findings suggest that unlike L-LA under eubiotic conditions, which elicited a significant immunomodulatory effect, vaginal microbiota organic acid metabolites representative of those found in BV conditions do not alter the inflammatory response of cervicovaginal epithelial cells when added to cells for comparable periods of time.

BV-Related Vaginal Microbiota Metabolites Have Immunomodulatory Effects on Cervicovaginal Epithelial Cells Only During Prolonged and Sustained Treatments

Given the above findings, and a previous report demonstrated a pro-inflammatory effect of SCFAs on peripheral blood cells, including neutrophils, after longer treatment periods

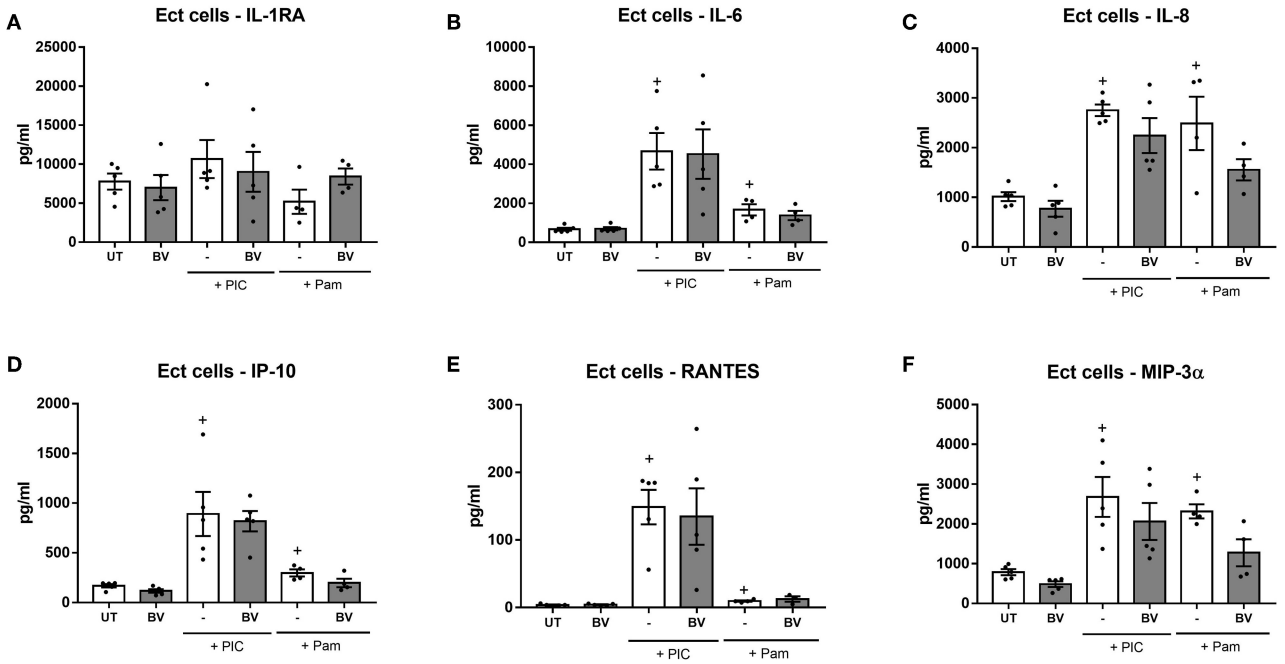

FIGURE 2 | A BV vaginal microbiota metabolite combination does not alter the production of immune mediators from Ect cells treated in a transwell system. Ect cells seeded in transwells were apically treated with a mixture of vaginal microbiota metabolites at pH 7 recapitulating bacterial vaginosis (BV; 6 mM L-LA, 100 mM acetic acid, 20 mM succinic acid, 4 mM propionic acid, and 4 mM butyric acid) in the absence or presence of TLR agonists PIC (TLR3) and Pam (TLR1/2) as indicated. Cells were stimulated for 1 h and supernatant collected from the apical compartment after an additional 18 h of culture. Production of IL-1RA **(A)**, IL-6 **(B)**, IL-8 **(C)**, IP-10 **(D)**, RANTES **(E)**, and MIP-3α **(F)** was quantified using a luminex multiplex assay. Graphs show mean and standard error of the mean from $n = 4$ independent assays. Mann-Whitney tests were used to estimate statistical significance between treatment groups. + denotes a significant difference ($p < 0.05$) as compared to untreated (UT) cells. TLR, toll-like receptor; PIC, polyinosinic:polycytidylic acid; Pam, Pam3CSK4.

(Mirmonsef et al., 2012b), we hypothesized that vaginal microbiota metabolites may need prolonged and sustained contact with lower female reproductive tract epithelial cells to elicit an observable effect. The transwell system allows the diffusion of molecules from the apical to basolateral compartments. Therefore, to sustain the concentration and pH of the established treatments for longer periods, cervicovaginal epithelial cells were seeded into 96-well plates. Cells were treated with the combination of vaginal microbiota metabolites representing BV conditions, with and without TLR stimulation at pH 7 for 24 h.

Prolonged treatments of Ect cells under these conditions with a mixture of vaginal microbiota metabolites representing BV caused an increase in the production of TNFα from unstimulated cells ($p = 0.03$, **Figure 3C**) and potentiated the TLR-elicited production of TNFα for Pam and BV-stimulated cells compared to cells treated with Pam alone ($p = 0.03$). A similar effect was also seen in VK2 cells (**Supplementary Figure 4B**). The BV vaginal microbiota metabolite mix also increased basal TNFα production from primary cervicovaginal cells, but intriguingly the opposite effect was observed in PIC-stimulated cells (**Supplementary Figure 4F**). The BV metabolite mixture also potentiated TLR-induced IL-8 production, with this effect being unique to Ect cells (**Figure 3B** and data not shown).

In contrast to this enhanced production of the pro-inflammatory mediators TNFα and IL-8, the BV vaginal microbiota metabolite mixture dampened the PIC and Pam-stimulated production of RANTES from all three cell types tested ($p < 0.05$ for all) (**Figure 3E** and **Supplementary Figures 4D,H**). Similarly, PIC-stimulated production of IP-10 was significantly inhibited by the BV vaginal microbiota metabolite mixture in all cell types (**Figure 3D** and **Supplementary Figures 4C,G**), while Pam-stimulated production was inhibited in VK2 and primary cells ($p = 0.03$ for both). Furthermore, the BV mixture inhibited basal production of IP-10, but this effect was only observed in primary cells (**Supplementary Figure 4G**). There was also a trend for the BV mixture to inhibit PIC-induced IL-6 production, although this was only significant in primary cells ($p = 0.03$) (**Figure 3** and **Supplementary Figure 4**). Similar results were observed when cells were treated with a BV mixture lacking L-LA (data not shown), consistent with our previous findings that the immunomodulatory effects of L-LA are only observed at a low pH (i.e., 3.9) (Hearps et al., 2017).

Taken together, these data demonstrate that prolonged and sustained treatments of cervicovaginal epithelial cells with a mixture of SCFAs and succinic acid recreating BV conditions results in a dysregulation of inflammatory responses, with a heightened basal and TLR-simulated production of the potent inflammatory cytokine TNFα, but a dampening of TLR-elicited production of IL-6 and the chemokines RANTES and IP-10. Although most of these effects were consistent between the three cell types tested, some effects appear to be cell-type

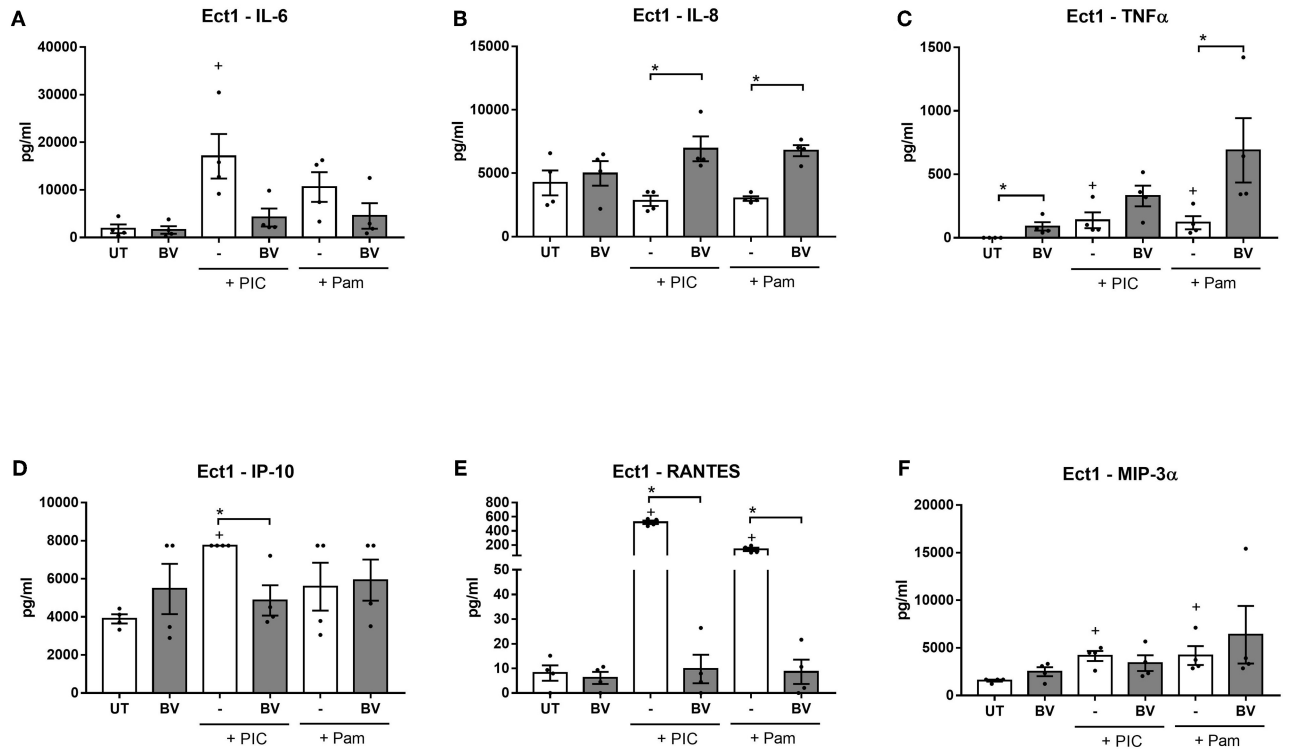

FIGURE 3 | Prolonged treatment of Ect cells with BV vaginal microbiota metabolite mixture at pH 7 elicits a dysfunctional inflammatory response. Ect cells seeded in a plate format were stimulated with a mixture of vaginal microbiota metabolites recapitulating BV (6 mM L-LA, 100 mM acetic acid, 20 mM succinic acid, 4 mM propionic acid and 4 mM butyric acid) for 24 h at pH 7 in the absence or presence of stimulation with the TLR agonists PIC and Pam and production of IL-6 **(A)**, IL-8 **(B)**, TNFα **(C)**, IP-10 **(D)**, RANTES **(E),** and MIP-3α **(F)** was quantified from supernatants. Graphs show mean and standard error of the mean from $n = 4$ independent assays. $P \leq 0.05$ is denoted as * as determined by Mann-Whitney test. + denotes a significant difference ($p < 0.05$) as compared to untreated (UT) cells. BV, bacterial vaginosis; TLR, toll-like receptor; PIC, polyinosinic:polycytidylic acid; Pam, Pam3CSK4.

dependant (e.g., TLR-induced TNFα production in primary cells and potentiation of IL-8 production in Ect cells), highlighting the subtle but potentially important differences in the immune response of different lower female reproductive tract epithelial cell types.

Individual Vaginal Microbiota Metabolites Elicit Dysfunctional Immunomodulatory Effects on Lower Female Reproductive Tract Epithelial Cells After Prolonged and Sustained Treatments

Having identified immunomodulatory effects of sustained treatments with a mixture of BV vaginal microbiota metabolites, we sought to identify which individual metabolite might be responsible for these dysfunctional effects. Lower female reproductive tract epithelial cells were therefore treated with individual SCFAs or succinic acid in 96 well plates under the same concentrations and pH conditions as above.

The pro-inflammatory effect of the BV mixture on increased TNFα production was largely recapitulated by treatment of cells with acetic acid alone (**Figures 4A–C**) with a consistent effect observed for all three cell types. Treatment with acetic acid alone also reproduced the potentiation of TLR-induced TNFα production in Ect and VK2 cells, and the converse effect in primary epithelial cells (**Figures 4A–C**). Butyric acid alone had a similar but non-significant effect on enhancing TLR-elicited TNFα production in Ect cells (**Supplementary Figure 5**), while no effects were observed for succinic acid or propionic acid alone in any cell type tested (data not shown). These data suggest that acetic acid, and to a lesser extent butyric acid, are likely the metabolites responsible for the pro-inflammatory effects of the BV mixture on TNFα production in these cells.

To identify the vaginal microbiota metabolites responsible for the potentiation of TLR-induced IL-8 production observed in Ect cells, we analyzed the effect of acetic, succinic, propionic, and butyric acids individually but were unable to identify any single metabolite which recapitulated this effect (data not shown). These data suggest the effects may be due to the combination of these metabolites. Interestingly, butyric acid alone showed a potentiation of TLR-induced IL-8 production in VK2 cells (**Supplementary Figure 6A**), which was not seen in Ect or primary cells, reinforcing the differences that exist between these cell types.

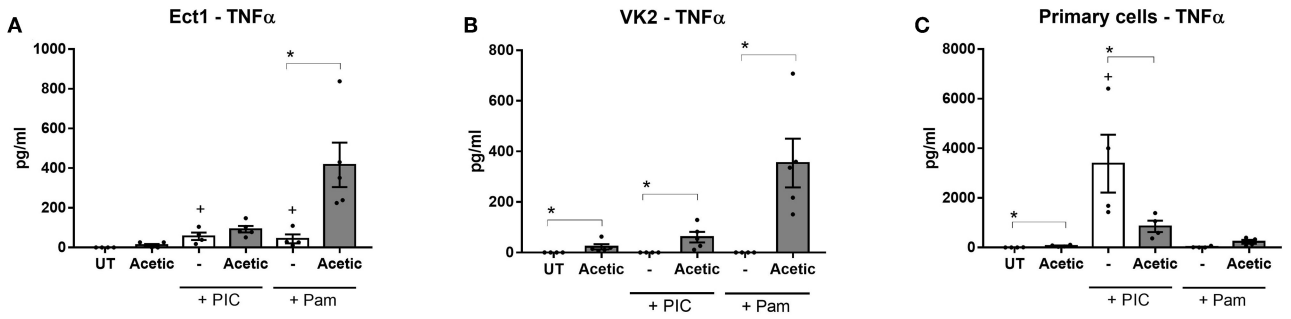

FIGURE 4 | Acetic acid alone increases basal and TLR-elicited TNF production from lower female reproductive tract epithelial cells. Ect **(A)**, VK2 **(B)**, and primary cervicovaginal epithelial cells **(C)** seeded into plates were treated with 100 mM acetic acid (Acetic) at pH 7 for 24 h in the absence or presence of stimulation with the TLR agonists PIC and Pam as indicated and production of TNFα was quantified from supernatants. Graphs show mean and standard error of the mean from $n = 4$ independent assays. $P \leq 0.05$ are denoted as * as determined by Mann-Whitney test. + denotes a significant difference ($p < 0.05$) as compared to untreated (UT) cells. TLR, toll-like receptor; PIC, polyinosinic:polycytidylic acid; Pam, Pam3CSK4.

When we interrogated the vaginal microbiota metabolites driving the dampening effect of the BV mixture on TLR-induced chemokine production we also found evidence implicating acetic acid. Treatment with acetic acid alone resulted in a striking inhibition of TLR3-induced RANTES, IP-10, and IL-6 production which was consistent across Ect, VK2 and primary epithelial cells (**Figures 5A–I**), suggesting this metabolite is contributing significantly to the immune dampening effect observed with the BV vaginal microbiota mixture. Butyric acid alone was also able to recapitulate the dampening effects on TLR-induced RANTES and IP-10 production, but primarily in VK2 cells (**Supplementary Figure 6**), and not in Ect cells (data not shown). Succinic acid also trended toward impairing TLR-elicited IL-6 production in Ect and VK2 cells, while no significant immunomodulatory effects were observed with propionic acid alone (data not shown). These experiments, which aimed to deconstruct the immune effects of a vaginal microbiota metabolite mixture reflecting a state of BV, identified acetic acid as a significant metabolite capable of eliciting seemingly conflicting immunomodulatory effects on a range of lower female reproductive tract epithelial cell targets. Our findings also suggest that butyric acid may be a potent immune modulating factor primarily for VK2 cells, indicating that different vaginal microbiota metabolites can have cell- and context- specific roles on modulating inflammation in the lower female reproductive tract.

DISCUSSION

A vaginal microbiota dominated by beneficial *Lactobacillus* spp. is associated with protection against viral and bacterial pathogens (Conti et al., 2009; Graver and Wade, 2011; O'Hanlon et al., 2011; Aldunate et al., 2013; Gong et al., 2014; Gosmann et al., 2017). Conversely, several studies have demonstrated a relationship between a non-optimal vaginal microbiota such as in BV and genital inflammation, and an increased risk of acquiring HIV (Sewankambo et al., 1997; Taha et al., 1998; Atashili et al., 2008; Masson et al., 2015; Gosmann et al., 2017). However, the vaginal microbiota produces a range of metabolites, peptides and proteins (Shaw et al., 2007; Cruciani et al., 2013; Vitali et al., 2015) and the discrete factors responsible for the aforementioned effects are not completely known, nor is it understood how these factors may interact with each other within the complex female reproductive tract milieu.

Here and previously, we have demonstrated a potent ability of L-LA, a major metabolite produced by lactobacilli, to elicit an anti-inflammatory effect as well as inhibit pro-inflammatory cytokine and chemokine production from cervicovaginal epithelial cells (Hearps et al., 2017). We describe for the first time the effect of L-LA on dampening TLR-elicited IP-10 production which is of special interest given the association of this cytokine with increased HIV transmission in women (Masson et al., 2015). In this study, we confirmed the anti-inflammatory activity of LA is maintained in the presence of an organic acid metabolite mixture relevant to vaginal eubiosis. This immunomodulatory effect was demonstrated by an increased production of the anti-inflammatory cytokine IL-1RA, which antagonizes the IL-1 receptor and inhibits pro-inflammatory signals from the IL-1 cytokines (Arend, 2002), and by a small decrease in the production of the pro-inflammatory factors IL-6 and MIP-3α. Moreover, in TLR stimulated cervicovaginal epithelial cells a eubiotic vaginal microbiota metabolite mixture containing L-LA dampened the production of pro-inflammatory factors associated with vaginal inflammation and recruitment of HIV target cells to the female reproductive tract epithelium (Sturm-Ramirez et al., 2000; Narimatsu et al., 2005; de Jong et al., 2008; Masson et al., 2015; Arnold et al., 2016; Gosmann et al., 2017).

Cervicovaginal epithelial cell secretion of MIP-3α has been shown to attract Langerhans cells (Cremel et al., 2005; Berlier et al., 2006), which although controversial, have been proposed to act as a "Trojan horse" facilitating HIV dissemination to lymph nodes (Cavrois et al., 2008; Matsuzawa et al., 2017). In this regard, the effect of L-LA on dampening MIP-3α production may also be important for inhibiting HIV transmission. The ability of L-LA to inhibit pro-inflammatory factors associated with HIV transmission, even when present within a complex vaginal microbiota metabolite mix, suggests this metabolite may be an important driver of the protective

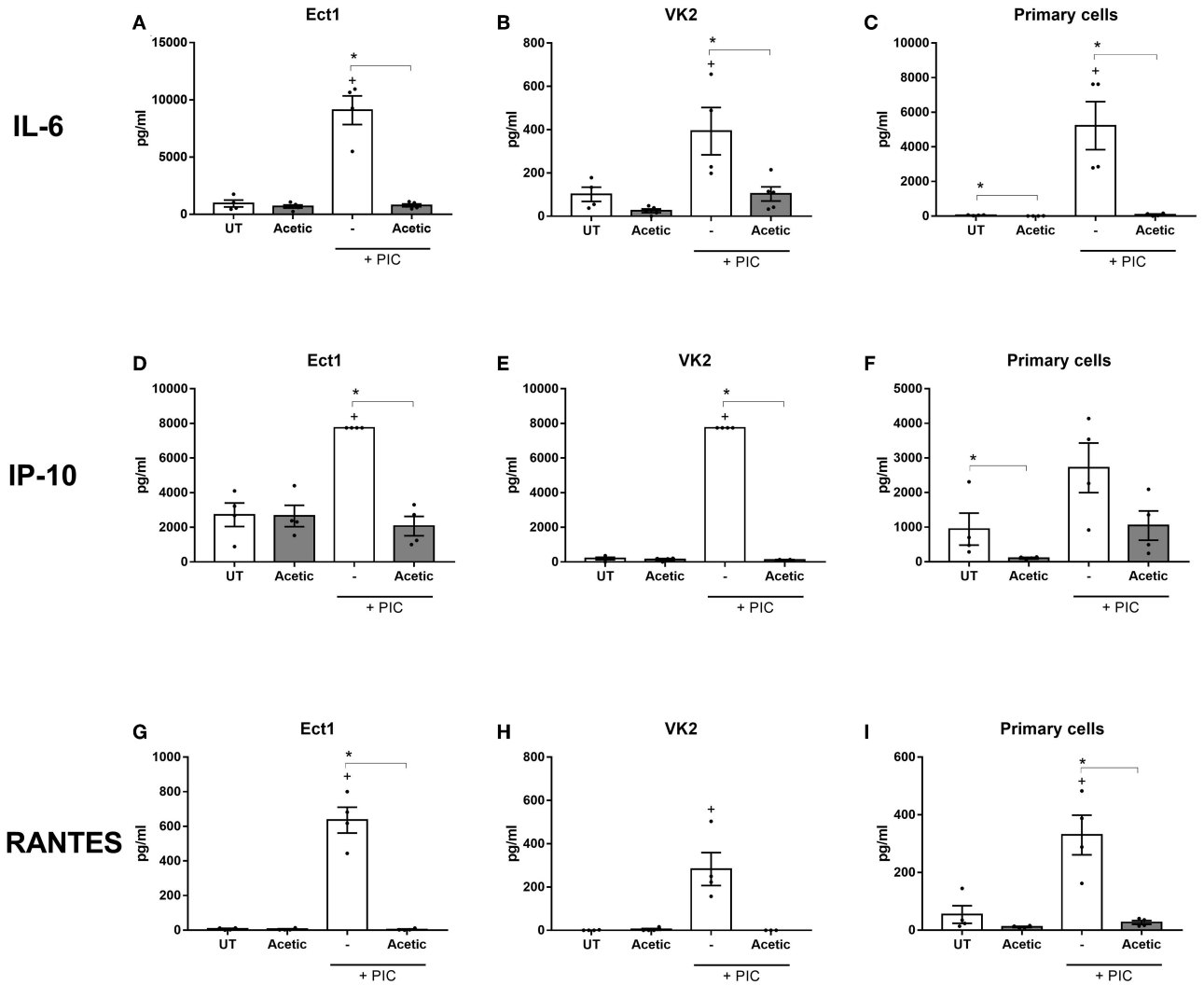

FIGURE 5 | Acetic acid alone inhibits TLR-elicited IL-6, IP-10, and RANTES production from lower female reproductive tract epithelial cells. Ect (left panels), VK2 (center panels), and primary cervicovaginal epithelial cells (right panels) seeded into plates were treated with 100 mM acetic acid (Acetic) at pH 7 for 24 h in the absence or presence of stimulation with the TLR agonist PIC. Production of IL-6 **(A–C)**, IP-10 **(D–F)**, and RANTES **(G–I)** was quantified from supernatants. Graphs show mean and standard error of the mean from $n = 4$ independent assays. $P \leq 0.05$ are denoted as * (as determined by Mann-Whitney test). + denotes a significant difference ($p < 0.05$) as compared to untreated (UT) cells. PIC, polyinosinic:polycytidylic acid.

effect of lactobacillus-dominated vaginal microbiota against HIV transmission observed *in vivo*. These L-LA-elicited effects also support the potential use of protonated LA administered vaginally, or vaginal probiotic treatment using lactobacilli to sustain the production of LA, to maintain an anti-inflammatory state *in vivo* and help protect against HIV infection.

In contrast to the striking immunomodulatory effects observed after a brief 1 h incubation of epithelial cells with L-LA and L-LA-containing metabolite mixtures, stimulation of cells for a similar time with a combination of vaginal microbiota metabolites representing BV conditions had no such effect. We also assessed the effect of individual SCFA and succinic acid, at the higher concentrations and pH reflective of BV, on cervicovaginal epithelial cells and similarly found no significant effects, even when metabolites were added at a lower pH of 5.5 (data not shown). This highlights the unique and potent effect of L-LA, and lends further weight to its potential therapeutic use to promote optimal female reproductive tract health. We did however find that sustained and prolonged treatments of epithelial cells with BV-relevant metabolites elicited somewhat conflicting and potentially dysfunctional changes in the inflammatory response of these cells. Consistent with previous findings demonstrating a pro-inflammatory effect of BV-relevant SCFA on peripheral blood mononuclear cells and neutrophils (Mirmonsef et al., 2012b), we found a combination of vaginal microbiota metabolites representing BV increased basal and TLR-induced production of TNFα, which was largely consistent across all cell types tested. Further analyses suggested this effect was due at least in part to the action of acetic acid. However, this pro-inflammatory effect was contrasted by

a significant impairment of TLR-induced production of other inflammatory factors, which again appeared to be mediated by acetic acid, and to a lesser extent butyric acid. These findings indicate that sustained treatment with BV-relevant vaginal microbiota metabolites dysregulates the immune response of cervicovaginal epithelial cells, with the net effect of these anti- and pro-inflammatory responses remaining unclear. However, it is worth noting that a similar dichotomous pattern in the levels of pro-inflammatory mediators was observed in the cervicovaginal fluid of HIV-uninfected women in South Africa with BV (Masson et al., 2014). In this study elevated levels of the pro-inflammatory cytokines IL-1α, IL-1β and TNFα but decreased levels of several pro-inflammatory chemokines including IP-10 and MIP-α was reported (Masson et al., 2014). While the *in vivo* cervicovaginal milieu is more complex than our *in vitro* analysis of BV-associated SCFAs on cervicovaginal epithelial cells, it is possible that the elevated levels of TNFα and decreased levels of IP-10 may in part be mediated by the action of SCFAs, and in particular acetic acid, on the cervicovaginal mucosa.

These experiments also identified cell-specific immunomodulatory effects, with the metabolite butyric acid appearing to have an immunomodulatory effect in VK2 but not Ect or primary epithelial cells, while acetic acid showed a largely consistent effect on all cell types. This is consistent with other studies reporting a cell-type dependent response to SCFAs (Park et al., 2007; Vinolo et al., 2011; Mirmonsef et al., 2012b; Kim et al., 2013) and highlights the importance of using physiologically relevant cell types in studies of vaginal microbiota metabolites. Our data also suggest there may be differences in the epithelial microenvironment at various sites within the female reproductive tract due to variations in the response of resident epithelial cells to microbiota products. Similar site-specific variations have been shown regarding the effect of the antiretroviral drug tenofovir, which increases pro-inflammatory cytokine production from endometrial and ectocervical, but not endocervical epithelial cells (Biswas et al., 2014). Another intriguing observation, which was unique to primary cells, was the conflicting effect of the BV metabolite mixture on TNFα production, in that it enhanced basal TNFα production but inhibited PIC-induced TNFα production. This effect obviously requires further investigation in different types of primary cells, but alludes to the fact that unlike LA, which exhibits a more consistent and universal anti-inflammatory effect on basal and stimulated cells, vaginal microbiota metabolites present in BV may have a more variable and nuanced effect which is influenced by cell and pathogen-specific factors.

The inflammatory state present in BV may be driven by vaginal microbiota metabolites other than those assessed here, or by other vaginal microbial products. A number of studies have profiled the BV metabolome and proteome and identified substantial metabolic and proteomic changes in vaginal fluid from BV-affected women (Srinivasan et al., 2015; Arnold et al., 2016; Borgdorff et al., 2016) with one study identifying 17 previously unreported molecules enriched in BV samples (Vitali et al., 2015). The impact of these factors on vaginal inflammation remains to be determined. However, the cause of female reproductive tract inflammation may not only be due to the presence of pro-inflammatory BV-related organisms (and the effect of their metabolites), but also the additional effect of an absence of anti-inflammatory factors such as LA.

A limitation of this study was that it analyzed the effect of vaginal microbiota metabolites on cervicovaginal epithelial cell monolayers. The cervicovaginal epithelium *in vivo* is multilayered and contains various cell types including immune cells. Thus, it will be important to confirm the effects of eubiotic and BV metabolites in a more physiologically relevant system, such as vaginal tissue, or the 3D EpiVaginal^{TM} tissue model (MatTek, Ashland, MA) we have previously used to validate the anti-inflammatory effects of LA (Hearps et al., 2017). Furthermore, we assessed concentrations of vaginal microbiota metabolites reflective of those found in the vaginal lumen; cells present deeper within the vaginal epithelium may be exposed to different, and likely lower, concentrations of metabolites than those analyzed here. The effects of eubiotic metabolite mixtures were assessed here at pH 3.9, and although we did not find a significant immunomodulatory effect of pH 3.9 media alone on cervicovaginal cells in a previous study (Hearps et al., 2017), acidity has been shown to dampen inflammatory responses of other types of epithelial cells (Hackett et al., 2016). It therefore remains possible that low pH may be contributing to the anti-inflammatory properties of the eubiotic metabolites shown here.

In this study we focused on L-LA due to our previous findings regarding its superior virucidal activity against HIV (Aldunate et al., 2013), and a similar anti-inflammatory effect of both L- and D-LA (Hearps et al., 2017), but it would be of interest to confirm the current findings with D-LA and/or various ratios of L- and D-LA reflective of those found in women colonized with different lactobacilli. We assessed responses to TLR3 and TLR1/2 agonists here given our previous findings of similar anti-inflammatory effects of LA irrespective of TLR agonist used (Hearps et al., 2017), thus potential differences regarding effects of the vaginal microbial metabolites studied here on responses to other TLR agonists remain to be confirmed. Although this study investigated five predominant organic acid metabolites detected in women with optimal and non-optimal microbiota, other metabolites including pyruvic and isobutyric acid are detected in the female genital tract and may also have immunomodulatory effects (Stanek et al., 1992). Notwithstanding these limitations, this study advances our knowledge on the effects of single vaginal microbiota metabolites that comprise the complex cervicovaginal environment and support our previous findings showing the beneficial effects of LA in preventing inflammation known to be associated with increased risk of HIV acquisition. Future *in vivo* studies assessing the efficacy of topically applied LA in maintaining a eubiotic vaginal state and preventing BV and associated genital inflammation will be important for informing new prevention strategies to mitigate transmission of HIV and other sexually transmitted infections.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

GT and AH conceived the study. DD-D and DT generated experimental data. AH, DD-D, JH, RG, and GT provided input into study design. DD-D performed data analysis and wrote the manuscript with assistance from AH and GT. All authors reviewed the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00446/full#supplementary-material

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Conflict of Interest: GT and AH are coinventors on patent application AU201501042 and United States Patent No. US 9,801,839 B2 claiming the anti-inflammatory effects of lactic acid.

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

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