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HUMAN HEALTH AND DISEASE IN A MICROBIAL WORLD

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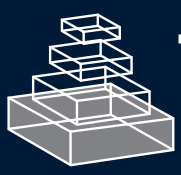

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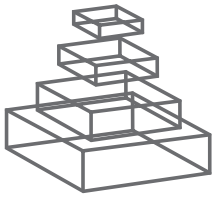

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HUMAN HEALTH AND DISEASE IN A MICROBIAL WORLD

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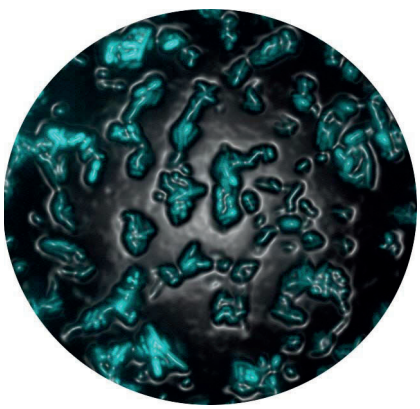

Humans and other mammals have co-evolved with trillions of microorganisms, whose aggregate genomes (the human microbiome) contribute functions not encoded by our own human genes. Recent developments in the emerging field of metagenomics have enabled an unprecedented description of the organismal, genetic, transcriptional, proteomic, and metabolic diversity across a wide range of body habitats, spawning multiple ongoing international human microbiome projects. These diverse studies, along with mechanistic research using *in vitro* and animal models, have emphasized the role the human microbiome plays in health and disease, including inflammatory bowel disease, metabolic syndrome, multiple sclerosis, and even cancer.

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EDITORIAL
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Human health and disease in a microbial world

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Humans and other mammals have co-evolved with trillions of microorganisms (the microbiota), whose aggregate genomes (the microbiome) contribute functions that are not encoded by our own human genome. Recent developments in the emerging field of metagenomics have enabled an unprecedented description of organismal, genetic, transcriptional, proteomic, and metabolic diversity across a wide range of body habitats, spawning multiple ongoing international human microbiome projects. These largely sequencing-driven studies, along with mechanistic studies *in vitro* and in animal models, have emphasized the role the human microbiome plays in health and disease, including inflammatory bowel disease (IBD), metabolic syndrome, autoimmune disorders, and even cancer.

Here, in this "Research Topic" collection of reviews, perspectives, and original research articles, leading researchers in the field have highlighted recent developments in our appreciation of the structure and function of the human microbiome, spanning a wide range of topics including immune development (Derrien et al., 2011; Kosiewicz et al., 2011; Pfeiffer and Sonnenburg, 2011; Reading and Kasper, 2011; Smith and Garrett, 2011), IBD (Young et al., 2011), nutrition, and obesity (Geurts et al., 2011; Li et al., 2011; Lin, 2011; Thomas et al., 2011), and novel methods for analyzing complex microbial communities (Moore et al., 2011; Parfrey et al., 2011).

First, we present a series of reviews highlighting that the human immune system is intimately linked to the gut microbiome; the gut microbiota influences immune development, susceptibility to infection from pathogens, and inflammation (Kosiewicz et al., 2011; Reading and Kasper, 2011; Smith and Garrett, 2011). Through the use of gnotobiotic mouse models colonized with cultured isolates from the human gut, recent studies have highlighted the role specific members of this complex community can play in programming the immune system. For example, segmented filamentous bacteria can promote the expansion of IL-17 cells, *Bacteroides fragilis* can alleviate colitis in animal models, and some *Clostridium* strains can promote the expansion of regulatory T-cells. Moving forward, the study of gnotobiotic mice colonized with more complex communities derived from human or animal donors, may provide insight into the mechanisms by which different microbial communities can alter host immunity (Reading and Kasper, 2011). In an original research article, Derrien et al. (2011) demonstrate that *Akkermansia muciniphila*, a bacterium specialized for the degradation of host mucin (a glycoprotein abundant in the mucosal layer of the gut), is able to alter mucosal gene expression, up-regulating genes for immune response and cell fate determination, with potentially important implications for immune tolerance.

Together, these interactions between the gut microbiome and immune system could contribute to multiple diseases involving aberrant inflammation. The most commonly studied case, although still poorly understood, is that of IBD. Young et al. (2011) argue that IBD should be considered a cluster of related diseases, that detailed time series analysis of luminal and mucosa-associated microbial communities are required, and that a comprehensive understanding of the relevant confounding variables in human clinical studies will be necessary. Perhaps most importantly, many technical aspects must be considered in designing a given study, spanning the methods used for sampling patients, the development of appropriate animal models, the platform used for sequencing, and the nascent development of bioinformatic methods for the concomitant analysis of DNA, RNA, proteins, metabolites, and spatial organization. Of note, recent studies are beginning to reveal the diversity of microbial eukaryotes living in the human gut, a largely underexplored component of our microbiome with potential implications for health and disease (Parfrey et al., 2011).

Another key method necessary to move beyond largely descriptive sequence-based analyses of the gut microbiome is functional metagenomics; the screening of metagenomic libraries created by cloning community DNA into a suitable host strain, allowing causal links between genes from uncultured organisms and their function (Moore et al., 2011). To date, studies of the human gut and other environments have already identified novel genes for antibiotic resistance, fiber degradation, bile salt hydrolysis, and immune modulation (Moore et al., 2011).

Pfeiffer and Sonnenburg (2011) highlight the role of the gut microbiome in regulating host susceptibility to infection from pathogenic bacteria and viruses. For example, infection by *Clostridium difficile* and *Salmonella* sp. can be triggered by antibiotic treatment, while other pathogens may alter the host immune response to promote persistent colonization. Furthermore, the gut microbiome may play an important role in viral infection: e.g., influenza, dengue, adenovirus, and HIV. Future studies promise to address the underlying mechanisms behind these phenomena, such as directly modifying viral particles, recruiting immune effectors that promote viral replication, or modulating host immunity to permit viral replication (Pfeiffer and Sonnenburg, 2011).

The human microbiome is also thought to play a critical role in both sides of the energy balance equation, allowing the digestion of complex substrates inaccessible to our own human enzymes and promoting the accumulation of body fat. Intriguingly, studies in livestock suggest that antibiotics can be used to alter weight gain (Lin, 2011). Thomas et al. (2011) review their recent results highlighting the potential for a new type of impact of the diet on our gut microbiome – the lateral transfer of genes from transient microbes ingested from the diet to our resident microorganisms. These genetic swaps could potentially have a lasting impact on the ability to digest a given diet, enabling an entrenched community to adapt to changes in diet. In two original research articles published

here, animal models are used to further explore this relationship between the gut microbiome and energy balance. Geurts et al. (2011) demonstrate that the gut microbiota and endocannabinoid system are modified in obese and diabetic leptin-resistant *db/db* mice, although follow-on studies are necessary to determine if the gut microbiota is contributing to or responding to this phenotype. Finally, Li et al. (2011) use rats to show that bariatric surgery results in an altered microbiota and metabolome, coupled to increased cytotoxicity.

Together, these original research articles, reviews, and perspectives emphasize the importance of the microbiome across many aspects of human health and disease. Although many important questions remain unanswered, the pace of discovery continues to accelerate, as a wide range of scientists across many disciplines are beginning to turn their expertise toward studying this complex and fascinating ecosystem. In the near future, these studies may lead to new diagnostic tools and therapies targeting our microbial communities. In a broader sense, they also provide a new perspective of our own human body, as an often imperceptible, but always ongoing, dialog with our trillions of microbial partners.

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Modulation of mucosal immune response, tolerance, and proliferation in mice colonized by the mucin-degrader *Akkermansia muciniphila*

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Epithelial cells of the mammalian intestine are covered with a mucus layer that prevents direct contact with intestinal microbes but also constitutes a substrate for mucus-degrading bacteria. To study the effect of mucus degradation on the host response, germ-free mice were colonized with *Akkermansia muciniphila*. This anaerobic bacterium belonging to the Verrucomicrobia is specialized in the degradation of mucin, the glycoprotein present in mucus, and found in high numbers in the intestinal tract of human and other mammalian species. Efficient colonization of *A. muciniphila* was observed with highest numbers in the cecum, where most mucin is produced. In contrast, following colonization by *Lactobacillus plantarum*, a facultative anaerobe belonging to the Firmicutes that ferments carbohydrates, similar cell-numbers were found at all intestinal sites. Whereas *A. muciniphila* was located closely associated with the intestinal cells, *L. plantarum* was exclusively found in the lumen. The global transcriptional host response was determined in intestinal biopsies and revealed a consistent, site-specific, and unique modulation of about 750 genes in mice colonized by *A. muciniphila* and over 1500 genes after colonization by *L. plantarum*. Pathway reconstructions showed that colonization by *A. muciniphila* altered mucosal gene expression profiles toward increased expression of genes involved in immune responses and cell fate determination, while colonization by *L. plantarum* led to up-regulation of lipid metabolism. These indicate that the colonizers induce host responses that are specific per intestinal location. In conclusion, we propose that *A. muciniphila* modulates pathways involved in establishing homeostasis for basal metabolism and immune tolerance toward commensal microbiota.

Keywords: *Akkermansia muciniphila*, mucin, germ-free mice colonization, host responses

INTRODUCTION

The human gut is colonized by a complex, diverse, and dynamic community of microbes that continuously interact with the host (Hooper et al., 2002; Kelly et al., 2005). Considerable attention has focused on pathogen recognition at the intestinal epithelium (Cummings and Relman, 2000; Kagnoff and Eckmann, 2001). Remarkably, the host response to intestinal commensals that are abundant in number and diversity have not been studied at a similar level. Our intestinal tract is colonized by thousands of bacterial species, most of which belong to the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*. *Proteobacteria* and *Verrucomicrobia* (Zoetendal et al., 2008). Pioneering studies on the impact of commensals on host gene responses have been performed using gnotobiotic germ-free animals (Hooper et al., 2002). It was convincingly shown that following colonization of germ-free mice *Bacteroides thetaiotaomicron*, a well-characterized member of the intestinal Gram-negative bacteria, activated the host immune system, stimulated angiogenesis (Stappenbeck et al., 2002), and promoted increased fat storage (Backhed et al., 2004). In contrast, murine colonization with *Bifidobacterium longum* that belongs to the Gram-positive *Actinobacteria*, showed a different transcriptional response as it resulted in down-regulation of several host genes that were up-regulated by *B. thetaiotaomicron* during co-colonization of both bacteria (Sonnenburg et al., 2006). Recently, the effect was studied of co-colonization of *B. thetaiotaomicron* and *Eubacterium rectale*, a butyrate-producer, belonging to the Firmicutes (Mahowald et al., 2009). This co-colonization induced

Abbreviations: GF, germ-free; IPA, ingenuity pathway input; qRT-PCR, quantitative RT-PCR.

more significant regulated genes on colonic cells than when species were inoculated alone. Significant regulation of genes involved in cellular growth and proliferation, and cell death were found after co-colonization. These observations were instrumental to demonstrate that (i) there exists a dynamic interaction between host and bacteria that affects homeostasis, (ii) the host response is specific for each bacterium studied, and (iii) intestinal bacteria contribute to host physiology. However, there is a need to expand these studies and address the host response of major intestinal phyla, notably those that are expected to have a direct interaction with their animal host.

Recently, several studies have addressed the response of human cells to *Lactobacillus* species, lactic acid bacteria that degrade sugars derived from plants and other components of our diet. Lactobacilli are abundant in our early life microbiota while in adults they colonize notably the upper intestinal tract (Heilig et al., 2002). Lactobacilli are abundantly present in a variety of animals and show specific and differential responses at various sites in the murine and human GI tract (Bron et al., 2004; Marco et al., 2007, 2010). Transcriptional analysis of duodenal biopsies from healthy adults exposed to *Lactobacillus plantarum* showed specific modulation of mucosal gene expression and pathways involved in immune tolerance (van Baarlen et al., 2009). *In vitro* studies already had suggested a modulation of the immune response by *Lactobacillus* spp that were found to stimulate polarization of immune T cells toward regulatory T cells (Mohamadzadeh and Klaenhammer, 2008). A molecular mechanism for this was recently provided by the finding that the cell envelope S-layer of *L. acidophilus* directly signals to the immune system by binding to dendritic cells that are present in the host mucosa (Konstantinov et al., 2008).

A direct interaction between host and bacteria in the intestinal tract is prevented by the presence of a thick mucus layer covering the intestinal cells that protects the epithelium against toxins, acids, and bacterial invasion. Major components of this intestinal mucus are the mucins, heavily glycosylated proteins that form a network via cross-linking of disulfide bridges. Mucin-associated bacteria have been studied previously and are found among the main intestinal phyla (Derrien et al., 2010). Molecular studies based on 16S rRNA sequence analysis indicated that communities that are strongly associated with the colonic mucosa are different from those that are frequently sampled from the feces (Zoetendal et al., 2002; Nielsen et al., 2003; Lepage et al., 2005), with an overrepresentation of bacteria that degrade mucins (Mackie et al., 1999). Recently, we have isolated a strictly anaerobic, Gram-negative bacterial species, *Akkermansia muciniphila*, that is specialized in the utilization of mucin as a carbon and nitrogen source (Hooper et al., 2002; Derrien et al., 2004). *A. muciniphila* was the first cultured representative of the *Verrucomicrobia* and is, so far, the sole representative of this phylum that is present in the human intestinal tract (Derrien et al., 2008). Moreover, its characteristic 16S rRNA signatures have been consistently identified in mucosal clone libraries and may make up over 5% of the retrieved sequences (Eckburg et al., 2005; Wang et al., 2005). Based on analysis of fecal samples, the numbers of *A. muciniphila* start to increase in newborns and reach a level close to that observed in adults within a year (Collado et al., 2007). This suggests that *A. muciniphila* is a relatively late colonizer of the human intestine compared to early infant colonizers such as *Bifidobacterium* and *Lactobacillus* species (Mackie et al., 1999; Favier et al., 2002). Remarkably, both *Bifidobacterium* spp. and *A. muciniphila* made up an important fraction of the microbial cells in pregnant woman but were numerically decreased in those that were overweight (Santacruz et al., 2010). Moreover, recent studies have indicated that *Akkermansia*-like bacteria are abundantly present in the mucosa of appendices of healthy subjects but strongly reduced during appendicitis (Swidsinski et al., 2011). The same finding was observed in mucosal samples from IBD patients suggesting that *A. muciniphila* is associated with healthy mucosa (Png et al., 2010).

A recent molecular inventory revealed that *Akkermansia* species are widely distributed amongst mammals, with a strong predominance in herbivores (Ley et al., 2008). In mice, *Akkermansia* species may constitute over 1% of the cecal microbial community (Ley et al., 2005; Turnbaugh et al., 2006). In hamsters and pythons their numbers were found to be high and increased during fasting, suggesting a relation with mucus production (Sonoyama et al., 2009; Costello et al., 2010). The frequent presence and high abundance of *A. muciniphila* in both human and other mammalian intestines underlines the relevance to address the role of this mucin-degrading commensal in the gut as well its impact on the host. As bacterial colonization may induce both general and species-specific responses in the host, we colonized germ-free mice with *A. muciniphila* and compared and contrasted its distribution, location, and impact on host transcriptional response with that of *L. plantarum*, a Gram-positive bacterium that principally ferments dietary sugars.

MATERIALS AND METHODS

ANIMALS

The study protocol was reviewed and approved by the Northern Stockholm Ethics Committee for Animal Experiments. Adult germ-free female NMRI–KI mice (45–65 days) were used ($n = 18$) for bacterial mono-association. The germ-free animals were inbred for >60 generations at the Laboratory of Medical Microbial Ecology at Karolinska Institute and they were housed in light-weight stainless-steel isolators (Gustafsson, 1959). All mice had free access to a steam-sterilized standard mouse chow (R36; Lactamin, Vadstena, Sweden) and to sterilized water. Artificial light was available between 6 a.m. and 6 p.m.; the temperature was 24 ± 2.2°C, and the humidity was 55 ± 10%. The germ-free status was checked weekly by inoculating fecal samples in different media incubated both aerobically and anaerobically at 20 and 37°C for up to 4 weeks.

BACTERIA AND GROWTH CONDITIONS

Two bacterial strains were used in this study, *A. muciniphila* Muc^T (ATTC BAA-835) and *L. plantarum* WCFS1 (NCIMB 8826). *A. muciniphila* was grown anaerobically in a basal mucin-based medium as previously described (Derrien et al., 2004) and *L. plantarum* was grown anaerobically at 37°C in Man–Rogosa–Sharpe broth (MRS; Le Pont de Claix, France).

MONO-ASSOCIATION

The germ-free mice were mono-associated using established protocols (Cardona et al., 2001). In brief, 10 ml of cultures in late

log phase of *A. muciniphila* Muc^T and *L. plantarum* WCFS1 were centrifuged (4500 rpm, 10 min). Pellets were resuspended in 1 ml of sterile anaerobic phosphate buffer saline (PBS) and dispensed into sterile ampoules which were heat-sealed. The external surface of each ampoule was sterilized with chromsulfuric acid before transfer into respective isolators. Inside the isolators, the ampoules were broken, and 0.2 ml (109 cfu/ml) of the strictly anaerobic *A. muciniphila* ($n = 6$) was inoculated intragastrically; *L. plantarum* ($n = 6$) was inoculated orally. Germ-free control mice ($n = 6$) were housed in separate isolators.

ESTABLISHMENT OF *A. MUCINIPHILA* IN MICE

To verify that mice were colonized with *A. muciniphila*, fecal samples (collected at day 3 and day 7) were diluted in mucin medium, incubated anaerobically at 37°C and inspected daily for growth for 6 days as previously described (Derrien et al., 2004). Exact enumeration of bacteria in intestinal samples was examined by a 16S rRNA quantitative PCR (qPCR) approach. In short, the genomic DNA from pure culture, ileal, cecal, and colonic contents was isolated using the Fast DNA Spin kit (Qbiogene, Inc., Carlsbad, CA, USA). PCR amplification of bacterial 16S rRNA genes was performed on genomic DNA of *A. muciniphila* using specific primers set AM1 (5′-CCT TGC GGT TGG CTT CAG AT-3′) and AM2 (5′-CAG CAC GTG AAG GTG GGG AC-3′; Collado et al., 2007).

PREPARATION OF SPECIMENS

After 7 days of colonization mice were killed by cervical dislocation and terminal ileum, cecum, and ascending colon specimens were sampled. Luminal contents were separated from the epithelium and were kept at −20°C for DNA extraction and bacterial enumeration by qPCR. Tissues were flushed with PBS. For RNA isolation, tissues were immediately preserved in five volumes of RNAlater (Ambion, Austin, TX, USA) and stored at 4°C until use. For histology, biopsies were fixed for 18 h at room temperature in 4% paraformaldehyde, pH 7.3 and subsequently processed for fluorescent *in situ* hybridization (FISH).

HISTOLOGY AND FLUORESCENT *IN SITU* HYBRIDIZATION

After preservation in paraformaldehyde, tissue samples were washed in phosphate buffer, dehydrated in an ethanol gradient, and embedded in paraffin. Five micrometer thick sections were mounted on Superfrost coated slides, dried, and incubated at 37°C for 16 h. For FISH, slides were deparaffinized in xylene and dehydrated in an ethanol gradient. Sections were overlaid with 100 μl hybridization buffer [0.9 M NaCl, 0.02 M Tris–HCl (pH 8.0), 0.01% sodium dodecyl sulfate] containing an oligonucleotide mixture (5 ng/μl) consisting of the *A. muciniphila* Cy3-labeled MUC-1437 (5′-CCTTGCGGTTGGCTTCAGAT-3′) and total bacterial FITC-labeled EUB-338 (5′-GCTGCCTCCCGTAGGAGT-3′) probes (Biolegio BV, Nijmegen4, The Netherlands). Hybridization was carried out at 50°C for 16 h in a humid chamber. After hybridization, the tissue sections were washed with a washing buffer (0.02 M Tris–HCl pH 8, 0.9 M NaCl) for 10 min at 50°C. Counterstaining was carried out with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich), and the slides were analyzed with a Nikon E600 epifluorescence microscope equipped with appropriate filter sets.

RNA EXTRACTION

Total mouse RNA from the intestinal tissue segments was extracted with Trizol® following supplier's protocol (Trizol reagent, Invitrogen). RNA was purified, treated with DNase, and concentrated using RNeasy mini kit (Qiagen). RNA quantity and quality were assessed spectrophotometrically (ND-1000, NanoDrop Technologies, Wilmington, USA) and Bioanalyzer nano chips (Bioanalyzer 2100; Agilent).

DNA MICROARRAY HYBRIDIZATIONS AND DATA ANALYSIS

Affymetrix GeneChip mouse genome 430 2.0 arrays (Affymetrix) containing 45,000 probe sets for the analysis of around 39,000 transcripts and variants of the approximately 22,000 mouse genes, were used to assess the transcriptional response to *A. muciniphila* and *L. plantarum* in the ileum, cecum, and colon. For each group and location, 2 μg of total RNA per mouse was subsequently pooled per group, and 10 μg were used for one cycle cDNA synthesis. Hybridization, washing, and scanning of GeneChip Mouse Genome 430 2.0 Array were done according to the manufacturer's protocol[1]. Normalization and probe-level intensities analysis were performed using the multi-mgMOS model (Liu et al., 2005) and a novel intensity-based Bayesian moderated T-statistic (IBMT; (Sartor et al., 2006) was used to identify all genes with a significantly altered transcriptional activity in response to the bacterial treatments. Gene significance cut-offs were cM/sM > 1, and PPLR values > 0.975 or < 0.025, corresponding to P-values < 0.05. Gene ontologies describe gene function in relation to a large and growing context of biological knowledge at three levels: biological process, molecular function, or cellular component, using a systematic classification and a formal vocabulary. Differential datasets were analyzed using the ingenuity pathways analysis (IPA) software tool[2]. Basically, IPA annotations follow the gene ontology (GO) annotation principle, but are based on a proprietary knowledge base of over 1,000,000 protein–protein interactions. GO annotations are used by Ingenuity in order to find overrepresented cellular functions and canonical (conserved in human, mouse, and rat) pathways. The IPA output includes metabolic and signaling pathways with statistical assessment of the significance of their representation being based on Fisher's Exact Test. Here, this test calculates the probability that genes participate in a given pathway relative to their occurrence in all other pathway annotations. Array data have been submitted to the Gene Expression Omnibus, accession number: GSE18587.

QUANTITATIVE REVERSE TRANSCRIPTION PCR

cDNA was synthesized using 500 ng of total RNA employed for microarray analyses using Superscript III reverse transcriptase; random primers according to supplier's protocol (Invitrogen). qPCR on 6 ng cDNA derived from pooled RNA used for microarray analysis was performed on a Bio-Rad iCycler (Bio-Rad Laboratories) using gene-specific primers (0.2 μM) for selected genes (**Table 1**) and 1× SYBR Green PCR Master Mix buffer (Bio-rad). Each pair was designed to generate a 100 to 200-bp amplicon

[1] http://www.affymetrix.com
[2] http://www.ingenuity.com

Table 1 | Primers sequences used in this study to validate arrays data.

Gene name	Gene symbol	Function	Accession number	Sequence (5′ → 3′)
18S rRNA	*18S*	Ribosome biogenesis and assembly	X00686	F: CATTCGAACGTCTGCCCTATC R: CCTGCTGCCTTCCTTGGA
S100 calcium binding protein g	*S100g*	Cell adhesion	NM 009789	F: ATGTGTGCTGAGAAGTCTCCT R: CGCCATTCTTATCCAGCTCCTT
Glycosylation dependent adhesion molecule 1	*Glycam1*	Cell adhesion	NM 008134	F: GTCCTGCTATTTGTCAGTCTTG R: CCTGGTCTTGATTCTCTG
Lipoprotein lipase	*Lpl*	Lipid metabolism	NM 008509	F: GGGAGTTTGGCTCCAGAGTTT R: TGTGTCTTCAGGGGTCCTTAG
Carbonic anhydrase 1	*Car1*	Nitrogen metabolism	BC011223	F: ACTGGGGATATGGAAGCGAA R: TGCAGGATTATAGGAGATGCTGA
Regenerating islet-derived family, member 4	*Reg4*	Vascularization	NM_026328	F: TGAGCTGGAGTGTCAGTCATA R: CAATCCACACAGGCAGGTTTC
Small proline-rich protein 2a	*Sppr2a*	Vascularization	AV371678	F: CCTTGTCGTCCTGTCATGT R: GGCATTGCTCATAGCACACTAC

were derived from Primerbank[3] and primers were purchased from Biolegio BV (Nijmegen, The Netherlands). The specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. All data were normalized to an internal 18S mRNA control ($\Delta\Delta$CT analysis), of which the expression did not differ between germ-free and mono-associated mice (data not shown).

RESULTS

AKKERMANSIA MUCINIPHILA AND *L. PLANTARUM* COLONIZE DIFFERENT INTESTINAL REGIONS OF GERM-FREE MICE

Efficient and reproducible colonization of germ-free mice was obtained by a single intragastric dose of 10^9 *A. muciniphila* cells grown anaerobically on mucin-containing medium. To verify that *A. muciniphila* had colonized the intestinal tract, DNA was extracted from intestinal regions (ileum, cecum, and colon), and used for 16S rRNA qPCR quantification. In all mice that were mono-associated with *A. muciniphila*, bacterial numbers present in the intestinal lumen reached 1.87 ($\pm$2.37) $\times$ 10^8 cells/g of ileal content, 3.12 ($\pm$0.7) $\times$ 10^{10} cells/g cecal content, and 1.73 ($\pm$0.6) $\times$ 10^9 cells/g colonic content. All *A. muciniphila*-associated mice were healthy throughout the study period of 7 days. Subsequently, FISH analysis was performed on all three intestinal regions using the specific oligonucleotide probe MUC-1437 that is complementary to part of a hypervariable region of *A. muciniphila* (Derrien et al., 2008). Aggregations of *A. muciniphila* cells were found in all sections of formaldehyde fixed samples, within a distance of 50 μm to the epithelium (**Figure 1**). This analysis indicated that *A. muciniphila* is closely associated with the mucus layer covering the epithelial cells and support the notion that *A. muciniphila* is utilizing mucin *in situ* to sustain growth (Derrien et al., 2004). To further evaluate this correlation between intestinal location and use of carbon sources, we studied the *in vivo* location of *L. plantarum*, capable of growing on dietary carbohydrates but a non-mucin-degrader. Based on 16S rRNA-gene qPCR quantification, *L. plantarum* colonized germ-free mice at levels similar to *A. muciniphila*, with the exception of the small intestine where the luminal colonization was 10-fold higher (10^9 cells/g) than in cecum or colon, consistent with previous studies (Marco et al., 2007). DAPI-stained sections from the three intestinal regions showed presence of non-aggregated, long cells with the morphology of *L. plantarum* cells (**Figure 1H**). Such cells were not observed in control biopsy sections from germ-free mice, indicating an exclusive luminal location of *L. plantarum* that contrasts with that of the mucosa-location of *A. muciniphila*.

COLONIZATION BY *A. MUCINIPHILA* AND *L. PLANTARUM* INDUCE SPECIFIC TRANSCRIPTIONAL RESPONSES IN THE THREE INTESTINAL REGIONS

To examine the impact of *A. muciniphila* and *L. plantarum* colonization on host mucosal gene expression profiles, microarrays were used to determine the transcriptome of the host before and after colonization using Bioconductor and third-party software (**Figure 2**). The total number of significantly regulated genes was highest in the ileum of mice mono-associated with *L. plantarum* (1233) followed by the colon (608) and cecum (449). In *A. muciniphila* mono-associated mice, the numbers of differentially expressed genes was highest in the colon (442), followed by the ileum (253), and the cecum (211; **Figure 3**). The percentage of shared genes between *A. muciniphila* and *L. plantarum* was highest in the colon (17.0%), followed by the cecum (9.9%), and the ileum (9.1%). After colonization by *A. muciniphila*, less than 10% of the differentially expressed genes occurred in all three intestinal regions (**Figure 4**). The percentage of common regulated genes between the cecum and colon was 8.3%, while 4.8% between the ileum and cecum, and 4% between the ileum and colon were shared. In *L. plantarum* mono-associated mice, percentages followed the same tendency but were twice as high. Together, the expression data and histology show that the mouse intestines undergo region-specific, differential transcriptome changes following colonization by different bacteria. Array data were validated by quantitative reverse transcription PCR (qRT-PCR) for

[3]http://pga.mgh.harvard.edu/primerbank/

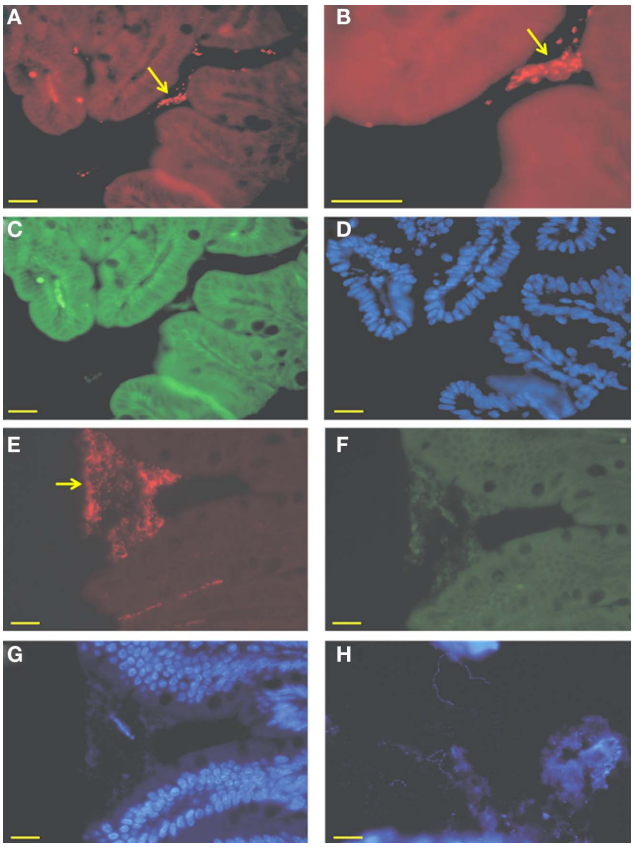

FIGURE 1 | Micrograph of intestinal biopsy samples from mono-colonized mice. (A–D) Ileal biopsies and colonic **(E–G)** of mice mono-associated with *A. muciniphila*. Cells were hybridized with the Cy3-labeled MUC-1437, specifically staining *A. muciniphila*. **(A,B,E)**, aggregates of *A. muciniphila* cells are zoomed and closely associated with the epithelial cells **(B)**, FITC-labeled EUB-338, targeting all bacteria **(C,F)** and a DAPI staining **(D,G)**. Arrows indicate aggregates of *A. muciniphila*. **(H)** Cecal biopsy from *L. plantarum* mono-associated mouse stained with DAPI. Bar represents 10 μm.

six selected genes harboring diverse fold change in ileum and colon from mono-colonized mice. qRT-PCR confirmed the expression levels for the selected genes (**Table 2**).

COLONIZATION BY *A. MUCINIPHILA* INDUCES EXPRESSION OF GENES INVOLVED IN REGULATION OF IMMUNE RESPONSES AND HOMEOSTASIS

Although bacteria are formally seen as "non-self" by the host immune system, colonization of the mammalian gut by bacteria leads in healthy individuals to a more or less neutral, non-inflammatory balance between pro- and anti-inflammatory immune responses, a condition that is known as gut immune homeostasis (Artis, 2008). Since mice colonized by the two different commensal bacteria did not show any sign of inflammation or discomfort, we assumed that the transcriptomes altered after colonization did show part of the transcriptional regulation of immune homeostasis. In order to extract as much biological function and context out of the altered transcriptomes in the mouse

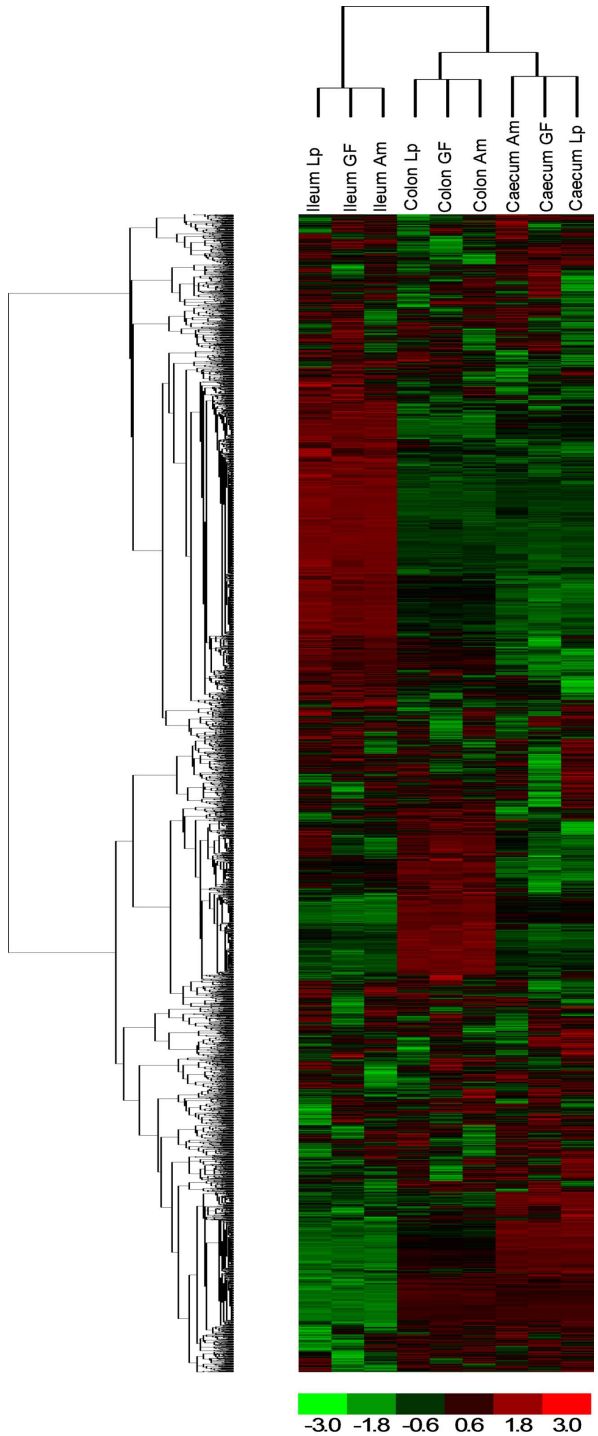

FIGURE 2 | Hierarchical clustering of genes obtained using D-Chip software. Hierarchical clustering was performed on intensity values generated by Affymetrix MAS 5.0 software, showing differential responses in the three locations (ileum, cecum, and colon) and with bacteria [*A. muciniphila* (Am) and *L. plantarum* (Lp) compared to germ-free (GF) mice]. Each column corresponds to one array (pool of six RNA) and each frame corresponds to one probe set. Red corresponds to an up-regulation of the gene expression and green to a down-regulation. Black corresponds to no change.

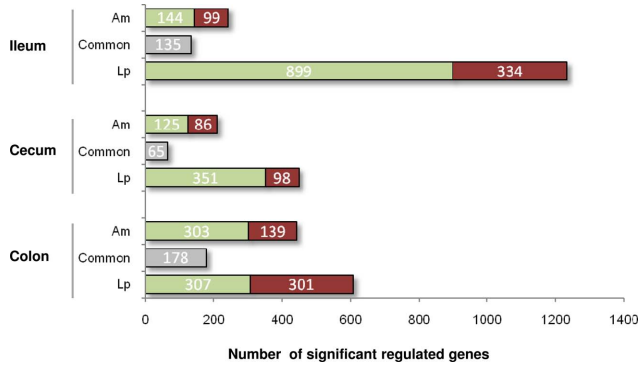

FIGURE 3 | Number of significantly up- (red) and down- (green) regulated genes in the ileum, cecum, and colon of mice mono-associated with *A. muciniphila* and *L. plantarum* vs. germ-free mice. Gray represents common regulated genes.

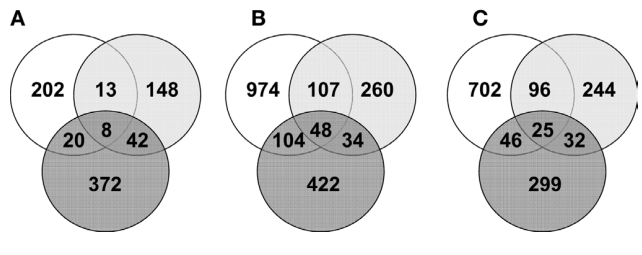

FIGURE 4 | Venn diagrams representing the number of common significantly regulated genes in the ileum (white), cecum (light gray), and colon (dark gray) of mice mono-associated with *A. muciniphila* (A) and *L. plantarum* (B) interaction compared to germ-free mice data and *L. plantarum* compared to *A. muciniphila* data (C).

Table 2 | Expression levels of selected genes in intestinal samples from *A. muciniphila* and *L. plantarum* mono-associated mice compared to the germ-free mice.

Gene	Location	*A. muciniphila*		*L. plantarum*	
		Arrays	qRT-PCR	Arrays	qRT-PCR
Reg4	Colon	−1.07	−5.02	−4.29	−12.01
Sppr2a	Ileum	1.06	1.65	−3.16	−4.29
Car1	Ileum	−2.33	−3.10	−29.62	−7.38
Lpl	Ileum	−1.72	−1.64	2.83	1.53
Glycam1	Colon	4.50	3.14	1.59	2.08
S100g	Colon	2.48	2.51	−2.08	−3.03

Assays were performed in triplicate. Data were normalized to 18S ribosomal RNA and results are expressed in fold change.

intestines after colonization by *A. muciniphila* and *L. plantarum*, IPA was used.

In the cecum of mice mono-associated with *A. muciniphila*, the region that was most efficiently colonized by this bacterial species, colonization of the mucosa apparently led to an altered expression of genes participating in membrane metabolism and signaling and antigen presentation pathways (**Figure 5**; **Table 3**). The major cellular functions that were modulated after cecal colonization by *A. muciniphila* were involved in the regulation of epithelial homeostasis and cell fate (**Table 3**). Cecal colonization by *A. muciniphila* did also result in up-regulation of genes involved in antigen presentation of leukocytes (**Figure 5**). In the colon, *A. muciniphila* induced multiple immune response-related pathways, both innate and adaptive (**Figure 6**; **Table 3**). Finally, ileal colonization by *A. muciniphila* led to differential expression of genes involved in metabolic and signaling pathways, mainly via modulation of PPARα-dependent processes. Most of these genes and pathways participate in lipid metabolism, small molecule biochemistry, and metabolic homeostasis (**Table 3**).

We noted that several categories of those mouse genes that showed the strongest alterations in transcription after intestinal colonization by *A. muciniphila* were implicated in regulation of the immune response and cell death and proliferation, the latter category especially in the colon (**Figure 7**). Immune response-associated genes in the colon were involved in chemotaxis and complement cascade, parts of the innate immune response, but also in cell adhesion and the maturation of B and T cells. This might suggest that colonization of the mouse intestine by *A. muciniphila* modulates the host immune system. Genes involved in vitamin and mineral metabolism were also differentially expressed in the colon, possibly reflecting a modulation of xenobiotic metabolism.

The expression profiles suggest that colonization by *A. muciniphila* leads to coordinated differential expression of genes involved in metabolic and immune response-regulatory processes. The balance between the involved pathways and processes differs per intestinal region; upon colonization, immune-regulatory processes were most pronounced modulated in the colon, followed by cecum, and ileum (**Table 3**).

COLONIZATION BY *L. PLANTARUM* STIMULATES TRANSCRIPTION OF GENES INVOLVED IN CELL SIGNALING, LIPID METABOLISM, AND GROWTH AND PROLIFERATION

Compared to *A. muciniphila*, a smaller proportion of genes that were differentially expressed after colonization of the intestines by *L. plantarum* were involved in immune response. Only a few genes encoding immune-regulatory molecules were differentially expressed; among these were the acute phase-response genes Reg3α and Reg3γ. According to IPA, the only immune response-related pathway that was significantly modulated was the B cell receptor signaling pathway, in the cecum and ileum. In the ileum, colonization by *L. plantarum* mainly led to regulation of genes participating in activation and regulation of lipid and fatty acid metabolism (**Figures 8** and **9**; **Table 3**). In the cecum, genes involved in metabolic and immune response signaling and involved in normal and xenobiotic metabolism were differentially expressed after colonization by *L. plantarum*. In the colon, genes involved in stress response signaling and tissue development and function were differentially regulated after colonization. Summarizing, after colonization of mouse intestines by *L. plantarum*, most of the differentially expressed genes and pathways in ileum, cecum, and colon were involved in regulation of lipid and fatty acid metabolism, cellular signaling, molecular

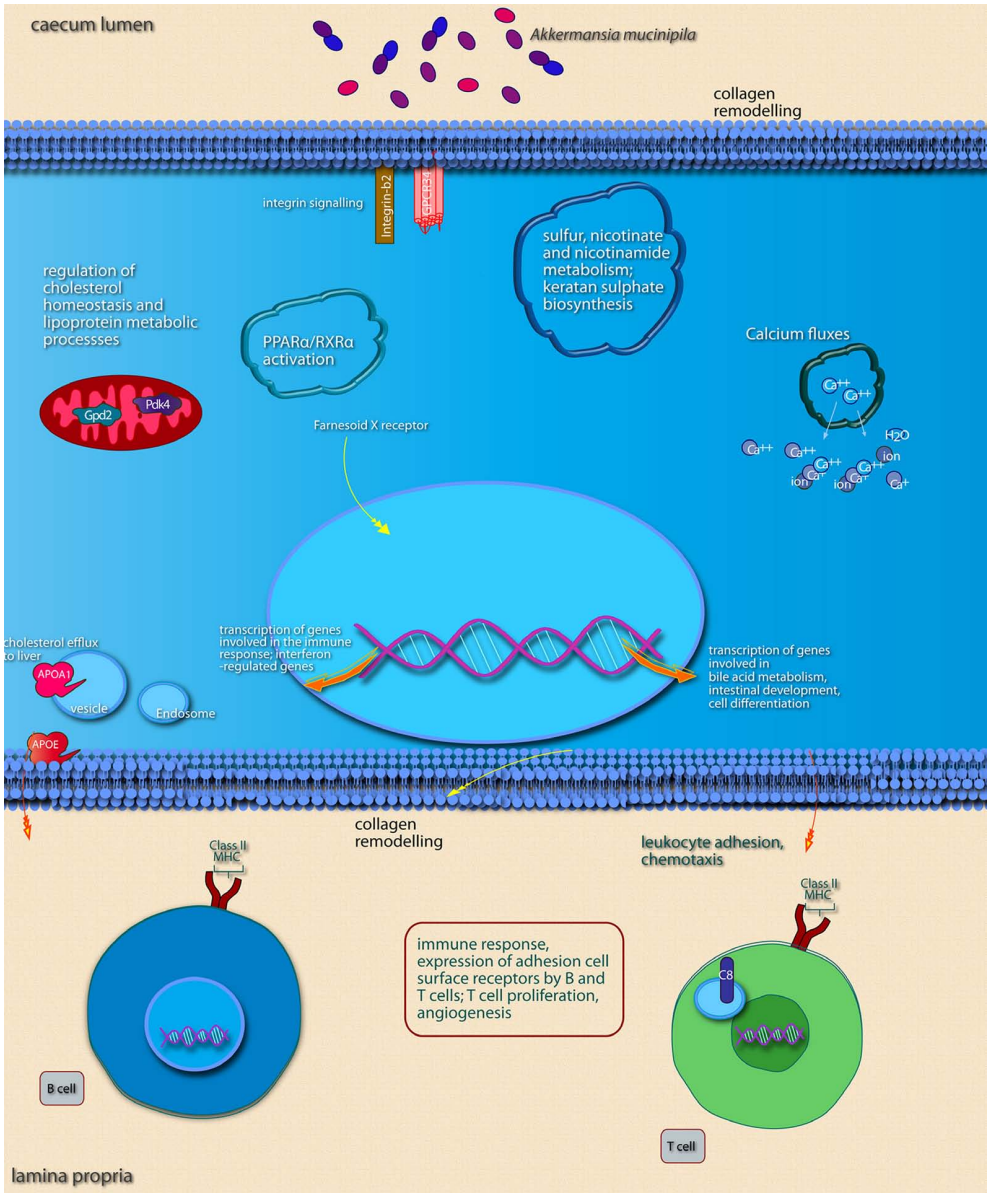

FIGURE 5 | Main cellular pathways and processes inferred to be modulated or activated after colonization of mouse cecum by *A. muciniphila*. Only induced pathways are shown. Distinguishing features are the induction of pathways involved in (epithelial) cell membrane modifications, sulfur metabolism, and pathways involved in the maturation of immune cells via induction of surface receptors. Pathways were considered to be induced if (nearly) all of the genes belonging to the pathway were up-regulated. The upper part of the image shows pathways and processes that are expected to occur in epithelial cells as well as pathways and processes with undefined or non-specific localization. Pathways and processes named in text "balloons" were among the most significantly modulated pathways according to IPA canonical pathway analysis. The lower part of the image shows pathways known to be regulated in immune cells of the lamina propria. In order to determine in which cell type and subcellular compartment a gene product was localized, the Entrez Gene GO annotation, Entrez Gene GeneRIFs, and scientific literature information were used. Interacting pathways and processes are grouped together as well as possible, both in terms of annotation as well as within the graphical space limitations.

transport, and xenobiotic metabolism (**Table 3**). In the colon, colonization by *L. plantarum* did also stimulate genes that regulate tissue development.

BACTERIAL COLONIZATION LEADS TO SPECIFIC EXPRESSION PROFILES THAT LEAD TO TOLERANCE TO MICROBIOTA

A direct comparison of differential gene expression in response to *L. plantarum* and. *A. muciniphila* is of interest since this may show if intestinal colonization of mouse hosts by different bacteria shows common or more specific expression profiles. So far, most of the described comparisons involve colonized and germ-free hosts. These comparisons may not be suitable to find processes that are exclusively altered by one specific bacterial species; specific processes may be obscured by the consequences of microbial colonization *per se*. For instance, genes involved in antigen presentation pathways were mainly down-regulated

Table 3 | Canonical pathways in the mouse ileum, cecum, and colon that are more significantly modulated upon colonization by *A. muciniphila* or *L. plantarum*.

Intestinal region	Comparison	
	***A. muciniphila* vs. GF**	***L. plantarum* vs. GF**
Ileum	PPARα–RXRα activation	PPARα–RXRα activation
	Tryptophan metabolism	LPS/IL-1 mediated inhibition of RXR function
	Serotonin receptor signaling	Propanoate metabolism
	Synthesis and degradation of ketone bodies	Protein ubiquitination pathway
	Dopamine receptor signaling	Aryl hydrocarbon receptor signaling
	Death receptor signaling	Pyruvate metabolism
Cecum	Keratan sulfate biosynthesis	LPS/IL mediated inhibition of RXR function
	PPARα/RXRα activation	Aryl hydrocarbon receptor signaling
	Integrin signaling	Glutathione metabolism
	Antigen presentation pathway	Beta-adrenergic signaling
	Sulfur metabolism	Xenobiotic metabolism signaling
	Nicotinate and nicotinamide metabolism	p53 signaling
Colon	Antigen presentation pathway	NRF2-mediated oxidative stress response
	B cell receptor signaling	p38 MAPK signaling
	Leukocyte extravasation signaling	Circadian rhythm signaling
	T cell receptor signaling	
	IL-4 signaling	
	Complement and coagulation cascades	

Significance was calculated by Fisher's Exact Test using ingenuity pathway analysis (IPA). These pathways may be interconnected since they may share common elements, e.g., NF-κB and G-protein coupled receptor (GPCR) signaling can play roles in multiple pathways.

in the cecum following colonization by *L. plantarum*. In contrast, colonization by *A. muciniphila* tended to up-regulate genes that are involved in antigen presentation pathways (**Table 3**). In direct comparisons of gene transcription after bacterial colonization, gene expression profiles of mice colons colonized by *A. muciniphila* showed relatively higher (compared to colonization by *L. plantarum*) up-regulation of genes participating in immune response signaling and ERK/MAPK signaling. Interestingly, a few genes that encode cell proliferation factors such as epidermal growth factor (Egf), early growth receptor 1 (Egr1), and connective tissue growth factor (Ctgf) were down-regulated in colons colonized by *A. muciniphila*, compared to expression of the same genes in colons colonized by *L. plantarum*. Furthermore, ilea colonized by *A. muciniphila* showed a relatively lower expression of genes involved in lipid and fatty acid metabolism compared to ilea colonized by *L. plantarum*. In addition, genes involved in cellular growth and developmental pathways were differentially regulated in ilea, with the inference that growth and proliferation were less stimulated in *A. muciniphila*-colonized ilea compared to ilea colonized by *L. plantarum*.

The comparative analysis of the response to *A. muciniphila* and *L. plantarum* show that colonization of germ-free mice by bacteria that utilize different carbon sources and are located in different sites leads to a differential expression of genes involved in cellular processes and pathways such as the immune response, lipid metabolism, and cell fate. Differential gene expression was not only dependent on the colonizing bacterial species but also on the intestinal region. Despite the up-regulation of genes involved in the immune responses, colonized mice did not develop microscopically visible inflammation nor did they show any sign of discomfort. This suggests that the transcriptional profiles we obtained are involved in the regulation of immune tolerance toward the Gram-negative *A. muciniphila* and the Gram-positive *L. plantarum* together with metabolic homeostasis of the colonized intestine. Although colonization by *A. muciniphila* did lead to a higher number of differentially expressed genes involved in regulation of the immune system, there were no strong indications that these genes participated in driving a pro-inflammatory response (e.g., up-regulation of a pro-inflammatory p50–p56 NF–κB complex (Wietek and O'Neill, 2007). Both in cecum and ileum, after intestinal colonization by *A. muciniphila*, and compared to GF control mice, immune response-associated genes were not enriched apart from genes involved in the antigen presentation pathway (**Table 3**). Likely, immune homeostasis had already been reached here, within the 7-days before RNA was extracted, and maintained via immune regulators such as the gene CEBPD (NF–IL6–beta), a regulator of genes involved in immune and inflammatory responses and genes associated with activation and/or differentiation of macrophages. In the colon, where the regulation of immune response-associated genes was highest, over 60 genes, including 16 genes encoding CD antigen markers and 10 genes encoding immune cell membrane receptors were up-regulated, nearly 14% of the total of regulated genes. These increased gene transcripts likely reflect presence of a larger number of immune-competent leukocytes in the lamina propria and are not themselves indicative of a pro-inflammatory immune response. Disproportionate immune responses in the colon may have been directly inhibited through activity of up-regulated serine protease inhibitors (serpins) and indirectly by up-regulation of genes encoding factors that modulate pro-inflammatory signaling, such as IκBε, an inhibitor of nuclear translocation of NF–κB. We compared our expression data with those obtained by profiling wound healing-associated inflammation in mice as reported by Cooper et al. (2004) and found less than 5% similar genes between our colonization experiments and their inflammation datasets. We propose that in mice, establishment of tolerance is achieved fast, especially in the small intestine, and is maintained through the activity of transcription factors in the small intestine, and through protection from overt cytotoxic proteolytic processes and avoidance of pro-inflammatory signaling in the colon.

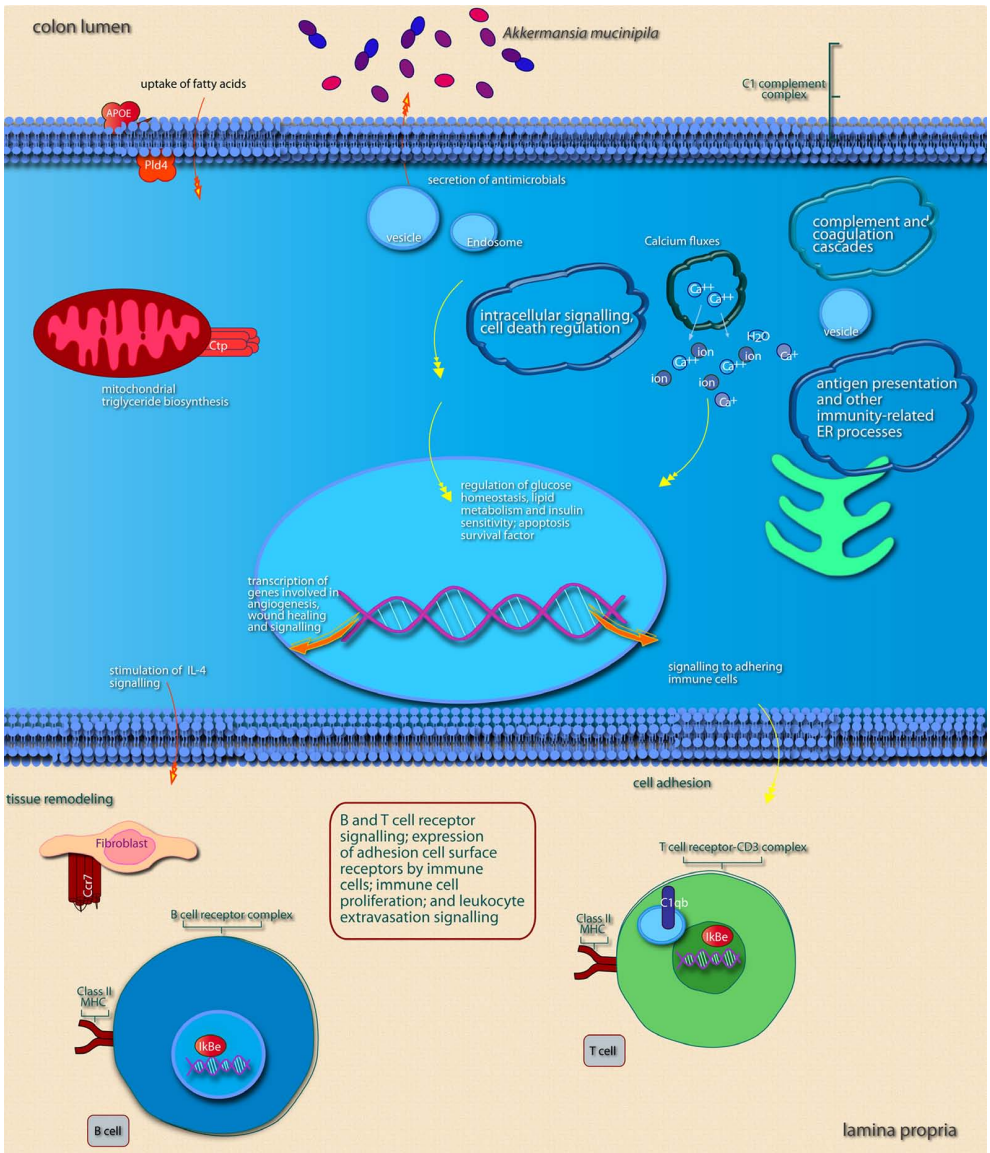

FIGURE 6 | Main cellular pathways and processes inferred to be modulated or activated after colonization of mouse colon by *A. muciniphila*. Distinguishing features are the induction of pathways involved in intracellular signaling, induction of secretion of (antimicrobial) factors, and of pathways that promote expression of immune cell receptors that mediate adhesion and T or B cell receptor signaling. For further explanation, see legend of **Figure 5**.

DISCUSSION

In this study we determined the transcriptional profiles of the mouse intestinal mucosa to examine the balanced changes in the mucosal transcriptome of a germ-free host following colonization by commensal intestinal bacteria. We colonized germ-free mice with bacteria that differed notably in physiology and phylogeny, viz. the Gram-negative, strictly anaerobic mucin-degrading *A. muciniphila* and the Gram-positive utilizer of dietary carbohydrates *L. plantarum*, which is incapable of utilizing mucin. Both bacteria colonized the intestinal tract within 7 days after a single inoculation. We used microarray profiling since this has been shown to be a useful tool for studying *in vivo* interactions between eukaryote hosts and their intestinal bacteria (Di Caro et al., 2005; Ukena et al., 2005; Baken et al., 2006).

Transcriptional changes upon colonization by *A. muciniphila* involved a large amount of genes participating in the immune response in comparison with germ-free mice or those colonized by *L. plantarum*. Colonization by *L. plantarum*, on the other hand, mainly stimulated expression of genes involved in the metabolism. Differentially expressed genes differed depending on bacterial colonizer and intestinal site to such an extent that only few genes were differentially expressed in all three intestinal regions.

The largest changes in gene transcription after colonization by *A. muciniphila* were observed in the colon; for *L. plantarum*, the largest changes were found in the ileum. This may be partly

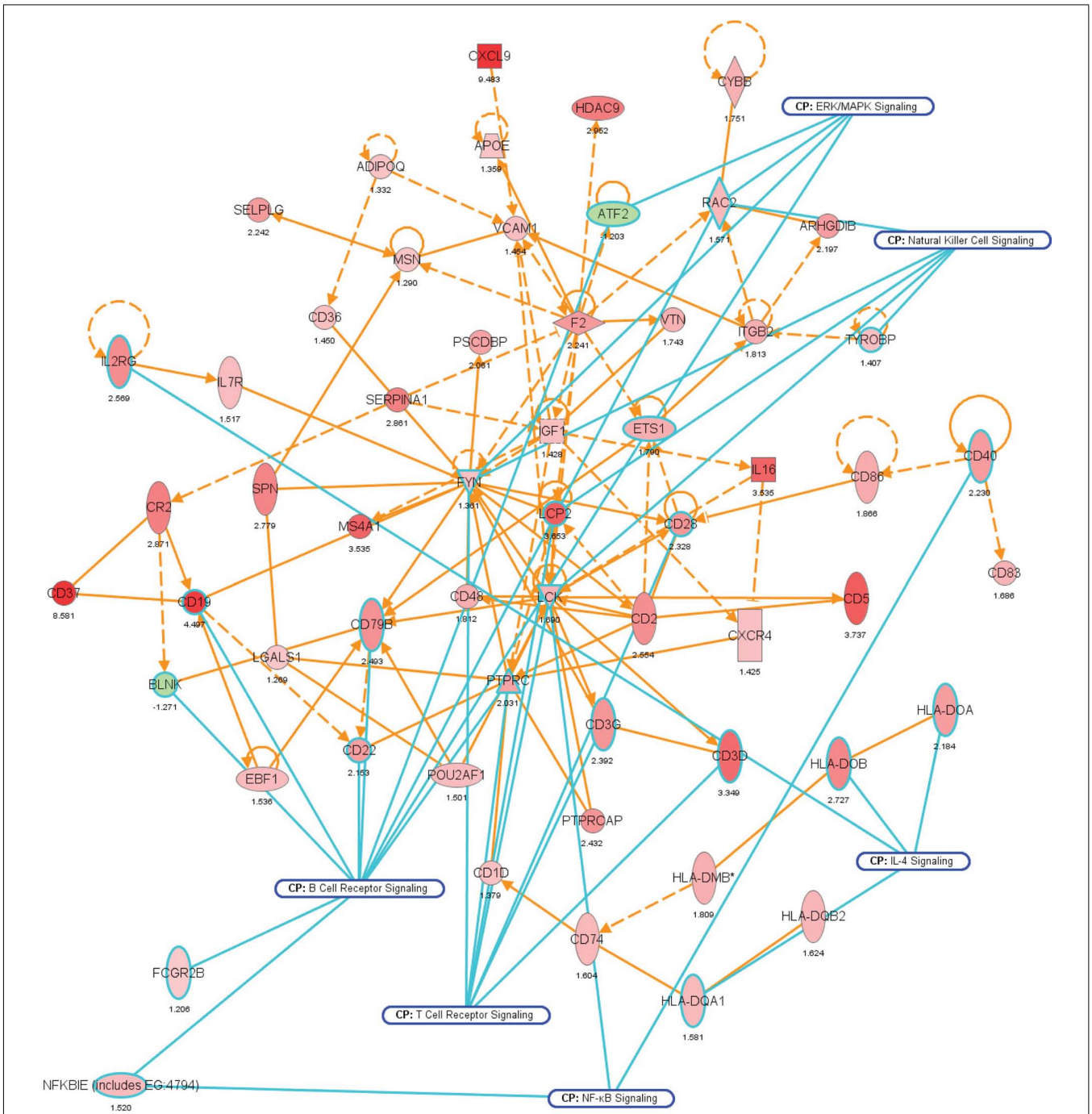

FIGURE 7 | Network representation of altered cellular functions during colonization of the mouse colon by *A. muciniphila* for the gene ontology (GO) category "immune response." The network shows that most of the genes regulating the immune response are up-regulated during colonization. Central regulatory genes encode growth factors (e.g., Igf1), cell surface molecules (Cd19, Cd37), (receptor) phosphatases (Ptprc or Cd45), and protein kinases (Lck, Fyn) that regulate (immune) cell proliferation and differentiation. The protein interaction network was derived from the transcription data using ingenuity pathway analysis (IPA). Nodes indicate proteins, the yellow lines connecting them represent interactions (e.g., protein–protein binding or phosphorylation events) that are mapped by Ingenuity's knowledge base (see Materials and Methods). Colors indicate altered transcription of the encoding genes, with deeper shades of red indicating stronger up-regulation, and deeper shades of green indicating stronger down-regulation. The genes and their encoded proteins that are used to reconstruct specific networks were selected based on overrepresentation of GO categories ("cellular functions" in IPA) and involvement in significantly (see Materials and Methods) modulated canonical pathways. The overlays with blue radiating lines represent some of these canonical pathways (CP) and are useful to indicate in which cellular pathways differentially regulated genes and their interacting products participate for the given GO categories. Note that in this network exemplifying the genetic regulation of the mouse colonic immune response to *A. muciniphila*, most of the genes are up-regulated, indicating that the immune response was induced following colonization of these bacteria.

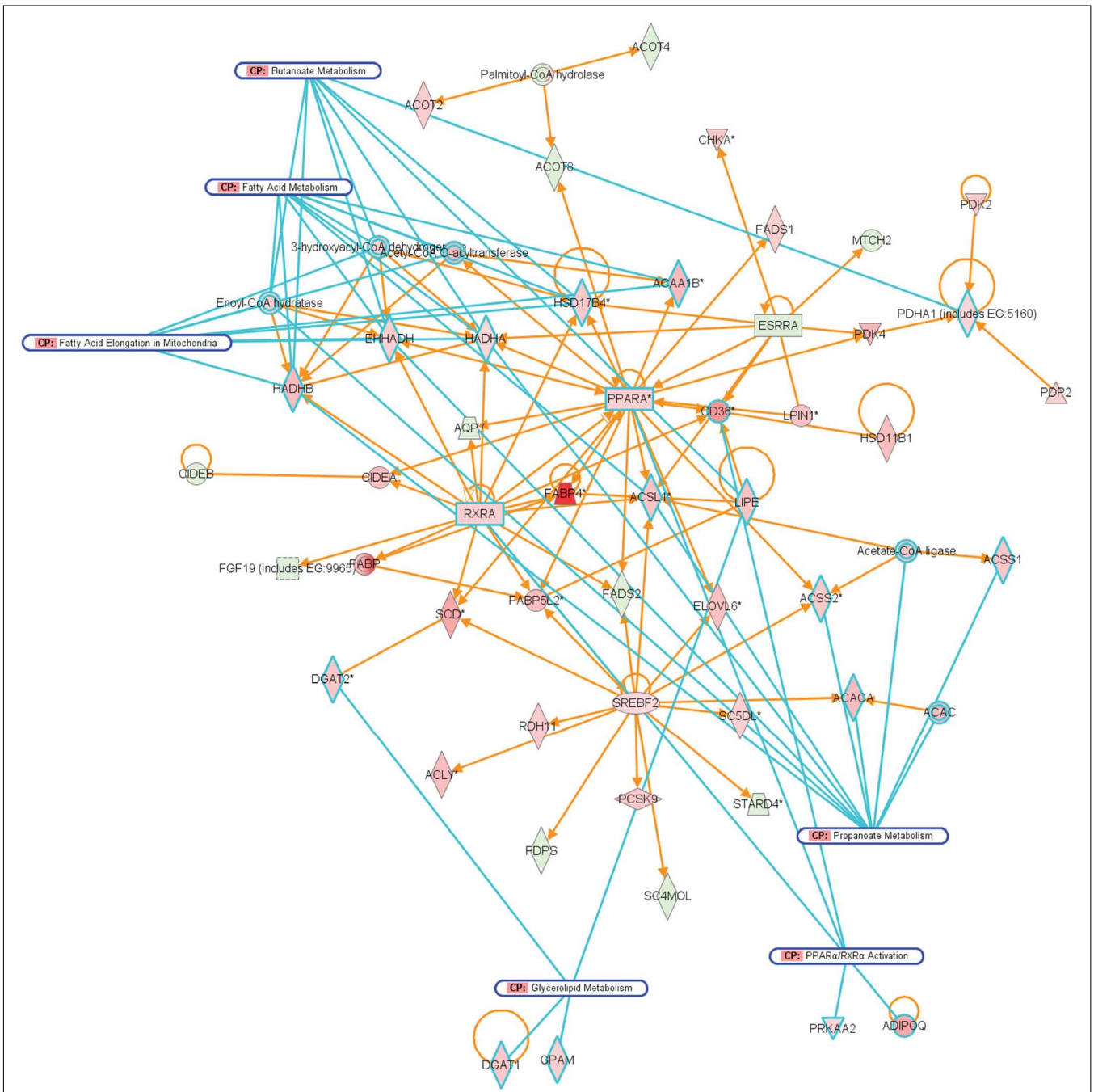

FIGURE 8 | Network representation of altered cellular functions during colonization of the mouse ileum by *L. plantarum* for the category "lipid metabolism." The network shows that most of the genes that are co-expressed with the major regulators, PPAR and RXR are up-regulated, or up-regulated through the activity of the sterol regulatory element binding transcription factor 2 (Srebf2).

explained by the different bacterial numbers reached in the three intestinal regions. *L. plantarum* reached ten times higher numbers in the ileum compared to *A. muciniphila*, whereas both bacteria colonized the colon and cecum to the same extent. In the cecum, where the highest bacterial colonization level was observed, similar responses were observed as in the colon. Interestingly, keratan sulfate biosynthesis was among the major regulated pathways in mouse cecal epithelia following colonization by *A. muciniphila*. This pathway is a feature of epithelial tissues responding to physiological changes such as typically result from development and wounding but it is also involved in mucin production (Funderburgh, 2000). It is possible that these changes are involved in establishing novel biosynthesis levels of epithelial cell components including mucins, since presence of *A. muciniphila* in the

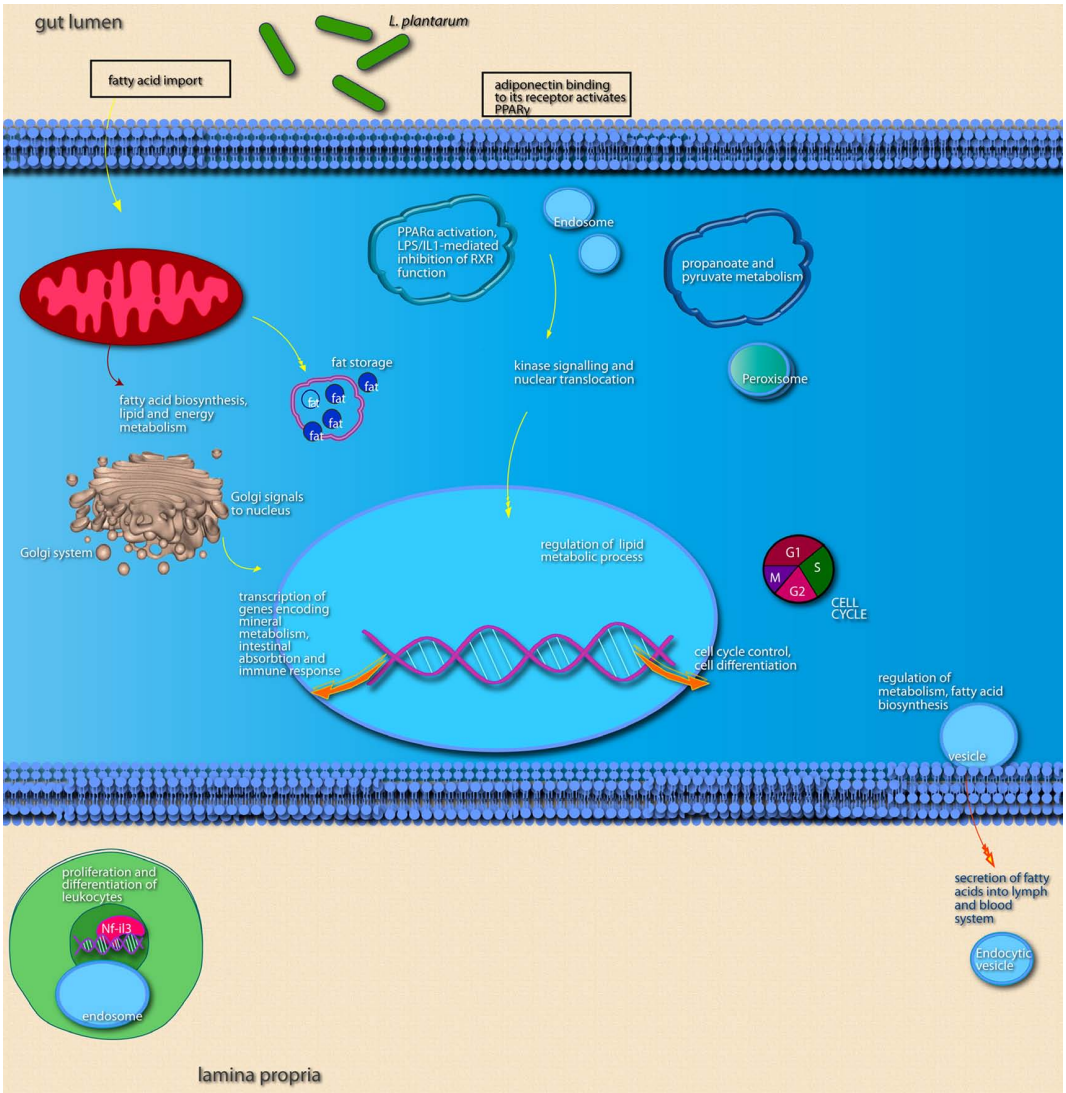

FIGURE 9 | Main cellular pathways and processes inferred to be modulated or activated after colonization of mouse ileum by *L. plantarum*. Distinguishing features are the up-regulation of relatively high numbers of genes encoding factors involved in lipid metabolism and of genes encoding transcription factors that regulate intestinal absorptive functions and the immune response.

mucus layer may lead to an increased turn-over of, and demand for, extracellular components. The genome of *A. muciniphila* has been recently elucidated and contains various genes that are predicted to code for secreted sulfatases (Derrien et al., 2010; van Passel et al., 2011), compatible with the fact that mucin serves as carbon, nitrogen, and sulfur source for *A. muciniphila*.

In the ileum, five canonical pathways that showed significant modulation after colonization by *L. plantarum* were also modulated following colonization by *A. muciniphila*. These pathways were mainly involved in regulation of cell signaling and lipid metabolism, small molecule biochemistry, and molecular transport (**Table 3**). Note that no enrichment of genes that may be involved in the regulation of cellular responses to cellular damage (such as the keratan sulfate biosynthesis pathway) was found in the ileal response to *L. plantarum*. These comparative differences became more obvious when transcriptional profiles of intestines colonized by *A. muciniphila* or *L. plantarum* were compared to each other rather than the response in germ-free animals. Indeed, direct comparisons of genes expressed in intestines colonized by *A. muciniphila* vs. intestines colonized by *L. plantarum* revealed that, in general, the intestinal regions colonized by *L. plantarum* showed enriched expression of genes that participate in cellular lipid metabolism, growth, and proliferation, whereas intestinal regions colonized by *A. muciniphila* tended to show enriched expression of genes involved in immune responses, death receptor signaling, and responses to cellular compromise. As the *L. plantarum* is a Gram-positive and *A. muciniphila* a Gram-negative species, these differential responses could be due to specific components from the bacterial cell envelopes involved in host signaling via cognate receptors. Alternatively, the differential host response could

be established because of a more intensive contact of the mouse mucosa with *A. muciniphila* cells that were found in aggregates at less than 50 μm distance from epithelial cells (**Figure 1**). Aggregated colonies were also observed in cultures of *A. muciniphila* growing in a mucin-based medium. In contrast, *L. plantarum* cells were more randomly distributed in the gut lumen (**Figure 1H**) and are expected to have far less opportunities for direct contact with the mouse mucosa.

In a recent study, the mouse colonic mucus layer has been investigated for the presence of bacteria (Johansson et al., 2008). Interestingly, no bacteria were present in the proximal 50 μm layer that mainly consisted of Muc2, while bacteria were found in the more accessible outer layer. This may suggest that in a germ-free background, *A. muciniphila* is able to colonize the inner mucus layer while in conventionalized mice it has to compete with other bacteria. Alternatively, it is possible that the method used for fixing the biopsies in the studies vary and explain the results while it is also possible that *A. muciniphila* may have escaped earlier detection (Johansson et al., 2008) as a general eubacterial probe was used that only poorly reacts with *Akkermansia* cells (Derrien et al., 2008).

Overall, our data show that colonization of germ-free mouse intestines by different bacteria leads to differentially altered host transcriptomes with a bias toward balanced immune responses indicative of tolerance for *A. muciniphila*, and toward a promotion of lipid and fatty acid metabolism, growth, and proliferation for *L. plantarum*. Since the germ-free mice did not develop any microscopic symptoms of intestinal disease, stress, or any other sign of discomfort following microbial colonization, we conclude that the colonization by these two very different bacteria led to a non-inflammatory, commensal interaction and to intestinal tolerance. In two different studies, human mucosal *in vivo* transcriptomes were obtained after 6 h exposure of the proximal part of the small intestine to the same *L. plantarum* strain as used in the present study (Troost et al., 2008). Comparison of these human and mouse transcriptomes obtained after colonization by *L. plantarum* shows that this bacterial isolate induces similar transcriptional changes (including modulation of genes participating in lipid metabolism, a non-inflammatory immune response, and cellular proliferation) in these two mammalian hosts. This is of great interest since mice are frequently used as a model system to gain a better understanding of the function of the intestinal microbiota. Further studies will be aimed at exploration of the cellular processes described here, and at identification of the bacterial products that mediate the contact (direct contact or soluble factors) with host cells in the intestine.

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Altered gut microbiota and endocannabinoid system tone in obese and diabetic leptin-resistant mice: impact on apelin regulation in adipose tissue

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Growing evidence supports the role of gut microbiota in the development of obesity, type 2 diabetes, and low-grade inflammation. The endocrine activity of adipose tissue has been found to contribute to the regulation of glucose homeostasis and low-grade inflammation. Among the key hormones produced by this tissue, apelin has been shown to regulate glucose homeostasis. Recently, it has been proposed that gut microbiota participate in adipose tissue metabolism via the endocannabinoid system (eCB) and gut microbiota-derived compounds, namely lipopolysaccharide (LPS). We have investigated gut microbiota composition in obese and diabetic leptin-resistant mice (*db*/*db*) by combining pyrosequencing and phylogenetic microarray analysis of 16S ribosomal RNA gene sequences. We observed a significant higher abundance of Firmicutes, Proteobacteria, and Fibrobacteres phyla in *db*/*db* mice compared to lean mice. The abundance of 10 genera was significantly affected by the genotype. We identified the roles of the eCB and LPS in the regulation of apelinergic system tone (apelin and APJ mRNA expression) in genetic obese and diabetic mice. By using *in vivo* and *in vitro* models, we have demonstrated that both the eCB and low-grade inflammation differentially regulate apelin and APJ mRNA expression in adipose tissue. Finally, deep-gut microbiota profiling revealed that the gut microbial community of type 2 diabetic mice is significantly different from that of their lean counterparts. This indicates specific relationships between the gut microbiota and the regulation of the apelinergic system. However, the exact roles of specific bacteria in shaping the phenotype of *db*/*db* mice remain to be determined.

Keywords: gut microbiota, type 2 diabetes, inflammation, LPS, endocannabinoid, apelin, APJ, metabolic endotoxemia

INTRODUCTION

Obesity and associated metabolic disorders (e.g., insulin resistance, type 2 diabetes, and cardiovascular diseases) are closely associated with a low-grade inflammatory state (Olefsky and Glass, 2010). Numerous studies have proposed that, in obese conditions, both the endocrine activity of adipose tissue and macrophage infiltration participate in the development of a low-grade inflammatory tone (Olefsky and Glass, 2010). Compelling evidence supports the idea that adipokines participate in the regulation of glucose homeostasis and low-grade inflammation (Deng and Scherer, 2010; Federico et al., 2010). Among the recently discovered adipokines, apelin has been proposed as a novel key peptide involved in the regulation of several physiological functions. Apelin and APJ mRNA are widely expressed in mammals and exert functional effects in both the central nervous system and the periphery (Sorli et al., 2006). Apelin plays a key role in the cardiovascular system by acting on heart contractility, blood pressure, fluid homeostasis, vessel formation, and cell proliferation (Maenhaut and Van, 2011). Apelin serum levels are linked to the nutritional status and plasma insulin levels in both rodents and humans (Dray et al., 2008; Duparc et al., 2011a). Furthermore, apelin plasma concentrations are increased in obese subjects and in hyperinsulinemic obese mice compared to lean subjects (Dray et al., 2010). Interestingly, apelin has been shown to control glucose homeostasis by AMP-kinase- and nitric oxide (NO)-dependent mechanisms (Dray et al., 2008; Duparc et al., 2011b). Recently, it has been proposed that inflammation could participate in the production of apelin and the modulation of its receptor expression (Daviaud et al., 2006; Han et al., 2008a,b). Nevertheless,

the mechanisms involved in the regulation of apelin and its specific G protein-coupled receptor APJ have not been completely elucidated.

Growing evidence supports the role of gut microbiota in the development of obesity, type 2 diabetes, insulin resistance, and low-grade inflammation (Backhed et al., 2004; Ley et al., 2006; Turnbaugh et al., 2006; Cani et al., 2007a, 2008, 2009; Martinez et al., 2009; Vijay-Kumar et al., 2010). However, the composition of the gut microbiota and the exact role of the microorganisms present in the gut remain poorly defined. Nonetheless, advances in culture-independent methods for characterizing microbial diversity have helped to evaluate the functional contribution of this large collection of microbes in host metabolism (Martin et al., 2008; Turnbaugh et al., 2009). For instance, recent data suggest that changes in gut microbiota composition and gut barrier functions play a critical role in the development of obesity-associated inflammation (Brun et al., 2007; Cani et al., 2008, 2009; De La Serre et al., 2010). Accordingly, we have proposed that obesity-associated low-grade inflammation and adipogenesis processes may be related to the gut microbiota by mechanisms involving the endocannabinoid system (eCB) and bacterially derived lipopolysaccharide (LPS; Cani et al., 2007a,b, 2008, 2009; De La Serre et al., 2010; Muccioli et al., 2010).

The eCB system is composed of endogenous lipids that activate specific G protein-coupled receptors termed cannabinoid receptors 1 and 2 (CB_1 and CB_2). Among these lipids, *N*-arachidonoylethanolamine (anandamide, AEA), and 2-arachidonoylglycerol (2-AG) are the most studied (Lambert and Muccioli, 2007). AEA and 2-AG are both widely present throughout the body, and their tissue levels are regulated by the balance between synthesis and inactivation. Briefly, *N*-acylphosphatidylethanolamines (NAPEs) are precursors for AEA through the activity of *N*-acylphosphatidylethanolamine-selective phospholipase-D (NAPE-PLD), whereas 2-AG can be synthesized mainly through diacylglycerol lipase (DAGL; Muccioli, 2010). Compelling strong evidence has shown that dysregulation of the tight control of endocannabinoid levels may result in pathological situations such as obesity and related metabolic syndromes or neurological disorders (Lambert and Muccioli, 2007). The main enzymes responsible for their degradation are fatty acid amide hydrolase (FAAH; Cravatt et al., 1996) and monoacylglycerol lipase (MGL; Dinh et al., 2002), respectively. Obesity and type 2 diabetes are associated with greater eCB system tone (altered expression of NAPE-PLD, CB_1 mRNA, and higher eCB levels in plasma or adipose tissue; Muccioli et al., 2010). Moreover, LPS is known to stimulate eCB synthesis (Liu et al., 2003; Muccioli et al., 2010), and the gut microbiota participate in this complex regulation (Muccioli et al., 2010).

Although changes in the gut microbiota of obese mice (*ob*/*ob*) or mice with high-fat diet-induced obesity were clearly demonstrated (Ley et al., 2005; Cani et al., 2007a,b; Turnbaugh et al., 2008, 2009; Hildebrandt et al., 2009), the exact composition of the gut microbiota of type 2 diabetic mice (*db*/*db*) remains unknown. In light of these recent findings, we also decided to investigate the potential implication of these three key players (eCB, LPS, and the gut microbiota) in the regulation of apelin and APJ expression in adipose tissue. Therefore, we combined multiple molecular methods, including bar-coded pyrosequencing and phylogenetic microarray analysis of 16S ribosomal RNA (rRNA) genes, to compare the gut microbial communities in genetic obese and diabetic mice with their lean littermates. The data presented herein demonstrated that apelin and APJ expression were down-regulated by the eCB in physiological conditions and were up-regulated by the bacteria-derived compound LPS in pathological situations. In addition, gut microbiota profiling revealed specific relationships between the gut microbiota and regulation of the apelinergic system and also potential novel bacterial targets that are essential to the host metabolism.

MATERIALS AND METHODS

MICE

Six-week-old *db*/*db* mice or lean littermates ($n = 6$/group; C57BL/6 background, Jackson-Laboratory, Bar Harbor, ME, USA) were housed in a controlled environment (12-h daylight cycle, lights-off at 6-pm) in groups of two mice/cage. Both groups of mice were given free access to similar control diet (A04, Villemoisson sur Orge, France) and tap water for 1 week.

All mouse experiments were approved by and performed in accordance with the guidelines of the local ethics committee. Housing conditions were specified by the Belgian Law of November 14, 1993, regarding the protection of laboratory animals (agreement number LA1230314).

TISSUE SAMPLING

Seven-week-old mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine after a 5-h fasting period. Blood samples were harvested for further analysis. Mice were sacrificed by cervical dislocation. Subcutaneous adipose deposits were dissected and immediately immersed in liquid nitrogen and stored at −80°C for further analysis.

PLASMA APELIN

Apelin was measured using an Apelin EIA kit (Phoenix peptides) according to the manufacturer's instructions.

MEASUREMENT OF ANANDAMIDE (AEA) TISSUE LEVELS.

Tissues were homogenized in $CHCl_3$ (10 ml), and a deuterated standard (d-AEA; 200 pmol) was added. Methanol (5 ml) and H_2O (2.5 ml) were added, and lipids were then extracted by vigorous mixing. Following centrifugation, the organic layer was recovered, dried under a stream of N_2 and purified by solid-phase extraction using silica, followed by elution with an EtOAc–Acetone (1:1) solution (Muccioli and Stella, 2008; Muccioli et al., 2010). The resulting lipid fraction was analyzed by HPLC–MS using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to an Accela HPLC system (Thermo Fisher Scientific). Analyte separation was achieved using a C-18 Supelguard pre-column and a Supelcosil LC-18 column (3 μM, 4 × 150 mm; Sigma-Aldrich). Mobile phases A and B were composed of MeOH–H_2O–acetic acid 75:25:0.1 (v/v/v) and MeOH–acetic acid 100:0.1 (v/v), respectively. The gradient (0.5 ml/min) was designed as follows: transition from 100% A to 100% B linearly over 15 min, followed by 10 min at 100% B and subsequent re-equilibration at 100% A. We performed MS analysis in the positive mode with an APCI ionization source. The capillary and APCI vaporizer temperatures were

set at 250 and 400°C, respectively. Anandamide was quantified by isotope dilution using its respective deuterated standard with identical retention. The calibration curves were generated as previously described (Muccioli and Stella, 2008), and the data were normalized by tissue sample weight.

DNA ISOLATION FROM MOUSE CECAL SAMPLES

The cecal contents of mice collected *post mortem* were stored at −80°C. Metagenomic DNA was extracted from the cecal contents (five *db*/*db* and five lean) using a QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two cecal contents were not included in further gut microbiota analyses for technical reasons.

16S RRNA GENE AMPLIFICATION AND SEQUENCING

For each sample, we amplified the V1–3 region of the bacterial 16S rRNA gene corresponding to *Escherichia coli* 16S rRNA gene positions 28–514, excluding primer sequences. PCRs included 1 μl of 50× diluted purified DNA, 0.5 μM of forward B-8fhomd (5′-gccttgccagcccgctcag-*ac*-GAGTTTGATCMTGGCTCAG-3′), 0.05 μM of forward B-8f-Bifido (5′-gccttgccagcccgctcag-*ac*-AGGGTTCGATTCTGGCTCAG-3′), and 0.5 μM of a barcoded reverse A-534RhomdDEGa primer (5′-gcctccctcgcgccatcag-NNNNNNNN-*at*-CCGCGGCTGCTGGCAC-3′) in 50 μl of PrimeStar HS Premix (Takara). These primers included (i) the 454 Life Science 19-base adaptors A (lowercase underlined) or B (lowercase plain); (ii) an eight-base sample-specific barcode sequence (NNNNNNNN; designated 692–701 in Hamady et al. (2008); **Table A1**); (iii) the sequence of the broad-range 16S forward or reverse primer (uppercase); and (iv) a dinucleotide sequence (lowercase italic) introduced between the 16S primer and the barcode sequence designed to prevent pairing of different barcodes with rDNA targets.

PCRs were performed for 29 cycles at 98°C for 10 s, 56°C for 15 s, and 72°C for 1 min. One microliter of the sample was run on the Agilent 2100 Bioanalyzer using a DNA1000 lab chip to confirm the proper size. Two replicate reactions were pooled and cleaned using the QIAquick PCR purification kit (Qiagen). DNA concentration was assessed using a NanoDrop 1000 spectrophotometer. One hundred nanograms of each of the purified samples were pooled and sequenced on a Genome Sequencer FLX system (Roche).

Bar-coded 16S rDNA amplicons associated with several unrelated projects were included in the same sequencing run. A total of 39,548 reads, which had a quality score <20, corresponded to *cecal* samples from the 10 mice presented in this study.

INFORMATIC ANALYSIS

Sequences containing uncalled bases, incorrect primer sequences or runs of ≥10 identical nucleotides were removed. Reads with the 16S rDNA forward oligonucleotide sequence CCGCGRCTGCTGGCGC, containing G instead of A at the penultimate position of the 3′ end, were likely due to a primer synthesis or sequencing artifact (Lazarevic et al., 2010) and were not removed from the dataset provided other quality criteria were met. After trimming primer sequences, reads <200 or >290 nt and those that incompletely covered the *E. coli* 16S rRNA gene positions 288–514, determined using the RDP pyrosequencing tool Aligner (Cole et al., 2009), were discarded, leaving 31,577 sequences. Sequences were examined for potential chimeras using the MG-RAST server (Meyer et al., 2008).

Sequences were assigned to representative phylotypes at 97% identity (97%-ID phylotypes) using CD-HIT (Huang et al., 2010). Distances between 97%-ID phylotypes aligned by MUSCLE (Edgar, 2004) were calculated using FastTree (Price et al., 2009). Hierarchical clustering and principal coordinates analyses were performed using UniFrac (Lozupone et al., 2006). The taxonomic composition was assigned using the RDP Classifier (Wang et al., 2007) with a recommended 50% confidence cut-off. The sequences (31,577 reads) are publicly available at the MG-RAST repository (Meyer et al., 2008) under ID 4455129.

MITCHIP: PCR PRIMERS AND CONDITIONS

The Mouse Intestinal Tract Chip (MITChip) is a phylogenetic microarray consisting of 3,580 different oligonucleotides specific for the mouse intestinal microbiota (Derrien et al., in preparation). Both the design and analysis of the MITChip were performed as previously described for the human counterpart (Rajilic-Stojanovic et al., 2009). In short, 20 ng of cecal DNA extract was used to amplify the 16S rRNA genes with the primers *T7prom*-Bact-27-for (5′-TGAATTGTAATACGACTCACTATAGGGgtttgatcctggctcag-3′) and Uni-1492-rev (5′-CGGCTACCTTGTTACGAC-3′). Subsequently, an *in vitro* transcription and labeling with Cy3 and Cy5 dyes was performed. Fragmentation of Cy3/Cy5-labeled target mixes was followed by hybridization on the arrays at 62.5°C for 16 h in a rotation oven (Agilent Technologies, Amstelveen, The Netherlands). The slides were washed and dried before scanning. Signal intensity data were obtained from the microarray images using Agilent Feature Extraction software, version 9.1[1]. Microarray data normalization and further analysis were performed using a set of R-based scripts[2] in combination with a custom-designed relational database12, which operates under the MySQL database management system[3].

RNA PREPARATION AND REAL-TIME QPCR ANALYSIS

Total RNA was prepared from tissues using TriPure reagent (Roche). Quantitation and integrity analysis of total RNA was performed by running 1 μl of each sample on an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent). cDNA was prepared by reverse transcription of 1 μg total RNA using a Reverse Transcription System kit (Promega, Leiden, The Netherlands). Real-time PCRs were performed with the StepOnePlus™ real-time PCR system and software (Applied Biosystems, Den Ijssel, The Netherlands) using Mesa Fast qPCR™ (Eurogentec, Seraing, Belgium) for detection according to the manufacturer's instructions. RPL-19 RNA was chosen as the housekeeping gene. Primer sequences for RPL-19, IL-1, F4-80, CD68, MCP-1, TNF-α, Apelin, APJ, CB1, MGL, FAAH, and NAPE-PLD were previously described (Cani et al., 2008,

[1]http://www.agilent.com
[2]http://r-project.org
[3]http://www.mysql.com

2009; Dray et al., 2008; Muccioli, 2010). The primer sequences for CD11c were F-ACG-TCA-GTA-CAA-GGA-GAT-GTT-GGA and R-ATC-CTA-TTG-CAG-AAT-GCT-TCT-TTA-CC. All samples were run in duplicate in a single 96-well reaction plate, and data were analyzed according to the $2^{-\Delta CT}$ method. The identity and purity of the amplified product was checked through analysis of the melting curve carried out at the end of amplification.

ADIPOSE TISSUE EXPLANT CULTURES

Subcutaneous adipose depots from 10 C57BL/6J (wild-type lean) mice were precisely dissected, and all visible vessels, particles, and conjunctive tissue were removed. The fat tissue was then cut with scissors into small pieces (4 mm^3), pooled, and placed in Krebs buffer (pH 7.4) containing 2% (w/v) fatty acid-free BSA, penicillin (100 IU/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μl/ml; Invitrogen). A total of 250 mg of adipose tissue was rinsed in phosphate-buffered saline and incubated in 100-mm Petri dishes containing 10 ml MEM with Earle's salts (Invitrogen) supplemented with 0.5% fatty acid-free BSA, penicillin (100 IU/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μl/ml; Invitrogen). All conditions were repeated in four to five different dishes ($n = 4$–5). The dishes were cultured for 24 h at 37°C in a 5% CO_2 atmosphere. The basal concentration of glucose in fresh media was 5 mmol/l, whereas the basal levels of cortisol and insulin were extremely low (∼0.5 nmol/l and 3 pmol/l, respectively). Different pharmacological agents were used in various combinations in accordance with the experimental protocols. LPS (*E. coli* 055:B5, 100 ng/ml, Sigma) and HU-210 (100 nM, Tocris) were diluted in DMSO, which also served as a control. Cell viability did not change over the course of the experiment (data not shown). At the end of the experiment, the adipose material was rinsed in phosphate-buffered saline, collected, immediately frozen in liquid nitrogen, and stored at −80°C until subsequent mRNA analysis.

STATISTICAL ANALYSES

The data are expressed as the mean ± SEM. Differences between two groups were assessed using an unpaired, two-tailed Student's *t*-test. Statistical comparisons of bacterial communities were performed using a two-tailed Student's *t*-test, treating variances as equivalent. Correlations were analyzed using Pearson's correlation. Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) and JMP 8.0.1 (SAS Campus Drive, Cary, NC, USA). The results were considered statistically significant at $P < 0.05$.

RESULTS

16S RRNA ANALYSIS OF GUT BACTERIAL POPULATIONS IN OBESE AND DIABETIC MICE COMPARED TO LEAN MICE

Obese (*ob*/*ob*) mice and diet-induced obese mice are characterized by an altered composition of the gut microbiota (Ley et al., 2005; Cani et al., 2007a,b; Turnbaugh et al., 2008, 2009). To date, the composition of the gut microbiota of leptin-resistant obese and type 2 diabetic (*db*/*db*) mice has never been reported. The main differences between *ob*/*ob* and *db*/*db* mice are, first, in the leptin system, *ob*/*ob* mice are leptin-deficient (they lack the *ob* gene), whereas *db*/*db* mice are leptin-resistant (the CNS has altered their "leptin receptors"), and, second, by phenotype, both mice are hyperphagic, severely obese and have developed fatty livers. However, *db*/*db* mice become type 2 diabetic early after birth (a few weeks). This major difference between the two models confers to *db*/*db* mice a clear advantage for investigating the role of the gut microbiota in this phenotype. The exact composition of the gut microbiota in this model of obesity and diabetes remains unknown. Therefore, we characterized the gut microbiota composition of obese and diabetic *db*/*db* mice by two complementary approaches: pyrosequencing and phylogenetic microarray analysis of the 16S rRNA genes isolated from mouse cecal samples.

To explore bacterial diversity in cecal samples, we targeted the 16S rDNA hypervariable region V3. A total of 31,577 sequence reads passed all quality control steps. We observed a significant increase of Proteobacteria (32,227%) in *db*/*db* mice compared to lean mice (**Table 1**). The abundance of 10 genera was significantly affected by the genotype (**Table 1**). Interestingly, the genera *Odoribacter, Prevotella*, and *Rikenella* were identified exclusively in the *db*/*db* group, whereas *Enterorhabdus* was identified exclusively in lean mice (**Table 1**). Importantly, the abundance of the *Tannerella* genus was increased by approximately 17,112% in *db*/*db* mice. The representatives of the class Deltaproteobacteria and the three genera of the phylum Bacteroidetes, all found exclusively in *db*/*db* mice, are Gram-negative bacteria. **Table A2** in Appendix shows the significant differences in 96 phylotypes (defined at 97%-ID) enriched or depleted in *db*/*db* mice. Furthermore, the results of the UniFrac analysis (Lozupone et al., 2006) clearly showed that *db*/*db* cecal communities were more similar to each other than to the communities of the control lean mice (**Figure 1A**). Principal coordinate analysis (PCoA) of UniFrac-based pairwise comparisons of community structures revealed two clusters corresponding to the two dietary conditions (**Figure 1B**). Importantly, we found a marked positive relationship between the genus *Oscillibacter* and the expression of apelin and APJ ($r = 0.88$, $P = 0.0006$, and $r = 0.87$, $P = 0.0011$, respectively). We also performed deep and global microbiota analysis using the MITChip, a high-throughput phylogenetic microarray designed after the human counterpart (Rajilic-Stojanovic et al., 2009), which was previously compared with 454 pyrosequencing (Claesson et al., 2009). The profiles of the cecal microbiota were obtained based on the hybridization to over 3,500 oligonucleotide probes and visualized the presence or absence of all targeted operational taxonomic units (OTUs). MITChip analysis revealed a clear separate clustering of the two treatment groups (**Figures 2C,D**) that shared 81.2 (±2.6)% similarities (Pearson product-moment correlation coefficient). We observed a lower relative and absolute abundance of the phyla Bacteroidetes and Deferribacteres in *db*/*db* mice compared to the lean group (**Figures 2A,B,D**), whereas we found a higher abundance of Firmicutes, Proteobacteria, and Fibrobacteres in *db*/*db* mice compared to the lean group (**Figures 2A,B,D**). In accordance with the pyrosequencing analysis, both groups were found to cluster in a genotype-specific manner. In addition to specific changes observed in the pyrosequencing analyses, we found several previously unidentified modifications at a lower taxonomic level,

Table 1 | Taxa enriched or depleted in *db*/*db* mice.

Rank	RDP classification	Abundance (percent of total sequences)*		Change in *db*/*db* group** (%)	*P* value***
		Lean	*db*/*db*		
Genus	Actinobacteria; Actinobacteria; Coriobacteriales; Coriobacteriaceae; Enterorhabdus	0.019 ± 0.008	0	LEAN [3]	0.0449
Genus	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Barnesiella	0.464 ± 0.148	8.204 ± 1.322	1668	0.0004
Genus	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Butyricimonas	0.091 ± 0.022	0.006 ± 0.006	−94	0.0061
Genus	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Odoribacter	0	0.150 ± 0.040	DB [5]	0.0053
Genus	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Parabacteroides	0.756 ± 0.122	0.235 ± 0.070	−69	0.0060
Genus	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Tannerella	0.008 ± 0.008	1.346 ± 0.328	17112	0.0036
Family	Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae	0	3.590 ± 0.748	DB [5]	0.0014
Genus	Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella	0	2.443 ± 0.463	DB [5]	0.0008
Family	Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae	4.262 ± 0.802	9.061 ± 0.798	113	0.0028
Genus	Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; Alistipes	4.262 ± 0.802	9.042 ± 0.794	112	0.0029
Genus	Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; Rikenella	0	0.019 ± 0.008	DB [3]	0.0409
Family	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	7.259 ± 1.231	14.029 ± 2.216	93	0.0283
Genus	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Oscillibacter	5.831 ± 0.858	10.539 ± 1.482	81	0.0251
Phylum	Proteobacteria	0.006 ± 0.006	2.036 ± 0.570	32227	0.0074
Class	Proteobacteria; Deltaproteobacteria	0	1.227 ± 0.292	DB [5]	0.0030

**Data are the mean ± SE.*

***Change in average taxa abundance in db/db mice subset when compared to the lean mice subset. Only changes associated with a P value < 0.05 are given.*

****P value was determined by a two-tailed Student's t-test with equal variance.*

[Number] following LEAN or DB corresponds to the number of mice in the indicated subset in which the given phylotype was found.

especially the absolute and relative increase of *Prevotella* spp. (not shown).

OBESE AND DIABETIC MICE EXHIBITED A HIGHER ENDOCANNABINOID SYSTEM TONE IN ADIPOSE TISSUE

We have previously demonstrated that the gut microbiota contribute to the regulation of eCB system tone in adipose tissue in both physiological and pathological situations (Muccioli et al., 2010). In addition, obesity is characterized by higher eCB system tone in both humans and rodents (Engeli et al., 2005; Bluher et al., 2006; Matias et al., 2006; Cote et al., 2007; D'Eon et al., 2008; Starowicz et al., 2008; Izzo et al., 2009; Muccioli et al., 2010). Consistent with these findings, we discovered that *db*/*db* mice, fed with a standard diet, exhibited an increased eCB system tone (AEA levels and mRNA expression). Anandamide (AEA; Devane et al., 1992) is an endogenous CB_1 and CB_2 ligand. Here we found a significant increase in AEA levels (about 50%) in adipose tissue (**Figure 3A**). In accordance with this result, NAPE-PLD and CB_1 mRNA expression were significantly increased, whereas mRNA expression for the AEA-degrading enzyme FAAH was reduced (**Figures 3B,C,D**). Note that expression of monoacylglycerol lipase (MGL), the main enzyme that degrades endocannabinoid 2-arachidonoylglycerol (2-AG), tended to be higher in the adipose tissue of *db*/*db* mice (**Figure 3E**), which is consistent with the reduced 2-AG levels observed in adipose tissue (**Figure 3F**). Altogether, these data demonstrate for the first time that *db*/*db* mice display an altered eCB system tone in adipose tissue, and more specifically, they display increased signaling for the endocannabinoid AEA.

INCREASED APELIN AND APJ EXPRESSION IN THE ADIPOSE TISSUE OF OBESE AND DIABETIC MICE

Recent evidence suggests that, in pathophysiological conditions (obesity and type 2 diabetes), the peripheral apelinergic system seems to be deregulated (Dray et al., 2008, 2010; Duparc et al., 2011a). To date, the mechanisms of regulation of apelin and APJ expression in the adipose tissue of obese and diabetic mice are not fully understood. Therefore, we investigated apelinergic system tone in this model and found that apelin and APJ mRNA levels were significantly increased by two- and threefold, respectively, in the adipose tissue of *db*/*db* mice compared to lean littermates (**Figures 4A,B**). Plasma apelin was increased by approximately 30%, but did not reach significance (*db*/*db*: 7.1 ± 1, lean: 5.8 ± 0.8 pg/ml).

Interestingly, we found several positive and negative correlations between the abundance of specific bacteria and apelin/APJ expression, suggesting a potential relationship between specific gut bacteria and the regulation of the apelinergic system (**Table 2**).

INCREASED LOW-GRADE INFLAMMATION IN THE ADIPOSE TISSUE OF DB/DB MICE

We have previously demonstrated that the higher eCB system tone observed in the adipose tissue of obese and diabetic mice was directly dependent on low-grade inflammatory tone (Muccioli et al., 2010). In addition, *db*/*db* mice are considered a widely used model to investigate the metabolic features associated with obesity and type 2 diabetes. These mice are characterized by a low-grade inflammatory tone with higher plasma LPS levels

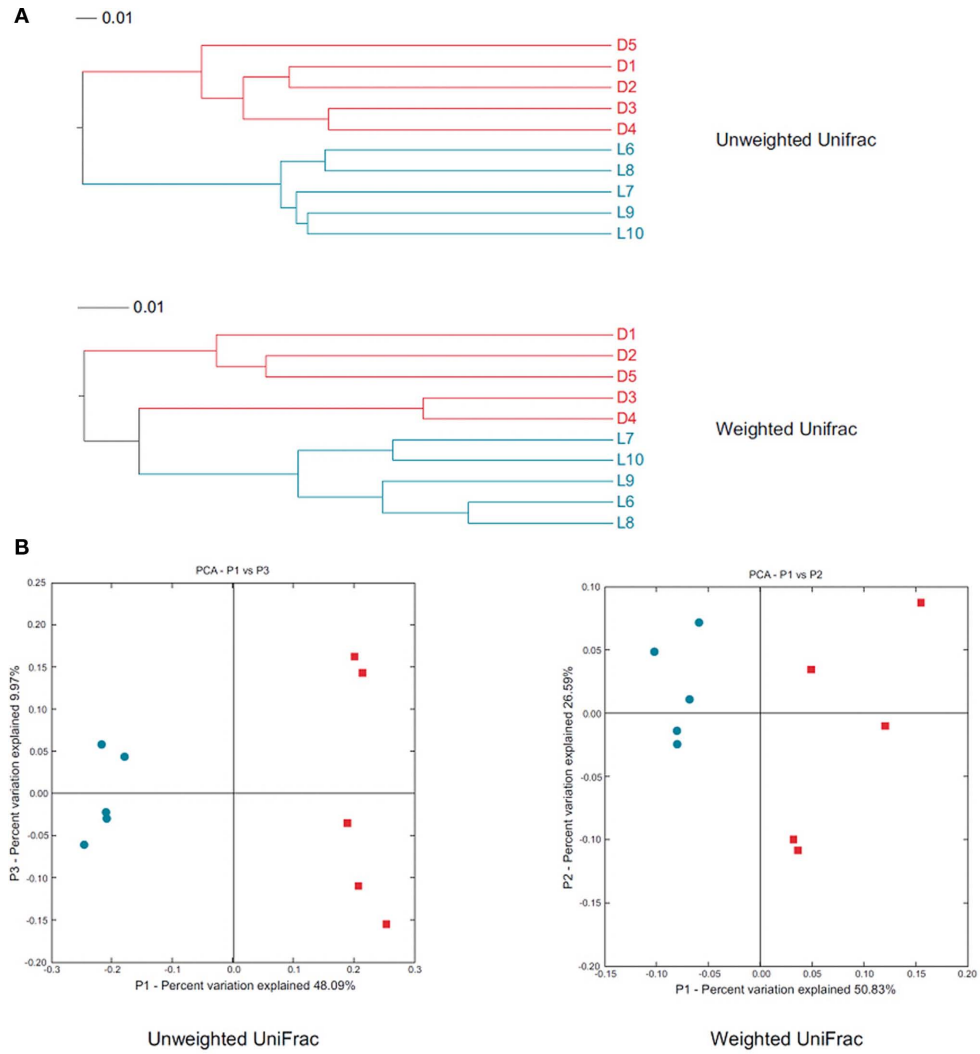

FIGURE 1 | Analysis of gut bacterial communities by 16S rRNA pyrosequencing from obese diabetic mice or lean mice. **(A)** Clustering of mouse cecal microbial communities in the two tested groups based on unweighted UniFrac analysis and 97%-ID phylotypes. Red indicates the diabetic mice (*db*/*db*), and blue indicates the lean mice. Branch length represents the distance between environments in UniFrac units, indicated by the scale bar ($n = 5$/group). **(B)** PCoA analysis based on unweighted (presence/absence) UniFrac analysis and 97%-ID phylotypes. Each symbol (filled circle or filled square) representing a single sample is colored according to dietary conditions: red corresponds to *db*/*db*, and blue corresponds to lean ($n = 5$/group).

and significantly higher plasma levels of IL-1β, IL-6, TNF-α, and INF-γ (Brun et al., 2007). Moreover, the assessment of adipose tissue inflammation revealed an increased number of crown-like structures corresponding to proinflammatory macrophages that are F4/80 and CD11c positive (Mihara et al., 2010; Hellmann et al., 2011). Consistent with numerous previous reports investigating meta-inflammation in this model, we found a significant increase in both inflammation and macrophage infiltration markers in our *db*/*db* mice. The markers investigated in this study (IL-1, Monocyte Chemoattractant Protein-1 (MCP-1), F4/80, CD11c, and CD68 mRNA expression) were all significantly increased by 50 to 400% in *db*/*db* mice compared to lean mice (**Figures 5A–E**).

BOTH THE ECB SYSTEM AND LOW-GRADE INFLAMMATION PARTICIPATE IN THE REGULATION OF ADIPOSE TISSUE APELIN AND APJ EXPRESSION

Understanding the molecular mechanisms responsible for the altered apelinergic system is essential to find putative novel targets for modulating adipose tissue metabolism. We have previously shown that both the low-grade inflammatory tone and the higher eCB system tone observed in obesity and related disorders could be linked to gut microbiota composition (Cani et al., 2009; Muccioli et al., 2010; Cani and Delzenne, 2011). More importantly, it has been previously demonstrated that inflammation regulates both apelin and APJ mRNA expression (Daviaud et al., 2006; Melgar-Lesmes et al., 2011). In addition, our *in vivo* experiments and

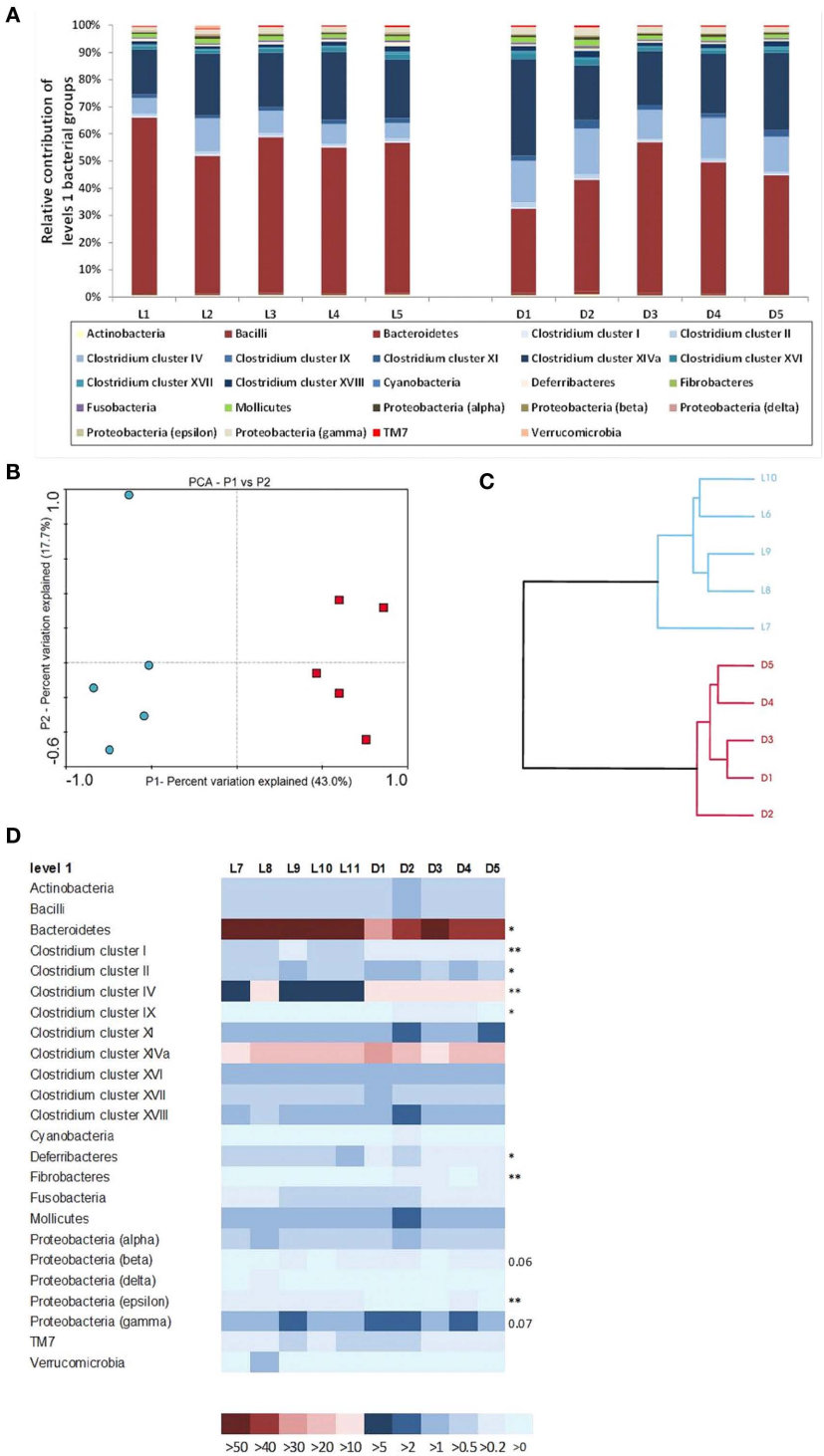

FIGURE 2 | Phylogenetic microarray analysis of gut bacterial communities from obese diabetic mice or lean mice. (A) Percentages of each community contributed by the indicated level 1 bacterial groups. **(B)** PCoA analysis based on MITChip phylogenetic fingerprints of the gut microbiota from the cecal contents of *db*/*db* and lean mice. **(C)** Hierarchical clustering visualizing the similarities of the phylogenetic MITChip fingerprints. Samples were hierarchically clustered based on the Pearson correlation. **(D)** Relative abundance of different bacterial classes expressed as the percentage of total probe signals ($n = 5$/group). $^{*}P < 0.05$, $^{**}P < 0.01$ determined by a two-tailed Student's *t*-test.

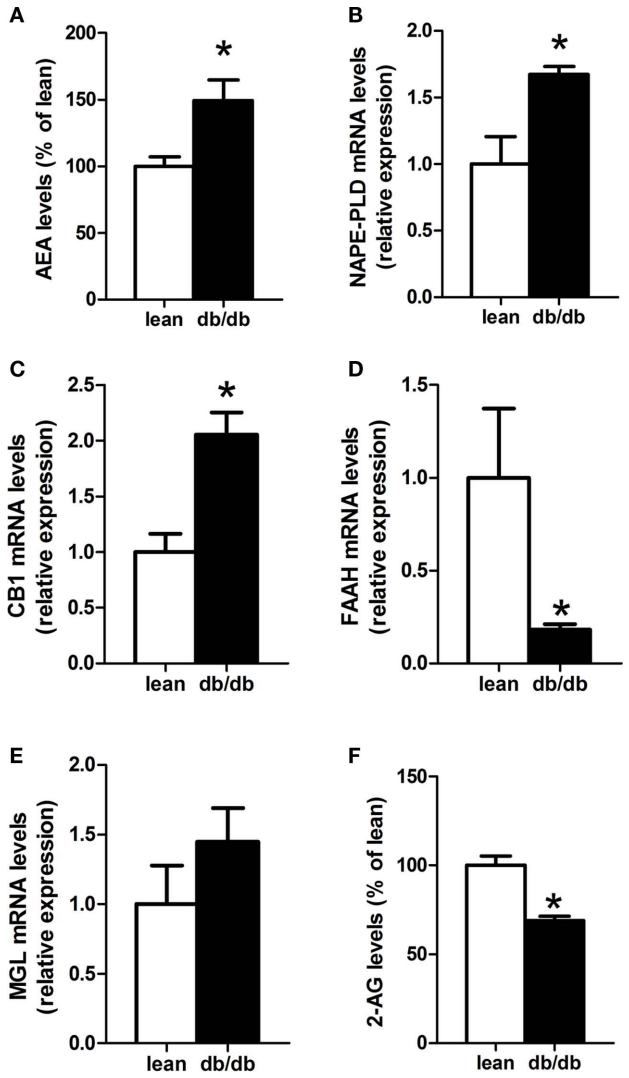

FIGURE 3 | Higher endocannabinoid system tone in the adipose tissue of *db/db* mice. (A) AEAtissue content (% of lean), **(B)** NAPE-PLD, **(C)** CB_1, **(D)** FAAH; **(E)** MGL mRNA expression, and **(F)** 2-AG tissue content (% of lean) measured in adipose tissue. Mean ± SEM, $n = 6$/group, $^*P < 0.05$ determined by a two-tailed Student's *t*-test.

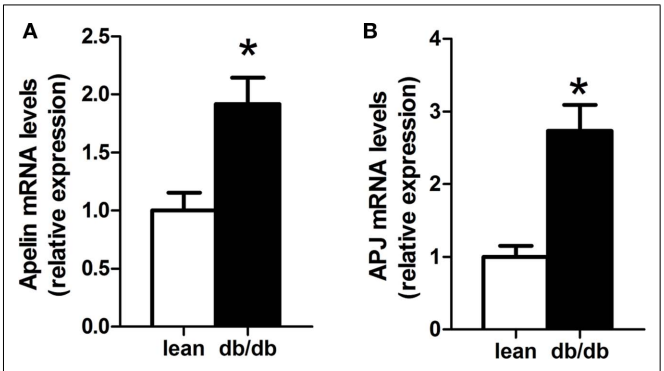

FIGURE 4 | Increased apelin and APJ mRNA expression in the adipose tissue of *db/db* mice. (A) Apelin and **(B)** APJ mRNA expression measured in adipose tissue. Mean ± SEM, $n = 6$/group, $^*P < 0.05$ determined by a two-tailed Student's *t*-test.

multiple correlation analyses suggest a link between inflammation and the regulation of the apelinergic system (**Figure A1**). However, the causal link between these parameters and the regulation of apelin and APJ expression in the adipose tissue remain to be demonstrated.

Given the difficulty of deciphering the impact of low-grade inflammation (i.e., plasma LPS or cytokines) and eCB system tone on the apelinergic system *in vivo*, we decided to explore these mechanisms on *in vitro* cultured adipose tissue explants. Of note, these explants came from healthy, lean C57BL/6 mice, allowing us to investigate physiological-like or pathological-like situations, that is, eCB system tone modulation with or without a concomitant inflammatory situation.

Here, we found that the activation of the eCB system by a cannabinoid receptor agonist (HU-210) significantly decreased apelin and APJ mRNA expression (**Figures 6A,B**). In line with the results obtained using the cannabinoid receptor agonist *in vitro*, in lean wild-type mice, we found that *in vivo* inhibition of AEA degradation by the administration of a potent FAAH inhibitor (URB597; Kathuria et al., 2003) significantly decreased apelin and APJ mRNA expression (**Figure A2**). These data support the idea that the eCB system down-regulates the apelinergic system in physiological situations. However, we found that low-dose LPS significantly increased these two markers. More importantly, the adipose tissue explants treated with both cannabinoid receptor agonist and LPS exhibited a significant increase in apelin and APJ mRNA expression (**Figures 6A,B**). Altogether, this set of experiments suggests that both the eCB system and low-grade inflammatory tone contribute to the regulation of apelin and APJ in adipose tissue. Interestingly, concomitant stimulation with eCB agonist and LPS resulted in up-regulation of the apelinergic system. In parallel, we found that two key inflammatory markers (IL-1 and TNF-α) were similarly up-regulated following LPS-eCB treatment (**Figures 6C,D**). These data further confirm that (i) both the eCB system and low-grade inflammation might be overactive during obese and type 2 diabetic situations, and (ii) that LPS and/or inflammation act as a master switch in the regulation of the apelinergic system.

DISCUSSION

This study provides new evidence for an altered gut microbiota in type 2 diabetes, independent of fat feeding. Indeed, we demonstrate that the gut microbial community is profoundly impacted by genotype and not necessarily diet because *db/db* mice, compared to their littermates, exhibited a profound alteration of the gut microbiota composition. In addition, we found that some taxa are present or rather absent in diabetic mice compared to their lean littermates. These important findings question the potential impact of these bacteria in the onset of the phenotypic alterations characterizing this model. Although the microbiota analysis relied on complementary technologies and classification methods, data showed similar patterns of specific clustering of bacterial communities. We observed a decrease in the abundance of the phylum Bacteroidetes and an increase of Firmicutes. Importantly, we found

Table 2 | Correlations between changes in the abundance of specific bacteria and adipose tissue apelin or APJ mRNA levels.

	r	*p*-Value
Apelin		
Acholeplasma et rel.	0.73	0.0162
Aerococcus urinaeequi et rel.	0.89	0.0005
Atopobium	0.70	0.0255
Bryantella et rel.	0.68	0.0296
Butyrivibrio crossotus et rel.	0.67	0.0344
Catenibacterium	0.66	0.0364
Cl. lactifermentans et rel.	0.75	0.0120
Cl. perfringens et rel.	−0.84	0.0024
Dialister et rel.	0.81	0.0043
Eggerthella et rel.	−0.65	0.0403
Eub. hallii et rel.	0.74	0.0135
Helicobacter	−0.72	0.0181
Lachnospira pectinoschiza et rel.	0.76	0.0110
L. salivarius et rel.	−0.64	0.0478
Mucispirillum schaedleri et rel.	−0.69	0.0276
Porphyromonas asaccharolytica et rel.	−0.80	0.0057
Prevotella	−0.68	0.0319
R. obeum et rel.	0.76	0.0106
Sporobacter termitidis et rel.	0.89	0.0005
Unc. Porphyromonadaceae	−0.86	0.0016
APJ		
Acholeplasma et rel.	0.74	0.0148
Aerococcus urinaeequi et rel.	0.84	0.0023
Atopobium	0.68	0.0319
Bryantella et rel.	0.71	0.0206
Butyrivibrio crossotus et rel.	0.68	0.0321
Catenibacterium	0.72	0.0197
Cl. difficile et rel.	0.65	0.0416
Cl. lactifermentans et rel.	0.66	0.0363
Cl. perfringens et rel.	−0.79	0.0070
Dialister et rel.	0.79	0.0065
Eub. hallii et rel.	0.76	0.0116
Fibrobacter succinogenes et rel.	0.65	0.0407
Helicobacter	−0.76	0.0113
Labrys methylaminiphilus et rel.	0.70	0.0236
Lachnospira pectinoschiza et rel.	0.72	0.0179
L. gasseri et rel.	0.67	0.0341
L. salivarius et rel.	−0.67	0.0333
Porphyromonas asaccharolytica et rel.	−0.76	0.0101
R. obeum et rel.	0.64	0.0479
Sporobacter termitidis et rel.	0.85	0.0017
Unc. Porphyromonadaceae	−0.86	0.0013

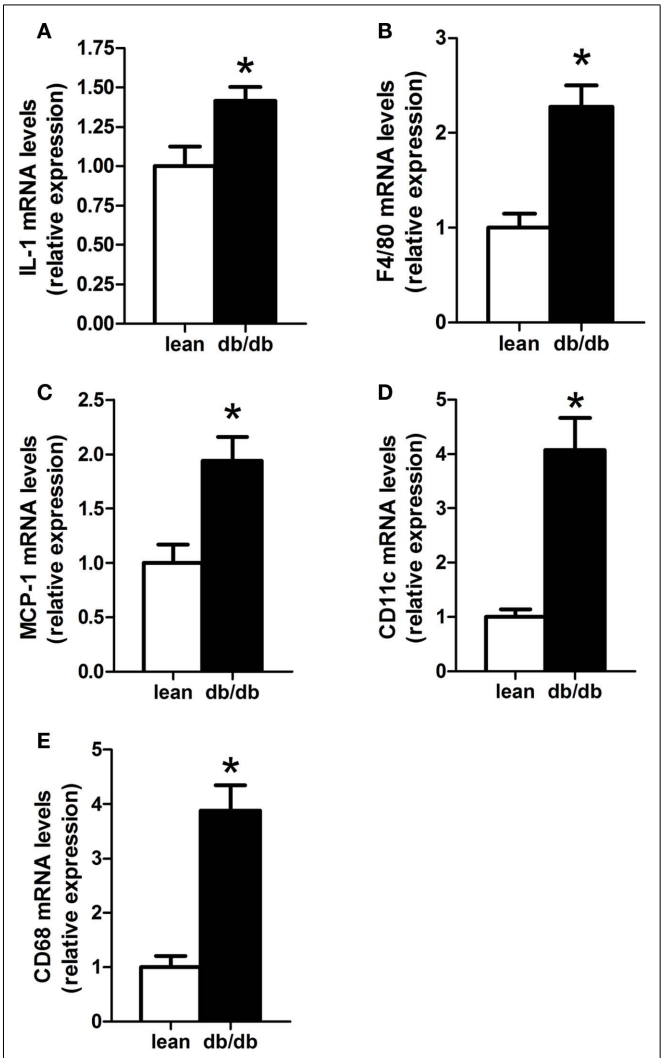

FIGURE 5 | Increased inflammatory markers in the adipose tissue of *db/db* mice. (A) IL-1, **(B)** F4/80, **(C)** MCP-1, **(D)** CD11c, and **(E)** CD68 mRNA expression measured in adipose tissue. Mean ± SEM, $n = 6$/group, $^*P < 0.05$ determined by a two-tailed Student's *t*-test.

a dramatic increase of Proteobacteria following the pyrosequencing method. Moreover, ten genera whose abundance was significantly affected by the genotype have been identified. Interestingly, we identified several taxa of Gram-negative bacteria at significantly higher frequencies or exclusively in *db*/*db* mice. Because LPS from Gram-negative bacteria triggered low-grade inflammation and stimulated apelin and APJ expression, we postulate that these specific changes contributed to the phenotype observed in *db*/*db* mice compared to their lean counterparts. In addition to the putative higher content of LPS within the intestinal lumen, it has been previously shown that *db*/*db* mice are characterized by greater intestinal inflammation and gut permeability (Brun et al., 2007; Duparc et al., 2011b). Therefore, in addition to the dysfunction of the leptin action characterizing this mouse model, we propose that the shift in the gut microbial community observed also contributed to the development of the complex phenotype found in *db*/*db* mice. Nevertheless, given the complexity of these modifications and the number of correlations found, the direct association between one or more specific taxa and the changes affecting host physiology remain to be investigated. Hence, further work is required to understand the functional links between the metabolic/catabolic activities of gut bacteria and their impact on host metabolism. For instance, it would be of interest to investigate this genetic model in a germ-free situation to identify the specific impact of the gut microbes on the onset of type 2 diabetes. Importantly, when we compared the classification results from pyrosequencing to those obtained from phylogenetic microarrays,

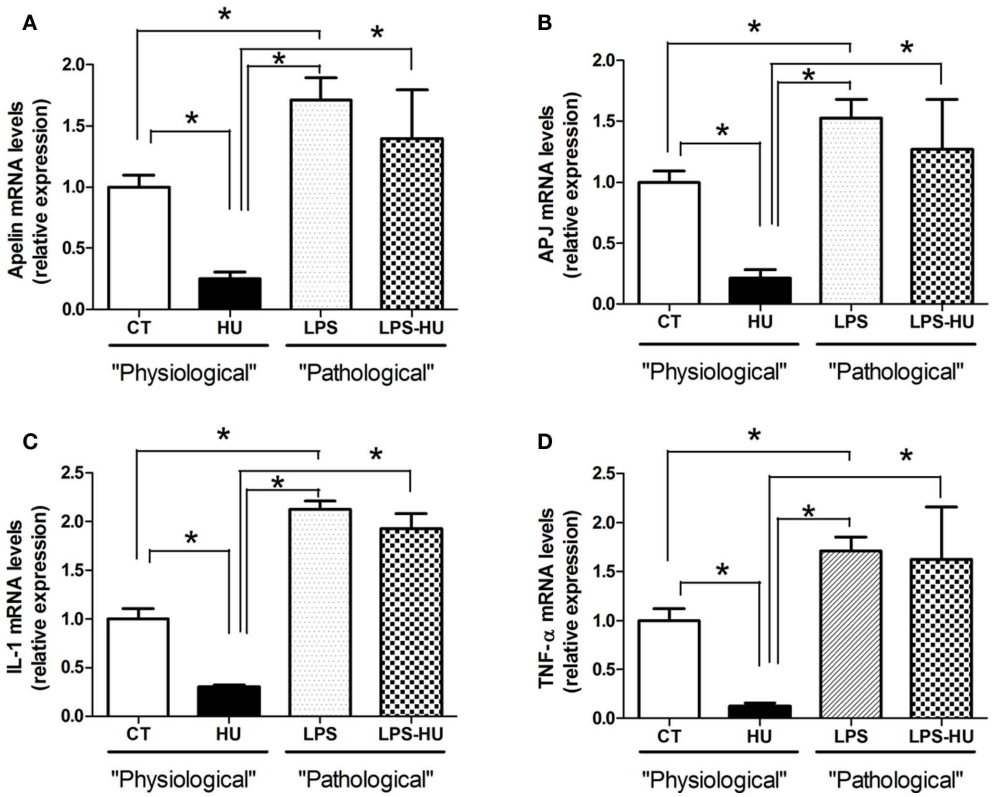

FIGURE 6 | The eCB system down-regulates LPS-induced inflammation (IL-1 and TNF-α mRNA expression) and the apelinergic system (apelin and APJ mRNA expression) in adipose tissue explant cultures. mRNA expression levels of **(A)** Apelin, **(B)** APJ, **(C)** IL-1, and **(D)** TNF-α in cultured adipose tissue explants from lean mice exposed to vehicle (CT), LPS (LPS) or cannabinoid receptor agonist HU-210 (HU; 100 nM) alone or in combination with LPS (100 ng.ml, LPS-HU) for 24 h. * indicates $P < 0.05$ for the drug effect; by a two-tailed Student's *t*-test.

we observed more differences attributed to a deeper analysis of the phylogenetic microarrays (<0.1% of the community; Rajilic-Stojanovic et al., 2009) than pyrosequencing. Recently the impact of use of different primers was assessed by 454 pyrosequencing and could impact the microbial profiling (van den Bogert et al., 2011), off note, deep pyrosequencing and phylogenetic microarrays provided similar biological conclusions, although the classification may be different from that in previously reported systems (Claesson et al., 2009).

This study also provided novel mechanisms involving the important roles of the eCB and low-grade inflammatory tone in the regulation of one specific adipokine and its receptor, namely, apelin and APJ. Hence, multiple correlation analyses suggest that specific gut microbes positively or negatively correlate with apelin and APJ mRNA. This study also supports the interest of combining high-throughput culture-independent approaches with physiological assessments to define the relationships between gut microbial communities and host metabolism. Nevertheless, given the complexity of these biological systems, the direct association between one or more bacterial groups and the changes affecting host physiology remain to be confirmed.

During the last years, numerous studies have demonstrated a clear relationship between energy metabolism and peripheral apelin action. Under pathophysiological conditions, the peripheral apelinergic system seems to be deregulated (Dray et al., 2008, 2010; Duparc et al., 2011a). While some researchers found that levels of plasma apelin in humans and mice were increased in obese diabetes (Heinonen et al., 2005; Dray et al., 2008) and in the insulin resistance state (Soriguer et al., 2009), others demonstrated that plasma apelin was reduced in type 2 diabetes patients (Erdem et al., 2008). Recently, it has been highlighted that the severity of insulin resistance might influence the regulation of apelin and APJ expression (Dray et al., 2010).

In this study, we first demonstrated the presence of an increase in eCB system tone (higher AEA levels, NAPE-PLD, and CB_1 expression and lower FAAH expression) in the adipose tissue of *db*/*db* mice. Here we show for the first time that AEA levels are increased in adipose tissue and are in accordance with our previous findings (Muccioli et al., 2010). In parallel, we found that both apelin and APJ expression were significantly increased. By using multiple correlation analyses, we observed very strong relationships between the eCB system and the apelinergic system, supporting the potential implication of the eCB system in apelinergic tone. However, *db*/*db* mice were also characterized by a higher inflammatory tone (Brun et al., 2007), which is, according to our results, positively and significantly correlated with apelin/APJ. Given that *db*/*db* mice also exhibited better eCB system tone and inflammation, we investigated the

direct impact of the endocannabinoids. To clarify this issue, we increased endogenous AEA levels in the adipose tissue of lean mice following the inhibition of FAAH (Muccioli et al., 2010), the main AEA-degrading enzyme (Muccioli, 2010). Strikingly, we found that this procedure significantly decreased apelin and APJ expression. Similarly to these *in vivo* data, the stimulation of adipose tissue explants with cannabinoid agonist profoundly reduced the expression of apelin and APJ. Furthermore, knowing that *db*/*db* mice developed a low-grade inflammatory tone, possibly linked to gut microbiota-derived LPS, we investigated the role of LPS in the regulation of these peptides. Interestingly, we demonstrated that LPS directly increased apelinergic system tone in adipose tissue. We also found that LPS treatment completely counteracted the eCB-dependent down-regulation of apelin and APJ. Taken together, all these data strongly support the concept that both systems are implicated in the regulation of apelinergic tone. However, both our *in vivo* and *in vitro* data point to inflammation as a master control in this cross-talk. Based on these data, we postulate that, in physiological conditions, eCB system tone participates in the down-regulation of the apelinergic system, whereas in pathological conditions, the low-grade inflammatory tone acts as a master switch to increase both eCB system tone and the apelinergic system. Although our data relied on different regulatory mechanisms (eCB tone and metabolic endotoxemia), both are clearly associated with gut microbiota composition (Cani and Delzenne, 2011). Thus, understanding the complexity of gut microbiota is increasingly important for deciphering potential interactions between hosts and microbes.

In conclusion, we have demonstrated that the eCB and low-grade inflammatory tone are closely involved in the regulation of apelin and APJ expression in adipose tissue. Moreover, we found that genetic obese and type 2 diabetic mice harbored profound changes in their gut microbiota compositions compared to their lean counterparts. Although we found strong positive and negative relationships between specific bacteria and the apelinergic system, the exact implications of several bacteria for shaping the phenotype of these mice remain to be studied.

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APPENDIX

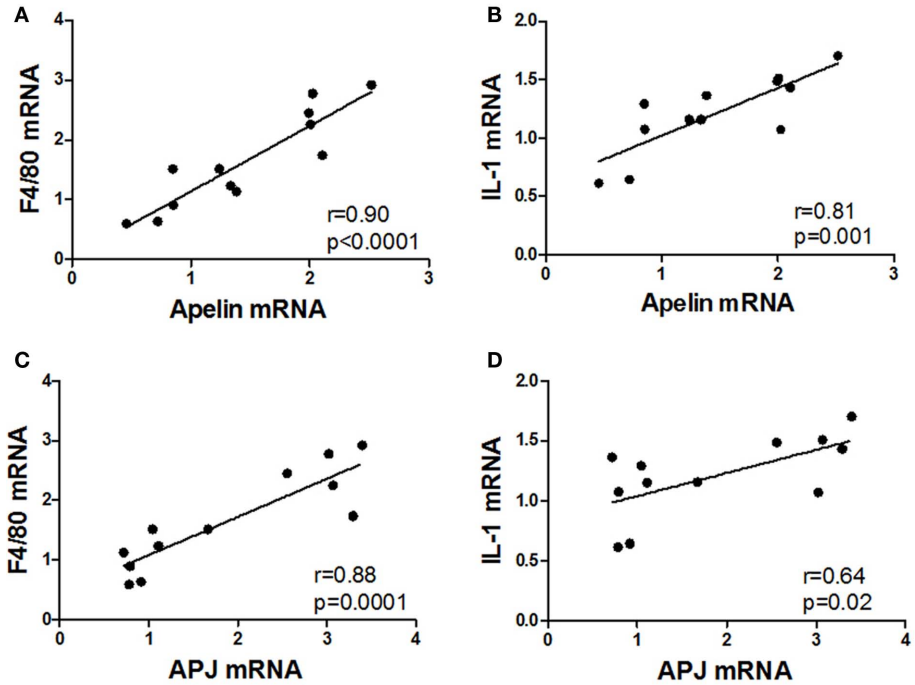

FIGURE A1 | Multiple correlation analyses between the apelinergic system and inflammation. Pairwise correlations between F4/80 or IL-1 and apelin **(A,B)** or APJ **(C,D)** mRNA expression measured in the adipose tissue of *db*/*db* mice. *Insets* in each graph depict Pearson's correlation value of the coefficient (r) and *P* values.

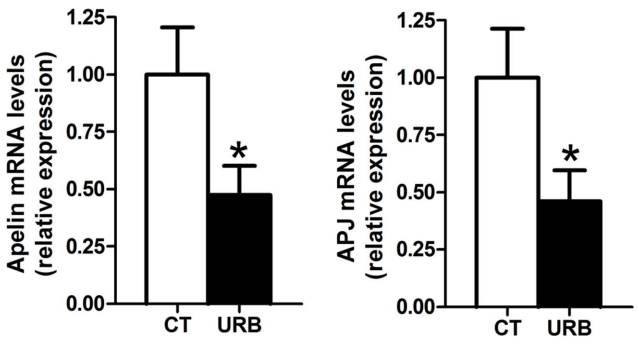

FIGURE A2 | FAAH inhibition decreased Apelin and APJ mRNA expression in the adipose tissue of mice. Apelin and APJ mRNA expression measured in the adipose tissue of lean mice treated with a single dose of the FAAH inhibitor URB. Mean ± SEM, $n = 6$/group, $^*P < 0.05$ determined by a two-tailed Student's *t*-test.

TABLE A1 | Sequencing statistics for 10 cecal microbiomes.

Primer barcode	Number of filtered sequences	Mouse ID	Group
692	3470	1	*db*/*db*
693	2754	2	*db*/*db*
694	3077	3	*db*/*db*
695	3038	4	*db*/*db*
696	3564	5	*db*/*db*
697	2558	6	lean
698	3314	7	lean
699	2920	8	lean
700	3707	9	lean
701	3175	10	lean

Reverse PCR primers contained barcode sequences described by Hamady et al. (2008).

Table A2 |The 97%-ID phylotypes enriched or depleted in *db/db* mice.

Lean*	*db/db***	Representative sequence	Phylum	Class	Order	Family	Genus	Change in *db/db* group*** (%)	*P*-value
0.0192 ± 0.0081	0	>F33FQ7E02I4JJM_129	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	*Enterorhabdus*	LEAN [3]	0.044906
12.0893 ± 3.5947	1.8624 ± 0.8334	>F33FQ7E01C600C_126	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	−85	0.024243
0.0068 ± 0.0068	1.7903 ± 0.4877	>F33FQ7E01A2OF5_121	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	26039	0.006437
0	0.4454 ± 0.1490	>F33FQ7E01CGOVF_123	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	DB [5]	0.017368
0	0.2853 ± 0.0857	>F33FQ7E02H3E41_121	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	DB [5]	0.010394
0.2629 ± 0.0783	0.0332 ± 0.0200	>F33FQ7E02I8QUO_122	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	−87	0.021732
0.0875 ± 0.0340	0	>F33FQ7E01E3D9N_126	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	LEAN [4]	0.032851
0	0.0450 ± 0.0124	>F33FQ7E01DXJI5_123	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	DB [4]	0.006838
0	3.6365 ± 0.7975	>F33FQ7E01A0YO6_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Barnesiella*	DB [5]	0.001851
0	2.6817 ± 0.6854	>F33FQ7E01BBPQI_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Barnesiella*	DB [5]	0.004465
0.2900 ± 0.1058	0.8761 ± 0.2243	>F33FQ7E01A6RU1_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Barnesiella*	202	0.045730
0	0.3336 ± 0.0743	>F33FQ7E01AQUIX_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Barnesiella*	DB [5]	0.002029
0	0.1618 ± 0.0619	>F33FQ7E02JC51O_123	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Barnesiella*	DB [5]	0.030955
0.1166 ± 0.0314	0.0251 ± 0.0065	>F33FQ7E02JY7FW_122	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Barnesiella*	−78	0.021467
0	0.0627 ± 0.0215	>F33FQ7E01ESHB2_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Barnesiella*	DB [4]	0.019226
0	0.0307 ± 0.0090	>F33FQ7E01CL9NZ_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Barnesiella*	DB [4]	0.009230
0	0.0187 ± 0.0077	>F33FQ7E02GS3UF_123	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Barnesiella*	DB [3]	0.040921
0.0738 ± 0.0118	0.0058 ± 0.0058	>F33FQ7E02IN2XO_129	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Butyricimonas*	−92	0.000848
0	0.1167 ± 0.0364	>F33FQ7E01ALRJX_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Odoribacter*	DB [5]	0.012401
0.7043 ± 0.1259	0.2073 ± 0.0547	>F33FQ7E02FWNJN_12 6	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Parabacteroides*	−71	0.006770
0	1.1969 ± 0.2696	>F33FQ7E02IVXTK_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Tannerella*	DB [5]	0.002167
6.3752 ± 0.8801	1.2372 ± 0.4627	>F33FQ7E01CNJ30_125	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−81	0.000856
4.6642 ± 0.2789	1.2713 ± 0.2025	>F33FQ7E01E3FCL_129	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−73	0.000010
5.0688 ± 0.4022	0.5212 ± 0.0994	>F33FQ7E02FZN3W_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−90	0.000004
1.7177 ± 0.1814	0.2086 ± 0.0710	>F33FQ7E02GRXIH_124	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−88	0.000055
0	1.3093 ± 0.1395	>F33FQ7E01B29HO_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.000014
0	0.6324 ± 0.1115	>F33FQ7E01C3L4J_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.000469
0	0.1000 ± 0.0100	>F33FQ7E02HEYTH_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.000008
4.7315 ± 0.7697	0.9255 ± 0.2360	>F33FQ7E01A18K9_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−80	0.001487
2.2515 ± 0.4312	0.2271 ± 0.0648	>F33FQ7E01ANWA4_128	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−90	0.001661
0	2.1072 ± 0.5609	>F33FQ7E01BILQZ_123	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.005567
0	1.3335 ± 0.2954	>F33FQ7E01ASH5F_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.001964
0	1.0818 ± 0.3838	>F33FQ7E01A0TCD_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.022548
0	0.9768 ± 0.2686	>F33FQ7E01A3Z7I_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.006618

(Continued)

Table A2 | Continued

Lean*	*db/db***	Representative sequence	Phylum	Class	Order	Family	Genus	Change in *db/db* group*** (%)	*P*-value
0.4385 ± 0.0257	0.2444 ± 0.0521	>F33FQ7E01EDHYP_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−44	0.010208
0.5135 ± 0.1094	0.1153 ± 0.0555	>F33FQ7E01DHOIX_126	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−78	0.011764
0	0.5304 ± 0.1648	>F33FQ7E01BKMLI_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.012275
0	0.4636 ± 0.1270	>F33FQ7E01CK5EY_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.006491
0.2777 ± 0.0459	0.0940 ± 0.0334	>F33FQ7E01D8VQW_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−66	0.011976
0.2491 ± 0.0575	0.0559 ± 0.0229	>F33FQ7E01DCG2L_129	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−78	0.014184
0	0.3102 ± 0.1254	>F33FQ7E01DCCQ5_122	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.038424
0.1928 ± 0.0376	0.0305 ± 0.0189	>F33FQ7E01ESAZ9_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−84	0.004808
0	0.2219 ± 0.0733	>F33FQ7E01C8TRS_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.016389
0.1610 ± 0.0267	0.0370 ± 0.0174	>F33FQ7E02JDUL3_123	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−77	0.004563
0	0.1798 ± 0.0502	>F33FQ7E01EGUX5_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.007194
0	0.1560 ± 0.0425	>F33FQ7E02F8ZOW_125	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.006319
0	0.1001 ± 0.0331	>F33FQ7E01AKVVR_121	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [5]	0.016490
0.0698 ± 0.0219	0	>F33FQ7E02G9TBG_129	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		LEAN [5]	0.013009
0	0.0687 ± 0.0222	>F33FQ7E02H89Q9_124	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [4]	0.014827
0.0724 ± 0.0199	0	>F33FQ7E01A2NC7_129	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−100	0.006663
0	0.0550 ± 0.0237	>F33FQ7E01DZNQX_123	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [4]	0.049216
0.0516 ± 0.0079	0.0066 ± 0.0066	>F33FQ7E02FI9M2_127	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		−87	0.002383
0.0522 ± 0.0154	0	>F33FQ7E01EMBT6_130	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		LEAN [4]	0.009508
0	0.0302 ± 0.0092	>F33FQ7E02GEE19_124	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		DB [4]	0.010932
0.0192 ± 0.0081	0	>F33FQ7E02IRC4P_129	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		LEAN [3]	0.044906
0.0185 ± 0.0077	0	>F33FQ7E02FIWV8_128	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae		LEAN [3]	0.041773
0	2.3778 ± 0.4610	>F33FQ7E02FKEY5_121	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*	DB [5]	0.000866
0	0.0327 ± 0.0105	>F33FQ7E01DGOVW_121	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*	DB [4]	0.014218
0	0.0203 ± 0.0083	>F33FQ7E01C3YFS_124	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*	DB [3]	0.040453
0	0.9523 ± 0.3752	>F33FQ7E01A2XH5_121	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae		DB [4]	0.034809
1.8525 ± 0.1536	0.9054 ± 0.0629	>F33FQ7E02I2HAW_125	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	−51	0.000451
0	2.0120 ± 0.3326	>F33FQ7E01B7G3N_122	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	DB [5]	0.000306
0	1.2598 ± 0.1877	>F33FQ7E01ECJMT_121	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	DB [5]	0.000151
0	0.3096 ± 0.0239	>F33FQ7E01D10PO_121	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	DB [5]	0.000001
0	0.0438 ± 0.0071	>F33FQ7E02HM2Q2_121	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	DB [5]	0.000260
0	0.9561 ± 0.2985	>F33FQ7E01DV3E4_125	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	DB [5]	0.012559

0.0192 ± 0.0081	04489 ± 0.0977	>F33FQ7E01BPSG1_121	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	2232	0.002335
0	0.1718 ± 0.0358	>F33FQ7E02HL6E5_121	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	DB [5]	0.001354
0	0.1270 ± 0.0356	>F33FQ7E02FW8RL_123	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	DB [4]	0.007337
0	0.0971 ± 0.0284	>F33FQ7E01CI251_124	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	DB [5]	0.009102
0	0.0627 ± 0.0198	>F33FQ7E01BPHML_121	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	DB [5]	0.013358
0.1365 ± 0.0202	0.0121 ± 0.0074	>F33FQ7E01D8XEK_128	Bacteroidetes	Bacteroidia	Bacteroidales			−91	0.000415
11.4169 ± 1.5069	3.6840 ± 1.3411	>F33FQ7E02G32BC_122	Bacteroidetes	Bacteroidia	Bacteroidales			−68	0.004994
1.4719 ± 0.1867	0.3390 ± 0.1286	>F33FQ7E02I8FKV_127	Bacteroidetes	Bacteroidia	Bacteroidales			−77	0.001058
0	1.2048 ± 04604	>F33FQ7E01AQVVG_121	Bacteroidetes	Bacteroidia	Bacteroidales			DB [5]	0.030802
0	0.0978 ± 0.0205	>F33FQ7E01D7P9O_124	Bacteroidetes	Bacteroidia	Bacteroidales			DB [5]	0.001401
0	0.0834 ± 0.0345	>F33FQ7E02GLPUM_123	Bacteroidetes	Bacteroidia	Bacteroidales			DB [4]	0.041894
0	0.0520 ± 0.0152	>F33FQ7E01AZMCB_121	Bacteroidetes	Bacteroidia	Bacteroidales			DB [5]	0.008918
0.0551 ± 0.0181	0.0058 ± 0.0058	>F33FQ7E01DIWOC_128	Bacteroidetes					−90	0.031791
0.0480 ± 0.0174	0.0056 ± 0.0056	>F33FQ7E02H64OA_130	Bacteroidetes					−88	0.048434
0.0729 ± 0.0549	1.3321 ± 0.3500	>F33FQ7E01DDMOM_121	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	1728	0.007464
0.6410 ± 0.1434	0.0534 ± 0.0378	>F33FQ7E02GQQO9_121	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	−92	0.004156
0.3824 ± 0.1115	0.0640 ± 0.0192	>F33FQ7E01EC512_127	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		−83	0.022693
0	0.2492 ± 0.0886	>F33FQ7E01DMCSH_121	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		DB [5]	0.022742
0.0549 ± 0.0144	0.0065 ± 0.0065	>F33FQ7E02HWP6Z_123	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		−88	0.015639
0.0614 ± 0.0262	0	>F33FQ7E02GZ92D_130	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		LEAN [3]	0.047037
0.0368 ± 0.0117	0	>F33FQ7E02H234E_129	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		LEAN [4]	0.013502
0	0.0180 ± 0.0074	>F33FQ7E02GU66L_121	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		DB [3]	0.040941
1.4801 ± 0.2784	4.5705 ± 1.1523	>F33FQ7E01A2P5 Q_121	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillibacter*	209	0.031277
0.0129 ± 0.0079	0.3393 ± 0.0881	>F33FQ7E01A0MY3_121	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillibacter*	2533	0.006123
0.0530 ± 0.0168	0.2026 ± 0.0618	>F33FQ7E01DZQUH_125	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillibacter*	282	0.047669
0	0.2003 ± 0.0687	>F33FQ7E02GQMSD_123	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		DB [4]	0.019376
0	0.0830 ± 0.0206	>F33FQ7E01CWU7L_122	Firmicutes	Clostridia	Clostridiales			DB [5]	0.003790
0	1.1947 ± 0.2830	>F33FQ7E01AOJ8E_121	Proteobacteria	Deltaproteobacteria				DB [5]	0.002912
0	0.1342 ± 0.0254	>F33FQ7E01C4Q69_123	Proteobacteria					DB [5]	0.000751
0	0.9103 ± 0.3114	>F33FQ7E01DFMOY_123						DB [5]	0.019191

Data are the mean ± SE.

**Change in average relative 97%-ID phylotype abundance in the db/db mice subset when compared to the lean mice subset.*

****Only significant ($P < 0.05$) differences between the two groups are indicated. P-values are based on the two-sample t-test assuming equal variances. DB, phylotypes found only in prebiotic-fed mice; LEAN, phylotypes found only in the lean group; [number] following DB or LEAN corresponds to the number of samples in the subset in which the given phylotype was found.*

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Gut microbiota, immunity, and disease: a complex relationship

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Our immune system has evolved to recognize and eradicate pathogenic microbes. However, we have a symbiotic relationship with multiple species of bacteria that occupy the gut and comprise the natural commensal flora or microbiota. The microbiota is critically important for the breakdown of nutrients, and also assists in preventing colonization by potentially pathogenic bacteria. In addition, the gut commensal bacteria appear to be critical for the development of an optimally functioning immune system. Various studies have shown that individual species of the microbiota can induce very different types of immune cells (e.g., Th17 cells, Foxp3$^+$ regulatory T cells) and responses, suggesting that the composition of the microbiota can have an important influence on the immune response. Although the microbiota resides in the gut, it appears to have a significant impact on the systemic immune response. Indeed, specific gut commensal bacteria have been shown to affect disease development in organs other than the gut, and depending on the species, have been found to have a wide range of effects on diseases from induction and exacerbation to inhibition and protection. In this review, we will focus on the role that the gut microbiota plays in the development and progression of inflammatory/autoimmune disease, and we will also touch upon its role in allergy and cancer.

Keywords: commensal bacteria, microbiota, autoimmunity, allergy, cancer, Treg, Th17, probiotics

INTERACTION BETWEEN THE GUT MICROBIOTA AND THE IMMUNE SYSTEM

The trillions of commensal microorganisms that constitute the intestinal microbiota are primarily composed of five bacterial phyla, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*. *Bacteroidetes* and *Firmicutes* predominate and represent ∼90% of the total gut microbiota (i.e., 25 and 65%, respectively; Rajilic-Stojanovic et al., 2007). However, these proportions can vary greatly between individuals and even within single individual over time. Although commensal bacteria inhabit the entire gut, greater numbers reside in the distal part of the small intestine and in the large intestine or colon. Acquisition of the intestinal microbiota occurs in the first year of life and is influenced by the maternal bacteria obtained during vaginal delivery (Bennet and Nord, 1987; Mandar and Mikelsaar, 1996; Penders et al., 2006), versus Cesarean section (Dominguez-Bello et al., 2010), and breastfeeding (Yoshioka et al., 1983; Balmer and Wharton, 1989; Harmsen et al., 2000; Hopkins et al., 2005). Early in life the major shifts in the gut microbiota can be caused by antibiotics, dietary changes, etc. (Koenig et al., 2011) as opposed to a purely stochastic model (Palmer et al., 2007). However, there is a lack of large-scale studies of temporal variation in adults.

The gut microbiota plays a critical role in the breakdown of indigestible complex plant polysaccharides, and provides an important layer of defense against invasion by pathogenic microorganisms. It is also required for proper development of the immune system, as indicated by the fact that germ-free (GF) mice have poorly developed lymphoid tissues. GF mice have spleens with few germinal centers and poorly formed T and B cell zones, hypoplastic Peyer's patches, lower numbers of lamina propria CD4$^+$ cells and IgA-producing plasma cells (Macpherson and Harris, 2004), and aberrant development and maturation of isolated lymphoid follicles (Bouskra et al., 2008). The lymphoid organs of GF mice exhibit aberrant development of T and B cell subsets, and more specifically, contain CD4$^+$ T cells that are skewed toward an T helper 2 (Th2) phenotype and produce little, if any, IFNγ (Mazmanian et al., 2005), and have fewer and smaller germinal centers (Bauer et al., 1963). There is also evidence that the microbiota plays a key role in the induction of IgA (Klaasen et al., 1993b; Talham et al., 1999) and maintenance of homeostasis of various T cell populations in the gut, including regulatory T cells (Tregs), and T helper 1 (Th1) and 17 (Th17) cells (Gaboriau-Routhiau et al., 2009).

The commensal bacteria that reside in the gut are diverse, and individual species appear, in some cases, to have distinct and opposing roles. Some commensal bacteria appear to drive Treg development preferentially, while others promote Th17 development. In a comparative analysis of terminal ileal tissue from mice maintained in conventional (CV) versus GF housing, tissue from CV mice exhibited 5- to 10-fold higher IL-10 and Foxp3 mRNA expression compared to GF mice, suggesting that the microbiota can drive Foxp3$^+$Treg induction and/or expansion in the intestine (Gaboriau-Routhiau et al., 2009). Furthermore, in the Peyer's patches and mesenteric lymph nodes (LN) of naive GF mice, Tregs were at a decreased frequency, were less effective at suppressing responder cell proliferation *in vitro*, and produced lower levels of IL-10 and TGFβ than naive mice housed under specific pathogen-free (SPF) conditions (Ishikawa et al., 2008). The colonization of

GF mice with *Bacteroides fragilis* increases the suppressive capacity of Tregs and induces anti-inflammatory cytokine production by Foxp3$^+$ T cells in the gut (Mazmanian et al., 2008). Similarly, colonization of GF mice with a cocktail of *Clostridium* strains increased dramatically the frequency of IL-10-producing Helios$^-$ Tregs (induced Tregs) in the colonic lamina propria (Atarashi et al., 2011). Numerous studies have also found that Tregs are induced following gavage with gut commensal bacteria of either the *Lactobacillus* or *Bifidobacterium* genus (Di Giacinto et al., 2005; O'Mahony et al., 2008; Karimi et al., 2009; Livingston et al., 2010; Zhang et al., 2010). In contrast, Th17 cell responses appear to be induced by a restricted number of microbial species, e.g., segmented filamentous bacteria (SFB). *Candidatus arthromitus*, commonly called SFB, are Gram-positive bacteria of the *Firmicutes* phylum and most closely related to the *Clostridium* genus (Snel et al., 1995). These bacteria colonize the small intestine and have been shown to stimulate the production of secretory IgA (Klaasen et al., 1993b; Talham et al., 1999) and to activate CD8αβ$^+$TCRαβ$^+$ intraepithelial cells in the small intestine (Umesaki et al., 1995). The SFB are thought to play a key role in the maturation of the adaptive mucosal immune response in the gut. Indeed, SFB colonization of GF mice has been shown to induce differentiation of primarily IL-17$^+$ T cells (Ivanov et al., 2008, 2009; Gaboriau-Routhiau et al., 2009) and to a far lesser extent, IFNγ$^+$Th1 and IL-10$^+$Treg cells (Gaboriau-Routhiau et al., 2009). These data indicate that SFB can promote the development of intestinal Th17 cells, a population of T cells that is not only important for fighting bacterial infection, but is also involved in the pathogenesis of a number of inflammatory and autoimmune diseases. A microbiota favoring SFB could, therefore, have an impact on the immune response, and consequently, on the development of Th17-mediated inflammatory/autoimmune diseases in the gut and at distant sites in predisposed individuals. The specific mechanisms that lead to the preferential induction of Tregs versus Th17 cells by various commensal bacteria are currently unknown. However, DNA derived from commensal bacteria has been shown to play a major role in intestinal homeostasis through toll-like receptor 9 (TLR9) engagement, and appears to be involved in controlling the balance between Tregs and effector cells. Indeed, TLR9 deficient (TLR9$^{-/-}$) mice exhibit increases in intestinal Foxp3$^+$ Tregs, and decreases in IL-17 and IFN-γ production by comparison to wild-type mice (Hall et al., 2008). One could speculate that specific commensal species may differentially modulate the T effector/Treg cell balance via this mechanism, since the ability of gut commensal bacteria to stimulate TLR9 varies and depends on the frequency of CG dinucleotides (Dalpke et al., 2006).

The composition of the microbiota can be influenced by various factors, including diet and exposure to antibiotics. Analysis of the fecal microbiota of humans and 59 other mammalian species has shown that the gut microbiota of humans living a modern lifestyle is typical of omnivorous primates (Ley et al., 2008). Moreover, feeding GF mice colonized with human fecal microbial communities a high-fat, high sugar "Western" diet instead of a low-fat, plant polysaccharide-rich diet significantly altered the microbiota composition, resulting in an increase in *Firmicutes* and decrease in *Bacteroidetes* (Turnbaugh et al., 2009). In contrast, a diet very high in fiber has been associated with increases in *Bacteroidetes* and a much lower abundance of *Firmicutes* in humans (De Filippo et al., 2010), although other factors may confound these comparisons. Interestingly, fluctuations in the proportions of these types of commensal bacteria have been found in patients and animals with inflammatory/autoimmune diseases (see below). In addition, antibiotic treatment is usually followed by a decrease in the diversity of the microbiota. Although the composition of the microbiota is similar in the days or weeks following the termination of treatment, some bacterial members are lost from the community indefinitely (Jernberg et al., 2007; Jakobsson et al., 2010). Furthermore, different types of antibiotics differentially affect bacteria and, consequently, immune cell development. Antibiotic treatment (ampicillin, gentamicin, metronidazole, neomycin, and vancomycin) that reduced bacteria from the *Firmicutes* phylum while increasing the proportion of bacteria from the *Bacteroidetes*, phylum was associated with a reduction in mucosal CD4$^+$T cells expressing IFNγ and IL-17 (Hill et al., 2010). Similarly, vancomycin or ampicillin treatment, both of which inhibit Gram-positive bacteria, dramatically decreased the numbers of Th17 cells in the lamina propria. In contrast, metronidazole or neomycin, which target anaerobes and Gram-negative bacteria, had little effect on the Th17 cell population (Ivanov et al., 2008). Because the microbiota profoundly affects immune system development and maturation, it is not surprising that modulation of the balance between the various commensal bacteria can influence inflammatory/autoimmune disease development and/or progression.

INVOLVEMENT OF COMMENSAL BACTERIA IN PROTECTION AGAINST AUTOIMMUNITY

The less than 100% concordance rate for autoimmune diseases in monozygotic twins (e.g., <40% for type 1 diabetes; Hyttinen et al., 2003), suggests that environmental factors can have a strong influence on inflammatory/autoimmune disease development. The composition of the microbial community that comprises the microbiota varies between individuals including monozygotic twins. Because the gut commensal bacteria influences the development of the immune system, the microbiota could be one of the environmental factors that affects inflammatory/autoimmune disease development in genetically susceptible individuals, i.e., differences in its composition could contribute to the lower than expected concordance rate in monozygotic twins. The composition of the gut microbiota may, therefore, either confer protection or trigger disease in genetically susceptible individuals.

Considerable evidence indicates that the composition of the microbiota can influence the development of intestinal inflammation. Some commensal bacteria may induce intestinal inflammation while others control it. The commensals capable of controlling inflammation in the gut mediate their effect either by balancing the immune response in favor of regulation or by controlling bacteria that may directly mediate intestinal inflammation. With regard to balancing the immune response, commensal bacteria from the *Bacteroidetes* and *Firmicutes* phyla appear to induce Tregs that can control Th17 cells that are responsible for intestinal inflammation. In a recent report, colonization of GF mice with the human commensal, *B. fragilis*, was shown to induce IL-10 production and Foxp3 expression, decrease Th17 cells and

prevent colitis development. Furthermore, injection of polysaccharide A (PSA) isolated from *B. fragilis* was found to recapitulate the effects of colonization with *B. fragilis* (Mazmanian et al., 2008; Round and Mazmanian, 2009), with PSA-induced signaling through TLR2 expressed by Tregs promoting tolerance (Round et al., 2011). In addition, mice orally inoculated with murine *Clostridium* species belonging to clusters IV and XIVa (*Firmicutes* phylum) were resistant to colitis development and exhibited an increase in the frequency of colonic Tregs (Atarashi et al., 2011). Various human *Lactobacillus* strains (of the *Firmicutes* phylum) have also been shown to be protective in several animal models of colitis, including the TNBS-induced and IL-10 deficient mouse models of colitis, and were found to mediate their effect via induction of Tregs (Di Giacinto et al., 2005; O'Mahony et al., 2008; Livingston et al., 2010). Commensal bacteria can also affect other potentially pathogenic bacteria; for example, commensal bacteria from the *Actinobacteria* phylum appear to control the levels of other commensal bacteria that cause intestinal inflammation. Administration of *Bifidobacterium animalis* subsp. *Lactis* (*Actinobacteria* phylum) reduced intestinal inflammation in the T-bet$^{-/-}$Rag2$^{-/-}$ model of colitis. This was associated with a concomitant reduction in *Enterobacteriaceae* levels, the commensal bacteria shown to initiate intestinal inflammation in this colitis model. Moreover, the authors reported that the fecal levels of *Bifidobacterium* in untreated T-bet$^{-/-}$Rag2$^{-/-}$ mice were lower compared to control mice, and found that the *Bifidobacterium* fecal levels were inversely related to the colitis score (Veiga et al., 2010). Taken together, these studies demonstrate that several different species of commensal bacteria can control inflammation in the gut via different mechanisms, e.g., by directly regulating the immune response or by affecting the composition of the microbiota and reducing other disease-mediating commensals.

Commensal bacteria can also have a protective effect at sites distant from the gut. The results of one recent study suggest that the microbiota plays an important role in type 1 diabetes in genetically susceptible mice. Type 1 diabetes-susceptible NOD mice that were crossed with MyD88$^{-/-}$ mice (NODMyD88$^{-/-}$) were found to be protected from type 1 diabetes. This protection appeared to involve changes in the composition of the microbiota, since protection was abrogated when NODMyD88$^{-/-}$ mice were treated with antibiotics or derived and maintained under GF conditions. A comparison of the gut microbiota between NODMyD88 sufficient and NODMyD88$^{-/-}$ mice confirmed that there were differences in the microbiota composition which could account for the differences in disease susceptibility. The NODMyD88$^{-/-}$ mice had a lower *Firmicutes*/*Bacteroidetes* ratio than the NODMyD88 sufficient mice, and an enrichment in cecal *Lactobacillacae*, *Rikenellaceae*, and *Porphyromonadaceae*. Finally, wild-type NOD mice that were colonized at birth with microbiota from NODMyD88$^{-/-}$ mice exhibited significantly reduced islet infiltration compared to untreated NOD mice (Wen et al., 2008), i.e., the microbiota from the NODMyD88$^{-/-}$ mice conferred some protection from diabetes to wild-type NOD mice. Moreover, a recent publication has reported that female NOD mice colonized with SFB exhibited a large Th17 population in the lamina propria of the small intestine and were protected from developing type 1 diabetes (Kriegel et al., 2011). These findings corroborate studies showing that Th17 cells do not directly mediate type 1 diabetes in NOD mice (Bending et al., 2009; Martin-Orozco et al., 2009), and in fact, appear to act as regulatory cells that protect against disease development (Han et al., 2010; Nikoopour et al., 2010). Interestingly, a recent study has shown that Th17 cell pathogenicity can be controlled in the small intestine, where pro-inflammatory Th17 cells can acquire a regulatory phenotype (rTh17) that includes *in vivo* immunosuppressive properties (Esplugues et al., 2011). Taken together, these data suggest that gut microbiota has a significant, if complex, influence on the development of inflammatory/autoimmune diseases. Another study has found that the microbiota plays a protective role in a model of collagen-induced arthritis. The study found that rats maintained in a GF environment developed more severe rheumatoid arthritis (RA) than rats maintained in a conventional environment, suggesting that the microbiota had a suppressive effect on RA development (Breban et al., 1993). Lastly, a series of studies has shown that the microbiota can protect against central nervous system (CNS) disease. Oral administration of antibiotic, leading to a reduction in bacteria from the *Firmicutes* phylum, but a relative increase in bacteria from the *Bacteroidetes* phylum, impaired the development of myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein-induced experimental autoimmune encephalitis (EAE) in mice. In this study, protection was associated with diminished pro-inflammatory responses, higher numbers of Tregs in mesenteric LN and potent IL-10-producing Foxp3^{+} Tregs that were present in the LN (Ochoa-Reparaz et al., 2009, 2010). Moreover, oral administration of PSA isolated from *B. fragilis* (of the *Bacteroidetes* phylum) was able to prevent EAE development via enhancement of CD103^{+} DC that converted naïve T cells into IL-10-producing Foxp3^{+} Tregs, suggesting that antibiotic-mediated protection is mediated the increase in *Bacteroidetes* (Ochoa-Reparaz et al., 2009, 2010). Finally, oral administration of probiotics, including the *Lactobacillus* and *Bifidobacterium* species, has been shown to protect against development of various autoimmune diseases such as type 1 diabetes (Matsuzaki et al., 1997; Calcinaro et al., 2005), experimental autoimmune encephalomyelitis (Lavasani et al., 2010), experimental RA (Kato et al., 1998; Baharav et al., 2004; So et al., 2008), and systemic lupus erythematosus (personal observation; Alard et al., 2009), via induction of IL-10-producing Tregs and attenuation of Th1 and Th17 cytokines. It is not clear whether the Tregs are induced upon direct interaction with the probiotics and/or via stimulation with tolerogenic antigen presenting cells that have been induced by the probiotics. We have, however, shown recently that dendritic cells cultured with *Lactobacillus casei* shift their cytokine profile in favor of IL-10, and are able to prevent type 1 diabetes development upon injection into NOD mice. Since a single injection of *L. casei*-treated DC is sufficient to mediate long-term protection, the mechanism in this case most likely involves induction of regulatory cells (Manirarora et al., 2011). Altogether, these data emphasize the importance of a balanced gut microbiota that can induce a protective immune response capable of suppressing inflammation in organs distant from the gut, via induction of tolerogenic DC and Tregs. How these cells traffic from the gut to the distal lymph nodes and possibly to the peripheral tissue (CNS, joint, pancreas) is currently unknown and remains to be determined.

Studies have indicated that the composition of the microbiota can have a significant effect on the development of inflammatory/autoimmune disease in humans. The composition of the gut microbiota differs greatly in subsets of patients with inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, compared to non-IBD individuals. The microbiota of IBD patients is characterized by depletion of *Bacteroidetes* and *Firmicutes* (Frank et al., 2007). More specifically, recurrence of Crohn's disease was associated with decreases in the relative proportion of *Faecalibacterium prausnitzii*, a bacterium belonging to *Clostridium* cluster IV, in these patients (Sokol et al., 2009). In animal studies, *F. prausnitzii* has been shown to have anti-inflammatory effects, and oral administration of *F. prausnitzii* has been found to reduce the severity of TNBS-induced colitis (Sokol et al., 2008). These data suggest that restoration of the microbial balance in IBD patients may be a good strategy to treat this disease, and in fact, administration of VSL#3, a cocktail of several *Lactobacillus* species (*L. Casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Lactobacillus delbrueckii*), *Bifidobacterium* species (*Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium infantis*) and a *Streptococcus salivarius* subsp., *Thermophilus*, has been shown to decrease ulcerative colitis in patients with mild to moderately active ulcerative colitis (Sood et al., 2009; Tursi et al., 2010). Studies in patients with early RA have also found that *Bifidobacteria* and *B. fragilis* in the gut are decreased (Vaahtovuo et al., 2008), suggesting that alteration in the abundance of these two commensal species may influence the pathogenesis of RA. However, in patients with established disease, the possibility that an alteration in the composition of the microbiota may be a consequence rather than a cause of the disease cannot be ruled out.

In conclusion, commensal bacteria belonging to the *Bifidobacterium*, *Bacteroides*, *Clostridium*, and *Lactobacillus* genera are associated with inflammatory/autoimmune disease protection. Therefore, establishing a balanced microbiota in favor of these protective commensal bacteria may be a good strategy for the prevention and/or treatment of inflammatory/autoimmune diseases in autoimmune-prone individuals.

INVOLVEMENT OF COMMENSAL BACTERIA IN TRIGGERING INFLAMMATORY/AUTOIMMUNE DISEASE

Microbial pathogens have long been implicated in the etiology of a variety of autoimmune/inflammatory diseases, including IBD (Sanderson and Hermon-Taylor, 1992; Chen et al., 2000), RA (Toivanen, 2003), experimental autoimmune encephalomyelitis (Cermelli and Jacobson, 2000; Buljevac et al., 2005; Gilden, 2005; Farrell et al., 2009), type 1 diabetes (Filippi and von Herrath, 2005), and systemic lupus erythematosus (Cavallo and Granholm, 1990; Zandman-Goddard and Shoenfeld, 2005; Poole et al., 2006). However, a growing body of evidence suggests that specific commensal bacteria may also negatively impact inflammatory and autoimmune diseases in genetically susceptible individuals. Interestingly, rendering mice germ-free (GF) can dramatically alter inflammatory/autoimmune disease incidence and severity, and/or the kinetics of disease onset and progression in animal models of disease. This is not surprising since we know that commensal bacteria play an important role in shaping the host systemic immune response. However, the mechanisms underlying the relationship between commensal microorganisms and the induction/exacerbation of autoimmune/inflammatory diseases are poorly understood. Furthermore, it is unclear how individual microbial species comprising the microbiota contribute to the effects on these diseases. Recent studies focusing on the relationship between the microbiota and induction of autoimmunity have begun to shed some light on these issues.

Most mouse models of colitis are antibiotic-responsive, and patients with IBD often benefit from treatment with antibiotics, suggesting that bacteria play a role in disease pathogenesis. The general consensus is that IBD may be driven by aberrant pro-inflammatory host responses to the commensal microbiota (Packey and Sartor, 2008). A recent study has found that the microbiota can induce inflammation in models of colitis by activating T cells via both innate and adaptive immune mechanisms (Feng et al., 2010). In that study, homeostatic proliferation of transferred T cells was inhibited in GF RAG$^{-/-}$ mice, but restored after reconstitution with Altered Schaedler's Flora (a standardized cocktail of mouse microbiota), indicating that T cells require the presence of the microbiota to proliferate. Moreover, microbiota-induced production of IL-6 by DC was required to reach the threshold number of IFNγ and IL-17-producing microbiota-specific T cells (i.e., induction of T cell proliferation) that was needed to induce colitis (Feng et al., 2010). In other studies, a number of bacterial species isolated from the intestines of IBD patients have been shown to be capable of inducing intestinal inflammation in rodents, including *Helicobacter hepaticus*, enterotoxigenic *B. fragilis*, and *Bacteroides vulgatus*. *H. hepaticus* infection is associated with the spontaneous development of colitis in some types of immunodeficient, but not immunocompetent mice (Ward et al., 1996). Furthermore, colonization with enterotoxigenic strains, but not non-toxigenic strains, of *B. fragilis* have been shown to induce colitis. Enterotoxigenic *B. fragilis* of human origin mediates colitis in multiple intestinal neoplasia (Min) transgenic mice in a Th17 cell-dependent manner (Wu et al., 2009). Similarly, enterotoxigenic *B. fragilis* of piglet origin has been shown to enhance DSS-induced colitis in mice (Rabizadeh et al., 2007), and the *B. fragilis* toxin appears to be essential for disease pathogenesis (Rhee et al., 2009). In an older study, colitis severity was found to be increased in a carrageenan-based model of colitis in guinea pigs after oral administration of *B. vulgatus* isolated from IBD patients, but not healthy individuals. This disease exacerbation could be transferred to recipients via spleen cells (Onderdonk et al., 1984), suggesting that bacteria isolated from IBD patients were inducing a cell-mediated (most likely T cell) immune response capable of enhancing inflammation. Interestingly, another study has found that a single species of commensal bacteria, filamentous segmented bacteria (SFB), can induce intestinal inflammation in the presence of a limited microbiota (Stepankova et al., 2007). In this study, GF SCID mice were colonized with individual or combinations of strains of anaerobic and aerobic bacteria and SFB, including *Enterococcus faecalis*, SFB, *Fusobacterium mortiferum*, *Bacteroides distasonis*, *F. mortiferum* + SFB, *B. distasonis* + SFB, and a SPF bacterial cocktail with limited diversity (SPF cocktail) ±SFB. Using the CD45^{high} transfer model of intestinal inflammation, the study found that only the mice colonized with the SPF cocktail +SFB developed clinical and histological signs of intestinal inflammation

following transfer of CD45RB^{high} cells (Stepankova et al., 2007). These data indicate that, unlike SFB, most gut bacteria cannot or do not trigger intestinal inflammation in the presence of a limited microbiota, such as the typical SPF bacteria.

The involvement of SFB in inflammatory disease pathogenesis is not limited to the intestine. Two recent studies have shown that SFB may also be involved in development of experimental autoimmune encephalomyelitis (EAE) (Lee et al., 2011) and rheumatoid arthritis (RA) (Wu et al., 2010). In the first study, the authors found that GF mice exhibited decreased severity of MOG-induced EAE compared to mice raised under SPF conditions. The reduced severity was associated with reduced IL-17A and IFNγ production and RORγt transcripts, but elevated levels of $CD4^+CD25^+Foxp3^+$ cells. Interestingly, whereas transfer of $CD4^+$ T cells from MOG-immunized GF mice into $RAG^{-/-}$ GF mice only induced very mild EAE, transfer of $CD4^+$ T cells from MOG-immunized standard pathogen free (SPF) mice induced severe disease, suggesting that the gut microbiota plays a role in activating T cells capable of inducing EAE. Finally, intestinal colonization of GF mice with SFB alone restored susceptibility to severe EAE induction, and correlated with elevated IL-17 and IFNγ expression in T cells in the spinal cord and small intestine lamina propria as well as a reduction of $Foxp3^+$ T cell levels. This study demonstrates that intestinal colonization with SFB can induce Th17 cells that attack the central nervous system (CNS) (Lee et al., 2011). In the second study, arthritis-prone K/BxN mice raised under GF conditions exhibited attenuated RA that was associated with a reduction in serum autoantibody titers, splenic autoantibody-secreting cells, germinal centers, and IL-17. Interleukin-17 was found to be critical for the pathogenesis of the disease in these mice, as treatment with anti-IL-17 antibodies abrogated RA development in mice raised under SPF conditions. Interestingly, colonization of GF K/BxN mice with a single bacterial species, SFB, resulted in the rapid development of arthritis, which correlated with restoration of IL-17-producing T cells in the lamina propria. These cells could in turn migrate to the spleen where they provided help for germinal center formation and antibody production (Wu et al., 2010). Therefore, SFB, a bacterium known to induce IL-17 production (Ivanov et al., 2009), is capable of triggering RA in arthritis-prone K/BxN mice via activation of Th17 cells (Wu et al., 2010). Colitis, EAE, and RA are all mediated by IL-17-producing cells, and it is perhaps not surprising that a commensal bacteria that induces Th17 cells may be involved in the etiology of these diseases. However, it is surprising that a commensal gut bacteria (such as SFB) can play a role in the development of autoimmune disease at a distant site, i.e., CNS (Lee et al., 2011) or joints (Wu et al., 2010). In fact, evidence of SFB-induced Th17 cells could be found not only in the intestine, but also in the spinal chord and spleen, demonstrating that a single intestinal commensal bacterial species is capable of priming immune responses that are extra-intestinal and extend to peripheral lymphoid organs and tissues. However, the role that the intestinal SFB-induced Th17 cells play at the disease sites, i.e., in the spinal chord or the joints, remains to be determined. Several studies have shown that the presence of IL-17 in the joints enhanced disease (Lubberts et al., 2004; Jacobs et al., 2009), suggesting that Th17 cells could amplify the inflammatory process in the joints. Indeed, IL-17 receptor is expressed by a wide range of synovial cells (Yao et al., 1995; Silva et al., 2003), and IL-17 drives these cells to produce pro-inflammatory mediators such as cytokines (GM-CSF) and chemokines (CXCL2, CXCL8), and induces recruitment of neutrophils (Laan et al., 1999, 2003). Moreover, one could speculate that Th17 cells may also participate in the inflammatory process by producing other cytokines capable of promoting the inflammatory response. For instance, IL-23 and GM-CSF have been shown very recently to play a crucial role in EAE induction. Interleukin-23 appears to induce Th17 or $ROR\gamma t^+$ T cells to produce GM-CSF that is required for disease induction; this was demonstrated in experiments showing that GM-CSF-producing IL-17-deficient T cells, but not GM-CSF-deficient Th17 cells, were able to induce EAE (Codarri et al., 2011; El-Behi et al., 2011). Furthermore, GM-CSF seems to target the myeloid cells that are responsible for sustaining inflammation in the CNS (El-Behi et al., 2011). Depending on the type of autoimmune diseases or the site of inflammation, SFB-induced Th17 cells may therefore promote/exacerbate inflammation in tissue through the production of GM-CSF and/or IL-17.

Understanding the role that commensal bacteria play in disease development in humans has been challenging. Studies about the composition of the gut microbiota in patients have identified bacteria with the potential to cause disease. Nonetheless, it is also possible that these microorganisms colonize the gut after disease is established and are not involved in the development of the disease. There is, however, a general consensus that IBD arises from an abnormal immune response to bacterial components of the intestinal microbiota (Packey and Sartor, 2008). Indeed, it has been reported that a subset of people with Crohn's disease harbor a strain of adherent–invasive *Escherichia coli* in their small intestine that is potentially pro-inflammatory (Pineton de Chambrun et al., 2008). In addition, RA patients treated with antibiotics such as minocycline exhibit significant disease improvement (Stone et al., 2003), suggesting that the commensal bacteria contributes, at some level, to disease development. Although it has been shown that humans can be colonized with SFB (Klaasen et al., 1993a), there is little evidence yet for involvement of SFB in inflammatory/autoimmune diseases in humans. It would be of great interest to determine whether SFB is more prominent in the microbiota of patients exhibiting certain types of autoimmune diseases, i.e., Th17-mediated. Moreover, multiple issues remain to be addressed concerning the involvement of SFB in autoimmune diseases, including identifying the molecules that mediate Th17 cell induction as well as determining whether other Th17-mediated autoimmune diseases can be triggered by SFB.

ROLE OF THE MICROBIOTA IN ALLERGIC DISEASES

In addition to inflammatory and autoimmune diseases, there are other chronic diseases that may be impacted by the gut microbial community. In particular, allergic disease development has been associated with alterations in the intestinal microbiota. The incidence of allergic diseases has increased over the past 40 years in industrialized countries including the United States, Canada, United Kingdom, Ireland, New Zealand, and Australia, but not in developing countries. These data suggest that environmental changes are a major factor in the development of allergic diseases (Asher, 1998; Mannino et al., 1998; Beasley et al., 2000).

The "hygiene hypothesis" suggests that the increase in the prevalence of allergic diseases observed in developed countries could be due to decreased early exposure to infectious agents that may alter the immune response and, in particular, the immunoregulatory compartment (Okada et al., 2010). However, the "microflora hypothesis" has been put forth as an alternative explanation and is based on changes in the intestinal microbiota that are due to antibiotic use and dietary differences (Noverr and Huffnagle, 2005). In either case, the regulatory mechanisms that normally control the Th2 responses generally associated with allergic diseases and asthma would not develop properly. Indeed, treatment of normal 3-week-old mice with the broad-spectrum antibiotic, kanamycin, results in the elimination of all Gram-negative bacteria in the stool, and a subsequent shift to a Th2 response (Oyama et al., 2001), suggesting that intestinal bacteria are involved in controlling the Th2 response. This was further supported in a study showing that treatment of mice with antibiotics combined with oral administration of the commensal fungus, *Candida albicans*, triggers pulmonary allergic responses (Noverr et al., 2004). Taken together, these studies suggest that the microbiota plays an important role in controlling allergic diseases.

Several studies have reported differences in the composition of the microbiota of infants who develop allergic diseases. Infants with food allergies were found to exhibit an imbalance between "beneficial" and potentially harmful bacteria, i.e., *Lactobacilli* and *Bifidobacteria* species were lower while coliforms and *Staphylococcus aureus* were higher (Bjorksten et al., 1999). Decreased levels of *Bifidobacteria* were also observed in infants with atopic eczema (Kirjavainen et al., 2001). Moreover, *Bifidobacteria* and *Enterococcus* were decreased while *Clostridium* was increased, resulting in a reduced ratio of *Bifidobacteria* to *Clostridia* in infants that developed atopic dermatitis (Bjorksten et al., 2001). These data suggest that specific species of intestinal commensal bacteria can play either a pathogenic or protective role in allergies that occur in the gut or at sites distant from the gut, such as lung and skin.

Prophylactic approaches based on the administration of probiotics to newborns at high risk for allergies have proven successful. The World Health Organization has defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" FAO/WHO (2002). Administration of *L. casei* GG to the mothers before and after (via breastfeeding) delivery prevents atopic eczema, as well as other atopic diseases that develop later, in children at risk (Kalliomaki et al., 2001, 2003). Protection from allergic diseases provided by oral administration of non-pathogenic *E. coli* in early life was shown to extend to adult life (Lodinova-Zadnikova et al., 2003). Finally, a number of studies have been performed using probiotics to treat pre-existing atopic disease in infants. These studies found that administration of *Lactobacilli* was able to decrease the severity of various allergic diseases, including atopic eczema, atopic dermatitis, and food allergy in these children (Majamaa and Isolauri, 1997; Kirjavainen et al., 1999; Rosenfeldt et al., 2003; Viljanen et al., 2005). There is no evidence to date indicating that probiotic therapies have a beneficial effect on the incidence or severity of asthma in children at high risk (Vliagoftis et al., 2008). However, studies using animal models have found that oral administration of certain *Lactobacilli* and *Bifidobacterium* species were able to modulate allergic responses in the respiratory tract apparently via induction of regulatory T cells, suggesting that it may be possible to treat asthma using an optimal combination of probiotics (Feleszko et al., 2007; Forsythe et al., 2007; Karimi et al., 2009; Lyons et al., 2010).

As described above for autoimmune diseases, microbiota that favor certain species of commensal bacteria appears to also predispose or protect susceptible individuals to allergy development at various extra-intestinal sites, including lung and skin. The mechanisms by which intestinal immune responses affect inflammatory responses at distant sites are still unknown and remain to be established.

ROLE OF THE MICROBIOTA IN CANCER

The intestinal microbiota does not only influence autoimmune and allergic diseases, but has also been implicated in the development of cancer, especially colon cancer. Colorectal cancer is highly prevalent in the United States, with an estimated 1 in 20 men and women receiving a diagnosis of colon or rectum cancer sometime during their lifetime (National Cancer Institute Website: http://seer.cancer.gov/statfacts/html/colorect.html). Similarly, colorectal cancer is the second and third most common cancer in women and men, respectively, in European Union countries (Ferlay et al., 2007). This is an important heath problem in developed countries, where the primary risk factor is diet in up to 80% of all colorectal cancers (Bingham, 2000).

Interestingly, accumulating evidence in the last 3 years has underscored the importance of the intestinal microbiota in the development of colorectal cancer (Bingham, 2000; Davis and Milner, 2009; O'Keefe et al., 2009; Uronis et al., 2009). In fact, diet and the composition of the microbiota strongly correlate with the risk of developing colorectal cancer in certain groups of individuals (Bingham, 2000; Davis and Milner, 2009; O'Keefe et al., 2009). One mechanism by which commensal bacteria contribute to the initiation of colorectal cancer involves the induction of inflammation by commensal bacteria. A study using an animal model of colorectal carcinoma, IL-10$^{-/-}$ mice exposed to the carcinogen, azoxymethane (AOM) tested this hypothesis. In this study, AOM-exposed IL-10$^{-/-}$ mice developed colorectal carcinomas when housed conventionally, but not when housed under GF conditions. Furthermore, MyD88 (an adaptor molecule necessary for most TLR signaling) was found to be essential in this process, suggesting that bacterial-induced inflammation in the intestine can play a crucial role in the development of the carcinomas (Uronis et al., 2009). Interestingly, ingestion of lactic acid-producing bacteria has been shown to prevent carcinogen-induced lesions and tumors in animal models (Goldin and Gorbach, 1980; Goldin et al., 1996; Pool-Zobel et al., 1996; Challa et al., 1997; Rowland et al., 1998). However, the epidemiologic studies are controversial in humans; some studies have found no association (Kampman et al., 1994a,b; Kearney et al., 1996), whereas others have found a significant association (Malhotra, 1977; Peters et al., 1992; Boutron et al., 1996) between consumption of fermented milk products and occurrence of colon cancer. Further studies are, therefore, needed to determine if probiotics can be used as protective agents for the prevention of human colon cancer.

Unlike the situation in autoimmune and allergic diseases, there is little evidence that the microbiota directly affects the immune response against tumors, and consequently, somehow

impacts tumor progression. One can, however, speculate that the type of immune response generated by the gut commensal bacteria could potentially influence tumor immunity. For example, mice colonized with enterotoxigenic *B. fragilis* exhibit colonic Th17 inflammatory infiltrates that are involved in induction of colon tumors, as indicated by inhibition of colon tumor formation following treatment with anti-IL-17 antibody (Wu et al., 2009). Moreover, Th17 cells have been found in cellular infiltrations in a variety of tumors, including prostate (Sfanos et al., 2008), pancreatic (Gnerlich et al., 2010), and ovarian cancer (Kryczek et al., 2009a), and in stomach cancer and the lymph nodes draining gastric cancer sites (Zhang et al., 2008). The frequency of Th17 cells correlates with the clinical stage of gastric cancer, i.e., Th17 cells are found at higher levels in late stage versus early stage (Zhang et al., 2008). On the other hand, Th17 cells appear to have a beneficial role in human ovarian cancer (Kryczek et al., 2009a) and murine melanoma, pancreatic, and colon cancer (Muranski et al., 2008; Kryczek et al., 2009b; Gnerlich et al., 2010). It is, therefore, possible that a microbiota favoring commensal bacteria that induces a Th17 response could have differential effects on tumors depending on the type of tumor or the stage of tumor development, and as found for autoimmunity and allergy, could alter the immune response to tumors at extra-intestinal as well as intestinal sites.

CONCLUSION

Genetic and environmental factors appear to shape the composition of the gut microbiota, which in turn plays a very important role in shaping the immune response at both intestinal and extra-intestinal sites, and in controlling the development of some types of autoimmune and allergic diseases as well as some forms of cancer. Therefore, any external factor that can alter the gut microbiota balance, such as diet or antibiotic treatment, should be viewed as a potential risk factor for development of these inflammatory diseases. Any alteration of the microbiota that leads to (1) a reduction in commensal bacteria favoring regulatory cells ($Foxp3^+$ or other, e.g., rTh17) or (2) an enrichment in commensal bacteria favoring the induction of potential pathogenic cells may elicit disease in genetically susceptible individuals (**Figure 1**). However, the relationship between gut microbiota, immunity, and disease is very complex, since the same commensal bacteria can induce a protective response or a pathogenic response depending on the susceptibility of the individual. SFB is the perfect example, since it is protective in type 1 diabetes, but causes disease in mouse models of EAE or arthritis. The specific commensal microorganisms that contribute to either the etiology of or protection from many different types of diseases have not yet been fully identified and described. Future studies aimed at determining the impact of specific commensal bacteria on the immune response and induction of inflammatory diseases are crucial for a better understanding of pathogenesis of these diseases. Furthermore, an understanding of the genetic and environmental factors that shape the composition of the microbiota will provide the basis for strategies that will allow for the manipulation of microbiota in individuals at risk of developing disease. With this information in hand, it should be possible to design novel tailored therapies capable of preventing disease development in high risk individuals and treating established disease in already sick patients. Although not discussed in this review, other microorganisms, such as fungi and

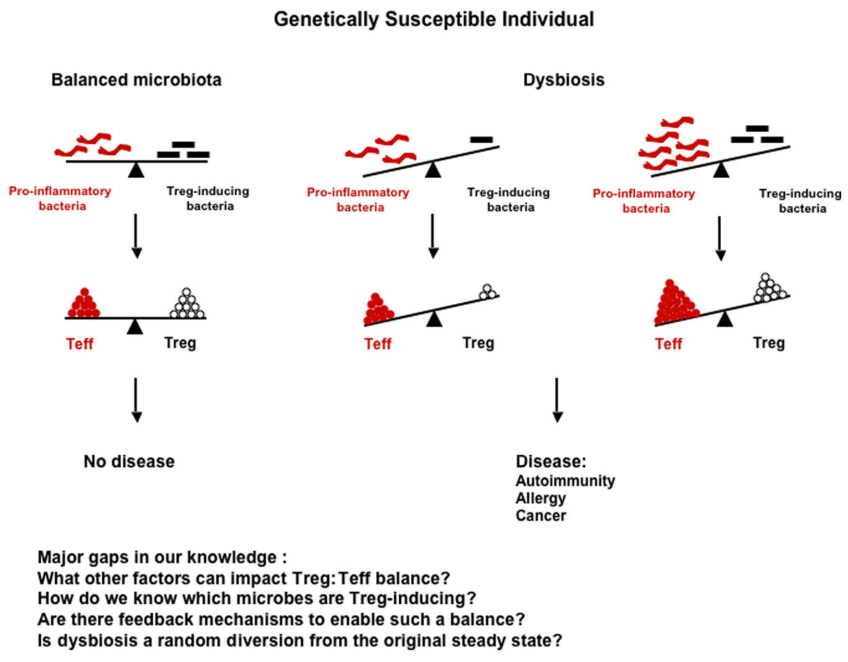

FIGURE 1 | Impact of the composition of the flora on the immune response and disease development. Dysbiosis can arise from either (1) a reduction in Treg-inducing bacteria (e.g., *Bifidobacteria* or *Bacteroides*) that results in decreased Tregs, but unchanged levels of Teff cells; or (2) an enrichment in pro-inflammatory bacteria (e.g., SFB) that results in increased Teff cells, but unchanged levels of Tregs. In both cases, the Treg:Teff cell balance is biased toward the Teff cells, and the outcome of this imbalance can be development of disease in genetically susceptible individuals.

viruses, are present in the intestine, and have also been shown to be involved in autoimmune disease or allergic disease pathogenesis in susceptible animals (Noverr et al., 2004; Cadwell et al., 2010). It would, therefore, be very important in the future to extend the studies cited above to these microorganisms rather than limiting them to commensal bacteria only. It may eventually be possible to establish profiles of the microbiota in humans based on the bacterial species composition or enterotypes (Arumugam et al., 2011). This "biological fingerprint" of the future, similar to blood or tissue typing, may be used to predict responses to drugs or diet as well as aid in disease diagnosis/prognosis, and could ultimately lead to the development of personalized therapies.

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Experimental bariatric surgery in rats generates a cytotoxic chemical environment in the gut contents

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Bariatric surgery, also known as metabolic surgery, is an effective treatment for morbid obesity, which also offers pronounced metabolic effects including the resolution of type 2 diabetes and a decrease in cardiovascular disease and long-term cancer risk. However, the mechanisms of surgical weight loss and the long-term consequences of bariatric surgery remain unclear. Bariatric surgery has been demonstrated to alter the composition of both the microbiome and the metabolic phenotype. We observed a marked shift toward *Gammaproteobacteria*, particularly *Enterobacter hormaechei*, following Roux-en-Y gastric bypass (RYGB) surgery in a rat model compared with sham-operated controls. Fecal water from RYGB surgery rats was highly cytotoxic to rodent cells (mouse lymphoma cell line). In contrast, fecal water from sham-operated animals showed no/very low cytotoxicity. This shift in the gross structure of the microbiome correlated with greatly increased cytotoxicity. Urinary phenylacetylglycine and indoxyl sulfate and fecal gamma-aminobutyric acid, putrescine, tyramine, and uracil were found to be inversely correlated with cell survival rate. This profound co-dependent response of mammalian and microbial metabolism to RYGB surgery and the impact on the cytotoxicity of the gut luminal environment suggests that RYGB exerts local and global metabolic effects which may have an influence on long-term cancer risk and cytotoxic load.

Keywords: obesity, bariatric surgery, Roux-en-Y gastric bypass, microbial profile, 16S rRNA, metabolic profile, cytotoxicity, gammaproteobacteria

INTRODUCTION

Obesity is worldwide epidemic with 1.5 billion adults (age > 20 years) and 43 million children under the age of 5 overweight, of which approximately 500 million are clinically obese (BMI > 30; WHO, 2006). Obesity and its comorbidities impose a substantial healthcare burden which are associated with mounting international healthcare costs (Eckel et al., 2005; Ashrafian et al., 2011c). Although several treatment strategies such as lifestyle modification (Wing and Phelan, 2005) and medications have been used to combat obesity, their long-term efficacy has yet to be demonstrated. Bariatric surgery, also called weight loss or metabolic surgery, was introduced 50 years ago to provide drastic weight loss in morbidly obese patients (Kremen et al., 1954; Ashrafian et al., 2010) and is now increasingly used to treat patients with lower adiposity, or occasionally purely for metabolic benefit (Ashrafian et al., 2011b). Currently the three most common types of bariatric surgery are the Roux-en-Y gastric bypass (RYGB), Adjustable Gastric Band and the Sleeve Gastrectomy. Of these, the RYGB is considered to be the "gold standard" procedure due to its established efficacy, metabolic benefits, and safety from side effects. Typically the RYGB operation is performed laparoscopically and consists of the creation of a small stomach pouch (30–50 ml) that is divided from a larger gastric remnant. The small bowel (jejunum) is bypassed approximately 50 cm below the stomach outlet ("biliopancreatic limb") in a Y-structured configuration. This anatomical rearrangement allows food from the outlet of the small stomach pouch to enter directly into the small intestine ("alimentary limb"). Both biliopancreatic and alimentary limbs are attached to a "common channel" to complete the Y-configuration (Ashrafian et al., 2010). This surgical procedure induces a series of physical and metabolic sequelae characterized as the BRAVE effects: Bile flow alteration; Reduction of gastric size; Anatomical gut rearrangement and altered flow of nutrients; Vagal manipulation; and Enteric gut hormone modulation (Ashrafian et al., 2010) leading to effective weight loss and resolution of type 2 diabetes.

Modulation of the enteric gut microbiota has been reported in both animal and human clinical studies post-RYGB surgery, although whether these changes are etiopathogenic or consequential is not yet clear. A direct effect on the microbiota post-RYGB surgery (Zhang et al., 2009) where a dramatic increase in the gammaproteobacteria population compared with control and obese subjects was demonstrated in a clinical study. This

finding was supported by similar observation in an experimental RYGB study in rats. Other studies have characterized the indirect effects of RYGB in altered gut microbial metabolites or microbial–mammalian co-metabolites in biofluids. Serum sphingosines, unsaturated fatty acids, and branched chain amino acids altered significantly in obese patients within 3 months post-RYGB (Mutch et al., 2009). This study also found a strong negative correlation between serum nervonic acid concentrations and insulin resistance (HOMA-IR). In a separate weight loss control study, amino acids and branched chain amino acids were found to decrease post-RYGB but not after dietary intervention, which suggested the down-regulation of amino acids may contribute to the glucose homeostasis and thus improve the metabolic status (Laferrere et al., 2011). Using a rat RYGB model, we previously characterized the correlation between the metabolic and metagenomic profiles following RYGB surgery (Li et al., 2011). Metabolic effects of surgery included a reduction in levels of TCA cycle intermediates, increased amine production (methylamine, trimethylamine, and putrescine), and increased production of microbial-derived metabolites such as propionate and 5-aminovalerate, or microbial–host co-metabolites (e.g., hippurate, 4-cresyl sulfate, 4-cresyl glucuronide, 4-hydroxyphenylacetate, and phenylacetylglycine). These microbial-related metabolites were positively associated with *Gammaproteobacteria*, particularly, with *Enterobacter hormaechei* and negatively associated with *Clostridia* (Li et al., 2011).

Epidemiological analyses suggest that reduced risks of cardiovascular disease and cancer (especially colonic) are long-term benefits of bariatric surgery, but the molecular mechanisms of these associations are poorly understood (Ashrafian et al., 2011a). As there is a general reduction in the incidence of cancer following RYGB surgery in man (Adams et al., 2007; Sjostrom et al., 2009) and there is a marked and long-term stable alteration in fecal bacterial composition following surgery we hypothesized that the protective effects of bariatric surgery could be mediated by the altered bioavailability of microbial cytotoxins and genotoxins. Fecal matter has been shown to be genotoxic (Lee et al., 2005), and it is well-established that diet-related fecal cytotoxicity and genotoxicity are strongly correlated with colon cancer risk (de Kok and van Maanen, 2000). Thus, it is possible that bariatric surgery modifies diet-associated exposure to cancer-inducing chemicals and this might be expected to influence the diet-borne genotoxic burden in the exposome. Hence, in order to determine the influence of bariatric surgery on the cytotoxic and genotoxic burden of fecal material, fecal water extracts obtained from RYGB and sham-operated rats were analyzed using a well-established *in vitro* mammalian cell mutation assay. Contrary to our initial hypothesis we found dramatically increased cytotoxicity in the fecal water of rats post-RYGB surgery, which prompts further questions as to the suitability of the rat as a model for RYGB surgery.

MATERIALS AND METHODS

ANIMAL MODEL

The animal experiment was carried out under a license authorized by the UK Home office (PL 70-6669) and the experiment details have been previously described in Li et al. (2011). Briefly, 12 male Wistar rats (non-obese) were housed in individual cages and kept under a 12/12-h light/dark cycle at a room temperature of $21 \pm 2^{\circ}$C. All rats were administered intraperitoneally with 1 ml amoxicillin/flucloxacillin solution (both at 12.5 mg/ml) pre-operation as standard prophylaxis against post-operative sepsis, both sham and RYGB rats received antibiotics to control antibiotic-related variations including the modulation of gut microbiota. This is consistent with common practice in human clinical operations. A total of six rats were subject to RYGB surgery RYGB whilst the others underwent a sham procedure and served as the control group. Fecal and urine samples were collected for 24 h pre-operation and at 2, 4, 6 and 8 weeks post operation.

CELL CULTURE

Mouse lymphoblastoid L5178Y cells were obtained from ATCC. L5178Y cells were cultured in RPMI 1640 media supplemented with $10\%_{v/v}$ heat inactivated horse serum, 2 mM L-glutamine, $0.1\%_{v/v}$ F68 pluronic, 100 units/ml penicillin, and 100 μg/ml streptomycin (Clements, 2000).

FECAL WATER EXTRACTION

In order to ensure the reproducibility of the fecal extraction and cell treatment, two separate experiments were performed. Firstly, a fecal pellet from each of six sham and six RYGB-operated rats at weeks 2 and 8 post operation were weighed (∼200 mg) and homogenized in distilled water by vigorously vortexing. A separate pellet for each animal was pipetted into a 2-ml Eppendorf™, containing 1.4 ml of 0.2 M sodium phosphate buffer (pH = 7.4) containing 20% deuterium oxide (D_2O) as a magnetic field lock, 0.01% 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]propionic acid sodium salt (TSP) as a spectral reference, and 3 mM sodium azide to terminate any bacterial activity, and homogenized. The sample was then vortexed for 15 s, sonicated for 30 min at 25°C and centrifuged at 10392 *g* for 20 min. A total of 700 μl supernatant was taken into a 1.5-ml Eppendorf™tube and centrifuged again under the same conditions after which the supernatant (600 μl) was taken into an NMR tube. A third pellet was used to generate the 16S rRNA data.

In the second experiment, four of the RYGB and four sham-operated rats were randomly selected for further investigation. Here, fecal pellets collected for each rat at each time point (pre-surgery and 2, 4, 6, and 8 weeks post surgery) were homogenized and extracted to follow a more refined time course. Samples were then split into two aliquots for cytotoxicity/genotoxicity assays and NMR analysis. All fecal extracts were then sterile filtered through a 0.2-μm membrane and the same volume of fecal extract equivalent to 20 mg were used in cytotoxicity and genotoxicity assays. Fecal pellet extracts for NMR analysis were mixed with 1.4 ml of the aforementioned phosphate buffer. The mixture was centrifuged at 1.0×10^4 *g* for 20 min and 600 μl of supernatant transferred into a 5-mm outer-diameter NMR tube (Beckonert et al., 2010).

CYTOTOXICITY AND GENOTOXICITY ASSAY

Cytotoxicity and genotoxicity evaluation was performed as described by Clements according to OECD guidelines (OECD, 1997; Clements, 2000). In brief, four million exponentially growing L5178Y cells were treated with sham or RYGB-operated fecal

water extract for 24 h at 37°C, 5% CO_2. The final treatment concentration was 1 mg fecal material per 1 ml cell suspension media. As a negative control, fecal water extract was replaced with water. Ethyl methanesulfonate (EMS, 20 μg/ml) and/or etoposide (73.5 ng/ml) were used as positive controls, unless otherwise stated. Following treatment, L5178Y cells were washed, counted, and the concentration of cells adjusted to 1.5×10^5/ml to maintain exponential growth. Treated L5178Y cells were counted and subcultured daily for a further 2 days to determine the relative suspension growth (RSG; a measure of cell death and proliferative ability following treatment relative to the negative control (Clements, 2000). On the third day, L5178Y cells were plated at 1.6 cells per well in 96 well plates to determine the cloning efficiency and 2,000 cells per well in trifluorothymidine (4 μg/ml) to determine the *Thymidine kinase* (*tk*) mutant frequency. Plates were incubated for 14 days at 37°C, 5%CO_2 and then colonies were scored.

NMR-BASED METABOLIC PROFILING OF FECAL WATER AND URINE

For each fecal pellet extract and urine sample 1-Dimensional proton NMR spectra of urine and fecal extracts were acquired using a Bruker 600 MHz spectrometer (Bruker, Rheinstetten, Germany) with a standard NMR pulse sequence (recycle delay-90-t_1(3 μs)-90-t_m(100 ms)-90-acquisition) at 27°C. Water suppression was achieved using selective irradiation during a recycle delay of 2 s and t_m, and a 90° pulse was 10 μs (Beckonert et al., 2010). A total number of 128 scans were accumulated into 64 k data points for each urine spectrum with a width of 20 ppm, whereas for fecal water extracts, 512 scans were acquired due to the low concentration of the samples.

MICOBIAL PROFILING VIA PYROSEQUENCING

The composition on the gut microbiota was determined by undertaking a survey of the 16S rRNA genes in each animal. A modified protocol based on the Qiagen Stool Kit (Qiagen, Crawley, UK) with an additional bead beating step to homogenize and lyse bacteria in the samples (0.1 g 0.1 mm sterile glass beads, 3 × 4500 rpm for 30 s with 5 min on ice in between cycles) was used to extract DNA from 250 mg fecal pellets (Cole et al., 2009; Schloss et al., 2009). The extracted DNA was quantified using the Invitrogen Qubit platform and diluted to a working concentration of 10 ng μl^{-1}. PCR was used to amplify the V1–V3 regions of the 16S rRNA gene from each DNA sample using the primers as published in (Li et al., 2011). PCR mixtures (25 μl) contained 1 × Buffer (20 mM Tris pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1.25 U of *Taq* polymerase (NEB, UK), 5 pmol of each primer, and 10 ng of DNA. PCR was performed in triplicate on all DNA extracts using a MJ Research PTC-200P Thermal Cycler (MJ Research, USA). The PCR conditions were: 95°C for 5 min initial denaturation, followed by 25 cycles of amplification at 95°C denaturation for 30 s, annealing at 55°C for 40 s, and extension of 72°C for 1 min, with a final extension of 72°C for 5 min. Pooled PCR products for each sample were purified using a Qiagen PCR purification kit, quantified and equimolar amounts pooled prior to running on a 1/4 of a PTP (Pico titer plate) using titanium chemistry (AGOWA, Berlin, Germany). The sequences were binned by sample source and any reads that were less than 250 bp were removed. The filtered sequences were classified using the RDP classifier and the relative proportions of phyla and families determined. Community analysis of the data was undertaken using MOTHUR.

STATISTICAL CORRELATION ANALYSIS

^{1}H NMR spectra were phased, calibrated to TSP δ 0.0, and baseline-corrected using an in house developed MATLAB script. The pre-processed NMR spectra were then imported into MATLAB (R2010b) platform with a resolution of 0.0005 ppm and water peak (δ 4.7–4.9) and noise containing regions (δ 0–0.3, 9.4–10.0) were removed to reduce the number of data points. The remaining spectral data were normalized using probabilistic quotient normalization method and then performed multivariate statistical data analysis (Trygg et al., 2007) including principal component analysis (PCA) and projection on latent structures (PLS) regression analysis. Statistical analysis used to calculate significance in cytotoxicity and genotoxicity assays was one way ANOVA with Dunnett's *post hoc* test compared to the negative control Correlation of the cytotoxicity values with the 16S rRNA profiles was also carried out.

RESULTS

CYTOTOXICITY AND GENOTOXICITY OF FECAL EXTRACTS

Fecal samples from a total of 12 rats at weeks 2 and 8 post operation were used for cytotoxicity, genotoxicity, and microbial composition evaluation. Cytotoxicity is reported as the RSG of the cells following treatment; a measure of cell cycle arrest, cell death, and clonogenicity. Unexpectedly, all RYGB samples exhibited strong cytotoxic responses compared with sham samples ($p < 0.001$, **Figure 1A**). No significant difference in toxicity was observed between the 2 and 8-week time points within the same group. However, the mean RSG of RYGB-operated rats was markedly lower than the sham-operated controls ($p < 0.001$; **Figure 1A**). Fecal water samples were also examined for genotoxicity using the *TK* locus in the regulatory acceptable Mouse Lymphoma L5178Y mutation assay. Genotoxicity above the spontaneous background rate (85×10^{-6}) was not observed with fecal water extracts from RYGB or sham-operated rats (**Figure 1B**), whereas the positive genotoxic control, EMS (20 μg/ml) gave a mutation frequency of 240 mutant clones per 10^6 cells.

In order to ensure the reproducibility of the experiments, 8 of the 12 rats were randomly selected (four sham and four RYGB) and the analysis repeated using additional sampling time points (pre-op, 2, 4, 6, and 8 weeks post-op). The RSG values of L5178Y mouse lymphoma cells exposed to RYGB fecal water extracts for 24 h was markedly lower than sham extracts (**Figure 2**), indicating that RYGB fecal water was toxic to the cells compared with sham fecal water, which is consistent with the results derived from the initial observation using six rats at two post-operative time points. However, cell survival following sham operation was more variable than RYGB surgery (**Figure 2**). Neither the sham nor the RYGB group pre-surgery fecal water extracts were cytotoxic. Thus, the cytotoxicity observed in our experiments directly resulted from RYGB surgery. As expected, the positive cytotoxic control (etoposide) induced cytotoxicity.

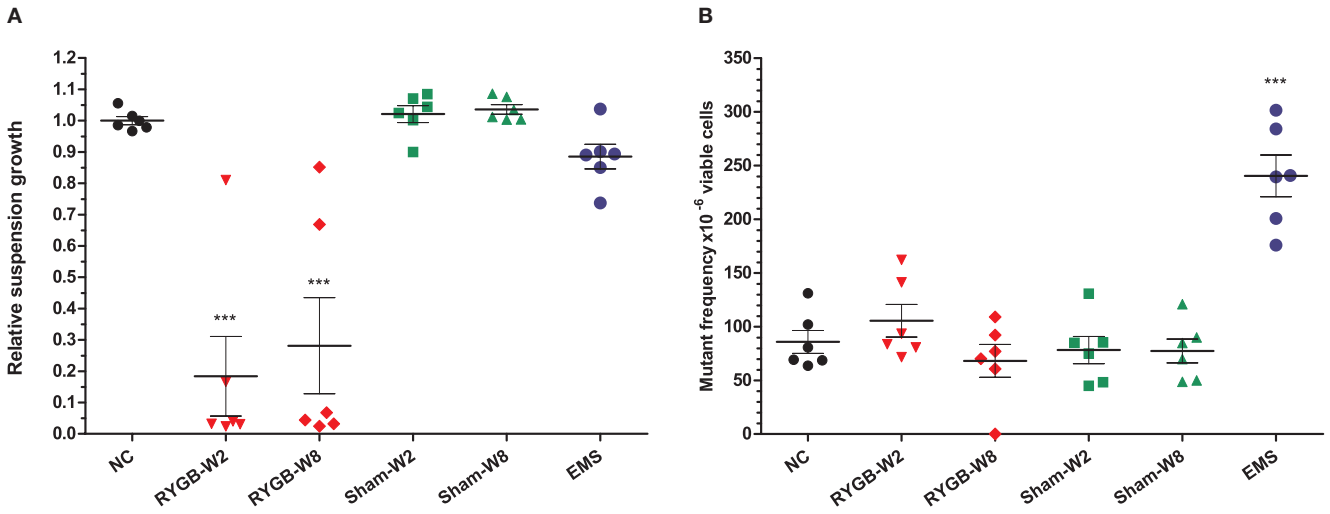

FIGURE 1 | Determination of the relative suspension growth as a measure of cytotoxicity (A) and genotoxicity (B) of fecal water extracts following treatment of L5178Y mouse lymphoblastoid cells for 24 h. Data represent mean ± SEM of six independent samples. The negative control (NC) replaced fecal material with water and EMS (20 μg/ml) was used as a positive genotoxin. ***$p < 0.001$ vs. negative control by one way ANOVA test.

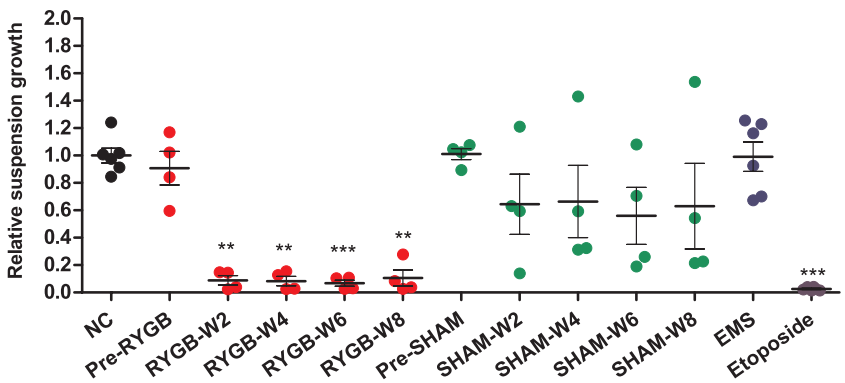

FIGURE 2 | Determination of the relative suspension growth (RSG) of L5178Y mouse lymphoblastoid cells after 24 h exposure to individual fecal water extracts from four sham and four RYGB-operated rats at pre-surgery, 2, 4, 6, and 8 weeks post surgery. The negative control (NC) replaced fecal material with water and EMS (20 μg/ml) was used as a positive genotoxin whilst etoposide (73.5 ng/ml) was used as a positive cytotoxin. **$p < 0.01$, ***$p < 0.001$ vs. negative control by one way ANOVA test.

MICROBIAL-METABOLIC-CYTOTOXICITY AXIS

To understand the relationship between the microbiota, metabolites, and the extent of cytotoxicity observed in our cell culture system, a statistical integration method was applied to correlate multiple datasets. O-PLS regression analysis was performed to identify correlation between (i) the NMR fecal and urine extracts and RSG values (**Figures 3A,B**) and (ii) the RSG values and selected bacterial families (**Figure 4**). The fecal aromatic amino acid tyrosine was found to be positively correlated with cell survival whereas fecal tyramine, uracil, glycine, gamma-aminobutyric acid (GABA) and putrescine, and urinary indoxyl sulfate, phenylacetylglycine, 4-cresyl glucuronide, 4-cresyl sulfate, 4-hydroxylphenylacetate, formate, and 5-aminovalerate appeared to be associated with lower RSG levels.

To enhance the correlation strength and minimize the inter-animal variation, the fecal water extracts from four rats sampled at five time points as described above were subjected to NMR spectroscopic analysis in the second experiment. A direct O-PLS correlation analysis was applied based on fecal water NMR profiles and RSG from cytotoxicity profiles (**Figure 3C**). The metabolic-cell growth correlation was consistent with aforementioned results from the initial dataset but with a stronger ($Q^2Y = 0.55$) and clearer model, where tyramine, methylamine, pyruvate, GABA, and putrescine were directly associated with cell death whereas butyrate, known as a major nutrient of colonic cells, was positively correlated with cell survival.

To investigate the association between the observed changes in the microbiome at a gross level and cytotoxicity levels, 22 bacterial familial levels were correlated with RSG of the cells following fecal water treatment. *Enterobacteriaceae*, *Pasteurellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* levels were found to be significantly ($p < 0.01$) correlated with RSG of the cells following

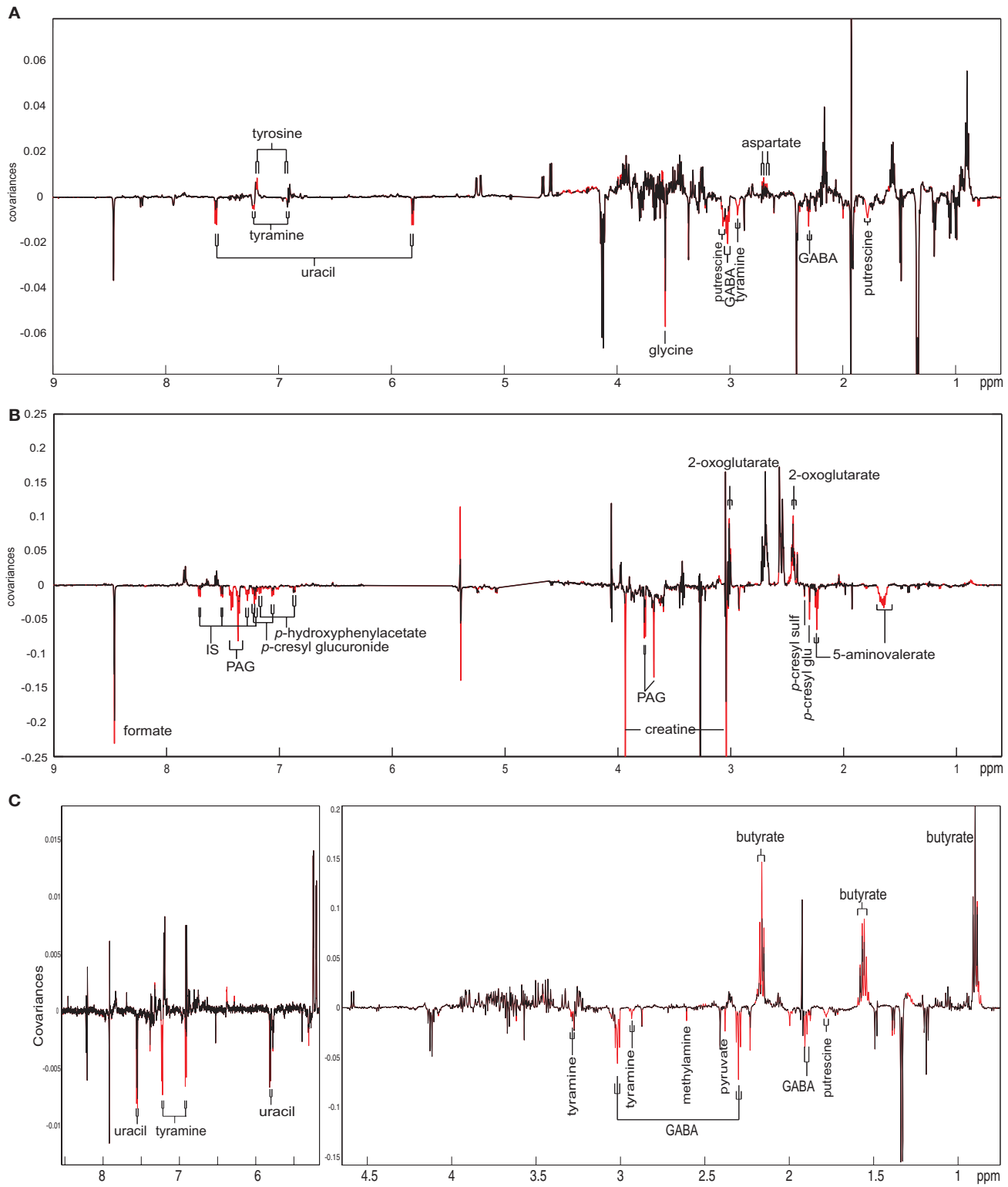

FIGURE 3 | O-PLS regression analyses of fecal water (A) and urine extracts (B) against relative suspension growth values obtained from a 24-h treatment of L5178Y cells with sham or RYGB-operated rat fecal extracts (week 2 and week 8) or O-PLS regression analyses of fecal water extracts against relative suspension growth obtained from 24 h treatment of L5178Y cells with two sham or two RYGB fecal water extracts [pre-surgery and 2, 4, 6, and 8 weeks post surgery (C)]. Peaks pointing upward in the loadings plots represent the metabolites are positively correlated to the cell growth and *visa versa*. Red peaks reach a significance level of $p < 0.005$. Keys: GABA, gamma-aminobutyric acid; IS, indoxyl sulfate; PAG, phenylacetylglycine; *p*-cresyl sulf, *p*-cresyl sulfate; *p*-cresyl glu, *p*-cresyl glucuronide.

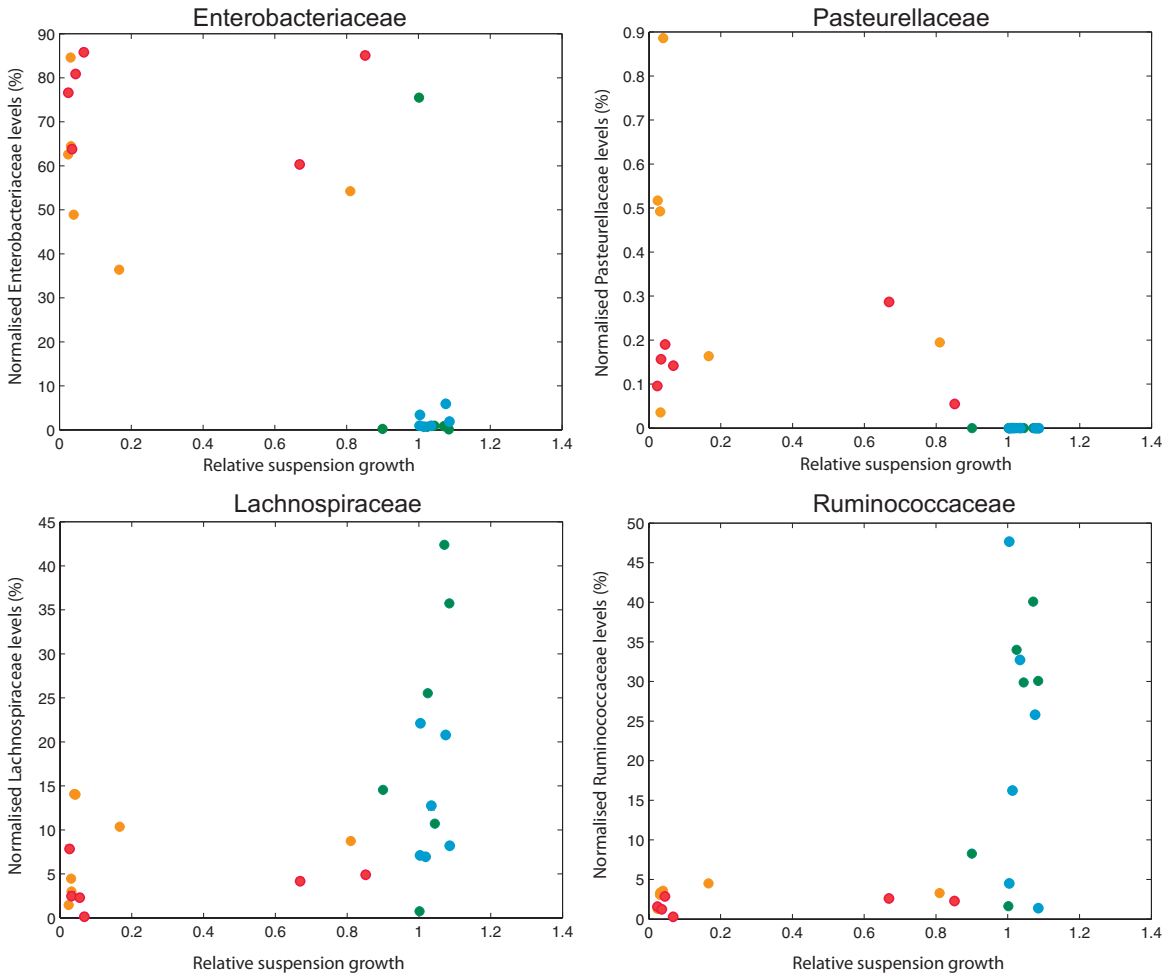

FIGURE 4 | Scatter plots of normalized bacterial levels (*Enterobacteriaceae*, *Pasteurellaceae*, *Lachnospiraceae, and Ruminococcaceae)* and relative suspension growth of L5178Y mouse lymphoblastoid cells exposed to fecal water extracts of six sham and six RYGB rats at weeks 2 and 8 post intervention. Orange: RYGB-2 weeks post op; Red: RYGB-8 weeks post op; Green: Sham-2 weeks post op; Blue: Sham-8 weeks post op.

treatment and the scatter plots of normalized bacterial levels and RSG values are illustrated in **Figure 4**. Levels of *Enterobacteriaceae* and *Pasteurellaceae* appear to be inversely correlated with cell survival, whereas levels of *Lachnospiraceae* and *Ruminococcaceae* were positively related to the RSG.

DISCUSSION

Contrary to our initial expectations, based on recent epidemiological findings that bariatric surgery is associated with lower cancer risk, this exploratory study has clearly shown that RYGB surgery in a Wistar rat model increases the cytotoxicity of fecal matter as indicated by marked reduction in (RSG) as a measure of cell death in a well-established rodent reporter cell line. Furthermore, this cytotoxicity profile of fecal water post-RYGB operation was strongly correlated with the fecal and urinary metabolic phenotypes with fecal concentrations of putrescine, uracil, tyramine, GABA, and methylamine being inversely correlated with cell survival, whereas butyrate was positively associated with cell survival. In addition to the correlation between fecal amines and cell death, several urinary metabolites also demonstrated an inverse correlation with cell survival, including indoxyl sulfate, phenylacetylglycine, 4-cresyl glucuronide, 4-cresyl sulfate, 4-hydroxylphenylacetate, formate, and 5-aminovalerate.

UPREGULATION OF AMINE METABOLISM

Putrescine is a polyamine found ubiquitously in eukaryotic cells and is vital for cell growth (Tabor and Tabor, 1984; Pegg, 1988). However, high intracellular polyamine accumulation, either from an imbalance in the cellular homeostatic mechanisms or exogenous supply, could have a deleterious effect on cell survival (Marton et al., 1991; Davis et al., 1992; Casero and Pegg, 1993; Buckton and Machiste, 1997). For example, treating polymorphonuclear cells with 1 mM putrescine for 30 min resulted in the induction of apoptosis, possibly through a caspase specific pathway (Mariggio et al., 2004). Further to this, administration of an exogenous supply of putrescine has been shown to result in intracellular accumulation with ensuing apoptosis in a hepatoma cell line (Tome et al., 1997) and in a mouse myeloma cell line (Tobias and Kahana, 1995).

Our study revealed a post-operative increase in fecal putrescine levels that correlated with the degree of fecal cytotoxicity based on a cell based assay, and may contribute to the induction of cell death following RYGB.

One likely mechanism for the rise in fecal putrescine after surgery may reflect the microbial catabolism of incompletely digested proteins reaching the colon as a result of surgical bypass of the foregut (Welters et al., 1999; Li et al., 2011). Alternatively the increase in post-operative fecal putrescine levels may occur as a result of its anti-inflammatory properties. Intracellular polyamines such as putrescine are known to possess powerful anti-inflammatory properties (Lovaas and Carlin, 1991) and have been demonstrated as a response to gut and liver inflammation (Bird et al., 1983). Using a carrageenan-induced edema rat model, putrescine was found to possess a tenfold higher anti-inflammatory activity than spermidine (Bird et al., 1983). The increase in polyamines is likely to negatively modulate the immune response through its action on leukocyte activity (Ferrante et al., 1986; Quemener et al., 1994). We and others (Evrard et al., 1993) have noted that bariatric surgery induces significant macroscopic trophic changes in the intestinal mucosa of animal models (more than two-fold increase in mucosal mass) that may reflect an increased post-operative gut inflammatory state. Consequently a post-operative increase in fecal putrescine may occur as an anti-inflammatory response to surgically induced inflammation.

Gamma-amino butyric acid, an inhibitory neurotransmitter in the mammalian central nervous system, is also increased in fecal samples post-RYGB and was found to inversely correlate with cell survival in our cytotoxicity assays. The increased GABA concentration in fecal water could be due to the high putrescine level as GABA can be derived from the microbial processing of putrescine (Kurihara et al., 2010). Increased expression of fecal GABA is consistent with the well-defined increase in glucagon-like peptide (GLP-1) after RYGB surgery (Ashrafian and le Roux, 2009). GABA stimulates GLP-1 release from intestinal cells (Gameiro et al., 2005), and raised GLP-1 concentrations in turn promote GABA formation by pancreatic β cells, with autocrine and paracrine effects, including immunomodulatory actions on infiltrating T cells to suppress autoimmune damage (Urbain et al., 1990).

Although we have suggested the cytotoxicity observed in our system following RYGB surgery may be a result of putrescine, it may be likely to involve both multiple monoamines such as methylamine, as well as polyamines. Horse serum used in the cell culture medium this study contains high semicarbazide-sensitive amine oxidase (SSAO) activity (Tipnis and He, 1998) which can convert methylamine to formaldehyde, ammonia, and hydrogen peroxide, all of which are cytotoxic (Lyles, 1996; Gubisne-Haberle et al., 2004). SSAO mediated degradation of methylamine has been shown to be cytotoxic to cultured endothelial cells (Yu and Zuo, 1993). Increased tyramine and decreased tyrosine levels were observed in RYGB-operated rats. Tyramine is a monoamine, derived from the microbial enzymatic decarboxylation of tyrosine by various lactic acid bacteria (Komprda et al., 2008) and has also been reported to be produced by *Enterobacter* spp. strains from food studies on cheese and meat (Marino et al., 2000; Curiel et al., 2011). This is consistent with the marked increase in *Enterobacter* observed following RYGB surgery. Although tyramine was reported to be mutagenically inactive using the mouse lymphoma assay (McGregor et al., 1988), mutagenesis was only observed after the combined treatment of tyramine and nitrite *in vitro* (Fujie et al., 1990).

URACIL METABOLISM

One of the strongest correlates with cytotoxicity following RYGB surgery was fecal uracil levels. Possible rationales for this phenomenon relate to apoptosis and inflammation. There is an increased incidence of apoptosis in inflamed colonic mucosa in cases of inflammatory bowel disease (Afford and Randhawa, 2000) that is associated with impaired mucosal healing (Iwamoto et al., 1996). Uracil has been previously reported to possess anti-apoptotic activity in a human colon epithelial cell line (Evans et al., 2005) and may be produced as a consequence following RYGB surgery in rats to compensate for the ensuing inflammation. An alternative explanation would be modulation of inflammation. Nitric oxide (NO) can be produced from the surrounding tissue and macrophages during inflammatory process, to modulate the extent of inflammation (Nussler and Billiar, 1993) and can react with oxygen to form the nitrosating agent nitrous anhydride which can, in turn, react with nucleophiles, such as amines and thiols (Caulfield et al., 1998). This propensity to react with nucleophiles can lead to nitrosation of primary amine groups in a DNA base leading to direct DNA damage, for example, the deamination of cytosine to uracil (Barnes and Lindahl, 2004). Uracil in genomic DNA can be repaired through excision by Uracil-DNA glycosylase leaving an abasic site creating a substrate for base excision repair (Hagen et al., 2008). It is plausible that uracil may be released during cell lysis caused by the inflammation following RYGB surgery.

PROTECTIVE FUNCTION OF BUTYRATE

The short chain fatty acid butyrate is produced by bacterial fermentation of dietary fiber and has important physiological roles in maintaining health and integrity of colonic mucosa (Clarke and Mulcahey, 1976). It also regulates colonic epithelial cell proliferation and differentiation. Increased carbohydrate fermentation can divert ammonia, which is toxic to colonic epithelial cells (Lin and Visek, 1991), into *de novo* bacterial protein synthesis. Butyrate enema treatment has been shown to reduce inflammatory colitis in rats (Butzner et al., 1996). Indeed, the anti-inflammatory role of butyrate through the inhibition of NF-κB activation in cultured human cells from patient biopsies has also been demonstrated (Segain et al., 2000).

CONCLUSION

The current study has for the first time integrated measures of cytotoxicity with metabolic and microbial phenotypes following bariatric surgery, which may shed some light on the reduced incidence of colonic cancer following weight loss surgery. Various fecal metabolites were found to correlate with cytotoxicity measures. In essence, cytotoxicity was dramatically increased following RYGB in a rat surgical model and this increase was associated with alterations in both the metabolic and the microbial phenotypes with particularly strong correlations between RSG and the *Enterobacteriaceae* and *Pasteurellaceae*. The metabolites suggested to be responsible for the cytotoxic effect, based on statistical associations, can be predominantly associated with an inflammatory

response following surgery, which was persistent over the 8-week study. Although it would have been interesting to follow the cytotoxic profile for a longer period, ethical considerations did not allow this.

In contrast to reports from large-scale human epidemiological cohorts suggesting a reduced cancer risk following bariatric surgery (Ashrafian et al., 2011a), we have demonstrated increased fecal cytotoxicity in this Wistar rat model following RYGB surgery; which can be associated with increased colonic cancer risk (de Kok and van Maanen, 2000). Two possible explanations for the conflicting RYGB effects between humans and rats include: (i) the rat model is not translational to humans with regard to RYGB surgery, or more controversially that the RYGB surgery increases the cytotoxicity of feces, however human colonocytes, are subject to a high degree of oxidative stress and possess enzymes capable of detoxifying H_2S and other toxins (Ramasamy et al., 2006), are resistant to this toxicity. The latter explanation however does not clarify the nature of the decreased cancer risk after bariatric surgery. As these operations are being increasingly performed worldwide within a framework of increased global obesity and metabolic dysfunction (Ashrafian et al., 2011b), an increased understanding of the mechanisms and consequences of bariatric surgery are essential, specifically in terms of cancer risk. The future of this field will require an integrated metabolic, metagenomic, and cytotoxic profiling approach in bariatric patients that have undergone metabolic surgery within robust clinical trials. This can strengthen our understanding of the cancer modifying mechanisms of bariatric surgery to improve the cancer related outcomes associated with metabolic dysfunction and obesity.

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Effect of antibiotic growth promoters on intestinal microbiota in food animals: a novel model for studying the relationship between gut microbiota and human obesity?

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As more children and adults become obese in the U.S. and other countries, obesity-associated diseases are becoming more prevalent worldwide (Poirier et al., 2006). Major chronic diseases linked to obesity include heart disease, stroke, and type 2 diabetes (Kahn et al., 2006; Poirier et al., 2006; Van Gaal et al., 2006). Thus, development of effective preventive and therapeutic strategies against obesity will ultimately reduce the burden of cardiovascular diseases and diabetes. Factors contributing to obesity development are complex. Although it is obvious that human genetics plays an important role in determining body weight, it is widely accepted that the increase in the prevalence of obesity over the past 30 years cannot be attributed to changes in human genome so other factors are responsible for obesity. Recent human and mice studies (Reviewed in DiBaise et al., 2008; Tilg et al., 2009) strongly support a concept that the gut microbiota together with host genotype and lifestyle contribute to the development of obesity. These studies suggest manipulating the microbial populations in the gut may be one means to control body weight. To develop such microbiota-manipulating strategies to aid in weight loss, it is critical to identify potential keystone microorganisms from more than 1,000 different species in the gut. However, there are three major limitations in these pioneering studies which ultimately slow progress in this field. First, previous studies on the relationship between microbiota and obesity (Ley et al., 2005, 2006; Turnbaugh et al., 2006) only analyzed fecal samples which represent the microbiota from the large intestine. However, the small intestine is the principal site for digestion, nutrient assimilation and energy harvest, which is directly relevant to body weight gain. In addition, the microbiota have been observed to vary significantly between small intestine and large intestine (Hayashi et al., 2005; Dumonceaux et al., 2006). Thus, use of fecal sample from small intestine is critical to reveal the direct relationship between gut microbiota and obesity development. Second, the potential role of microbiota in obesity development has focused on the utilization of indigestible polysaccharides in colon. However, many metabolic functions of microbiota, such as fat digestion, are not captured by only considering polysaccharide utilization. For example, the deconjugation of bile salt complexes by bile salt hydrolases, which are produced by many commensal bacteria (e.g., *Lactobacillus*), could reduce lipid solubilization and absorption and even lower cholesterol levels in humans (Begley et al., 2006; Ridlon et al., 2006). Lastly, due to technical difficulties, these studies used fecal biota as a surrogate for the entire gut microflora. However, fecal biota may not contain the mucosa-associated microbial populations that are in close contact with the underlying gut epithelium and play a different but important role in nutrient assimilation (Zoetendal et al., 2002; Eckburg et al., 2005). Thus, to identify specific obesity-associated microorganisms, it is essential to develop appropriate animal model and organ systems to overcome the above limitations in studying the relationship between gut microbiota and human obesity.

Although humans are interested in manipulating microbiota to aid in weight loss, the food animal industry has been engaged for decades in manipulating microbiota to increase in weight gain through the use of low-dose antibiotics, usually called antibiotic growth promoters (AGPs) as feed additives (Frost and Woolcock, 1991). At present, the precise mechanisms of growth-promoting effects of AGPs are still unknown. However, it is widely accepted that the growth-promoting effect of AGPs is mediated by the interaction between the AGPs and the intestinal microbiota (Chapman and Johnson, 2002; Dibner and Richards, 2005) because oral antibiotics do not have growth-promoting effects in germ-free animals (Coates et al., 1955, 1963). Use of AGPs may change the diversity and structure of microbial communities in the animal intestine and ultimately result in an optimal and balanced microbiota for increased energy harvest capacity and better growth performance of food animals. Given that both above agricultural and human biomedical issues rely on the relationship between gut microbiota and body weight gain, we propose examining the effect of AGPs on intestinal microbiota in food animals may provide an innovative model system for us to study the relationship between obesity and gut microbiota and identify obesity-associated microorganisms. Notably, a recent human clinical study showed that a significant weight gain can occur in human after a 6-week treatment of vancomycin plus gentamicin for infective endocarditis with a risk of obesity (Thuny et al., 2010), which further supports our hypothesis to study human obesity using a new model system that builds on more than 50 years of consistent observation of the growth-promoting effect of AGPs on food animal production.

While microbiota in the intestine of food animals have been broadly investigated, very limited information is available concerning the response of intestinal microbiota to AGP treatment (Engberg et al., 2000; Knarreborg et al., 2002; Collier et al., 2003; Smirnov et al., 2005; Dumonceaux et al., 2006; Guban et al., 2006; Wise and Siragusa, 2007). With the aid of culture-independent molecular approaches, the investigations on the effect of AGPs on intestinal microbiota have been initiated in poultry (Knarreborg et al., 2002; Dumonceaux et al., 2006; Wise and Siragusa, 2007) and swine (Collier et al., 2003). As expected, in both swine and poultry the composition of intestinal

microbiota was influenced by AGP treatment (Knarreborg et al., 2002; Collier et al., 2003; Dumonceaux et al., 2006; Wise and Siragusa, 2007). Particularly, lactobacilli populations were significantly affected by AGPs; AGP treatment could either reduce or increase the abundance of specific lactobacilli species in intestine (Engberg et al., 2000; Knarreborg et al., 2002; Collier et al., 2003; Dumonceaux et al., 2006), which is consistent with the dual effects (beneficial and detrimental) of lactobacilli on the host (Gaskins et al., 2002; Reid et al., 2003; Dibner and Richards, 2005; Heselmans et al., 2005). Although these findings greatly improve our understanding of intestinal microbiota change in response to AGPs, these studies either failed to show the growth-promoting effect of AGPs (Dumonceaux et al., 2006; Wise and Siragusa, 2007) or lacked any growth/nutritional measurements in conjunction with microbial ecology analysis (Knarreborg et al., 2002; Collier et al., 2003), which greatly reduced the relevance of observed microbial structure shift to animal growth. The lack of a growth promotion response of the food animal to AGPs in previous studies is likely due to the use of highly sanitized research facility environments that limits the establishment of diverse microflora in intestine. Thus, to use food animals as a model for studying human obesity, it is critical to simulate industrial conditions for observation of growth promotion in an experimental system, consequently obtaining high quality, growth-relevant intestinal samples for microbial ecology study using complementary genomics and metagenomics approaches.

Similar to the commonly used mice model, food animals also have different normal gut microbiota from human in terms of diversity and relative abundance, which may challenge the identification of obesity-related microorganisms using the proposed food animal model system. However, intestinal microflora in humans, mice as well as food animals are primarily derived from the surrounding environment and diets. So human and food animals share many common gut microflora (Gong et al., 2002; Leser et al., 2002; Lu et al., 2003; Backhed et al., 2005; Eckburg et al., 2005; Zoetendal et al., 2006). Therefore, the findings from the study using the food animal model should provide insights into the relationship between gut microbiota and obesity development in humans. In addition, despite differences in the host genetic makeup, human and animals should share many common themes with respect to the symbiosis relationship between host and gut microbiota for food digestion, nutrient utilization, and energy harvest during evolution. Thus, to better understand host–bacterial mutualism in the intestine, in addition to revealing phylogeny information, it is essential to examine functional and metabolic diversity of gut microbiota affected by AGP usage using recently developed high-throughput approaches, such as metagenomic sequencing of gut microbiota (Turnbaugh et al., 2006) and GeoChip (He et al., 2010).

Among various food animals, chicken appears to be the most appropriate animal model to test our hypothesis for identifying specific obesity-associated microorganisms due to following major reasons. First, compared to the previous human and mice studies (Ley et al., 2005, 2006; Turnbaugh et al., 2006), the ease of handling and the low cost of chickens provide obvious advantages for collecting various samples from different intestinal sites (e.g., jejunum, ileum, mucosa). This enables us to examine microbiota from various intestinal sites and provides stronger statistical power for analyzing complex gut microbiota through greater replication. Second, compared to other food animals (e.g., pig) that have long production cycle and demanding rearing environment, it is much easier to experimentally simulate poultry industrial conditions and thus replicate industrial growth promotion, consequently obtaining growth-relevant intestinal samples for analysis (Waldroup et al., 2005; Hunkapillar et al., 2009). Finally, the chicken has been an important model organism in the research on human genetics, disease, nutrition, immunology, and development (Dodgson, 2003; Stern, 2005; Burt, 2007; Kohonen et al., 2007; Bahr, 2008). The high similarities between the chicken and human genomes with respect to the genes involved in the cell's basic structure and function (Hillier et al., 2004) further supports the generalizability of using chicken as a model organism. Together, examining the effect of AGPs on chicken gut microbiota may offer several significant advantages over mice as an experimental system, and provide a complementary alternative to identify specific obesity-associated microbes in humans.

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Functional metagenomic investigations of the human intestinal microbiota

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The human intestinal microbiota encode multiple critical functions impacting human health, including metabolism of dietary substrate, prevention of pathogen invasion, immune system modulation, and provision of a reservoir of antibiotic resistance genes accessible to pathogens. The complexity of this microbial community, its recalcitrance to standard cultivation, and the immense diversity of its encoded genes has necessitated the development of novel molecular, microbiological, and genomic tools. Functional metagenomics is one such culture-independent technique, used for decades to study environmental microorganisms, but relatively recently applied to the study of the human commensal microbiota. Metagenomic functional screens characterize the functional capacity of a microbial community, independent of identity to known genes, by subjecting the metagenome to functional assays in a genetically tractable host. Here we highlight recent work applying this technique to study the functional diversity of the intestinal microbiota, and discuss how an approach combining high-throughput sequencing, cultivation, and metagenomic functional screens can improve our understanding of interactions between this complex community and its human host.

Keywords: functional metagenomics, human intestinal microbiota, antibiotic resistome

INTRODUCTION

A growing body of evidence indicates that human microbial communities play a role in the pathogenesis of diseases as diverse as neonatal necrotizing enterocolitis, asthma, eczema, inflammatory bowel disease, obesity, atherosclerosis, insulin resistance, and neoplasia. Because the composition of the intestinal microbiota is highly variable in early infancy and largely stabilizes by the end of the first year of life, understanding the determinants of the composition of the infant enteric microbial community is of particular interest (Vael and Desager, 2009). The decreased rates of early childhood infections, atopic disease, diabetes, and obesity in breastfed infants have been well-documented (Oddy, 2004; Bartok and Ventura, 2009; Duijts et al., 2009; Le Huerou-Luron et al., 2010; Gouveri et al., 2011), as have the differences in the composition of the intestinal microbiota between breast- and formula-fed infants. In breastfed infants, *Bifidobacterium* spp. rapidly become the predominant group of organisms (Harmsen et al., 2000), while formula-fed infants develop a different microbial community comprised of some *Bifidobacteria* and large proportions of other potentially pathogenic organisms, including *Bacteroides*, *Staphylococcus*, *Enterobacteria*, *Clostridia*, and *Enterococcus* spp. (Yoshioka et al., 1983; Rubaltelli et al., 1998). Fermentative metabolites generated by *Bifidobacterium* and other saccharolytic species decrease stool pH, inhibiting the growth of potential pathogens in breastfed infants (Bullen et al., 1976). Relative decreases in the proportion of *Bifidobacteria* and concomitant increases in other enteric flora in infancy have been linked to disease states later in life: increased numbers of *Escherichia coli* and *Clostridium difficile* are associated with the development of atopic disease such as asthma and eczema (Penders et al., 2007), while lower Bifidobacterial counts and greater numbers of *Staphylococcus aureus* are associated both with overweight mothers (Collado et al., 2010) and an increased risk of the infant becoming overweight in early childhood (Kalliomaki et al., 2008). *Bifidobacteria* may also enhance intestinal barrier function, decreasing the likelihood of bacterial translocation during periods of metabolic stress (Wang et al., 2006; Ruan et al., 2007). The gastrointestinal microbiota appear essential to the development of the immune system (Round and Mazmanian, 2009), can act as a reservoir for antibiotic resistance genes (van der Waaij and Nord, 2000), and may contribute to chronic inflammatory states (Erridge et al., 2007; Ghanim et al., 2009). Together, these data suggest that understanding the interactions between microbial communities and their human hosts may illuminate the pathogenesis of complex human diseases such as obesity and the metabolic syndrome, atopic disease, and autoimmune disorders,

and thereby provide a rich source for mining novel therapeutic approaches.

To understand microbial community effects on human health, both the phylogenetic profile of human microbial communities and the functional capacity of their members must be characterized. Much progress has been made toward these ends using direct bacterial culture, 16S sequencing, shotgun metagenomic sequencing, PCR probing for specific genes, and chemical profiling of microbial metabolites. These approaches have yielded incredible insights ranging from shifts in prevalent bacterial phylotypes and altered metabolic profiles in human subjects with inflammatory bowel disease, variations in the composition of the intestinal microbiota with human diet and functional differences in the gut microbiota related to host body habitus, developmental changes in the composition of the gastrointestinal microbiota during infancy and childhood, and the genetic epidemiology of antibiotic resistance in the intestinal microbiota. (Rimbara et al., 2005; Qin et al., 2006; Turnbaugh et al., 2006; Bezabeh et al., 2009; Jansson et al., 2009; Paliy et al., 2009; Gillevet et al., 2010; Kang et al., 2010; Koenig et al., 2011; Rigsbee et al., 2011). In this perspective, we will focus on the emerging application of functional metagenomic screens, a technique developed for investigating unculturable environmental microbes that neatly complements the aforementioned techniques currently used to characterize the human microbiota.

Direct culture, historically the *sine qua non* of microbiology, readily provides information on the functional characteristics of the species being investigated. The majority of gastrointestinal microbiota, however, are obligate anaerobes recalcitrant to culture. Traditional estimates are that only 15–20% of the gastrointestinal microbiota are culturable, precluding direct characterization of the majority of bacterial species (Langendijk et al., 1995; Eckburg et al., 2005; Gill et al., 2006). A recent report by Goodman et al. (2011) showed, using high-throughput 16S sequencing in combination with extensive anaerobic culturing, that up to 56% of gastrointestinal microbial species are culturable. Although this represents a dramatic improvement over standard culturing techniques, there remains a significant proportion of unculturable organisms that must be characterized using complementary techniques. Different approaches have been employed to overcome this problem ranging from simple PCR-based screens to large metagenomic sequencing analyses and functional metagenomic screens. Together, these methods have expanded our knowledge about the fraction of the GI tract microbiota that cannot be characterized by culture-based approaches.

FUNCTIONAL METAGENOMICS: AN EMERGING TECHNIQUE FOR CHARACTERIZING UNCULTURABLE ORGANISMS

Functional metagenomic screens, originally proposed as a method to characterize the unculturable fraction of soil microbiota (Handelsman et al., 1998; Rondon et al., 2000) and successfully used for years to characterize the functional diversity of microbes in a variety of environments (Warnecke et al., 2007; Allen et al., 2009b; Berlemont et al., 2009; Torres-Cortes et al., 2011), has relatively recently been adapted to characterize the functions of human microbial communities, representing an interesting cross-pollination between environmental microbiology and biomedical science. The functional metagenomic screening method, based on clone libraries containing genomic DNA from a microbial community, does not require direct culture of fastidious organisms. Instead, clone libraries are constructed by first extracting and shearing DNA from a sample of a microbial community, then cloning the fragmented DNA into a relevant vector, and subsequently transforming this vector into a suitable host strain (**Figure 1**). Once a library is constructed, it can be functionally screened by cultivation on selective media or by employing a reporter system. Using this approach, it is possible to identify

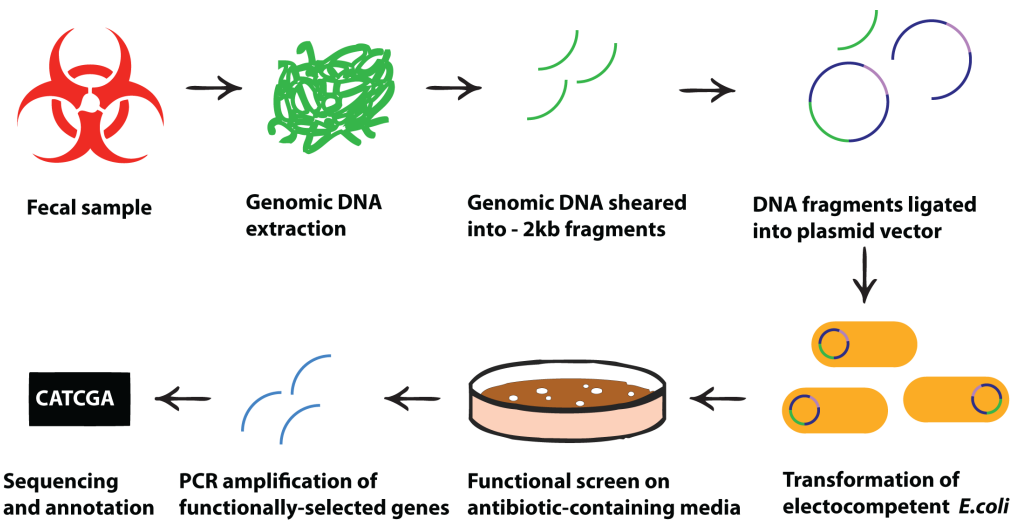

FIGURE 1 | Schematic presentation of the processes leading from fecal microbial sample to functional selection of antibiotic resistance genes. Metagenomic DNA is directly extracted from any microbial community (e.g., from a fecal sample) and cloned into an expression system in a cultivable, genetically tractable host strain (e.g., *E. coli*). Metagenomic transformants harboring DNA fragments that encode antibiotic resistance genes are selected by subjecting the library of clones to specific antibiotics at concentrations which inhibit the growth of the untransformed indicator strain. Selected DNA fragments can then be sequenced to identify the specific resistance genes.

genes encoding a variety of functions such as antibiotic resistance, metabolism of complex compounds, and modulation of eukaryotic cells. Subsequent sequencing and *in silico* analysis of the DNA inserts from isolated clones provides information about the source of the genes and the putative mechanisms of action of their products.

INTERACTIONS WITHIN MEMBERS OF THE INTESTINAL MICROBIOTA: ANTIBIOTIC RESISTANCE

One area of early success for functional metagenomic screens is in the discovery of new antibiotic resistance genes in the human gastrointestinal microbiota. Multidrug resistant bacteria are increasingly prevalent in both hospitals and the community, and pose a growing threat to human health (Boucher et al., 2009; Högberg et al., 2010). Infections with antibiotic resistant organisms are associated with increased mortality and cost of treatment (Maragakis et al., 2008), and novel antibiotic discovery has not kept pace with the emergence of microbial resistance to existing agents (Högberg et al., 2010). In order to develop a rational approach to curtail the emergence of antibiotic resistance in human pathogens, a deeper understanding of the flow of resistance genes within microbial communities is required. Pathogenic organisms present in the environment may acquire resistance genes from soil or water microbes, while commensal gastrointestinal organisms that are continuously exposed to the outside environment via host ingestion of food, may also come in contact with pathogenic bacteria during the course of an infection. Although great strides have been made in recent years documenting genetic resistance reservoirs and patterns of gene flow within and between environmental and human commensal microbiota, fully characterizing the diversity and mobility of the environmental resistome will be crucial to control the emergence of ever more resistant organisms (Aminov and Mackie, 2007; Martinez, 2008; Aminov, 2009; Allen et al., 2010).

Multiple studies demonstrate the efficacy of simple PCR screens in the detection and quantification of antibiotic resistance genes present in the gastrointestinal microbiota. PCR assays have been used to detect the presence of known tetracycline resistance genes (*tet*) in fecal samples from antibiotic-naive infants (Gueimonde et al., 2006). Similarly, qPCR has been used to quantify the levels of *tet* and *erm* genes, which confer resistance to tetracycline and macrolide, lincosamide, and streptogramin B antibiotics respectively, in animal and human waste water (Smith et al., 2004; Auerbach et al., 2007; Chen et al., 2010). The extraordinary specificity of PCR-based studies is also an important limitation of the technique: because PCR can only be used to interrogate a sample for known genes, it is an ineffective method for identifying novel resistance genes.

Functional metagenomic screens obviate this problem by identifying genes by their function in an expression vector rather than by a specific sequence used for PCR probing. Using this approach, novel antibiotic resistance genes have been identified in different environments including oral microbiota, soil microbiota, and moth gut flora (Diaz-Torres et al., 2003; Riesenfeld et al., 2004; Allen et al., 2009a). Sommer et al. (2009) demonstrated the power of metagenomic functional screens to identify novel antibiotic resistance genes in fecal samples from two healthy adults. Metagenomic libraries with a total size of 9.3 Gb (gigabases) and an average insert size of 1.8 kb (kilobases) were screened for resistance against 13 different antibiotics, revealing 95 unique inserts representing a variety of known resistance genes as well as 10 novel beta-lactamase gene families (Sommer et al., 2009). Genes identified using metagenomic functional screens were, on average, 61% identical to known resistance genes from pathogenic organisms, while genes identified via aerobic culturing of isolates from the same individuals had greater than 90% sequence identity to previously described resistance genes. One of the novel resistance genes identified with the functional metagenomic screen had 100% sequence identity to a protein of unknown function, demonstrating the power of metagenomic functional screens to identify novel resistance genes even in fully sequenced and apparently well-annotated organisms. Antibiotic resistance with high sequence identity to known genes were more likely than novel genes to be flanked by mobile genetic elements such as transposases, possibly indicating that the novel genes represent a potential resistance reservoir that has not yet become widely disseminated. Recent work by Goodman et al. (2011) demonstrated that interindividual differences in gastrointestinal antibiotic resistance genes can be detected by subjecting both uncultured fecal samples as well as pools of phylogenetically representative fecal culture collections to metagenomic functional screens. Notably, the presence or absence of specific resistance genes (e.g., those encoding amikacin resistance) in uncultured samples, as determined by functional metagenomics, correlated with the fraction of cultured isolates phenotypically resistant to those compounds, and the presence of the exact genes identified by functional metagenomic screens was reconfirmed by PCR assay in phenotypically resistant cultured strains. These authors also found that the nearest genome-sequenced phylogenetic neighbors of the resistant strains isolated from the the gastrointestinal microbiota of the sampled individuals lacked similar resistance genes, further highlighting the diversity and individualized nature of antibiotic resistomes. Together, these studies indicate that the gastrointestinal microbiota are likely to harbor many more resistance genes that will continue to be revealed by further investigation.

Functional metagenomic screens have also been used to mine the resistance reservoir in the intestinal microbiota of farm animals. Livestock are frequently dosed with antibiotics to treat infections and promote growth, and mounting evidence indicates that these practices lead to increased antibiotic resistance not only in the microbiota of the treated animals but also in their human caregivers (Sorum et al., 2006). The scope of this problem is highlighted by the findings of Kazimierczak et al. (2009), who employed metagenomic functional screens to identify both known and novel tetracycline resistance genes in fecal samples from organically farmed pigs that had not been exposed to antibiotics. Most of these genes were associated with mobile genetic elements, possibly explaining their persistence in an environment without any obvious selection pressure. The clinical and epidemiologic relevance of resistance genes present in the intestinal microbiota must be further defined by examining secondary effects of these genes, such as fitness costs or benefits associated with their expression, as well as by demonstrating the potential for direct transfer of the resistance gene to pathogenic organisms.

FUNCTIONAL METAGENOMICS FOR UNDERSTANDING THE GENETIC DETERMINANTS OF METABOLIC FUNCTION IN THE GASTROINTESTINAL MICROBIOTA

As previously noted, specific variations in the composition of the gastrointestinal microbial community have been linked to important states of human health and disease. Recent advances in understanding the interactions between bacterial metabolites and the host cellular machinery have begun to illuminate the physiologic basis of microbial contributions to human pathology. Metabolites generated either directly or indirectly by saccharolytic species may provide a mechanistic explanation for the observed human health outcomes associated with different compositions of the enteric microbial community. Conjugated linoleic acids, which are generated by some *Bifidobacterial* species (Rosberg-Cody et al., 2004), modulate tumorigenesis in animal models (Kelley et al., 2007), and are being investigated for a role in modulating inflammation and risk for neoplasia in humans (Bhattacharya et al., 2006; Coakley et al., 2009). Short-chain fatty acids (SCFAs) are bacterial metabolites that have wide-ranging effects on human physiology. In animal models of prematurity, some SCFAs (acetic and butyric acid) directly injure colonic mucosa in a dose-dependent fashion in the most immature age groups (Lin et al., 2002), an effect that disappears with increasing postnatal age (Nafday et al., 2005). This suggests a possible role for bacterial metabolites in the complex pathogenesis of necrotizing enterocolitis, a necroinflammatory disease commonly seen in preterm infants but non existent in older age groups. Butyrate, a SCFA that is produced by fermentation of dietary fiber, has a variety of effects modulating inflammation and risk for neoplasia (Hamer et al., 2008). Butyrate is taken up by colonocytes via the MCT1 and SMCT1 transporters (downregulated in cancer cells), and is protective against colon cancer in animal models. It also inhibits histone deacetylase and inhibits TNF-κB activation, which may explain its role in modulating inflammation. Acetate and propionate, two other SCFAs, have opposing effects on cholesterol biosynthesis (Wong et al., 2006). Microbe-generated SCFAs also may contribute to host obesity via interaction with fasting-induced adipocyte factor (Fiaf), AMPK, and Gpr41, which modulate pathways regulating fatty acid storage in adipocytes, fatty acid oxidation, gastrointestinal motility, and nutrient absorption (Backhed et al., 2007; Samuel et al., 2008).

Functional metagenomic screens offer a powerful means for detecting the genetic determinants of microbial metabolism. Jones et al. (2008) employed a functional screen using a large-insert metagenomic library to identify bile salt hydrolases within the human gastrointestinal microbiota. End-sequencing of clones displaying bile salt hydrolase activity revealed a broad phylogenetic distribution of bile salt hydrolase enzymes suggesting that this metabolic capacity is a conserved trait among bacteria adapted to life in the human gastrointestinal tract. Since bile salts play important roles in the processing and uptake of dietary fats in the intestines, microbial catabolism of these compounds may affect the amount of energy extracted from the diet.

Catabolism of fibers indigestible by the human host, another significant activity of the human intestinal microbiota, has been investigated using successive rounds of functional screens to enrich the metagenomic library with carbohydrate-metabolizing enzymes followed by high-throughput sequencing to identify genetic determinants of carbohydrate metabolism within the human gastrointestinal microbiota (Tasse et al., 2010). They identified 73 carbohydrate-metabolizing enzymes from the enriched library, representing a fivefold increase in active genes identified compared to metagenomic sequencing without enrichment. This highlights the strong potential of serial functional screens combined with high-throughput sequencing to identify novel genes and yield increasingly comprehensive information on the metabolic potential of a given microbial community.

INTEGRATING FUNCTIONAL SCREENS WITH SHOT-GUN METAGENOMIC SEQUENCING ANALYSIS

The advent of convenient applications for metagenomic data analysis such as MG-RAST and MEGAN have simplified annotation and comparative analysis of functionally selected genes, which together with the declining cost of high-throughput sequencing, offer an efficient complement to functional screens (Huson et al., 2007; Meyer et al., 2008). Several studies have used this approach to connect functional genes with metabolic capacities and to identify pathways, such as metabolism of sugars, amino acids, and nucleotides, that are enriched in the gastrointestinal microbiota relative to representative genome-sequenced strains (Gill et al., 2006; Kurokawa et al., 2007; Turnbaugh et al., 2009; Arumugam et al., 2011). Moreover, by ranking functional gene clusters according to their frequencies, a minimal gut genome and a minimal gut metagenome have been described (Qin et al., 2010). The former reflects the minimal set of genes required by a single member of the gastrointestinal microbiota, while the latter indicates the minimal set of genes required to sustain the aggregate gastrointestinal microbiota. The minimal gut genome includes genes essential to all bacteria (e.g., replication, transcription, translation) as well as gut-specific genes such as those facilitating adhesion to epithelium. In contrast, the minimal gut metagenome includes genes necessary for metabolism of complex sugars, underscoring the importance of coupled metabolism in sustaining the GI tract microbiota. The importance of confirming gene function *in vitro* and *in vivo* to ensure reliable annotation is illustrated by Hess et al. (2011), who used metagenomic sequencing to identify >20,000 carbohydrate active genes from the cow rumen microbiota. From this gene set, they selected 90 *in silico* predicted carbohydrate-metabolizing genes, expressed them, subjected them to functional assays, and found that 51 genes were enzymatically active *in vitro* (Hess et al., 2011). These studies exemplify how metagenomic sequencing, automated annotation of large data sets, and functional screening comprise a powerful toolkit capable of characterizing functional networks in highly complex environments such as the GI tract microbiota.

FUNCTIONAL MAPPING OF INTERACTIONS BETWEEN HUMANS AND THEIR INTESTINAL MICROBIOTA

Functional metagenomic screens may also illuminate the genetic determinants of microbial interactions with host cells. The intestinal microbiota have long been known to modulate intestinal epithelia, for instance, by stimulating intestinal cell differentiation (Bry et al., 1996). In order to identify specific bacterial gene products that directly influence the fate of human cells, Gloux et al.

(2007) used cell lysate from individual clones in a gastrointestinal metagenomic library to screen for modulation of cell growth in CV-1 kidney fibroblast and HT-29 human colonic tumor cells. Using this approach, they identified 30 growth-stimulating and 20 growth-inhibiting clones, with Bacteroidetes as the dominant phylum among both sets. Using transposon mutagenesis on these sets of clones, they identified seven candidate genes with putative growth modulation effects.

Functional metagenomic screens have also been designed to investigate the immune-modulatory capacity of the gastrointestinal microbiota. To identify clones modifying the host immune response, Lakhdari et al. (2010) constructed an NF-κB activated reporter system from a human colorectal carcinoma cell line. By screening metagenomic libraries of GI tract microbiota from patients with Crohn's disease, in which NF-κB activity is frequently elevated (Ellis et al., 1998), they identified several clones either inducing or inhibiting NF-κB activity. Together, these studies demonstrate the potential for functional metagenomic screens to illuminate the genetic mechanisms for microbial community contribution to the development of the human immune system and the pathogenesis of atopic, autoimmune, and neoplastic disease, which may provide novel therapeutic targets for these conditions.

In addition to interacting with human cells, commensal bacteria can also use quorum-sensing to convey signals over distances and thereby coordinate community gene expression. Guan et al. (2007) used a metabolite regulated expression(METREX) screen based on a quorum-sensing inducible promoter fused to *gfp* to identify genes encoding a new class of quorum-sensing inducing molecules in moth gut microbiota, demonstrating the power of functional metagenomics for characterizing the determinants of community behavior in uncultured organisms.

FUNCTIONAL METAGENOMICS FOR REFINING PRE- AND PRO-BIOTIC INTERVENTIONS

Increased understanding of the effects of gastrointestinal microbiota on human health has generated interest in targeting these communities for therapeutic intervention (Cani and Delzenne, 2011). Short-chain carbohydrates that are indigestible by humans but are fermentable by some microbes have demonstrable efficacy in increasing the populations of *Lactobacilli* and *Bifidobacteria* in the human gastrointestinal tract (Wang and Gibson, 1993). Investigations of galactose oligosaccharides (GOS) and fructose oligosaccharides (FOS) as additives to infant formula have demonstrated increased *Bifidobacterial* populations, decreased stool pH, generation of a stool fatty acid profile more similar to that found in breastfed infants, and reduced populations of potential pathogens such as *Clostridia* spp., *Bacteroides* spp., and *E. coli* (Fanaro et al., 2005; Knol et al., 2005; Costalos et al., 2008; Magne et al., 2008; Rao et al., 2009). Prebiotic supplementation with oligosaccharides may promote blooms of beneficial bacteria more effectively than direct administration of pro-biotic organisms: a study directly comparing infant formula containing *Bifidobacterium animalis* with GOS/FOS-supplemented formula revealed a significantly greater proportion of *Bifidobacterial* species in the infants fed oligosaccharide-containing formula but no difference between the *Bifidobacterial* supplemented formula and control formula groups (Bakker-Zierikzee et al., 2005). Administration of prebiotics such as inulin and oligosaccharides in adult humans have shown some effect on hunger and satiety mechanisms (Whelan et al., 2006; Cani et al., 2009) but inconsistent results when applied to pathologies such as atopy and inflammatory bowel disease (Guarner, 2005; Roberfroid et al., 2010). Functional metagenomics has the potential to refine current prebiotic therapies by more completely defining the genetic determinants of metabolism for given constituents of a microbial community, providing a rational basis for more precise design of prebiotic agents intended to promote blooming of a specific subset of organisms.

TOWARD A COMPLETE FUNCTIONAL REPRESENTATION OF THE GASTROINTESTINAL MICROBIOTA

Functional metagenomic screens have been successful in elucidating novel genes encoding microbial antibiotic resistance, metabolic machinery, and immune-modulatory elements. Despite their demonstrable utility, functional metagenomic screens have several important limitations. First, the DNA insert must be compatible with the host's expression machinery and the gene product must be non-toxic and functional in the host (for an in-depth review, see Uchiyama and Miyazaki, 2009). Second, the host must be suited for the screen: when screening for antibiotic resistance genes, a host sensitive to the antibiotic of interest must be chosen. Third, the insert size may restrict the diversity of functions portrayed in a screen; a small insert library cannot reveal the function of genes organized in large operons such as many metabolic pathways or some efflux pumps associated with antibiotic resistance. Finally, the expression level of the insert can significantly affect the result of a functional screen. Using a high-copy plasmid as vector or a strong synthetic promoter can result in an overestimation of functionality. Conversely, overexpression of potentially lethal proteins may cause underestimation of functional genes, (e.g., cell lysis due to overexpression of efflux pumps). Despite these limitations, multiple studies demonstrate the potential for functional metagenomic screens to powerfully complement direct culture, 16S sequencing, shotgun metagenomic sequencing, and metabolomic analysis to offer new insight into the complex interactions between microbial communities and their human hosts. Used in concert, these techniques promise to expand our understanding of microbial community function, its impact on human health, and to provide novel targets for therapeutic development in the coming years.

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Microbial eukaryotes in the human microbiome: ecology, evolution, and future directions

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High throughput sequencing technology has opened a window into the vast communities of bacteria that live on and in humans, demonstrating tremendous variability, and that they play a large role in health and disease. The eukaryotic component of the human gut microbiome remains relatively unexplored with these methods, but turning these tools toward microbial eukaryotes in the gut will likely yield myriad insights into disease as well as the ecological and evolutionary principles that govern the gut microbiota. Microbial eukaryotes are common inhabitants of the human gut worldwide and parasitic taxa are a major source of morbidity and mortality, especially in developing countries, though there are also taxa that cause no harm or are beneficial. While the role microbial eukaryotes play in healthy individuals is much less clear, there are likely many complex interactions between the bacterial, archaeal, and eukaryotic microbiota that influence human health. Integrating eukaryotic microbes into a broad view of microbiome function requires an integrated ecological approach rather than one focused on specific, disease-causing taxa. Moving forward, we expect broad surveys of the eukaryotic microbiota and associated bacteria from geographically and socioeconomically diverse populations to paint a more complete picture of the human gut microbiome in health and disease.

Keywords: intestinal protozoa, host-associated communities, eukaryotic diversity

INTRODUCTION

Microbial eukaryotes are an important component of the human gut microbiome. Eukaryotes that reside in the human gut are distributed across the eukaryotic tree (**Figure 1**) and their relationship with the human host varies from parasitic to opportunistic to commensal to mutualistic. For the purposes of this review, we are focusing on the microbial eukaryotes and are not discussing metazoan parasites, which are thoroughly covered elsewhere (Stoll, 1947; Kassai, 1999; Muller, 2002; Bogitsh et al., 2005). We are also focusing on the gut microbiome as many eukaryotic microbes are found there, and it is the best-characterized human body site in terms of the bacterial communities.

Eukaryotes are one of the three domains of life and are defined by the presence of nuclei. Animals, plants, and fungi are the most visible clades of eukaryotes, but these are just three of the 70+ lineages (Patterson, 1999), most of which are microbial. In recent decades, perspectives on the organization of eukaryotic diversity have shifted away from a Five Kingdom view emphasizing plants, animals, and fungi (Whittaker, 1969; Margulis et al., 1990). Molecular systematics and evolutionary studies clearly demonstrate that the bulk of the eukaryotic tree of life is microbial (Adl et al., 2005; Keeling et al., 2005; Parfrey et al., 2010), and eukaryotes are now divided into a small number of higher-level clades, although the structure of the eukaryotic tree is still stabilizing (Adl et al., 2005; Burki et al., 2010; Parfrey et al., 2010). Microbial eukaryotes are sometimes colloquially referred to as protists, although this term is not phylogenetically meaningful because microbial and macroscopic organisms intermingle in the eukaryotic tree of life (Adl et al., 2005; Parfrey et al., 2010). Similarly, terms such as protozoa, amebae, flagellates, and algae do not have phylogenetic meaning but are useful as morphological descriptors.

APPROACHES TO STUDYING HOST-ASSOCIATED MICROBIAL EUKARYOTES

Microbial eukaryotes in the human gut have been studied primarily from a parasitological point of view and are generally considered to negatively impact human health. The methods typically used focus on elucidating the presence of specific parasitic taxa, traditionally with culture and microscopy-based approaches (Bogitsh et al., 2005; Church et al., 2010) and more recently with targeted molecular analyses such as PCR based assays (Stensvold et al., 2011). Broad surveys of the eukaryotic diversity in the gut microbiota have only been explored on a limited basis (Marchesi, 2010), but are likely to provide new insights into the role of microbial eukaryotes in the gut by providing data on the whole community, including uncultured organisms and potential interactions among taxa. Fewer than 1% of microbes are culturable, and culture independent (CI) studies of microbial communities have revealed abundant insights into the nature and function of bacterial and archaeal communities in diverse environments (Pace, 1997), including the human microbiome (Marchesi, 2010; Robinson et al., 2010). While studies on eukaryotes lag behind

those of bacteria, CI approaches in environmental microbiology have also revealed hidden diversity and ecological interactions within eukaryotes (Caron et al., 2009; Steele et al., 2011).

In recent years barcoded high throughput sequencing of marker genes like small subunit ribosomal DNA (Hamady et al., 2008) coupled with analytical tools to assess patterns in the resulting data (Caporaso et al., 2010) have made it possible to assess the composition of bacterial communities and compare communities across many samples. These approaches have successfully been applied to bacteria and archaea, and have revolutionized our understanding of the human microbiome (Costello et al., 2009; Marchesi, 2010; Robinson et al., 2010). Comparing eukaryotic communities across a diverse collection of individuals from different geographic regions, economic status, and disease states are the next steps in characterizing the human gut microbiome.

There is growing body of theoretical and experimental literature on co-infection within the infectious disease community that stresses the importance of considering the action of disease agents within the context of other microbial players (e.g., Cox, 2001; Graham, 2008; Lafferty, 2010; Telfer et al., 2010). This may provide a framework for understanding the broader community context of the gut microbiome. Taking advantage of analytical tools such as co-occurrence networks, which detect positive or negative correlations among taxa (Steele et al., 2011), will also be crucial in detecting important interactions among community members. Together, these approaches will provide a baseline of expected microbial eukaryotes in healthy individuals and yield a better understanding of community changes associated with disease states.

ROLES OF HOST-ASSOCIATED EUKARYOTES: PATHOGEN, COMMENSAL, BENEFICIAL

Understanding the prevalence and distribution of microbial eukaryotes in the human gut has large consequences for human health. This is especially true in the developing world, where microbial parasites represent a large source of morbidity and mortality (Kaplan et al., 2000; Haque, 2007). Major gastrointestinal pathogens include *Entamoeba histolytica* (amoebiasis), which infects millions of people and results in 40,000–100,000 deaths annually, and *Giardia intestinalis* (giardiasis), which is the most prevalent intestinal parasite (Haque, 2007). While intestinal protistan parasites are often considered a tropical malady, they are actually broadly distributed across the globe, and their prevalence within a population is often linked to poor sanitation of human waste (Bogitsh et al., 2005; Pritt and Clark, 2008).

While the focus is generally on pathogens (in terms of study effort, genome sequencing, etc.), most microbial eukaryotes that reside in the gut do not cause harm, being either beneficial or commensal. Some eukaryotic microbes are considered probiotics, in particular the yeast *Saccharomyces boulardii* was originally isolated to combat Cholera, and it now marketed as a cure for diarrhea (McFarland and Bernasconi, 1993). Other eukaryotes have been shown to be beneficial in certain context, for example the hypothesized reduction in asthma and allergies in conjunction with hookworm infection (Falcone and Pritchard, 2005). Many other eukaryotic microbes are present in the gut but do no harm (they are commensals), including *Pentatrichomonas* and *Entamoeba dispar* (Bogitsh et al., 2005).

Human-associated microbial eukaryotes found in the gut are the focus of this article; however, eukaryotes are also part of the microbial community in other locations of the human microbiome. There is a low-diversity fungal community associated with human skin dominated by the genus *Malassezia* (Paulino et al., 2006). Fungi are also members of the oral microbiota; CI studies of western populations reveal diverse fungal lineages (Ghannoum et al., 2010). The mouth can harbor microbial eukaryotes that are closely related to taxa found in the gut such as *Entamoeba gingivalis* and *Trichomonas tenax* in individuals with poor oral hygiene (Bogitsh et al., 2005). Parasites that cause malaria (*Plasmodium*) and sleeping sickness (*Trypanosoma brucei*) reside in the liver and bloodstream, and other taxa cause problems when in brain tissue (e.g., *Encephalitozoon, Toxoplasma, Naegleria*; Bogitsh et al., 2005).

CURRENT SURVEYS OF THE EUKARYOTIC COMPONENT OF THE HUMAN MICROBIOME

Comprehensive study of the eukaryotic component of the human microbiome is just beginning and lags far behind our understanding of the bacterial communities. Initial CI surveys that were done with low-throughput methods of community finger printing and clone library sequencing are intriguing and suggest that there are numerous parallels between the bacterial and eukaryotic components of the microbiome. Fungi and *Blastocystis* are the dominant (and in many cases the only) eukaryotes in the gut microbiome of healthy individuals (Nam et al., 2008; Ott et al., 2008; Scanlan and Marchesi, 2008). The diversity of microbial eukaryotes within an individual is low, with fewer than 10 phylotypes recovered per individual (Ott et al., 2008; Scanlan and Marchesi, 2008). The limited data available thus far suggests that eukaryotic communities are stable across time and unique to individuals (Scanlan and Marchesi, 2008). The apparent diversity will likely rise as more individuals from diverse populations are sampled with next generation sequencing technologies by that enable rare taxa within the microbiota to be detected.

EVOLUTIONARY CONTEXT

The evolutionary patterns of microbial diversity within the vertebrate gut are similar across eukaryotes, bacteria, and archaea. Across all taxa only a small number of lineages have adapted to the gut environments, but those lineages are successful in colonizing a wide variety of hosts. Reduced diversity at deep phylogenetic levels (e.g., the Bacteroidetes and Firmicutes divisions of bacteria) is a hallmark of host-associated bacteria and archaea (Ley et al., 2006). This pattern is also evident in eukaryotes, where a small subset of eukaryotic lineages have host-associated representatives (**Figure 1**). Within the bacteria, the lineages present in the gut have flourished and produced extensive species and strain level diversity (Ley et al., 2006). The genetic diversity of microbial eukaryotes at shallow phylogenetic levels is lower than observed for bacteria, although there is still extensive strain (or species) level variation in isolates from different individuals (Parkar et al., 2010; Stensvold et al., 2011). The second major trend is that the taxa that have adapted to host-associated environments are broadly distributed as parasites and commensals of many different animal hosts. For example, commensal flagellate genera *Chilomastix* and *Pentatrichomonas* are found both in the bovine rumen and in

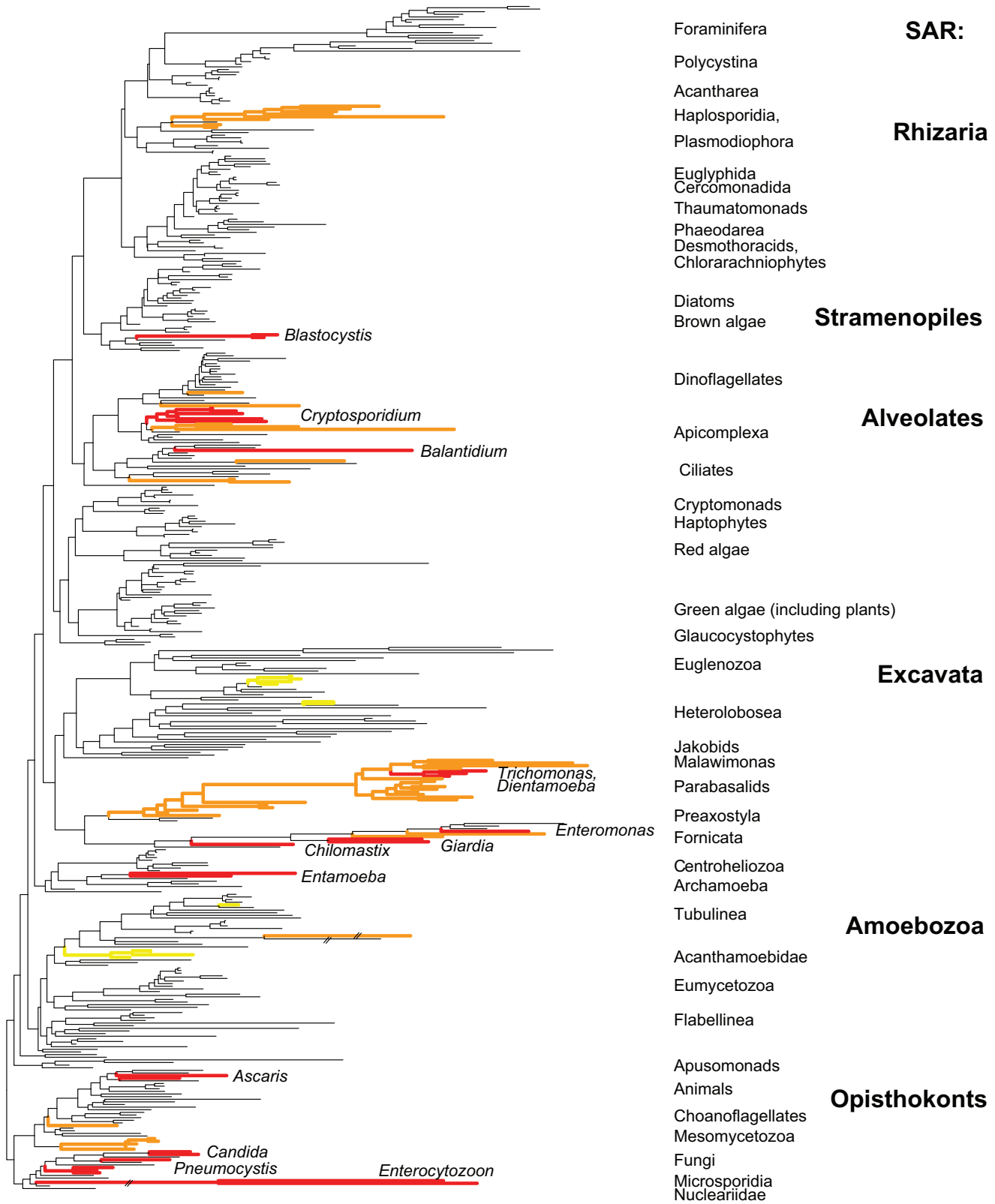

FIGURE 1 | Distribution of host-associated taxa across a broadly sampled eukaryotic tree of life. Red branches represent lineages found in the vertebrate gut with human-associated genera listed. Orange branches represent lineages found in invertebrates (some red lineages are also present in invertebrates). Yellow taxa are non-gut parasites of humans. Tree is based on Parfrey et al. (2010).

the human gut (Williams and Coleman, 1992), and *E. histolytica* and *E. invadens* are parasitic in humans and snakes, respectively (Barnard and Upton, l994). This suggests that relatively few taxa have adapted to host-associated environments, but these taxa are successful in a variety of hosts and have diversified at shallow phylogenetic levels.

The common patterns of diversity in the gut microbiome seen across the three domains of life are likely driven by the fact that host-associated environments, and especially the gut, are unique microbial habitats that are difficult to colonize because the stable, warm, low-oxygen, and eutrophic conditions represent an extreme environment (Ley et al., 2008). Eukaryotes that live in the gut are anaerobic or microaerophilic, and in most cases have highly reduced mitochondria (Hjort et al., 2010). Global analysis of bacterial communities demonstrate that host-associated communities are quite distinct from their free-living counterparts (Ley et al., 2008). While global patterns of eukaryotic diversity have yet not been examined quantitatively, it is likely that similar patterns to bacteria will emerge, as most gut taxa are known only from host-associated environments and are found across a range of hosts (Kreier and Baker, 1987). However, extensive CI studies of eukaryotes may reveal additional hidden diversity either within known lineages or in lineages not previously found within the microbiome.

EUKARYOTIC COMMUNITIES IN OTHER HOSTS

The eukaryotic microbiota has been extensively studied in animals where eukaryotes play a more central role, such as ruminants and termites, and these studies may provide expectations for the role of eukaryotes in the human microbiome. Culture independent studies have shown that mice harbor fungal communities that are much more diverse than those found in humans (Scupham et al., 2006; Marchesi, 2010), and animals that break down cellulose may generally harbor higher eukaryotic diversity. Eukaryotes make up the bulk of microbiota residing in the rumen and hindgut of many herbivores. Yet, even here the high-level phylogenetic diversity is limited: for example, the multitude of rumen ciliates all belong to a single order, the Litostomatea (Williams and Coleman, 1992). The human commensal *Balantidium* also belongs to this order. One of the best-characterized examples of mutualism occurs between symbiotic flagellates that reside in the hindgut of basal termites and the wood-eating cockroach *Cryptocercus* and break down cellulose (Radek, 1999). These insects rely on the parabasalid and oxymonad symbionts to break down cellulose and release energy, and these flagellates can constitute 15–30% of the body weight of the termite (Radek, 1999). Going a step further, parabasalids of the genus *Trichonympha* that inhabit *Cryptocercus* are themselves intimately associated with bacterial symbionts (Carpenter et al., 2009). There are also complex endosymbioses between rumen ciliates, methanogenic Archaea (related to *Methanobrevibacter smithii*) and proteobacteria (Irbis and Ushida, 2004).

Examining the eukaryotic microbiota associated with diverse animals has the potential to reveal new lineages of eukaryotes as several clades are known only from the microbiome of specific taxa. For example opalinids, a clade of stramenopiles, are only found in the hindgut of amphibians and hypermastigid parabasalids in the hindguts of termites and cockroaches. Parasites of invertebrate animals are more broadly distributed across the eukaryotic tree (Orange branches in **Figure 1**), and several novel lineages have been recently discovered (e.g., Hertel et al., 2002). This pattern likely stems from two factors: "invertebrates" are a far more diverse category phylogenetically than are vertebrates, and invertebrate-associated environments span a far broader range of conditions.

THE ECOLOGICAL CONTEXT OF THE GUT MICROBIOME

The species composition and general patterns of diversity found in the intestinal microbiota are best understood from an ecological perspective (reviewed in Ley et al., 2006; Robinson et al., 2010). Eukaryotes in the gut are not acting in isolation but are part of a complex community of microorganisms that includes bacteria, archaea, and viruses that interact through predation (Wildschutte et al., 2004), competition (Graham, 2008; Lafferty, 2010), and mutualism (Radek, 1999). The microbiota are also interacting and responding to the host immune system. Thus, it is important to determine changes in the bacterial and archaeal communities that correspond to the presence or absence of eukaryotic microbes. Next-generation sequencing of the total microbiota across diverse samples will enable this type of investigation. Placing eukaryotic microbes within the ecological context of the microbiome may be important for determining factors that influence pathogenicity of those that reside in the human gut. An ecological perspective will be particularly useful in understanding asymptomatic infections, opportunistic infections, and complex phenotypes such as IBS and malnutrition.

The range of the host response to many individual microbial eukaryotes varies greatly from asymptomatic to causing morbidity or mortality. For example, many people infected with known parasites, such as *E. histolytica* and *G. intestinalis,* are asymptomatic (Prado et al., 2005; Pritt and Clark, 2008). In the case of *E. histolytica,* only 10% of infections are invasive and result in amoebic dysentery, but these cases often lead to death (Pritt and Clark, 2008). Conversely, a number of microbial eukaryotes that are normally commensal or absent from the human microbiota can occasionally cause disease when they become abundant or infect immunocompromised people. Many factors underlie the differential manifestations of pathology, including host immune response, prior exposure to the pathogen, host genetics, and nutritional status, and co-infection with multiple parasites (e.g., Pritt and Clark, 2008). Differences in the bacterial and archaeal communities may be a further factor determining whether or not an individual manifests symptoms when their intestinal tract is colonized by protistan parasites. Building a large database of co-occurring eukaryotic and bacterial communities will allow one to test the hypothesis that changes in the bacterial community are responsible for the variance in the pathogenicity of eukaryotic parasites.

Opportunistic parasites are a significant source of morbidity in immune compromised patients, although these same taxa may be present in healthy people without apparent consequence. These emerging opportunistic pathogens include *Cryptosporidium parvum*, *Pneumocystis carnii*, and microsporidia, and all generally cause severe diarrhea (Kaplan et al., 2000). This shift in pathogenicity between healthy and immunocompromised individuals might result from changes in the microbial community in addition to changes in the immune system. These parasitic infections may

be similar to *Clostridium difficile* induced diarrhea, which has been shown to result from disruption of the gut microbial community (Walk and Young, 2008). A better understanding of the microbial ecology of opportunistic infections may lead to more effective treatment options, as it has with *C. difficile* (Walk and Young, 2008). There are also other organisms that may be present in a healthy microbiome community, but become problematic when their numbers increase, including the flagellate *Chilomastix* (Bogitsh et al., 2005) and the fungus *Candida albicans* (Schulze and Sonnenborn, 2009). In these cases it may be very important to look simultaneously at the bacterial and eukaryotic communities to determine whether a shift in the normal (bacteria dominated) microbiota is correlated with a rise in opportunistic microbial eukaryotes.

Eukaryotes have also been implicated as causative agents of diseases such as IBS (*Blastocystis*), IBD (fungi), and "leaky gut" (*Candida*; Boorom et al., 2008; Ott et al., 2008; Schulze and Sonnenborn, 2009). These diseases, which have become prevalent in western populations, are due to complex changes in the microbial community. Elucidating community-wide changes, rather than presence or absence of specific taxa, will be crucial to understanding the cause and potential treatment of in these complex polymicrobial diseases (Swidsinski et al., 2009). Samples taken from along the gastrointestinal tract may also be important to determine whether community changes associated with IBS or IBD are restricted to the site of disease or are systemic changes in the overall gut community, as has been the observed in bacterial communities (Peterson et al., 2008).

Several eukaryotic parasites are thought to interact with malnutrition and increase morbidity in parts of the developing world. For example, children infected with *Giardia* may experience reduced growth even when the infection is asymptomatic (Prado et al., 2005). Other eukaryotes that inhabit the gut are only pathogenic when their host is malnourished or otherwise stressed, as in the ciliate *Balantidium* (Schuster and Ramirez-Avila, 2008). These eukaryotes may interact with the bacterial community to impact nutrient cycling and absorption in the gut. Given the patchy distribution of these parasitic taxa in human populations it should be possible to assess whether the presence of these taxa correlate with differences in the bacterial community host malnourishment.

PROSPECTS FOR THE FUTURE

Incorporation of eukaryotes into human microbiome studies is just beginning, and will benefit from broad CI surveys of the eukaryotic communities in healthy and diseased individuals. These surveys should encompass diverse populations of healthy and diseased individuals, and should exploit within-subject time-series designs (to control for inter-individual diversity of the microbiome), and use identical twins (to control for genetic variability). Analyzing these data with emerging computational tools, such as co-occurrence networks, and pipelines for comparing large numbers of samples (Caporaso et al., 2010) will enable the patterns in these data to be elucidated. In-depth sequencing of the metatranscriptome of eukaryotes in the gut is another technique that will offer a window into the functioning of eukaryotes in the gut microbiome (Gianoulis et al., 2009). This could be targeted toward eukaryotes by selecting Poly(A) + RNAs for sequencing, although depletion of host transcripts in an unbiased way will be a major issue. Genomic surveys of uncultured host-associated eukaryotes will soon be possible using techniques such as single-cell genomics and transcriptomics (Heywood et al., 2011; Yoon et al., 2011). By connecting single-cell techniques, improved phylogenetic tree construction, broad surveys, and the parameters of host phenotypes, these studies have great potential to improve our understanding of the links between microbial eukaryotes and human health, and our understanding of the eukaryotic component of the tree of life.

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The intestinal microbiota and viral susceptibility

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Many infections start with microbial invasion of mucosal surfaces, which are typically colonized by a community of resident microbes. A growing body of literature demonstrates that the resident microbiota plays a significant role in host susceptibility to pathogens. Recent work has largely focused on the considerable effect that the intestinal microbiota can have upon bacterial pathogenesis. These studies reveal many significant gaps in our knowledge about the mechanisms by which the resident community impacts pathogen invasion and the nature of the ensuing host immune response. It is likely that as viral pathogens become the focus of studies that examine microbiota–host interaction, substantial effects of resident communities exerted via diverse mechanisms will be elucidated. Here we provide a perspective of the exciting emerging field that examines how the intestinal microbiota influences host susceptibility to viruses.

Keywords: intestinal microbiota, virus, pathogen, mucosal immune system, infection

THE INTESTINAL MICROBIOTA

A staggering number of microbes reside in and upon the human body (Dethlefsen et al., 2007). Our co-evolution with this microbiota has resulted in the integration of microbially derived developmental cues and metabolic capacities into human biology (Backhed et al., 2005). The vast majority of the 10–100 trillion microbial cells that inhabit the human body are found within the distal digestive tract. An altered intestinal microbiota composition has been linked to numerous pathologic states such as inflammatory bowel diseases and obesity in mouse models and in humans (Ley et al., 2005, 2006; Frank et al., 2007). In some cases an etiological relationship between the microbiota and disease state has been established (Turnbaugh et al., 2006; Garrett et al., 2007; Vijay-Kumar et al., 2010). Thus, human biology and health are intimately intertwined with the biology of our microbial inhabitants.

Ongoing large-scale sequencing efforts are providing a comprehensive sequence-based definition of this intestinal microbiota (Qin et al., 2010). Typically two bacterial phyla dominate the healthy Western adult intestine, the Firmicutes and Bacteroidetes compose >90% of the bacterial cells. Proteobacterium and Bifidobacterium and a handful of other bacterial phyla make up the remainder of the community, along with members of Archaea and Eukaryota. Despite the limited representation of bacterial phyla, at finer scales of phylogenetic resolution, such as species and strain, the intestinal microbiota is highly diverse and exhibits substantial compositional variability between people, and thus represents an "individualized fingerprint." In addition to inter-individual variability, the microbiota exhibits temporal variability, likely due to the numerous factors that continually perturb this dynamic microbial ecosystem, such as changes in host diet and introduction of orally acquired pathogens.

The tremendous foundation of microbiome sequencing data is propelling human microbiota studies into a second phase focused on function and mechanism. Recently published metagenomic data highlight the overlap and conservation of core functionalities within the intestinal microbiome of different individuals (Turnbaugh et al., 2007, 2009). This similarity embedded within the combined genomes of different consortia reveals that each person's gut is endowed with a core set of genes (a core microbiome) that carry out functions common to the human intestine. This new phase of investigation, which is focused on the emergent properties of the host–microbial super-organism, is attracting biomedical scientists from diverse fields, including virologists.

A WELL-SUPPORTED CASE FOR MICROBIOTA IMPACT ON PATHOGEN SUSCEPTIBILITY

The ability of an orally acquired pathogen to cause disease is dependant upon multiple steps that the microbiota may influence. A pathogen must navigate through the dense community of microbes, gain access to the epithelial surface, and manage the ensuing immune response. Commensal microbes present significant competition for nutrients (Sonnenburg et al., 2006), secrete microbicidal proteins (Corr et al., 2007), and elicit host responses that are fundamental to the development and maintenance of the mucosal innate and adaptive immune system (Cebra, 1999; Hooper et al., 2001; Ivanov et al., 2009). Despite this seemingly inhospitable environment, pathogens can gain access to host tissue and cause disease.

The role of the intestinal microbiota in reducing host susceptibility to enteric bacterial pathogens is commonly referred to as "colonization resistance," although the underlying mechanisms are poorly

understood. The increased susceptibility of oral antibiotic-treated mice or humans to enteric infection nicely illustrates the potential impact of microbiota perturbation on pathogen emergence (Pavia et al., 1990; Barthel et al., 2003; Doorduyn et al., 2006; Lawley et al., 2009). Furthermore, treating infectious diarrheas with live microbial supplements (i.e., probiotics) has proven successful in decreasing the duration of symptoms, but the mechanisms of action and whether such treatment is more effective for certain causative agents (e.g., bacterial vs. viral) remain obscure (Servin, 2004; Vandenplas et al., 2007; Guandalini, 2008; Allen et al., 2010). Together, these data support that alterations in host-associated microbial communities can impact an ensuing interaction with a pathogen.

INTESTINAL MICROBIOTA INFLUENCES IMMUNE FUNCTION AND PATHOGEN SUSCEPTIBILITY

The gut microbiota influences the status of the host immune system during development and throughout life. The host typically maintains a dynamic and attenuated physiological state of inflammation in the mucosa that is tuned to the membership of the adjacent microbial community (Cebra, 1999). Disruption of the microbiota-dependent homeostasis can be deleterious to the host. For example, in an experimental model of chemically induced intestinal epithelial injury, cell damage is exacerbated in the absence of a microbiota or if the mice are deficient in Myd88, an adaptor protein critical for toll-like, receptor-mediated recognition of the commensal microbiota (Rakoff-Nahoum et al., 2004). Similarly, *Salmonella*-induced inflammation becomes more severe in the absence of an intestinal microbial community (Stecher et al., 2005). Antimicrobial protein secretion (Hooper et al., 2001), induction of secretory IgA (Moreau et al., 1978; Shroff et al., 1995), Th1:Th2 bias (Mazmanian et al., 2005), and recruitment of the myeloid and lymphoid cell lineages to the gut (Cebra et al., 1998; Zaph et al., 2008) are impacted by microbial signals derived from the microbiota.

Therefore, aspects of mucosal and systemic immunity are similar in concept to a rheostat, with the microbiota playing a significant role in modulating the set-point. The concept that microbial modulation of the immune system can alter pathogen susceptibility is illustrated in a mouse model of latent herpesvirus infection. In this model, the latent virus is capable of elevating steady state levels of IFN-γ, effectively altering the set-point of the immune system and decreasing the host susceptibility to intracellular bacterial pathogens (Barton et al., 2007). The increased resistance to *Listeria monocytogenes* and *Yersinia pestis* of mice harboring latent herpesvirus implies that such dormant viruses (most humans possess several such herpesviruses) may be viewed as symbionts with mutualistic potential. These data are also suggestive that these same principles may apply to the microbiota: specifically, host immune status may be modulated by commensal microbes prior to pathogen exposure to alter host resistance to infection.

Since the vast majority of infections begin at the mucosal surface, it is not surprising that pathogen susceptibility may be impacted by the presence or absence of resident microbes that are capable of influencing local and systemic aspects of immunity. Segmented filamentous bacteria (SFB), an intestinal resident of mice, were recently identified as necessary and sufficient to induce Th17 development in the gut (Ivanov et al., 2009). In this same study, the presence of SFB proved critical in minimizing the inflammation caused by a colitis-inducing bacterial pathogen, *Citrobacter rodentium*. Similarly, alteration in host susceptibility to respiratory infection following intestinal exposure to a specific microbe has been reported. In an experimental model of *Cryptococcus neoformans*-induced pulmonary eosinophilia, the Th2-driven pathology can be abrogated by prior introduction of *C. rodentium* into the digestive tract (Williams et al., 2006). *C. rodentium* inflammation induces a Th1-response, which reduces the Th2-skewing and *C. neoformans*-associated pulmonary inflammation. This protective effect is lost if mice are colonized with a mutant strain of *C. rodentium* that is deficient in the intimin receptor, and therefore is incapable of attaching to the host colonic epithelium and unable to induce a Th1 response. Similarly, multiple studies have illustrated the ability of either chronic or acute parasitic infection in the intestine to influence immune responses in the lung, specifically decreasing allergic airway inflammation (Wilson et al., 2005; Kitagaki et al., 2006; Gibbons et al., 2009). The ability of a group of *Clostridium* species to induce regulatory T-cells in the colon and decrease the severity of colitis and IgE responses in a mouse model further affirms the role of gut microbes in modulating aspects of mucosal and systemic immune function (Atarashi et al., 2010).

THE MICROBIOTA'S DIRECT INFLUENCE ON PATHOGENS

In addition to acting through the immune system, the intestinal microbiota may impact pathogens more directly. The emergence of bacterial pathogens, such as *Clostridium difficile* and *Salmonella* spp., during or shortly after antibiotic treatment, suggests the loss of inhibitory effects of the microbiota upon antibiotic-induced disruption. Several bacterial pathogens, such as *Salmonella* and *Citrobacter*, appear to subvert the host immune response within the intestine causing persistent microbiota disruption thus aiding their own proliferation (Lupp et al., 2007; Stecher et al., 2007). Competing for nutrients, physically obstructing access to the mucosa, and producing antimicrobial proteins have all been proposed as mechanisms that contribute to colonization resistance (Stecher and Hardt, 2008). The bacteriocin, Abp118, produced by a *Lactobacillus salivarius* strain, is capable of killing *L. monocytogenes*, and its production *in vivo* is required to protect the host in a murine model of oral *Listeria* infection (Corr et al., 2007). This example provides a glimpse of the type of warfare that can take place amongst and between permanent and transient gut residents. Alternatively, egg hatching of *Trichuris muris*, a nematode that invades the intestinal epithelium of mice, is enhanced in the presence of the microbiota-derived microbes *in vitro*, and *T. muris* burden is decreased after antibiotic knock-down of the microbiota (Hayes et al., 2010). The ability of multiple pathogens to infect in the absence of apparent microbiota disruption (i.e., not all orally acquired pathogens require antibiotic treatment to emerge and cause disease) suggests that several infectious agents may be adapted to and benefit from the microbiota in a normal state.

POTENTIAL IMPACT OF THE MICROBIOTA ON VIRAL INFECTIONS

In general, the microbiota may enhance viral infection, reduce viral infection, or have no effect on viral infection. In instances when the microbiota influences viral infection, it may be via direct

mechanisms, such as virion modification, or indirect mechanisms, such as host cell modification. Direct effects of the microbiota on viruses are likely to occur locally, whereas indirect effects may occur locally or systemically.

Several studies have shown that the microbiota has the potential to reduce viral infection and disease. First, germ-free mice have enhanced susceptibility to influenza A virus (Dolowy and Muldoon, 1964), coxsackie B virus (Schaffer et al., 1963), and Friend virus (Mirand and Grace, 1963), which suggests that the microbiota directly or indirectly limits disease in mice with a conventional flora. Second, while dengue virus is a blood-borne pathogen in mammals, it initiates mosquito infection through the enteric route. Interestingly, dengue virus replication is enhanced in the midguts of antibiotic-treated mosquitoes, which suggests that the insect microbiota limits viral replication. These effects may occur indirectly via microbiota-mediated anti-viral immune activation through the Toll pathway (Xi et al., 2008). Third, adenovirus replication is inhibited by defensins, which are antimicrobial peptides produced by the host in response to the microbiota (Gropp et al., 1999). Defensins directly bind adenovirus virions and limit replication *in vitro* (Smith and Nemerow, 2008). Therefore, the microbiota may limit adenovirus infection through induction of host defense mechanisms, although the effects of defensins on enteric viruses *in vivo* is unknown.

Conversely, several studies have shown that microbial products can indirectly enhance viral replication and disease. First, persistence of mouse mammary tumor virus is enhanced by lipopolysaccharide (LPS), a component of Gram-negative bacteria. LPS activates the innate immune pathways culminating in IL-10-mediated tolerance and viral persistence in mice (Jude et al., 2003). Second, Theiler's murine encephalitis virus-induced replication and disease in the central nervous system is enhanced by treatment with LPS. These effects likely occur through indirect mechanisms involving increased inflammation in the central nervous system, which promotes viral replication (Pullen et al., 1995). In future work, it will be important to determine whether native microbiota-derived LPS, rather than experimentally administered LPS, is sufficient to promote viral infection. Third, human immunodeficiency virus (HIV) pathogenesis and progression to acquired immunodeficiency syndrome (AIDS) is enhanced by LPS-mediated chronic immune activation from microbial translocation through the intestinal wall (Brenchley et al., 2006). Therefore, the microbiota may indirectly enhance progression to AIDS through indirect immune activation mechanisms.

When considering effects of the microbiota on viruses, it is important to be mindful of the evolutionary potential of viruses, particularly RNA viruses. Viruses have error frequencies up to a million-fold higher than bacteria or mammals and likely evolved to replicate in their respective niches, including niches containing a microbiota. Thus, the hypothesis that some viruses will generally fare worse in the absence of the microbiota with which they have evolved will be important to test using microbiota-deficient mice.

GAPS THAT NEED TO BE ADDRESSED FOR THE FIELD TO MOVE FORWARD

Clearly, it is important to understand how the microbiota influences viral infections, but what are the major questions to address? The questions will be shaped by virus type. For example, the intestinal microbiota may have direct and indirect effects for enteric viruses that directly encounter them in the intestinal lumen (enterovirus, rotavirus, Norwalk virus, astrovirus, etc.). However, the intestinal microbiota may also impact non-enteric viruses through indirect mechanisms. Several viruses encounter host–microbiota at other body sites, such as skin and the genital tract (HIV, papilloma virus, herpes simplex virus, etc.). Therefore, microbiota may affect a wide variety of viruses through diverse mechanisms (**Figure 1**).

The first step is to determine the impact of the microbiota on various viruses by depleting the microbiota and quantifying viral replication and pathogenesis. Mouse models will be very useful for these experiments, provided that there is an appropriate mouse model for the virus of interest. Mice are naturally resistant to many pathogens, including viruses, often requiring the use of immunodeficient or neonatal mice for pathogenesis studies. Therefore, diverse systems may be required to overcome this "species barrier." Microbiota depletion can be accomplished with broad-spectrum antibiotic treatment or the use of germ-free animals. Antibiotic treatment is an attractive option since it is relatively simple and inexpensive and can be used with any animal model. However, in antibiotic-treated animals, microbiota clearance is incomplete, the ratios of various species are altered, and the antibiotics can indirectly alter the host via microbiota-independent effects (Rakoff-Nahoum et al., 2004). Another negative is that antibiotic-mediated microbiota clearance requires 1 week or more, and since neonatal mice are required for some viral pathogenesis models, the antibiotic model is not ideal. Using germ-free mice overcomes many of these obstacles. Germ-free mice are completely microbiologically sterile, eliminating background and allowing recolonization with specific microbes to examine specificity of microbiota effects. However, germ-free mice require highly specialized facilities, are expensive, and a limited number of germ-free mouse strains are available. Additionally, germ-free mice have immature immune systems and altered intestinal physiology, which may complicate interpretation of experiments (Smith et al., 2007). A combination of experiments with antibiotic-treated mice and germ-free mice may be necessary to paint a complete picture of microbiota effects on viruses. Retrospective and/or prospective studies on humans treated with antibiotics and their incidence of viral infections may also be useful in evaluating microbiota effects on viruses.

Once the impact of the microbiota has been established for a given virus, the second step is to determine whether effects occur through direct or indirect mechanisms. The microbiota may directly alter the infectivity of viral particles through virion modification or processing. Microbial components or products may interact with virions to enhance or reduce their infectivity. It is also possible that virions may "hitchhike" on motile bacteria to access host cells. Conversely, the microbiota may indirectly influence viral infection by altering the host. For example, the microbiota may promote or limit viral replication by altering innate and/or adaptive immune responses, changing the susceptibility of host cells, or altering viral receptor expression. A variety of experimental systems will be useful to determine the nature of microbiota effects on viruses. For example, direct effects of microbiota on virions can be examined through *in vitro* mixing experiments and infectivity assays. Indirect microbiota effects on viruses mediated by innate immune responses can be examined in mouse strains lacking

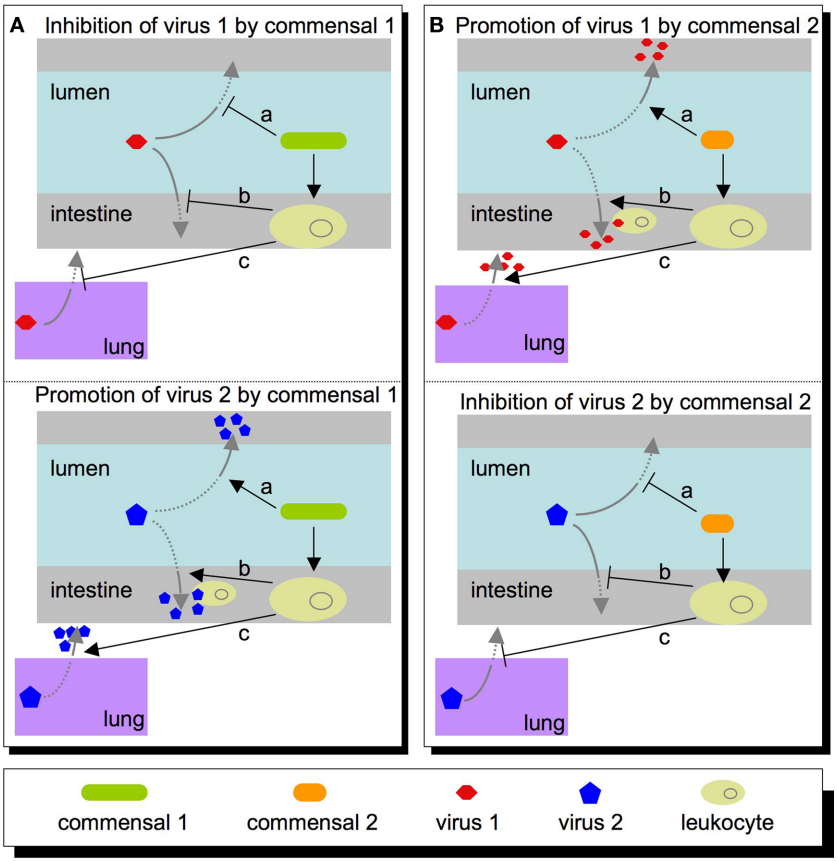

FIGURE 1 | Potential roles that commensal microbes within the intestine may play in host susceptibility to virus. (A) A commensal may inhibit a virus (top left panel) directly (a), or indirectly at a local site (b), or at a peripheral site such as the lung (c). Conversely, this same commensal may promote viral infectivity (lower left panel) via these same mechanisms, such as directly activating the virus (a), recruiting leukocytes that promote/permit viral replication (b), or setting systemic immune parameters that are permissive for viral replication at a distant site (c). **(B)** Other commensals may have opposite effects on the same viruses, and alterations in microbiota composition or functional state may shift the balance in favor of the first or second scenario.

components of the innate response. The specificity of microbiota effects can be examined by colonizing germ-free mice with specific types of bacteria to determine whether a particular type of bacteria is sufficient to affect the virus of interest. Together, these types of experiments will reveal the basics of microbiota–virus interactions and will provide information for detailed mechanistic studies in the future.

IMPLICATIONS FOR HUMAN HEALTH

It is clear that the microbiota provides a major source of biological variation between individuals, and it is a contributor to numerous biological processes. Microbiota plasticity, which is significantly greater than that of our own genome, may be harnessed to modulate aspects of human health, such as optimizing resistance to certain pathogens. The link between microbiota perturbation and enteric pathogen emergence illustrates that investigation into the factors that govern microbiota stability could have great significance for humans. Alternatively, if certain members of the microbiota, or certain functions encoded within the microbiota, are co-opted by viruses and are required for invasion of host tissue, this set of interactions presents new therapeutic targets.

The possibility of leveraging the microbiota's role in infectious disease as a platform to alter the course of infections presents many important questions. Can we rationally and reproducibly manipulate the composition and function of our intestinal microbiota? Can this manipulation be performed in ways that prevent or ameliorate human disease? To address these questions, it is critical to lay a foundation of basic research that will establish the fundamental principles that govern this complex set of interactions. Highly controlled studies in model system are needed to establish how the gut microbiota may be manipulated rationally through the use of orally administered microbes (probiotics), dietary supplementation (prebiotics), and/or microbiota-targeted small molecules to optimize host resistance to infectious disease.

As we expand our understanding of pathogens that possess diverse routes of invasion, tropisms, and/or toxins, and we investigate how this biology intersects with the host–microbiota interaction, strategies for using this information to improve human health will become evident. For example, interferon (IFN) signaling is an important part of the immune response to many viral infections; however, this system is a target for inhibition by many viruses. Identifying a microbiota manipulation (e.g., dietary change or

probiotic administration) that induces a mild anti-viral-like IFN response suggests a strategy for pre-conditioning the host immune response to provide increased resistance during periods of heightened vulnerability (e.g., in infancy or during epidemics). Tuning the immune rheostat to desired set-points using microbial exposure (i.e., colonization status) provides a promising avenue that may have broad applicability. Identification of biomarkers of these host responses, which can be surveyed in feces, urine, or blood, will enable these responses to be tracked and appropriately altered.

The ability to modulate the status of the mucosal immune system has implications for improving the efficacy of oral vaccination campaigns. One such example is the global effort to eradicate wild poliovirus, which has stalled in recent years due to the failure of oral polio vaccines to achieve seroconversion of children in developing nations. In the Indian states of Uttar Pradesh and Bihar, less than 71% of children are protected against type 1 poliovirus despite an average of 15 oral doses of trivalent vaccine per child before 5 years of age; 85% protection is achieved with 10 average doses across the rest of India (Grassly et al., 2006). This failure in protection is seen in several developing regions of the world with poor sanitation and has been linked to diarrhea and the presence of enteric viruses (Myaux et al., 1996). These studies lend further evidence that the status of the mucosal immune system and/or stability of the microbiota dictates responsiveness to newly encountered agents (whether vaccines or pathogens) and places additional importance on pursuing a mechanistic understanding of how the relationship between the microbiota and mucosal immune system may be optimized to reduce viral disease and to improve the efficacy of oral vaccines.

A logical and likely extension of personal genomes is the personal microbiome, elucidating the genes encoded by the core species or taxa within an individual's intestine. With ever the increasing understanding of how the biology of host and microbiota integrate, these two sets of DNA sequences will offer insight into current or looming pathologies by identifying potential deleterious combinations of polymorphisms in the genes of host and microbiota. The microbiome offers a set of genes much more amenable to manipulation compared to our human genome. A prerequisite for incorporating the microbiota into personalized, preventative medicine is to attain a mechanistic understanding of microbiota function and to understand how microbes within the intestine affect the resistance and susceptibility to pathogens (Nicholson et al., 2005; Zaneveld et al., 2008). The rational manipulation of our "microbial self" will soon be a reality, and it is imperative to understand which manipulations will promote human health.

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The starting lineup: key microbial players in intestinal immunity and homeostasis

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The complexity of microbiota inhabiting the intestine is increasingly apparent. Delicate balance of numerous bacterial species can affect development of the immune system, how susceptible a host is to pathogenic organisms, and the auto-inflammatory state of the host. In the last decade, with the increased use of germ-free mice, gnotobiotic mice, and animal models in which a germ-free animal has been colonized with a foreign microbiota such as humanized mice, it has been possible to delineate relationships that specific bacteria have with the host immune system and to show what role they may play in overall host health. These models have not only allowed us to tease out the roles of individual species, but have also allowed the discovery and characterization of functionally unknown organisms. For example, segmented filamentous bacteria (SFB) have been shown to play a vital role in expansion of IL-17 producing cells. Prior to linking their key role in immune system development, little was known about these organisms. *Bacteroides fragilis* can rescue some of the immune defects of gnotobiotic mice after mono-colonization and have anti-inflammatory properties that can alleviate colitis and experimental allergic encephalitis in murine models. Additionally, *Clostridium* species have most recently been shown to expand regulatory T-cell populations leading to anti-inflammatory conditions. This review will highlight and summarize some of the major findings within the last decade concerning the role of select groups of bacteria including SFB, *Clostridium*, *Bacteroides*, *Bifidobacterium*, and *Lactobacillus*, and their impact on host mucosal immune systems.

Keywords: mono-colonization, homeostasis, microbiome, germ-free, segmented filamentous bacteria, Bacteroides fragilis, Clostridium, probiotics

INTRODUCTION

Humans play host to 500–1000 different species of bacteria in the intestine and 100 times more bacterial cells than eukaryotic cells (Whitman et al., 1998). Increased appreciation for the crucial role that the intestinal microbiota plays in host health and immunity is continually surfacing. While mammalian hosts provide a nutrient rich niche for these bacteria, the bacteria provide the host with much more including: aid in digestion, protection against pathogenic enteric pathogens, and development of the immune system (Hooper and Gordon, 2001; Macpherson and Harris, 2004; Sekirov et al., 2008; Sekirov and Finlay, 2009). Indeed, a skewed microbiota balance can affect health in numerous ways and can lead to conditions that promote diseases such as obesity, diabetes, inflammatory bowel disorders, and multiple sclerosis (Round and Mazmanian, 2009; Ochoa-Reparaz et al., 2010b). Additionally, a skewed microbiota can leave a host susceptible to infection (Manichanh et al., 2006; Turnbaugh et al., 2006, 2009; Frank et al., 2007; Garrett et al., 2007; Peterson et al., 2008; Wen et al., 2008). The complexity of the mammalian intestinal microbiota has long been appreciated; however, our knowledge of this area has greatly expanded in recent years as more advanced sequencing methods have become available. Due to the harsh conditions of the intestine, many of the organisms which dwell there are fastidious and cannot be cultured *in vitro*. Modern sequencing techniques have enabled us to begin cataloging in detail the microbial life that exists within the intestine whether or not it can be cultured *ex vivo* (Turnbaugh et al., 2007). The importance of commensal bacteria in host development and health is most clearly demonstrated by germ-free mice, raised in the absence of any bacteria. These mice exhibit numerous developmental defects, which can be compensated for by microbial colonization (Macpherson and Harris, 2004).

Developmental problems and defects faced by germ-free mice are, in part, centered around immune system development and function (Smith et al., 2007). While not an exhaustive list, some of the immunological defects seen in germ-free mice include immature lymphoid follicles, an enlarged cecum, reduced plasma cells and reduced production of mucosal immunolobulin A (IgA), anti-microbial peptides, and adenosine tri-phosphate (ATP). The number of CD8+ intestinal epithelial cells (IELs) and αβ T-cell receptor (TCR) IELs is reduced as well as Thy1 expression and cytolytic activity. IEL expression of major histocompatibility complex (MHC) II, Toll-like receptor (TLR) 9, and interleukin (IL) 25 is also reduced. CD4+ T-cells in the lamina propria (LP), Foxp3+ regulatory cells in the colonic LP, and CD4+CD25+ T-cells in the mesenteric lymph nodes (MLNs) are reduced. Immune structures in germ-free animals are also compromised. The Peyer's patches are small compared to conventional animals, and the spleens and MLNs have depletion of lymphocyte zones (Lefrancois and

Goodman, 1989; Rothkotter and Pabst, 1989; Shroff et al., 1995; Umesaki et al., 1995; Smith et al., 2007; Round and Mazmanian, 2009). Finally, germ-free mice have increased susceptibility to infection from enteric pathogens. It has been shown that they have less resistance than conventional mice to infection with: *Shigella flexneri*, *Listeria monocytogenes, Clostridium difficile*, and *Salmonella enterica* (Sprinz et al., 1961; Zachar and Savage, 1979; Nardi et al., 1989). When germ-free mice are colonized with as little as one commensal bacterial species, the susceptibility to infection is reduced. This feat is not accomplished by every intestinal commensal organism, however, indicating that colonization resistance (CR) ability is specific (Maier and Hentges, 1972; Round and Mazmanian, 2009; Ivanov and Littman, 2010).

Given the number of bacterial species residing in the intestine, it is hard to imagine teasing out specific contributions by individual species. However, mono-colonization of germ-free mice has allowed exactly this and we have gained important knowledge by examining which components of the faulty germ-free mouse immune system can be re-constituted with the addition of one bacterial species. This review highlights several groups of bacterial species that have been shown to play an important role in immune development and homeostasis and were characterized in this manner. While not an exhaustive description of enteric bacteria that affect host immune systems, the groups we have picked form a representative sample of species that show varied impacts on their hosts. Each of these groups has been historically well studied; however, recent advances make them particularly relevant.

BACTEROIDES FRAGILIS

Although members of the *Bacteroidales* order are the most prominent gram negative bacteria in the intestine, the colonic bacteria *Bacteroides fragilis* make up only one percent of intestinal microbiota. While not numerically dominant amongst the *Bacteroidetes* in the intestine, this species of *Bacteroides* has been shown to have important effects on host health, both beneficial and detrimental (Polk and Kasper, 1977; Troy and Kasper, 2010). Normally symbiotic when contained within the intestine, in the event of bowel perforation, *B. fragilis* becomes pathogenic, inducing abscess formation throughout the peritoneal cavity (Polk and Kasper, 1977). *B. fragilis* is the most common clinically isolated anaerobic bacterial species, but in recent years, has become more well-known for the extent of positive effects it exerts on the immune system (Polk and Kasper, 1977; Mazmanian et al., 2005, 2008; Lassmann et al., 2007). *B. fragilis* and other *Bacteroides* have the genetic capability to produce multiple capsular polysaccharides, with *B. fragilis* producing eight. In *B. fragilis* these polysaccharides are important for commensal colonization of the intestine (Krinos et al., 2001; Coyne et al., 2008; Liu et al., 2008). At least two of these polysaccharides, polysaccharide A (PSA) and polysaccharide B (PSB) contain both positive and negative charges, making them zwitterionic (Tzianabos et al., 1993). Contrary to traditional characterization of carbohydrates as T-cell independent (Gonzalez-Fernandez et al., 2008), PSA and other zwitterionic carbohydrates evoke both CD4+ T-cell dependent and T-cell independent immune responses (Tzianabos et al., 2000; Cobb et al., 2004). Many characterized zwitterionic carbohydrates from bacteria have been shown to have immunomodulatory properties (Tzianabos et al., 1993, 2000, 2001; Cobb et al., 2004); however, PSA might be the most well-characterized microbial factor involved in commensalism. When germ-free mice are colonized with *B. fragilis*, many of the defects seen in these mice are corrected almost to the level of conventionally colonized mice (Mazmanian et al., 2005). Colonization of germ-free mice with *B. fragilis* promotes expansion of CD4+ T-cells, corrects depletion of splenic lymphocytic zones, and corrects Th1/Th2 imbalances by reduced IL-4 production and increased interferon (IFN)-γ production. Importantly, these rescue effects have been narrowed down to the activity of zwitterionic PSA. When *B. fragilis* defective in production of PSA is used to colonize germ-free mice, no correction of Th1/Th2 imbalance is seen. This was one of the first instances that showed correction of germ-free immunological defects by not just a commensal organism, but a specific surface molecule or symbiosis factor (Mazmanian et al., 2005). *B. fragilis's* immunomodulatory capabilities have been shown to directly play a role in health and disease. Numerous studies have shown that an imbalance of microbiota can lead to intestinal inflammation due to lack of mucosal immune tolerance (Frank et al., 2007; Round and Mazmanian, 2009; Ivanov and Littman, 2011). *B. fragilis* has been shown to be protective against both immune (CD4+CD45Rb transfer with *Helicobacter hepaticus* inoculation) and chemically (trinitrobenzene sulfonic acid, TNBS) induced colitis (Mazmanian et al., 2008). In addition, purified PSA itself is protective against chemically induced colitis. This protection is dependent on CD4+ production of IL-10, induced by PSA. In animals defective for IL-10 production, no protective effect is seen by *B. fragilis* (Mazmanian et al., 2008). *B. fragilis* has subsequently been shown to alleviate chemically induced colitis in mice post colitis induction, showing that it can have both a preventative and therapeutic role. IL-10 production induced by *B. fragilis* results from increased numbers of Foxp3+ T-regulatory cells and mono-colonization of mice with *B. fragilis* has shown that these bacteria alone are capable of mediating the development of Foxp3+ T-regulatory cells from CD4+ T-cells (Round and Mazmanian, 2010). The anti-inflammatory effects of *B. fragilis* are not limited to a role in mouse models of models of colitis, but also additional models of inflammatory disease. For example, it was recently shown that *B. fragilis* is protective against an experimental autoimmune encephomyelitis (EAE), a mouse model mimicking human multiple sclerosis (Ochoa-Reparaz et al., 2010b). In an initial study, Ochoa-Reparaz et al., showed that purified *B. fragilis* PSA given to mice orally could protect against EAE. The administration of PSA to these mice enhanced a population of dendritic cells which express CD103 and these cells were seen accumulating in the cervical lymph nodes. Similar to the proposed mechanism for PSA's protection against colitis, in IL-10 deficient mice, PSA offered no protection (Ochoa-Reparaz et al., 2010a). In an exciting follow-up study, the same group showed that oral colonization of mice with the entire organism, *B. fragilis,* could also protect against EAE and when a PSA deficient strain of *B. fragilis* was used, no protection was seen. Similar to purified PSA, the addition of *B. fragilis* to these mice stimulated the numbers of Foxp3+ T-regulatory cells accumulating in the cervical lymph nodes (Ochoa-Reparaz et al., 2010a,b). These studies are particularly ground-breaking since they show one of the first examples of systemic effects of *B. fragilis's* immunomodulatory capabilities.

B. fragilis and PSA's anti-inflammatory properties are currently being tested in other models of inflammation to determine how universal their effects are.

SEGMENTED FILAMENTOUS BACTERIA

Segmented filamentous bacteria (SFB) or *Candidatus* Arthromitus (Snel et al., 1995) were originally observed and morphologically characterized over three decades ago in the small intestine of rats, mice, and chickens. These segmented organisms uniquely attach to the apical epithelium of primarily the small intestine through an attachment point in which the host cell epithelium and apical cytoplasm is modified (Hampton and Rosario, 1965; Savage, 1969; Fuller and Turvey, 1971; Davis and Savage, 1974). The biology of these organisms is intriguing as they appear in numerous vertebrates after birth and disappear shortly after adulthood (Davis and Savage, 1974). Early reports indicate many influences over the colonization of SFB within a host organism including age, sex, and immune status (Davis and Savage, 1974; Jiang et al., 2001). The timing of the appearance of these organisms in their vegetative state has led to numerous studies as well as speculation about the environmental signal that induces sporulation or overall disappearance of SFB from their host. It is possible that this could be related to the establishment of microbial colonization in young vertebrates or possibly it is related to nutritional changes once mammals are weaned from their mothers. Microscopically, SFB have been observed in numerous laboratory and non-laboratory vertebrates, including chickens, dogs, cats, pigs, fowl, macaque, and humans (Klaasen et al., 1993); however, they have also been shown to be extremely species specific. Tannock et al. (1984) showed that when ileal homogenates containing SFB from mice were given to germ-free rats, the SFB failed to attach to the epithelium. Likewise, when ileal homogenates containing SFB from rats were given to germ-free mice, the SFB also failed to adhere to the epithelium, whereas when source SFB were given to the same species, they were able to adhere to the epithelium (Tannock et al., 1984). This indicates a co-evolutionary relationship between SFB and their hosts. Although SFB were visualized by light microscopy to be part of the human microbiota, definitive genetic identification of these microbes in humans has not been reported (Klaasen et al., 1993). Many of the aforementioned characteristics of SFB led researchers to believe they had an important role in the immunology of their hosts; however, the un-culturability of SFB has made many studies of this organism's unique features impossible. A break-through came with the ability to mono-colonize mice with SFB. Several groups were able to take fecal homogenates and limit them to only spore-forming bacteria using chloroform and ethanol washes (Klaasen et al., 1990). These mixes of SFB and some *Clostridium* species could be diluted to only contain SFB and were illeo-inoculated into mice establishing a mono-colonized species (Klaasen et al., 1991). The study of mice mono-colonized with SFB in combination with high powered sequencing methods has allowed many groups to observe the profound effects that SFB have on their host's immunity (Klaasen et al., 1991; Umesaki et al., 1995; Ivanov et al., 2008, 2009; Gaboriau-Routhiau et al., 2009).

Mono-colonization of mice with SFB leads to many immunological changes to germ-free mice particularly within the small intestine. In the small intestine, there is an induction of MHCII molecules on IECs as well as fucosylation of GM1 glyco-lipids (Umesaki et al., 1995). Significant changes are also seen on IELs such as an expansion of IELs bearing both αβ and γδ TCRs, increased CD8+ T-cells, increased cytolytic activity, and increased Thy1 expression. Crypt cell proliferation is induced as well as production of columnar cells. Upon mono-colonization, germinal center reactions in the Peyer's patches are stimulated and CD4+ and $CD45RB^{low}$ T-cells increase until they reach levels of conventional mice (Umesaki et al., 1995; Talham et al., 1999). IgA is also produced in significant amounts after SFB colonization of germ-free mice (Umesaki et al., 1995; Suzuki et al., 2004). While these changes are substantial, the levels of αβ TCRS do not fully come up to conventional levels nor are many of the morphological characteristics of germ-free mice such as an enlarged cecum normalized (Umesaki et al., 1995; Talham et al., 1999). Interestingly, in mice deficient in IgA, a prominent and persistent expansion of SFB is seen that returns to normal when the mice are compensated with IgA (Suzuki et al., 2004). IgA is seen as a major mechanism for maintaining intestinal homeostasis among commensal organisms (Duerkop et al., 2009), and its absence in these mice is compensated for by large amounts of IgM and normal expression of defensins and angiogenins (Suzuki et al., 2004). This reaffirms the paradigm in which an IgA feedback loop is a method of keeping commensal organisms near the epithelial surface in check (Hooper, 2009).

Most recently, several studies have pinpointed dramatic effects of SFB colonization based on their presence in some host species and not in others. A major indication of SFB's role in the induction of pro-inflammatory factors came with the report that mice from differing sources (Taconic Farms versus Jackson Laboratory) had differing levels of IL-17 producing cells and animals that had decreased Th17 cells had increased Foxp3+ regulatory cells (Ivanov et al., 2008). In a subsequent report, the authors were able to show, using 16S rRNA phylochip analysis, that SFB which were present in mice from some sources and not others accounted for the difference seen in Th17 cells. When the authors mono-colonized germ-free mice with SFB, they saw induction of Th17 cells that produce IL-17 and IL-22 in the lamina propria as well as up-regulation of genes associated with inflammation and anti-microbial defenses (Ivanov et al., 2009). In a similar deductive study, Gaboriau-Routhiau et al. noticed that they were able to conventionalize the transcriptome of germ-free mice with a murine microbiota, but not with a human fecal microbiota or cultured murine microbiota. This led them to believe an un-culturable organism must be the missing link to reconstituting conventional level gene responses in their germ-free mice. They deduced that SFB may be the missing link and upon mono-colonizing mice with these organisms, they saw expression of many mucosal genes equal to those of conventional mice including: RegIIIγ, IFNγ, IL-1β, IL-10, IL-17, inducible nitric oxide synthase (iNOS), and IL-12p40. They also saw local induction of IFNγ, IL-10, and IL-17. SFB increased production of IFNγ by CD4+ T-cells, IL-17 production by CD4+ T-cells and increased the total number of CD4+CD25+Foxp3+ T-regulatory cells in the lamina propria of the small intestine and colon (Gaboriau-Routhiau et al., 2009). This induction of both pro-inflammatory and anti-inflammatory factors is extremely interesting; however, in general, SFB have

been more frequently associated with pro-inflammatory Th17 cells (Ivanov et al., 2008, 2009; Wu et al., 2010). Importantly, while SFB were initially suspected to be a sole factor needed to conventionalize mice, none of these factors were increased to conventional levels, indicating that SFB works in concert with other commensal organisms (Gaboriau-Routhiau et al., 2009). Due to their stimulation of the immune system and immediate proximity to the host epithelium, many groups have hypothesized that SFB are involved in CR to host enteric pathogens. Several studies have already shown that in the presence of SFB, hosts are immune to organisms they might otherwise by susceptible to. For example, Heczko et al. (2000) showed that rabbits colonized with SFB were less likely to be infected by rabbit enteropathogenic *Escherichia coli* (REPEC) and conversely, all rabbits who did not have SFB were colonized by REPEC. SFB has also been shown to have protective effects against *Salmonella enteritidis* in rats and *Citrobacter rodentium* in mice (Garland et al., 1982; Ivanov et al., 2009). The culmination of these studies and the observation that SFB are present at a young age strongly supports a role for SFB in CR, possibly selective, during the establishment of a host's intestinal microbiota.

It is well-known that the composition of the microbiota can have strong impacts on health and any imbalance can cause either susceptibility to infection or on the other side can lead to autoinflammatory conditions. Although SFB have been designated as commensal organisms due to their generally non-pathogenic characteristics, it is possible that this classification may be pre-emptive as we learn more about the effects they exert on their hosts. Given SFB's strong pro-inflammatory capabilities, when not reined in by other regulatory factors, their effects could lead to diseased states. For example, recently it was shown that in a mouse model of autoimmune arthritis, for germ-free mice, arthritis is attenuated. Arthritis is quickly re-induced; however, with the addition of solely SFB (Wu et al., 2010). Similar phenomenon have been observed with SFB in EAE (Lee et al., 2011). Alternatively, SFB may exhibit pathogenic qualities in the presence of other bacteria. Work by Stepankova et al. indicates that SFB may require additional microbiota to exert its pro-inflammatory effects. This group showed that SFB could trigger chronic inflammation in SCID mice, which received $CD45RB^{high}$ CD4+ T-cells. However, colitis was only triggered in germ-free SCID mice which received a cocktail of specific pathogen free (SPF) microbiota as well as SFB and not in the mice which received either SPF microbiota or SFB alone (Stepankova et al., 2007).

No studies thus far have looked at the long-term effects of mono-colonization of mice with SFB. We are just uncovering the surface in characterization of these organisms and their mechanisms. While they have been identified in humans through light microscopy (Klaasen et al., 1993), they have not been identified in humans using 16S rRNA sequencing. Understanding the true niche of this organism will help elucidate their role in host biology. Current work is underway to sequence the genome of these species from fecal DNA isolation. In addition, numerous efforts to culture this organism *in vitro* are underway in multiple labs. Armed with the genome sequence and a way to grow these organisms to high numbers in culture, there is potential to understand the molecular mechanisms behind the fascinating SFB interaction with mammalian epithelial cells and the mechanisms responsible for their effects.

CLOSTRIDIUM

While SFB, located primarily in the small intestine of their hosts, induce effector T-cell function and pro-inflammatory conditions, a recent report shows that members of the genus *Clostridium*, most commonly located in the large intestine, do the exact opposite (Atarashi et al., 2011). This is may indicate that the commensal microbiota of the small and large intestine have compartmentalized effects on the resident T-cells. The effect of mixed *Clostridium* species on germ-free mice has been examined previously. In earlier, less controlled studies, the effect of colonizing germ-free mice with chloroform treated or, *Clostridium* rich feces from conventional mice was examined. Under these conditions, normalization of the enlarged germ-free cecum was seen and the mix of 46 *Clostridium* species was defined (Itoh and Mitsuoka, 1980, 1985). These studies were some of the original work to show that *Clostridium* species alone can have a big impact on the intestinal status of a mouse and were part of the initial efforts to tease apart the roles of individual genuses in the intestine. More recent studies build upon those initial efforts by examining specific parameters induced by this defined mix of *Clostridium* species. In the recent study by Atarashi et al., a combination of 46 spore-forming *Clostridium* species mainly composed of clusters IV (*leptum)* and XIVa (*coccoides)* induced a strong anti-inflammatory response in the intestine through expansion of Foxp3+ regulatory T-cells. This effect was partially mediated by the release of TGF-β from IELs. The group showed that pattern recognition receptors such as *Myd88, Rip2*, and *Card9* were not involved. A similar regulatory T-cell accumulation was seen when the *Clostridium* mix was enhanced in normal mice with a healthy immune system and these mice were also more resistant to animal models of inflammation (Atarashi et al., 2011). This response invites the use of *Clostridium* as an anti-inflammatory probiotic. In future studies, it will be interesting to see how individual species of *Clostridium* have an effect on mucosal immunology especially considering the opposite effect exerted by very closely related SFB. Compartmentalized effects of *Clostridium* species and SFB species have been illustrated by the mono-colonization of germ-free mice with SFB or the aforementioned 46 species of *Clostridium* or dual colonized with both. In the SFB mice, αβ IELs and MHC II were increased only in the small intestine while in the *Clostridium* mice, CD8+ T-cells and αβ IELs were increased only in the large intestine. In the co-colonized mice, the mice more closely resembled conventional mice indicating a distinct balance and localization of the effects of each group of species (Umesaki et al., 1999).

LACTOBACILLUS AND BIFIDOBACTERIUM

The beneficial health effects of the endogenous intestinal bacterial genera *Lactobacillus* and *Bifidobacterium* are reflected through their frequent use as probiotics. Species within these bacterial genera have anti-inflammatory properties as well as many other health benefits for hosts such as a contribution toward CR against pathogens, and aid in improved digestion, nutrient adsorption, and increased availability of nutrients in the intestine (Sanchez et al., 2010; Turpin et al., 2010). The genomes of *Bifidobacterium*

species reflect a large propensity for carbohydrate uptake and metabolism as well as the presence of many enzymes for the break-down of complex carbohydrates. These traits are thought to give *Bifidobacterium* species a competitive advantage within the intestine (Schell et al., 2002; Ryan et al., 2005; Kim et al., 2009). Meanwhile, *Lactobacillus* species encode numerous transporters and have a large capacity for sugar internalization and break-down as well as numerous mucus binding cell surface proteins (Kleerebezem et al., 2003; Boekhorst et al., 2006a,b; Siezen et al., 2006). *Bifidobacterium* were originally isolated from human baby feces and were identified as a substantial portion of the normal microbiota of humans. Their positive effects were seen through bottle fed babies that lacked *Bifidobacterium* and subsequently suffered from more diarrhea (Kleerebezem and Vaughan, 2009). Both *Bifidobacterium* and *Lactobacillus* species are among the first subsets of bacteria to colonize the human colon after birth and decrease in number into adulthood (Favier et al., 2003; Vaughan et al., 2005). Much of the characterization of *Bifidobacterium* and *Lactobacillus* and their effects on the mammalian host has come through the mono and co-colonization of germ-free mice and observation of immune and physiological responses from the host as well as bacterial transcriptome changes as they adapted to different niches within a host (Sonnenburg et al., 2006; Denou et al., 2007, 2008; Menard et al., 2008; Kleerebezem and Vaughan, 2009) and also through their probiotic effects on humans (Ouwehand, 2007; Kleerebezem and Vaughan, 2009). Among the many positive effects these groups of bacteria have on hosts is the ability to reduce inflammation. Skewed levels of microbiota are one important factor in inflammatory bowel disease as well as other inflammatory conditions like rheumatoid arthritis (Frank et al., 2007; Gueimonde et al., 2007; Round and Mazmanian, 2009). In mouse models of colitis, under germ-free conditions or after treatment of mice with antibiotics, intestinal inflammation cannot be readily induced (Bamias et al., 2002; Strober et al., 2002). *Bifidobacterium* and *Lactobacillus* are both important in the natural balance of the intestinal community and in cases of inflammatory bowel disease (IBD), both groups of bacteria are seen at decreased levels in fecal samples as opposed to *Enterococcus* and *Bacteroides*, which are seen elevated in the mucosa of patients (Frank et al., 2007). Both *Bifidobacterium lactis* and *Bifidobacterium infantis* have been shown to be protective against inflammation caused by chemically and *Salmonella* induced colitis respectively (Round and Mazmanian, 2009). Both species of bacteria can suppress the transcription of the inflammatory factors: IL-1β, tumor necrosis factor (TNF)-α, NFκB and translation of IL-1β and IL-6 (Turpin et al., 2010). Treatment of colitic mice with *Bifidobacterium infantis* induces the production of CD4+CD25+ regulatory T-cells and these cells can be adoptively transferred to another mouse and prevent activation of inflammatory factors (O'Mahony et al., 2008). Additionally, numerous species of *Lactobacillus* have exerted protective effects against chemically and *IL-10*−/− induced models of colitis (Round and Mazmanian, 2009). It has been suggested that *Lactobacillus rhamnosus* can also induce regulatory T-cell activity. Bone marrow dendritic cells (BMDCs) incubated with *Lactobacillus rhamnosus* offer protection from induction of intestinal inflammation in a CD4+CD25+ regulatory T-cells dependent fashion (Foligne et al., 2007).

In addition to their anti-inflammatory properties, *Bifidobacterium* and *Lactobacillus* species have been shown to play a role in exclusion of enteric pathogens. For example, the inflammatory effects of disease seen after infection of mice with *Salmonella* serotype Typhimurium can be countered by treating the mice with *Bifidobacterium infantis* through the induction of Foxp3+ T-regulatory cells (O'Mahony et al., 2008).

While the precise mechanisms behind the beneficial effects of *Lactobacillus* and *Bifidobacterium* are largely unknown, a significant amount of their activity can be attributed to cell surface associated structures and extracellular protein interaction with mucosal immune cells (Kleerebezem et al., 2010). Such cell surface structures include but are not limited to: exopolysaccharides, bacteriocins, lipoteichoic acid, and extracellular proteins (Sanchez et al., 2010). Many of these proteins from both *Bifidobacterium* and *Lactobacillus* are primarily identified using bioinformatics and most have yet to be fully characterized. For example, *Lactobacillus plantarum* are capable of adhering to mannosyl moieties on human mucosa and in doing so prevent ETEC infection (Adlerberth et al., 1996); however, the responsible mannose specific adhesion (Msa), a sortase dependent cell surface protein was only recently discovered thanks to bioinformatics (Pretzer et al., 2005). Informatics searches for potential adhesions, mucin binding domains, and secretory sequences have been very successful (Buck et al., 2005; Boekhorst et al., 2006a; Sanchez et al., 2008; Barinov et al., 2009). Secreted surface molecules have been shown to play a role in *Bifidobacterium* and *Lactobacillus* CR through the enhancement of the mucosal barrier and tight junctions, induction of anti-microbial peptides, and some secreted proteins are thought to interact directly with host epithelial cells possibly blocking niches for pathogenic bacteria (Sanchez et al., 2010). Schlee et al. (2008) showed that numerous species of *Lactobacillus* are able to induce anti-microbial peptide production, which in turn contributes to CR of pathogens. Pre-conditioned media from *Lactobacillus rhamnosus* GG contains peptides with anti-microbial activity against: *E. coli* EAEC, *Salmonella typhi* and *Staphylococcus aereus* (Lu et al., 2009). Anti-microbial peptide production by *Lactobacillus salivarius* can protect mice from *Listeria monocytogenes* while non-bacteriocin producing *Lactobacillus salivarius* do not confer protection (Corr et al., 2007). The *Lactobacillus crispatus* S-layer protein (SlpA) interacts directly with collagen on host epithelial cells (Antikainen et al., 2002) and has been shown to block the binding of pathogens such as *Escherichia coli* O157:H7 and *Salmonella typhimurium* (Chen et al., 2007), indicating that the CR ability of some probiotics may be directly mediated by adhesion molecules. As well as playing a role in CR, SlpA was shown by Konstantinov et al. to play a role in the induction of host immune responses. They showed that SlpA interacts directly with the non-integrin DC-SIGN, inducing IL-10 production and low IL-12p70 and that in an *slpA* mutant strain, which overexpresses *slpB*, the immune reaction is skewed toward a more pro-inflammatory response (Konstantinov et al., 2008). Another extracellular protein immune modulator is the serine protease inhibitor (Serpin) present in many species of *Bifidobacterium*. Ivanov et al. (2006) showed through a series of *in vitro* studies that serpin could inhibit pancreatic neutrophil elastases, thereby modulating host inflammatory responses. *Bifidobacterium* can play a

role in mucosal health partially through strengthening of tight junctions. Ewaschuck et al. have shown that *Bifidobacterium infantis* pre-conditioned media (BiCM) can increase production of epithelial cell tight junction proteins and increase transepithelial resistance (TER). This BiCm also had drastic effects *in vivo*, attenuating inflammation and colonic permeability in IL-10 deficient mice in part mediated through the Mapk pathway (Ewaschuk et al., 2008). Extracellular proteins secreted by *Lactobacillus* species also play a role in mucosal barrier maintenance through MapKs. Two of the better characterized *Lactobacillus* extracellular proteins are p40 (a hypothetical cell surface antigen) and p75 (a hypothetical cell wall-associated hydrolase). These proteins, when purified, promoted growth in human and murine colonic epithelial cells through activating protein kinase (akt) and were able to reduce TNF-α induced colonic injury in tissue explants (Yan et al., 2007; Seth et al., 2008).

While many *Lactobacillus* and *Bifidobacterium* species are considered culturable, there are still many uncultured species of each within the intestine indicating that we still have much to discover about *Bifidobacterium* and *Lactobacillus* mechanisms of action and surface molecules (Heilig et al., 2002; Ben-Amor et al., 2005). Also, while many extracellular and secreted proteins from *Bifidobacterium* and *Lactobacillus* have been characterized *in vitro*, their roles have yet to be confirmed *in vivo* (Kleerebezem et al., 2010). Full characterization of secreted and surface proteins from these groups of bacteria could further advance therapeutics in intestinal diseases and our knowledge of immune regulation by commensal bacteria.

DISCUSSION

In this review, we have highlighted several bacterial groups and specific species that have an immunomodulatory impact on their hosts (summarized in **Figure 1**). There are an incredible 10^{14} bacteria in the intestine and the mammalian immune system must be able to sustain these constant visitors without eliciting a strong reaction, yet at the same time, be primed to react to incoming and invading pathogens. We have described several different instances in which intestinal bacteria prime responses that mirror and enhance this vital balance by either promoting inflammatory (SFB and Th17 cells) or anti-inflammatory conditions (*Clostridium, Bacteroides fragilis, Bifidobacterium, and Lactobacillus*).

It is interesting that closely related groups of bacteria such as *Clostridium* and SFB can exert such different effects. This is also seen in the case of the pathogen *Clostridium difficile*. These differing effects could possibly be due to uncharacterized effector molecules present on the surface of SFB species versus *Clostridium*. The effects of *B. fragilis* on host immunology are clearly shown to be mediated through the symbiosis factor PSA, which has been well-characterized over the last two decades. Not as much is known about the active molecules from many other symbiotic bacteria. Discovery and characterization of these molecules will be extremely important for fully understanding immune system–bacterial cell interactions. In the case of SFB, the potential molecules that allow its association with epithelial cells and possible effector proteins or carbohydrates that elicit host immune responses from SFB are tantalizing. One of the proposed mechanisms of homeostasis within the intestine that allows residence of bacteria without a hyper-activated immune response is the sequestration of commensal bacteria in the mucus layer of epithelial cells and in the intestinal lumen (Hooper, 2009). SFB clearly break this rule. Actin accumulation by eukaryotic epithelial cells has been seen underneath the attachment points (Jepson et al., 1993) of SFB to eukaryotic cells. This is highly reminiscent of the manner in which many enteric pathogens such as enterohemorrhagic *E. coli* attach to host cells. These pathogens contain a secretion system that enable them to "inject" effector proteins into host cells (Coburn et al., 2007). Is it possible that SFB may have a similar system of their own? This also begs the question as to whether SFB truly are commensal, non-pathogenic organisms. The ability to grow these organisms *in vitro* as well as obtaining the genome sequence of these organisms will allow for studies that will begin to answer these questions.

While we have merely scratched the surface and new roles of different species are continually being discovered, the studies highlighted here give us an idea of how powerful the effects of single bacterial species can be within the intestinal framework. Given an environment of over 500 species, it is remarkable that individual species could have such strong effects. Although

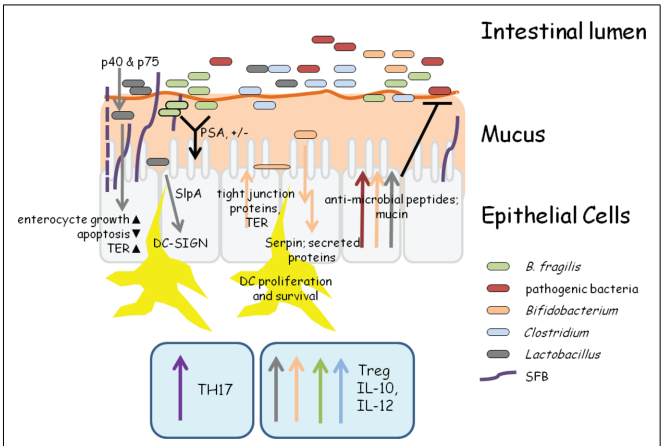

FIGURE 1 | Snapshot of bacterial–host activity in the intestine. This extremely simplistic view of intestinal activity highlights some of the major roles of different bacteria within the intestine. SFB attach to epithelial cells, induce pro-inflammatory responses, and expand TH17 cells (Davis and Savage, 1974; Gaboriau-Routhiau et al., 2009; Ivanov and Littman, 2010). *Bacteroides fragilis* and many *Clostridium* species induce IL-10 production and the expansion of T-regulatory cells (Mazmanian et al., 2005,2008; Atarashi et al., 2011). In *B. fragilis*, this is mediated through the surface polysaccharide, PSA (Mazmanian et al., 2008). Both *Lactobacillus* and *Bifidobacterium* can induce anti-inflammatory cytokine production, anti-microbial peptide, and mucin production, and may adhere to epithelial cells (Adlerberth et al., 1996; Pretzer et al., 2005; Kleerebezem et al., 2010; Sanchez et al., 2010; Turpin et al., 2010). The secreted proteins, p40 and p75 from many *Lactobacillus* species promote cell growth through a PI-3K and AKT pathway, inhibit apoptosis by causing decreased TNFα levels, and increase transepithelial resistance (TER) through increased tight junction protein production (Yan et al., 2007; Seth et al., 2008). *Lactobacillus* S-layer protein A (SlpA) binds to DC-SIGN which leads to increased IL-10 production. SlpA can bind directly to epithelial cells, which may play a role in colonization resistance (CR) against pathogenic bacteria (Antikainen et al., 2002; Chen et al., 2007). The *Bifidobacterium* serine protease inhibitor Serpin inhibits neutrophil elastase, thereby modulating acute inflammation in the intestine (Ivanov et al., 2006). Finally, undefined secreted proteins from *Bifidobacterium* species cause an increase in tight junction protein production and thereby TER, contributing to CR (Sanchez et al., 2010).

mono-colonization of germ-free mice is an excellent way to observe species specific immune modulation, it is also an extremely simplified view of an extremely complex ecosystem and as additional roles of individual species are discovered, investigating how bacteria interact with each other within the intestine will be the next direction. Taken from the simple environment of mono-colonization, it is hard to believe that the roles of bacteria would remain the same once placed in a melting pot of hundreds of species all competing for nutrients and space. However in many cases, such as with *Clostridium*, *Bifidobacterium*, *Lactobacillus*, SFB, and *Bacteroides*, the effects of these bacteria can be seen when their populations are increased within a conventional animal, not solely within a mono-colonized animal (Dasgupta et al., unpublished data) (Sonnenburg et al., 2005, 2006; Mazmanian et al., 2005, 2008; Stepankova et al., 2007; Kleerebezem and Vaughan, 2009; Round and Mazmanian, 2010). In some cases, such as SFB, immunomodulatory effects are seen more readily in the context of a complete microbiota rather than a mono-colonized animal (Stepankova et al., 2007). With such great success at teasing apart the individual contributions of many resident intestinal bacteria through mono-colonization experiments, another body of information will come from determining dynamic interactions between multiple groups of a host's natural inhabitants and trying to determine how they use symbiosis factors to communicate with not only their host tissues, but also each other. Already, many groups have looked at the effects of colonizing mice with a select microbiota that represents many of the abundant species normally present in the mammalian intestine (Hooper et al., 2001; Macpherson and Harris, 2004; Sonnenburg et al., 2006; Round and Mazmanian, 2009; Stecher and Hardt, 2011).

As described earlier, SFB from a rat cannot attach to the epithelium of mouse and vice versa (Tannock et al., 1984; Hooper et al., 2001; Macpherson and Harris, 2004; Sonnenburg et al., 2006). This is one example illustrating that colonization of germ-free animals by any bacteria is not always sufficient to conventionalize the animal. Rather, specific bacterial colonization is necessary. This implies a co-evolutionary relationship between a host and its microbiota. The study of humanized mice (germ-free mice colonized with human fecal samples) is one tool that has been utilized to answer whether hosts require a host-specific microbiota. While humanized mice have a "complete" microbiota, it is foreign. At least two studies have shown that humanized mice may more closely resemble germ-free mice in many immunological traits than conventionalized mice and that colonizing mice with a foreign microbiota cannot completely restore immune defects seen in germ-free mice, nor can it restore many other germ-free defects such as metabolism (Chung et al. submitted; Gaboriau-Routhiau et al., 2009). When comparing the composition of microbiota from mice colonized with human fecal matter and mice colonized with mouse cecal matter, the two groups of microbiota had a high degree of resemblance through the genus level. The majority of differences were seen on the species level (Chung et al. submitted) again emphasizing the dramatic effects on the host carried out by individual species.

The interest in the dynamics of microbes in the intestine has existed for decades, but has gained and lost momentum as new technology comes and goes. As we are starting to see definite trends in the composition of the intestine, gain knowledge through sequencing, and identify the diversity of intestinal bacteria through the Microbiome project, we are in a position to understand the gut like we never have before. This knowledge will expand our understanding of bacterial symbiosis factors, host regulation of the commensal microbiota, and likewise bacteria–host communication and bacterial–bacterial communication within the intestine. This will lead to a better understanding of and characterization of intestinal bacterial imbalances that lead to diseased states, giving us a better grasp of this previously mysterious world and the tools to greatly impact intestinal health in the future.

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The gut microbiota and mucosal T cells

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It is intuitive that immune cells in the gut may require microbiota-derived cues for their differentiation. The proximity between host and microbe in the intestine would seemingly necessitate co-adaptation. However, it has been challenging to determine the members and features of the gut microbiota that influence immune system development and function. The recent identification of immunomodulatory members of the commensal microbiota is providing insight into the dependence of select, intestinal immune cell subsets on specific microbial species. In this review, we focus on the gut microbiota's influence on the development and function of mucosal T cells subsets, specifically intraepithelial lymphocytes and lamina propria CD4 T cells.

Keywords: gut microbiota, T cells, mucosal immunity

OVERVIEW

The mucosal immune system of the intestinal tract faces the challenge of co-existing with a diverse and dynamic community of microbes while remaining poised to protect and defend against invasive pathogens. This microbiota is an active participant in host metabolism and also provides critical developmental cues for the immune system. Studies from animals models lacking all microbes, referred to as germ-free, have established that gut microbes are crucial for the development, maturation, and function of the intestinal immune system and many aspects of systemic immunity. Mucosal T cells that reside within the epithelial cell layer and the lamina propria (LP) of the intestine are especially reliant on direct and indirect microbial signals for their proper differentiation and function. Recently a few species, *Bacteroides fragilis*, *Candidatus* Arthromitus sp. segmented filamentous bacteria (SFB), and *Clostridium* spp. from Clostridium Clusters III, IV, and XIVa have been identified that drive both effector and/or regulatory mucosal T cell maturation. In this review, we provide an introduction to mucosal T cell subsets, specifically intraepithelial lymphocytes and LP CD4 T cells, with a focus on the gut microbiota's influence on their development and function.

INTRAEPITHELIAL T LYMPHOCYTES

Below the tight junctions that join the plasma membranes of epithelial cells and residing above the basement membrane, are specialized subsets of gut T cells aptly named intraepithelial cell lymphocytes (IELs; **Figure 1**). IELs promote barrier repair, rapidly accumulate at sites of injury and infection, and defend against intestinal pathogens, such as *Eimeria vermiformis* (Roberts et al., 1996) and *Salmonella* spp. (Dalton et al., 2006). The majority of IELs express the CD8 glycoprotein, that binds MHC class I molecules (van Wijk and Cheroutre, 2009). IELs are categorized by the two chains that compose their T cell receptor: either alpha beta ($\alpha\beta$) or gamma delta ($\gamma\delta$) heterodimers. $\alpha\beta$ IELs are primarily derived in the thymus and migrate into the intestine after development (Sheridan and Lefrancois, 2010). In contrast, $\gamma\delta$ T cells are derived extrathymically and while they constitute a small fraction of T cells in peripheral lymph nodes and the spleen, 50% of IELs in mice express $\gamma\delta$. In humans, 15% of IELs in the small intestine (SI) and upward of 40% in the colon are $\gamma\delta$ positive (Kagnoff, 1998). In response to bacteria or intestinal injury, $\gamma\delta$ IELs produce proinflammatory cytokines and chemokines that recruit neutrophils, eosinophils, and T cells. $\gamma\delta$ IELs also promote epithelial healing via production of keratinocyte growth factor, which stimulates epithelial cell proliferation and restoration of barrier function (Yang et al., 2004).

Despite the important role of $\gamma\delta$ IELs in the intestine and their close proximity to luminal and mucosal microbes, relatively little was known about their regulation by the gut microbiota until recently. Because of their small absolute numbers and propensity for apoptosis when cultured *ex vivo*, $\gamma\delta$ IELs have proven a challenging cell subset to study. The application of laser capture microdissection to the study of $\gamma\delta$ IELs by the Hooper laboratory has provided a major advance for investigating this cell subset (Ismail et al., 2009). While germ-free (GF) mice have equal numbers of $\gamma\delta$ IELs compared with conventionally raised mice (Bandeira et al., 1990), data have emerged on the significant role of the gut microbiota on $\gamma\delta$ IEL function. Ismail et al. (2009) have begun to unravel an elaborate and dynamic cross-talk between commensal bacteria and $\gamma\delta$ IELs during mucosal injury that both promotes wound healing and prevents invasion by opportunistic pathogens. By comparing the transcriptional profiles of colonic $\gamma\delta$ IELs isolated from conventional and GF mice both prior to and following injury with the mucosal disruptant, dextran sulfate sodium (DSS), Ismail et al. (2009) identified key effectors in the $\gamma\delta$ IEL microbiota-dependent response to injury. While upregulation of lysozyme was a microbiota-independent function of IELs, the

induction of numerous chemokines (including KC and MIP2α), proinflammatory cytokines (e.g., IL-1β), and the bactericidal lectin RegIIIγ was microbiota-dependent. Importantly, colonization of GF mice with a specific pathogen-free (SPF) microbiota restored γδ IELs' ability to promote wound healing. Furthermore, γδ IELs were also critical to protect against bacterial invasion immediately following intestinal injury as increased numbers of bacteria were detected in mesenteric lymph nodes in mice deficient in IELs (Ismail et al., 2009).

γδ IELs exhibit a multifaceted ability to promote intestinal homeostasis by responding directly to signals from the microbiota. The response of γδ IELs to gut microbes is only partially dependent on TLR signaling. Thus, it will be interesting to determine the other receptors, microbiota associated molecular patterns and specific bacterial species involved in orchestrating the IEL immune response to mucosal injury. Given that γδ IELs produce cytokines, such as IL-1β and TGFβ, that induce expansion of both antimicrobial and anti-inflammatory LP CD4 T cell subsets, respectively, it will also be interesting to determine how cross-talk between these two populations regulates intestinal homeostasis following injury and subsequent bacterial invasion.

LAMINA PROPRIA CD4 T LYMPHOCYTES

Beneath the epithelial cell layer's basement membrane is the LP region of the mucosa. The LP of the small and large intestine is home to 70–80% of the body's immune cells (Furness et al., 1999). As opposed to the intraepithelial lymphoid compartment, populated with CD8 T cells, the LP is dominated by T cells expressing the CD4 glycoprotein, which binds MHC class II. While there are CD8 αβ and γδ LP populations, we focus on the many helper and regulatory CD4 T cells that reside in the LP and the evolving understanding of their relationship with gut microbiota.

Helper CD4 T cells (Th) represent a diverse collection of subsets with specific cytokines and chemokine profiles that result in activation and growth of cytotoxic T cells, promotion of myeloid cell bactericidal activity, and B cell differentiation and antibody production. In general, T helper type 1 produce interferon-γ, TNF-α, and IL-12 and participate in host defense against intracellular pathogens; T helper type 2 produce IL-10, IL-13, IL-5, and IL-4 and defend against helminths; and T helper 17 produce IL-17, IL-21, and IL-22 and afford protection against extracellular bacteria and parasites. Interactions between LP CD4 T cells and the gut microbiota, often with epithelial cells or dendritic cells functioning as the intermediaries, are critical for shaping the adaptive immune response in the intestine (**Figure 2**).

In healthy individuals all of these Th subsets are present in the LP and exist in a dynamic balance with regulatory CD4 T cells. Regulatory T cells also have many subsets especially in the gut. The environmental exposures (dietary components, resident intestinal microbes, transiently ingested microbes, and invasive pathogens) that the gut continuously encounters necessitate a nimble, diverse, and versatile functional potential of the adaptive T lymphoid compartment. There is also an increasing understanding of the functional plasticity across CD4 subsets, especially for regulatory subsets and Th17 cells (Lee et al., 2009).

Both the gut and systemic immune system receive critical developmental cues from the intestinal microbiota. For example, GF rodents have marked abnormalities in mesenteric lymph nodes, peyer's patches, and splenic architecture (Smith et al., 2007). In addition to impacting lymphoid structures and cell numbers, the microbiota influence lymphoid function under steady state conditions and in response to injury without any direct physical interaction. Surveys of the gut bacterial communities are revealing that there are alterations in the microbiota in both system and gut-localized allergic and inflammatory disease (Cerf-Bensussan and Gaboriau-Routhiau, 2010). As T cells play critical effector roles in diseases like asthma, inflammatory bowel disease, type 1 diabetes, and multiple sclerosis, it is of great import to unravel the cause and effect relationships between the gut microbiota and LP T cell responses in allergic and autoimmune diseases.

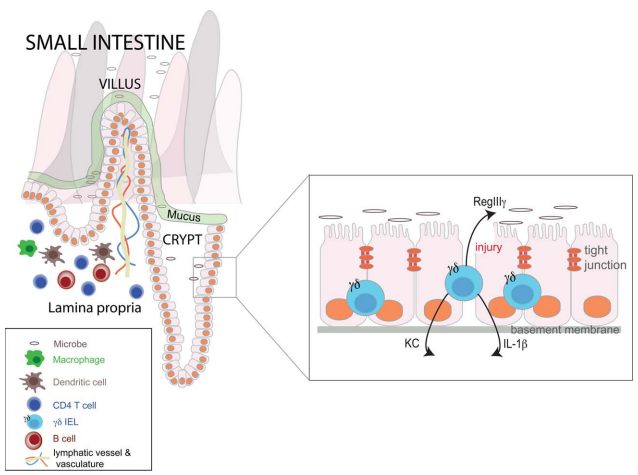

FIGURE 1 | A schema of the small intestinal mucosa. The crypt-villus axis of the small intestine is illustrated, as are its key immune subsets. Inset is an expanded view of the epithelium highlighting the γδ intraepithelial lymphocytes and several of the microbiota-dependent factors they produce in response to injury.

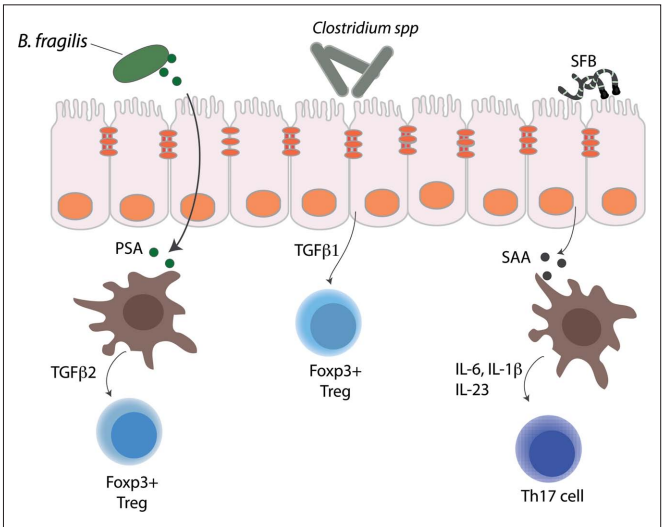

FIGURE 2 | Immunomodulatory members of the gut microbiota that regulate lamina propria CD4 T cell subsets. *Bacteroides fragilis,* segmented filamentous bacteria, and *Clostridium* spp. are depicted along with the mechanisms by which they influence lamina propria CD4 T cell subsets.

GUT MICROBIOTA AND Th1–Th2 CELL EQUILIBRIUM

T helper type 1 (Th1) and type 2 (Th2) balance has been a useful paradigm facilitating understanding of both how the immune system executes cellular and humoral immune responses and how imbalanced and over-exuberant responses underlie arthritis, type 1 diabetes and multiple sclerosis in the case of Th1 immunity or asthma and allergic diseases in the case of Th2 immunity (Mosmann et al., 1986; Zhu et al., 2010). With the discovery of other T helper subsets, e.g., T helper 17 and T helper 9 and also regulatory subsets, the binary model of Th1 vs. Th2 is overly simplistic. However, early focus on these subsets during development and in GF mice was instrumental in understanding how the microbiota influence immune cell development. During the neonatal period; the immature fetal immune system is biased toward a Th2 phenotype, which if not corrected predisposes rodents and humans to infections, allergic and inflammatory diseases (Wilson, 1986; Holt, 1995). Prior to weaning, rats are skewed toward a Th2 biased phenotype in the spleen and mesenteric lymph node and lack basic Th1 cell mediated functions. These defects can be corrected by a lyophilized combination of bacteria including *Staphylococcus*, *Streptococcus*, and *Klebsiella* strains (Bowman and Holt, 2001). Recent studies in lymphopenic mice have provided mechanistic insight demonstrating that TLR ligands derived from the intestinal microbiota drive spontaneous T cell proliferation via innate cell production of IL-6 (Feng et al., 2010). These data suggest that exposure to bacteria may be necessary to drive physiologic Th1 cell development. Subsequent studies have revealed that GF mice have reduced numbers of Th1 cells in both systemic and mucosal compartments (Smith et al., 2007). This reduction in basal Th1 immunity contributes to susceptibility to infection and morbidity with bacterial pathogens, including *Shigella flexneri* and *Listeria monocytogenes* (Round and Mazmanian, 2009). These early studies focused on Th1 and Th2 cells established an important role for the gut microbiota in CD4 T cell development systemically and within the gut.

While the human gut is home to trillions of bacteria cells representing a thousand species, *B. fragilis* was the first bacterial species identified that corrected the Th1 and Th2 cell imbalances observed in non-gut lymphoid tissues in GF mice. *B. fragilis* is a Gram-negative symbiont that inhabits the lower intestinal tract and colonizes an estimated 30–70% of humans (Sears and Pardoll, 2011). Monocolonization of GF mice with *B. fragilis* induced overall CD4 T cell expansion and increased numbers of Th1 cells to levels similar to those of conventionally raised mice. Mechanistically, the effect of *B. fragilis* was entirely dependent on a zwitterionic capsular polysaccharide, polysaccharide A (PSA; Mazmanian et al., 2005). Exposure to PSA *in vivo* and *in vitro* was sufficient to induce Th1 expansion and cytokine production and GF mice colonized with *B. fragilis* lacking PSA failed to correct their Th1/Th2 imbalance. As all humans and mice do not necessarily have *B. fragilis* in their gut, the intestinal microbiota undoubtedly harbors other symbiotic factors that facilitate systemic and intestinal Th1 cell development yet to be discovered.

GUT MICROBIOTA AND Th17 CELLS

Early studies aimed at understanding how the gut microbiota affect LP CD4 T cell populations were mainly focused on the restoration of Th1 responses; more recent work has shed light on how commensal bacteria also affect the development of Th17 cells. Th17 cells were identified as major mediators of pathology in several autoimmune and inflammatory disease models; however, Th17 cells play an important role in host defense against extracellular bacteria and parasites (Korn et al., 2009). Th17 cells are found both in the LP of the SI and the colon and have an established role from studies in mouse models in defense against *Shigella flexneri* and *Citrobacter rodentium* (Ivanov et al., 2009; Sellge et al., 2010). Similar to findings with LP Th1 cells, Th17 cell numbers in the gut are also substantially reduced in GF mice, illustrating their dependence on the gut microbiota. An astute observation about marked differences in LP Th17 cells between mice obtained from different commercial suppliers of laboratory animals garnered the attention of Dan Littman's laboratory and led to their observation that the composition of the microbiota may be an important factor in inducing LP Th17 cells. In contrast with Th1 cells, colonization of GF mice with "any old" SPF microbiota was not enough to drive the development of LP Th17 cells, suggesting that particular bacterial species were required for Th17 cell development. The laboratories of Nadine Cerf-Bensussan and Dan Littman identified a role for SFB in driving LP Th17 cell development (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). SFB are well known to mucosal immunologists interested in the role of microbiota and immune system development and have a well-established role in intestinal IgA and T cell responses (Talham et al., 1999). Colonization of GF mice with a SFB strain led to an increase in Th17 cell numbers, which restricted the growth and invasive capacity of *Citrobacter rodentium* providing a mechanism by which SFB can prevent invasion by opportunistic bacteria (Ivanov et al., 2009).

Many questions still remain about how SFB drive gut Th17 cell development. Transcriptional profiling of mice colonized with SFB identified a substantial increase in the acute phase response protein serum amyloid A (SAA). SAA induced dendritic cells to produce the cytokines IL-6 and IL-23, both of which are important for Th17 development (Korn et al., 2009). How SFBs mediate this effect is unclear, however, electron micrographs suggest that SFB are in close association with the epithelium of the terminal ileum (Klaasen et al., 1992). Although some intestinal dendritic cells can extend processes into the intestinal lumen (Chieppa et al., 2006), whether SFBs interact directly with dendritic cells or with epithelial cells as go-betweens remains unclear. A striking feature of SFBs, seen on some electron micrographs, are their terminal buds (tear-drop protuberances) that occur at attachment sites with the epithelium where the SFBs appear to perturb but not penetrate host epithelial cell membranes (Klaasen et al., 1992; Ivanov et al., 2009). The whole genome sequence of the strain used in Ivanov et al. (2009) will likely prove quite informative in facilitating understanding of the host–microbiota interactions mediated by SFBs.

Bacteroides fragilis and SFBs are but a mere two out of the thousands of species populating the mouse and human intestinal tracts. The identification of these bacteria raises many questions about microbiota-immune system co-evolution and adaptation. Have only a select number of gut microbes evolved immunomodulatory potential and can a few species modulate both helper and regulatory CD4 T cell behavior or have several bacterial species (or genera) evolved different effectors for different subsets? Who are the others and once identified, what are their immunomodulatory

molecules? Are immunomodulatory species equally effective for all individuals (mouse or human) and what host genes and signaling pathways are required for these bacterial-derived signals? Some of these questions have been answered for *B. fragilis*, which also appears to modulate immunity in both mice and humans, but much remains to be discovered.

LAMINA PROPRIA T REGULATORY CELLS

An important mechanism for maintaining intestinal homeostasis is the presence of CD4 regulatory T cells (Tregs) in the intestine. Regulatory CD4 T cells are important response modulators especially in the gut which is subject to: high luminal resident bacterial loads, incursions from pathogens, changing dietary components and also Th cells which require tuning of the intensity and duration of their inflammatory responses so that chronic inflammation does not ensue.

T regulatory cell subsets

There are two main subsets of CD4 Tregs present in the gut, naturally occurring Tregs that develop in the thymus before trafficking to other sites in the body and inducible Tregs that develop in the periphery (Feuerer et al., 2009). The transcription factor Foxp3 has been defined as a master regulator of Tregs and drives both differentiation and suppressive functions. However, there is also evidence, especially in the intestine, of Tregs that do not express Foxp3. Tregs typically are able to exert their effects through dampening inflammatory responses by secreting anti-inflammatory cytokines, such as IL-10 (Maynard and Weaver, 2008). The importance of IL-10 as an inflammatory damper is evidenced by the spontaneous intestinal inflammation observed in IL-10 knock-out mice (Powrie and Leach, 1995). Interestingly, neither GF *IL-10*$^{-/-}$ nor SPF *IL-10*$^{-/-}$ mice that are not colonized with *Helicobacter* spp. (Kullberg et al., 1998) develop spontaneous colitis. These observations have helped to establish the important role for IL-10 in the maintenance of homeostasis between the host and its gut microbiota.

Gut microbiota and T regulatory cells

Recent studies have identified a key role for the gut microbiota in the development of intestinal Tregs. The majority of Foxp3+ intestinal Tregs are found in the LP of the SI and colon (Atarashi et al., 2011b). Studies by the laboratory of Kenya Honda have revealed that in SPF mice the frequency of Foxp3+ Tregs in the SI and colon increased after weaning, whereas peripheral Treg populations remained stable (Atarashi et al., 2011a). Comparison of SPF mice to GF mice demonstrated that while these mice have similar numbers of Tregs in the SI, GF mice have a significantly lower percentage of CD4+Foxp3+ Tregs in the colon. Furthermore, antibiotic treated mice also had significantly reduced numbers of Tregs in the colonic LP but not the SI. These findings imply that accumulation of Tregs in the colon and SI is differentially regulated and the colonic Treg differentiation is more heavily influenced by the microbiota (Atarashi et al., 2011b). Furthermore, these observations correlate with the increased number of luminal microbes in the colon vs. the SI and may suggest that inducible Tregs and the density and spatial organization of bacteria in the intestine may have coadapted.

Use of GF mice has been particularly helpful in deconstructing how single organisms and defined bacterial communities are able to instruct Treg responses. Recent work has shown that colonization of GF mice with either a single bacterial species or collection of bacterial strains can induce the differentiation of Tregs in the colon. In addition to its effects on systemic Th1 cells, *B. fragilis* and its immunomodulatory molecule, PSA, are capable of driving Treg differentiation (Round and Mazmanian, 2010). Monocolonization of GF mice with *B. fragilis* resulted in increased T cell expression of Foxp3+ and IL-10 in the colon, which was entirely dependent on PSA (Round and Mazmanian, 2010). Furthermore, treatment of T cells with PSA induced the development of functional Foxp3+ Tregs with suppressor activity that was likely dependent on IL-10. Importantly, PSA induced Foxp3+ Tregs were able to alleviate experimental colitis after onset of symptoms, indicating a potential use in therapeutic interventions (Mazmanian et al., 2008).

Recently *Clostridium*, a genus of Gram-positive bacteria that often are spore formers, have gained attention as potent inducers of gut Tregs. Investigations from the laboratory of Kenya Honda showed that while GF mice had markedly reduced colonic LP Foxp3+ Treg numbers, colonization with a collection of 46 *Clostridium* species restored Treg levels to that of conventionally raised mice (Atarashi et al., 2011a). There was a spatial correlation between colonic Treg distribution and the *Clostridium* strains along the intestine as both reach their highest density in the proximal colon (Atarashi et al., 2011a). Interestingly, the majority of Foxp3+IL-10+ Tregs induced by *Clostridium* were negative for Helios, which is a marker of natural (thymic-born) Tregs (Thornton et al., 2010). Therefore, the diminished Treg numbers observed in GF mice are likely inducible Tregs. *Clostridium* induced Tregs were functionally robust and afforded protection from intestinal inflammation in SPF mice challenged with the mucosal disruptant DSS (Atarashi et al., 2011a). Both Treg development and Treg-mediated protection from colitis were from the taxonomic *Clostridium* clusters IV and XIVa, which have been observed to have a reduced relative abundance in human inflammatory bowel disease patients compared with healthy controls (Frank et al., 2007). Collectively, these studies suggest that the gut microbiota may contribute to a host's resiliency from mucosa injury mediated by DSS and potentially that the absence of specific groups of bacteria may increase susceptibility to chronic inflammation. In particular, the experiments where *Clostridium* strains provided protection from DSS-mediated colitis raise hope that these strains hold potential as therapeutic agents.

Bacteroides fragilis and Clostridium spp.: separate paths to gut Treg expansion

Bacteroides fragilis and *Clostridium* spp. elicit Foxp3+IL-10+ inducible Tregs through independent mechanisms. *B. fragilis*, through PSA, can act directly on CD4 T cells by inducing production of TGF-β2, which drives Treg differentiation (Round and Mazmanian, 2010). In contrast, *Clostridium* species influence Treg differentiation via epithelial cell-derived TGF-β1 (Atarashi et al., 2011a). There are also differences in the signaling pathways required for Treg development between these two bacteria. Tregs isolated from TLR2 deficient mice failed to expand in response to PSA and produced low levels of IL-10, consistent with a requirement for TLR2 signaling (Round and Mazmanian, 2010). Interestingly, the effect of *Clostridium* was independent of several bacterial associated pattern recognition receptors, and Treg induction occurred in mice deficient in MyD88, Rip2, and Card9-mediated signaling pathways

(Atarashi et al., 2011a). Taken together, this indicates that genera from genetically disparate phyla have evolved distinct mechanisms to influence the development of T cell subsets necessary for host homeostasis. These non-redundant mechanisms afford alternative opportunities for inducible Treg differentiation should genera be temporarily (or permanently) lost secondary to a course of antibiotics or an inflammatory process.

CONCLUSIONS AND FUTURE DIRECTIONS

It is intuitive that immune cells in the gut may depend on microbiota-derived cues for their development. The proximity between host and microbe in the intestine would seemingly necessitate co-adaptation. However, it has been challenging to determine the members and features of the gut microbiota that influence immune system development and function. Animal models, particularly those wherein the microbiota can be manipulated, are revealing how the gut microbiota profoundly influence the development of host intestinal immune responses. The use of GF mice, a tool that is over 50 years old (Reyniers, 1957), is providing fresh insight into the species-selectivity of gut T cell subset development. Much still remains to be understood about interactions of such select species (e.g., SFB and *Clostridium* spp.) with other gut microbial community members and the bacterial-derived signals sensed by the host. More nuanced ways to manipulate complex microbial communities will be essential to unravel host–immune interactions. Transposon-based mutagenesis strategies selectively targeting single organisms within a complex community (Goodman et al., 2009) or dynamic communities designed with auxotrophic mutant members (Hapfelmeier et al., 2010) offer new opportunities to deeply probe host–microbiota interactions and unravel co-adaptation in real time. Regarding the molecular cross-talk between the immune system and microbes, the discovery of the toll-like receptor and nod-like receptor signaling pathways represents a ground-breaking contribution toward understanding host–microbiota interaction. It is unlikely, however, that the dialog between the immune system and gut microbiota is limited to the current list of microbe associated molecular patterns. Gut microbes are efficient metabolic machines and their metabolites merit consideration as immune system cues. Both ATP and short chain fatty acids have emerged as microbiota-derived immune modulators – undoubtedly more bacterial immunomodulatory metabolites will be discovered (Maslowski and Mackay, 2011). Metagenomic surveys of the microbiota are providing vast amounts of information about the membership and function of the microbial world within the gut. Mucosal immunology is still discovering new T cell subsets and innate lymphoid cell lineages in the intestine. Thus, to paraphrase, the time is now and the place is the gut.

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Environmental and gut *Bacteroidetes*: the food connection

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Members of the diverse bacterial phylum *Bacteroidetes* have colonized virtually all types of habitats on Earth. They are among the major members of the microbiota of animals, especially in the gastrointestinal tract, can act as pathogens and are frequently found in soils, oceans and freshwater. In these contrasting ecological niches, *Bacteroidetes* are increasingly regarded as specialists for the degradation of high molecular weight organic matter, i.e., proteins and carbohydrates. This review presents the current knowledge on the role and mechanisms of polysaccharide degradation by *Bacteroidetes* in their respective habitats. The recent sequencing of *Bacteroidetes* genomes confirms the presence of numerous carbohydrate-active enzymes covering a large spectrum of substrates from plant, algal, and animal origin. Comparative genomics reveal specific Polysaccharide Utilization Loci shared between distantly related members of the phylum, either in environmental or gut-associated species. Moreover, *Bacteroidetes* genomes appear to be highly plastic and frequently reorganized through genetic rearrangements, gene duplications and lateral gene transfers (LGT), a feature that could have driven their adaptation to distinct ecological niches. Evidence is accumulating that the nature of the diet shapes the composition of the intestinal microbiota. We address the potential links between gut and environmental bacteria through food consumption. LGT can provide gut bacteria with original sets of utensils to degrade otherwise refractory substrates found in the diet. A more complete understanding of the genetic gateways between food-associated environmental species and intestinal microbial communities sheds new light on the origin and evolution of *Bacteroidetes* as animals' symbionts. It also raises the question as to how the consumption of increasingly hygienic and processed food deprives our microbiota from useful environmental genes and possibly affects our health.

Keywords: *Bacteroidetes*, adaptation to environmental niches, microbiota

THE PHYLUM BACTEROIDETES

The phylum *Bacteroidetes* is a very diverse bacterial phylum, the name of which changed several times over the past years. It is also known as the *Cytophaga–Flexibacter–Bacteroides* (CFB) group, an appellation that reflects the diversity of organisms found in this phylogenetic group (Woese, 1987; Woese et al., 1990). According to the Bergey's Manual of Systematic Bacteriology (Bergey's, 2011), the *Bacteroidetes* phylum comprises four classes: *Bacteroidia*, *Flavobacteria*, *Sphingobacteria*, and *Cytophagia*, representing around 7000 different species (NCBI, October 2010). The largest class is the *Flavobacteria*, grouping together around four times more species than the three others (**Table 1**). These bacteria are all Gram negative, cover a mixture of physiological types, from strictly anaerobic *Bacteroides* to strictly aerobic *Flavobacteria*. They are non-motile, flagellated, or move by gliding.

Members of the phylum *Bacteroidetes* have colonized many different ecological niches, including soil, ocean, freshwater, and the gastrointestinal tract (GIT) of animals, where they display various biological functions. In particular, they are well known degraders of polymeric organic matter. This review describes current knowledge on the role and mechanisms of polysaccharide degradation by *Bacteroidetes* in their respective habitats. We emphasize the features shared by members of the phylum that allow this functional specialization in various environments. We address the links between these different microbial communities through food consumption, which raise the question of the evolution of gut microbes.

BACTEROIDETES IN THE NORMAL MICROBIOTA OF ANIMALS

Microbes that live in and on humans (known as microbiota) can represent up to 100 trillion cells, 10 times more than the eukaryotic stem and somatic cells (Ley et al., 2006b; Turnbaugh et al., 2007). The large majority of these microbes reside in our GIT, and belong either to the *Firmicutes* or *Bacteroidete*s phyla (Marchesi, 2010). In fact, these two bacterial phyla account for >98% of the 16S rRNA sequences detected in the gut microbiota of mammals (Ley et al., 2006b). *Bacteroidetes* have colonized all the different parts of the GIT, despite the different conditions they have to face in terms of, e.g., pH, nutrients, and oxygen availability. Due to their easier accessibility, the mouth and colon microbiota have been the most studied. The large intestine is the most colonized compartment of the GIT, with bacterial densities reaching 10^{11}–10^{12} cells/ml (Whitman et al., 1998). Members of the *Bacteroidetes* phylum are well known colonizers of the colon. They account for about 50% of the 16S rRNA sequences detected from healthy human mucosal tissues (Eckburg et al., 2005). Among this phylum, members of the genus *Bacteroides*

Table 1 | Census of the phylogenetic divisions inside the *Bacteroidetes* phylum (NCBI, October 2010).

Class	No. of families	No. of genera	No. of species
Bacteroidia	5	28	858
Flavobacteria	3	110	3583
Sphingobacteria	3	29	787
Cytophagia	3	47	765
Unclassified strains	–	–	996

are the most abundantly represented in the fecal microbiota (Moore and Holdeman, 1974; Sghir et al., 2000). *Bacteroidetes* have also been found in the normal microbiota of the oral cavity, either in the saliva or dental plaque (Keijser et al., 2008; Nasidze et al., 2009a,b). In the throat, Andersson et al. (2008) found that *Bacteroidetes* account for ~20% of the reads using barcoding pyrosequencing, and identified *Prevotella* genus as the second most represented after *Streptococcus*. The same proportions were found in the distal esophagus (Pei et al., 2004). Due to its low pH, the stomach is a harsh ecological niche, quite different from the other GIT compartments. However, even if *Helicobacter pylori* represents a major part of the stomach microbiota, *Bacteroidetes* were still found to account for 10–20% of the bacteria (Bik et al., 2006), including members of the genera *Prevotella*, *Capnocytophaga*, *Bergeyella*, *Porphyromonas*, and *Tannerella*. In fact, *Prevotella* was the third most abundant genus in the stomach microbiota, after *Helicobacter* and *Streptococcus*.

Bacteroidetes are also encountered in the microbiota of other mammals, such as mice (Dubos et al., 1965; Savage et al., 1968), dogs (Middelbos et al., 2010), pigs (Leser et al., 2002), and ruminants (Tajima et al., 1999; Leng et al., 2010). They appear in the GIT of domesticated and wild birds, such as chickens (Zhu et al., 2002), turkeys (Scupham et al., 2008), goose (Lu et al., 2009), and ostrichs (Matsui et al., 2010). They have been demonstrated as dominant in the microbiota of echinoderms (Balakirev et al., 2008), millipedes (Knapp et al., 2010), and the last compartment of termite gut (Schmitt-Wagner et al., 2003).

The interaction between *Bacteroidetes* and their animal host is now known to be mutualism rather than commensalism since the fitness of both partners is increased (Backhed et al., 2005). Several studies have shown the implication of *Bacteroidetes* for the normal development of the GIT. For example, it has been shown that germ-free animals display an altered GIT compared to conventional ones, leading to changes in morphology and function (see for review, Abrams, 1983). Other contributions of *Bacteroidetes* to the health of their host include interactions with the immune system for the activation of T-cell mediated responses (Mazmanian et al., 2008; Wen et al., 2008), and limitation of the GIT colonization by potential pathogenic bacteria (Mazmanian, 2008). Gut *Bacteroidetes* generally produce butyrate, an end product of colonic fermentation which is thought to have antineoplastic properties and thus plays a role in maintaining a healthy gut (Kim and Milner, 2007). They are also involved in bile acid metabolism and transformation of toxic and/or mutagenic compounds (Smith et al., 2006).

However, their main and most studied biological function as symbionts is the degradation of biopolymers in the large intestine, and in particular polysaccharides. Carbohydrates represent the bulk of normal human and animal diets, thus forming a main source of nutrients for both the host and the microbiota. Mammals can easily absorb simple sugars (e.g., glucose, galactose) via active transport in the proximal small intestine (Ferraris, 2001) or degrade some disaccharides (e.g., lactose, maltose, sucrose) to monomers. They also possess enzymes which allow the degradation of starch to glucose. However, they are generally under-equipped for the degradation of other complex polysaccharides, which are resistant to the action of digestive enzymes and reach the colon relatively intact. The microbiota and especially *Bacteroidetes* are believed to complement eukaryotic genomes with degradation enzymes targeting resistant dietary polymers, such as plant cell wall compounds (e.g., cellulose, pectin, and xylan). *Bacteroidetes* also degrade host-derived carbohydrates, primarily coming from GIT secretions, such as *N*-glycans found in mucins or chondroitin sulfates (Salyers et al., 1977). With this respect, one well studied example is *Bacteroides thetaiotaomicron*, a prominent member of the human microbiota that has been shown to principally forage on host mucin *O*-glycans. This has an impact on their colonization and probably represents an important evolved component of their adaptation to the gut habitat (Martens et al., 2008). The bacteria-mediated fermentation of these food, and host-derived polysaccharides in the colon leads to the release of volatile, short-chain fatty acids (mainly acetate, propionate, and butyrate) that are reabsorbed by the host. Therefore these intestinal bacteria help the host to gain energy from otherwise refractory carbohydrate sources. In herbivorous animals such as ruminants, the diet consists largely of plant cell wall compounds resistant to the host's digestive enzymes. Thus, assimilation of short-chain fatty acids produced by microbial fermentation of polysaccharides can support more than 50% of the total caloric supply (Carroll and Hungate, 1954). In omnivorous mammals, notably humans, this extra-source of energy accounts for 7–10% of the daily allowance (Smith and Bryant, 1979; Hooper et al., 2002). In rats, it has been shown that germ-free animals excrete 87% more calories within the feces than their normal counterparts, and need to ingest 30% more food to maintain their body weight (Wostmann et al., 1983). Hence, the presence of the intestinal microbiota is required for the optimal uptake of energy from the diet.

ENVIRONMENTAL *BACTEROIDETES*

Members of the phylum *Bacteroidetes* have colonized virtually all types of environments encountered on Earth. This versatility is reflected by the diversity of sources from which cultivated strains have been isolated, such as soil, activated sludge, decaying plant material, compost, freshwater, and marine samples, algae, dairy products, and diseased animals (Bernardet and Nakagawa, 2006; Reichenbach, 2006). While the GIT microbiota is mainly composed of species from the *Bacteroidia* class, environmental *Bacteroidetes* belong primarily to the *Flavobacteria*, *Cytophagia*, and *Sphingobacteria* classes.

Over the past years, the diversity of environmental bacterial communities has been re-evaluated due to the use of culture-independent techniques. *Bacteroidetes* are increasingly recognized as an important compartment of the bacterioplankton in marine environments (Jooste and Hugo, 1999; Kirchman, 2002), especially in pelagic oceans. They are highly abundant as shown by fluorescent *in situ* hybridization (FISH) experiments using specific probes

on seawater samples. They account for as much as half of the identified cells potentially identified and outnumber alternative species (Glöckner et al., 1999; Simon et al., 1999; Cottrell and Kirchman, 2000). Using 16S rRNA gene clone libraries from nine different sampling sites (including coastal and oceanic waters at 5 m-depth in temperate, tropical, and polar regions), Pommier et al. (2007) found that *Bacteroidetes* and *Proteobacteria* were dominant. Marine *Bacteroidetes* are also often demonstrated as living on marine inert or living surfaces, either in sediments (Llobet-Brossa et al., 1998; Julies et al., 2010), in biofilms (Edwards et al., 2010), in hydrothermal vents (Sievert et al., 2000), associated with corals (Frias-Lopez et al., 2002; Rohwer et al., 2002) or on the surface of macroalgae (Beleneva and Zhukova, 2006; Staufenberger et al., 2008; Salaun et al., 2010) and angiosperms (Crump and Koch, 2008).

Bacteroidetes also colonize freshwater environments to a significant extent. Using FISH, they were found in all the water samples collected in lakes from different locations (Austria, Switzerland, Germany, and Siberia), with a median abundance ranging from 2 to 12% which tends to increase with depth (Glöckner et al., 1999). *Bacteroidetes* accounted for 40–60% of the detectable bacteria in river samples from Spain (Simek et al., 2001). An integrative analysis of the available bacterial 16S rDNA sequences recovered from freshwater lakes and rivers over the past 15 years concluded that these environments host a specific planktonic bacterial community, different from the adjacent terrestrial habitats and sediments (Zwart et al., 2002). *Bacteroidetes* were amongst the major bacterial lineages in this community, together with *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*.

Soil-associated bacterial communities also comprise members of the *Bacteroidetes* phylum. Using different techniques they have been detected in soil samples from various locations, including cultivated fields (Borneman et al., 1996; Martinez-Alonso et al., 2010), greenhouse soils (Kim et al., 2006) and unexploited areas (Buckley and Schmidt, 2003; Zhou et al., 2009). Recently, a study including soils from 88 different places revealed a positive correlation between the pH of the substrate and the relative abundance of *Bacteroidetes* (Lauber et al., 2009), ranging from 1.7% at low pH (<4) to 17% in basic soils (pH > 8). The surface of leaves, or phyllosphere, is another location of *Bacteroidetes* colonization in terrestrial environments. In a recent study, they accounted for 21.5% of the OTUs sequenced in samples from 56 tree species. Indeed, the most common group of bacteria found on the leaves was *Sphingobacteriales* which represented 21.3% of all sequences (Redford et al., 2010). Interestingly, *Bacteroidetes* tend to be more abundant on conifers (gymnosperms), reaching 70% of the sequences detected on the leaves, and less abundant on leaves from angiosperms.

Environmental *Bacteroidetes* are thought to be specialized in the degradation of complex organic matter in the biosphere, especially in the form of polysaccharides and proteins (Church, 2008). As a group, they are very versatile in the range of biopolymers they can use as carbon and energy source, e.g., plant, algal, or animal compounds. The description of new taxa often results from screening of environmental samples to discover original enzymatic activities with potential biotechnological applications (Barbeyron et al., 2001, 2008; Pankratov et al., 2006; Lee et al., 2010).

Several lines of evidence designate *Bacteroidetes* as crucial degraders of complex organic matter in the environment and not only in laboratory, many of them resulting from research on aquatic ecosystems (Kirchman, 2002). In the ocean, particulate and dissolved organic matter tends to aggregate to form particulate detritus (>0.5 mm) known as marine snow (Chin et al., 1998). These sinking particles are hotspots of organic carbon and thus play a key role in the export of matter from the euphotic surface to the sediment of the deep ocean (Azam and Malfatti, 2007). Marine snow hosts a considerable bacterial population, with densities reaching 10^6–10^8 cells per aggregate (Alldredge et al., 1986). Numerous studies characterizing these particles-associated populations have revealed that *Bacteroidetes* account for a large part (Rath et al., 1998; Ploug et al., 1999) and can even be the most abundant phylogenetic type detected (DeLong et al., 1993). Similar results have been found for estuarine particles, where *Cytophaga* spp. are amongst the dominant species in the bacterial assemblage (Crump et al., 1999). This enrichment of *Bacteroidetes* can be linked to their ability (i) to efficiently degrade complex and recalcitrant biopolymers sequestered in the particulate organic detritus and (ii) to colonize surfaces (Kirchman, 2002). Similarly, the immersion of insoluble cellulose for one month in the Irish Sea resulted in the colonization of the fibers by a specific bacterial biofilm, dominated by *Bacteroidetes* and *Gammaproteobacteria* (Edwards et al., 2010). In lakes, Glöckner et al. (1999) found that *Bacteroidetes* relative abundance in the water column increased with the depth of sampling. They proposed that this was due to the accumulation of slowly degradable, sinking macromolecules, a substrate that may have selected efficient degraders like *Bacteroidetes*.

Another evidence for consumption of high molecular weight (HMW) compounds by *Bacteroidetes* comes from several experiments where the bacterial community was followed after an increase of input of organic matter. Coastal and deep-sea environments typically receive seasonal pulses of organic matter after phytoplankton bloom events (Gooday, 2002; Kim et al., 2009). During such blooms of diatoms (Riemann et al., 2000) and dinoflagellates (Fandino et al., 2001), the population of *Bacteroidetes* increased significantly in a short time scale, concomitant with increasing activity of extracellular hydrolytic enzymes. Concordant with these results, a recent study of the communities of water masses from the North Atlantic Ocean revealed a specific enrichment of *Flavobacteria* in the space directly surrounding nanophytoplankton (Gomez-Pereira et al., 2010). In a freshwater lake, the virus-induced lysis of filamentous cyanobacteria led to the appearance of newly emerging bacterial species (van Hannen et al., 1999). The majority of these new microbial populations belonged to *Cytophagales* and *Actinomycetes*.

In alternative experiments, seasonal pulses were mimicked in laboratory conditions by an artificial increase of the organic carbon input. Gihring et al. (2009) simulated a phytodetritus deposition event by adding heat-killed *Spirulina* cyanobacteria on coastal sediments, resulting in an increase in the proportion of *Bacteroidetes* that was limited to the surface of the sediment (from 0 to 4 mm depth). This suggests a role of *Bacteroidetes* in the phytodetritus degradation in aerobic conditions (Gihring et al., 2009). The addition of exogenous protein to Californian coastal seawater mesocosms also triggered a change in the bacterial community, with an increase of the occurrence of several

Bacteroidetes species (Pinhassi et al., 1999). Finally, by combining microautoradiography and FISH (MICRO–FISH), Cottrell and Kirchman (2000) compared the uptake of biopolymers (protein and chitin) and their constituent monomers (amino acids and *N*-acetylglucosamine, respectively) by natural bacterial assemblages from coastal waters of Delaware. This approach showed an over-representation of *Bacteroidetes* in the portion of bacteria consuming polymers, whereas Alphaproteobacteria dominated uptake of monomers (Cottrell and Kirchman, 2000).

Taken together, these results show that environmental *Bacteroidetes* specialize in the mineralization of HMW organic matter. Thus, this phylum represents a key compartment for carbon fluxes and budgets in ecosystems (Nagata, 2008).

BACTEROIDETES AS PATHOGENS

As outlined above, *Bacteroidetes* are found in the normal bacterial community of many diverse habitats, where they play a beneficial role in the degradation of organic matter. On the contrary, some members of this phylum can have a strong pathogenic behavior toward different eukaryotic species. This is underlined by the number of *Bacteroidetes* strains isolated from clinical samples of human and animal origin (Hugo et al., 1999), including blood, urine, infected wounds, and feces. Some members of the *Bacteroides* genus, although belonging to the normal GIT microbiota, can cause opportunistic infections if the integrity of the intestinal mucosal barrier is broken (Smith et al., 2006). Indeed, the majority of species isolated from anaerobic infections falls into the *Bacteroides* genus, acting on different discrete sites such as peritoneal cavity, vagina, sinuses, skin, and heart (Wexler, 2007). These infections are usually polymicrobial, but *B. fragilis* and *B. thetaiotaomicron* are the most frequent species. *Bacteroides* are also associated with bacteremia, and *B. fragilis* is the most common blood isolate recovered from patients (Brook, 2010). Members of the genera *Porphyromonas*, *Prevotella*, and *Tannerella* are well known pathogens of the oral cavity, where they can notably cause periodontal disease and caries (Tanner et al., 1986; Shah and Collins, 1990; Gibson and Attardo Genco, 2006). The emergence of an infection seems to be linked to the assemblage of pathogens in bacterial consortia more than on the individual action of specific species (Jenkinson and Lamont, 2005).

The *Flavobacteria* class also contains opportunistic human pathogens, invading hosts with poor immune system (Bernardet and Nakagawa, 2006). One of them, *Elizabethkingia meningoseptica*, causes meningitis in infants (King, 1959; Ratner, 1984; Kim et al., 2005). *Myroides* has been reported in cases of surgery wound, urinary tract infections, septicemia, pneumonia, meningitis, fasciitis, ventriculitis, and nosocomial infections (Mammeri et al., 2002). The genera *Empedobacter*, *Bergeyella*, *Weeksella*, and *Capnocytophaga* also contain pathogenic species (Hugo et al., 2006a,b; Leadbetter, 2006).

Bacteroidetes infections can have devastating effects for farmed and wild fish (Austin and Austin, 2007), as reported a century ago and first observed in aquaria (Davis, 1922). *Flavobacterium psychrophilum*, *F. columnare*, *F. branchiophilum*, *Tenacibaculum maritimum*, and *Chryseobacterium scophtalmum* are some of the species with the most severe economic impact (Bernardet and Bowman, 2006). Members of the genera *Riemerella*, *Ornithobacterium*, and *Coenonia* can cause septicemia and respiratory tract infections in birds (Segers et al., 1993; Vandamme et al., 1994, 1999).

In addition, *Bacteroidetes* can affect various plants and algae. *F. johnsoniae* is incriminated as an opportunistic pathogen in the decay of fresh plants and vegetables called "soft rot" (Liao and Wells, 1986). Members of the genera *Zobellia*, *Cellulophaga*, and *Kordia* have algicidal activities (Skerratt et al., 2002; Sohn et al., 2004), and can therefore control blooms of microscopic algae. The Anaaki disease, severely damaging the red macroalga *Porphyra yezoensis*, is caused by *Flavobacterium* sp. LAD-1 (Sunairi et al., 1995). Uyenco (1977) isolated strains of *Flavobacterium* associated with "ice-ice disease" in decaying red alga *Eucheuma striatum*, characterized by whitening and hardening of the seaweed's tissues. Similarly, a *Cytophaga* sp. promotes the ice–ice disease in stressed branches of the farmed Rhodophytes *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Largo et al., 1995). Members of the *Flavobacterium* genus can also infect brown macroalga such as *Undaria pinnatifida*, where they are associated with "spot-rotting" disease and "shot hole disease" (Neill et al., 2008).

The pathogenicity of *Bacteroidetes* is at least partly due to the production of polymer-degrading enzymes targeting host cellular components. Animal pathogens produce a range of hydrolytic enzymes such as hyaluronidase, chondroitin sulfatase, heparinase, glucosidases, and *N*-acetylglucosaminidase (Rudek and Haque, 1976; Duerden, 1994). These enzymes damage the extracellular matrix of animals and thus are important both for tissue colonization and nutrients supply. In a study of the fish pathogen *F. columnare*, a chondroitin lyase was found to be related to the virulence (Suomalainen et al., 2006). Plant and algal pathogens are able to degrade cell wall compounds such as pectins and agars. Indeed, phytopathogenic strains of *F. johnsoniae* secrete much more pectate lyase than their non pathogenic counterparts (Liao and Wells, 1986). *Bacteroidetes* also secrete various proteases, and this has been documented as an important virulence factor. Proteases secreted by different pathogenic *Bacteroides* species can destroy human brush border digestive enzymes (Riepe et al., 1980). Greiner and Mayrand (1987) found that the pathogenic strains of *Porphyromonas gingivalis* had a higher collagenolytic activity than the non-virulent strains. In animal models, *P. gingivalis* mutants lacking the ability to produce various proteases lost their virulence (Loesche, 1993; Fletcher et al., 1995). Whatever their environment, it appears that the biological function of *Bacteroidetes* either as symbiont, environmental, or pathogen bacteria is connected with their propensity to degrade complex biopolymers, i.e., polysaccharides and proteins.

HABITAT EVOLVED ADAPTATION MEASURED BY THE VARIETY OF POLYSACCHARIDES THAT ARE DEGRADED

As a phylum, and especially due to their versatility in habitats, *Bacteroidetes* have access to an amazing diversity of carbon sources. Indeed, the chemical diversity of polysaccharides largely outnumbers the possibility for protein folds – it has been calculated that there are 1.05×10^{12} possible linear and branched forms of a single hexasaccharide (Laine, 1994). Moreover, these structural variations have been harnessed by living organisms to fulfill very different roles: e.g., structural, storage, specific signaling, specific recognition, host–pathogen interactions to name but a few

(Carpita and Gibeaut, 1993; Graham et al., 2000; Stahl and Bishop, 2000). Consequently, carbohydrates account for around 75% of the biomass on Earth, a natural resource that was not lost on competing organisms that were developing their own strategies to utilize this chemical energy for their own survival.

But even more importantly several classes of polysaccharides are niche specific. While cell walls are a characteristic feature of all plants, they are not exclusive to plants, with most bacterial and algal cells as well as all fungal cells also being surrounded by extracellular, macromolecular barriers (extracellular matrix or ECM). The macromolecular composition, however, is characteristically different among the major evolutionary lineages of the living world, linking specific life style or nutritional habits to specifically encountered biopolymers. A vivid example is provided by the polysaccharides of the marine environment that are typically and to a large majority sulfated (carrageenans and fucans) or highly ionic (alginates) and unique to this particular habitat (Michel et al., 2010a,b; Popper et al., 2011). In contrast, the basic polysaccharide components of plant cell walls are cellulose and hemicellulose (pectins, xylans, mannans, xyloglucans, etc.), whereas fungal cell walls primarily consist of chitin (Niklas, 2004). In metazoa, the ECM will predominantly consist of chondroitin or dermatan, which are essentially made of sulfated polysaccharides referred to as glycosaminoglycans (GAGs; Sugahara and Kitagawa, 2002) that are interconnected by fibrillar proteins (collagens). Other sources of carbohydrates in animals are glycosylation sites, such as mucin that contain a high proportion of sialic acid in addition to GAGs (Raman et al., 2005).

Reflecting this chemical diversity of the substrate, glycosidases, the enzymes responsible for the breakdown of di-, oligo-, and polysaccharides, as well as glycoconjugates, are ubiquitous through all domains of life (Turnbaugh et al., 2010). Carbohydrate processing enzymes (CAZymes), including glycosidases and glycosyltransferases (the enzymes which transfer saccharides to other saccharide moieties, small molecules, lipids, or proteins), constitute between 1 and 3% of the genome of most organisms (Davies et al., 2005). Noteworthy, the genomes of *Bacteroidetes* species have revealed that they are champions with respect to the diversity and number of CAZymes they contain, reflecting the molecular strategies evolved by this microbial community to differentiate, capture, and degrade complex glycans. Consequently and as a result of this ability to degrade host and plant glycans, cultured (environmental or gut) species are often used to isolate specific enzymes for polysaccharide degradation (Berg et al., 1980; Tierny et al., 1994; Bernardet and Nakagawa, 2006; Reichenbach, 2006).

THE GENOMIC PERSPECTIVE

The first sequenced genome of a *Bacteroidetes* representative was published in 2002 for the human symbiont *B. thetaiotaomicron* (Xu et al., 2003). Since then, many sequencing projects have been conducted to increase the genomic knowledge on this phylum. To date, 33 *Bacteroidetes* genomes are complete, publicly available and published (**Table 2**). Many others are in a draft state or have not yet been published (total of 125 sequences censed on NCBI). During the last 10 years, sequencing efforts have indiscriminately concerned environmental, pathogen, and symbiotic/commensal species with the aim to better understand their biological functions, including their capacity to interact with their habitats. This data allows comparing the different enzymatic capabilities of various genera, which sheds new light on the specialization of *Bacteroidetes* toward degradation of organic matter. A striking common feature revealed by this comparative genomic approach is the trend of *Bacteroidetes* genomes to encode many polymer-degrading enzymes, acting either on proteins or carbohydrates. The census of Carbohydrate-Active enzymes (CAZ Ymes) in the CAZY database[1] (Cantarel et al., 2009) eases the comparison of the number of glycosylhydrolases (GH) and polysaccharide lyases (PL) in an increasing number of sequenced species. In each of the four classes of the *Bacteroidetes* phylum, there are examples of CAZYme-enriched species. In *Bacteroidia*, the proteomes of *B. thetaiotaomicron*, *B. fragilis*, and *P. ruminicola* comprise 272, 137, and 130 GH and PL respectively (Xu et al., 2003; Kuwahara et al., 2004), much more than other members of the gut microbiota, or outside the *Bacteroidetes* phylum, such as *Clostridium perfringens* (57 GH and PL) and *Bifidobacterium longum* (49 GH). The same is true for *Flavobacteria* [e.g., *G. forsetii* with 48 GH/PL (Bauer et al., 2006), *Z. profunda* with 120 GH (Qin et al., 2010), *F. johnsoniae* with 152 GH/PL (McBride et al., 2009)], *Cytophagia* [e.g., *S. linguale* with 151 GH/PL (Lail et al., 2010), *D. fermentans* with 98 GH/PL (Lang et al., 2009), and *Sphingobacteria*, e.g., *C. pinensis* with 184 and *P. heparinus* with 163 GH/PL, respectively (Han et al., 2009; Del Rio et al., 2010)]. In some cases, the prediction of CAZYme-encoding genes in newly sequenced organisms can even unveil unexpected catabolic capabilities toward specific substrates (McBride et al., 2009). Moreover, the predicted enzymatic battery of a bacterial species will help characterize its natural habitat (i.e., the available substrates) and its ecological function in organic matter recycling. Recently, this feature has been used for genome and habitat comparison, linking the number and occurrence of specific CAZyme-families to the environmental niche (Pope et al., 2010; Purushe et al., 2010). With this respect, the presence of a vast majority of exo-acting enzymes in the genome of *B. thetaiotaomicron*, suggests that the organism is able to use the saccharide decorations appended to the backbone of structural polysaccharides and glycoproteins (Xu et al., 2003). Another recent study elegantly demonstrates that this particularly evident expansion in exo-GHs of family GH92 enzymes (23 members) is indeed related to the α-mannosides present in the *N*-glycans of host and dietary glycoproteins (Zhu et al., 2010).

However, one has to keep in mind that a rough analysis of the number of degradation enzymes in general is insufficient; one needs to go down to the enzymatic sub-family to infer a putative metabolism. Indeed, the CAZyme classification based on sequence similarity[1] has the consequence that gene families group together enzymes with widely different substrate or product specificities (Henrissat, 1991). Therefore, to derive knowledge useful for subsequent functional predictions, phylogenetic analyses defining subgroups that contain biochemically characterized representatives are needed to perform unambiguous assignments (Turnbaugh et al., 2010).

In spite of the general trend of *Bacteroidetes* to possess numerous degradation enzymes, there are several noteworthy exceptions. The fish pathogen *F. psychrophilum* genome harbors only 13 proteases, i.e., 4.5 proteases per megabase (Mb) and 3 GH

[1]http://www.cazy.org

Table 2 | List of *Bacteroidetes* with a fully sequenced and published genome, and their respective habitats.

Species	Genome size (CDS number)	Habitat (functional role)	Reference
***FLAVOBACTERIA* CLASS**			
Capnocytophaga ochracea DSM 7271	2.6 Mb (2193)	Animals and humans (S/P)	Mavrommatis et al. (2009)
Gramella forsetii KT0803	3.8 Mb (3585)	North Sea surface water during phytoplankton bloom (E)	Bauer et al. (2006)
Robiginitalea biformata HTCC2501	3.5 Mb (3211)	Sargasso Sea (E)	Oh et al. (2009)
Flavobacterium johnsoniae UW101	6.1 Mb (5056)	Soil and freshwater (E)	McBride et al. (2009)
Flavobacterium psychrophilum JIP02/86	2.9 Mb (2432)	Fish (P)	Duchaud et al. (2007)
Croceibacter atlanticus HTCC2559	3.0 Mb (2715)	Sargasso Sea (E)	Oh et al. (2010)
Zunongwangia profunda SM-A87	5.1 Mb (4653)	Deep-sea sediments (E)	Qin et al. (2010)
Candidatus Sulcia muelleri GWSS	0.3 Mb (228)	Sap-feeding insects flora (S)	McCutcheon and Moran (2007)
Maribacter sp. HTCC2170	3.9 Mb (3411)	Surface sea water, Oregon coast (E)	Oh et al. (2011)
Periplaneta americana	0.6 Mb (581)	Cockroach endosymbiont (S)	Sabree et al. (2009)
Blattella germanica	0.6 Mb (586)	Cockroach endosymbiont (S)	Lopez-Sanchez et al. (2009)
Polaribacter dokdonensis MED152	3.0 Mb (2646)	Northwestern Mediterranean sea surface water (E)	Gonzalez et al. (2008)
Dokdonia donghaensis MED134	3.3 Mb (2284)	Korean sea water (E)	Gomez-Consarnau et al. (2007)
Leeuwenhoekiella blandensis MED217	4.2 Mb (3735)	Mediterranean sea water (E)	Gomez-Consarnau et al. (2007)
***SPHINGOBACTERIA* CLASS**			
Chitinophaga pinensis DSM 2588	9.1 Mb (7302)	Pine litter in Australia (E)	Del Rio et al. (2010)
Pedobacter heparinus DSM 2366	5.2 Mb (4287)	Soil (E)	Han et al. (2009)
Salinibacter ruber M8	3.6 Mb (3086)	Saltern crystallizer pond, Mallorca (E)	Pena et al. (2010)
Salinibacter ruber M31	3.6 Mb (2934)	Saturated thalassic brines (E)	Mongodin et al. (2005)
Rhodothermus marinus R-10	3.4 Mb (2914)	Submarine hot spring, Iceland (E)	Nolan et al. (2009)
***CYTOPHAGIA* CLASS**			
Dyadobacter fermentans NS114	7.0 Mb (5804)	Stems from *Zea mays* (E)	Lang et al. (2009)
Cytophaga hutchinsonii ATCC 33406	4.4 Mb (3790)	Soil (E)	Xie et al. (2007)
Spirosoma linguale 1	8.5 Mb (7069)	Soil, freshwater (E)	Lail et al. (2010)
***BACTEROIDIA* CLASS**			
Candidatus Azobacteroides pseudotrichonymphae CfPt1-2	1.1 Mb (758)	Endosymbiont of termite gut protist (S)	Hongoh et al. (2008)
Porphyromonas gingivalis ATCC 33277	2.4 Mb (2090)	Deep periodontal pockets (P)	Naito et al. (2008)
Porphyromonas gingivalis W83	2.3 Mb (1990)	Deep periodontal pockets (P)	Nelson et al. (2003)
Parabacteroides distasonis ATCC 8503	4.8 Mb (3867)	Human gastrointestinal tract (S)	Xu et al. (2007)
Bacteroides vulgatus ATCC 8482	5.2 Mb (4088)	Human gastrointestinal tract (S)	Xu et al. (2007)
Bacteroides fragilis NCTC 9343	5.2 Mb (4274)	Human gastrointestinal tract (S/P)	Cerdeno-Tarraga et al. (2005)
Bacteroides fragilis YCH46	5.3 Mb (4578)	Human gastrointestinal tract (S/P)	Kuwahara et al. (2004)
Bacteroides thetaiotaomicron VPI-5482	6.3 Mb (4779)	Human gastrointestinal tract (S)	Xu et al. (2003)
Prevotella ruminicola 23	3.6 Mb (2763)	Bovine rumen (S)	Purushe et al. (2010)
Prevotella bryantii $B_1$4	3.6 Mb (2780)	Bovine rumen (S)	Purushe et al. (2010)
UNCLASSIFIED			
Candidatus Amoebophilus asiaticus 5a2	1.9 Mb (1557)	Obligate intracellular ameba symbiont (S)	Schmitz-Esser et al. (2010)

The functional role is given in brackets: S, symbiont; P, pathogen; E, environmental.

per Mb (Duchaud et al., 2007). This is far less than its closely related soil-associated cousin *F. johnsoniae*, which possesses 20.5 proteases and 23 GH per Mb and has a genome more than twice as large. The reduced number of degradation enzymes in the fish pathogen can be explained by its dedication to the infection of animal tissues. For such a species, the relative restricted diversity of substrates (compared to a soil-associated species) would diminish the need of multiple families of hydrolases. A second, similar example is *P. gingivalis* that has a relatively lower number of hydrolases (only 24 GHs) compared to other members of the *Bacteroidia* class (Nelson et al., 2003). Again, the most plausible explanation is the high speciation of this species to dental plaque degradation that results in the restricted diversity of substrates utilized by this bacterium.

Some marine representatives of the *Flavobacteria* class seem to alternate between two life strategies depending on the abundance of carbon sources. This is notably the case of *Polaribacter dokdonensis* (strain MED152), *Leeuwenhoekiella blandensis* (strain

MED217^T), and *Dokdonia donghaensis* (strain MED134). On the one hand, commonly with other *Bacteroidetes*, they are very well equipped to attach to surfaces and depolymerize organic matter. For example, *P. dokdonensis* and *D. donghaensis* genomes encode many enzymes to degrade proteins (93 and 120 peptidases, respectively) and polysaccharides (30 and 22 GH, respectively; Gonzalez et al., 2008; Kirchman, 2008; Woyke et al., 2009). On the other hand, when polymeric substrates become scarce, these species switch to a free-living lifestyle adapted to carbon-poor environments. Genome analysis showed the presence of proteorhodopsin, a light-dependent H^+ pump that can drive ATP synthesis (Beja et al., 2000). This protein allows the phototrophic production of sufficient energy to maintain the population growth when the concentration of organic carbon decreases (Gomez-Consarnau et al., 2007). Additionally, *P. dokdonensis* is enriched in enzymes involved in anaplerotic reactions, and assimilates CO_2 faster in light conditions than in the dark (Gonzalez et al., 2008). Altogether, these results suggest that marine *Bacteroidetes* may cope as well with feast and famine, and complement the understanding of their role in carbon cycles (DeLong and Beja, 2010).

Beyond the prediction of numerous polysaccharide-degrading enzymes, two large paralogous families of proteins have been found in *Bacteroidetes* genomes, which likely participate in polysaccharide uptake. These include homologs of the outer membrane proteins SusC and SusD from *B. thetaiotaomicron*, which are involved in starch utilization. Seminal work of the Salyers' group showed that this human gut symbiont degrades starch via a dedicated starch utilization system (Sus) with several proteins acting in coordination to sense, bind, and hydrolyze the substrate (Anderson and Salyers, 1989a,b; Shipman et al., 2000). The genes encoding these proteins cluster on the bacterial chromosome into typical polysaccharide utilization loci (PUL).

POLYSACCHARIDE UTILIZATION LOCUS: FROM THE ARCHETYPIC SUS SYSTEM TO NEW SUBSTRATES

In *B. thetaiotaomicron*, the Sus locus comprises eight genes, *susRABCDEFG* (Martens et al., 2009). SusR is an inner membrane regulatory protein which activates the transcription of the other genes in the presence of maltose or starch (D'Elia and Salyers, 1996). SusC, SusD, SusE, and SusF are outer membrane proteins involved in the binding of the polysaccharide (Shipman et al., 2000). In fact, analyses of mutant strains have shown that SusC and SusD account together for 70% of the starch-binding capabilities of the wild type (Reeves et al., 1997). Surface-bound starch is hydrolyzed by the outer membrane α-amylase SusG, which acts endolytically and releases oligosaccharides larger than maltotriose (Shipman et al., 1999; Martens et al., 2009). These degradation products are then channeled to the periplasm through the TonB-dependent receptor, β-barrel-type SusC, where they are further cleaved by the neopullulanase SusA and α-glucosidase SusB. The atomic structures of several protein members have been resolved, namely the starch-binding SusD (Koropatkin et al., 2008), the α-glucosidase SusB (Kitamura et al., 2008), and the α-amylase SusG (Koropatkin and Smith, 2010). The Sus locus is organized into two transcriptional units under the control of SusR, one containing *susA* and the other containing *susB* to *susG* (D'Elia and Salyers, 1996; Reeves et al., 1997). This allows a co-regulation of the PUL (Anderson and Salyers, 1989b).

The characterization of this first starch-specific PUL was followed by the discovery of numerous PULs in *Bacteroidetes*. *susC*-like and *susD*-like genes are strikingly frequent in *Bacteroidetes* genomes, often appearing in tandem and as the central units of substrate-specific PULs. *B. thetaiotaomicron* possesses 107 paralogs of *susC*, of which 101 are paired to a *susD*-like gene. 62 of these pairs are part of larger clusters, together with polysaccharide-degrading enzymes (Xu et al., 2003). In addition, some of these PULs comprise enzymes targeting glycan decorations, such as sulfatases or acetyl esterases. Thus, depending on the specificity of the predicted enzymes, one can infer the favorite substrate(s) of a given PUL. In total, PULs represent 18% of the genome of *B. thetaiotaomicron* (Martens et al., 2008). The closely related *B. fragilis*, *B. vulgatus*, and *Parabacteroides distasonis* also possess numerous PULs (Kuwahara et al., 2004; Xu et al., 2007). These PULs likely favor the success of *Bacteroides* spp. in the uptake of dietary and host-derived polysaccharides in the highly competitive gut habitat, and may explain their evolution as symbionts.

Interestingly, environmental species also harbor plenty of specific PULs. The annotation of the *F. johnsoniae* genome revealed 42 pairs of *susC*-like and *susD*-like genes, among which many were associated with CAZymes (McBride et al., 2009). The authors were notably able to predict PULs targeting starch (homologous the *B. thetaiotaomicron* Sus locus), chitin, and hemicelluloses. The genome of the marine *Flavobacteria G. forsetii* encodes 40 paralogs of SusC, and 14 clusters of *susCD*-like genes were detected (Bauer et al., 2006), often in the vicinity of CAZyme genes. This suggests that environmental *Bacteroidetes* as well as their gut-associated cousins use a unique and similar strategy to bind and degrade polymeric organic matter. Indeed, SusD homologs are only found in *Bacteroidetes* representatives. Thus, we speculate that the appearance of PULs including a *susD-like* gene in the ancestral *Bacteroidetes* could have at least partly driven the emergence of the phylum, and allowed its evolution as a group specialized in carbohydrate degradation.

THE HIGH PLASTICITY OF *BACTEROIDETES* GENOMES

The size of *Bacteroidetes* genomes varies considerably between species (**Table 2**). Among published sequencing projects, *Chitinophaga pinensis* has the largest genome (9.1 Mb) whereas *Candidatus Sulcia muelleri* has the smallest (0.3 Mb). This great discrepancy can be at least partly associated with the different ecological niches colonized and the biological functions played by *Bacteroidetes* (**Figure 1**). Obligate intracellular symbionts have a more reduced genome size, due to their peculiar lifestyle. These species have evolved through successive inactivation and loss of genes, affecting virtually every cellular process. The possible causes of this genome reduction are multiple, including the unusual stability and metabolic richness of the cytoplasmic compartment they inhabit (McCutcheon and Moran, 2010). Pathogenic *Bacteroidetes*, such as *P. gingivalis* and *F. psychrophilum*, have a small genome typically around 2 Mb. This can be linked to the dedication of their metabolic capabilities toward the infection of specific sites. Living

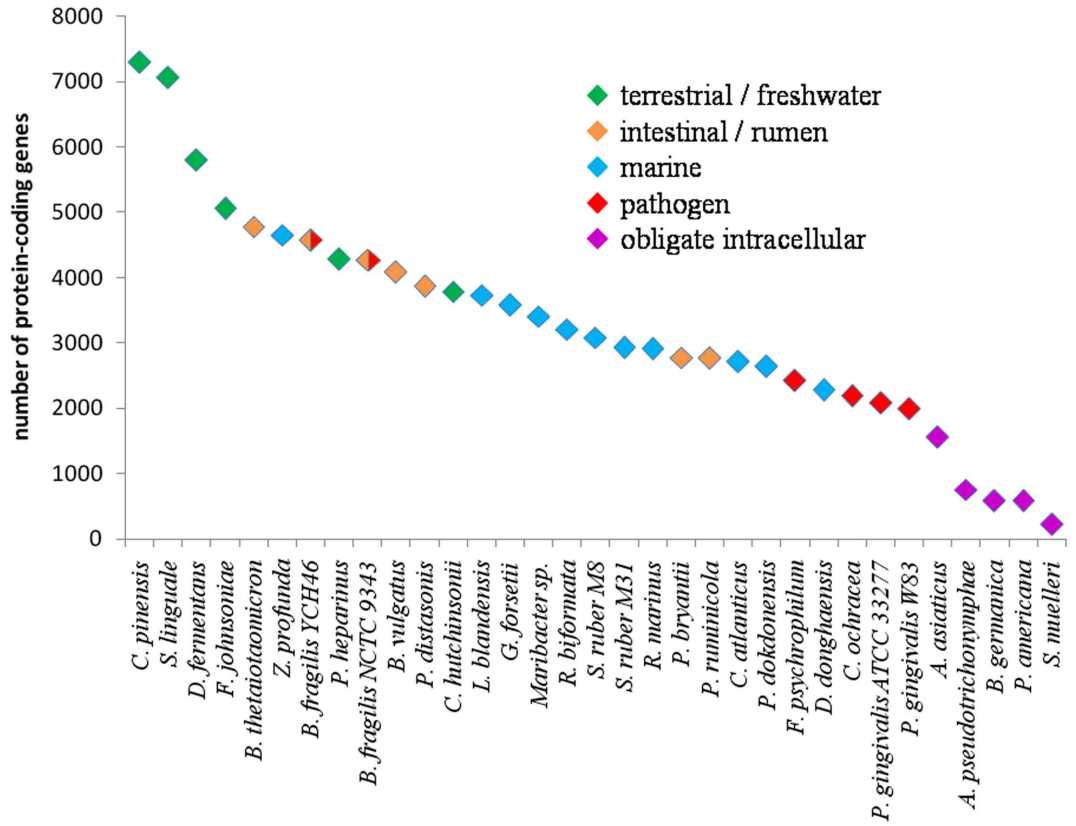

FIGURE 1 | Schematic diagram representing the total number of coded proteins as a function of genome size of *Bacteroidetes* species. Only complete and published genomes of the *Bacteroidetes* phylum have been included (see also **Table 2**).

in complex habitats and metabolizing a lot of different substrates, environmental, and intestinal species tend to have larger genomes (**Figure 1**), correlating with their broader catabolic capabilities. This is also the case of the opportunistic pathogen *B. fragilis*, which is part of the normal human gut microbiota but can cause infections at many other sites. To date, marine representatives harbor smaller genomes than their intestinal or terrestrial counterparts, but this will certainly progress as the number of complete genome sequences increases. For example, our group has recently annotated the genome of the marine *Flavobacteria Zobellia galactanivorans*. It comprises 5.5 Mb and encodes 4738 proteins, representing one of the largest genomes for a marine *Bacteroidetes* (Barbeyron et al., unpublished data). Interestingly, the proteorhodopsin-containing *P. dokdonensis* and *D. donghaensis* possess the smallest proteome among environmental species (2646 and 2284 proteins, respectively). Thus, the genome size fits the ecological niche. Big genomes increase the metabolic capacities, and hence broaden the spectrum of potential substrates for bacteria living in complex environments. In more stable habitats, bacteria tend to specialize toward specific functions and harbor smaller genomes. This raises the question of the nature of the ancestral *Bacteroidetes* genome. Indeed, the present variations in genome size could be due either to massive loss of genes from a large ancestral genome, or to successive acquisitions completing a small genome with genes representing a selective advantage.

In this respect, the sequencing era unraveled the plasticity of *Bacteroidetes* genomes, which evolved, and probably still evolve, through dynamic processes. The outcome of this plasticity reflects in the rapid deterioration of the global synteny between evolutionary-related species living in the same environment, as revealed for gut and rumen *Bacteroidetes* (Xu et al., 2007; Purushe et al., 2010). Their evolution is driven by highly frequent genetic rearrangements, gene duplications, and lateral gene transfers (LGT) between species. Genome analysis of two *B. fragilis* strains revealed extensive DNA inversions affecting the expression patterns of several genes (Kuwahara et al., 2004; Cerdeno-Tarraga et al., 2005). These events control the antigenic composition of bacterial surface structures and likely help *B. fragilis* evading the immune system and colonize novel sites. Notably, the expression of 20 SusC-like proteins, most of them coupled with SusD homologs, is regulated through DNA inversions (Kuwahara et al., 2004). This may participate in the cell adaptation to degrade specific polysaccharides found at the infection sites. *Bacteroidetes* evolution is also characterized by frequent gene duplications and further divergence in sequence and function, leading to considerably expanded paralogous groups. Notably, GH and SusC/SusD-like proteins are amongst the largest paralogous families in several sequenced *Bacteroidetes* (Xu et al., 2003, 2007; Bauer et al., 2006). In addition to this intra-strain plasticity, *Bacteroidetes* genomes evolve through inter-species exchange of genetic material (Thomas and Nielsen, 2005). Using a phyloge-

netic approach, Xu et al. (2007) showed that around 5.5% of the genes in gut *Bacteroidetes* genomes were laterally acquired from non gut-associated bacteria, among which glycosyltransferases (GT) where significantly over-represented. These LGT events could partly explain the niche specialization of different species. The authors suggest that acquisition of new genes from outside the gut brought novel metabolic pathways to intestinal *Bacteroidetes* and broaden the spectrum of digestible substrates. Furthermore, it has been shown that the convergence of GT and GH repertoires in gut *Bacteroidetes* sharing the same habitat is largely due to massive LGT rather than gene duplications (Lozupone et al., 2008). Conjugative LGT events are also demonstrated to be responsible for antibiotic resistance spreading in natural communities of gut *Bacteroides* (Shoemaker et al., 2001). The exchange of genetic material is not necessarily restricted to closely related species and can overcome phylogenetic barriers. Indeed, glyceraldehyde-3-phosphate dehydrogenase genes have been horizontally transferred from a β-proteobacteria to a *Bacteroidetes* (Figge et al., 1999). Another example is the transfer of genes between an Archeae and the hyperhalophilic *Sphingobacteria S. ruber* (Mongodin et al., 2005).

Taken together, recent analyses of *Bacteroidetes* genome sequences have shown that: (i) there is a gradation in the size of the genomes correlated with the functional specialization; (ii) genomes can undergo massive reorganizations; (iii) highly frequent LGT events allow spreading of novel metabolic capabilities inside *Bacteroidetes* populations. As already mentioned, intestinal *Bacteroidetes* are specialized in the degradation of plant-derived polymers, a feature shared with environmental relatives. In the last part of this review, we will discuss the potential connections between these two communities that are not *prima facie* obviously interacting.

THE FOOD CONNECTION: *TELL ME WHAT YOU EAT, I WILL TELL YOU WHAT YOUR BACTERIA CAN DO*

Several studies have shown that the diet strongly influences the intestinal microbiota. Early research focused on the comparison of fecal microbes retrieved from individuals with different nutritional habits. Benno et al. (1986) showed significant variations in the cultivable microbiota of rural Japanese and urban Canadians. They proposed that this discrepancy relates to the contrasted diet of the two populations. Similarly, in a rat model transplanted with human microbiota, the consumption of resistant starch changed the bacterial composition compared to a sucrose diet (Silvi et al., 1999). Yet, the use of cultivable bacterial counts is a limited method (Amann et al., 1995) and no statistical difference was found when comparing the fecal microbiota of strictly vegetarians and individuals consuming a general diet (Goldberg et al., 1977). The development of culture-independent techniques to assess bacterial abundance and diversity helped testing the influence of diet on the GIT microbiota. In a mouse model reproducing the human intestinal microbiome, the bacterial community composition and the representation of metabolic pathways was strongly dependant on the nature of the diet (Turnbaugh et al., 2009). The proportion of *Bacteroidetes* representatives decreased drastically when animals were switched from a chow containing low levels of fat and high level of plant polysaccharides to a Western diet (high fat, high sugar). Feeding on the diet rich in plant polysaccharides resulted in an enriched set of pathways including *N*-glycan, glycosaminoglycan, and starch degradation that are typical for *Bacteroidetes* (Turnbaugh et al., 2009). An independent study on a murine model showed that a high-fat diet was associated with a decrease in more than 30 lineages within the *Bacteroidetes* phylum, including in the *Bacteroidaceae*, *Prevotellaceae*, and *Rikenellaceae* families (Hildebrandt et al., 2009). Recent studies have investigated the diet impact on the human gut microbiota. The consumption of chemically modified resistant starch (RS4) instead of normal, digestible starch led to a shift in the bacterial community (Martinez et al., 2010). Even if results varied substantially between the 10 considered subjects, RS4 consumption was notably followed by enrichment in *Bacteroidetes*, among which *Parabacteroides distasonis* increased sevenfold. The observed changes were completely reversible within 1 week, demonstrating the high population dynamics (Martinez et al., 2010). In a recent comparison of the fecal microbiota of children from Burkina Faso, and Italy, De Filippo et al. (2010) showed a significant difference in the community composition. African children showed a higher proportion of *Bacteroidetes* (57 vs. 22%) and a lower proportion of *Firmicutes* (27 vs. 63%) than Europeans. The authors explained this difference by the higher dietary fiber content of the rural African food, mainly composed of cereals, legumes, and vegetables, which would favor the development of the polysaccharide-degrading *Bacteroidetes*. Interestingly, the genera *Prevotella*, *Xylanibacter*, *Cytophaga*, and *Paludibacter* were found exclusively in African microbiota. This is probably due to their increased fitness to grow on polysaccharides abundant in the Burkina Faso diet, such as xylan or cellulose (De Filippo et al., 2010). The control of gut microbiome composition by the diet quality likely denotes a selection of the population that optimally degrades the available substrates. In the case of *Bacteroidetes*, the selection criteria would primarily be based on the ability to digest complex polymers. Some species may have acquired specific catabolic pathways that others lack. This hypothesis has been recently tested in a mouse model. Germ-free mice were inoculated with two *Bacteroides thetaiotaomicron* and *B. caccae* strains of which the latter one can grow with inulin as carbon source. When the mice where fed with an inulin rich diet the ratio of the two species changed toward *B. caccae*. The ability to use inulin was associated with a GH32 absent in *B. thetaiotaomicron*. This clearly showed that diet selects species composition in the animal intestine (Sonnenburg et al., 2010).

The question arises if different human populations with different diets contain specific food adaptations on the genetic level of their gut microbes. Recently, our group has shown that gut *Bacteroidetes* were able to get gene updates from environmental species to acquire novel functions (Hehemann et al., 2010; Rebuffet et al., 2011). Indeed, in the marine flavobacterium *Zobellia galactanivorans*, we have discovered and characterized the first porphyranases (Hehemann et al., 2010) as well as a 1,3-α-3,6-anhydro-L-galactosidase (Rebuffet et al., 2011). These enzymes are used by marine bacteria to degrade agarocolloïds, sulfated galactans only found in the cell walls of red algae, such as *Porphyra* or *Gracilaria*. When using these new sequences as lead sequences to probe publicly available databases, homologs were identified not only in other marine bacteria, but surprisingly also in the human gut isolate *Bacteroides plebeius*. The genome of *B. plebeius* (DSM 17135) contains a porphyran/agar degradation locus, transferred from an ancestral marine *Bacteroidetes* (Hehemann et al., 2010; Rebuffet et al., 2011). This PUL was identified as a result of biochemical and

structural characterization of the first two porphyranases belonging to the family GH16 (PorA and PorB; Hehemann et al., 2010) and the 1,3-α-3,6-anhydro-L-galactosidase AhgA belonging to the family GH117 (Rebuffet et al., 2011). The PUL contains sequences coding for two putative β-agarases (GH86), one β-agarase (GH16), two β-galactosidases (GH2), a sulfatase, a carbohydrate-binding module, and a susD-like gene associated with its TonB-dependant receptor (**Figure 2**). Altogether these enzymes form a complete system of detection and degradation for porphyran and agar, which provides *B. plebeius* with the set of utensils to use these polysaccharides as carbon source.

Metagenomic data revealed that porphyranases and 1,3-α-3,6-anhydro-L-galactosidases are absent in North American and Danish population but present in Spanish and Japanese populations with proportions of 10 and 38% respectively (Hehemann et al., 2010; Rebuffet et al., 2011). The biological rationale of "marine" enzymes in gut microbes could be linked to the high input of sea-derived products in the diet of these two populations. Indeed, the Japanese consume about 14.2 g seaweed per day and person (Fukuda et al., 2007), and the most popular seaweed is Nori (*Porphyra* spp.) used to make maki-sushi (Nisizawa et al., 1987). Similarly, Spain is the second largest consuming nation of seafood in the world (Manrique and Jensen, 2001). In both populations, contact between human-associated microbes and non-sterile seaweed or seafood could have created a favorable condition for a LGT from marine bacteria to human gut *Bacteroidetes*. Noteworthy, Nori is the only food that contains porphyran, which allowed associating the transfer of these genes to one special food source. This first evidence of a life style-associated adaptation of the genetic repertoire of the human gut microbiome could be detected due to the unique signature of

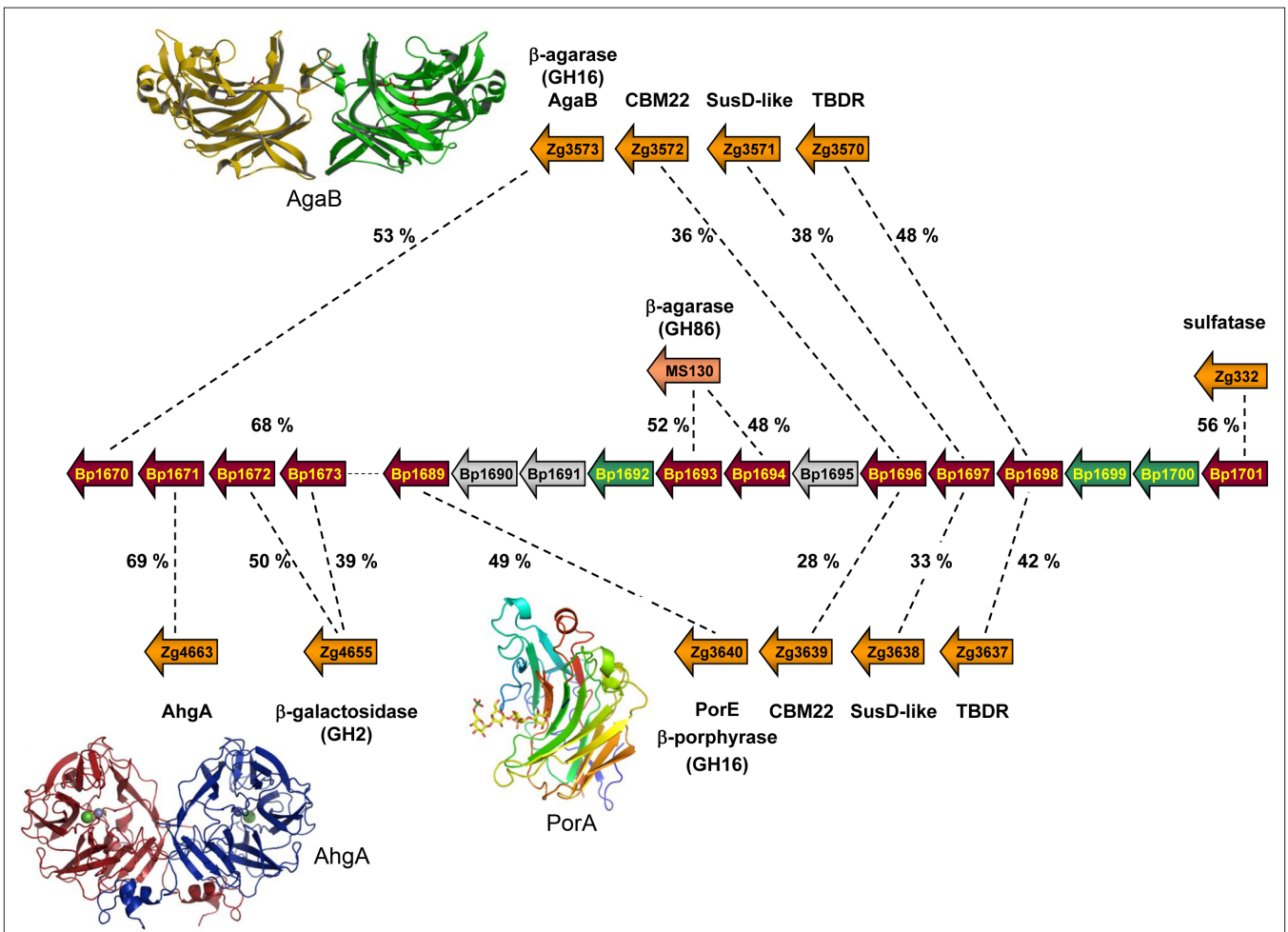

FIGURE 2 | Schematic representation of a PUL region, present in the genome of *Bacteroides plebeius* and first identified as interesting because of the presence of a porphyranase gene (Bp1689; Hehemann et al., 2010). Besides the conserved *Sus*-like genes, the locus also contains carbohydrate-related genes which share highest identity with proteins used for red algal galactan degradation in two marine *Bacteroides*. Shown are the sequence identities between *B. plebeius* and one Microscilla sp. PRE1 protein, as well as with several *Zobellia galactanivorans* proteins. Six of these genes (Bp1670, Bp1671, Bp1689, Bp1693, Bp1694, and Bp1696) are conserved only with marine bacteria, and are absent in genomes of other gut *Bacteroides*. The crystal structures of marine homologous enzymes, coded by three of these genes (namely Bp1670, Bp1671, and Bp1689) have recently been determined and are illustrated as ribbon representations (PDB codes: AgaB – 1O4Z; AghA – 3P2N; and PorA – 3ILF). The 3D structures help determine the crucial residues for activity and substrate specificity that are all verified and present in the sequences of the *B. plebeius* proteins.

seaweed degrading CAZymes. Therefore marine CAZymes may be particularly good probes to reveal such adaptations in the human gut microbiome.

These examples of LGT show that gut bacteria are able to acquire new functions via transfer of a complete degradation pathway from food-associated environmental bacteria. Interestingly, the *Firmicutes Epulopiscium* sp., which lives in the gut of grazer surgeonfish (Clements and Bullivant, 1991), also possesses a putative agar degradation locus acquired from marine bacteria (Rebuffet et al., 2011). One can therefore extrapolate that similar events occurred in omnivorous and herbivorous animals. During the course of evolution, the metabolic repertoire of their gut microbes could have been influenced by contacts with food-associated environmental bacteria. Thus, acquisition of selectively advantageous genes by successive LGT events could explain how gut symbionts acquired CAZymes involved in green plant polysaccharide degradation.

In conclusion, some crucial criteria seem to have particularly favored the adaptation of *Bacteroidetes* to such contrasting environments, rendering their distribution/dominance close to ubiquitous. Among these, we note the presence of specific Sus-like PULs that are dedicated to polysaccharide degradation (Xu et al., 2003; Flint et al., 2008) and their extreme genome flexibility (Xu et al., 2007) that assures the capacity to gain new functions by LGT from species living in different habitats. Consequently, the recently revealed LGT linking environmental and gut *Bacteroidetes* (Hehemann et al., 2010) raises the question as to how the ancestral members of the intestinal microbiota might have evolved from plant-degrading species, settled in the GIT of early herbivores, such as marine protists. Evidently, gut symbionts have co-evolved with their hosts (De Filippo et al., 2010), notably in response to the consumption of specific food, and this interdependence likely shaped and still shapes the lifestyle of human populations (Ley et al., 2006a). This "food connection" points toward the fact that recurrent contacts between environmental and gut microbes can have beneficial effects (Rook and Brunet, 2005). At the same time, it underlines the potential problem of our modern lifestyle and the consumption of hyper-hygienic, extensively processed food for human health, depriving us of the environmental reservoirs of microbial genes that allow adaptation by lateral transfer (De Filippo et al., 2010; Sonnenburg, 2010). On the other hand, global travel and trade are providing contact to new types of food and to diverse populations, perhaps giving access to new microbes harboring novel genes destined for integration into our microbiome.

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Studying the enteric microbiome in inflammatory bowel diseases: getting through the growing pains and moving forward

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In this commentary, we will review some of the early efforts aimed at understanding the role of the enteric microbiota in the causality of inflammatory bowel diseases. By examining these studies and drawing on our own experiences bridging clinical gastroenterology and microbial ecology as part of the NIH-funded Human Microbiome Project (Turnbaugh et al., 2007), we hope to help define some of the "growing pains" that have hampered these initial efforts. It is our sincere hope that this discussion will help advance future efforts in this area by identifying current challenges and limitations and by suggesting strategies to overcome these obstacles.

Keywords: inflammatory bowel diseases, human enteric microbiome, microbial dysbiosis, microbial ecology, new generation DNA sequencing

The notion that the indigenous enteric microbiota play an important role in the etiopathogenesis of inflammatory bowel diseases (IBD; Annese et al., 2005) is now well accepted. This realization has prompted a flurry of research activity that is aimed at determining the mechanisms by which changes in the gut microbiota contribute to the development and progression of IBD. Much of this research has been made possible by advances in culture-independent microbial ecology. Microbial ecologists who have been studying complex communities in natural environments such as soil and seawater have leveraged advanced molecular microbiological techniques to profile the structure and dynamics of these microbial consortia. However, the application of these techniques to human-associated microbial communities has not been particularly straightforward. Most clinicians and researchers who study clinically related problems are not familiar with the concepts and techniques employed by microbial ecology. Conversely, most microbial ecologists have not had a great deal of experience interpreting their data in a clinical context, therefore, both sets of investigators need to understand and develop new paradigms. By working together, clinical experts and microbial ecologists can design studies that will leverage their respective expertise and avoid potential pitfalls.

CLINICAL CONSIDERATIONS IN APPROACHING THIS AREA

IBD IS NOT A SINGLE DISEASE

Inflammatory bowel diseases is not a single disease, nor is it fully or accurately represented by the commonly used classifications of Crohn's disease and ulcerative colitis (UC). Crohn's disease includes a wide range of presentations and can affect any part of the gastrointestinal tract from the mouth to the rectum. Crohn's disease causes a transmural inflammation that can be associated with structuring, penetrating, and perianal disease. By definition, UC is limited to the colon and is characterized by confluent mucosal inflammation starting at the rectum and extending proximally to various lengths in the colon. The remaining 10–15% of patients are given the diagnosis of "indeterminate colitis," a classification based on having features of both Crohn's disease and UC, but also reflecting the ambiguities of clinical descriptors. None of the terms take into account the many types of genetic and pathophysiological processes that can lead to disease having similar clinical phenotypes. Thus, studies aimed at establishing a role of enteral microbial dysbiosis in the etiopathogenesis of "IBD" are not likely to be useful without recognition or consideration of the complexity and heterogeneity of IBD patient populations. The solution must involve designing studies where more homogeneous patient subsets can be defined. Unfortunately, this task remains a difficult as few molecular and genetic markers have emerged that identify a clear disease subset. Thus, the challenge remains in developing better metrics to identify IBD patients that share genetic and/or pathophysiological features. When this is achieved, we can truly begin to understand the relationship of enteric microbiomes to etiopathogenesis and clinical outcomes.

MICROBIAL DYSBIOSIS IN IBD: CAUSE OR CONSEQUENCE?

Dysbiosis of the intestinal microbiota is commonly found in patients with IBD (Peterson et al., 2008), but in almost all cases, it

cannot be determined whether these changes are causal or merely consequences of the activated immune and inflammatory condition. Typically, large changes in 16S rRNA gene-based profiles are observed at the phylum level. These changes are characterized by blooms of Proteobacteria and decline in Firmicutes and Bacteroidetes (Frank et al., 2007), the latter typically associated with the microbiota of a normal, healthy colon (Eckburg et al., 2005). Similar patterns have been observed in other non-IBD inflammatory states (Lupp et al., 2007; Sekirov et al., 2008) and in experimental colitis (McKenna et al., 2008; Hoffmann et al., 2009; Nagalingam et al., 2011), suggesting that these changes are largely a consequence of the altered immune and inflammatory state. Similarly, studies of healthy and IBD-associated microbial functional metagenomes have shown significant differences (Manichanh et al., 2006). In the final analysis, however, these studies have resulted in the accumulation of large, descriptive datasets that have shed little light on fundamental mechanisms of IBD etiopathogenesis. What is needed are prospective studies, initiated at a time point before the onset of disease in order to capture information on functional and structural characteristics of the enteric microbiome that correlate with eventual development of disease or with maintained health. Unfortunately, these types of studies are difficult to do in IBD, because the means to identify subjects at risk do not yet exist. Moreover, long term, population-based studies that include microbiome analysis are impractical and cost-prohibitive. Nevertheless, a few opportunities exist for longitudinal studies of the enteric microbiome in IBD risk, particularly when the study questions and types of test subjects can be more precisely defined. As one example, the development of pouchitis in patients with UC is a condition where the incidence of disease is high and predictable (Hurst et al., 1996; Ferrante et al., 2008). Pouchitis is an inflammatory complication of the surgically created ileal pouch that serves as a pseudo-rectal vault in patients with UC who have undergone total colectomy. The condition is unique to UC, as it is rarely seen in patients with familial adenomatous polyposis (FAP) who undergo the same surgical procedure (Salemans et al., 1992). The study of pouchitis offers several advantages: (1) a clear "time-zero" can be defined when all patients are absent of disease and off medications, (2) each patient serves as his/her own control, (3) sampling of both luminal and mucosa-associated pouch microbiota is feasible, eliminating potential confounders such as the need for lavage preparation, and (4) the analysis of microbiome and host responses can be easily correlated with clinical outcomes.

CONFOUNDING VARIABLES ASSOCIATED WITH CLINICAL RESEARCH

Very few studies to date that have examined the relationship between IBD and intestinal microbial dysbiosis have taken into account confounding variables such as age of onset, disease duration, patient age, gender, life style, smoking history, ethnic background, diet, environmental exposures, surgical history, and medications. Each of these factors may be important in IBD patients and can independently impact host biology and enteric microbiota, directly or indirectly. Conclusions drawn from data analysis in absence of careful multivariate analysis or patient stratification can be misleading and lead to both type I and type II errors. As one example, dietary modifications are common in the management or clinical course of IBD patients. Dietary components such as fat and carbohydrates have also been shown to have dramatic effects on the enteric microbiota in animal models (Hildebrandt et al., 2009; Turnbaugh et al., 2009). Since the diets of patients in remission versus those that have active disease are likely to be quite different, this introduces a variable that confounds interpretation of data aimed at defining a causal relationship between changes in enteric microbiota and disease activity. Few studies have taken into consideration the types of medications patients may be taking. IBD patients take a number of medications which are aimed at altering the immune response and promoting intestinal healing. Although no data exists to indicate how the various classes of medications impact the intestinal microbiome in IBD, preliminary data from animal models suggest that medications may have a dramatic and sustained impact (Croswell et al., 2009; Hill et al., 2010; Manichanh et al., 2010; Robinson and Young, 2010). The nature of these confounding variables are perhaps more significant in complex immune-based disorders and challenge our ability to design studies that can yield unambiguous results. They underscore the importance of a team effort that involves clinician investigators, microbial ecologists, and biostatisticians in the design of microbiome-IBD studies in compiling, reviewing, and analyzing these important patient factors.

TECHNICAL NUANCES AND CHALLENGES OF MICROBIOME RESEARCH RELATED TO IBD

Researchers who are considering embarking on studies of the microbiome in IBD need to consider numerous factors before initiating their studies. The planning and design phase are essential and arguably the most critical aspects of any research endeavor. The explosion of microbiome-based technologies provides numerous research opportunities and emphasizes the need for collaboration between clinical researcher and microbiologist, particularly in the early stages of study development.

These considerations can be divided into two main categories. The first involves the clinical (e.g., patient-oriented) aspects of the research. The second major category involves consideration of the technical aspects of microbiome research. Although most researchers who study clinical aspects of IBD might consider the latter category to be of prime importance, it is critical to realize that the nature of microbiome research requires re-evaluation of the patient-oriented components of IBD research.

CLINICAL CONSIDERATIONS (SAMPLING, REPLICATES, ETC.)

Microbiome research involves characterizing complex microbial communities that inhabit a particular ecological niche. A major concern with regards to this question is exactly what samples should be studied. Current evidence suggests that in a genetically susceptible individual, IBD results from an abnormal interaction between the indigenous microbiota and the host epithelium/immune system (Sartor, 2008; Round and Mazmanian, 2009; Garrett et al., 2010). As such, consideration has to be given as to whether it is appropriate to sample luminal contents versus the microbes that are associated with the mucosa. It has been proposed that examination of fecal material is an appropriate surrogate for all of the microbial communities that are upstream of the rectum. In this manner, stool can be considered to be the "summary statement" of the entire gastrointestinal (GI) tract. However, while many of the organisms that inhabit the GI tract can be detected

in feces, the relative abundance of these organisms in fecal material is likely to be quite different from that found either luminally or mucosal-associated in more proximal sections of the GI tract. Since the relative abundance of microbes in a community is important to the actual function of that community, stool may not be the most appropriate analyte for microbiota research in all types of IBD (Lepage et al., 2005). For example in patients with ileal Crohn's disease, analysis of fecal material is likely to be a poor surrogate for the microbial community found in the terminal ileum.

Given the realization that fecal material may not be the most suitable sample for assessing microbial communities associated with IBD, despite the ease of obtaining this material and its relative abundance, many microbiome studies related to IBD utilize material retrieved via endoscopy. With regards to endoscopically harvested material, it should be noted that there is also an axial variation in the microbiota. Studies have demonstrated that the intestinal microbiota have both a structural and spatial organization that may be altered in patients with IBD and vary with disease activity (Swidsinski et al., 2002; Hu et al., 2010; Wang et al., 2010a,b). In addition to the axial variation, the variation in communities along the GI tract, coupled with the known regional variation in IBD from region to region, makes it important to take these variables into consideration when designing studies. A comprehensive experimental design might therefore involve sampling from both mucosal and luminal communities.

Another important consideration arises when obtaining samples via endoscopy; the standard bowel preparation regimens can have a significant impact on the gut microbiome. The washout of luminal intestinal contents can skew or abolish both the longitudinal and axial gradients that are normally present within the GI tract (Wang et al., 2010a,b) and thus affect the entire gut microbial community. Although bowel preparation is required for standard diagnostic and interventional endoscopic procedures, its routine use may adversely affect research endoscopies performed to understand the role of the indigenous microbiota in IBD. Even local endoscopic washes prior to sample collection could alter the community structure of the associated microbes and should be avoided or taken into consideration when interpreting findings. Furthermore, the exact sampling technique needs to be considered. Biopsies are commonly used, but cause local trauma (which may affect subsequent longitudinal samples) and survey a limited area. Brushings can be used, but it needs to be established whether these two techniques yield similar samples. Regardless of what types of specimens are obtained, investigators should carefully follow established protocols for collection, handling, and storage of samples. Inconsistencies in these steps may dramatically alter microbial communities thereby decreasing the reliability and accuracy of the results.

In addition to these considerations regarding sample acquisition from an individual patient, there are multiple considerations relating to overall planning and establishing a study population. It is becoming abundantly clear that although certain features of the gut microbiome are encountered in many normal individuals, there is also considerable person-to-person variation (Costello et al., 2009; Benson et al., 2010; Willing et al., 2010; Walker et al., 2011). As such, considerations related to replication, study size, and selection of controls related to microbiome research require significant attention. The analytic methods related to processing microbiome data involve statistical methods that previously were not commonly applied to biomedical research (see below). Therefore, the standard power analyses that are applied to clinical research are not always easily translated to microbiome study design. Given that this field is in its infancy, there is no consensus yet on which statistical methods and power analyses are most appropriate. However, given the ongoing work in this area, clarification of which statistical methodology to use is forthcoming. In addition, because many early microbiome studies have been aimed at finding associations between aspects of the microbiome and health or disease, these studies have been "hypothesis generating"; once they are completed, appropriately powered confirmatory studies can be designed.

Experimental replication also requires significant consideration (Prosser, 2010). Replication can be in the form of multiple samples from the same region from a given individual at a specific time, longitudinal sampling from an individual patient, or obtaining samples from multiple individuals stratified by specific clinical criteria. Each form of replication may be important depending on the actual clinical question posed. Selection of appropriate controls can also be problematic given the inherent individual variation in microbiome structure. Comparative studies of the microbiome in IBD have used "healthy" controls that included patients with irritable bowel syndrome (IBS), diverticulosis, acute self-limited colitis, and a variety of other gastrointestinal conditions. Clearly many of these controls may also have perturbations in their microbiome, which must be accounted for in the data analysis and interpretation of results. In some cases, patients might be able to serve as their own controls, which will be useful for understanding how the microbiota changes correlate with the course of disease. With such a study design the status of the microbiome within a given patient prior to a particular intervention, for example treatment with a biologic agent or antibiotics or surgery, could serve as the controls for subsequent samplings that were done in a longitudinal manner.

Finally, although study of human patients is a necessary component of IBD research, there is a distinct utility for using animal models of disease (Wirtz and Neurath, 2007). Animal models, including the wide variety of murine models of IBD, can be used in conjunction with human clinical and translations studies to address questions mechanism and causality in IBD pathogenesis. Specific hypotheses that arise from observations in human patients can be directly tested in appropriate animal models. The same considerations with regards to sampling need to be applied to animal model studies, especially to provide comparability with observations made in patients.

ANALYSIS OF THE MICROBIOME: WHERE TO BEGIN?

For investigators who have not conducted or even considered performing microbiome research the variety of analytic methods that are available can appear daunting (Robinson et al., 2010). Many of the culture-independent methods for studying microbial ecology have benefited from the advances in next-generation sequencing platforms (Andersson et al., 2008; Huse et al., 2008). Additionally advances in mass spectroscopy and other means for large-scale analysis of complex mixtures of proteins and metabolites have been applied for microbiome research in order to try and make sense of which methods might be appropriate for specific

biomedical questions. Investigators can start by understanding what types of questions each specific method is best suited to address. One way to consider the suite of methods for microbiome analysis available is to divide them into groups based on the specific types of information they provide about a given microbial community. There are several useful reviews that describe the available technologies (Zoetendal et al., 2008; Simon and Daniel, 2011). The first type of information available is information about the structure of a specific consortium of microbes. This can be thought of as a census of microbes both in terms of the number of different types of microbes and their relative abundance. The next type of information goes beyond community structure and provides a cataloging of functional capacity of the entire community. The final type of information that can be gained by certain analytic methods gives information regarding the *in situ* activity of the given microbial community. We will discuss each of these platforms in more detail as well as describe in general terms specific analytic methods that can provide each of these types of data.

16S SEQUENCE RETRIEVAL: CHOOSING THE APPROPRIATE PLATFORM

Landmark ideas and research from Woese et al. (1990) and Pace (2009) established a common metric for identifying microbes – the nucleotide sequence of the small subunit (SSU) ribosomal RNA. The gene encoding the SSU rRNA has a sedimentation coefficient of 16S which is unique to bacteria and archaea and allows distinction from the SSU rRNA from human eukaryotic cells. Initially, SSU sequences were obtained by amplification and sequencing of SSU genes from complex microbial communities and then compared to databases containing more than 2 million aligned rRNA gene sequences (DeSantis et al., 2006; Pruesse et al., 2007; Cole et al., 2009) to provide a census of microbes in each sample. More recently, the application of "next-generation" sequencing platforms has increased the number of sequences that can be obtained, as well as lowering the cost of analysis (Sogin et al., 2006; Huse et al., 2008).

Analysis of the data obtained by SSU sequence analysis continues to evolve, but two general approaches are used to bin or classify the sequences into microbial populations. Sequences can be compared to reference taxonomic outlines and binned based on similarity to references sequences ("phylotyping") or the sequences can be assigned to operation taxonomic units (OTUs) based on similarity to other sequences within a given dataset. There are relative advantages and disadvantages of each method (see Schloss and Westcott, 2011 for a discussion). It should be noted however, that the desire to "name" a given community member based on SSU analysis can be complicated by the fact that most existing taxonomies for bacteria are based solely on cultured organisms.

The number of sequences required to assess microbial communities depends both on the questions being asked as well as the spatial and temporal variability in a community. Deeper sequencing will uncover less common members of a community, which may be necessary to enumerate a particular pathogen, but shifts in overall community structure can be detected identified with many fewer sequences (Young and Schmidt, 2004; Antonopoulos et al., 2009). A critical factor in determining the depth of sequencing required to address a question is to assess variability within replicate samples and determine if the variability is less than that found in treatment level comparisons. Pilot studies with either clone libraries or high-throughput sequencing methods are essential to identify the degree of variability and will establish the extent of sequencing required in a full-scale experimental design.

The next step is to consider how the structure of the microbial community might relate to its function. 16S sequences on their own do not provide specific functional information. However, if there is a genome sequence available corresponding to a bacterium with a given 16S with a known function, it may be possible to infer the functional capacity. It should be noted that inference of the metabolic potential of an organism based on its SSU rRNA gene sequences may also be complicated by the lateral transfer of genes between microbes.

LOOKING AT THE "BIG PICTURE": METAGENOMES, METATRANSCRIPTOMES, AND *IN SITU* ANALYSIS

Rather than inferring metabolic potential from 16S rRNA gene sequences, the genetic diversity of the microbiome can be accessed directly through shotgun metagenomes (Handelsman, 2004; Riesenfeld et al., 2004; Streit and Schmitz, 2004; Gill et al., 2006). In this approach, DNA extracted from a sample of the microbiome is sequenced directly, rather than following amplification of a specific gene (e.g., 16S rRNA). The absence of a specific amplification step to recover microbial genes often means that suitable amounts of DNA from microbial communities are difficult to obtain, particularly without interference from host DNA. Physical methods for separating microbial communities from host tissue, including the user of lasers to remove attached microbes from epithelial cells in the GI tract (Wang et al., 2010a), can be effective, but typically provides insufficient DNA for direct sequencing. Fortunately, there are approaches for whole genome amplification that can be employed to produce sufficient DNA for metagenomic sequencing (Binga et al., 2008). Understanding the biases and variability introduced by each of these steps is essential for a meaningful analysis of the resulting sequences.

When sequences derived from metagenomes are compared to previously characterized genes, using platforms such as MG-RAST (Glass et al., 2010), a picture of the metabolic potential of a community emerges. Millions of sequences from shotgun metagenomes from the human GI tract (Qin et al., 2010) have been generated in an effort to identify those that are consistent with health and various disease states. It has been suggested that while the taxonomic structure of microbiomes can fluctuate considerably, the composition of metabolic genes remains consistent (Turnbaugh et al., 2009). The definition of OTUs for both rRNA genes and protein-encoding genes will certainly influence this interpretation of the data: defining the appropriate level of resolution in sequence analysis is central to future analysis of microbiome sequences.

A logical extension of the metabolic potential suggested by community metagenomic sequencing is insight into the actual activity of a community gained through metatranscriptomic sequencing (Gilbert and Hughes, 2011; Gosalbes et al., 2011). In this case, total RNA is isolated and structural RNAs removed to enrich for mRNA, which is then reverse transcribed into cDNA for sequence analysis. Rather than just revealing the potential activity, this will indicate which of the potential metabolic

pathways are actually being used on the basis of their transcription within the community. To move even closer to actual function, metaproteomics employs high-throughput, high-resolution mass spectroscopy to determine which proteins are actually present in a given community (Verberkmoes et al., 2009). This approach generally requires some knowledge of the coding potential of a community in order to make predictions about potential proteins based in mass/charge ratios, and thus is often combined with metagenomic sequencing. A final approach used to assess *in situ* function, often via mass spectroscopy, is to measure the complement of metabolites (e.g., short chain fatty acids, lipids, small molecules) associated with a community. This so-called metabolomics or metabonomics approach assesses function based on the presence of metabolites, many of which will be produced by specific members of the community (Martin et al., 2007; Kinross et al., 2011).

The three dimensional structure of microbial communities in the GI tract, particularly those in close proximity to epithelial cells may also provide useful information about the function of the community, including cell–cell interactions among microbes and between microbes and their host. Extraction and purification of DNA for microbiome analysis obliterates the architecture of microbial communities, but fortunately the sequence data gathered as part of a SSU microbial census can be used to design fluorescently labeled probes that permit visualization of the structural organization of microbes in preserved samples. The recent application of combinatorial labeling of probes and spectral imaging (Valm et al., 2011) offers the potential to visualize dozens of microbes in a community and holds considerable promise for microbiome studies.

SELECTING THE APPROPRIATE METHODOLOGY; AN ARGUMENT FOR THE TEAM APPROACH

With this immense armamentarium of tools for microbiome analysis, the decision as to which method to employ must return to the most basic considerations, namely, what is the scientific and/or clinical question(s) to be addressed? In some cases, associations with disease based on 16S sequence retrieval are an appropriate first step, in an exercise as we discussed earlier that can be thought of as being hypothesis generating. However, in order to specifically test a given hypothesis or to monitor the physiologic effects of specific microbiome alterations, functional assessments via metagenomics or metabolomics might be more appropriate. To help in such decision making, a "team science" approach is often necessary, bringing together clinicians with expertise in IBD with microbial ecologists, bioinformatics specialists, statisticians, and microbial physiologists. As demonstrated by the NIH Human Microbiome Project (HMP) and the European MetaHIT projects, collaborative teams of scientists from a broad range of disciplines working together to address questions of the microbiome in health and disease are an important and effective approach. Similarly, the study of IBD using a "systems science," with interdisciplinary teams and expertise will be essential for discovering the etiopathogenesis of these diseases, novel therapies, and potentially a cure.

SUMMARY

We propose that collaboration between microbial ecologists and clinician investigators is critical and should be considered an essential component for translational studies of the role of the microbiome in IBD. We caution against overzealous claims about the significance of the findings based on our current evidence. Existing descriptive studies should be used to generate hypotheses and help us move toward mechanistic studies that will truly help us discover the causes and potential cures for IBD and other gastrointestinal diseases. As our molecular techniques for evaluating the microbiome evolve and become more refined, the field needs to move beyond the descriptive studies which constitute the current state of microbiome-IBD research and toward mechanistic studies that will fundamentally improve and expand our understanding of IBD.

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