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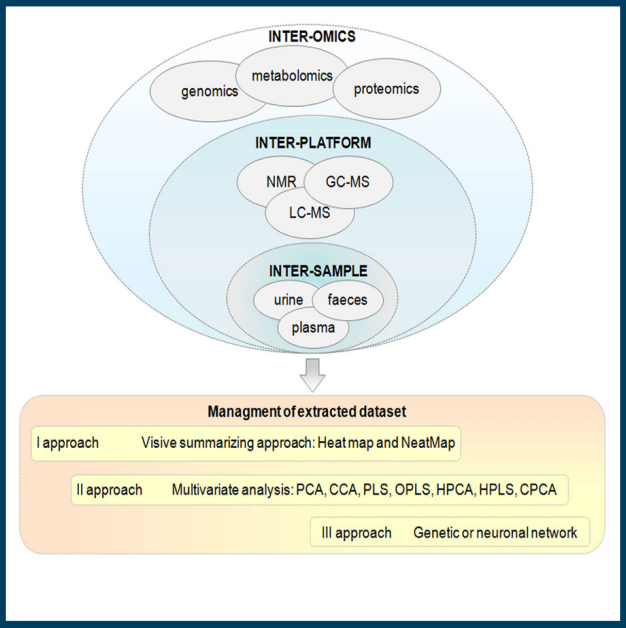
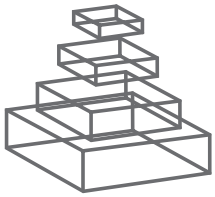


HUMAN GUT MICROBIOTA: ONSET AND SHAPING THROUGH LIFE STAGES AND PERTURBATIONS, 2ND EDITION

Topic Editor
Lorenza Putignani

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY



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HUMAN GUT MICROBIOTA: ONSET AND SHAPING THROUGH LIFE STAGES AND PERTURBATIONS, 2ND EDITION

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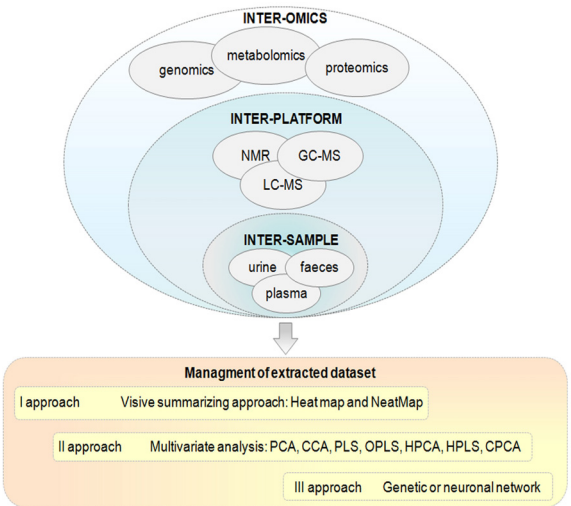

Schematic representation of statistical data integration methods in the area of inter-omics, inter-platform, and inter-sample integration. Image from Vernocchi P et al. (2012) Integration of datasets from different analytical techniques to assess the impact of nutrition on human metabolome. *Front. Cell. Inf. Microbio.* 2:156. doi: 10.3389/fcimb.2012.00156

Taxa distribution and density of gut microbiota habitants diverge in different individuals and is modulated by diverse determinants of temporal and spatial variability. Until the moment of birth, the gastrointestinal tract (GI) of a normal foetus is sterile. During birth and thereafter, bacteria from mother and surrounding environment colonize the infant's gut. Rapidly after the birth, bacteria start to appear in the faeces in a few hours and reach 108 to 1010 per gram of faeces within a few days. The epithelium at the interface between intestinal microbiota and lymphoid tissue plays a critical role in shaping the mucosal immune response. When commensal/pathogenic bacteria homeostasis is broken up, the perturbation leads to immunological impairment and disease.

Gut imbalance occurring during early life can lead to diseases such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), Hirschsprung's disease, respiratory and chronic pulmonary disease, immunological impairment, obesity and metabolic syndrome,

cardiovascular risks, etc., along all life stages. Investigation on individuality of gut microbiota onset and modulation requires to speculate on genetic and epigenetic affecting factors. Indeed, relationship between breast feeding, gut microbes, and immune system response appears crucial. However, classical microbiology is underpowered by its inability to provide unbiased representation of gut microbiota. Failure to cultivate in vitro the majority (60–80%) of microbiota taxa hampers a fulfilling description. The advent of high-throughput-omics-based methods, through the holistic view of the "systems biology", is opening new avenues in the knowledge of the gut ecosystem. In the coming years, the plasticity of the microbiota will be even exploited to provide new categories of therapeutics reflecting specific microbe-microbe modulation and microbe-host interaction.

Modern microbiology may concur in addressing one of the most complicated challenges of current medicine, which relies on the delivery of effective therapies tailored to exact biology or biological state of an individual, in order to enable the so called "personalized healthcare solutions".

This Research Topic covers several aspects of gut microbiota, mainly focused on the microbiologist's point of view, but extending to complementary approaches coming from clinical research, immunology, genetics, genomics and proteomics.

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EDITORIAL
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Human gut microbiota: onset and shaping through life stages and perturbations

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Microbial taxa distribution and density of gut microbiota habitants diverge in different individuals and is modulated by diverse determinants of temporal and spatial variability. Until the moment of birth, the gastrointestinal (GI) tract of a normal fetus is almost sterile. During birth and thereafter, bacteria from mother and surrounding environment colonize the infant's gut by vertical and horizontal transmission. Rapidly after the birth, bacteria start to appear in the feces in a few hours and reach 10^8–10^{10} per gram of faeces within a few days. The epithelium at the interface between intestinal microbiota and lymphoid tissue plays a critical role in shaping the mucosal immune response. When commensal/pathogenic bacteria homeostasis is broken up, the perturbation leads to immunological impairment and disease, crucial in "programming early phases" and dysbiosis establishment. Gut imbalance occurring during perinatal and neonatal life can lead to diseases such as irritable bowel syndrome (IBS), early-inflammatory bowel disease (IBD), respiratory and chronic pulmonary disease, immunological impairment, obesity and metabolic syndrome, hence triggering cardiovascular risks and nutritional impairment further along life stages. Investigation on individuality of gut microbiota onset and modulation requires to speculate on genetic and epigenetic affecting factors. Indeed, relationship between breast feeding, gut microbial taxa, and immune system response appears crucial in early life. However, classical microbiology is underpowered by its inability to provide unbiased representation of gut microbiota. Failure to cultivate *in vitro* the majority of microbiota taxa hampers a fulfilling description. The advent of high-throughput-omics-based methods, through the holistic view of the "systems biology," is opening new avenues to the knowledge of the gut ecosystem. In the coming years, the plasticity of the gut microbiota will be even exploited to provide new categories of therapeutics, providing therapeutic modification of the gut microbiota, on the basis of specific microbe-microbe modulation and microbe-host interaction, aiming to correct and improve life style conditions, and medical management of chronic patients such as cystic fibrosis-affected people. Modern microbiology may really concur nowadays in addressing one of the most complicated challenges of the current medicine, the achievement of effective patient-tailored therapies to exactly depict the biology and physiology of the microbiota "organ" of each person. This volume aims to highlight several "frontiers" aspects of gut microbiota studies, through the contribution of 12 articles describing the microbiologist's, the "omics" people point of view, but also the clinician's complementary approaches coming from different expertises and research areas, such as obstetrics, neonatology and hepatometabolic diseases. The first article, by Pessione (2012), presents a spectacular overview on Lactic Acid Bacteria (LAB), ancient microorganisms that, modulating sugar fermentation and decarboxylation/deimination, ensures their survival and colonization in the buffered environments of the GI trait, by a complex molecular cross-talk between LAB and host. LAB proteins, produced in response to gut, promote bacterial adhesion to mucosa and stimulate immune cells. Furthermore, LAB antagonistic relationships with other microorganisms constitute the basis for their anti-infective role. Thus, interesting perspectives for their utilization as antioxidant nutraceutical vectors are hypothesized. The second article, by Rigon et al. (2012) focuses on vertical determinants of gut variability associated to vaginal or cesarean delivery in the mother-child pair, and discuss breast- or formula feeding, also thoroughly discussed in the article by Guaraldi and Salvatori (2012). The two articles are particularly remarkable because they focus, by employing the point of view of the clinician, on the very early phases of gut microbiota programming, still "mysterious" and difficult to be unveiled. A cluster of three outstanding articles addresses, in a fascinating way, the gut "programming" and modification through life stages up to senescence (Kolling et al., 2012; Lagier et al., 2012; Ottman et al., 2012), with special emphasis on gut pathogens occurring during different ages (Kolling et al., 2012), or on data obtained by metagenome, metatranscriptome, and metaproteome integrated approaches (Ottman et al., 2012). Interestingly, Lagier et al. (2012), introduce the novel concept of "culturomics," a breakthrough in gut microbiota research, with the microbial identification and characterization performed by MALDI-TOF technology. In the review by Kolling et al. (2012), the process of aging is discussed in term of changes due to environmental exposures that subsequently affect the immune system and host-associated microbiota. Within the host's shifting setting, infections by enteric pathogens likely exploit these shifts with resultant initiation of pathogenesis and/or establishment of a mutualistic relationship with the host leading to potential dissemination of the pathogen. There is still much to be learned about how life stages and perturbations shape the gut microbial population, and how these changes influence health and disease and predominant enteric pathogens. Future insights into the interdependency between environment-host-microbe network will be essential for development of novel therapeutic approaches that treat or prevent enteric disease. Understanding the roles of these factors within the host is complementary to external approaches (*e.g.*, sanitation,

water treatment) for controlling or eradicating pathogen dissemination. The paper by Berrilli et al. (2012) strengthen the approach undertaken by Kolling et al. (2012), on gut ecosystems and pathogenicity and deal with the inter-play between parasites and microbial gut ecology. The paper has been strongly desired in the e-book *Human gut microbiota: onset and shaping through life stages and perturbations*, because of the enormous novelty of the topic, still not properly approached by the current literature. Indeed, complex communities, different from bacterial gut ecosystems, including parasites ("parasitome") and the even less studied fungi ("micetome"), surely co-evolve and interact with the gut microbiome modulating its ecology and physiology, reacting in a different way with and against the host. Indeed, interesting are the parasite immune-modulations at the gut enterocyte level on host immune reaction. On the other site, "omics" technology and their paramount effects on gut microbiota studies have been also deeply described in the articles by Shen et al. (2012) (genomics, metagenomics, and gut modifications), Vernocchi et al. (2012), and Masotti (2012) (gene expression, transcriptomics). Metabolic profiling has a wide potential to understand the complex interactions amongst components of the gut microbiota and to elucidate the relationships (cause/effect) between specific nutritional choices and related shifts in microbiota taxa composition. Particularly, the identification of gut metabolic signatures is extremely challenging, due to its linkage with nutritional choices. In fact, the whole set of metabolites, which can be detected in body fluids and characterizes the metabolic gut-associated phenotypes, can be named "metabotype" (Vernocchi et al., 2012). Dealing with a differential expression of miRNAs in different areas of GI tract can be considered as a function of microbiota composition. In these cases, intestinal microbiota are the "actors." Conversely, we should also think to miRNAs as "actors" when, under proper conditions, influence the regulation of goblet-cell differentiation. Therefore, an interconnected cycle could be envisaged, as suggested by the Opinion article by Masotti (2012), where miRNAs and gut microbiota are the two main partners.

Finally, two remarkable papers by Alisi et al. (2012), and Manco (2012) open new avenues on non-alcoholic fatty liver disease (NAFLD), one of the most common causes of chronic liver disease worldwide, and on the gut-brain axis in obesity endophenotypes, respectively. In NAFLD, several observations suggest a potential role of microbiota in NAFLD development, authors suggest that probiotics, for their excellent tolerability, may affect gut microbiota, hence representing promising therapeutic agents to revert NASH-related liver damage. Furthermore, the interesting relationships between gut microbiota and developmental programming of the brain, discussed in the Opinion article by Manco (2012), are providing unpredictable evidence in studies on mental retardation, autism and role of gut dysbiosis through the route to the final outcome of the overt disease.

In summary, the articles herein presented, discuss data and matters concerning current investigation and future perspective on gut microbiota studies. In our opinion, further insights will come from interdisciplinary approaches progressively provided by enlarged consortia, including researchers and clinicians, able to exploit high-throughput technological platforms to apply translational workflows in diagnostic pipelines and, finally, in patient care and treatment programs.

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OPINION ARTICLE
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Maternal factors pre- and during delivery contribute to gut microbiota shaping in newborns

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Normally at birth, the human infant gut is sterile, but it becomes fully colonized within a few days with bacteria from the mother and the environment (Salminen and Isolauri, 2008).

An altered gut microbiota composition has been associated with attenuated immune responses to inflammation in experimental models and humans (Fanaro et al., 2003; Ly et al., 2011; Vebø et al., 2011).

The pioneer microbiota of the neonate may affect future actions of the immune system (Conroy and Walker, 2008; Karlsson et al., 2011; Vael et al., 2011).

The relation between the neonatal gut microbiota and the development of allergic diseases and obesity has led to several clinical trials of probiotics (live bacteria given orally that allow for intestinal colonization) in human subjects both during pregnancy or in the neonatal period. Probiotic trials thus far have failed to show a consistent preventive effect (Litonjua, 2012).

Many factors contribute to the shaping of this complex ecosystem, and all must be taken into account. Maternal gut microbiota is a factor in neonatal colonization. Reduced concentrations of *Bifidobacterium* and *Bacteroides* and increased numbers of *Staphylococcus*, Enterobacteriaceae were detected in overweight compared with normal-weight pregnant women (Santacruz et al., 2010).

Increased Enterobacteriaceae numbers were related to increased ferritin and reduced transferrin, while *Bacteroides* numbers were related to increased HDL-cholesterol and folic acid levels (Santacruz et al., 2010; **Table 1**).

Population related factors are significant (Bäckhed, 2011). Karlsson et al. (2011) observed *Lactobacillus* in all neonates, other bacterial groups were detected only in 14–30% of the subjects (*Bifidobacterium*, *Enterococcus*, and the *Bacteroides fragilis* group). Fallani compared neonatal fecal samples from Sweden, Scotland, Germany, Italy, and Spain. *Bifidobacterium* genus was predominant (40% average proportion of total detectable bacteria), followed by *Bacteroides* (11.4%) and Enterobacteria (7.5%; Fallani et al., 2010). Differences in colonization pattern can be observed between infants in industrialized and developing countries (Adlerberth, 2008). Siblings increase the numbers of Bifidobacteria, while pets and country residence show no significance (Penders et al., 2006). Dominguez observed a neonatal colonization corresponding to maternal skin population in case of cesarean section and coincident with maternal vaginal flora in case of vaginal delivery (Dominguez-Bello et al., 2010).

Breastfeeding is a significant factor in the determination of neonatal gut microbiota. During lactation, cells from gut-associated lymphoid tissue travel to the breast via the lymphatics and peripheral blood (Donnet-Hughes et al., 2010).

Breast milk gives a flora rich in *Bifidobacterium* spp. Other obligate anaerobes, such as *Clostridium* spp. and *Bacteroides* spp., are more rarely isolated and also Enterobacteria and Enterococci are relatively few. Formula-fed babies are often colonized by other anaerobes in addition to Bifidobacteria and by facultatively anaerobic bacteria; the development of a "*Bifidus* flora" is unusual (Fanaro et al., 2003). Breastfeeding leads to higher *Lactobacillus* and lower count of *E. coli*, *Clostridium difficile*, *B. fragilis* (Penders et al., 2006; Fallani et al., 2010). After delivery, breastfeeding continues to enhance the original inoculum by specific lactic acid bacteria and Bifidobacteria and bacteria from the mother's skin enabling the infant gut microbiota to be dominated by Bifidobacteria. Modifying this exposure can take place by probiotic bacteria when breastfeeding is not possible (Conroy and Walker, 2008; Salminen and Isolauri, 2008). Fecal *Bifidobacterium* and *Lactobacillus*/*Enterococcus* spp. counts were higher in breastfed than formula-fed infants at 6 months (Rinne et al., 2006; **Table 1**). Maternal and neonatal medical treatment is an issue. Newborns from mothers treated with antibiotics perinatally had lower proportions of *Bacteroides* and members of the *Atopobium* cluster. Antibiotics lower the count of Bifidobacteria and *B. fragilis* group, according to Penders et al. (2006). Gut microbiota is influenced by perinatal conditions. Ley et al. (2005) found that obese pregnant mice have a 50% reduction in *Bacteroidetes* and a proportional increase in Firmicutes compared to normal controls on the same diet. One mechanism here could lie in the ability of specific gut microbes to induce excessive energy harvests (Collado et al., 2008). Overweight women show increases of *Clostridium*, *Bacteroides*, *Staphylococcus*, and *Akkermansia* during pregnancy according to the author. Their infants' fecal microbial composition was related to the weight and weight gain of their mothers during pregnancy (Collado et al., 2010; **Table 1**).

In extremely low-birth-weight infants characterized by antibiotic therapy, parenteral nutrition, delayed oral feedings, and intubation the gut is colonized by a small number of bacterial species; *Lactobacillus* and *Bifidobacterium* spp. are seldom identified (Fanaro et al., 2003). Rougé et al. (2010) indicated that the gastrointestinal tract of preterm infants, born less than 33 weeks, has a low biodiversity. According to Dai and

Table 1 | Effects of neonatal physiological and pathological conditions on gut microbiota onset and distribution.

Bacterial species	Prevalence/ abundance	Decrease/ abatement	Physiological conditions	Pathological conditions	Reference
Escherichia coli	+++		Increased ferritin/reduced transferrin		Santacruz et al. (2010)
Bacteroides spp.	+++		Increased HDL-cholesterol/folic acid		Santacruz et al. (2010)
Staphylococcus spp., *E. coli*	+++			Overweight pregnant women	Santacruz et al. (2010)
Bifidobacterium spp., *Bacteroides* spp.		+++		Overweight pregnant women	Santacruz et al. (2010)
Bifidobacterium spp., *Lactobacillus* spp.	+++		Baby breast feeding		Fanaro et al. (2003), Rinne et al. (2006)
Clostridium, Bacteroides, Enterococcus spp.		+++	Baby breast feeding		Fallani et al. (2010), Penders et al. (2006)
Bacteroides spp., *Atopobium* spp., *Bifidobacterium* spp.		+++	Perinatal antibiotics treatment		Penders et al. (2006)
Bacteroides spp.		+++		Mouse obesity	Ley et al. (2005)
Firmicutes	+++			Mouse obesity	Ley et al. (2005)
Clostridium spp., *Bacteroides* spp., *Staphylococcus* spp., *Akkermansia* spp.	+++			Overweight pregnant women	Collado et al. (2010)
Lactobacillus spp., *Bifidobacterium* spp.		+++		Extremely low-birth-weight infants	Fanaro et al. (2003), Rougé et al. (2010), Dai and Walker (1999)
Clostridium spp., *Bacillus* spp.	+++		Vaginal delivery		Penders et al. (2006), Lif Holgerson et al. (2011), Huurre et al. (2008)
Bacteroides spp., *Atopobium* spp., *Bifidobacterium* spp.		+++		Cesarean section	Fallani et al. (2010)
Bifidobacterium spp.	+++		Probiotic administration during pregnancy		Gueimonde et al. (2006)
Lactobacillus spp., *Enterococcus* spp., *Clostridium* spp.		+++	Probiotic administration during pregnancy		Rinne et al. (2006)

Walker (1999), premature infants requiring intensive care acquire intestinal organisms slowly, and the establishment of bifidobacterial flora is retarded. The aberrant colonization of the premature infant may contribute to the development of necrotizing enterocolitis.

Neonatal gut is related to mode of delivery. In infants born by cesarean section (C-section) the establishment of a stable flora is delayed (Fanaro et al., 2003). Significantly more bacterial taxa were detected in the infants delivered vaginally (79 species/species clusters) compared with infants delivered by C-section (54 species/species clusters; Lif Holgerson et al., 2011). Newborns delivered by C-section had lower proportions of *Bacteroides* and members of the *Atopobium* cluster (Fallani et al., 2010). Infants delivered by C-section had fewer bifidobacteria at an early age and were shown to mount a stronger humoral immune response (Huurre et al., 2008). At 1 month of age, the total gut bacterial cell counts per 1 g feces were higher in vaginally delivered infants. This distinction was mainly due to the greater number of Bifidobacteria in vaginally delivered infants. During the first year of life, the total number of immunoglobulin (Ig) A, IgG-, and IgM-secreting cells was lower in infants born by vaginal delivery than in those born by C-section, possibly reflecting different antigen exposure (Huurre et al., 2008).

Dominguez observed a neonatal colonization corresponding to maternal skin population in case of c-section and coincident with maternal vaginal flora in case of vaginal delivery (Dominguez-Bello et al., 2010). An extensive Netherlands study shows conclusively that vaginal delivery brings on a faster colonization by all species, mostly Bifidobacteria, with high *B. fragilis* and low *C. difficile* counts (**Table 1**). High *Clostridium* counts were associated with clinical complications and hospital admittance (Penders et al., 2006). Most significantly things change with age. The bacterial flora is usually heterogeneous during the first days of life, independently of feeding habits. After the first week of life, a stable bacterial flora is usually established (Fanaro et al., 2003).

The first bacteria to establish in the neonatal gut are usually aerobic or facultative anaerobic bacteria, like Enterobacteria, Enterococci, and Staphylococci. During their growth, they consume oxygen and change the intestinal milieu making it suitable for the proliferation of anaerobic bacteria. *Bifidobacterium*, *Clostridium,* and *Bacteroides* are among the first anaerobes establishing in the microbiota. As more oxygen-sensitive species establish and the complexity of the microbiota increases, the population sizes of aerobic and facultative bacteria decline (Adlerberth, 2008).

Vebo showed a decrease in Staphylococci from 10 days to 4 months and a peak of Bifidobacteria and *Bacteroides* at 4 months (Gueimonde et al., 2006; Vebø et al., 2011). Clinical effects of an altered neonatal colonization have been noticed. In the past 20–30 years, the prevalence of atopic diseases, particularly among children in the Western world, has increased. It has been suggested that Western lifestyle may have reduced the overall exposure to microbial stimulation early in life (Øien et al., 2006).

The pioneer microbiota of the neonate may affect future actions of the immune system (Karlsson et al., 2011). A close relationship between allergic sensitization and the development of the intestinal microflora may occur in infancy. Intestinal microorganisms could down-regulate the allergic inflammation by counterbalancing type 2 T-helper cell responses and by enhancing antigen exclusion through an immunoglobulin Ig-A response (Kirjavainen and Gibson, 1999). According to Vael et al. (2011), early colonization by *Clostridium coccoides* or *B. fragilis* could lead to asthma in later life.

Altered gut colonization could lead to obesity in later life. Germ-free mice are protected against developing diet-induced obesity. The gut microbiota affects expression of secreted proteins in the gut, which modulate lipid metabolism in peripheral organs and is a source of pro-inflammatory molecules that augment adipose inflammation and macrophage recruitment by signaling through the innate immune system (Bäckhed, 2011). An intriguing observation is that neonates treated with antibiotics during the first 6 months of life had an increased risk of overweight among children of normal-weight mothers (OR: 1.54, 95% CI: 1.09–2.17) with a decreased risk of overweight among children of overweight mothers (Ajslev et al., 2011). Trials with probiotic precursors administered to the mother have been reported. Maternal administration of *Lactobacillus rhamnosus* GG (L-GG, ATCC 53103) during late pregnancy promotes a Bifidobacteria profile of infant gut microbiota, similar to that of a healthy breastfed infant. Microbial diversity in neonatal gut microbiota was not influenced by this probiotic administration at 1 week postpartum (Ismail et al., 2012). In a prospective randomized study Gueimonde et al. (2006) showed that maternal *L. rhamnosus* administration during late pregnancy is associated in the neonates at 5 days of age with a higher occurrence of *Bifidobacterium brevis* and lower of *Bifidobacterium adolescentis*. Probiotic supplementation has been tried in newborns. One-hundred thirty-two neonates were randomized in a placebo group and the others were treated with *L. rhamnosus*. For 6 months after delivery, mothers had been treated prenatally for 6 months in the treatment group. At 6 months, there were less Clostridia in feces in the placebo compared with the probiotic group ($P = 0.026$), whereas after long-term follow-up at 2 years, there were less Lactobacilli/Enterococci and Clostridia in feces in the probiotic group than in the placebo group (Rinne et al., 2006; **Table 1**).

Rinne showed in another randomized trial of neonatal *L. rhamnosus* administration that at 3 months IgG-secreting cells in breastfed infants supplemented with probiotics was higher (Rinne et al., 2005). Chierici underlined the importance of a probiotic diet with bifidogenic activity of non-digestible but fermentable carbohydrates (Chierici et al., 2003).

In conclusion, we infer that many observations indicate the significance of bacterial neonatal colonization of the gut. A tighter control of factors influencing this phenomenon is warranted if results of preventive or therapeutic measures, or effects of maternal, or perinatal conditions is to be identified.

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Effect of breast and formula feeding on gut microbiota shaping in newborns

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INTRODUCTION

The gastrointestinal microbiota is a complex and dynamic ecosystem consisting of several hundreds of different microbes, mainly bacteria (10^{11-12} bacteria/g of colonic content, forming 60% of total fecal mass; Eckburg et al., 2005; O'Hara and Shanahan, 2006). Total number of bacteria exceeds 10 times the number of human cells, and the collection of microbial genome (microbiome) contains 100 times more genes than the human genome (Vael and Desager, 2009). Gut microbiota influence the growth and differentiation of gut epithelial cells, and play pivotal nutritive, metabolic, immunological, and protective functions (O'Hara and Shanahan, 2006). Its deregulation is involved in the pathogenesis of immunological, cardiovascular, and metabolic diseases (Hammer, 2011; Maslowski and MacKay, 2011; Harris et al., 2012).

The investigation on microbiota composition started in 1900 (Tissier, 1900) and has been performed by culturing methods since the recent advent of DNA sequence-based methods, that, thanks to their ability to identify a large number of species that cannot be cultivated, have allowed a more complete and rapid assessment of the gastrointestinal ecosystem (Palmer et al., 2007; Adlerberth and Wold, 2009). On the basis of 16S ribosomial – RNA encoding gene, more than 7000 distinct phylotypes have been detected in the human distal gut (Vael and Desager, 2009), with high inter-individual and age variability, but belonging to a limited number of broad taxonomic divisions (mainly the anaerobes *Bacteroides*, *Eubacterium*, *Clostridium*; Hayashi et al., 2002; Eckburg et al., 2005; Zoetendal et al., 2008). In a very recent study, Arumugam et al. (2011), by combining fecal metagenomes of individuals from different countries, identified three different enterotypes (with the prevalence of *Bacteroides*, *Prevotella*, and *Ruminococcus* species, respectively) that are not nation or continent specific, and showed that intestinal microbiota variation is stratified, not continuous, indicating further the existence of a limited number of well-balanced host-microbial symbiotic states. These enterotypes do not seem to differ in functional richness and apparently do not correlate with nationality, gender, age, or body mass index; at the same time, they seem to characterize and be quite stable in individuals, so that they can be restored after perturbations.

Gut microbiota composition and concentration physiologically varies throughout the gastrointestinal tract (increasing gradient from the stomach to the colon and characteristic gut-compartment distribution of microflora) and life stages, progressing from the newborn sterility to the extremely variable and dense colonization of adult gut, under the influence of various internal host-related and external factors (Mackie et al., 1999; Palmer et al., 2007).

ESTABLISHMENT AND DEVELOPMENT OF INTESTINAL MICROFLORA

The fetal intestine is sterile and bathed by amniotic fluid. The establishment of the gut microbial population is a continuous and complex process which starts at delivery and proceeds for several years through successive stages under the influence of several internal and external factors (Mackie et al., 1999; Fanaro et al., 2003; Adlerberth and Wold, 2009; Arumugam et al., 2011). Due to the abundance of oxygen in the neonatal gut, facultative aerobes (mainly Enterobacteriaceae, *Enterococcus*, and *Streptococcus* species) represent the first colonizers. *Escherichia coli*, *Enterococcus fecalis*, and *faecium* are the most represented, followed by *Klebsiella* and *Enterobacter*, and, more rarely and transiently, *Aeromonas*, *Pseudomonas*, *Acinetobacter*, alpha-haemolyticus Streptococci, and coagulase-negative Staphylococci (Penders et al., 2006; Adlerberth and Wold, 2009; Vael and Desager, 2009). Their expansion leads to a gradual consumption of oxygen, so to a more reduced environment, which favors the proliferation of obligately anaerobic bacteria, with the dominance of *Bifidobacterium*, *Bacteroides*, and *Clostridium*, followed by *Veillonella*, *Eubacterium*, and *Ruminococcus* species (Penders et al., 2006; Adlerberth and Wold, 2009; Vael and Desager, 2009). With time, anaerobic species will expand and outnumber facultative bacteria (Penders et al., 2006; Adlerberth and Wold, 2009; Vael and Desager, 2009), toward an adult-like microbiota profile, characterized by the preponderance of *Bacteroides* and *Firmicutes*, common occurrence of *Verrucomicrobia* and very low abundance of Proteobacteria and aerobic Gram negative bacteria (Palmer et al., 2007).

Colonizing bacteria derive from the mother (mainly vaginal and intestinal microflora), breast milk (for breast-fed babies), and surrounding environment (which includes equipment, air, other infants, and nursing staff). The pattern and level of exposure during the neonatal period is likely to influence the microbial

succession and colonization in the GI tract. Factors influencing microbial colonization can be grouped in two main categories: *extrinsic*, which include geographic area, maternal, and surrounding environment bacteria, mode of delivery, hygiene measures, and feeding habits, and drug therapies; and *intrinsic*, which include the neonatal genetics, bacterial mucosal receptors, and interactions, intestinal pH and secretions, peristalsis, and immune response (Mackie et al., 1999; Penders et al., 2006; Adlerberth and Wold, 2009; Fallani et al., 2010).

Diet has a dominant role over other possible variables such ethnicity, sanitation, hygiene, geography, and climate, in shaping the gut microbiota (De Filippo et al., 2010).

THE IMPACT OF BREAST-FEEDING ON MICROBIOTA COMPOSITION

Human milk presents a complex and dynamic composition, influenced by gestational age at parturition, lactation period, and woman's diet, which differs from formula feeding for nutrients concentrations and composition, and, more importantly, for the exclusive presence of growth factors, cytokines, immunoglobulins, and digestion enzymes (Le Huerou-Luron et al., 2010; Roncada et al., 2012).

Feeding type has been demonstrated to influence microbiota composition directly, by providing the substrates for bacterial proliferation and function (Le Huerou-Luron et al., 2010) and sources of bacterial contamination (originating from the nipple and surrounding skin, and milk ducts for breast milk; from the dried powder, the equipment used for preparation and the water used for suspension for formula milk; Mackie et al., 1999), and indirectly, by modulating the morphology, cell composition and physiology of the intestinal mucosa, and the pancreatic function (Le Huerou-Luron et al., 2010).

Studies performed in the last two decades on large populations of neonates aged ≥4 weeks, using both culturing and molecular methods, demonstrated that Bifidobacteria were the most represented species in both breast- and formula-fed infants (Balmer and Wharton, 1989; Mackie et al., 1999; Harmsen et al., 2000; Fanaro et al., 2003; Bezirtzoglou et al., 2011; Fallani et al., 2011). In most of the cases, no significant count differences were found between breast- and formula-fed infants (Mackie et al., 1999; Harmsen et al., 2000; Fanaro et al., 2003; Fallani et al., 2011). Conversely, Bezirtzoglou et al. (2011) observed more than two times increased numbers of bacteria cells in breast-fed infants, compared to formula-fed ones. Among Bifidobacteria, *Bifidobacterium breve*, *B. adolescentis*, *B. longum*, and *B. bifidum* are isolated in both formula- and breast-fed infants, whereas *B. infantis* is typical of breast-feds, *B. fragilis* of formula-fed infants (Mackie et al., 1999; Penders et al., 2006). In most of the studies, *Bacteroides* and Enterobacteria represent the two most frequently found species after Bifidobacteria (Balmer and Wharton, 1989; Mackie et al., 1999; Harmsen et al., 2000; Fanaro et al., 2003; Fallani et al., 2011). Palmer et al. (2007) and Favier et al. (2002) failed to demonstrate *Bacteroides* as part of the dominant microbiota; this finding could be due to the interfering action of other environmental factors (Penders et al., 2006).

Breast-fed newborns have been demonstrated to carry a more stable and uniform population when compared to the formula-fed ones (Bezirtzoglou et al., 2011). Relatively small amounts of formula supplementation of breast-fed infants will result in shifts from a breast-fed to a formula-fed pattern (Mackie et al., 1999), characterized by a wider microbiota spectrum. In particular, the counts and incidences and counts of *Clostridium* (*C. paraputrificum*, *C. perfringens*, *C. clostridiiforme*, *C. difficile*, and *C. tertium*) and *Streptococcus* (*S. bovis*, *S. faecalis*, and *S. faecium*) species, *Bacillus subtilis*, *Bacteroides vulgatus*, *Veillonella parvula*, *Lactobacillus acidophilus*, *Escherichia coli*, *Pseudomonas aeruginosa* (Benno et al., 1984; Mackie et al., 1999; Penders et al., 2006; Adlerberth and Wold, 2009; Fallani et al., 2010; Bezirtzoglou et al., 2011), *Enterococcus faecalis* (Jimenez et al., 2008; Adlerberth and Wold, 2009), and *Atopobium* (Bezirtzoglou et al., 2011) in the bottle-fed infants were significantly higher than those in the breast-fed infants. On the other hand, *L. rhamnosus* and *Staphylococci* prevailed in breast-fed infants (Adlerberth and Wold, 2009), with *Staphylococcus epidermidis* representing the distinctive tract of the feces of lactating woman and their infant, while it was almost absent in samples from feces of formula-fed infants.

The introduction of solid food profoundly impacts on the microbial ecology of breast-fed infants (Stark and Lee, 1982; Mackie et al., 1999; Adlerberth and Wold, 2009). Once dietary supplementation begins, microbiota profile of breast-fed infants changes toward formula-fed-infants profile, with the significant increase in the count of Enterococci and Enterobacteria, and the appearance of *Bacteroides*, Clostridia, and other anaerobic Streptococci (Stark and Lee, 1982; Mackie et al., 1999; Adlerberth and Wold, 2009). Between the first and the second year of life, differences between breast- and formula-fed infants are lost, and the microbiota profile resembles that of the adult for composition and microbiota counts (Stark and Lee, 1982; Mackie et al., 1999; Adlerberth and Wold, 2009).

THE IMPACT OF BREAST-FEEDING ON IMMEDIATE AND LONG-TERM HEALTH-EFFECTS

Numerous studies have been performed in the last decades with the aim to define short- and long-term effects related to the initial microbial gut colonization.

The nature of mucosal microflora acquired in early infancy has been proven to be critical in the determination of mucosal immune response and tolerance, so that alterations of gut environment are directly responsible for mucosal inflammation and disease, autoimmunity, and allergic disorders in childhood and adulthood (Gronlund et al., 2000; Ogra and Welliver, 2008). The type of feeding, through its selective action on bacterial colonization and growth, which, in turn, induce specific T cell responses and modulates substrates oxidation and consumption, has a major impact on the development of immune functions and oral tolerance (Palma et al., 2012). Systematic revisions of available data, pointed out the protective role of breast-feeding against the development of diarrhoea and necrotizing enterocolitis in the newborn (Mackie et al., 1999), and allergic and autoimmune diseases in childhood, including coeliac disease (Akobeng et al., 2006; Palma et al., 2012), type I diabetes and atopic dermatitis, whereas no clear risk reduction was evident in relation with asthma or allergic rhinitis (Bjorksten, 2005; Kramer, 2011). Later in life, breast-feeding has been associated to a reduced risk of inflammatory bowel diseases, cardiovascular diseases, obesity, and type-2 diabetes.

POTENTIAL PROTECTIVE ROLE RELATED TO THE ADDICTION OF PREBIOTICS AND PROBIOTICS TO FORMULA FOOD

Because of the recognized healthy properties, exclusive breast-feeding has been recommended by the World Health Organization for the first 6 months of life and supplemental breast-feeding up to 2 years and beyond (Le Huerou-Luron et al., 2010). According to a recent analysis by Le Huerou-Luron et al. (2010), the prevalence of exclusive breast-feeding in the world between 2000 and 2005 was 90% in the early postpartum, but only 41% at 4–6 months of age, with the highest percentages in Africa, followed by East and South Asia, Latin America and The Pacific, and, finally, Europe.

Considerable efforts have been made to mimic the composition of human milk by the addition to formula feeding of living bacteria (*probiotics*), non-digestible fibers, nucleotides and oligosaccharides (*prebiotics*), and bovine lactoferrin in order to induce a breast-fed-similar microbiota colonization in formula-fed infants, with the final aim to stimulate the maturation and proper function of the immune system (Fanaro et al., 2003; Rinne et al., 2005; Singhal et al., 2008; Vael and Desager, 2009). Overall, the implementation of formula food with prebiotics and probiotics has been demonstrated to be effective in changing microflora composition toward the desired breast-feeding pattern and stimulating immune response (Rinne et al., 2005; Sherman et al., 2009). No definitive results are available regarding the real health improvement related to their use (Bjorksten, 2005; Sherman et al., 2009; Vael and Desager, 2009) although in preterm infants their supplementation is associated with a reduced incidence of necrotizing enterocolitis and sepsis (Mackie et al., 1999; Lee, 2011).

CONCLUSIONS

Several studies performed in the past decades have clearly demonstrated the complexity of gut microbiota composition and the modulatory effect played by several endogenous and exogenous factors on it. Type of feeding in the first months of life appears as one of the most important determinants of the child and adult well-being, and its protective action seems to rely mainly on its ability to modulate intestinal microflora composition at early stages of life. In recent years, the implementation of milk formula with prebiotics, probiotics, and lactoferrin has been demonstrated to change newborns' microflora composition toward breast-feeding pattern and stimulate immune response. At the same time, no definitive results are available regarding the real health improvement, so that breast milk, whose beneficial health-effects are undoubtedly unique, has to be considered the food of choice for infants in the first 6 months of life.

For the same reasons, breast-feeding should be encouraged and, at the same time, new researches are advised in order to better define the composition of intestinal microbial ecosystem and the specific interactions amongst diet, microbiota composition, and children health.

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The function of our microbiota: who is out there and what do they do?

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Current meta-omics developments provide a portal into the functional potential and activity of the intestinal microbiota. The comparative and functional meta-omics approaches have made it possible to get a molecular snap shot of microbial function at a certain time and place. To this end, metagenomics is a DNA-based approach, metatranscriptomics studies the total transcribed RNA, metaproteomics focuses on protein levels and metabolomics describes metabolic profiles. Notably, the metagenomic toolbox is rapidly expanding and has been instrumental in the generation of draft genome sequences of over 1000 human associated microorganisms as well as an astonishing 3.3 million unique microbial genes derived from the intestinal tract of over 100 European adults. Remarkably, it appeared that there are at least 3 clusters of co-occurring microbial species, termed enterotypes, that characterize the intestinal microbiota throughout various continents. The human intestinal microbial metagenome further revealed unique functions carried out in the intestinal environment and provided the basis for newly discovered mechanisms for signaling, vitamin production and glycan, amino-acid and xenobiotic metabolism. The activity and composition of the microbiota is affected by genetic background, age, diet, and health status of the host. In its turn the microbiota composition and activity influence host metabolism and disease development. Exemplified by the differences in microbiota composition and activity between breast- as compared to formula-fed babies, healthy and malnourished infants, elderly and centenarians as compared to youngsters, humans that are either lean or obese and healthy or suffering of inflammatory bowel diseases (IBD). In this review we will focus on our current understanding of the functionality of the human intestinal microbiota based on all available metagenome, metatranscriptome, and metaproteome results.

Keywords: human intestinal microbiota, functional metagenomics, metatranscriptomics, metaproteomics

INTRODUCTION

The human intestinal microbiota is known to play a key role in several metabolic, nutritional, physiological, and immunological processes, and recent years have seen a rapid development in the techniques for studying this previously overlooked organ (O'Hara and Shanahan, 2006). The human microbiota is established after birth and starts out as a dynamic ecosystem, dominated by bifidobacteria, that stabilizes during the first 2–3 years (Koenig et al., 2011; Scholtens et al., 2012). During life the microbial composition increases in both diversity and richness (Scholtens et al., 2012) (**Figure 1**) and reaches highest complexity in the human adult, with several hundred species-level phylotypes dominated by the phyla *Bacteroidetes* and *Firmicutes* (Rajilic-Stojanovic et al., 2009). Each human individual reaches a homeostatic climax composition, which likely remains relatively stable during most of a healthy adult's life. Although the individual microbial composition has an "individual core" that varies at the bacterial phylotype level and depends on the depth of the analysis (Zoetendal et al., 2008; Jalanka-Tuovinen et al., 2011), the overall phylogenetic profile can be categorized into a limited number of well-balanced host-microbial symbiotic states, the so-called enterotypes (Arumugam et al., 2011). At the late stages of life the microbiota composition becomes again less diverse and more dynamic, characterized by a higher *Bacteroides* to *Firmicutes* ratio, increase in *Proteobacteria* and decrease in *Bifidobacterium* (Biagi et al., 2010) (**Figure 1**).

The establishment of the bacterial ecosystem in early life is suggested to play a role in the microbial composition and disease susceptibility throughout life (Scholtens et al., 2012). A different microbiota composition is associated with chronic intestinal disorders and the severity of perturbation during disease and after antibiotic use (Sekirov et al., 2010). Diet is another important factor in microbiota composition development. Early in life there is already an impact of the diet on the microbiome: the microbiota of breast-fed and formula-fed infants was found to differ significantly in both composition and diversity. Breast-fed babies contain a microbiota that is more heterogeneous than that of formula-fed babies and contain a higher taxonomic diversity (Schwartz et al., 2012) (**Figure 1**). In addition, food habits can influence microbiota composition, and malnutrition results in lower abundance of *Bacteroidetes* that are shown to be specialized in breaking down the carbohydrates in energy rich western diet

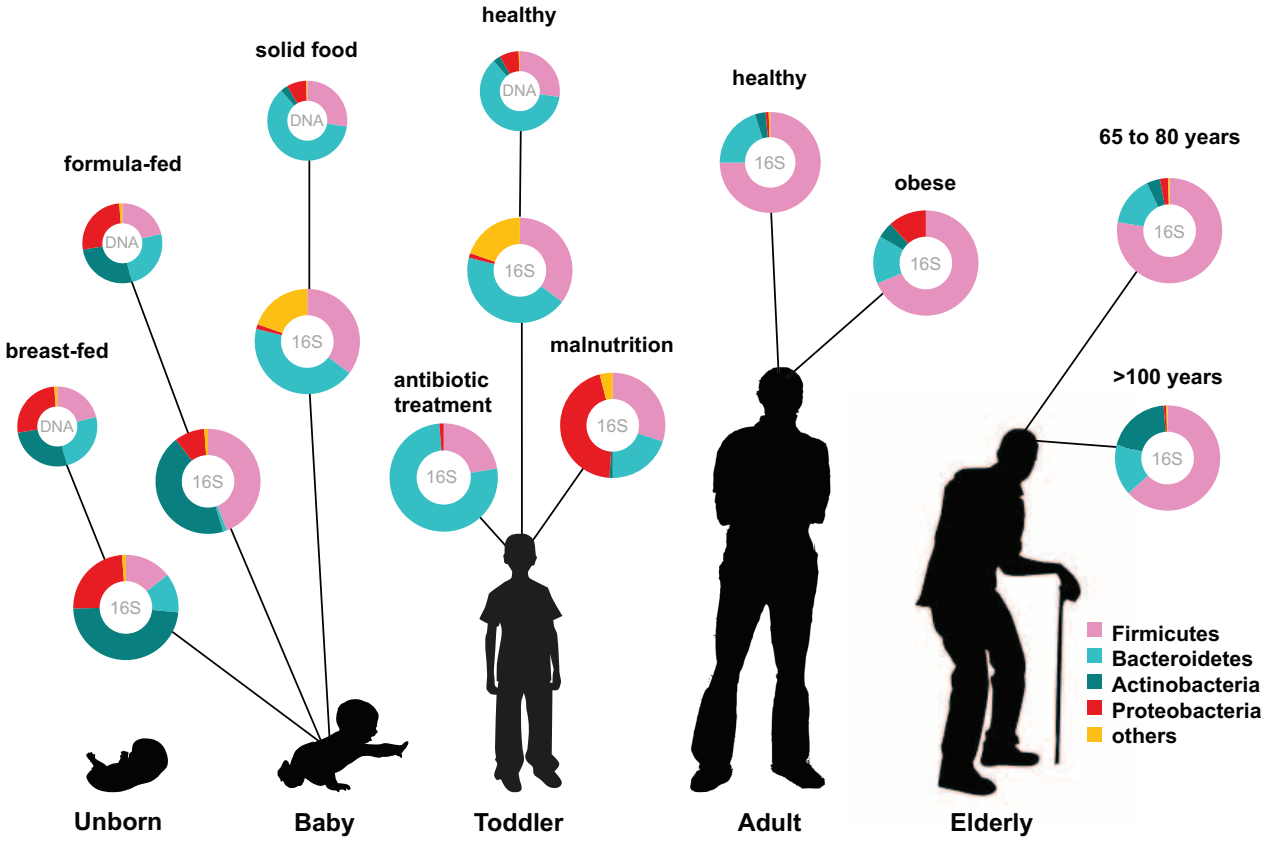

FIGURE 1 | Human microbiota: onset and shaping through life stages and perturbations. The graph provides a global overview of the relative abundance of key phyla of the human microbiota composition in different stages of life. Measured by either 16S RNA or metagenomic approaches (DNA). Data arriving from: Babies breast- and formula-fed (Schwartz et al., 2012), baby solid food (Koenig et al., 2011), toddler antibiotic treatment (Koenig et al., 2011), toddler healthy or malnourished (Monira et al., 2011), adult, elderly, and centenarian healthy (Biagi et al., 2010), and adult obese (Zhang et al., 2009).

foods. Diet-related diseases such as allergies and obesity are also characterized by microbiota changes. Obesity is characterized by a typical *Firmicutes* to *Bacteroides* ratio. Energy harvest potential and short chain fatty acids (SCFA) are determined by the microbiota composition and have a direct effect on the host epithelial cell energy availability. A microbiota stimulated with probiotic microbes can even decrease the incidence of infant diarrhea and atopic eczema due to host immune stimulation (Niers et al., 2009; Sjogren et al., 2009).

Numerous meta-omics approaches have vastly increased the knowledge available on the genome, activity and functionality of the complex ecosystem residing in the human gut. By far the most commonly applied technique is metagenomics, which is based on direct isolation and, in most cases, sequencing of the complete genetic material obtained from an environmental sample, such as the intestine. However, one of the biggest drawbacks of this technique is its inability to display the actual metabolic activity due to the fact that it detects both expressed and non-expressed genes. In addition, it may generate information from dead cells as it is known that more than half of the cells in fecal samples are non-viable or heavily damaged (Ben-Amor et al., 2005). Instead of focusing on microbiota composition the purpose of this review is to combine the available knowledge on microbial genomics with reports on the functional metagenomics, i.e., transcriptomics and proteomics approaches. This combination is expected to provide a refined understanding of the role of the microbiota and its capabilities in regulating human health.

ROLE OF THE MICROBIOTA IN EARLY AND LATE LIFE

EARLY LIFE

During natural birth, a newborn is exposed to the environmental, mainly maternal, microbiota which commences the acquisition of what we assume is a normal microbiota. The mode of delivery strongly affects the composition of the microbiota. In the case of caesarean delivery (C-section), other environmental bacteria form the basis for the microbiota instead of vaginal and faecal bacteria from the mother, reportedly resulting in a substantial reduction of bifidobacteria (Biasucci et al., 2008). In a comparison of the microbiota of babies delivered either vaginally or via C-section, it was shown that the newborns harbored undifferentiated bacterial communities across skin, oral, nasopharyngeal, and gut habitats regardless of delivery mode, and that the microbiota of C-section babies was similar to the skin communities of the mothers whereas vaginally delivered infants acquired bacterial communities resembling the vaginal microbiota of their mothers (Dominguez-Bello et al., 2010). Other factors influencing the

microbiota are the type of infant feeding, gestational age, infant hospitalization, and antibiotic use by the infant. The microbiota of breast-fed infants is dominated by bifidobacteria whereas the counts of *Escherichia coli*, *Clostridium difficile*, *Bacteroides fragilis* and lactobacilli are higher in exclusively formula-fed infants (Penders et al., 2006).

The composition of the intestinal microbiota plays an important role in immune system development, and it is possible that childhood allergies are related to differences in the microbiota (Sjogren et al., 2009). The intestinal defense of the preterm infant is rather immature and exaggerates inflammatory responses that can be evoked by both commensal and pathogenic bacteria (Nanthakumar et al., 2000). Thus, the first microbes colonizing the intestinal tract hold a pivotal role. Once the core microbiota has developed, it stabilizes and is expected to become less sensitive to modification. The question is at what age does the microbiota become adult-like and recent data with large cohorts of babies in various parts of the world indicate that this is at ages after at least 3 years (Yatsunenko et al., 2012).

The succession of the microbial ecosystem in the intestinal tract of newborns is a complicated process, which is not yet fully understood. The increasing diversity of the microbiota is believed to have an effect on the functional gene content over time. Several studies have provided insight in the infant gut community structure and its perturbations during early development and highlighted the impact of weaning (Favier et al., 2002, 2003). Moreover, a recent 2.5-year case study was reported, where sixty fecal samples were collected from a healthy infant (Koenig et al., 2011) (**Figure 1**). The results of this study showed a gradual increase in the phylogenetic diversity of the microbiome over time. Life events such as changes in diet, illnesses, and antibiotic treatments were associated with large shifts in the abundances of major groups in this single infant. Assignment of gene functions to the metagenomic data from this study revealed an enrichment of carbohydrate-metabolizing genes involved in lactate utilization from the very beginning of life. Interestingly, during an exclusive breast-milk diet, genes facilitating the breakdown of plant-derived polysaccharides were already present, suggesting that the microbiota is metabolically prepared to receive simple plant-derived foods. This is consistent with other observations that showed high similarity in the proportions of Clusters of Orthologous Groups (COG) encoding proteins specialized for the transport and metabolism of plant polysaccharides or COGs encoding proteins transporting and metabolizing human milk oligosaccharides (HMO) between infant and maternal microbiota samples (Vaishampayan et al., 2010) (**Table 1**). Baby gut microbiomes are also enriched in functions involved in using glycans represented in breast milk and the intestinal mucosa, even more so in the microbiomes of Amerindian and Malawian babies compared with US babies, possibly reflecting differences in the glycan content of breast milk (Yatsunenko et al., 2012).

Recently it has been shown that the use of specific human milk-derived glycans such as HMO utilization is not exclusive to certain well-known infant colonizers, such as *Bifidobacterium* species, since members of the genus *Bacteroides* can also use milk glycans as a sole carbon and energy source (Marcobal et al., 2010). Moreover, it has been shown that *Bacteroides thetaiotaomicron* responds to common structural motifs found in oligosaccharides from mother's milk and intestinal mucin glycans, suggesting that HMOs may mimic mucus glycans to attract mucin-adapted resident mutualists to an infant microbiota (Marcobal et al., 2011). However, specific HMO components select for HMO-adapted species such as *Bifidobacterium longum* subsp. *infantis*, and provide a selective advantage to this species *in vivo* when biassociated with *B. thetaiotaomicron* in the gnotobiotic mouse gut. The complex oligosaccharide mixture within HMOs thus attracts both mutualistic mucus-adapted species and

Table 1 | Percentages of COG categories expressed in the gut microbiota.

Population	Sample size (n)	COG categories (percentage of all genes)							Reference
		C	E	G	L	M	J	O	
mother 1m	1	5.0	6.0	11.0	10.0	8.5	3.0	3.0	Vaishampayan et al., 2010
mother 11m	1	6.0	10.0	12.0	5.5	6.0	5.0	2.5	"
infant 1m	1	7.0	7.5	11.5	6.0	6.0	4.5	4.0	"
infant 11m	1	4.0	11.0	12.0	7.5	6.0	5.0	3.0	"
female twin pair[a]	2	14.0	n/a	16.0	n/a	n/a	19.0	12.0	Verberkmoes et al., 2009
healthy volunteers	10	6.0–13.0	3.5–7.0	9.5–22.0	3.5–11.0	1.5–8.0	9.0–15.0	2.5–14.0	Gosalbes et al., 2011
female cotwin (TS28)[b]	1	8.0	7.0	8.0	6.0	6.0	9.0	6.0	Turnbaugh et al., 2010
female cotwin (TS29)[b]	1	8.0	6.0	9.0	5.0	5.0	10.0	7.0	"
female cotwin (TS28)[c]	1	5.0	6.0	8.0	9.0	6.0	7.0	5.0	"
female cotwin (TS29)[c]	1	5.0	7.0	9.0	9.0	6.0	6.0	4.0	"

COG descriptions: C, Energy production and conversion; E, Amino acid transport and metabolism; G, Carbohydrate transport and metabolism; L, DNA replication, recombination, and repair; M, Cell envelope biogenesis, outer membrane; J, Translation, ribosomal structure, and biogenesis; O, Post-translational modification, protein turnover, chaperones

[a] *out of the core proteome*

[b] *out of genes with high relative expression*

[c] *out of genes with low relative expression.*

HMO-adapted bifidobacteria to the infant intestine that likely facilitate both milk and future solid food digestion.

Little is known of the effect of diet on the composition and in particular the activity of the developing gut microbiota. Comparison of host epithelial cell gene expression and microbiota profile between breast- and formula-fed infants demonstrated that differences in the diet of infants can have an influence on the host gene expression via the gut microbiota (Schwartz et al., 2012). Virulence characteristics of the microbiota were the only functional properties that were found to differ among these two groups. Further analysis of the host transcriptome revealed a subset of eleven immunity and mucosal defense-related genes exhibiting evidence of a multivariate relationship with microbiome virulence characteristics. This provides additional proof for the capability of human milk to promote the mutualistic interactions between the mucosal immune system and the microbiome in maintaining intestinal homeostasis. Gene content analysis of the gut microbiome of 110 individuals including both adults and babies from Venezuela, Malawi, and the US revealed age-related changes in the metabolism of vitamins B12 (cobalamin) and folate (Yatsunenko et al., 2012). Genes involved in *de novo* biosynthesis of folate decreased with age whereas genes encoding most enzymes associated with cobalamin biosynthesis increased, correlating with previous data of blood levels of these vitamins in different age groups (Monsen et al., 2003).

The key players in the neonate gut are the bifidobacteria, which dominate the microbial community of human milk-fed infants. A number of studies using metagenomic approaches have also demonstrated the importance of this genus in the developing gut (Turroni et al., 2012; Yatsunenko et al., 2012), while at the same time other studies have reported low abundance or even absence of bifidobacteria (Palmer et al., 2007; Koenig et al., 2011), most likely due to technical biases related to DNA extraction protocols or the selected PCR primers. Genome analysis of *Bifidobacterium longum* subsp. *infantis* revealed a nutrient-utilization strategy targeting milk-derived molecules which are not of nutritional value to the infant (Sela et al., 2008). The proteomic profile of the organism grown on HMOs confirmed the activity of these genes (Sela et al., 2008). This suggests *B. longum* subsp. *infantis* coevolved with its infant host and under the presence of human milk compounds.

Furthermore, the type of milk, either mother's milk or formula, determines the colonization with different types of bifidobacteria. Breast-fed infants contain a high abundance of *Bifidobacterium breve*. In contrast, faecal samples from standard formula-fed infants lacked detectable amounts of this *B. breve* but contained *B. longum*. Remarkably, infants that received breast milk and later a prebiotic formula consisting of a standard formula milk containing a mixture of specific galacto- and fructo-oligosaccharides, continued to harbor a *B. breve*-dominant faecal population (Boesten et al., 2011).

Transcriptional analysis of the response of *B. longum* to human milk and formula milk indicated upregulation of genes involved in carbohydrate metabolism in breast milk, endorsing the concept that the bifidogenic effect of breast milk is primarily based on its oligosaccharides (Gonzalez et al., 2008). Moreover, the same study found upregulation of putative genes for cell surface type 2 glycoprotein-binding fimbriae associated with attachment and colonization in the intestine in both breast milk and formula milk when compared to semisynthetic medium with glucose. Transcriptome analysis of *B. breve* in a mouse model also showed differential expression of genes encoding for the production of type IVb tight adherence pili, which are essential for efficient *in vivo* murine gut colonization (O'Connell Motherway et al., 2011).

Another study comparing the bifidobacterial transcriptome of breast-fed infants and prebiotic-containing formula-fed infants showed that in the beginning of the intervention breast-fed infants had higher counts of bifidobacteria compared to the formula-fed infants (Klaassens et al., 2009). However, during the intervention the bacterial numbers and species diversity of *Bifidobacterium* increased significantly in the formula-fed infants, possibly on account of the galacto- and fructo-oligosaccharides in the formula. These prebiotics have also previously been shown to shift the bifidobacterial quantities toward those of breast-fed infants (Knol et al., 2005). The metatranscriptome analysis in babies revealed that the most prominent functions of the transcripts were related to carbohydrate metabolism, with higher expression of genes encoding these functions in breast-fed infants compared to formula-fed infants (Klaassens et al., 2009). This included significant expression of genes involved in HMO degradation. Moreover, the expression of genes involved in folate production was observed in all babies indicating that intestinal bifidobacteria produced this important vitamin involved in neural development. In the same study, a gene for bifidobacterial transaldolase, which is a key enzyme of the non-oxidative phase of the pentose phosphate pathway, was expressed in samples from all infants. Bifidobacterial transaldolase was also found in the only metaproteome study thus far to look at the infant gut microbiota (Klaassens et al., 2007). Production of the protein spot on a 2D-gel corresponding to this protein was increased over time, suggesting an increase in the numbers and activity of bifidobacteria in the infant's gut. Understanding the factors relating to the existence and host interactions of bifidobacteria and linking the functionality of this early intestinal colonizer to specific diets and groups of healthy or diseased individuals may eventually lead to the possibility of guiding the development of the microbiota. This can be achieved with pro- and prebiotic supplemented infant formulas that are aimed at increasing the bacterial diversity and a more optimal bifidobacterial community composition.

LATE LIFE

In addition to the beginning of life, the microbiota also undergoes significant changes toward the other extremity of life, old age. These alterations, however, are not clear-cut partially due to the various physiological changes that the elderly go through. These include factors such as modifications in lifestyle, nutritional behavior, increase in infection rates and inflammatory diseases, and therefore the need for more medication. All of these issues will certainly also affect the composition and activity of the microbiota, but the course and mechanisms behind these changes are not yet completely understood.

The process of ageing has been demonstrated to have a negative effect on the diversity of the microbiota, but different studies

have reported conflicting results on the age-related changes with regard to the two major phylogenetic groups. Assessment of the gut microbiota of the elderly with quantitative PCR revealed high levels of *Escherichia coli* and *Bacteroidetes* as well as a significant difference in the *Firmicutes* to *Bacteroidetes* ratio for adults (10.9) and elderly individuals (0.6) (Mariat et al., 2009). In this study the total bacterial counts for adults and seniors were comparable whereas another study, employing cytosine (%G + C) profiling and 16S rRNA gene sequencing, described a significant reduction in overall numbers of microbes in elderly subjects compared to young adults (Makivuokko et al., 2010). They also observed lower numbers of *Firmicutes* and an increase in *Bacteroidetes*, with lowered amounts of known butyrate producers belonging to *Clostridium* cluster XIVa.

Another study, which included young (20–40 years old), elderly (60–80 years old) and an additional group of centenarian citizens (∼100 years old), clearly demonstrated that the process of ageing coincides with decreasing microbiota diversity (Biagi et al., 2010) (**Figure 1**). By using the Human Intestinal Tract Chip (HITChip) and qPCR, they observed that the composition of microbiota was quite similar between the young and the elderly groups represented by dominant portions of *Firmicutes* and *Bacteroidetes* (95% of total bacteria). The centenarian group also showed a dominant portion of *Firmicutes* and *Bacteroidetes* (93% of total bacteria). The *Firmicutes/Bacteroidetes* ratios obtained for the centenarians, elderly and young adults were 3.6, 5.1, and 3.9, respectively. However, there was a significant decrease in the *Firmicutes* subgroup *Clostridium* cluster XIV and an increase in *Bacilli* in the centenarian group. Furthermore, there was a significant increase in several facultative anaerobes, members of the *Proteobacteria* phylum, many of which constitute opportunistic pathogens. This rearrangement of the microbiota does not seem to be in favor of the aging subjects that showed an increased level of circulating inflammatory cytokines. These were inversely associated with bacteria belonging to *Clostridium* cluster XIV and *Clostridium* cluster IV that include the main butyrate-producers in the gut. Butyrate has been associated with a range of health effects from anti-inflammatory properties to enhancement of intestinal barrier function (Macfarlane and Macfarlane, 2011).

Recently, pyrosequencing of tagged PCR-amplified 16S rRNA genes was applied to characterize the fecal microbiota of 161 seniors aged 65 years and older in the ELDERMET consortium (Claesson et al., 2011). In this extensive study the elderly microbiota was observed to be dominated by the phylum *Bacteroidetes* (57%) compared with *Firmicutes* (40%). However, the proportions of the major phyla showed extraordinary variation between individuals, with the proportion of *Bacteroidetes* ranging from 3 to 92% and *Firmicutes* from 7 to 94%. In addition to the general composition, also the core microbiota of the elderly differed substantially with that of young adults, characterized by a shift to a more *Clostridium* cluster IV-dominated community in the elderly. The microbiota of the elderly showed temporal stability for the majority of subjects as revealed by analysis of 3-month follow-up samples.

These studies indicate that there undoubtedly are fluctuations in the elderly microbiota, but both the threshold for an "aged" microbiota and the trends for these changes seem to be highly variable. Some of these differences may be explained by country-specific dietary habits, as the most recent studies used separate cohorts from two different European countries, Italy (Biagi et al., 2010) and Ireland (Claesson et al., 2011). The living environments of elderly people are highly dependent on their health status, with healthier seniors living independently and subjects with medical issues often living in nursing homes. These factors can also influence the aging gut microbiota. Follow-up studies assessing the function of the elderly gut microbiota by functional metagenomic techniques already applied for the infant and adult microbiota will shed more light on these issues and reveal prospects for possible dietary interventions aimed at improving the health of the elderly.

MICROBIOTA ACTIVITY IN RESPONSE TO DIET

Host dietary habits appear to affect gut microbiota composition, but the actual association between different diets and the microbial community composition as well as the underlying causes for this are still unclear. Although there was no clear environmental or genetic explanation found for the initial clustering of the enterotypes (Arumugam et al., 2011), these were found to be strongly associated with long-term diets, with protein and animal fat correlating with the enterotype characterized by high levels of *Bacteroidetes*, and carbohydrates with the *Prevotella* enterotype (Wu et al., 2011). Differences in microbiota composition as a result of diverging dietary habits was also shown in a comparison of the microbiota of European children, who consumed a diet high in animal protein, sugar, starch and fat and low in fiber, and children from Burkina Faso, where the predominantly vegetarian diet consists mainly of carbohydrates, fiber and non-animal protein (De Filippo et al., 2010). The European microbiome was enriched with *Firmicutes* and *Proteobacteria*, whereas *Actinobacteria* and *Bacteroidetes* were more represented in the African children. Interestingly, *Xylanibacter* and *Prevotella* were only present in the children from Burkina Faso, leading the authors to hypothesize that members of these genera could improve the ability to extract calories from indigestible polysaccharides commonly consumed in rural Africa indicating a coevolution of the microbial community with the polysaccharide-rich diet. Malnourished children from poor socio-economic status families in Bangladesh were found to have lower diversity of gut microbiota compared to healthy children from moderate to high income families in the same region, characterized by lower relative abundance of *Bacteroidetes* and a dominance of *Proteobacteria* (Monira et al., 2011). The authors suggest that the low presence of *Bacteroidetes*, which are known to digest complex dietary material and thus improve energy extraction from various foods, and the higher presence of potentially pathogenic *Proteobacteria* might contribute to explaining the poor health of the malnourished children.

In a metagenome study, short-term dietary intervention (high-fat/low-fiber or low-fat/high-fiber diets) lead to rapid changes in the microbiome composition but was not sufficient to shift individuals between the two enterotypes described in the same study (Wu et al., 2011). Few functional gene categories, including bacterial secretion system, protein export, and lipoic acid metabolism, differentiated between the two test diets suggesting

a shift in selected bacterial functions in response to the dietary changes. Microbiome analysis of subjects on a diet rich in protein, typically consumed in the US, showed enrichment of multiple Enzyme Commission (EC) groups when compared with Malawian and Amerindian subjects consuming a diet high in carbohydrates (Yatsunenko et al., 2012). These included degradation of glutamine and other amino acids, catabolism of simple sugars, vitamin biosynthesis, and bile salt metabolism. Degradation of glutamine has earlier been found to be overrepresented in carnivorous mammalian microbiomes, while glutamate synthase, which was enriched in Malawian/Amerindian microbiomes, was present in higher proportions in herbivorous mammalian microbiomes (Muegge et al., 2011).

Several metatranscriptome and metaproteome studies describing the human intestinal microbiota have confirmed the importance of bacterial functions related to carbohydrate metabolism in the colon. Enrichment of these genes has earlier been shown in metagenomic studies of the human gut (Gill et al., 2006; Kurokawa et al., 2007; Turnbaugh et al., 2009a). Metatranscriptome analysis of fecal samples from two healthy volunteers found that most expressed genes (26% of all sequenced and annotated transcripts) were involved in the metabolism of carbohydrate (Booijink et al., 2010). Recently the majority of bifidobacterial transcripts within the fecal community of adults were also reported to be involved in metabolism of carbohydrates of plant origin (Klaassens et al., 2011).

Similar results were seen in a transcriptional analysis of fecal samples from a monozygotic, obese twin pair (Turnbaugh et al., 2010) (**Table 1**), and metatranscriptomics analysis of fecal samples from ten healthy volunteers (Gosalbes et al., 2011) (**Table 1**). Metatranscriptomic data from the less studied small intestinal microbiota showed enrichment in sugar phosphotransferase (PTS) and other carbohydrate transport systems, as well as energy- and central metabolic, and amino acid conversion pathways as compared with the metagenome (Zoetendal et al., 2012). This suggests rapid uptake and fermentation of available simple sugars by the small intestinal microbiota, compared to the degradation of more complex carbohydrates by the bacteria in the colon. The importance of carbohydrate metabolism is also evident from the enormous amount of carbohydrate-active enzymes (CAZymes) present in the gut microbiome. By applying a multi-step functional screening procedure of a metagenomic library from the feces of volunteer following a fiber-rich diet, 73 CAZymes from 35 different families were recently discovered (Tasse et al., 2010).

Shotgun metaproteomics approach used to identify microbial proteins in fecal samples from a female twin pair identified several COG categories more highly represented in the microbial metaproteome compared to the average metagenome (Verberkmoes et al., 2009) (**Table 1**). A high proportion of the proteins that were equally abundant in both samples were from common gut bacteria, such as *Bacteroides*, *Bifidobacterium,* and *Clostridium*. These included proteins involved in translation, carbohydrate metabolism and energy production. In another study, two human fecal samples were analyzed and the functions of the identified proteins were predicted (Rooijers et al., 2011). The most abundantly present COGs were involved in translation, energy production, and conversion as well as carbohydrate transport and metabolism, which supports the findings of studies linking the microbiota with carbohydrate metabolism (Kovatcheva-Datchary et al., 2009). The study also pointed out the abundance of *Akkermansia muciniphila*, the only intestinal member of the *Verrucomicrobia*, within the microbiota and showed that most of the proteins produced by these bacteria are involved in carbohydrate transport and metabolism as well as amino acid transport and metabolism. This is in line with observation that *A. muciniphila* can use mucin as the sole carbon and nitrogen source (Derrien et al., 2008). The fecal samples were also subject to metagenome sequencing and the phylogenetic diversity was determined with two approaches, 16S rRNA sequence analysis of the metagenomic data sets and an abundance analysis of the metagenomic sequences using a synthetic metagenome as reference set. The results showed that *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Verrucomicrobia,* and *Proteobacteria* were the dominant groups in the microbiota of the study subjects.

These results were further confirmed by analysing the gut metaproteome of three healthy subjects over a period of 6–12 months (Kolmeder et al., 2012). In this study, proteins involved in carbohydrate transport and metabolism accounted for over 10% of the detected proteins, forming a part of the core metaproteome found in all the test subjects. The glycolysis pathway, in particular, was noticeable with several related enzymes identified. After assigning the spectral hits for each COG functional category per phylum, it was apparent that *Firmicutes* and *Actinobacteria* were responsible for the active carbohydrate metabolism, while *Bacteroidetes* showed more mixed functions. Both *Firmicutes* and *Bacteroidetes* were found to have an active carbohydrate metabolism on a transcriptional level in an earlier report (Gosalbes et al., 2011). Furthermore, Kolmeder et al. (2012) observed that the majority of the identified actinobacterial peptides were predicted to be involved in sugar metabolism. The importance of carbohydrate metabolism has been shown also previously for the core genome of bifidobacteria (Bottacini et al., 2010). Temporal analysis showed that the metaproteome is stable over time, as is the microbial composition of the gut, suggesting that homeostasis in function and composition of the intestinal microbiota are tightly linked (Kolmeder et al., 2012).

Recently, a metatranscriptomics approach with RNAseq has been applied to investigate the effect of a fermented milk product (FMP) containing several probiotics on the gut microbiome of gnotobiotic mice colonized with a model human gut microbiota and monozygotic twins (McNulty et al., 2011). There were no or minimal changes observed in the bacterial species composition in mice and humans after consumption of FMP. Still, transcriptional analysis revealed significant changes in numerous metabolic pathways, especially in carbohydrate metabolism, in both mice and human subjects. The question, however, is whether this reflects a functional difference in the colon or is a result of technical or biological effects such as variations in the transit time of the fecal material used for this analysis.

Metagenomic approaches combined with studies using gnotobiotic animals colonized with only a few known microorganisms or even the entire human fecal microbiota provide a

powerful tool for examining the relationship between the host and the functionality of the microbial community under controlled conditions. A study of humanized gnotobiotic mice transplanted with either fresh or frozen adult human fecal microbial communities into germ-free C57BL/6J mice revealed a stable and heritable colonization which enabled a diet intervention, where the mice were switched from a low-fat, plant polysaccharide to a high-fat, high-sugar diet (Turnbaugh et al., 2009b). This diet change induced a structural shift in the microbiota within one day and presented an enrichment for various KEGG pathways involved in nutrient processing compared to the control diet. Metatranscriptome analysis of rRNA-depleted RNA isolated from the ceca of the humanized mice demonstrated a clear difference in the gene expression of the mice on the Western diet compared to the control group, with upregulation of clusters containing *Clostridium innocuum* strain SB23 genes encoding Western diet-associated transcripts (pyruvate formate-lyase, PTS, phosphoglycerate kinase) and *Firmicutes* gene clusters encoding ABC-type sugar transport systems.

A shift in the microbial community was also seen after switching both wild-type and RELMβ-deficient mice to a high-fat diet, indicating that the diet itself was responsible for the detected changes independent of obesity (Hildebrandt et al., 2009). RELMβ is a colonic goblet cell-specific gene, whose expression is dependent on the presence of the gut microbiome. After the dietary switch the amounts of *Proteobacteria*, *Firmicutes*, and *Actinobacteria* increased whereas *Bacteriodetes* decreased, as measured from fecal samples. Analysis of gene functions revealed a decrease in the number of metabolic genes under the high-fat condition, possibly as a result of nutrient deficiency. However, as also noted by Turnbaugh et al. (2009b), a group of genes for ABC-transporters increased in abundance, indicating adaptation to the high-fat diet by enhancing nutrient intake in an environment with limited substrate availability.

Mice colonized with 10 sequenced human gut bacteria, and fed with a series of refined diets showed that casein concentration was highly correlated with the yield of total DNA per fecal pellet in all 17 test diets (Faith et al., 2011). The abundance of all of the ten species was significantly associated with casein, with seven of them positively correlated with casein concentration and three negatively correlated. None of the diets caused significant changes in the gene expression of the bacterial species, analyzed by RNA-sequencing, but high expression of genes predicted to be involved in pathways using amino acids as substrates for nitrogen, as energy and/or carbon sources were found for the species positively correlated with casein.

In conclusion, the studies to date endorse the concept that the intestinal microbiota thrives on using polysaccharides and peptides, which are indigestible to human (Guarner and Malagelada, 2003). The metagenomic data are confirmed on a functional level by the metatranscriptomics and metaproteomics data. The composition of the microbiota in the colon is dominated by *Firmicutes* that appear to be active in carbohydrate metabolism whereas *Bacteroidetes* show activity in a number of functions like energy production and conversion as well as amino acid transport and metabolism, in addition to carbohydrate metabolism. The complex polysaccharides are degraded by a specialized microbial community and the released oligosaccharides can in turn be used by other commensal bacteria. In this manner, diet is has a crucial influence on the intestinal microbial activity.

MICROBIAL IMBALANCES AND DISEASE

INFLAMMATORY BOWEL DISEASES

The gut microbiota has been connected to several diseases, with obesity and inflammatory bowel diseases (IBD) representing the most studied disorders to date. Most research about potential differences of microbiota related to different disease states has so far focused on describing the composition and diversity of the microbiome in patients compared to healthy subjects, and consequently revealing interesting associations between them. In order to get a better understanding of the underlying mechanisms of the relationship between the microbial communities and specific disorders, functional microbiomic approaches need to be employed.

Despite exhaustive research efforts, the etiology and pathogenesis of IBD, including Crohn's disease (CD) and ulcerative colitis (UC) have stayed unclear. The causes of these intestinal diseases are most likely linked with both human gene- and microbiome-associated factors (Pflughoeft and Versalovic, 2012). CD and UC patients seem to harbor separate microbial communities both from each other and healthy subjects, and also have lower bacterial diversity compared to healthy people (Manichanh et al., 2006; Dicksved et al., 2008; Qin et al., 2010). Several bacterial groups have been implied to be either increased or decreased in association with IBD. However, it is not clear whether this dysbiosis is the reason for the inflammation in IBD, or simply something caused by the disturbed environment in the GI tract.

Metagenomic studies and microarray analyses have demonstrated a reduction of *Firmicutes*, such as *Faecalibacterium prausnitzii*, in CD (Manichanh et al., 2006; Kang et al., 2010). A 16S rRNA gene pyrosequencing study of twin pairs who were concordant or discordant for CD or UC showed a clear division in the microbial composition between CD and healthy individuals but not between UC and healthy individuals (Willing et al., 2010). There were more *Firmicutes* detected for colonic involvement CD and less for ileum localized CD (ICD) compared to healthy subjects. In addition to *F. prausnitzii*, also other core members of the microbiota, such as *Roseburia*, were less abundant in ICD. Interestingly, a separate study analyzing the same samples showed clear shifts in metabolic profiles corresponding to the same bacterial groups (Jansson et al., 2009). Pathways with differentiating metabolites included those involved in the metabolism and or synthesis of amino acids, fatty acids, bile acids, and arachidonic acid.

A recent analysis of the fecal microbiota of UC patients in relapse and remission further confirmed the reduction of bacterial diversity in these patients and showed that this mainly affects members of the *Clostridium* cluster IV within the phylum Firmicutes (Rajilic-Stojanovic et al., 2012). The authors also speculated on the role of SCFA in UC as they reported reduced numbers of butyrate-producing bacteria, along with other studies (Frank et al., 2007), and a disturbed abundance of typical propionate producers. A depletion of one propionate producer, *A. muciniphila*, was observed in the fecal samples while another one, *Megamonas* sp. was increased. *A. muciniphila* was previously

found to be decreased in biopsies of patients with UC with an associated increase in *Ruminococcus* sp. (Png et al., 2010). The role of butyrate and propionate, both of which have anti-inflammatory properties (Tedelind et al., 2007), in UC is still under debate (Chapman et al., 1994; Roediger et al., 1997). These and forthcoming studies will eventually help in screening and diagnosing IBD patients.

OBESITY AND METABOLIC SYNDROME

Obesity and obesity-associated metabolic disorders, such as metabolic syndrome and type 2 diabetes have been suggested to be associated with the composition and function of the intestinal microbiota. Initial research showed an increase in the relative abundance of *Firmicutes* and decrease in *Bacteroidetes* in both obese mice (Ley et al., 2005) and humans (Ley et al., 2006), but later studies have failed to endorse these findings and showed inconsistent results with respect to the changes in the microbiota of obese people (Nadal et al., 2009; Santacruz et al., 2009, 2010; Zhang et al., 2009; Schwiertz et al., 2010) (**Figure 1**). In addition, the transfer of the gut microbiota of obese (*ob/ob*) mice to germ-free wild-type mice causes an increase in fat mass in the recipients, indicating that the obese microbiota has an increased capacity to harvest energy from the diet (Turnbaugh et al., 2006).

Departing from these findings, scientists are now trying to unravel the mechanisms behind the observations. One study found that loss of Toll-like receptor (TLR) 5, which is a transmembrane protein recognizing bacterial flagellin, in a mouse model results in a phenotype resembling human metabolic syndrome (Vijay-Kumar et al., 2010). The authors speculated that the loss of this receptor alters the microbiota inducing low-grade inflammatory signalling, which eventually leads to hyperphagia and metabolic syndrome. In another study, TLR 2-deficient mice, which are protected from diet-induced insulin resistance under germ-free settings, developed a condition reminiscent of metabolic syndrome after colonization (Caricilli et al., 2011). The microbiota of the mice showed notable increase in *Firmicutes* and slight increase in *Bacteroidetes* compared to controls. The authors suggested that the mechanisms by which the TLR 2-deficient mice became insulin resistant and, later, obese could be related to increased capacity for energy harvesting from the diet or alternatively to increased level of LPS caused by increased gut permeability and LPS absorption. Recently, it was shown that antibiotic treatment with vancomycin for diet-induced obese mice significantly reduced the proportions of *Firmicutes* and *Bacteroidetes*, and increased *Proteobacteria* (Murphy et al., 2012). These changes were associated with improvement in the metabolic abnormalities associated with obesity, by reducing body weight gain and improving inflammatory and metabolic health of the host. Based on these studies, it seems plausible that the ability of the gut microbiota to regulate inflammatory responses play an important role in the complex mechanisms behind obesity and metabolic syndrome. Still, more long-term studies in animal models and humans are required to acquire a clearer picture of the relationship between the intestinal microbiota and different diseases.

CONCLUDING REMARKS

The complexity of the microbiota–host interactions has been the prime obstacle in defining microbial functionality at a post-genomic level. The recent technical advances in analyzing genomes, transcriptomes and proteomes of complex bacterial consortia and intra- and interspecies metabolic networks help to tackle this problem and will enable systems-level analyses of the crosstalk between the microbiota and the host.

There are multiple reports providing circumstantial evidence to support the concept that microbiota composition and activity influence host metabolism and disease development. These examples include the differences in microbiota composition and microbiota expressed proteins of breastfeeding as compared to formula-fed babies (Schwartz et al., 2012), differences between microbiota composition and activity between healthy and malnourished infants (Monira et al., 2011), differences in the microbiota composition of elderly and centenarians as compared to youngsters (Biagi et al., 2010), and differences in microbiota composition and activity between humans that are either lean or obese (Ley et al., 2005; Zhang et al., 2009) and healthy or suffering of IBD (Willing et al., 2010). The data suggest that the activity and composition of the microbiota is affected by food intake and genetic background of the host. Most findings are supported by animal studies but there is also data on human subjects. The field of functional microbiomics is still rapidly advancing with continuously emerging new techniques and results. Nevertheless, a lot of times the high throughput techniques fail to correlate bacterial species and genome content to function due to the lack of characterized isolates and genes. It is important to identify the regulating parameters of the functioning intestinal ecosystem to gain insight into the influence of the microbiota on human development, aging, and disease.

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Human gut microbiota: repertoire and variations

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The composition of human gut microbiota and their relationship with the host and, consequently, with human health and disease, presents several challenges to microbiologists. Originally dominated by culture-dependent methods for exploring this ecosystem, the advent of molecular tools has revolutionized our ability to investigate these relationships. However, many biases that have led to contradictory results have been identified. Microbial culturomics, a recent concept based on a use of several culture conditions with identification by MALDI-TOF followed by the genome sequencing of the new species cultured had allowed a complementarity with metagenomics. Culturomics allowed to isolate 31 new bacterial species, the largest human virus, the largest bacteria, and the largest Archaea from human. Moreover, some members of this ecosystem, such as Eukaryotes, giant viruses, Archaea, and Planctomycetes, have been neglected by the majority of studies. In addition, numerous factors, such as age, geographic provenance, dietary habits, antibiotics, or probiotics, can influence the composition of the microbiota. Finally, in addition to the countless biases associated with the study techniques, a considerable limitation to the interpretation of studies of human gut microbiota is associated with funding sources and transparency disclosures. In the future, studies independent of food industry funding and using complementary methods from a broad range of both culture-based and molecular tools will increase our knowledge of the repertoire of this complex ecosystem and host-microbiota mutualism.

Keywords: gut microbiota, culturomics, metagenomics, archaea, transparency disclosures, antibiotics

INTRODUCTION

The exhaustive description of human microbiota and their relationship with health and disease are major challenges in the twenty-first century (Turnbaugh et al., 2007). To assess the importance of this challenge, we used the ISI Web of Knowledge to demonstrate the dramatically renewed interest of scientists in this subject. To extend the chart presented by Sekirov et al. (2010); Marchesi (2011), which lists the number of publications per year involving human gut microbiota, we found that in 2011, there were more than 4 times as many citations referencing human gut microbiota than in 2005 (**Figure 1A**), when Eckburg et al. (2005) published the seminal large-scale gut metagenomics study. In addition, in 2011, there were approximately as many published items investigating human gut microbiota than during the 10 years between 1993 and 2002 (**Figure 1B**).

The human gut microbiota is composed of approximately 10^{11-12} microorganisms per gram of content, including diverse populations of bacteria, mainly anaerobes (95% of the total), which is 10 times higher than the total number of human cells (Ley et al., 2006a). In the study of human gut microbiota, two major technological periods can be distinguished: schematic microscopic observation and culture-based methods before 1995 followed by the advent of culture-independent methods. This technology-driven progress led to suggest relationships between gut microbiota composition and diverse diseases, such as irritable bowel syndrome (Kassinen et al., 2007), polyposis or colorectal cancer (Scanlan et al., 2008), necrotizing enterocolitis (Siggers et al., 2008), Crohn's disease (De Hertogh et al., 2006; Manichanh et al., 2006; Scanlan et al., 2006), and metabolic diseases such as type II diabetes (Larsen et al., 2010) and obesity (Ley et al., 2006b; Turnbaugh et al., 2006, 2009; Armougom et al., 2009; Santacruz et al., 2009).

Based on these early data and to complete the description of the human gut composition, considerable funds have been granted. Among the projects pursuing this line of research, the human microbiome project is an international consortium with the aim of sequencing 1,000 bacterial genomes and multiplication by metagenomic analysis to characterize the complexity of microbial communities at several body sites, including the human gut, to determine whether there is a core microbiome (Turnbaugh et al., 2007). Despite these advances in knowledge of gut microbiota composition, the relationships of the microbiota with their host and, consequently, with health and disease are still largely unknown, as reflected in several contradictory results (Sekirov et al., 2010). Moreover, molecular tools and by extension, experimental models, often reflect a reductionist approach as opposed to a holistic approach (Fang and Casadevall, 2011). Nevertheless, an appealing approach that was recently applied to the study of oral microbiota will allow us to detect the minor bacterial populations, which are usually neglected, using dilution to obtain a threshold below 10^6 bacteria per ml or DNA >1 pg per µl (Biesbroek et al., 2012).

We propose here an inventory of current knowledge regarding gut microbiota composition, the techniques used for this study and the relationships with the host. Finally, further research on

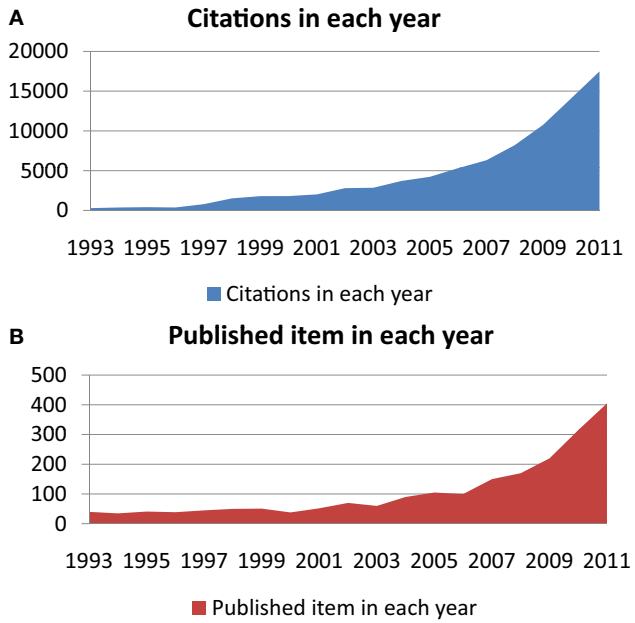


FIGURE 1 | Using the key words "human gut microbiota" or "human fecal flora" and using the ISI Web of Knowledge database, (A) shows citations in each year regarding this subject, and (B) shows the number of published items each year, both between 1993 and 2011.

human gut microbiota is the subject of considerable funding by the food industry. Consequently, to perform an efficient analysis of this subject, the design and/or interpretation of the results of each study can be associated with a conflict of interest. For example, it has recently been shown that published papers in obesity research in which the authors were funded by the food industry were more likely than other papers to contain results or an interpretation that favored the industry or company that was producing the product or service that was being studied (Thomas et al., 2008).

REPERTOIRE

CULTURE

Culturing has been the first method used to characterize a bacterial ecosystem (Finegold et al., 1974, 1977; Moore and Holdeman, 1974a). Gut composition was first studied by microscopic observation and axenic culture. Gram staining has been widely used by microbiologists to describe stool composition. Using this technique, gram-positive bacteria accounted for only 2–45% of the cells observed (Gossling and Slack, 1974). However, a discrepancy arises because culture counts reveal a predominance of gram-positive bacteria in human feces. Indeed, one of the first culture studies of human stools showed that anaerobes always constitute the major component of the culturable flora of children and adults (Mata et al., 1969), with a predominance of gram-positive cells. Moore and Holdeman (1974a), in a study of 20 individuals, revealed 113 different bacteria, including more gram-positive bacteria (*Bifidobacterium*, *Eubacterium*, *Peptostreptococcus*, *Ruminococcus*, *Lactobacillus*, and *Clostridium* genera) than gram-negative bacteria (*Bacteroides*, *Fusobacteria* genera;). Nevertheless, these studies attempted especially to culture anaerobic bacterial species whereas some gut bacteria preferentially grown in microaerophilic conditions.

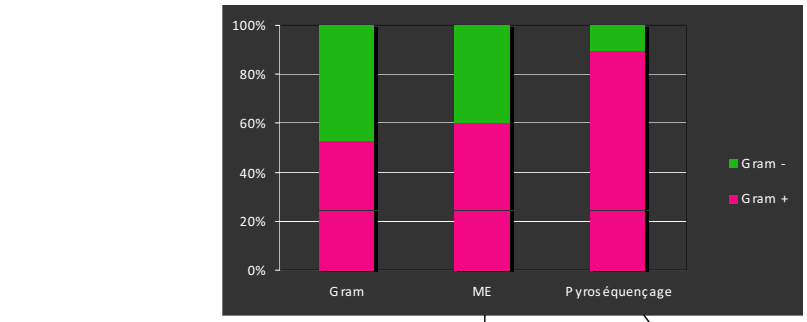
Among other unique problems associated with bacterial culture, Moore have also observed a major discordance between the culture counts and the microscopic counts of species (Moore and Holdeman, 1974b); these discrepancies have been named by Staley and Konopka (1985) as the "great plate count anomaly". Indeed, it is generally accepted that only 1% of bacteria can be easily grown *in vitro* (Vartoukian et al., 2010). Consequently, the major population easily isolated from stools is composed of bacteria that grow quickly in classical high-nutrient growth media, with the usual carbon or electron sources at mesophilic temperatures (Hugenholtz, 2002), and this constitutes the most studied bacteria. It is estimated that approximately 75% of published studies by microbiologists before the advent of molecular tools focused on only nine bacterial genera among four phyla (*Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*; Galvez et al., 1998), whereas we know now that more than 30 different phyla compose the gut microbiota (**Figure 2**; Rajilic-Stojanovic et al., 2007). Nevertheless, studies of these fast-growing and easily cultured bacteria neglect the minority bacterial populations, including potentially pathogenic bacteria such as *Salmonella typhi*.

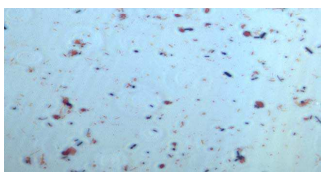
Finally, considering the main first culture-based studies the number of bacterial species was estimated at approximately 400–500 (Mata et al., 1969; Moore and Holdeman, 1974a; Finegold et al., 1977). In addition to the necessary use of stringent anaerobic conditions to culture bacteria from human stools, the usual phenotypic identification methods are time consuming and expensive (Seng et al., 2009). Indeed, the exponential technological advances in molecular tools led microbiologists to progressively abandon the culture-based approach for studies of the gut microbiota ecosystem.

METAGENOMICS AND PYROSEQUENCING

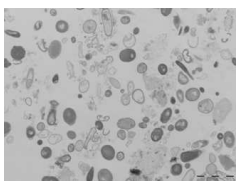
As often occurs during scientific progress, technological advances in microbiology allowed scientists to revisit the knowledge base (Rajilic-Stojanovic et al., 2007). Since 2000, large-scale 16S rRNA or metagenomic studies have allowed scientists to dramatically expand the known diversity of the human gut microbiome, illuminating new ways (Eckburg et al., 2005; Andersson et al., 2008). It is now commonly accepted that approximately 80% of the bacteria found by molecular tools in the human gut are uncultured, and hence can be characterized only by metagenomic studies (Eckburg et al., 2005). Whereas the number of species was limited in the seminal studies using culture-based methods (Finegold et al., 1974; Moore and Holdeman, 1974b), Turnbaugh et al. (2010) estimated 473 phylotypes using V2 pyrosequencing. There is a significant discrepancy between bacterial observations with a microscope and most of the molecular studies, which observe a striking dominance of gram-positive bacteria (Eckburg et al., 2005; Andersson et al., 2008; Turnbaugh et al., 2010; **Figure 2**).

Indeed, these recent methods generate contradictory results reflecting the biases in every step of the Polymerase Chain Reaction procedure. A dramatic divergence in the proportion of the different phyla was observed depending of the type of extraction kit used, notably for the *Fusobacteria* (2–40%) and *Bacteroidetes*





Gram staining (×100 oil immersion)
53% bacteria Gram-positive
47% bacteria Gram-negative



Electron microscopy (×7100)
60% bacteria Gram-positive
40% bacteria Gram-negative

Phylum	Reads	%
Firmicutes	44769	71.12
Actinobacteria	5797	9.21
Other	6627	10.53
Bacteroidetes	3983	6.33
Proteobacteria	1747	2.78
Cyanobacteria	21	0.03
Verrucomicrobia	4	0.01
Total	62948	100

Pyrosequencing
80% bacteria Gram-positive
9% bacteria Gram-negative
11% not available

FIGURE 2 | A comparison of Gram staining, electron microscopy, and pyrosequencing to determine the proportion of Gram-positive/Gram-negative bacteria in the same stool sample (personal data).

(40–60%) phyla (Wu et al., 2010). In addition, the relative abundance of a phylum depends significantly on the 16S hypervariable region, independent of pyrosequencing chemistry. For example, the 454 titanium and Illumina next-generation sequencing (NGS) methods reveal a dominance of the *Bacteroidetes* phylum using 16S rDNA v4v5 region primers, whereas *Firmicutes* was predominant using v3v4 primers on the same gut microbiota (Claesson et al., 2010). Using 454 titanium, *Ralstonia* genera have been detected only by V4/V5 primers, whereas *Bifidobacteria* have been detected only by V3/V4 primers (Turnbaugh et al., 2010). In parallel, Hong et al. (2009) have described that the rRNA approach misses half of the bacteria in environmental microbiology.

Although controversial, the higher taxonomic level analyses (as phylum level) have suggested an association between obesity and *Firmicutes/Bacteroidetes* proportion (Ley et al., 2005). The genus-level analysis has allowed to hypothesize specific enterotypes compositions despite controversies (Arumugam et al., 2011). In addition, Murphy et al. (2012) has recently observed in a study from the manipulation of the mice gut microbiota in diet-induced obesity that a better separation of lean and diet-induced obese mice was observed at the family and genus-level than at the phylum level. However, the large inter-individual variability leads the analysis of lower taxonomic-level to complex results because of small number of samples. Finally, the optimization of primers able to detect genera often misdetected by pyrosequencing, as *Bifidobacteria* (Sim et al., 2012), and technology progress in pyrosequencing, will allow to more quickly analyze longer reads sequenced to study larger cohort samples in low taxonomic level.

Finally, molecular methods detected bacteria present at concentrations greater than approximately 10^6 and neglected minority populations. Among these neglected populations are potentially pathogenic bacteria such as *S. typhi*, *Yersinia enterocolitica*, and *Tropheryma whipplei*, which may be present in human stools at concentrations below 10^5 cfu per ml (Raoult et al., 2010), the current threshold of the latest NGS method (Turnbaugh et al., 2010; Lagier et al., 2012a; **Figure 3**). The depth is directly correlated with the number of generated sequences, and no plateau was obtained in the number of phylotypes observed, although close to 1,000,000 16S rRNA gene amplicons have been sequenced by Turnbaugh et al. (2010).

VIRUSES

Research in the human gut has been focused on bacterial composition (Walker, 2010). Early studies suggested that most DNA viruses found in the intestine were phages and that most RNA viruses were plant viruses (Breitbart et al., 2008). Nevertheless, a recent metagenomic study carried out over 1 year, with three stools analyzed from each monozygotic adult twin and their mother, revolutionized virome knowledge (Reyes et al., 2010). The authors carried out shotgun pyrosequencing to generate over 280 Mb of sequence and, at the same time, a pyrosequencing of 16S rRNA genes to identify the bacterial species. Approximately

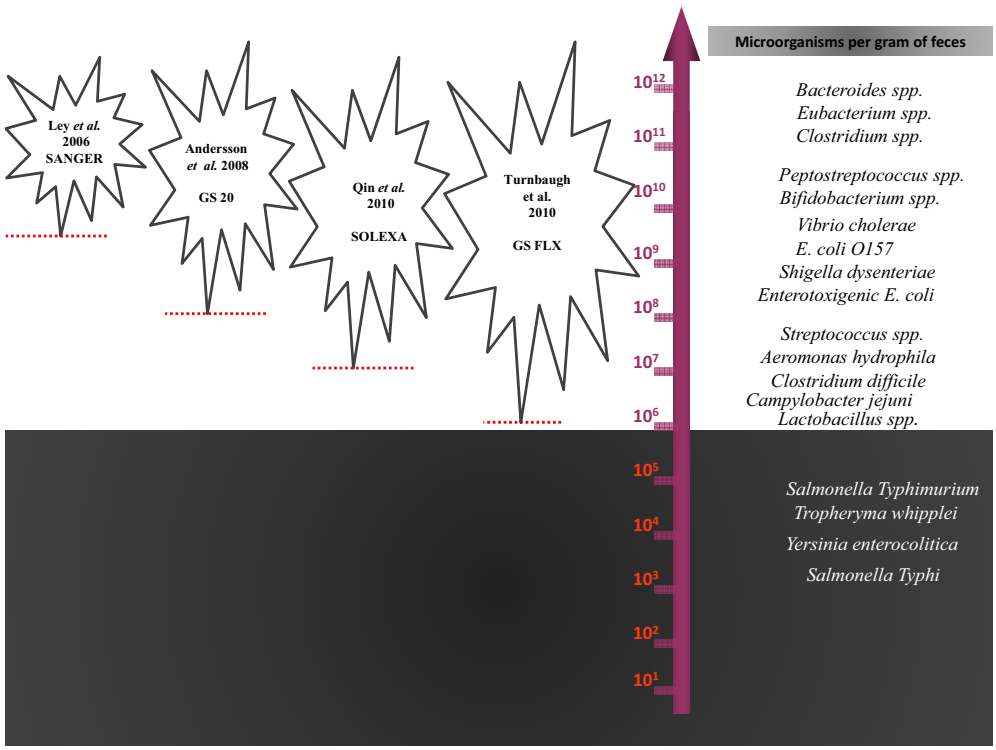


FIGURE 3 | The statistical detection thresholds of metagenomic methods. The statistical detection thresholds of metagenomic methods are correlated with the number of bacteria in the ecosystem studied by the number of sequences generated.

80% of sequencing reads did not match any known viruses in the database corresponding to prophages or temperate phages. These populations were persistent in each individual, with no significant clustering between co-twins or between twins and their mothers, contrasting with the bacterial similarity between twins (Turnbaugh et al., 2009). In addition, Minot et al. (2011) observed that a change of diet is associated with a change in virome composition.

CULTUROMICS

There has been a renewed interest in culture methods for these "non-cultivable" species (Vartoukian et al., 2010). Initially, environmental microbiologists were confronted with the fact that the majority of bacteria do not grow in classical Petri dishes. These first studies used prolonged incubation and stringent anaerobic conditions, notably, diffusion chambers (Kaeberlein et al., 2002; Bollmann et al., 2007), with the aim of simulating the natural environment of these "uncultivable" microorganisms (Kaeberlein et al., 2002). This technique enlarged the diversity of the environmental microorganisms that were isolated (Epstein, 2009). In parallel, a recently published study proposed an anaerobic culture of a single stool sample to complement 16S rRNA sequencing, using rumen fluid or an extract of fresh stools to mimic the natural environment of the gut bacteria. Goodman et al. (2011) have recovered 36 cultured species: four uncultured described species and 53 unknown isolates with different v2 sequences. However, these authors used the most probable number (MPC) technique for creating arrayed species collections that do not detect minority populations.

In addition to the stringent culture conditions, some of the difficulties linked to culture include the cost and the amount of time required for bacterial identification (Seng et al., 2009). These difficulties have recently been overcome by mass spectrometry, which enables quick and effective identifications in routine bacteriology (Seng et al., 2009, 2010) and allow the researcher to quickly check the major population and to concentrate interest on the minority population. We have recently reported a breakthrough in this field of research with the microbial culturomics concept (Lagier et al., 2012a). We applied 212 different culture conditions in two African stools and a French obese stool samples, including enrichment techniques, *Escherichia coli* phage cleaning, and innovative conditions (using rumen fluid, sterile human stools). We analyzed 32,500 colonies by MALDI-TOF, allowed us to culture 340 different bacterial species among seven phyla and 117 genera. This included 174 species never described in the human gut. Moreover 31 new species were found, including five new genera, as well two species from rare phyla (*Deinococcus-Thermus* and *Synergistetes*). Genome sequencing and description of each new species is in progress (Kokcha et al., 2012; Lagier et al., 2012b,c; Mishra et al., 2012a,b,c,d). By comparison, pyrosequencing of 16S rDNA amplicons from the three stools noted a dramatic discrepancy with culturomics as only 51 species identified by 16S rDNA amplification and sequencing were also found among the 340 cultured species highlighting the renewed interest for the culture in the gut

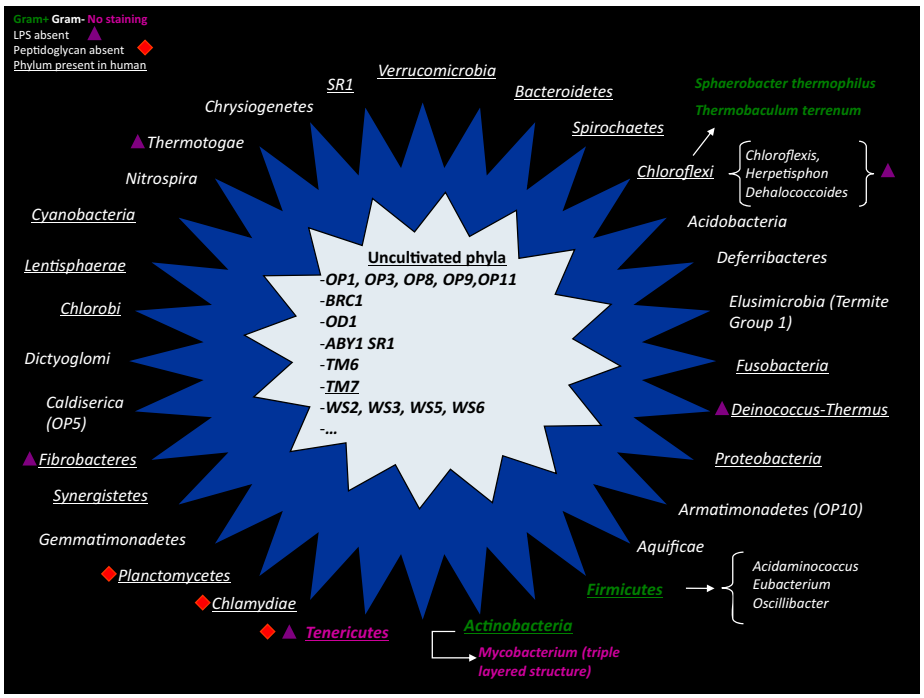


FIGURE 4 | A non-exhaustive representation of different bacterial phyla found in culture (outer star in blue) or phyla with no representative in culture (inner star in gray). Gram-positive bacteria are colored in green, and Gram-negative bacteria are colored in white. Bacteria with an atypical cell wall (triple-layered structure of *Mycobacterium*) or without a cell wall (*Tenericutes*) have abnormal Gram staining and are shown in pink. The purple triangle represents the absence of lipopolysaccharide in the outer membrane of Gram-negative bacteria. The red square symbolizes phyla that do not have a peptidoglycan structure.

microbiota study. Culturomics allowed us break several "records" with the largest number of bacteria cultured from a single stool (219 species), the first bacteria from *Deinococcus-Thermus* phylum isolated from human, the largest human virus and the largest bacteria from human (Lagier et al., 2012a).

COMPARISON OF THE TECHNIQUES

There are currently no rational explanations for the typical observed proportions of gram-positive/negative bacteria, which are highly divergent microscopically (Turnbaugh et al., 2007) with culture, (Gossling and Slack, 1974) and the proportions obtained by sequence detection (Eckburg et al., 2005; **Figures 2** and **4**). In 2002, Hayashi compared the digestive microbiota of three individuals by cloning/sequencing and anaerobic culture using the "plate-in-bottle" method. These researchers isolated between 48 and 65 phylotypes in the cloning of individuals and 48 species, of which three individuals were potentially three new species (Hayashi et al., 2002b). In light of the phylogenetic tree described in this publication, these authors found significant discrepancies between these two techniques, which were somewhat surprising given the low number of species and phylotypes identified. Several species in culture had no equivalent in cloning. A previous study compared these same techniques, but the number of species and phylotypes was even lower (Wilson and Blitchington, 1996). In this study, of 48 species, 25 were detected only by cloning, nine were common to both techniques, and 14 were identified only by culture. In addition, in our microbial culturomics study, by comparison with the 340 bacteria cultured, pyrosequencing of 16S rDNA amplicons from the three stools identified 698 phylotypes including 282 phylotypes of known bacterial species and 416 phylotypes of uncultured bacteria. We noted a dramatic discrepancy with culturomics as only 51 species identified by 16S rDNA amplification and sequencing were also found among the 340 cultured species. Consequently, microbial culturomics increased by 30% the microbial repertoire of the human gut studied by pyrosequencing (Lagier et al., 2012a).

GAPS IN KNOWLEDGE

In addition to the bias previously described, some components of human gut microbiota have been partially neglected by the current tools (**Figure 5**).

EUKARYOTES

Eukaryotes are an important part of the human gut microbiome and play different beneficial or harmful roles. Some species may be commensal or mutualistic, whereas others may be opportunistic or parasitic (Parfrey et al., 2011). The eukaryotic component of the human gut microbiome remains unexplored because these organisms are of limited interest (Marchesi, 2010). Culture-dependent techniques and microscopy-based approaches have been mainly used to explore eukaryotes in the human gut, and identification has frequently been based on morphological and physiological techniques with numerous biases. Moreover, this approach detects only a small fraction of microorganisms, including *Candida* and *Saccharomyces* spp., but the growth requirements for many eukaryotic species remain unknown.

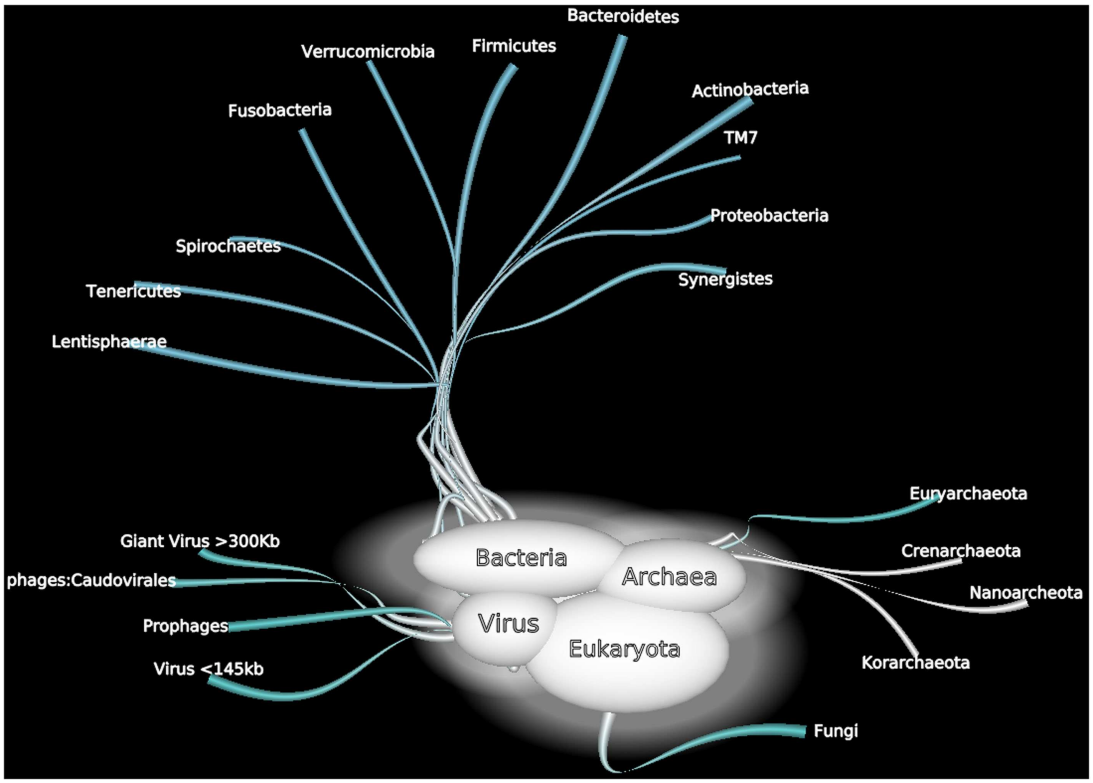


FIGURE 5 | A non exhaustive overview of human gut microorganisms among bacterial, Archaea, viral, and *Eukaryota* domains.

Using culture-independent methods, Scupham et al. (2006) have identified a large number of fungi, including *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota* phyla, in studies of mouse feces. Furthermore, Scanlan and Marchesi (2008), studying the human distal gut, have shown that the diversity and abundance of eukaryotes is low relative to those of bacteria. Only members of the genera *Gloeotinia*/*Paecilomyces* and *Galactomyces* have been identified as the most abundant. Nevertheless, we have shown that due to a large variety of primers used, the human gut contains a broader eukaryotic diversity than predicted (Hamad et al., 2012). In parallel, applying traditional and modern laboratory techniques (using intergenic spacers for 18S rDNA), the repertoire of intensive care unit pneumonial microbiota has been considerably extended, notably regarding fungal microbiota and plants (Bousbia et al., 2012).

Giant viruses

Giant viruses growing in amoebae have previously been isolated in the environment, e.g., in the water of cooling towers, in rivers and lakes, in seawater, in decorative fountains, and in soil (Pagnier et al., 2008). Mimivirus DNA has been obtained from the bronchoalveolar lavage of patients (Raoult et al., 2007; Lysholm et al., 2012), and a laboratory infection was documented by serology (Raoult et al., 2006). In addition, Lysholm et al. (2012), in a viral microbiome metagenomic study performed in 210 children and adults with lower respiratory infections, recently identified Mimivirus. Because the authors used two pools and filtered with 0.22 and 0.45 μm pore-size disk filters, they were able to isolate a giant virus that is frequently missed by large-scale virome metagenomics studies that use only 0.22 μm filters, making giant virus detection unlikely (Willner et al., 2009; Reyes et al., 2010).

In our laboratory, in an effort to obtain fastidious bacteria from an African stool sample by amoeba (*Acanthamoeba polyphaga*) co-culture, we obtained a new giant virus strain named Senegal virus (Lagier et al., 2012a), which we sequenced (Genbank JF909596–JF909602). These findings indicate that giant viruses may be a part of the gut microbiota and that virome metagenomic studies should use different filter sizes. Because the potential pathology of the giant viruses is currently unknown, it is unreasonable to neglect them (Boyer et al., 2009).

Archaea

Nottingham and Hungate (1968) isolated a previously unidentified methanogenic Archaea from human feces using a non-selective medium and a stringent anaerobic atmosphere composed of 80% H_2 and 20% CO_2. Miller et al. (1982) isolated *Methanobrevibacter smithii* from human stool specimens from four healthy adults using anaerobic cultures enriched with the same H_2–CO_2 anaerobic atmosphere pressurized to two bars. Illustrating the technical limitations of the fastidious Archaea culture, in our laboratory, we have recently achieved the isolation of the fourth methanogenic Archaea species in humans and the first cultured representative of a new order of Archaea (*Methanomassiliicoccus luminyensis*) after a 16-month tentative culturing procedure. We obtained this strain after subtle modifications in the composition of the culture medium (enzyme co-factors) and adaptation of the

atmospheric pressure (the culture medium is patented; Dridi et al., 2012a). In addition, the genome sequencing of this new species represents the largest genome of a methanogenic euryarchaeota isolated from humans (Gorlas et al., 2012).

In addition, recent molecular studies indicated that human Archaea constitute an expanding world (Dridi et al., 2011). Using 16S rDNA sequencing, many studies confirmed the presence of *M. smithii* and *M. stadtmanae* in the human gut, with variable and low prevalence (Dridi et al., 2009). Nevertheless, in our study, our new Archaea was detected in stools in 4% of individuals, and its prevalence increases with age, although its role in human health is unknown (Dridi et al., 2012b). Regarding the influence of Archaea on human health, a recent meta-analysis compared the number of sequences of *Methanobrevibacter* spp. in stools. Obese individuals had fewer *Methanobrevibacter* genera by quantitative polymerase chain reaction (qPCR) than non-obese subjects (Angelakis et al., 2012a). Previous studies had reported discordant results concerning the levels of detection of *M. smithii* in the obese gut (Zhang et al., 2009; Schwiertz et al., 2010; Million et al., 2011). In addition, the detection of Archaea in the vaginal flora of pregnant women allowed us to hypothesize a possible mother-to-child transmission (Dridi et al., 2011).

Planctomycetes

The phylum Planctomycetes, phylogenetically closely related to Verrucomicrobia and Chlamydiae, is composed of environmental microorganisms characterized by a peptidoglycan-free cell wall and cell compartmentalization (Fuerst and Sagulenko, 2011). The culture is fastidious and requires the addition of appropriate antibiotics (peptidoglycan synthesis inhibitors) and amphotericin B to prevent contamination of the culture medium. Undetected by conventional 16S rRNA PCR or standard culture techniques, this phylum has been reported in black-and-white colobus monkey stools (Yildirim et al., 2010) and, in one instance, in the human gut microbiota, using metagenomics (De Filippo et al., 2010). In our laboratory, preliminary results (unpublished data) confirmed the presence of specific Planctomycetes DNA in human stools. Several species of Planctomycetes and, more generally, of species including the superphyla Verrucomicrobia, Planctomycetes, and Chlamydiae, are undergoing genome sequencing. It is expected that this sequencing will increase our knowledge of this specific branch of the tree of bacterial life (Wagner and Horn, 2006).

The variability depending of the gut samples

"The gut microbiota is non-homogenous with a progressive increase of bacterial concentration from the stomach (approximately 10^3 bacteria per gram to the colon (approximately 10^{11} bacteria per gram; O'Hara and Shanahan, 2006). Nevertheless, most of studies explored stools samples reflecting mainly the colonic composition. However, differences in compositions have been reported between small intestine biopsies (most of Streptococcaceae belonging to Firmicutes phylum and Actinomycinaeae and Corynebacteriaceae belonging to Actinobacteria phylum) whereas colonic biopsies were enriched by Bacteroidetes phylum and Lachnospiraceae among the Firmicutes phylum (Frank et al., 2007). Intestinal analysis of tiered samples will allow to exhaustively describe the gut composition."

COMPOSITION

The composition of the human gut ecosystem is influenced by multiple and diverse factors, some physiological (age, origin, environment) and others linked to external factors, such as dietary habits, antibiotics, and probiotics (**Figure 6**).

AGE

In a pioneering study using microarrays to detect small rRNAs, Palmer followed a cohort of newborns, including a pair of twins, during the first year of life. It was shown that despite considerable temporal variations and environmental influences, the composition of the intestinal ecosystem tended to be characteristic of adulthood at the end of this period (Palmer et al., 2007). The proportion of *Bacteroides fragilis* increased from 1 month to 1 year (Vael et al., 2011). In a 2.5-year case study, Koenig analyzed >300,000 16S rRNAs from 60 fecal samples from healthy children and showed that infant gut variation is associated with life events. The phylogenetic diversity of the microbiome increased gradually over time with progressive temporal changes but, inversely, the major phyla, genera, and species composition showed rough shifts in abundance corresponding to modifications in diet or health (Koenig et al., 2011).

Nevertheless, using microbiota array to analyze gut microbiota composition in adolescent subjects, Agans et al. (2011) found a statistically significantly higher abundance of *Bifidobacterium* and *Clostridium* genera, contrary to current knowledge, suggesting that the gut microbiome of adolescents is different from that of adults. At the other extreme of life, using pyrosequencing of 16S rRNA gene V4 region amplicons, the gut microbiota composition of elderly subjects was distinct from that of younger adults, with a greater temporal stability over a limited time, particularly in the proportion of *Bacteroides* spp. (Claesson et al., 2011).

GEOGRAPHICAL PROVENANCE AND ENVIRONMENT

Discordant results have also been published regarding a geographic signature of the gut microbiota depending on the technique used. To investigate the hypothetical association between gut composition and cancer, early culture-dependent studies compared populations at high-risk (western countries) and at low risk (Japan, Uganda, India) and reported different compositions of microbiota (Hill et al., 1971; Drasar et al., 1973; Finegold et al., 1974). The high-risk population had a microbiota composed primarily of *Bacteroidetes*, and there were specific differences, including patterns of food consumption, between western countries and Asian or African populations, although multiculturalism and population exchanges have reduced these differences. Only a few large-scale molecular studies have used stool samples collected from Asia or Africa (De Filippo et al., 2010; Lee et al., 2011), where approximately 75% of the population of the world lives; nevertheless, the findings have suggested a possible signature of biogeography (Lee et al., 2011). Indeed, most of the large-scale metagenomic or pyrosequencing studies used stools collected from American or European individuals (Ley et al., 2006b; Turnbaugh et al., 2006; Claesson et al., 2011).

Lay, characterizing 91 European gut microbiota using FISH combined with flow cytometry, did not observe a significant grouping with regard to country of origin (France, Netherlands,

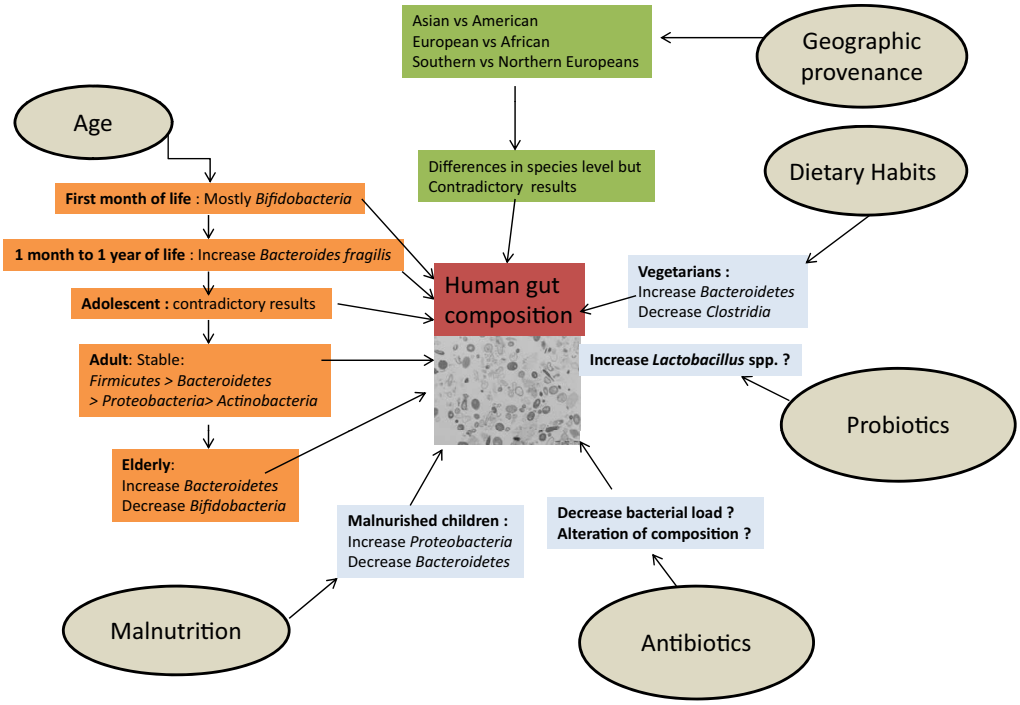


FIGURE 6 | The influence of external factors determining the composition of the human gut microbiota.

Denmark, UK, and Germany; Lay et al., 2005). With the same technology, Mueller et al. (2006) found differences in *Bifidobacteria* species between European individuals. Grzeskowiak et al. (2012), using flow cytometry-FISH and qPCR, have shown that several species (*Bifidobacterium adolescentis*, *Staphylococcus aureus*, and *Clostridium perfringens*) were absent in Malawian children but present in 6-month-old Finnish infants. Fallani comparing infants living in northern or southern European countries by 16S rDNA pyrosequencing, have found that geographical provenance is important, with a higher proportion of *Bifidobacteria* in northern infants and more *Bacteroidetes* and *Lactobacilli* in southern European countries.

Finally, Arumugam studied 22 fecal metagenomes of individuals from four different countries and identified three different enterotype clusters, which were independent of geographic provenance. The three different enterotypes were, respectively, richer in *Bacteroides*, in *Prevotella*, and in *Ruminococcus* for the Enterotype 3. Arumugam suggested that each enterotype used a different route to generate energy (Arumugam et al., 2011).

DIETARY HABITS

Dietary habits are thought to be a major factor contributing to the diversity of the human digestive microbiota (Backhed et al., 2005). Part of the geographic diversity of the gut microbiota seems to be explained by differences in diet. For example, African children from a rural area in Burkina Faso showed a specific abundance of *Prevotella* and *Xylanibacter*, known to contain a set of bacterial genes for cellulose and xylan hydrolysis, completely lacking in European children (De Filippo et al., 2010). The authors hypothesized that the abundance of these genera could be a consequence of the high intake of fiber, similar to the diet of early human settlements at the time of the birth of agriculture, maximizing the extraction of metabolic energy from the polysaccharides of ingested plants (De Filippo et al., 2010). A vegetarian diet affects the intestinal microbiota, specifically by decreasing the amount and modifying the diversity of *Clostridium* cluster IV (Liszt et al., 2009). Based their studies on RFLP analysis, Hayashi et al. (2002a) Hayashi found that the digestive microbiota of vegetarians harbored Clostridium rRNA clusters XIVa and XVIII. Recently, Walker et al. (2011) tested overweight people successively with a control diet, a diet high in resistant starch (RS) or non-starch polysaccharides (NSP) and a reduced carbohydrate weight loss (WL) diet for 10 weeks by two different methods: large-scale sequencing and quantitative PCR. No significant effect was observed at the phylum level, but at finer taxonomic level, *Eubacterium rectale* and *Ruminococcus bromii* showed significant and dramatic (fourfold) increased proportions in the RS diet, whereas the proportion of *Collinsella aerofaciens*-related sequences was decreased significantly on the WL diet (Walker et al., 2011). In this study, reproducible changes were found only at the phylotype level, whereas no differences were significant at a broader taxonomic level (the phylum or family Ruminococcaceae level), and the analysis suggested that the amplified 16S rRNA sequence clustered more strongly by individuals than by diet. These changes are entirely in agreement with studies using RNA-based stable isotope probing, which showed that *R. bromii* was the first starch degrader in the human gut (Kovatcheva-Datchary et al., 2009). Wu analyzed stool samples from 98 individuals and found that

enterotypes were strongly associated with long-term diets, especially for animal fat and protein (*Bacteroides*) vs. carbohydrate (*Prevotella*). Changes in gut microbiota related to short-term diet modifications occurred rapidly, were detectable within 3–4 days, and were rapidly reversed (Walker et al., 2011; Wu et al., 2011). Conversely, Wu et al. (2011) suggested that long-term dietary interventions might allow the pervasive modulation of an individual's enterotype to improve health. In animal models of obesity induced by diet (DIO), Turnbaugh et al. (2008) showed that a high-fat diet could significantly alter the intestinal flora of experimental models with a bloom in a single uncultured clade within the *Mollicutes* class of the *Firmicutes*. Hildebrandt et al. (2009) suggested that a high-fat diet altered the intestinal flora regardless of weight change. These authors observed a bloom of *Clostridia* and *Proteobacteria* associated with the high-fat diet. The major group of *Proteobacteria* that increased in abundance was the Delta-*Proteobacteria* phylum, order *Desulfovibrio*. Finally, Monira et al. (2011) have recently published a study comparing the gut flora of malnourished children with that of well-nourished children in Bangladesh and found a decrease in *Bacteroidetes* and an increase in *Proteobacteria* phyla, including *E. coli* and *Klebsiella* spp.

OBESITY AND GNOTOBIOTIC MICE

Beginning in 2005, obesity has been associated with a specific profile of bacterial gut microbiota, including a decrease in the *Bacteroidetes/Firmicutes* ratio (Ley et al., 2005, 2006b; Turnbaugh et al., 2006, 2009) and decreased bacterial diversity (Turnbaugh et al., 2009). Since these pioneering studies, significant associations have been found between obesity and an increase in some bacterial groups, including *Lactobacillus*, *S. aureus, E. coli*, and *Faecalibacterium prausnitzii* (Collado et al., 2008; Kalliomaki et al., 2008; Armougom et al., 2009; Santacruz et al., 2009; Balamurugan et al., 2010). In a recent review, we found no reproducible and significant alteration linking obesity and gut microbiota at the phylum level (Angelakis et al., 2012a). Conversely, meta-analysis at the genus-level found decreased levels of bifidobacteria (Collado et al., 2008; Kalliomaki et al., 2008; Santacruz et al., 2009; Balamurugan et al., 2010; Schwiertz et al., 2010) and *Methanobrevibacter* spp. (Armougom et al., 2009; Schwiertz et al., 2010; Million et al., 2011), the leading known representative of Archaea in the human gut, in overweight/obese people. To date, controversial studies show that the connection between the microbiome and excess weight is complex (Pennisi, 2011). We found a difference at the species level, with *L. reuteri* enriched in obese gut microbiota, whereas *L. plantarum* was increased in lean individuals (Million et al., 2011). At the gene level, obesity has been associated with an altered representation of bacterial genes and metabolic pathways. Turnbaugh et al. (2009) showed that diversity of organismal assemblages yields a core microbiome at a functional level and that deviations from this core are associated with different physiological states (obese compared with lean), with obese gut microbiota having an increased capacity for energy harvest.

As a theoretical basis for the causal link between alterations in the gut microbiota and obesity, several mechanisms have been suggested. First, the gut microbiota may interact with weight regulation by hydrolyzing indigestible polysaccharides to easily absorbable monosaccharides and by activating lipoprotein lipase. Consequently, glucose is rapidly absorbed, producing substantial elevations in serum glucose and insulin, both factors that trigger lipogenesis. In addition, fatty acids are stored excessively, with *de novo* synthesis of triglycerides derived from the liver. Together, these phenomena cause weight gain (Backhed et al., 2007). Using *Fasting-Induced Adipocyte Factor* (Fiaf) knockout mice, Backhed et al. (2007) showed that gut microbiota suppressed intestinal Fiaf, consequently increasing the storage of calories.

Second, the composition of gut microbiota has been shown to selectively suppress angiopoietin-like protein 4/fasting-induced adipose factor in the intestinal epithelium. This molecule is a circulating lipoprotein lipase inhibitor and a regulator of peripheral lipid and glucose metabolism (Backhed et al., 2004). Backhed et al. (2004) showed that when the microbiota of normal mice were transplanted into germ-free mice, after 2 weeks, body fat increased by 60% without increased food consumption, modifications of energy expenditure, or relative insulin resistance, and there was a 2.3-fold higher production of triglycerides in the liver, suggesting that the gut operates in host energy homeostasis and adiposity.

Third, it has been suggested that bacterial isolates of gut microbiota may have pro- or anti-inflammatory properties, impacting weight. Obesity has been associated with a low-grade systemic inflammation corresponding to higher plasma endotoxin lipopolysaccharide (LPS) concentrations, defined as metabolic endotoxemia (Bastard et al., 2006; Hotamisligil, 2006; Sbarbati et al., 2006; Fogarty et al., 2008). Cani et al. (2008) showed that antibiotics can lower LPS levels in mice fed a high-fat diet and in *ob/ob* mice and, consequently, can reduce glucose intolerance, body weight gain, and fat mass. Conversely, some *Bifidobacterium* and *Lactobacillus* species have been reported to deconjugate bile acids, which may decrease fat absorption (Shimada et al., 1969).

ANTIBIOTICS AND PROBIOTICS

Antibiotics and total bacterial count

According to the literature, oral or intravenous antibiotics tend to decrease the bacterial load in the digestive tracts of infants (Palmer et al., 2007) and elderly patients (Bartosch et al., 2004). However, other studies reported that only the microbiota composition is altered, and the total biomass is not modified by antibiotics (Sekirov et al., 2008). In contrast to amoxicillin and metronidazole or cefoperazone, Robinson noted that the alterations in community structure associated with vancomycin specifically occurred without a significant decrease in the overall bacterial biomass (Robinson and Young, 2010).

Structural disruption

Antibiotic administration has a reproducible effect on the community structure of the indigenous gastrointestinal microbiota in mice (Robinson and Young, 2010). A very recent study found that the administration of a commercial growth-promoting antibiotic combination (ASP250: chlortetracycline-sulfamethazine and penicillin) entailed a reproducible bloom in proteobacteria (1–11%) in swine gut microbiota (Looft et al., 2012). This shift was driven by an increase in *E. coli* populations. In humans, analysis of the fecal microbial populations of infants after antibiotic therapy showed a major alteration as measured by SSU rDNA microarray analysis (Palmer et al., 2007) or culture-based methods

(Savino et al., 2011). In adults, the same dramatic shift has been reported, depending on the antibiotic. Clindamycin (Donskey et al., 2003; Jernberg et al., 2005a) has the strongest effect compared to oral cephalosporin, which is responsible for minor or no changes (Swedish Study Group, 1991a,b). Of note, the extremely moderate effect of cephalosporin on gut microbiota (Donskey et al., 2003) has been linked with the low activity of this molecule on intestinal anaerobes. Moreover, the fecal elimination of carbapenems is very limited, explaining why changes in the intestinal microflora are only moderate, whereas these agents have the broadest spectra of the beta-lactam antibacterial agents (Sullivan et al., 2001). The characterization of gut microbiota alteration by metagenomic analysis of the v3–v6 region has been studied in three patients on ciprofloxacin (Dethlefsen et al., 2008). Ciprofloxacin decreased to one-third the abundance of taxa [number of ref Operational taxonomic units (OTU)], their diversity and distribution. However, comparing gut microbiota alterations by DGGE analysis, the rate of similarity with the pre-treatment profile was 73% with ciprofloxacin but only 11–18% with clindamycin (Donskey et al., 2003). In addition, ciprofloxacin has been reported to have little or no impact on anaerobic intestinal microbiota (Nord, 1995; Edlund and Nord, 1999b). Glycopeptides, used widely in agriculture as growth promoters, are associated with natural resistance of most of the lactobacilli and have no effect on gram-negative bacteria, including *Enterobacteria* (Barna and Williams, 1984). Analyzing vancomycin-associated gut microbiota alterations in mice by cloning sequencing, Robinson found that vancomycin increased members of the *Proteobacteria* and *Tenericutes* phyla and the Lactobacillaceae family, whereas members of the Lachnospiraceae family decreased (Robinson and Young, 2010). Using a continuous-culture colonic model system, Maccaferri et al. (2010) demonstrated that rifaximin, reported to induce clinical remission of active Crohn's disease while not altering the overall structure of the human colonic microbiota, increased *Bifidobacterium*, *Atopobium*, and *F. prausnitzii* and led to a variation of metabolic profiles associated with potential beneficial effects on the host. The effects of tetracycline on gut microbiota in humans are of particular interest because this antibiotic is commonly used in poultry production as a growth promoter, suggesting dramatic changes in intestinal microbial populations. One notable effect of tetracycline is a decrease in bifidobacteria (Nord et al., 2006; Saarela et al., 2007). Overall, specific gut microbiota changes are associated with specific antibiotics (**Table 1**).

The effects of three growth-promoting antibiotics (avilamycin, zinc bacitracin, and flavomycin) on broiler gut microbial community colonization and bird performance were investigated (Torok et al., 2011). OTU linked to changes in gut microbiota in birds on antimicrobial-supplemented diets were characterized and identified. Lachnospiraceae, *L. johnsonii*, Ruminococcaceae, and Oxalobacteraceae genera were less prevalent in the guts of chicks fed antimicrobial-supplemented diets. *L. crispatus*, *L. reuteri*, *Subdoligranulum*, and Enterobacteriaceae were more prevalent in the guts of chicks raised on the antimicrobial diet (Torok et al., 2011). These results suggest that antibiotic effects on gut microbiota may be relevant at the species level because different *Lactobacillus* species-related OTUs showed paradoxical changes.

The reversibility of structural gut microbiota modification

The recovery of the gut community toward baseline after short-term antibiotic therapy has been reported in animal models (Robinson and Young, 2010), but pervasive disturbance to the community has been observed several weeks after withdrawal of certain antibiotics, including cefoperazone (Robinson and Young, 2010) and quinolones (Dethlefsen et al., 2008). Changing the intestinal microbiota of termites with antibiotics offers a privileged experimental model and has shown that prolonged antibiotic treatment with rifampicin has an irreversible effect not only on microbial diversity but also on longevity, fecundity and the weight (weight gain compared to controls) of two termite species, *Zootermopsis angusticollis* and *Reticulitermes flavipes.*

Probiotics

Probiotics were initially used in agriculture to prevent diarrhea in poultry because they reduce intestinal colonization by *Salmonella* spp. and *C. perfringens* (Angelakis and Raoult, 2010), but the use of probiotics such as *Lactobacillus* spp. can led to a rapid weight increase in chickens (Angelakis and Raoult, 2010). *L. acidophilus*, *L. plantarum*, *L. casei*, *L. fermentum*, and *L. reuteri* are the most commonly used *Lactobacillus* species in agriculture (Anadon et al., 2006). The inoculation of *L. ingluviei* in mice is responsible for gut flora alterations associated with an increase in weight gain and liver enlargement (Angelakis et al., 2012b). In parallel, probiotics are increasingly used in human foods, notably in the milk industry (Raoult, 2008). Although the mechanisms are not yet known, many studies suggest that probiotics function through direct or indirect impacts on colonizing microbiota of the gut (Sanders, 2011). Million et al. (2011) recently found that different *Lactobacillus* species may have a paradoxical effect, with higher levels of *L. reuteri* and lower levels of *L. plantarum* and *L. paracasei* in obese gut microbiota. A recent systematic meta-analysis reported that the administration of *L. acidophilus* is responsible of weight gain in human and animals and that the use of *L. fermentum* and *L. ingluviei* resulted of weight gain in animals (Million et al., 2012). Thuny et al. (2010) observed a weight gain in patients treated with vancomycin and hypothesized that the gain was induced by the growth-promoting effect of *Lactobacillus* spp., as these species are resistant to glycopeptides. In contrast, symbiotics (the combination of prebiotics and probiotics) have been proposed for the management of malnutrition, with promising results on mortality (Kerac et al., 2009). After gavage of gnotobiotic mice with a combination of bacteria, including *B. animalis* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *cremoris*, and *Streptococcus thermophilus*, only anecdotal changes were noted in microbiota composition, whereas significant changes were observed in the expression of microbiome-encoded enzymes involved in metabolic pathways, notably, carbohydrate metabolism (McNulty et al., 2011). However, these suggestions of a relationship between probiotics and obesity remain controversial (Delzenne and Reid, 2009). In addition, the reports of the anti-diabetic and anti-inflammatory effects of *Lactobacilli* should be considered cautiously because the translation of findings based on animal models to humans is hazardous (Kootte et al., 2012). Finally, all these results should be interpreted with caution in view of the substantial funding of obesity research by the food industry, creating conflicts of interest.

Table 1 | Modifications of gut flora linked to antibiotics.

Antibiotic	Method	References
PENICILLINS		
Ampicillin		
Decrease in enterococci	Cultivation	Black et al. (1991)
Decrease in streptococci	Cultivation	Black et al. (1991)
Decrease in *E. coli* strains	Cultivation	Black et al. (1991)
Slight decrease in anaerobic Gram-positive bacteria	Cultivation	Black et al. (1991)
Amoxicillin		
Increase in aerobic Gram-negative rods, such as enterobacteria, other than *E. coli* (*Klebsiella*, *Enterobacter*)	Cultivation	Brismar et al. (1993), Floor et al. (1994), Stark et al. (1996)
Increase in anaerobic Gram-positive rods	Cultivation	Swedish Study Group (1991b)
Increase in *Bacteroides*	Cultivation	Swedish Study Group (1991b)
Decrease in streptococci and Staphylococci	Cultivation	Brismar et al. (1993)
Decrease in anaerobic Gram-positive cocci, such as eubacteria	Cultivation	Brismar et al. (1993), Stark et al. (1996)
Amoxicillin/clavulanic acid		
Increase in enterococci and *E. coli*	Cultivation	Lode et al. (2001)
Decrease in lactobacilli, clostridia, bifidobacteria	Cultivation	Lode et al. (2001)
Disappearance of *Clostridium* cluster XIVa (cloning/sequencing)	Cloning/sequencing	Young and Schmidt (2004)
Decrease in *Faecalibacterium* spp.	Cloning/sequencing	Young and Schmidt (2004)
Piperacillin/tazobactam*		
Decrease in enterobacteria	Cultivation	Nord et al. (1993)
Decrease in bifidobacteria, eubacteria, lactobacilli	Cultivation	Nord et al. (1993)
Decrease in anaerobic Gram-positive cocci like clostridia	Cultivation	Nord et al. (1993)
CEPHALOSPORINS		
Cefepime		
Decrease in *E. coli* and bifidobacteria	Cultivation	Bacher et al. (1992)
Increase in clostridia and *Bacteroides*	Cultivation	Bacher et al. (1992)
Ceftriaxone		
Decrease in the total numbers of anaerobes	Cultivation	Welling et al. (1991)
Dramatic decrease in clostridia, lactobacilli, bifidobacteria	Cultivation	Vogel et al. (2001)
Dramatic decrease in Gram-negative rods (enterobacteria)	Cultivation	Cavallaro et al. (1992), Vogel et al. (2001), Welling et al. (1991)
Increase in enterococci	Cultivation	Vogel et al. (2001), Welling et al. (1991)
Carbapenems		
Meropenem		
Decrease in enterobacteria and streptococci	Cultivation	Bergan et al. (1991)
Increase in enterococci	Cultivation	Bergan et al. (1991)
Decrease in clostridia, Gram-negative cocci, and bacteroides	Cultivation	Bergan et al. (1991)
FLUOROQUINOLONES		
Ciprofloxacin		
Dramatic decrease in enterobacteria	Cultivation	Bergan et al. (1986), Borzio et al. (1997), Brismar et al. (1990), Brumfitt et al. (1984), Enzensberger et al. (1985), Esposito et al. (1987), Holt et al. (1986), Krueger et al. (1997), Ljungberg et al. (1990), Rozenberg-Arska et al. (1985), Van Saene et al. (1986), Wistrom et al. (1992)
Decrease in aerobic Gram-positive cocci	Cultivation	Bergan et al. (1986), Brismar et al. (1990), Brumfitt et al. (1984), Ljungberg et al. (1990), Van Saene et al. (1986)
Decrease in streptococci	Cultivation	Brismar et al. (1990), Brumfitt et al. (1984), Ljungberg et al. (1990)
Decrease in enterococci	Cultivation	Bergan et al. (1986), Brismar et al. (1990), Ljungberg et al. (1990), Van Saene et al. (1986)

(Continued)

Table 1 | Continued

Antibiotic	Method	References
Increase in enterococci	Cultivation	Borzio et al. (1997)
Decrease in anaerobic bacteria	Cultivation	Bergan et al. (1986), Brismar et al. (1990), Rozenberg-Arska et al. (1985)
Suppression of *Bacteroides putredinis*, *Ruminococcus torques*	DGGE	Donskey et al. (2003)
Norfloxacin		
Dramatic decrease in enterobacteria	Cultivation	de Vries-Hospers et al. (1985), Edlund et al. (1987), Leigh et al. (1985), Pecquet et al. (1986)
Decrease in aerobic Gram-positive cocci	Cultivation	de Vries-Hospers et al. (1985), Pecquet et al. (1986)
Decrease in streptococci	Cultivation	Pecquet et al. (1986)
Decrease in enterococci	Cultivation	de Vries-Hospers et al. (1985)
Ofloxacin		
Dramatic decrease in enterobacteria	Cultivation	Edlund et al. (1988), Edlund et al. (1997b), Pecquet et al. (1987)
Decrease in aerobic Gram-positive cocci	Cultivation	Edlund et al. (1988), Edlund et al. (1997b), Pecquet et al. (1987)
Decrease in enterococci	Cultivation	Edlund et al. (1988), Pecquet et al. (1987)
Decrease in lactobacilli, bifidobacteria, eubacteria	Cultivation	Edlund et al. (1988)
Decrease in anaerobic bacteria	Cultivation	Edlund et al. (1988)
Decrease in *Veillonella* and *Bacteroides* spp.	Cultivation	
Levofloxacin, Gatifloxacin, Trovafloxacin, Moxifloxacin		
Dramatic decrease in enterobacteria	Cultivation	Edlund et al. (1997b), Edlund and Nord (1999a), van Nispen et al. (1998)
Strong decrease in aerobic Gram-positive cocci	Cultivation	Edlund et al. (1997b), Edlund and Nord (1999a), van Nispen et al. (1998)
Levofloxacin, gatifloxacin: decrease in clostridia	Cultivation	Edlund et al. (1997b), Edlund and Nord (1999a)
Gatifloxacin: decrease in fusobacteria	Cultivation	Edlund and Nord (1999a)
GLYCOPEPTIDS		
Oral vancomycin		
Decrease in enterococci	Cultivation	Edlund et al. (1997a), Lund et al. (2000)
Decrease in staphylococci	Cultivation	Van der Auwera et al. (1996)
Overgrowth of lactobacilli (and pediococci)	Cultivation	Edlund et al. (1997a), Lund et al. (2000), Van der Auwera et al. (1996)
Strong suppression or elimination of bacteroides	Cultivation	Edlund et al. (1997a), Lund et al. (2000)
Decrease in clostridia and bifidobacteria	Cultivation	Lund et al. (2000)
Oral teicoplanin		
Increase in Gram-negative aerobic rods and total numbers of aerobes	Cultivation	Van der Auwera et al. (1996)
Increase in lactobacilli and pediococci	Cultivation	Van der Auwera et al. (1996)
LINEZOLID		
Reduction of enterococci	Cultivation	Lode et al. (2001)
Reduction of bifidobacteria, lactobacilli, clostridia, and bacteroides	Cultivation	Lode et al. (2001)
Increase in *Klebsiella*	Cultivation	Lode et al. (2001)
TETRACYCLINES		
Doxycycline		
Decrease in bifidobacteria	Cultivation	Saarela et al. (2007)
Tigecycline		
Decrease in enterococci	Cultivation	Nord et al. (2006)
Decrease in *E. coli*	Cultivation	Nord et al. (2006)
Increase of other enterobacteria (*Klebsiella* and *Enterobacter* spp.)	Cultivation	Nord et al. (2006)

(Continued)

Table 1 | Continued

Antibiotic	Method	References
Marked reduction of lactobacilli and bifidobacteria	Cultivation	Nord et al. (2006)
Increase in yeasts	Cultivation	Nord et al. (2006)
MACROLIDES, LINCOSAMIDES, SYNERGISTINS		
Erythromycin		
Dramatic decrease in streptococci and enterobacteria	Cultivation	Brismar et al. (1991)
Decrease in clostridia, lactobacilli, bifidobacteria, and bacteroides	Cultivation	Brismar et al. (1991)
Clarithromycin		
Reduction of enterobacteria, *E. coli*, and streptococci	Cultivation	Brismar et al. (1991), Edlund et al. (2000b)
Dramatic decrease in clostridia, and bacteroides	Cultivation	Brismar et al. (1991)
Reduction of lactobacilli and bifidobacteria	Cultivation	Brismar et al. (1991), Edlund et al. (2000b)
Telithromycin		
Decrease in *E. coli* but overgrowth of non-*E. coli* enterobacteria	Cultivation	Edlund et al. (2000a)
Reduction of lactobacilli and bifidobacteria	Cultivation	Edlund et al. (2000a)
Clindamycin		
Increase in enterobacteria	T-RFLP	Jernberg et al. (2005b)
Decrease in total anaerobic bacteria	Cultivation	Nord et al. (1997)
Decrease in lactobacilli and *Bacteroides*	Cultivation	Nord et al. (1997), Sullivan et al. (2003)
Decrease in clostridia	Cultivation	Nord et al. (1997)
Disappearance of bifidobacteria	Cultivation	Jernberg et al. (2005b), Nord et al. (1997)
Dramatic decrease in *Bifidobacterium*, *Clostridium* (particularly *C. coccoides* subgroup as *Eubacterium*) and *Bacteroides*	T-RFLP	Jernberg et al. (2005b)
Suppression of *B. vulgatus*, *B. acidofasciens*, *F. prausnitzii*, *C. indolis*, and *C. leptum* cluster	DGGE	Donskey et al. (2003)
No change in *B. thetaiotaomicron* and *B. uniformis*	DGGE	Donskey et al. (2003)
Streptogramins: Quinupristin/dalfopristin		
Decrease in anaerobic Gram-negative bacteria	Cultivation	Scanvic-Hameg et al. (2002)
Increase in enterococci and enterobacteria	Cultivation	Scanvic-Hameg et al. (2002)
OTHERS		
Cotrimoxazole		
Suppression of Enterobacteriaceae	Cultivation	Mavromanolakis et al. (1997)
Metronidazole		
No significant change but not enough data available	Cultivation	Sullivan et al. (2001)
Nitrofurantoin		
No impact on intestinal microflora	Cultivation	Mavromanolakis et al. (1997)

REDUCTIONIST APPROACH AND BIASES

In their attempts to reduce ignorance and in contrast to the holistic approach based on the combination of conventional techniques and technology-driven methods, which enable researchers to study and make sense of a complex ecosystem, diverse studies based on experimental models have induced reductionism in the understanding of human microbiota and have generated contradictory results (Raoult, 2010; Fang and Casadevall, 2011).

HYPOTHESIS-DRIVEN RESEARCH VERSUS HOLISTIC-DRIVEN RESEARCH

Because it has been suggested that gut microbiota play a role in health and disease, it has been attractive to find a stable model to help scientists to understand host-gut microbiota mutualism, but this relationship is very complex and involves control diets, genetics, and environmental conditions. The first germ-free model was used by Pasteur in 1885. Since that time, various models have been used to study gut microbiota, including germ-free neonatal pigs (Meurens et al., 2007), zebrafish (Rawls et al., 2004), and gnotobiotic mice, which is the most effective tool (Backhed et al., 2007; Goodman et al., 2011).

Conversely, based on observations and not supported by any preconceived hypothesis, several findings by different research teams have shown a significant reduction in *Bacteroidete*s proportions in obese patients (Armougom et al., 2009; Turnbaugh et al., 2009; Million et al., 2011). Comparing the composition of the gut microbiota between young adult female monozygotic or dizygotic twins who are obese or lean and their mothers, Turnbaugh found that obesity was associated with reduced bacterial diversity and, notably, a reduced proportion of *Bacteroidetes*. Moreover, the

genes over-represented within obese individuals exclusively belong to the *Firmicutes* phylum (Turnbaugh et al., 2009). Nevertheless, previous investigations based on genomic data and experimental models have shown that co-colonization of *B. thetaiotaomicron* and *M. smithii* in the digestive tract of xenobiotic mice was responsible for significant weight gain, with discordance between sequence analysis results and the initial hypothesis (Xu et al., 2003; Samuel and Gordon, 2006).

CONFLICTS OF INTEREST

Finally, to exhaustively review the topic of human gut microbiota composition and mutualism with the host, it would be morally objectionable not to address the central influence of funding sources and transparency disclosures (Million and Raoult, 2012).

A transparency declaration of conflict of interest is important for publication in the medical literature. Lundh et al. (2010), based on the articles published in six of the most prestigious medical journals, showed that the publication of studies financed by industries was associated with an increase in the impact factor of the journal. Regarding the economic aspect and the payment of physicians by five manufacturers of hip and knee prostheses, a recent study confirmed that only approximately 80% of direct payments and 50% of indirect payments to physicians have been disclosed (Okike et al., 2009). Some authors even consider that publications in medical journals are a marketing tool for the pharmaceutical industry (Smith, 2005). In the beverage and food industry, Levine studied the financial relationships between industry and authors who have published research on alimentary substitutes. Classifying these publications as neutral, critical, or supportive toward the alimentary substitutes, the authors suggested a significant association between the authors who support the efficiency of the substitute and the authors with financial relationships with the industrial company (Levine et al., 2003).

Finally, Thomas et al. (2008) has recently shown that of 63 randomized trials published regarding nutrition and obesity, 67% were supported by the food industry. Moreover, the results of industry-supported trials were significantly associated with a higher quality of reporting score associated with long-term WL. Moreover, compounding this problem, some scientists do not declare their conflicts of interest. Based on these data, and the considerable financial involvement associated with human gut microbiota research, notably in obesity, we regret that there is not more public funding (Smith, 2005), and that conflict of interest with food industry are not actively required as for pharmaceutical industry.

CONCLUDING REMARKS

Factors affecting the composition of the gut microbiota and the relationship with the hosts are of considerable complexity. Both physiological and external factors are often unstable over time, influencing the gut microbiota. Despite the contribution of recent technologies, the repertoire of this ecosystem remains incomplete. As a striking example, despite the dramatic increase in the number of publications regarding gut microbiota, simplistic anomalies persist, such as the discordance among microscopic observation, pyrosequencing, and culture results. We regret that fewer studies are based on observation and description in opposition to studies performed to confirm a hypothesis. Indeed, it is paradoxical to design experiments and models to confirm a hypothesis because the ecosystem is only partially described. Finally, the central problem of funding sources and transparency declarations lead us to hope that public funding will develop food-industry-independent research to increase confidence in the results.

In the future, we think that culturomics followed by the high-throughput genome sequencing and its applications as the exploration of host-pathogen interactions will allows to capture the relationships in the gut microbiota. In addition, technology advances in pyrosequencing with higher reads fragment analysis, may facilitate the analysis to low taxonomic level (genera, species) reducing consequently the depth bias.

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Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows

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Lactic Acid Bacteria (LAB) are ancient organisms that cannot biosynthesize functional cytochromes, and cannot get ATP from respiration. Besides sugar fermentation, they evolved electrogenic decarboxylations and ATP-forming deiminations. The right balance between sugar fermentation and decarboxylation/deimination ensures buffered environments thus enabling LAB to survive in human gastric trait and colonize gut. A complex molecular cross-talk between LAB and host exists. LAB moonlight proteins are made in response to gut stimuli and promote bacterial adhesion to mucosa and stimulate immune cells. Similarly, when LAB are present, human enterocytes activate specific gene expression of specific genes only. Furthermore, LAB antagonistic relationships with other microorganisms constitute the basis for their anti-infective role. Histamine and tyramine are LAB bioactive catabolites that act on the CNS, causing hypertension and allergies. Nevertheless, some LAB biosynthesize both gamma-amino-butyrate (GABA), that has relaxing effect on gut smooth muscles, and beta-phenylethylamine, that controls satiety and mood. Since LAB have reduced amino acid biosynthetic abilities, they developed a sophisticated proteolytic system, that is also involved in antihypertensive and opiod peptide generation from milk proteins. Short-chain fatty acids are glycolytic and phosphoketolase end-products, regulating epithelial cell proliferation and differentiation. Nevertheless, they constitute a supplementary energy source for the host, causing weight gain. Human metabolism can also be affected by anabolic LAB products such as conjugated linoleic acids (CLA). Some CLA isomers reduce cancer cell viability and ameliorate insulin resistance, while others lower the HDL/LDL ratio and modify eicosanoid production, with detrimental health effects. A further appreciated LAB feature is the ability to fix selenium into seleno-cysteine. Thus, opening interesting perspectives for their utilization as antioxidant nutraceutical vectors.

Keywords: selenium, bioactive molecules, proteolysis, exopolysaccharides, CLA, short chain fatty acids, bacteriocins, diet

SHORT HISTORY OF LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) include a large number of bacterial genera among which the best known are lactobacilli, lactococci, enterococci, streptococci, leuconostoc, and pediococci. These genera differ for morphology, pH and salt tolerance, temperature optimum, habitats, and pathogenic potential. At present, it is very difficult to establish a clear demarcation line between beneficial and virulent species, being some problematic traits more linked to strain than to species. However, Lactobacilli and Lactococci are considered GRAS (generally regarded as safe).

LAB were among the first living organisms on the earth: they appeared about three billions years ago, in the transition period from anaerobiosis to aerobiosis. Apparently, they seem to be well adapted to both anaerobic and aerobic life conditions, since they bear all the necessary proteins for respiration and several enzymes involved in fermentative pathways. Nevertheless, during evolution they never acquired the ability to biosynthesize heme, an essential component of cytochromes, so they can shift to respiratory metabolism only with exogenous hemine supplementation. In the case of pathogenic streptococci, they can subtract heme from host hemoglobin by means of hemolysin, a protein considered a virulence factor. Therefore, most LAB species are compelled to live on fermentation metabolism with lower energy yields (Carr et al., 2002).

Furthermore, LAB inability to synthesize heme molecules implies that they cannot get functional heme enzymes such as peroxidases and catalases that are the most efficient proteins involved in hydroxyl radical scavanging. This results in an impaired resistance to O_2 toxicity. In spite of this, LAB can tolerate oxygen and are defined microaerophylic organisms (Brioukhanov and Netrusov, 2007).

Some strategies have been set up by these bacteria to replace heme enzyme anti reactive oxygen species (ROS) efficacy, for instance the use of metals as radical scavengers: zinc constitutes an ion trap for oxygen radicals, (Salvatore et al., 2007) manganese can act as a superoxide dismutase-like system (De Angelis

and Gobbetti, 1999), while selenium acts in the selenocysteine-harboring proteins (Calomme et al., 1995). It is known that LAB species are able to accumulate high intracellular amounts of manganese (up to 25 mM), selenium, and zinc.

During evolution, due to the low energy gain by fermentation and to the harsh life condition in an "oxidant" world (like the one in which we now live), LAB have been compelled to specialize their metabolism rather toward stress defence than to acquire strategic biosynthetic abilities. Therefore, they developed symbiont/parasite relationships with plants and animals which can supply vitamins, proteins, and amino acids. It is worth noting that LAB had acquired the ability to recognize several sugars, such as for instance xylose, cellobiose, ribose, arabinose, glucose, and fructose, before they developed the ability to ferment lactose to lactate, which was made possible only after mammal's expansion on the Earth. Therefore, they firstly colonized fruits and vegetable ecological niches, and later cheese, wine, and especially milk, which constitute their election habitat being rich in lactose (Carr et al., 2002).

It is possible that, in early life, sugar catabolism was not as predominant as in current LAB metabolism and that parallel pathways, like acid and amino acid decarboxylations and arginine deimination (ADI), could simultaneously be activated to get energy on poor media such as cell lysis products (ribose, fatty acids, amino acids) (Konings, 2006).

However, commensal LAB living in both gut and other mucosal ecological niches, although fed with abundant nutrients, still have a stressful life and often are compelled to cope with antagonistic host factors as well as with yeast or bacteria sharing the same habitat. These harsh conditions allowed the evolution of interesting metabolic and cross-talk features.

LAB BIOCHEMISTRY

Most LAB biochemical pathways have been fully elucidated so far, due to the strategic importance of these bacteria in the food industry both as starters and as biocontrol agents for food deterioration. Recent interest on LAB as ascertained probiotic agents has completed the information about LAB metabolism and its role in the context of the gut ecosystem.

LAB ENERGY METABOLISM

LAB energy metabolism is chiefly based upon lactic fermentation, ADI, acid, and amino acid decarboxylation. While sugar fermentation has a true energetic role, the other pathways can solve different and more complex functions. Lactic fermentation is an appreciated feature in both gut and industrial LAB since its end-product, lactate, by causing acidification, ensures a control over less friendly bacteria.

Sugar fermentations

Homolactic fermentation. Homolactic fermentation always results from glycolysis. Obligate homofermentative LAB (pediococci, lactococci, streptococci, and some lactobacilli) produce 100% lactic acid through the Embden-Mayeroff route from different hexoses, which are internalized by means of specific membrane transporters such as permeases and symporters, and then isomerized to glucose or fructose. Galactose constitutes an exception since it can be either isomerized to glucose by the Leloir reaction mediated by high energy compounds (UDPGlu-UDPGal) or drifted to glycolysis through the D-tagatose 6-phosphate route, generating diidroxiacetonphosphate, hence glyceraldehyde phosphate (**Figure 1**).

When a disaccharide, such as lactose or saccharose, is present it can be hydrolyzed into the two monosaccharides in the external environment or rather be uptaken as disaccharide and then hydrolyzed inside the cell. From each exose, two moles of lactic acid are produced at the end of the glycolytic process as the result of pyruvate reduction to lactate, through NADH re-oxidation to NAD^+. Lactic acid can be either in the D or in the L optical form, depending on each species' genetic determinants encoding either D-lactate (D-LDH) or L-lactate (L-LDH) dehydrogenase, respectively. Some species can produce both D and L lactate as the result of racemase activity, or due to the presence of genetic determinants for both LDH isoforms. In this case the second genetic determinant can derive by horizontal gene transfer. The most common catabolic pathway, i.e., the conversion of the disaccharide lactose into lactate, generates therefore four moles of lactic acid and four moles of ATP. No gas is produced in the process. This low energy gain can sometimes be improved by proton-substrate symport, i.e., lactic acid excretion, generating a proton gradient: since this system is electrogenic it can increase the energy yield of LAB.

Facultative homofermenters can direct part of the pyruvic acid that is generated by glycolysis toward the production of formate, acetate, and ethanol. Pyruvate-formate-lyase can convert pyruvate (C3) into formate (C1) and acetylCoA (C2). The latter can undergo transferase reaction into acetyl phosphate and then conversion into acetate leading to ATP synthesis. Acetate can be either accumulated in the growth medium or alternatively reduced to ethanol *via* acetaldehyde with NADH consumption, depending on the pH, and reduced pyridine coenzymes availability. This route allows one additional ATP mole gain, but less lactic acid is produced. Since formate can be decarboxylated/oxidized, an additional CO_2 mole can be produced by this pathway (gas producing bacteria).

Heterolactic fermentation. Heterolactic fermentation concerns LAB which lack the glycolytic enzyme fructose 1,6 bisphosphate aldolase (*Leuconostoc*, *Oenococcus,* and some *Lactobacillus* species) so they cannot metabolize hexoses through the Embden-Mayeroff pathway. Therefore, they utilize the pentose-phosphate route with the transketolase reaction joining the glycolysis with the three carbon metabolite glyceraldehyde 3-phosphate. The remaining C2 unit, acetyl phosphate, is then converted into ethanol or into acetate (the latter reaction resulting in an additional ATP mole gain), depending on NAD^+/NADH ratio, as previously described for the facultative homofermenters. Nevertheless, acetate production is not so frequent due to the necessity of NADH re-oxidation. The energetic yield of the transketolase pathway is lower than the homolactic fermentation giving rise only to 1 ATP mole, 0.5 moles of lactate, and 0.5 moles of ethanol or acetate, per mole of consumed hexose, plus one CO_2 mole deriving from the hexose/pentose conversion (by decarboxylation). So, the heterofermentative route is gas producing.

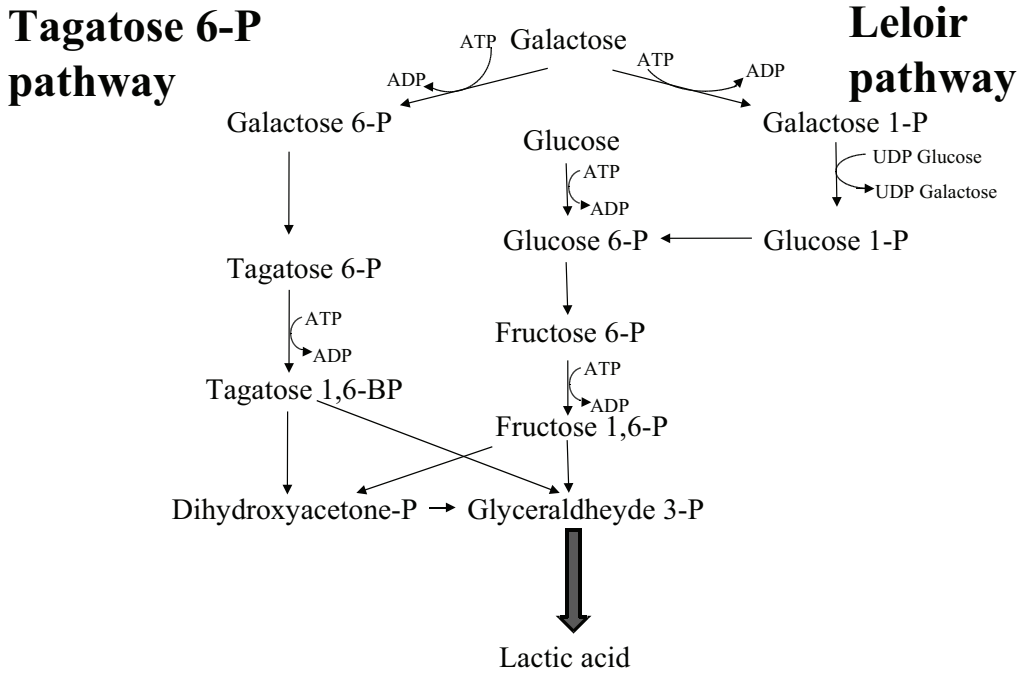

FIGURE 1 | Galactose metabolism in Lactic Acid Bacteria.

All heterofermentative LAB can also degrade pentoses, while not all homofermenters are also pentose degraders.

Arginine deimination

Besides sugar level phosphorylation, LAB can get energy from ADI to citrulline and citrulline cleavage to ornithine plus carbamoyl phosphate. The latter is then converted into ATP and carbon dioxide, or rather used for pyrimidine biosynthesis. This route also generates two moles of ammonia (one from the arginine-citrulline transition, the second from carbamoyl phosphate hydrolysis) useful to compensate the acidity generated by sugar catabolism to lactic acid, acetic acid, and formic acid, in both homo- and hetero-fermenting conditions (De Angelis et al., 2002).

On the other hand, ornithine is exchanged with extracellular arginine in an ornithine/arginine antiport system ensuring the continuity of the reactions. In wine, LAB possessing this pathway have been selected, since the wine environment is still rich in arginine after yeast alcoholic fermentation. Although the ADI route is somehow similar to an inverted urea cycle, from an evolutionary view point these pathways are not related at all (Liu et al., 1996).

Acid and amino acid decarboxylations

Apart from its secondary involvement in pH buffering, the arginine deiminase route is chiefly an ATP generating pathway useful to complement substrate level phosphorylation. On the contrary, acid and amino acid decarboxylations solve more complex roles and their evolutionary history is worth to know. Decarboxylations are coupled with electrogenic antiport systems generating a proton gradient across the cytoplasm membrane and the overall system supports a double role: acidity control and proton motive force (PMF) generation, i.e., supplementary energy in organisms unable of respiration. In the case of acid decarboxylations, the reaction concerns dicarboxylic and tricarboxylic acids, like malate and citrate, and the reaction products are not informational molecules.

It has been hypothesized that, early in evolution, acid decarboxylase activities were mainly directed toward acidification control. Later, free energy conservation mechanisms have been acquired by coupling decarboxylation with electrogenic antiport systems, which allowed more sophisticated cellular responses, like generation of metabolic energy and a better overall acid stress resistance. These pathways are believed to having evolved through horizontal gene transfer allowing antiporter proteins genes to be located in the same operon as decarboxylase genes, thus undergoing the same transcriptional control (Makarova and Koonin, 2007) On the other hand, LAB genomes are known to encode a highly variable number of genes that suggests that during evolution gene acquisition/loss has played a crucial role in determining the present situation. LAB genome size ranges from the 1.8 Mb of *L. gasseri* to 3.3 Mb of *L. plantarum* and also the G+C content vary significantly (from 34 to 46%) (Siezen et al., 2004), suggesting frequent recombination events. Furthermore, the presence of soluble acid decarboxylases and membrane bound precursor/product exchangers in LAB (Lucas et al., 2005), constitutes an intermediate event between the simple decarboxylation occurring in the cytoplasm of strictly anaerobes (like *Clostridia* and *Fusobacterium*), where no free energy is conserved, and the membrane bound complexes (including decarboxylases and transporters) found in gamma proteobacteria

in which active sodium extrusion occurs (Makarova et al., 2006).

For what concerns amino acid decarboxylases, their physiological roles are similar to those of acid decarboxylases, (Molenaar et al., 1993). Nonetheless, some additional functions like bacteria–bacteria interactions and host-symbiont communication were found, being some products informational molecules. Glutamate can be decarboxylated to gamma-aminobutyrate (GABA), histidine to histamine, phenylalanine to β-phenylethylamine, tyrosine to tyramine, triptophane to triptamine, ornithine to putrescine, lysine to cadaverine (Konings, 2006).

Amino acid decarboxylations are catalyzed by PLP or pyruvoil dependent enzymes that can be either soluble (Pessione et al., 2005) or membrane-located. In *Enterococcus* a membrane bound tyrosine and phenylalanine decarboxylase has been described (Pessione et al., 2009): this cellular location can allow a better interaction between decarboxylase enzyme and transporter protein. This may constitute a further evolutive step toward a more efficient system to get energy and to counteract acidic stress in a less acid-tolerant genus than other LAB (**Figure 2**).

Some produced amines are bio-active molecules (histamine, tyramine, triptamine, beta-phenylethyl amine, GABA), acting at vascular or central nervous system level of human host (Moreno-Arribas et al., 2003), while others are informational messengers for microbial cross-talk and for stress control (at least for putrescine in some bacterial species). All of them can undergo further bio-transformations by monoamino- and diamino-oxidases. These routes can thus be considered as "escape-pathways" during sugar depletion or excessive acidification (i.e., stationary phase stress) but also as means to interact with the external environment. It has to be considered that most LAB genomes harbor genes for both the ADI pathway enzymes and amino acid decarboxylases: often these two routes are reciprocally regulated (*Lactobacillus, Lactococcus*) (Lamberti et al., 2011a,b) but sometimes they do not compete and are parallely used (*Enterococcus*), underlining their similar but different physiological function. Proteomic studies can add useful information about the physiological and environmental modulations acting on these foundmental pathways. From a human point of view, while acid decarboxylations are appreciated because they reduce acidity (i.e., in wine), amino acid decarboxylations, and ADI are both regarded as problematic since they can give rise to ammonia and undesired bio-active amines such as histamine, that is an allergy mediator, or tyramine which is involved in hypertension and more severe syndromes such as cerebral hemorrhage (Millichap and Yee, 2003). Conversely GABA and beta-phenylethylamine, are appreciated molecules since they can control appetite, mood, and smooth muscle activity, as it will be better elucidated in the next paragraphs (Inoue et al., 2003).

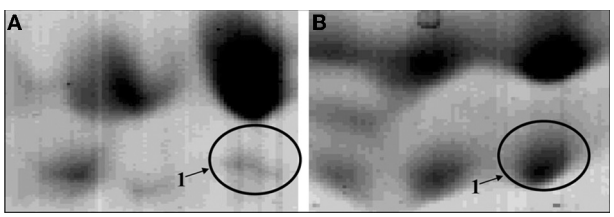

FIGURE 2 | Zoom of the proteomic maps of the membrane-enriched protein fraction of a tyramine and beta-phenylethylamine producer *Enterococcus faecalis* grown in absence (A) or presence (B) of tyrosine. The circle indicated the enzyme TDC (tyrosine/phenylalanine decarboxylase).

LAB proteolytic system

The choice to use amino acids not only as a nitrogen source but also for energy metabolism, requires high amino acid availability, and, for this reason, LAB have evolved complex proteolytic and peptidolytic systems. These include three components: (1) extracellular or membrane bound proteases (mainly PrtP and CEP), hydrolyzing proteins in oligopeptides; (2) membrane transport proteins (Opp, Dtp, and Dpp ATP-binding cassette transporters), catalyzing intracellular uptake of oligopeptides, di/tripeptides, and dipeptides, respectively; (3) intracellular peptidases, which cleave peptides into free amino acids (Konings, 2002).

In Lactococci, proteases are prevalently cell-envelope anchored (CEP proteases), except in *Lactococcus lactis* subsp. *cremoris* which is able to secrete proteinases. In Lactobacilli some of them have an extracellular location (*Lb. lactis* and *Lb. helveticus*) while others are cell-wall associated (*Lb. delbruekii* ssp *bulgaricus*). Most LAB proteolytic enzymes are synthesized as pre-pro-enzymes, whose signal peptide is cleaved upon membrane translocation: the resulting membrane bound pro-proteinase undergoes autocatalytic cleavage to obtain its mature, active, conformation. These proteases are serine proteinases, with high molecular weight and 5.5–6.5 pH optima, which is compatible with the environment in which LAB live.

However, other systems could be present to allow free amino acid availability at the external side of the cytoplasmic membrane. Actually, some peptidases have been detected on the inner face of the cytoplasmic membrane by antibody recognition (PepX in Lactococci) or have been supposed to be associated to an anionic specific permease, although they had been cytosolic-predicted on the basis of genome sequences data. For instance, PepA does not bear a signal peptide or a true hydrophobic domain that could justify its membrane location. Nevertheless, its high substrate specificity in releasing N-terminal acidic residues from peptides could account for its role in supplying free glutamate, for the glutamate-GABA pathway antiporter, at membrane level. In *E. faecalis* a similar function can be performed by ClpP: this protease is able to hydrolyze tetra- and tri-peptides containing the recognition sites tyrosine-tryptophan or tyrosine-leucine (Katayama et al., 1988). This may result in free tyrosine availability for the tyrosine-tyramine decarboxylation route.

An important role that is performed by both released and cell-wall associated LAB proteinases in the human gut are the digestion of not fully hydrolyzed proteins. They may shorten long and medium-sized peptides into smaller peptides. This role can also be played by intracellular peptidases that are released by LAB cell autolysis. Since the LAB proteolytic system has evolved in the milk ecological niche, is particularly suitable for diary protein hydrolysis. Apart from their digestive-assimilative function,

these enzymes have received great attention because they are able to generate bio-active peptides from both caseins and milk whey proteins (Law and Haandrikman, 1997). Diary proteins are digested in the human stomach and tenuous intestine by endogenous proteolytic enzymes. This digestion is completed by proteases supplied by the gut microflora. Casein is made up of four main proteins: alpha s1 casein, alpha s2 casein, beta-casein, and k-casein, differing in amino acid sequence, phosphorylation, and glycosylation degree, hydropaticity index. The hydrolysis of all these components, whose ratio is about 38:11:38:13, can give rise to bioactive molecules acting at different biological levels. Similarly, hydrolytic cleavage of milk whey proteins (alpha lacto albumine, beta lactoglobulin, lactoferrin, and immunoglobulins) can also generate bioactive peptides. The proteolytic enzymes that are released by LAB have been analyzed by 2DE-MS and proved to be very different in the different LAB species and strains, giving rise to a different pool of bioactive peptides. However, their activity, although very high at the normal intestinal pH (6.5–7.0), proved to be different toward alphas1 and beta casein (Hébert et al., 1999). The produced peptides are generally stable, although more investigations are needed to assess their real life span *in vivo*. The most interesting bioactive peptides resulting by LAB proteolytic activity are involved in immune system, cardiovascular system and central nervous system regulation, nutritional supplementation (such as metals assumption), and antimicrobial functions (antibiotic-like compounds). Sometimes each single peptide can accomplish two or more different physiological functions (Clare and Swaisgood, 2000).

LAB BIOSYNTHETIC CAPABILITIES

In spite of their limited biosynthetic abilities (especially for amino acid and vitamin synthesis), LAB can produce molecules of interest among which the most interesting are exopolysaccharides (EPS) and fructooligosaccharides (FOS), short chain fatty acids (SCFA), conjugated linoleic acids (CLA), and selenoproteins (**Figure 3**).

EPS and FOS

Like most bacteria, LAB can synthesize cell-wall structural polysaccharides (PS) such as peptidoglycan and lipoteichoic acids and exocellular polymers. The latter include both capsular PS where the PS is covalently bound to the cell surface and the EPS which form a loosely bound slime layer that can also be secreted into the environment.

EPS can be divided in homopolysaccharides (Homo-EPS) and heteropolysaccharides (Hetero-EPS). Homo-EPS consist of either D-glucose (glucans) or D-fructose (fructans) residues, with different types of linkage and branching degree. Hetero-EPS are constructed from multiple copies of an oligosaccharide and show little structural similarity to one another: glucose, galactose, xylose, mannose, arabinose, and rhamnose are the most represented sugars but also amino-sugars and polyols can be

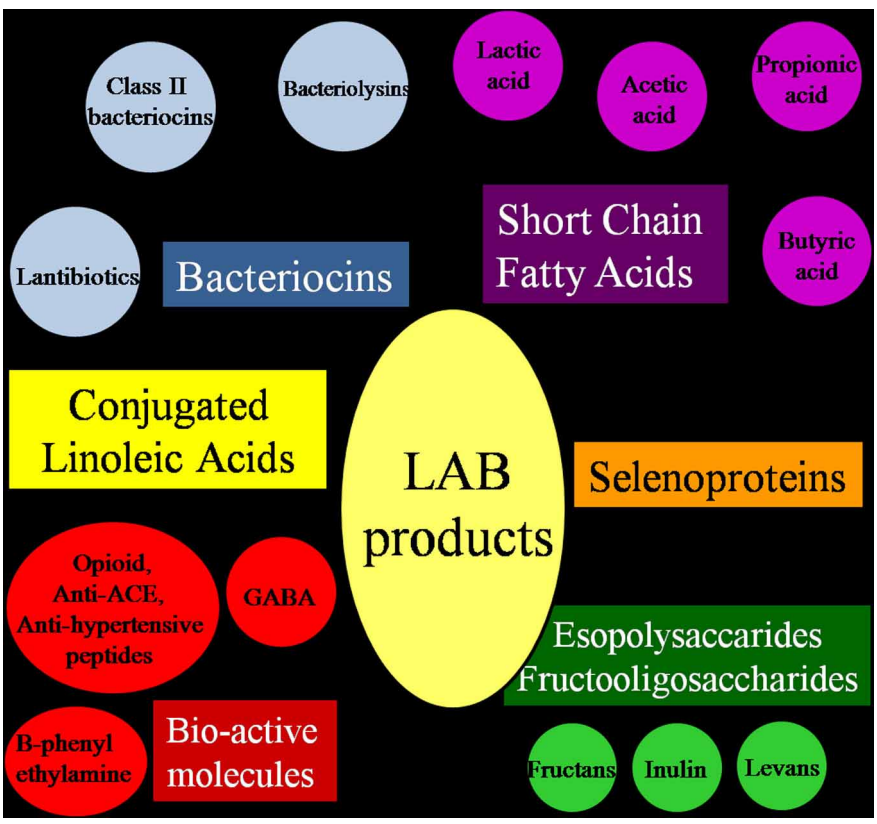

FIGURE 3 | Molecules contributing to the nutraceutical and probiotic potential of Lactic Acid Bacteria.

occasionally present as well as glucuronic acid. They are often highly branched with different binding types. From a biochemical point of view the synthesis of Hetero-EPS is an energy-intensive process that requires four separate reactions: (1) sugar internalization; (2) synthesis of sugar nucleotide precursors, providing the energy for the polymerization reaction; (3) assembly of the monosaccharide repeating unit by the combined action of different types of glycosyltransferases located at the cytoplasmic membrane level; (4) export of the EPS. Glucose-6-phosphate is the key metabolite that can be diverted from the glycolytic catabolic pathway to be addressed to the synthesis of UDP-glucose and dTDP-glucose, but also the Leloir route, when present, can be used to convert galactose to UDPGal and UDPGlu. In mesophylic LAB, the enzymes required for sugar uptake and for the synthesis of sugar nucleotides are chromosomally encoded, while the genetic determinants for EPS specific enzymes are plasmid located (Laws et al., 2001). Conversely, homo-EPS synthesis requires only one extracellular glycosyltransferase and the sucrose is used as substrate instead of activated sugars. This enzyme can thus synthesize alpha-glucans (dextran, reuteran, mutan) or beta-fructans (levans and inuline). The low-molecular mass oligosaccharides are known as FOS and glucooligosaccharides (**Figure 4**).

Based on the identification of sucrose binding boxes, several putative encoding genes for both glucansucrases and fructansucrases have been identified in the genome sequences of LAB (van Hijum et al., 2006).

Generally, LAB divert a small percentage of their sugar substrates toward the biosynthesis of EPS, whose physiological role is probably diverse and complex: due to their high water-binding properties, EPS constitute a protection factor against dessication and osmotic stress (Kumar et al., 2007). Their role in protecting the bacterial cell from phage attack has also been suggested (Moineau et al., 1996), but more recent experiments failed to reveal a significant phage resistant phenotype in EPS producer strains (Deveau et al., 2002). The same is true for the firstly hypothesized role as extracellular energy/carbon reserve, since most producers species lack the onset of genes necessary for their own EPS degradation (Badel et al., 2011). Therefore, this reserve would constitute a benefit only in the case of sintrophyc/symbiotic life with other bacteria. Actually EPS are biosynthesized by most LAB species under quorum-sensing control and they are related with biofilm formation and adhesion to solid surfaces. In the biofilm EPS also play a crucial role in sequestering essential cations (Kumar et al., 2007). In this context, EPS role as carbon reserve makes sense considering the overall population. However, their main biological function *in vivo* is to protect bacterial cells from toxic compounds (ethanol, sulphur dioxide, and toxic metal ions), from antibiotics and from host immune system, especially phagocytosis. A second important role is connected with adhesion to eukaryotic cells (plants, tooth surface), cellular recognition, *via* binding to lectins (Cerning, 1990), and immune system modulation. Krinos and co-workers (2001) demonstrated that, like capsular PS, also EPS can affect the surface antigenicity of the different strains (different EPS giving rise to different combinations) ultimately resulting in maintenance or elimination of specific strains in the gut ecological niche.

In the past decade fructo-oligosaccharides (FOS = inulin and levans), have found growing interest as prebiotics, i.e., compounds supporting growth of probiotic organisms. Prebiotics are molecules that are not metabolized by humans but favor both adhesion and persistence of probiotic bacteria in the gut environment. Inulin production has been observed in *Lactobacillus reuteri* and all inulosucrases share a common C-terminal motif (LPXTG) for cell-wall anchoring. Surface display of these enzymes results in enhanced adhesion of bacteria to human tissue cells, contributing to the maintenance of a healthy urogenital mucosa. Therefore, the presence of fructansucrase enzymes in commensal LAB can solve two distinct problems: from one side helping tissue colonization and persistence and from the other side supply with "self made" prebiotics (e.g., fructans) the commensal flora.

SCFA

Although SCFA are not produced by anabolic reaction but rather as the final catabolites of the energy metabolism, their importance in host-bacteria interactions is of such growing interest that will be treated in this paragraph. The most studied SCFA are butyrate, propionate, and acetate. *Lactobacilli* can produce SCFA by the fermentation of pyruvate, which is generated during the glycolytic pathway, but also by the phosphoketolase route in heterofermenting conditions. Acetate can contribute to ennvironment acidification and this is an appreciated feature since LAB can compete with other less acidophylic organisms (most pathogenic bacterial species) present in the same ecosystem. *In vivo* acetate enters the peripheral circulation to be metabolized by muscles and other tissues, while propionate is taken up by the liver. Both molecules can modulate sugar metabolism after a standard meal, by lowering glycaemia and improving

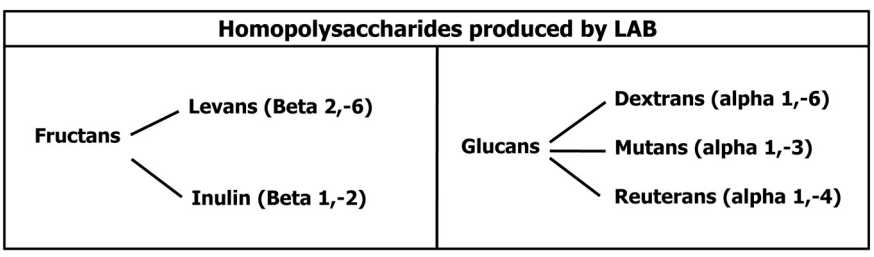

FIGURE 4 | Homopolysaccharides biosynthesized by Lactic Acid Bacteria.

insulin sensitivity. Butyrate has been claimed to have detrimental effects on body weight and obesity, since it represents an additional, easy available, energy source which is not directly introduced with the diet, but present as the result of commensal bacteria metabolism (non-digestible PS fermentation in the colon) (Turnbaugh et al., 2006). Once this SCFA is absorbed in the large intestine, it is not only converted to fat within the liver but it also positively regulates host genes promoting lipogenesis and fat storage into adipocytes (DiBaise et al., 2008). However, butyrate is readily absorbed by colonocytes where it exerts a control over uncontrolled proliferation and also stimulates differentiation, thus promoting the switch from neoplastic to normal phenotypes. It is known that butyrate differently affects normal colonocytes and tumor colon cells. On the former it exerts a trophic action inducing growth and proliferation, on the latter it rather induces cell-cycle arrest, differentiation, and apoptosis (Iacomino et al., 2001). Butyrate selectivity on the two different cell populations is considered a good weapon to treat cancer. The reason of the different effects of butyrate is probably linked to its different concentrations within the two type of cells: normal colonocytes rapidly utilize (through mitochondrial beta-oxidation) butyrate as the major carbon/energy source thus supporting ATP synthesis and proliferation. The remaining low levels cannot suppress cell cycle progression or induce apoptosis (Boosalis et al., 2001). On the contrary, colorectal tumor cells, due to the switch toward anaerobic metabolism, display an impaired capability to oxidatively catabolize butyrate. Therefore, in this type of neoplastic cells, its intracellular concentration is higher. This high level of butyrate may constitute a critical threshold responsible for the activation of signals that induce cell cycle arrest, differentiation, and apoptosis (Lupton, 2004). The effect on cell cycle arrest is partly due to inhibition on specific histone deacetylase (Davie, 2003), and this action can also be obtained by the use of propionate, but not acetate. Some authors also hypothesized that butyrate and propionate act in a synergistic way (Minucci and Pelicci, 2006). Furthermore, comparative transcriptomic and proteomic studies on butyrate-treated and control colonocytes, underlined a butyrate effect on extracellular matrix components which are important in cell–cell interactions, and on the angiogenetic process controlling the expression level of VEGF (vascular endothelial growth factor) and HIF-1 (hypoxia-inducible-factor-1) (Pellizzaro et al., 2002). Finally, butyrate can control oxidative and metabolic stress at molecular level, by enhancing repair responses (Sauer et al., 2007) and it can inhibit the synthesis of pro-inflammatory (IL12, TNF, gamma IF) cytokines, also contributing to tumor development control (Inan et al., 2000).

It is reasonable to conclude that butyrate can contribute to the host health when a neoplastic risk is present, while it is a problematic metabolite in case of obesity and metabolic disorders.

CLA

LAB can also biosynthesize CLA that are positional and geometric isomers of the cis9, cis12 octa-deca-dienoic acid (linoleic acid). So far, the best producing strains belong to the genera *Lactobacillus* and *Streptococcus*. The most frequently found isomers of linoleic acid have the unsaturation site at positions 9, 11 or 10, 12, namely, Δtrans 9, cis11 octa decadienoic acid, and, in lower amounts, Δcis10, trans 12 linoleic acid.

CLA can have positive effects on inflammation, cancer (apoptosis induction), metabolic disorders (insulin resistance, body weight control), and cardio-vascular diseases. Nevertheless, some detrimental influences on mammalian health have been observed, like a tumor-stimulating action or deleterious effects on glucose metabolism and lipid profiles. There is some evidence suggesting that different CLA isomers can exert differential effects, the more dangerous being the trans 10, cis 12 isomer. Also CLA concentrations play a role in the balance toward beneficial or detrimental effects (Rose, 1997): anti-carcinogenic effects are observable at CLA dosages of 0.5–1% (w:w) of the total diet and studies showed that humans generally excrete 20 mg of linoleic acid per day. This substrate can then be available for CLA producing bacteria in the intestine. Higher dietary intake of linoleic acid may imply risks (Ewaschuk et al., 2006). All these considerations suggest that further studies are needed to better characterize CLA producing LAB and their metabolic products.

LAB selenoproteins biosynthesis

The capability to produce metal-fixing enzymes is a further appreciated feature in LAB, that render them interesting commensal organisms. Actually, several *Lactobacillus* species are able to intracellularly fix sodium selenite (**Figure 5**) into selenocysteins and selenomethionines, thus providing a more bio-available form of this metal, which is generally poorly adsorbed by human cells in its inorganic form (Calomme et al., 1995).

The selenocysteine is defined as the 21st amino acid, is encoded by the UGA codon, and possess its own tRNA, which uses serine as an intermediate (SerSec t-RNA). Selenomethionine is non-specifically incorporated into proteins at the place of methionine and it is also important for trans-selenation reactions. Several bacterial enzymes have been demonstrated to contain selenocysteines in their active site and all belong to the oxidoreductase class: among these glutathione peroxidase, an enzyme of crucial importance to control oxidative stress and all related diseases in both bacteria and eukaryotic cells. *Lactobacillus reuteri,* for instance, express a selenocysteine-lyase (Lamberti et al., 2011a,b), a PLP-dependent enzyme having a key role for new

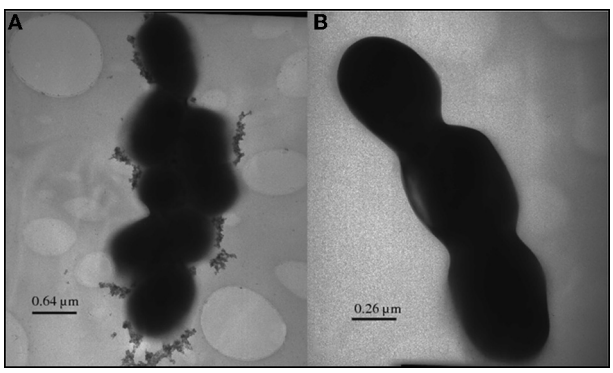

FIGURE 5 | Inorganic selenium granules displayed by TEM on the surface of a selenium-fixing *Lactobacillus reuteri* strain.

seleno-proteins biosynthesis, supplying the optimal substrate (H_2Se) to selenophosphate synthetase, which catalyze the production of the "activated" selenium form useful to selenocysteine sinthetase to produce L-selenocysteinil-tRNA (Lacourciere and Stadtman, 1998). LAB can also function as chelators for other nutraceutical important metals at the colon level. Investigations carried out in different prokaryotes leaded to the characterization of many mechanism for "channeling" zinc (Znll) ions, including zinc-binding, zinc-importing proteins, and zinc export systems (Blencowe and Morby, 2003). Zinc-bearing and zinc-extruding LAB can thus function as immunomodulators useful to control viral gastroenteritis (Salvatore et al., 2007).

LAB ECOLOGY

The contribution of LAB to the overall intestinal ecosystem has been only recently elucidated. Traditional culture methods failed to shed light on the actual microbial populations resident in the gut of adult humans, but the application of culture-independent techniques such as PCR combined with DGGE (Denaturing gradient gel electrophoresis) resulted in a more detailed knowledge of what really happens at gut level. First of all it is important to differentiate resident and transient bacteria: Vaughan and co-workers (2002) reported a list of *Lactobacillus* species permanently colonizing the intestine: *L. acidophilus*, *L. brevis*, *L. casei*, *L. crispatus*, *L. delbrueckii*, *L. fermentum*, *L. fructivorans*, *L. gasseri*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*, *L. ruminis*, *L. sakei*, *L. salivarius*, *L. vaginalis*. It is worth mention that LAB represent the 0.01–1.8% of the total bacterial community living in the human gut (Louis et al., 2007).

LAB–GUT MICROBIOTA INTERACTIONS AND LAB ANTIBACTERIAL POTENTIAL

Both antagonism and cooperation contribute to the reciprocal relationships between LAB and other intestinal microorganisms. Biofilm formation, phage- and pheromones-mediated genetic exchange and synthrophies are well established strategies for cooperating with other gut microorganisms. Nonetheless, the antagonistic behavior is prevalent.

Nutritional, enzymatic, and metabolic competition

Cell wall targeted enzymes such as N-acetylmuramidase and N-acetylmuramoyl-L-alanine amidase are proteins involved in peptidoglycan renewal, acting as general cell-wall lysis factors (Salazar and Asenjo, 2007). They are often produced to control bacterial populations sharing the same ecological niche. Similarly, chitinase, a protein hydrolyzing the prominent component of yeast and fungi cell walls, i.e., chitin, has been found in the exoproteome of LAB (Genovese, unpublished results).

However, nutrient competition is the best known aspecific interference, common to all microbial populations, set up by LAB. Since LAB are predominantly saccharolytic, but also amino acid utilizers, they can subtract nutrients to both saccharolytic and proteolytic species living in the gut environment. Furthermore, their peculiar fermentative metabolism, generating acids, constitutes a specific metabolic competition for coping with other endogenous or exogenous microbial species together with carbon dioxide, ethanol, hydrogen peroxide (produced and fastly eliminated by the NADH oxidase activity). The direct antimicrobial effects of organic acids (lactic, acetic, and propionic acid) is due to their interference with bacterial cytoplasm membrane potential and inhibition of active transport, while carbon dioxide (formed during LAB heterolactic fermentation) and hydrogen peroxide prevent the growth of some bacteria by creating an unfavorable environment.

It is worth noting that the mentioned LAB protelytic activity toward caseins can also give rise to antimicrobial peptides such as k-casecidin, a pentapeptide (Phe-Phe-Ser-Asp-Lys) derived by k-casein hydrolysis, and isracidin, an oligo peptide originated from alpha s1 casein. Both molecules proved to be very active against *Staphylococus aureus,* and the latter also stimulates phagocytosis. Artificial removal of the Lys C-terminal and Phe N-terminal from k-casecidin strongly reduces the original activity of the natural pentapeptide (Matin and Otani, 2002). *L.acidophylus, L.plantarum, L.helveticus, L.rhamnosus,* and *Lactococcus lactis* produce other alpha s1 casein-derived peptides also, which have a completely different amino acid sequence. The latter proved to be active against *Enterobacter sakazakii*, a gram negative species causing infections in newborns (Hayes et al., 2006).

Bacteriocin production

A promising feature of LAB is the production of interference molecules, the bacteriocins (Montalbán-López et al., 2011). Bacteriocins are proteinaceous molecules that are synthesized at ribosomal level (and not as secondary metabolites) which can interfere with the growth of most bacteria. They have bactericidal action and are selective for prokaryotes. Bacteriocins are produced at the end of the exponential growth-phase and their spectrum of action can vary, depending on the producing species. Producer strains are immune to their own bacteriocins because they possess genes that encode immunity mechanisms, which enable a distinction between "self" and "non-self." Protection can be provided by a dedicated immunity protein and/or by a specialized ABC-transporter system that pumps the bacteriocin outside the producer membrane (Draper et al., 2009).

LAB are particularly prolific in bacteriocins production and can biosynthesize different types of antagonistic molecules. The lantibiotics, so named because they contain post-translationally modified aminoacids such as lanthionine (two alanines linked by a sulphur), β-metyl-lanthionine, dehydroalanine, and dehydrobutyrine, are small peptides (19–38 amino acids in length) mainly active against Gram positive bacteria (**Figure 6**).

They can be divided into two subclasses on the basis of their structure and mode of action: the elongated amphiphilic cationic lantibiotics (Nisin, Lactococcin, and Pediocin) are active through the formation of pores, leading to the dissipation of membrane potential and the efflux of small metabolites from target cells. By contrast, the globular lantibiotics (for example, Mersadicin) act through enzyme inhibition, interfering with cell-wall biosynthesis. The class II bacteriocins are small (<10 kDa) heat-stable peptides, not undergoing extensive post-translational modifications, although they may contain D-amino acids. The best known class II bacteriocin, the so-called pediocin-like, has a narrow but very specific activity against the food pathogen *Listeria monocytogenes* (Kazazic et al., 2002). Finally, bacteriolysins are large,

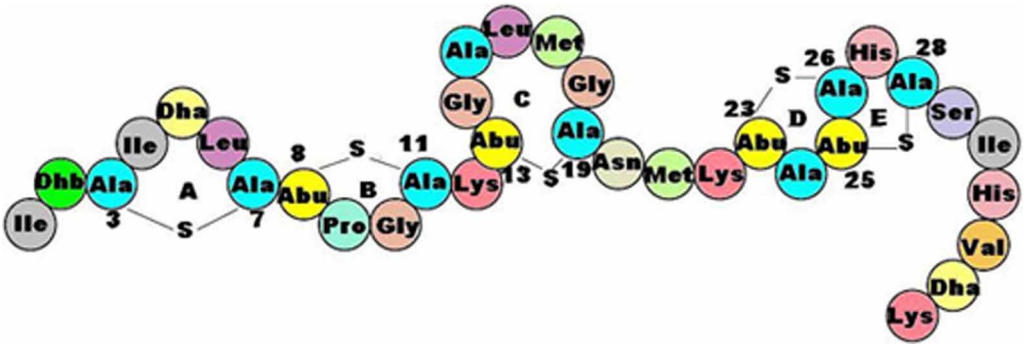

FIGURE 6 | General structure of a lantibiotic: covalently bound alanines and post-translationally modified amino acids are peculiar features of these anti-microbial peptides.

heat-labile antimicrobial proteins causing the lysis of sensitive cells by catalyzing cell-wall hydrolysis. Their structure contains a catalytic N-terminal domain that shows homologies to endopeptidases, and a C-terminus that represents the target recognition site (Lai et al., 2002).

All these natural antimicrobial compounds are the most interesting antagonist metabolic end-products synthesized by LAB. Last years' research has been focused on the study of their isolation, structure, and mechanism of action (Millette et al., 2007).

LAB-HUMAN HOST INTERACTIONS

LAB colonization and diet

LAB, especially Lactobacilli, colonize the new-born as soon as the infant is breast-fed. On the contrary, artificially-fed new-borns generally host different bacterial species such as Clostridia and Bacteroides. The presence of LAB, that are prevalently saccharolytic, acidogenic and bacteriocin producers, acts as a selective agent, protecting the gut environment from putrefactive and sulphate-reducing bacteria known to be involved in the production of gas, thiols, phenols, indole, histolesive proteases, and carcinogens (Holzapfel et al., 1998). While sugar fermentations chiefly occur in the proximal colon (pH 5–6), proteins and peptides putrefaction is typical of the distal colon (pH 7). LAB presence is maximal in the tenue and proves to be absolutely beneficial for the health and the well being of the host, but, to be maintained is necessary a specific diet.

Actually, it is well established that diet plays a crucial role in selecting microbial populations in the complex and dynamic ecosystem of human gut bacteria. Food containing LAB, such as yogurt and cheese, can enrich gut LAB populations since these bacteria can easily survive to the gastric transit. Actually, LAB are naturally acidophilic organisms (growth pH optimum 3.5–6.5) and they can further improve their acidic pH resistance by setting up strategies that produce alkaline metabolites (the referred ADI pathway and amino acid decarboxylations routes). Furthermore, as human or artificial milk can select LAB or Clostridia in the new-born, similarly, a "meat-fat" rich diet contributes to select and expand Clostridia in the adult while a non-metabolizable sugar diet (especially fructose-, inuline-, and cellulose-rich nutrients that escape regular digestion in the human body) favors LAB. The epidemiological link between the "meat-fat" rich diet and colon cancer (Hill, 1975) can partly be explained by the capability of Clostridia to metabolize bile acids to carcinogenic molecules responsible for neoplastic transformation of the gut mucosa (Murray et al., 1980). Thus, the role of LAB is not only confined to preventing colonization by exogenous bacteria but also to cope against toxins and mutagenic molecules of endogenous origin.

LAB host cross-talk and stress chaperones

It has recently been established that "symbiosis" can trigger adaptive responses in both bacteria and host, which are detectable by transcriptomic and proteomic studies (host-microbiota interactomics), and concern specific metabolic pathways activation, immune system stimulation/depression, and stress responses.

From the host side, activation of important genes involved in cell signaling pathways and immunomodulation has been observed in an *in vitro* experiment using CaCo-2 epithelial cells exposed to *Lactobacillus acidophilus*, a natural inhabitant of the human small intestine. The use of other food-derived LAB cannot trigger the same biological response, thus demonstrating that the long symbiosis has selected peculiar genetic characters for host-microbe interaction (O'Flaherty and Klaenhammer, 2011). Furthermore, the importance of LAB cell wall glycome in cytokine induction in intestinal epithelial cells has been proved with the use of mutants knock-out for protein glycosylation ability. These mutants not only failed to stimulate cytokines, but they were also unable to adhere to $CaCo_2$ cells and showed a significant impaired persistence in the mice gut environment (Tytgat et al., 2011).

From the bacterial side, adaptation responses, promoting persistence in the host have been described. In LAB, genetic loci specifically induced by gut transit are those involved in nutritional adaptation (hydrolases, ABC transporters, and PTS systems) but also in anti-stress mechanisms such as membrane modification, surface glycosylation and anti-oxidative defences (Lebeer et al., 2011). Bacterial stress protein production (enhanced synthesis of chaperones GroEL, GroES, DnaK, Hrc, Clp, Cts) in response to host factors has also been established (Henderson et al., 2006). It is now well demonstrated that, besides helping correct protein

folding, chaperones can perform multiple functions such as preventing denaturation, rearranging proteins after oxidative and pH stress and performing targeted proteolysis on the irreversible denaturated proteins. Among these functions is the adhesion to extracellular matrix proteins of the host tissues, and the stimulation of the host immune system. To perform these additional functions chaperones should be surface or extracellularly located.

This phenomenon is known as moonlighting and several moonlighting proteins have been described so far, both in eukarya and in bacteria, including LAB. They include not only stress chaperones (GroEL, DNAk) but also glycolytic and TCA cycle enzymes. Moonlighting proteins are extracellularly transferred without any export signal and without specific cell-wall or membrane anchoring motif: thus the surface or extracellular localization can be related to the environmental pH. Antikainen and co-workers (Antikainen et al., 2007a) demonstrated that higher extracellular pH can cause release of the weakly linked proteins in *Lactobacillus crispatus* but cell-wall renewal, that is frequent during the exponential growth phase, can also be involved in the detachment of such proteins (Sánchez et al., 2008). Generally surface-exposed and released moonlighting proteins exert different biological functions, namely adhesion (cell-wall bound) and immune system modulation (extracellular chaperones).

LAB adhesion to host tissues and immunomodulation

Adhesion. The bacteria-human mucosa interaction is a dynamic equilibrium and several factors can alterate this balance. Intestinal movements and bacterial chemotaxis cause a weak association between bacteria and the surface of the gut epithelium where electrostatic and hydrophobic bonds are prevalent. In a following step, a more specific interaction between molecular determinants on bacterial surface and specific gut mucosa receptors occurs. Specific targets on human tissues are collagen type I and IV, fibronectin, and laminin. In LAB, good binding-effectors are PS, lipoteichoic acids as well as proteins such as lectins and adhesines. The adhesion potential of a certain LAB strain is therefore crucial for survival and persistence in the gut environment.

Several surface located moonlighting proteins can act as adhesines. Both the chaperone GroEL and an elongation factor, EF-Tu, display adhesive capabilities, toward human tissues in *Lactobacillus johnsonii.* EF-Tu is a guanosine nucleotide binding protein playing a central role in protein synthesis (when expressed inside the cell) but able to bind fibronectin or mucine and epithelial cells when exposed on the cell surface (Granato et al., 2004). Trigger factor is also connected with adhesion: it has been described to be exposed on the surface of *Lactobacillus plantarum* and, in *L. reuteri* NCIB11951, a collagen I binding protein, shares high sequence homology with *E. coli* trigger factor (Aleljung et al., 1994). Apart from these proteins, also glycolytic enzymes can have adhesive properties: GAPDH is able to bind fibronectin, plasmin, and mucine in several bacterial species (Alvarez et al., 2003) and a similar role may be performed by PGK (phosphoglycerate kinase) that is referred to be extracellularly located in *Lactobacillus rhamnosus* GG (Sánchez et al., 2008). Some of these proteins will be treated in a separated chapter for their peculiar attitude to behave also as plasminogen receptors.

Immune system modulation. About 70% of the immune system is localized in the gastro-intestinal tract as GALT (gut-associated lymphoid tissue). A reciprocal relationship exists between LAB and immune system. From one side the immune system selects the LAB species to be accepted from the other side LAB can modulate immunological functions. The most evident LAB's effects concern the enhancement of the ratio between anti-inflammatory (IL-10, β-TGF) and pro- inflammatory (IL-1 beta, IL-3, IL-4) cytokines (Pessi et al., 2000) and the selection of T-lymphocyte populations (Karimi et al., 2009). Different LAB components, such as teichoic and lipoteichoich acids, lipoproteins, and EPS may be the inducers of the immune response (Weidenmaier and Peschel, 2008). Some of the referred adhesive proteins also exhibit immunomodulatory properties when they are secreted. This is particularly true for the chaperone GroEL which has been demonstrated to interact with macrophages and stimulate cytokines secretion. In *L. johnsonii* GroEL also induces a strong aggregation of the pathogen *Helicobacter pylori*, contributing to decrease of the bacterial load and thus facilitating clearance of the aggregated pathogens by the mucus (Granato et al., 2004). In *L. reuteri* a strict link between adhesion and regulatory T-cell induction has been demonstrated as well (Smits et al., 2005). Last but not least, an indirect immune stimulating activity can be triggered by proteolytic intestinal LAB species through the production of immunomodulating peptides from casein. These molecules primarly enhance lymphocyte proliferation and macrophage phagocytosis but they are probably important in regulating the development of the immune system in new borns and might contribute to attenuate allergic reaction and tumor development as well (Korhonen and Pihlanto, 2006).

LAB interaction with the plasminogen-plasmin system

Lactobacillus crispatus, *Lactobacillus acidophilus, L. amylovorus, L. gallinarum, L. gasseri,* and *Lactobacillus johnsonii* display the ability to bind plasminogen on their cell wall (Hurmalainen et al., 2007). Several adhesion-involved glycolytic enzymes (enolase, GAPDH, phosphoglycerate kinase, and phosphoglycerate mutase), bile salts hydrolase and the stress protein DnaK, have been demonstrated to behave as plasminogen (Pg) receptors. They all share a common feature, a C-terminus lysine. Some reports suggest that plasminogen binding capability in LAB can constitute a risk because plasminogen can be activated to plasmin thus triggering further proteolytic cascades, resulting in the degradation of the gut extracellular matrix. Nevertheless, LAB lack the intrinsic potential for plasminogen activation typical of pathogenic species. Actually, pathogenic bacteria like *Staphylococcus aureus* and *Streptococcus pneumoniae* can convert plasminogen into plasmin by specific virulence factors (staphylokinases and streptokinases, respectively) (Bergmann et al., 2003) that, so far, were never found in the genome of LAB. According to some authors (Antikainen et al., 2007b) LAB can exploit the endogenous system of the host (urokinase and tissue plasminogen activators) to trigger proteolysis, according to others (Sánchez et al., 2008). LAB can quench plasminogen, subtracting it from pathogenic bacteria. At present, this is still a controversial question concerning LAB and more research is needed to fully shed light on this bacteria-host interaction aspect.

LAB PROVED BENEFICIAL ACTIONS ON HUMAN HEALTH

The most important beneficial interactions between gut LAB and humans include metabolism regulation, infection control, and inflammation/allergy modulation. The last three effects are in some way connected with the immune system regulatory action exterted by LAB.

METABOLISM

Energy/mineral recovery

LAB can enhance energy recovery from nutrients by degrading non-metabolizable sugars and supply the host with their own β-galactosidase thus by-passing enzymatic lactose intolerance. Some casein phosphopeptides (CPP) show the capacity of maintaining in solution calcium ions, even in alkaline pH. This results in increased absorption of calcium useful in osteoporosis, but also in enhanced absorption of iron, zinc, and manganese, useful as enzyme co-factors or prostetic groups. To perform this function is necessary that LAB digest casein, especially alpha s1 and alpha s2 caseins, releasing CPP phosphorylated on Ser and Thr residues. Since beta and k-caseins are poor in hydroxylated amino acids, they are less suitable for phosporylation, and the peptides derived from them are less active in metal binding (Meisel, 1998).

Cholesterol-lowering

When released in the gut by LAB, SCFA can cause a decrease in the hepatic cholesterol synthesis and a redistribution of cholesterol from blood to the liver (Pereira and Gibson, 2002). Also deconjugation of bile acids by LAB can play a similar role in plasma cholesterol-lowering. Deconjugated bile acids are not well absorbed by the gut mucosa and therefore excreted. As a consequence, new cholesterol is driven from blood to liver, for *de novo* bile acid synthesis (St-Onge et al., 2000). In *Lactobacillus reuteri* a specific choloylglycine hydrolase, catalyzing the initial gateway reaction for bile acids deconjugation, is biosynthesized (Martoni et al., 2008). Cholesterol-lowering capability has also been demonstrated by LAB-produced EPS (Pigeon et al., 2002). Benefits on cardiovascular pathologies and hearth disease are thus expected.

INFECTION AND IMMUNOMODULATION

LAB have longly been known as being able to control infections because of their direct antibacterial action (mediated by bacteriocins and acid production) and to their indirect (immunologically mediated) action against viruses. The role of Lactobacilli in preventing traveller's diarrhea, growth of *Helicobacter pylori* and toxin producing *E. coli* has been demonstrated in both *in vitro* and *in vivo* studies. *Lactobacillus rhamnosus* GG has also been successfully employed for preventing *C. difficile* colitis, as well as to treat atopic eczema by means of modulation over Interleukin-10 (Pessi et al., 2000), while *Lactobacillus reuteri* can control IgE-mediated allergies by acting at regulatory T cells level (Karimi et al., 2009). Antiinflammatory (Kitazawa et al., 1998) and antitumoral activities have also been reported to be due to LAB-produced EPS. Rhamnose Hetero-EPS proved to be effective against gastric ulcer (Badel et al., 2011) and a stimulation of TNF has been demonstated (DeVuyst et al., 2007). Experiments using cocktails of *Lactobacillus* and *Bifidobacterium* also revealed the ability to cause apoptosis of colon carcinomas.

INFORMATIONAL MOLECULES AND OXIDATIVE STRESS

A very cutting-edge evidence is the possibility that LAB can control both the human antioxidant defences and the production of informational molecules acting on mood, blood pressure, and more generally on the gut-brain axis.

Lactobacillus helveticus, *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1, and *Lactococcus lactis* subsp. *cremoris* FT4 can all modulate blood pressure by producing angiotensin 1-converting enzyme inhibitory peptides (ACE inhibitors) from milk proteins. The most active molecules are tripeptides made up of Val-Pro-Pro and Ile-Pro-Pro, but other, casein-derived, antihyperthensive peptides like alpha s1 casokinin-5 (Phe-Phe-Val-Ala-Pro), alpha s1 casokinin-6 (Thre-Thre-Met-Pro-Leu-Trp), beta-casokinin (Lys-Val-Leu-Pro-Val), and a dipeptide Tyr-Pro, proved to be effective in blood pressure control as well (Yamamoto et al., 1999). Since their production is the result of the proteolytic activity of some LAB strains over casein, it is dependent upon casein availability in the intestine and from the proteolytic potential of each single strain. Generally LAB peptidases, by shortening the poly/oligopeptide chain, contribute to enhance the anti ACE potential. Interestingly, the final active short peptides are resistant to both pH variations and human digestive enzymes (Sipola et al., 2002; Gobbetti et al., 2004). Antithrombotic peptides derived from human and bovine k-caseinglycopeptides have also been found in five-days-old newborns after breast and formula feeding, respectively (Chabance et al., 1995). Even if these molecules can be produced as the result of human proteolysis on food, LAB proteolytic activity on casein can for sure contribute to their release *in vivo*. These molecules, named casoplatelin and thrombin inhibitory peptide, are 6–11 amino acids long oligopeptides, preventing the aggregation of ADP-activated platelets and the binding of human fibrinogen (lambda chain) on the platelet surface receptor. A shorter molecule, casopiastrin, also displayed fibrinogen binding inhibiting activity (Jollès et al., 1986).

The most interesting molecules produced with the contribution of LAB protease system on dairy food are the opioid peptides (exorphins) and opioid antagonist peptides (casoxins). In mammals' central and peripheral nervous system there are receptors (K, delta, and mu) for endogenously produced opioid peptides (endorphins) which have inhibitory activity on adenylate cyclase enzyme. Protein fragments with opioid-like activity have been found as the result of both human digestive pH/proteases and microbial proteases over casein molecules. These peptides are made-up of 4–10 aminoacids and can trigger a myorelaxant effect by acting on the mu receptors (beta-casomorphins) or on the delta receptor (alpha s1 casein-derived peptide). The N-terminal residue, which is crucial for triggering biological activity, is Tyr in beta-casomorphins and Arg in alpha s1 casein-derived peptide (Loukas et al., 1983). Nevertheless, the two molecules display different half-life due to their different structure and the presence of several proline residues (six) in beta-casomorphins seem to be the factor enhancing peptide resistance to enzymatic digestion in human digestive tract. Here they can affect intestinal transit time, water balance, and aminoacid uptake. Once absorbed into blood,

they reach the brain and peripheral receptors where they exert their relaxing action inducing sleeping and calmness (Chabance et al., 1998). Opposite to this action is the anti-oppioid activity displayed by peptides derived from k-casein hydrolysis. These molecules called casoxins function as oppioid-antagonists over both the mu and k-type receptors. Their possible physiological action on human is counteracting the life-threatening depression of the CNS and respiratory system (Chiba and Yoshikawa, 1986). Finally, some peptides derived from casein by LAB proteolys can exert mixed functions such as morphine-like and immunostimulating (Kayser and Meisel, 1996).

Some *Lactobacillus* (Cho et al., 2007) and *Lactococcus* species (Mazzoli et al., 2010) are also able to produce, by glutamate decarboxylation (see paragraph on energy metabolism), GABA, the most widely distributed neurotransmitter in the vertebrate central nervous system, that also acts as modulatory effector at the gut level. Although GABA cannot by-pass the blood-brain barrier, it can act as a relaxing molecule over the gut smooth muscles, can lower the blood pressure in mild hypertensive patients (Inoue et al., 2003) and also plays regulatory and trophic roles on pancreas (Erlander et al., 1991). Even if not all the cited LAB are stable inhabitants of human gut, they can be acquired by food and can persist in suitable conditions.

For what concerns the antioxidant potential, as referred in a previous paragraph, some LAB strains are able to fix inorganic selenite into seleno amino acids thus opening the possibility to biosynthesize *in vivo* selenoproteins, essential factors for oxidative stress control. Selenium has been demonstrated to be a nutritionally essential trace element since 1957 (Schwarz and Foltz, 1957). However, dietary selenium intake vary widely among different countries, from 7 mcg per day in China up to 100–200 mcg of Canada, USA, and Venezuela (Rayman, 2008). The reason of such differences lies in the different soils in which vegetables are grown and animal are raised. It is now evident that the selenium content of food is strictly linked to soil selenium availability and where this availability is scarse severe endemic selenium deficiencies occur (Zhao et al., 2007). Furthermore, serum selenium concentrations reflect food selenium content, thus demonstrating that no alternative endogenous way to get this metal is possible (Combs, 2001).

Even if it is widely accepted that selenium deficiency (exception made for very specific and rare syndroms) does not cause illness by itself, nevertheless, this metal appears to be a key nutrient for countracting bacterial and viral infections (Beck et al., 2003), for thyroid hormone balance (Tinggi, 2008), for limiting oxidized LDL associated atherogenesis and consequent heart disease (Furman et al., 2004), and for controlling age-related disorders such as oxidative status, immunodeficiency, general inflammation (Méplan, 2011), and prostate cancer (Klein et al., 2003). Many evidences suggest a cancer-protective effect of selenium supplementation since this metal proves to be an effective tool for controlling DNA damage risk associated with neoplastic degeneration. An inverse correlation between selenium blood levels and cancer mortality has also been observed.

The recommended dietary intake of selenium for humans is 55 mcg per day (Alzate et al., 2008). The *Lactobacillus* biosynthesized selenium proteins not only represent a more bioavailable dietary source but there is also a correlation between this organic form and the concentration of this metal in the external environment, opening interesting perspectives on metal release in the gut environment by LAB, with consequent oxidative stress control.

LAB LIGHTS AND SHADOWS

LAB, like every organism on this earth, are not perfect. The described metabolic and biochemical features underline the risk that they can produce toxic metabolites suh as ammonia and bio-active undesired amines like histamine and tyramine. Their ability to produce butyrate can be severely evaluated when a metabolic syndrome is present since this metabolite, although having a protective effect against neoplastic degeneration, can increase the human calories gain and also inhibit lypolytic processes. Similarly, some CLA isomers produced by LAB could be deleterious, so a careful typing of gut LAB single strains and their metabolites can help to avoid undesiderd effects. For what concerns the ability to activate plasmin-mediated proteolytic cascades, by using endogenous factors, this risk is counterbalanced by the possible quenching effect of released palsminogen receptors on the plasminogen itself.

A separate comment is needed for the Genus *Enterococcus*. While some species like *E. faecium* are currently considered safe and even employed as starters in the food industry and as probiotics in both food and pharmaceutical preparations, other species like *E. faecalis* can cause problems due to different reasons. It should be remembered that enterococci are more tolerant to salt, less acidophilic, and less acydogenic than other LAB, thus constituting a group *per se*. First of all they are naturally resistant to many antimicrobial agents, and also bear transmissible antibiotic resistance factors. Furthermore, their pheromone production is very high thus enhancing the frequency of recombination. A second risk is linked to the fact that most pathogenicity characters expressed by enterococci are not species but rather strain related. Among these are worth mentioning gelatinase, serine proteases, endocarditis antigen, and the aggregation factor. A further problem is connected with the oxidative catabolism of steroid molecules. During their enterohepatic circulation, bile acids are bio-transformed by the intestinal microbiota into a variety of metabolites some of which are carcinogenics, especially the C7 dehydroxy- and the C7 keto-derivatives (Murray et al., 1980). Although dehydrogenation, epimerization, and dehydroxylation are mainly peformed by *Clostridia*, also some *Enterococcus faecalis* strains have been reported to have C7 dehydroxylating activity. These reactions are directed not only toward bile acids or cholesterol but also to different steroid molecules present in the gut (Groh et al., 1993).

It is always difficult to establish once and for all when the benefits are higher than the risks, especially when dealing with bacteria, whose frequency of mutation, genetic recombination, and evolution can change the scenario in few years. Only some decades ago *E. coli* was considered safe and *Clostridia* highly pathogenic. Nowadays, some *E. coli* strains have been proved to cause death and *Clostridia* have even been proposed as probiotics (Cartman, 2011). For sure the "omics" approach can help us, now and in the near future, to shed light on some controversial aspects still concerning LAB biology, and on how

environmental condition can affect their biology. Nevertheless, considering the absence of the main bacterial pathogenicity characters (toxins, invasion potential) and all the beneficial traits supplied by LAB in the context of the gut ecosystem (acidification, prebiotic FOS biosynthesis, lactose recovery, bacteriocins release, positive bio-active molecules production acting on mood and appetite, immune system modulation, and selenium organication) we can conclude that this group of bacteria, is not only well adapted to live as commensal with humans, but can also help the human host to maintan his/her healty status and, may be, to enhance his/her performances and longevity.

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Enteric pathogens through life stages

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Enteric infections and diarrheal diseases constitute pervasive health burdens throughout the world, with rates being highest at the two ends of life. During the first 2–3 years of life, much of the disease burden may be attributed to infection with enteric pathogens including Salmonella, rotavirus, and many other bacterial, viral, and protozoan organisms; however, infections due to *Clostridium difficile* exhibit steady increases with age. Still others, like *Campylobacter* infections in industrialized settings are high in early life (<2 years old) and increase again in early adulthood (called the "second weaning" by some). The reasons for these differences undoubtedly reside in part in pathogen differences; however, host factors including the commensal intestinal microbial communities, immune responses (innate and acquired), and age-dependant shifts likely play important roles. Interplay of these factors is illustrated by studies examining changes in human gut microbiota with inflammatory bowel disease and irritable bowel syndrome. Recent gut microbial surveys have indicated dramatic shifts in gut microbial population structure from infants to young adults to the elders. An understanding of the evolution of these factors and their interactions (e.g., how does gut microbiota modulate the "inflamm-aging" process or vice versa) through the human life "cycle" will be important in better addressing and controlling these enteric infections and their consequences for both quality and quantity of life (often assessed as disability adjusted life-years or "DALYs").

Keywords: enteric pathogen, intestinal microbiota, malnutrition, diarrhea, age distribution

DEFINING ENTERIC "DISEASE"

Defining "pathogens" first requires defining "disease." We propose defining enteric disease as far more than simply passage of unformed stools (i.e., "diarrhea") from one end of a very long and functionally critical "tube" to sustaining healthy life. Therefore, a broader definition of enteric disease would include: ≥3 or more unformed stools per day and any documented intestinal infection associated with disrupted intestinal absorptive and/or barrier function. This may impair growth in young children or cognitive function. Implicit in this more comprehensive concept of enteric disease is an urgent need for improved biomarkers of impaired intestinal absorptive and barrier function.

Thus, an enteric pathogen may be classified as any microbe that is able to cause enteric "disease" as defined above. Microbial pathogenesis can involve direct invasion, signals triggering host inflammation or other changes, or secreted factors that damage the host directly (e.g., toxins), indirectly (e.g., microbial competition), and/or by exploiting other environmental host-associated factors to thrive.

HOST LOCALE OF ENTERIC DISEASE

The intestinal epithelium is a heterogeneous mixture of cells poised to respond to the closely positioned microbiota and luminal contents. The tight junctions that exist between intestinal epithelial cells provide a maintained barrier (Marchiando et al., 2010) that when breached by enteric pathogen or toxin can lead to leakage of luminal contents into the underlying lamina propria where immune cells reside and potentially instigate deleterious inflammatory and other physiological responses leading to diarrhea or disrupted absorptive function (Su et al., 2009). Conversely, nutritional status, intestinal microbiota, and stress have been shown to play roles in maintaining barrier function (Brewster et al., 1997; Yang et al., 2006; Zareie et al., 2006). Perturbations of nutritional status and alteration of the intestinal microbiota is evident in persistent malnutrition and enteric infections (Mondal et al., 2012). Changes in the intestinal architecture during a malnourished state include blunted villi, crypt hypertrophy, and altered levels of intraepithelial lymphocytes leading to impaired immune responses, nutrient absorption (**Figure 1**) and ultimately decline in early childhood growth (Guerrant et al., 2008; Moore et al., 2010).

Effects of intestinal flora on the brain and enteric nervous system are now being elucidated. Early childhood enteric disease and malnutrition have been linked with impaired cognitive development (Lorntz et al., 2006; Laus et al., 2011). The profound effect of childhood malnutrition and enteric disease on cognitive function ultimately affects individual productivity in life; moreover, stunting early in life may increase risk for later obesity and possibly other chronic diseases (Guerrant et al., 2008; Victora et al., 2008). Factors such as *APOE4* may affect enteric disease and cognitive function. The *APOE4* allele in Brazilian children has been correlated with lower diarrhea burdens, and improved

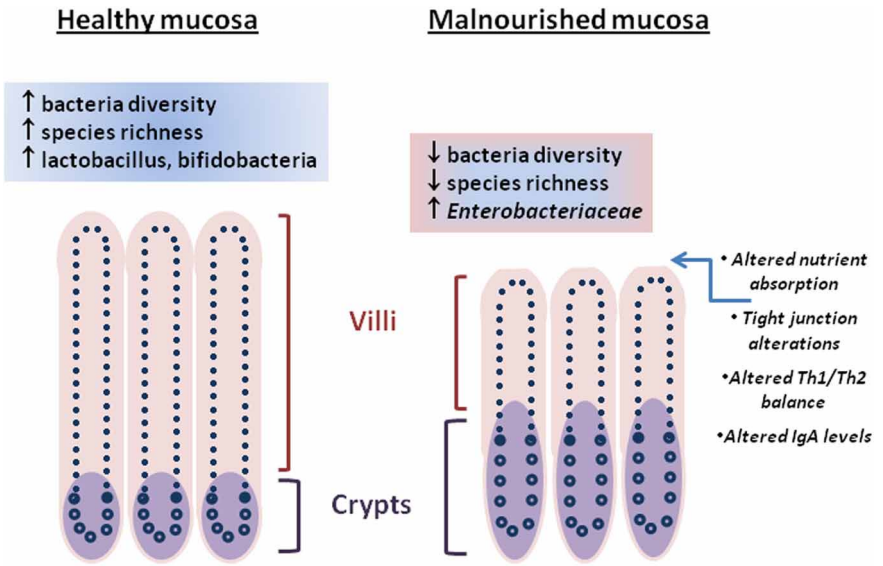

FIGURE 1 | Differences between "healthy" and malnourished intestinal mucosa are represented by architectural changes (villi height/crypt depth, crypt hypertrophy), molecular changes (tight junction alteration, nutrient absorption), immune changes, and the intestinal microbiota. Cross-talk between the intestinal microbiota and intestinal mucosa through metabolites is unclear.

cognitive performance (Oria et al., 2005); cellular effects of APOE in the intestine and brain are under study (Vitek et al., 2009; Azevedo et al., 2012). While *APOE4* provides protection to children with enteric disease, it is also linked with the neurodegenerative, Alzheimer's disease (Strittmatter et al., 1993). Currently, no apparent links appear to exist between *APOE4* and malnutrition in the elderly (Matera et al., 2010); furthermore, limited studies have found associations between cognitive impairment and malnutrition or infections in the elderly, although causality remains unclear (Orsitto et al., 2009; Fagerstrom et al., 2011). The long-term impact of enteric infections in children is a subject of intense research; however, parallel studies in elders are lacking. Increasingly, research addressing the connection(s) between intestine, microbiota, and brain (Grenham et al., 2011) should illuminate host-microbe processes.

LIFE STAGES, INCIDENCE, PATHOGENS

By expanding the definition of enteric disease, the role of the host:pathogen (H:P) balance throughout life stages can be addressed in several contexts. Globally, the highest rates of diarrhea mortality tend to occur during early and late-life stages (**Figure 2A**; WHO data); however, rates in countries with the lowest GNI remain high throughout life. The age-associated trend is evident in cases of salmonellosis with high indices in infants and elders ≥65 (Trevejo et al., 2003). *E. coli* infections impacting early and late life stages are pathogen dependent; furthermore, diarrheagenic *E. coli* segregate geographically (Qadri et al., 2005; Ochoa et al., 2009; Snedeker et al., 2009; Opintan et al., 2010). *Cryptosporidium* may have its greatest impact in children <1yo or in already stunted children, (Checkley et al., 1997, 1998, 2008; Putignani and Menichella, 2010) rotaviruses in the first 2 years of life; *Campylobacter* in early childhood and young adulthood (the "second weaning") (Ailes et al., 2008; da Silva et al., 2010; Soofi et al., 2011), then *C. difficile* infections clearly increasing steadily in both frequency and severity with increased age (Zilberberg et al., 2008). Given age-dependent changes in the incidence of enteric diseases, additional factors affecting the H:P balance include: intestinal microbiota, environment/exposure, and immune response.

AGE-DEPENDENT SHIFTS IN THE INTESTINAL MICROBIOTA

The gut microbiota play important roles in maintaining the human health, including energy and nutrient extraction (Deguchi et al., 1985; Cummings and Macfarlane, 1991, 1997; Turnbaugh and Gordon, 2009), host immune system modulation and protection against pathogens (van der Waaij et al., 1971; Rolfe RD, 1997). Understanding of the diversity of human gut microbiota has improved dramatically in recent years largely due to culture-independent 16S rRNA based surveys. Recent studies have shown large interpersonal variations in the gut microbiota as a function of age (**Figure 3**), health status and diet (Dethlefsen et al., 2006; Ley et al., 2006; Mueller et al., 2006; Woodmansey, 2007; Tannock, 2008; O'Toole and Claesson, 2010; Claesson et al., 2011; Wu et al., 2011). Nevertheless, population based studies show three "enterotypes" with potential diagnostic value (Arumugam et al., 2011). The enterotypes are based on the preponderance of three genera: *Bacteroides*, *Prevotella*, and *Ruminococcus*. It is likely that additional enterotypes will surface when other health-related factors are considered (De et al., 2010; Monira et al., 2011; Wu et al., 2011).

The gut microbiota during early **infancy** is relatively simple but highly dynamic (Favier et al., 2002; Palmer et al., 2007).

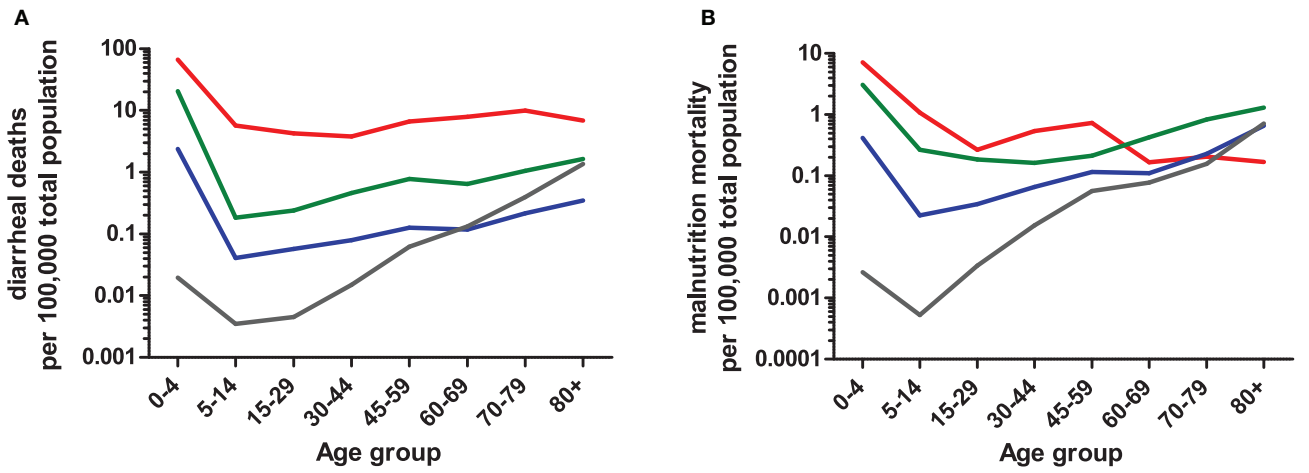

FIGURE 2 | Age-dependent mortality rates due to diarrhea (A) or protein-energy malnutrition (B) based on 2008 WHO data. Numbers of diarrheal or malnutrition from the Global Burden of Disease in 2008 cases were compared to the total population sum of each WHO region (2012). WHO regions were grouped into four categories (i.e., low, low-middle, upper-middle, and upper) based on Gross National Income (GNI) brackets established by the World Bank (2012). The GNI income bracket most represented within a WHO region was used to combine data. For example, the Region of Americas (AMRB) consists of 26 countries, the majority of which ($n = 18$) are classified as upper-middle income countries and were therefore included in the upper-middle income data. The low-income (red line) category consists of African Region (AFR D and E) and South East Asian Region (SEAR D); low-middle (green line) consists of Region of the Americas (AMR D), Eastern Mediterranean Region (EMR D), Western Pacific Region (WPR B), and SEAR B; upper-middle (blue line) consists of AMR B, EMR B, and European Region (EURB and C); upper (grey line) consists of AMR A, EUR A, and WPR A. The mortality rate data are limited by the use of all-age population data, so actual age-dependent rates may differ.

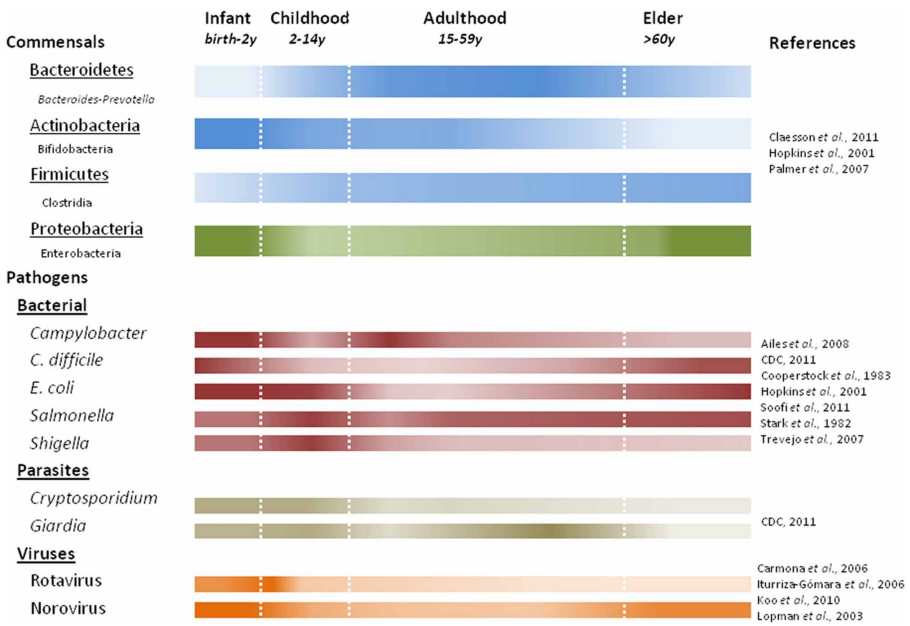

FIGURE 3 | Predominant commensal and pathogenic microbes associated with human life stages. Color intensity of each bar correlates with presence or absence of commensal bacteria at the phylum (underlined) with examples listed below using noted references. Studies characterizing fecal microbiota prior to 2007 quantify the viable counts of bacteria, while later studies use DNA based methods.

Incidental exposures (e.g., maternal microbiota, breastfeeding vs. formula) play a major role in seeding the neonatal gut (Bennet et al., 1991; Mandar and Mikelsaar, 1996; Penders et al., 2006; Adlerberth and Wold, 2009). As a result, the progression of early colonization is often chaotic and variable (Palmer et al., 2007). Facultative anaerobes, like *E. coli* (Hopkins et al., 2001; Salminen and Isolauri, 2006; Mariat et al., 2009) and Gram-negative obligate anaerobes (e.g., *Bacteroides-Prevotella*) dominate early followed by a predominance of bifidobacteria by three months of age (Mah et al., 2007; Mariat et al.,

2009). Infection with *Salmonella* and pathogenic *E. coli* pose the greatest risks in early infancy when facultative anaerobes are predominant. Over the first year, through a series of successions and replacements, the infant microbial communities become more similar to one another, converging toward a more stable adult-like profile (Favier et al., 2002; Palmer et al., 2007), characterized by a preponderance of *Bacteroidetes* and *Firmicutes*. In addition to the composition difference, quantitative PCR show total bacterial counts in infants to be nearly ten-fold lower than in adults and elders (Mariat et al., 2009).

Gut microbiota in young **adults** are dominated by *Bacteroidetes* and *Firmicutes* (making up approximately 95% of the microbiota) with smaller fractions of *Actinobacteria* and *Proteobacteria* (Ley et al., 2006; Andersson et al., 2008; Tap et al., 2009). The peak numbers and diversity in gut microbial composition is achieved near the end of adolescence. Still, each adult's gut appears to harbor a unique microbial community that remains relatively stable through adulthood (Franks et al., 1998; Zoetendal et al., 1998; Vanhoutte et al., 2004; Leser and Molbak, 2009). One recent study suggested that stability may last longer than expected, and that **aging** starts to affect the gut microbiota after 65 years of age (Biagi et al., 2010). In addition, decreased intestinal motility (Brocklehurst, 1972; Macfarlane et al., 1989), dietary changes (Flint et al., 2007), and "inflamm-aging" (a chronic low-grade inflammation in elders) (Franceschi, 2007; Guigoz et al., 2008) all affect homeostasis of the gut microbial ecosystem. Recent studies indicate dramatic shifts in the composition of gut commensals in elders. When elders were compared to young adults, a decrease in the relative abundance of *Bifidobacteria* and *Firmicutes* was observed, accompanied by a commensurate increase in *Bacteroides* and facultative anaerobes (Hopkins and Macfarlane, 2002; Mariat et al., 2009; Claesson et al., 2011), although large compositional variations were also found among elderly individuals and populations (Mueller et al., 2006; Claesson et al., 2011). The decline in beneficial bifidobacteria is one of the most marked changes in the aging gut, manifested in both microbial abundance and species diversity (Mitsuoka et al., 1974; Benno et al., 1992; Mitsuoka, 1992; He et al., 2001; Hopkins et al., 2001; Hopkins and Macfarlane, 2002). Such a population shift could increase the susceptibility of elders to *C. difficile* infections (CDI) (Woodmansey, 2007; Guigoz et al., 2008), as a similar but more pronounced composition change has been observed in CDI patients (Hopkins et al., 2001; Hopkins and Macfarlane, 2002). Furthermore, a stable gut microbiota is crucial in preventing *C. difficile* overgrowth. This is supported by studies showing significant reductions in the microbial diversity in patients with recurrent CDI (Chang et al., 2008), association of CDI onset with altered microbiota composition(s) before antibiotic treatment (De La Cochetiere et al., 2008), and prevention of primary or recurrent CDI through probiotic therapy or fecal transplantation, respectively (Hickson et al., 2007; Khoruts et al., 2010). *Bifidobacteria* and butyrate-producing bacteria (e.g., *Clostridium* clusters IV and XIVa) have been suggested to play important roles in providing colonization resistance against *C. difficile* (Fallani et al., 2006; Rousseau et al., 2011); however, more comprehensive studies are required to substantiate these claims (Gore et al., 2008).

In addition to CDI, changes in the gut microbial composition have been linked to childhood allergies and inflammatory bowel diseases (IBD). It has been proposed that the gut microbiota in infants modulates the mucosal immune response to environmental allergens (Tannock, 2007). Interestingly, differences in the bifidobacterial populations have been associated with atopic diseases and the allergic status of children (Sepp et al., 1997; Ouwehand et al., 2001; Gore et al., 2008; Hong et al., 2010). IBD, such as Crohn's disease and ulcerative colitis are usually diagnosed in adolescence or early adulthood. Several lines of evidence suggest a microbial etiology in the development of IBD (D'Haens et al., 1998; Sellon et al., 1998; Gionchetti et al., 2000; Shen et al., 2001; Sartor, 2004). Previous studies have led to the hypothesis that altered gut microflora, excluding a specific pathogen, contribute to the etiology of IBD. Loss of microbial diversity and richness, especially in members of clostridial cluster IV (e.g., *Faecalibacterium prausnitzii)*, was observed in Crohn's disease patients (Martinez-Medina et al., 2006; Scanlan et al., 2006; Frank et al., 2007; Sokol et al., 2008; Cucchiara et al., 2009). An increased *Bacteroidetes:Firmicutes* ratio and a predominance of opportunistic *Proteobacteria* have been reported in pediatric and adult IBD patients (Cucchiara et al., 2009). However, there is an ongoing debate on whether these changes are the cause or an effect of the disease (Sokol et al., 2008; Stecher and Hardt, 2008).

COMMON THEMES OF THE MICROBIOTA

As already mentioned, a loss of species diversity and richness within the microbiota has been observed in IBD and CDI; in addition, based on their relative abundance, facultative anaerobes including members of *Proteobacteria* appear to thrive. Increased levels of *Proteobacteria* are also seen in malnourished children or in patients with celiac's disease (Bonventre, 1990; Monira et al., 2011). On a gross level, these findings also tend to occur with older age when this microbiota "structure" could alter other host functions (e.g., immune response). For example, the relative abundance of *Bacteroidetes* accounted for <15% of the intestinal community in 71% of malnourished Bangladeshi children compared to >40% relative abundance in 71% of healthy cases.

EXPOSURE AND HOST RESPONSES

The environment plays a major role in acquisition of the pathogen; in many cases exposure to contaminated food and/or water are the main vehicles for pathogen transmission. Globally, countries in the low and low-middle income brackets face the highest rates of diarrhea-associated mortalities over much of the life cycle (**Figure 2**). The age-dependent mortality due to diarrhea evident at both ends of the life cycle has dramatic regional differences impacted in part by socio-economic category (e.g., developed vs. developing countries). Additional factors likely include polymicrobial infections, diet, host-microbiota relationship, waning maternal antibody and other regional specific co-morbidities. Indeed, this rationale could be applied to similar rates of diarrhea mortality among elders living in WHO regions falling into upper-middle and upper income categories in contrast to respective

regional infant rates. In the case of the USA and Canada (i.e., AMRA), infection with *C. difficile* in healthcare or long-term care facility environments contribute to age-associated increases in mortality (Zilberberg et al., 2008). Depending on the setting, a patient may be treated with antibiotics that disrupt the "normal" microbiota resulting in susceptibility to opportunistic pathogens. Furthermore, prolonged stay within a hospital environment favors the transfer of nosocomial pathogens, in particular *C. difficile* (McFarland et al., 1989).

Conversely, exposure to pathogens in early infancy is greatest as children are weaned. In areas with high diarrhea burdens, both introduction of weaning foods and cessation of breastfeeding was associated with increased risk of dehydrating diarrhea (Guerrant et al., 1983; Fuchs et al., 1996). Consequently, increased malnutrition-based mortality is coincident with increased diarrhea mortality (**Figure 2B**). Adaptations in host immune responses and intestinal microbiota are crucial for the host to survive constant exposure to malnutrition or undernutrition and diarrhea disease throughout much of the life cycle, and these adaptations offer protection in later life when immune status wanes. In neonates, colonization and stabilization of intestinal microbial communities is intertwined with immune system development (Shulzhenko et al., 2011) with similar processes, albeit senescent in nature, occuring in elders (Biagi et al., 2010) suggesting age-dependant alterations of these systems are closely linked to pathogen resistance/susceptibility.

The innate immune system consists of frontline defenses to pathogens (Hooper and Macpherson, 2010) with parallel or subsequent responses by the adaptive arm of the immune system. Maturation and senescence of components in the adaptive immunity have been reviewed elsewhere (Adkins et al., 2004; Cancro et al., 2009). Antibody-mediated protection *in utero* and postpartum occurs through maternal, passive transfer and is cleared by 5 months of age; the age at which growth shortfalls often start (Victora et al., 2010). The impact of diet affects the host and subsequent adaptive responses; during malnutrition, both B- and T-cell responses are altered. In young mice and children, protein energy malnutrition decreases mucosal IgA responses potentially impacting the course of infection (Green and Heyworth, 1980; McGee and McMurray, 1988; Oberhelman et al., 1991). Conversely, patients with little to no IgA due to common variable immunodeficiency were more likely to be malnourished (Muscaritoli et al., 2001) suggesting important links between the immune system and nutritional status, which have been recently tied to the intestinal microbiota (Shulzhenko et al., 2011). The effect of malnutrition on T-cell responses during enteric infection have been examined in mice and humans with divergent results. In mice, tissue IFNγ increased in response to malnutrition and infection with *Cryptosporidium* or *Heligmosomoides* (Ing et al., 2000; Coutinho et al., 2008). Other *in vivo* studies show that protein-energy malnutrition alone ↑ serum IL-10 and ↓ IFNγ (Hillyer et al., 2007; Monk and Woodward, 2009) suggesting that infection further alters the immune response during malnutrition. Isolation and subsequent *in vitro* activation of CD4+ cells from malnourished or well-nourished, infected children, resulted in more IL-10 or IFNγ release characteristic of Th2 or Th1 bias, respectively (Rodriguez et al., 2005). The infecting pathogen(s) were not classified in this study, but samples from both gastrointestinal and respiratory infections were analyzed.

CONCLUSIONS

The process of aging is associated with continuous changes in environmental exposures that subsequently affect the immune system and host-associated microbiota. Within the host's shifting landscape, infections by pathogens that cause enteric disease likely exploit these shifts with resultant initiation of pathogenesis and/or establishment of a mutualistic relationship with the host (e.g., carrier) leading to potential dissemination of the pathogen. There is still much to be learned about how life stages and pertubations shape the gut microbial population, and how these changes influence health and disease, and predominant enteric pathogens. For whatever population shifts we can associate with disease(s), it is necessary to demonstrate causality and not a mere effect or unrelated association with the diseases to fulfill Koch's postulates. Future insights into the interdependency between environment-host-microbe will be essential for development of novel therapeutic approaches that treat or prevent enteric disease. Understanding the roles of these factors within the host will be complementary to external approaches (e.g., sanitation, water treatment) for controlling or eradicating pathogen dissemination.

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Interactions between parasites and microbial communities in the human gut

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The interactions between intestinal microbiota, immune system, and pathogens describe the human gut as a complex ecosystem, where all components play a relevant role in modulating each other and in the maintenance of homeostasis. The balance among the gut microbiota and the human body appear to be crucial for health maintenance. Intestinal parasites, both protozoans and helminths, interact with the microbial community modifying the balance between host and commensal microbiota. On the other hand, gut microbiota represents a relevant factor that may strongly interfere with the pathophysiology of the infections. In addition to the function that gut commensal microbiota may have in the processes that determine the survival and the outcome of many parasitic infections, including the production of nutritive macromolecules, also probiotics can play an important role in reducing the pathogenicity of many parasites. On these bases, there is a growing interest in explaining the rationale on the possible interactions between the microbiota, immune response, inflammatory processes, and intestinal parasites.

Keywords: parasites, protozoans, helminths, microbiota, parasitome, pathogenesis, immune system, probiotics

THE HUMAN INTESTINAL MICROBIOTA

The human gut represents a complex ecosystem composed by a large microbial community associated with the human body (Human Microbiome Project Consortium, 2012). The species composition varies greatly between individuals, with each individual harboring a unique collection of bacterial species, which may change over time (Bäckhed et al., 2005; Eckburg et al., 2005; Qin et al., 2010). Genetic factors play an important role in gut microbiota development, although environment also drives species acquisition (Zoetendal et al., 2001). Recently, the human body together with its gut microbiota has been referred to as a "superorganism" where an extensive coordination of metabolic and physiological processes occurs (Nicholson et al., 2004). The presence of the intestinal microbiota enriches the human organism with important functions, particularly in regulating host fat storage (Bäckhed et al., 2004), stimulating intestinal epithelium renewal (Rakoff-Nahoum et al., 2004), and influencing the maturation of the immune system (Mazmanian et al., 2005).

As recently reviewed (Sekirov et al., 2010; Clemente et al., 2012), the balance among the gut microbiota and the human body is crucial for health maintenance, and perturbation of microbial composition has been supposed to be involved in a range of diseases (Bäckhed et al., 2005; Palming et al., 2006). Moreover, the commensal microbiota contributes to the "barrier effect" of the intestinal epithelium, which plays the primary role of protecting the host, representing a real obstacle to pathogens invasion (Bancroft et al., 2012). Within this complex scenario, intestinal parasites interact with the microbial community modifying the balance between host and gut microbiota. Each of these organisms metabolizes and modifies substrates interactively. Resident microbiota products may strongly interfere with the survival and the physiology of many parasites and, consequently, with the outcome of many parasitic infections. On the other hand, intestinal parasites, both protozoans and helminths, constantly excrete and secrete molecules that may change the environment determining alterations in gut microbiota compositions. Also part of the energy extracted from nutrient metabolism by resident microbes may be beneficial not only to the host (Sekirov et al., 2010) but also to parasitic organisms eventually present. It is therefore pertinent to consider the intestinal environment as an ecosystem where biological and chemical interactions occur at various organizational levels between host, parasites, and microbial communities (Nicholson et al., 2004; Bancroft et al., 2012).

PROTOZOANS

A wide range of protozoans are common parasites of human gastro-intestinal tract. They are a not homogenous group and their physiology and biochemistry are largely geared to the parasitic habit. They show different mechanisms of host invasion, some are intracellular (e.g., *Cryptosporidium* spp.) and host specialized (e.g., *Entamoeba histolytica*), many of them are adapted to more than one host (e.g., *Giardia duodenalis*). Few species do any real damage but some occasionally give rise to symptoms that usually include diarrhea related to damage in the wall of the bowel.

Among protozoans, the species *G. duodenalis* could represent a good model to highlight some mechanisms related to the existing interactions with the intestinal microbiota. This flagellate is recognized as one of the most common pathogenic gastrointestinal parasites in humans and in a wide range of animals (Thompson,

2000). The spectrum of clinical manifestations varies from a mild self-limiting illness to acute or chronic diarrhea and weight loss, with malabsorption lasting for several months (Farthing, 1996). Furthermore, people may be infected without any symptoms. The causes determining this variability in clinical picture are still poorly understood.

Numerous studies assessed that pathogenesis results from interaction between parasite products, such as proteinases that break the epithelial barrier, and host inflammatory and immunological responses as observed for *Cryptosporidium* (Chai et al., 1999; Guk et al., 2003) as well as for *Giardia* (Scott et al., 2004; Ankarklev et al., 2010). Recognition of protozoans parasitizing mucosal surfaces may involve innate immune system, e.g., toll-like receptors (TLRs), as demonstrated *in vivo* on infected humans for *Trichomonas vaginalis* (Zariffard et al., 2004), and *in vitro* on human monocytes for *E. histolytica* (Maldonado et al., 2000). Moreover, T cells (particularly involving CD8+ cells), macrophages, neutrophiles, and antibodies (IgM, IgG, and IgA) are major players of the acquired immune response necessary for the resolution of giardiasis.

PROTOZOAN INFECTION AND GUT MICROBIOTA

Gut microbiota represents an additional factor that may strongly interfere with the pathophysiology of the parasite infections. However, the existing interactions between the enteric flora and protozoan parasites are still poorly understood.

Based on mouse models, normal intestinal flora was shown to decrease susceptibility to infection by *Cryptosporidium parvum* (Harp et al., 1992). Conversely, in other studies, the presence of gut microbiota seems to be essential for the pathogenic expression of other enteric protozoans such as *E. histolytica* (Phillips et al., 1955), *Blastocystis hominis* (Phillips and Zierdt, 1976), and different species of *Eimeria* (Visco and Burns, 1972; Owen, 1975; Gouet et al., 1984).

Different hypotheses have been proposed to explain the mechanisms involved in this pathogenic stimulation by bacteria. Some of them are related to changes caused by axenisation of the protozoans. In this case, the surface saccharide ligands of the superficial membrane are altered by the presence of intracellular bacterial symbionts, so that in axenic protozoa cured of their endosymbionts, a possible decrease in adhesion or in invasive abilities can be observed (Phillips, 1973; Dwyer and Chang, 1976). Also in *Giardia*, in the past decade, ultrastructural observations of *Giardia muris* in a murine model revealed endosymbiotic microbes which, according to the authors, could be related to variation in the trophozoite pathogenicity, metabolism, range of infectivity, antigenic surface characteristics, and host specificity (Nemanic et al., 1979). More recently, the presence of *Giardia* trophozoites harboring peripheral bacterial endosymbionts was also demonstrated by El-Shewy and Eid (2005). Based on TEM examination, the authors found that only trophozoites with endosymbionts were lysed when in close vicinity of the activated Paneth cells, confirming the host protective role of the bacterial endosymbionts within *Giardia* trophozoites and further supporting the idea that gut microbiota may directly and indirectly interfere in the pathogenesis of giardiasis.

Similarly intriguing is the idea that axenisation of the host at the intestinal level can be involved in the virulence expression of protozoan parasites. Working with *E. histolytica*, Mirelman and colleagues (1982, 1983) evidenced that interactions of amoebae of low pathogenicity with a variety of Gram-negative bacteria, mainly *Escherichia coli* strains, may be responsible for the increase in amoebic virulence. More recently, Galván-Moroyoqui et al. (2008) demonstrated that phagocytosis of enteropathogenic bacteria strains (e.g., *E. coli* and *Shigella dysenteriae*) *in vitro* co-cultured with *E. histolytica* and *Entamoeba dispar* augmented the cytopathic effect of *E. histolytica* and increased expression of Gal/GalNAc lectin on the amoebic surface and the cysteine proteinase activity. *E. dispar* remained avirulent.

Also for *G. duodenalis*, several studies have shown that the intestinal microbiota can stimulate the pathogenic expression but not the multiplication of parasites (Torres et al., 1992, 2000). In a gnotobiotic animal model, Torres et al. (2000) provided evidence that the bacteria responsible for part of the stimulation of *G. duodenalis* pathogenicity are present in the dominant duodenal microbiota. In this work, facultative and strictly anaerobic micro-organisms of the duodenal microbiota were obtained from biopsy of five children with symptomatic giardiasis and tested for their ability to stimulate *G. duodenalis* pathogenicity in gnotoxenic mice. Quantification of cysts in faeces and of trophozoites in the small bowel was also performed to evaluate protozoan multiplication in the different groups of mice. As observed, germ-free animals did not develop intestinal pathological modifications during experimental *Giardia* infection; infected gnotoxenic mice showed intermediate pathological alterations between germ-free and infected conventional mice used as controls; finally, no pathological changes were observed in non-infected gnotoxenic or conventional animals. According to the authors, these results support the hypothesis that, as demonstrated also for other intestinal pathogenic protozoans, bacterial components from the intestinal microbiota represent stimulatory factors for *Giardia* pathogenicity but not for protozoan multiplication since faecal cyst levels remained similar among the three different groups of mice during the experimental infection.

HELMINTHS

The intestine represents the ideal habitat for a large number of parasitic worms. Among flatworms, cestodes of the genera *Diphyllobothrium, Taenia,* and *Hymenolepis* and digeneans such as *Fasciolopsis, Heterophyes,* and *Schistosoma*, live in close interaction with human gut mucosae and lumen. As for nematodes, the most common intestinal roundworms are geohelminths (*Ascaris, Trichuris,* Ancylostomatidae, and *Strongyloides*), as well as *Enterobius vermicularis*.

While in less-favored areas, the interest in intestinal helminthiases is mainly focused on the parasitic disease itself, in industrialized countries the intimate relationships between intestinal helminthes with gut microbiota and the putative down-regulation of self-pathogenic immune response have been the object of recent studies, as a consequence of the increasing concern regarding childhood allergies, atopic dermatitis and asthma (Patel et al., 2008), IBDs like Crohn's disease and ulcerative colitis, and autoimmune disorders (Weinstock and Elliott, 2009).

HELMINTH INFECTION AND GUT MICROBIOTA

The human intestinal microbiota is essential in providing nourishment, regulating epithelial development, and instructing innate immunity (Eckburg et al., 2005). A significant variability and differences between community compositions are often described, all consistent with a picture of a highly diverse ecosystem. It has been suggested that, in the course of helminth infections, significant changes in the abundance and composition of gastrointestinal tract microbiota are observed. Intestinal nematodes produce molecules that may alter the habitat for gut microbiota. Walk et al. (2010) showed that infection of mice with *Heligmosomoides polygyrus*, a parasite of the duodenum, induces changes in composition of bacteria communities in the ileum but not in the colon; the majority of bacteria within the infected ileum were Lactobacillae species. Interestingly, *H. polygyrus* is able to significantly reduce inflammation of colitis in mice (Elliott et al., 2004) and to determine alterations in epithelial barrier function in the colon (Su et al., 2011). Additionally, Li et al. (2012) demonstrated a significant alteration in the colon microbiota of pigs induced by *Trichuris suis* after 21 days from infection. As suggested by Wu et al. (2012), the initial infection, even when not followed by the persistence of the parasitosis, is able to determine changes in the abundance of up to the 13% of genera detected, in particular *Fibrobacter* and *Ruminococcus.*

A further major aspect related to the helminth infections is the potential interaction between macrofauna, microflora, and host immunity. It has been evidenced the overall decrease in proinflammatory cytokines associated with chronic inflammation observed in the course of helminth infections; moreover, autoimmune disorders have a reduced incidence in geographical regions where higher prevalence of parasitic infections are reported (Sewell et al., 2002). Reddy (2010) argued that a reduced exposure to pathogenic organisms in developed countries may determine a minor stimulation of the immune system and an increased incidence of autoimmune and allergic diseases in the human populations. Based on several studies from developing country settings, evidences have been provided for the role of intestinal nematodes in the prevention of allergic responses (van den Biggelaar et al., 2004; Summers et al., 2005a; Croese et al., 2006; Leonardi-Bee et al., 2006; Flohr et al., 2009). This phenomenon is known as "Hygiene hypothesis" (Wills-Karp et al., 2001; Weinstock and Elliott, 2009). In particular, the interactions between helminth infections and host immune system may prove to be beneficial for both, the parasite and the host, with regard to the control of autoimmune diseases (Maizels et al., 2009).

On this basis, there is a growing interest in explaining the rationale on the existing interactions between helminthes, gut microbiota and immune-mediated intestinal inflammatory status, e.g., in celiac patients, as recently reviewed by Bancroft et al. (2012). The authors, focusing mainly on infections due to *Trichuris* sp., considered the immunomodulation by parasitic helminths and the interaction between the microbiota and the immune system in an integrated manner, where Th17 (T-helper) and Tregs (regulatory T cells) are affected by the action of microbiota, and are in turn able to act on parasite survival. At the same time, parasitic worms produce molecules that may alter the habitat for intestinal microbiota.

Besides to nematodes, also digeneans such as *Schistosoma mansoni* have been described to induce microbial disturbance. In a metabonomic investigations in mice infected with *S. mansoni*, Wang et al. (2004) reported several complex outcomes to the metabolism disturbance due to *Schistosoma* infections, including impaired liver functions, perturbation of amino acids metabolism and of the tricarboxylic acid (TCA) cycle. Moreover, high excretion of urinary trimethylamine, phenylacetylglycine, and p-cresol glucuronide indicating disturbances in the gut microbiota are found in *S. mansoni*-infected mice, probably due to an increased production from microbial agents caused by alteration of the microbial ecosystem in the presence of the parasite. Analogous changes at the Nuclear Magnetic Resonance (NMR) metabolic profiles have been detected during the infections by *Fasciola hepatica, Necator americanus,* and other human helminth parasites, as reviewed by Wang et al. (2010).

Similarly, Balog et al. (2011), on the base of urinary response of rodent and human hosts to *S. mansoni* infection, demonstrated gross disturbance of metabolites associated with gut microbial community and microbial co-metabolism and Li et al. (2011) identified 12 urinary and five faecal metabolites as biomarkers of *Schistosoma* infection, able to differentiate infected and not infected mice, adding further evidence to the hypothesis that *S. mansoni* infection either directly or indirectly modulates host gut microbial activity.

NEMATODES AS A THERAPY

The positive results in the potential of therapeutic effect of worms or their molecules in animals have led to several human studies exploring presumptively harmless helminthes like *T. suis*, a whipworm that naturally infects pigs. Treatment of colitis patients with *T. suis* ova provided promising results and such therapies are currently under development (Summers et al., 2005b). However, a special attention should be paid to possible adverse effects. The first concern regards the zoonotic potential of *T. suis*. The systematics of the group is still controversial and a clearcut delineation of species infecting humans is actually under definition. The second aspect is related to the possibility that *T. suis* infection may play a role in the internalization of intestinal pig epithelial cells by bacteria (e.g., *Campylobacter jejuni*) and subsequent bacterial invasion (Wu et al., 2012). Finally, the effect of helminth infections on allergic diseases may vary depending on the parasite species, as it has been proposed that different species may act as immunosuppressant or as enhancers of allergic phenomena (Pinelli, 2012).

PROBIOTICS AGAINST PARASITES

Probiotics may also be a factor that can potentially inhibit the development of several pathogens. As reviewed by Travers et al. (2011), probiotics demonstrated to be efficient for the treatment of gastrointestinal disorders, respiratory infections, and allergic symptoms, and also can kill or inhibit pathogens by strain-specific mechanisms relying on competition, molecule secretion, and/or immune induction. Several studies have reported the effects of probiotics on parasites, both protozoans (e.g., *Cryptosporidium*, *Eimeria*) and helminths (e.g., *Ascaris*, *Trichuris*).

As regard *Giardia*, a large amount of data are now available, since the first study of Singer and Nash (2000) who provided preliminary evidences that the composition of the intestinal flora was likely involved in the highly variable manifestations in giardiasis in both humans and animals. Pérez et al. (2001) studied the *in vitro* effect of different probiotic bacteria (six *Lactobacillus acidophilus* strains, and *Lactobacillus johnsonii* La1) on *G. duodenalis* strain WB trophozoites demonstrating that only *L. johnsonii* La1 significantly inhibited the proliferation of *Giardia* trophozoites. The activity of *L. johnsonii* La1 (NCC533) was confirmed by Humen et al. (2005) in *in vivo* experiments where a protection against parasite-induced mucosal damage and a cellular response to *Giardia* antigens was stimulated in spleen cells from La1-treated animals, leading to a resolution of infection.

Moreover, *Lactobacillus casei* MTCC 1423 strain as well as *Enterococcus faecium* SF68 were both effective in eliminating *Giardia* infection in probiotic-fed mice by minimizing or preventing the adherence of *Giardia* trophozoites to the mucosal surface (Shukla et al., 2008) and stimulating an humoral response (Benyacoub et al., 2005).

Recently, the effectiveness of different lactobacilli species/strains to prevent and treat murine *Giardia* infection has been further assessed by several authors (Shukla et al., 2009, 2010; Goyal et al., 2011). The results obtained by Shukla and Sidhu (2011) and Shukla et al. (2012) showing the positive effect of *L. casei* in renourished *Giardia intestinalis* infected BALB/c mice confirm the role of probiotics to reduce the duration and severity of giardiasis through the morphological and physiological retrieval of the intestine.

As for worms, Bautista-Garfias et al. (2001) suggested that oral treatment with *L. casei* appears to reduce the parasite burden *Trichinella spiralis* in mice. Also *Enterococcus faecalis* CECT7121, a probiotic with inhibitory activity against Gram-positive and Gram-negative bacteria, possesses *in vitro* and *in vivo* larvicidal activity determining up to a 90% reduction of the number of *Toxocara canis* larvae in liver and lungs of laboratory mice (Basualdo et al., 2007; Chiodo et al., 2010). *Zymomonas mobilis*, a bacterium producer of bioethanol, was reported to provide over 60% protection from the infection of *S. mansoni* in mice (Santos Jde et al., 2004).

FUTURE PERSPECTIVES

The multidimensional linkages among human body, gut microbiota and parasites result in a complex ecosystem where alterations in one of the these components determine a counter response in the remaining ones. In this view, in order to achieve an advanced understanding of the ongoing processes determining the infections, an -omics approach which include comprehensive, multidisciplinary and combined actions from these different perspectives is needed. Many outstanding questions require further investigations, e.g., the existing interactions between the microbiota, immune response, inflammatory processes, and intestinal parasitic diseases as well as the mechanisms regarding how probiotics act against intestinal parasites and the possibility for their therapeutic use for humans. Finally, new exciting areas such as the study of the parasitome and the metabolome of gut microbiota during chronic parasite infection and their relationship with host immunoregulatory mechanisms have now to be approached in the framework of an integrated approach.

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Interplays between gut microbiota and gene expression regulation by miRNAs

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The gastrointestinal tract is one of the most colonized organs and harbors a large microbial population (10^{14} bacteria) that has co-evolved with us establishing a finely tuned symbiosis (Ley et al., 2008). As a result of their occurrence in complex mixtures, their combined genomes or metagenome, contain 150-fold more genes respect to humans, therefore providing us with novel functions (Qin et al., 2010). Increasing evidences show that the disruption of this symbiosis may lead to pathologies such as obesity or to an increased risk of developing inflammatory bowel disease (Ott et al., 2004). Since the intestine is constantly exposed to an almost limitless number of foreign antigens (i.e., food-derived materials, commensal microbes, pathogenic bacteria, viruses and parasites), it is of fundamental importance that an appropriate immune homeostasis in the gut mucosa is established and maintained. This requires a highly sophisticated immunological regulatory systems achieved by the cooperative interaction of intestinal epithelial cells (IECs) and mucosal cells of the immune response (Goto and Kiyono, 2011). The IECs comprise columnar epithelial cells, Paneth cells, endocrine cells and goblet cells (van der Flier and Clevers, 2009) and consist in the first physical barrier between the host and the external environment. Owing to many signals transmission from epithelial cells to the various innate and acquired types of mucosal cells of the immune response, these cells regulate each other resulting in intestinal immunological homeostasis.

To gain information about the composition of gut microbiota communities, their molecular interactions with the host and their impact on various host functional processes, several studies have been carried out on germ-free animals by coupling genomics and bioinformatics techniques (Hooper and Gordon, 2001; O'Hara and Shanahan, 2006). However, little is known about the molecular mechanisms of such modulations and the host post-transcriptional gene expression regulation by microRNAs.

MicroRNAs (miRNAs) are short (∼22 nt) non-coding RNAs that control gene expression by base pairing with 3′-untraslated regions (3′UTRs) of their regulated transcripts. MiRNA biogenesis occurs through various steps in which are involved Drosha and Dicer, two main RNase III endonucleases. Precursor miRNAs (pre-miRNAs) are ∼70 nucleotide-long RNA molecules with a characteristic hairpin structure. They originate in longer primary transcripts (pri-miRNAs) that are cleaved in animals by the Drosha endonuclease in the nucleus (Lee et al., 2003). Following the export of pre-miRNAs to the cytoplasm by Exportin-5, the loop region of the hairpin is removed by the Dicer endonuclease to produce a short, double-stranded RNA (dsRNA) (Cullen, 2004). Based on the thermodynamic stability of each end of this duplex (O'Toole et al., 2006), one of the strands is preferentially incorporated in the RNA-induced silencing complex (RISC), producing a biologically active mature miRNA (generally the -5p miR) (Bartel, 2004), while the inactive strand (the -3p miR) is degraded (Kim, 2005). The coupling of the active miRNA to the 3′UTR of its target gene, facilitates mRNA degradation or translation inhibition (Djuranovic et al., 2012). As a direct consequence, miRNAs regulate many biological processes and have critical roles in cell proliferation, differentiation and death (Shivdasani, 2006; Gomase and Parundekar, 2009).

However, the role of miRNAs in microbiota host interactions is beginning to be investigated (**Figure 1A**) (Dalmasso et al., 2011; Kaser et al., 2011). Dalmasso et al. used germ-free mice colonized with the microbiota from pathogen-free mice to study whether miRNAs are involved in microbiota-mediated regulation of host gene expression (Dalmasso et al., 2011). Their miRNA expression analysis revealed that nine miRNAs were differentially expressed in the ileum and colon of colonized mice compared to germ-free mice. By overlapping the predicted targets of deregulated miRNAs with DNA microarray gene expression profiling, they found that the up-regulation of miR-665 induced a significant down-regulation of the ATP-binding cassette sub-family C member 3 (*Abcc3*) gene (a target of miR-665). Abcc3 belongs to the multidrug resistance-associated protein family, which mediates the metabolism of xenobiotics and endogenous toxins (Hooper et al., 2001). A similar study by Singh et al. emphasized the emerging interplay between endogenous microbiota and caecal miRNA signature (Singh et al., 2012). In fact, intestinal miRNAs have been proven experimentally to have roles in the regulation of neonatal nutrient metabolism (Liao and Lonnerdal, 2010), in the control of intestinal fluid and electrolyte transport (Sansom et al., 2010) and permeability (Zhou et al., 2010), affecting also intestinal epithelial cell differentiation (Dalmasso et al., 2010) and maturation (Zeng et al., 2009). By using germ-free and conventionally raised mice, the impact of the endogenous microbiota on the global expression of caecal miRNAs

A

Aim of the study	Experimental model	Investigated miRNAs	Target genes	Ref.
To study whether miRNAs are involved in microbiota-mediated regulation of host gene expression.	Germ-free mice colonized with the microbiota from pathogen-free mice	miR-298; miR-128; miR-200c*; miR-342-5p; miR-465c-5p; miR-466d-3p/5p; miR-665;miR-683	Abcc3	(Dalmasso et al., 2011)
To study the impact of the endogenous microbiota on the global expression of caecal miRNAs *in vivo*.	Germ-free and conventionally raised mice	miR-21*; miR-351; miR-487b; miR-467a; miR-27b; miR-148a; miR-145; miR-183; miR-133a; miR-150; miR-672; miR-181a; miR-664; miR-455; miR-138*; let-7g*	34 genes among glycosylation enzymes, junctional proteins, proteins found in the mucus layers and in the immune regulation.	(Singh et al., 2012)
To study miRNAs affecting the intestinal epithelial monolayer.	Mice with an inducible intestinal epithelial cell–specific deficiency in *Dicer1* (*Dicer1* $^{\Delta gut}$)	miR-375	KLF5	(Biton et al., 2011)
To study the TLR-4-mediated transcriptional activation of intestinal epithelial cells (IECs).	Mice immediately after birth	miR-146a	IRAK-1	(Chassin et al., 2010)
To study microbiota regulation of miRNA expression and intestinal homeostasis.	C57BL/6 (B6), B6.IL-10$^{-/-}$, B6.MyD88$^{-/-}$ and B6.RAG$^{-/-}$ mice	miR-10a	IL-12/IL-23p40	(Xue et al., 2011)

B

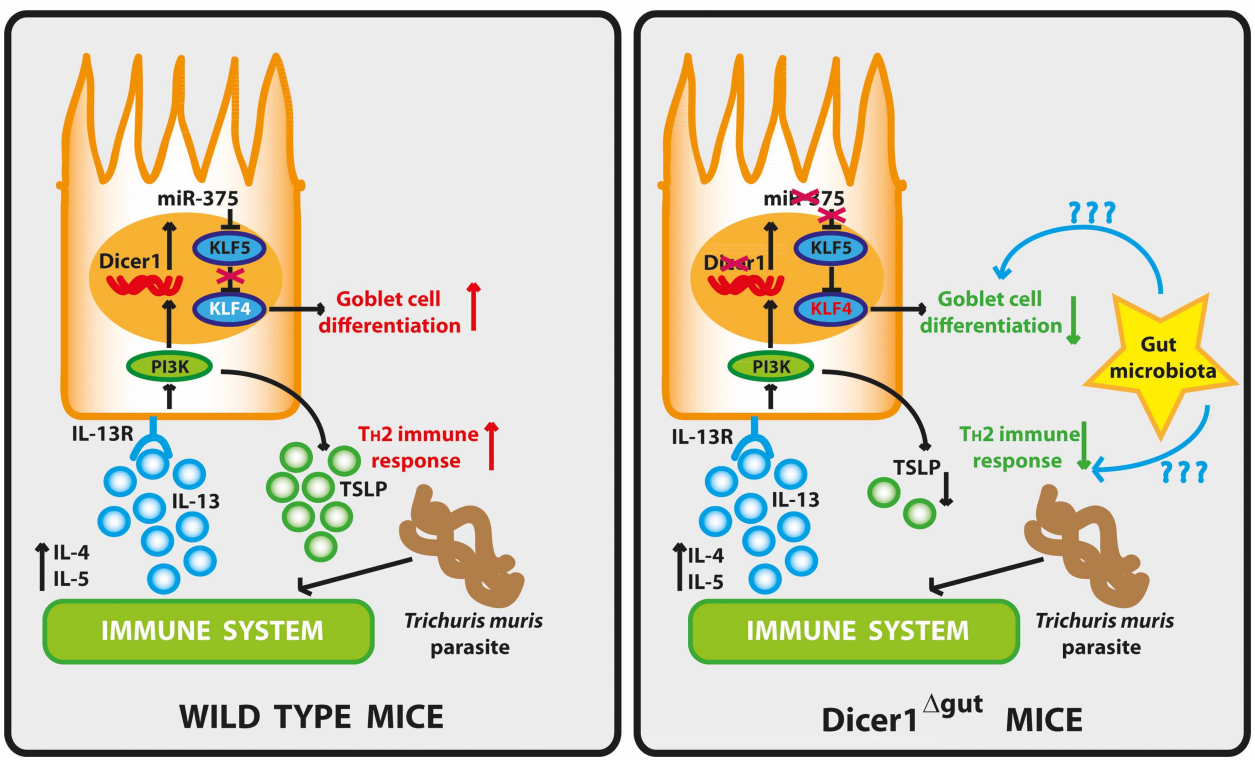

FIGURE 1 | (A) Research projects for the study of the interplays between gut microbiota and miRNAs. **(B)** Intestinal epithelial differentiation and T_H2 immune responses are regulated by miRNAs: in wild type mice, Dicer1 and miR-375 inhibit KLF5, a known antagonist of KLF4 that promotes the differentiation of goblet cells via KLF4. Helminth infection induces T_H2 cytokines, especially IL-13, which leads to epithelial expression of miR-375 and goblet-cell maturation via PI3K. Moreover, miR-375 also induces TSLP to accelerate T_H2 immune responses to parasite infections. In *Dicer1*$^{\Delta gut}$ mice, depletion of Dicer1 or miR-375 results in fewer goblet cells and diminished T_H2 responses. The gut microbiota can be involved in the induction and regulation of miRNA expression either for active or quiescent immunity. Likewise, other miRNAs can be involved in the generation of optimal protective immunity to various pathogens. This figure has been adapted from (Goto and Kiyono, 2011).

in vivo has been investigated, showing that the murine miRNA signature in the caecum is affected by the presence of the microbiota (Singh et al., 2012). Moreover, authors found that 34 putative miRNA target genes encode for proteins involved in the regulation of the intestinal barrier function (i.e., glycosylation enzymes, junctional proteins, proteins found in the mucus layers) and in the immune regulation (i.e., MHC I and II proteins). They found that the expression of miRNAs depends on the endogenous microbiota and that 16 unique miRNAs were deregulated between germ-free and conventional raised mice. By cross-matching the list of intestinal barrier genes predicted to be modulated by differentially expressed miRNAs, with genes already demonstrated to be deregulated in the jejunal mucosa of intestinal-specific Dicer knock-out mice (McKenna et al., 2010), the authors supported the hypothesis that gut commensals impact the intestinal barrier via miRNAs expression modulation. Therefore, the miRNAs modulation by gut microbiota may potentially affect the expression of a huge number of host genes, so far unexpected, especially in those diseases where the microbiota composition is altered toward less desirable species. In this context, the use of synthetic miRNAs could represent a potentially novel therapeutic perspective.

Another example of the role of miRNAs in affecting the intestinal epithelial monolayer, has been provided by Biton et al. by using mice with an inducible intestinal epithelial cell–specific deficiency in *Dicer1* (*Dicer1*$^{\Delta gut}$) (Biton et al., 2011). They found that *Dicer1* deletion in the mice gut lead to goblet-cell depletion and that the regulation of goblet-cell differentiation is dependent on the expression of miR-375. In fact, the expression of this miRNA is able to inhibit the translation of KLF5, an antagonist of the goblet cell–differentiation factor KLF4, supporting the differentiation of goblet cells. Moreover, they observed a lower expression of IL-4, IL-5, and IL-13 in *Dicer1*$^{\Delta gut}$ mice and an enhanced susceptibility to helminth parasite *Trichuris muris* infection (**Figure 1B**). IL-13, presumably supplied by T_H2 cells, induces miR-375 in IECs *in vitro* and a down-stream production of the T_H2-facilitating epithelial cytokine TSLP, indicating an appropriately balanced T_H2 feed-forward loop regulated by miR-375. On the basis of their results, the authors suggested that miR-375 directs the differentiation of goblet cells and the promotion of antiparasitic T_H2 immune responses. As miR-375 expression is very high in the human intestine (Wu et al., 2010), mucosal expression of this particular miRNA might also be important in the regulation of intestinal homeostasis and protection against parasite infection in humans (Goto and Kiyono, 2011). Further investigation should allow answering to many open questions still remaining, such as whether there are other miRNAs involved in this process or whether there are other miR-375 targets relevant to the differentiation of goblet cells or in the maintenance of gut immunological homeostasis. It is quite easily conceivable that in a near future we will assist to the development of innovative mucosal miRNA–targeted treatments and to the diagnosis of pathogenic mucosal conditions such as allergy, inflammatory bowel diseases and colon cancer, as well as infection by bacteria, viruses and parasites by employing specific miRNA-designed tests.

One of the most recently emerging and appealing concept is the role of toll-like receptors (TLRs) as potential mediators between gut microbiota and miRNAs/mRNAs modulation in humans. In fact, it has been recognized clearly that host gene expression is regulated by gut microbiota along the length of the gut and that microorganisms recognition is mediated by TLRs through the adaptor molecule MyD88 (Dalmasso et al., 2011; Larsson et al., 2012). Therefore TLRs, localized at the interface between the microbiota and the molecular machinery of host cells, may be key players in these relationships. In a recent study, Chassin et al. found that the TLR-4-mediated transcriptional activation of IECs observed in mice immediately after birth, was induced by oral ingestion of environmental endotoxin and induced a post-transcriptional down-regulation of epithelial IRAK1 protein expression, protecting further from bacteria-induced epithelial damages (Chassin et al., 2010). According to evidences showing that IRAK-1 expression is regulated by miR-146a (Taganov et al., 2006), miR-146a levels declined only in concomitance to the increase of IRAK-1 protein level, whereas miR-146a silencing induced IRAK1 protein expression. Moreover, authors demonstrated that the oral treatment with anti-miRNAs is a viable option to down-regulate the expression of miR-146a in intestinal epithelial cells.

Another study focusing on the microbiota regulation of miRNAs expression and on the maintenance of intestinal homeostasis, has been reported by Xue et al. who reported a connection between the expression of miR-10a and of its target IL-12/IL-23p40, a key molecule for innate immune responses to commensal bacteria (Xue et al., 2011). They also found that commensal bacteria down-regulated dendritic cell miR-10a expression via TLR–TLR ligand interactions through a MyD88-dependent pathway and that mice with colitis expressed higher levels of IL-12/IL-23p40 and lower level of gut miR-10a, compared to control mice, opening new perspectives for the study of miRNAs regulation in intestinal diseases.

In the field of RNA silencing, very close to that of miRNAs for their common mechanism of action, a novel approach exploiting engineered bacteria has been reported few years ago (Xiang et al., 2006). This approach holds great promise for functional genomics in mammalian systems and for other *in vivo* applications, since it demonstrates that the trans-kingdom RNA interference (RNAi) process is feasible both *in vitro* and *in vivo*. Authors employed *E. coli* engineered to produce short hairpin RNAs, by the use of a plasmid that they termed TRIP (trans-kingdom RNAi plasmid). This vector contains *Inv* and *HlyA* encoding for invasin, and listeriolysin O, respectively, enabling the entry into β1-integrin-positive mammalian cells and the release of genetic materials from internalized vesicles. By co-culturing human colon cancer cells (SW480) *in vitro* with the engineered *E. coli*, a significant down-regulation of a specific target gene has been observed, demonstrating the effectiveness of the trans-kingdom RNAi mechanism *in vitro*. By oral or intravenous administration of the engineered *E. coli*, the authors demonstrated also an efficient gene silencing in the intestinal epithelium and in human colon cancer xenografts in mice,

suggesting a clinically feasible approach to the *in vivo* application of RNAi technology. Most interestingly, this trans-kingdom RNAi approach not only can be exploited clinically to silence genes in the colonic mucosa and in other organs colonized by bacteria (i.e., oral cavity, urinary bladder, and female genital tract), but also suggest the speculative but intriguing possibility that such RNAi mechanism may occur also in natural interactions such as infections, commensal interaction and symbiosis.

In conclusion, I described two works (Dalmasso et al., 2011; Singh et al., 2012) dealing with a differential expression of miRNAs in different areas of the intestinal tract as a function of microbiota composition. In these cases, intestinal microbiota are the "actors." Conversely, we should also think to miRNAs as "actors" when, under proper conditions, influence the regulation of goblet-cell differentiation (Biton et al., 2011). Therefore, an interconnected cycle could be envisaged where miRNAs and gut microbiota are the two main partners. Two interesting approaches (use of engineered vectors and the oral delivery of anti-miRNAs) (Chassin et al., 2010; Xue et al., 2011) have emerged as interesting possibilities to study experimentally the interplays between miRNA and gut microbiota, and their mutual role in influencing host immune system and related processes. Therefore, although still a few, the studies reported so far emphasize that we cannot ignore that "our other genome" is intimately linked to "our natural miRNome."

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Causative role of gut microbiota in non-alcoholic fatty liver disease pathogenesis

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Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease worldwide (Milić and Stimac, 2012). NAFLD affects prevalently children and adults with particular risk factors including genetic susceptibility and inappropriate lifestyle (i.e., over-/mal-nutrition and physical inactivity). In fact, obesity, as well as some traits of metabolic syndrome, such as insulin resistance and dyslipidemia, are co-morbidities often associated to the presence of NAFLD (Vanni et al., 2010). In line with the increased obesity epidemics, epidemiological studies indicate that the estimated global prevalence of NAFLD ranges from 3–10% depending on age, sex, ethnicity, and risk factors. Interestingly, in obese children and adults this prevalence may raise up to 20–80% (Alisi et al., 2009; Vernon et al., 2011).

The term of NAFLD defines a series of hepatic pathologies that include the relatively benign steatosis that may, under the pressure of multiple triggering factors, progress to the more severe condition of non-alcoholic steatohepatitis (NASH), characterized by steatosis, necro-inflammation, and eventually fibrosis (Brunt, 2010).

The NAFLD development is still unclear, however, it is now largely accepted that, beside to the genetic background, the increased consumption of obesogenic foods may have a role in the NAFLD pathogenesis. In particular, diets enriched in fat and fructose may be steatogenic in two ways: favoring the occurrence of systemic insulin resistance closed to a dangerous accumulation of free fatty acid (FFA) in the liver; causing deposition of visceral fat and consequent hepatic insulin resistance responsible for steatosis development (Tilg and Moschen, 2008). Steatotic liver is susceptible to the action of next insults that may exacerbate steatosis and promote NASH. These NASH promoters include: imbalance of production/release of hormones derived from adipose tissue (adipocytokines) with consequent necro-inflammation, oxidative stress, activation of specific nuclear receptors activation, and fibrogenesis (Malaguarnera et al., 2009). In response to the systemic insulin resistance, pancreatic β-cells increase insulin hypersecretion accelerating liver fat accumulation and leading to NAFLD. Recently, it has been reported that also gut-liver axis may play a crucial role in this complex network of multiple interactions (Musso et al., 2010a). In fact, it has been suggested that the diet-dependent increase of gut microbiota products may influence intestinal permeability and activate molecular mechanisms of innate immune response, acting as possible inductor of necro-inflammatory lesions and severe fibrosis in NAFLD (Compare et al., 2012).

The gut microbiota having an extensive cross-talk with the liver represents an important source of hepatotoxic factors comprising bacteria and bacterial products. In such as scenario the intestinal microbiota composition can be affected and dynamically altered by diet regimen, lifestyle, genetic background, antibiotic usage, etc (Compare et al., 2012). The gut microflora exerts various central functions, among which fermentation of dietary components eluding the digestion and protection against possible invading pathogens (Othman et al., 2008; Cani and Delzenne, 2009). Therefore, the maintenance of the integrity of the intestinal barrier is of crucial importance to preserve an healthy gut-liver axis. In fact, a derangement of the homoeostasis between bacteria and host and a qualitative and quantitative alteration of gut microflora lead to an increased intestinal permeability. This promotes bacterial and endotoxin translocation triggering a production of pro-inflammatory molecules and cytokines and metabolic disorders. Notably, the intestinal flora, comprised small intestinal overgrowth (SIBO), has been found altered in many chronic liver diseases. On this regard it has been shown that NAFLD patients have an increased intestinal permeability and SIBO (Miele et al., 2009). Several lines of evidence have shown that NAFLD can be affected in different way by gut microbiota. From a side, the microbiota can directly affect the quantity of calories recovered from intestinal contents influencing the body weight possibly preceding the obesity occurrence. Further, gut microbiota and related endotoxemia can be implicated in the development of insulin resistance involved in NAFLD pathogenesis by various mechanisms (Cani et al., 2007a,b; Musso et al., 2010b). Also, the intestinal integrity can be lost by the alteration of the tight junctions causing an increase intestinal permeability which leads to bacterial translocation and their products into the systemic circulation, which in turn reach the liver being correlated to NAFLD (Miele et al., 2009; De Gottardi and McCoy, 2011). The gut liver-axis is the way by which the bacteria and their potential hepatotoxic products (LPS, DNA, RNA, etc.) can easily reach the liver. These microbial compounds can be generally classified as pathogen-associated molecular patterns (PAMPs) while the endogenous products are distinguished

in damage-associated molecular patterns (DAMPs). Interestingly, the serum levels of lipopolysaccharide (LPS), one of most studied PAMPs, are up-regulated in NASH patients with necro-inflammation and sever liver damage (Alisi et al., 2010). The final effect is the activation of the signaling cascade triggered by specific immune receptor resulting in the expression of pro-inflammatory cytokine genes including tumor necrosis factor (TNF)α and several interleukins (ILs) that may exacerbate hepatocyte damage (**Figure 1**).

The immune system has generated, along the evolution, a series of specific pattern recognition receptors (PRRs) comprising the Toll-like receptors (TLRs), which are evolutionary conserved type I transmembrane glycoproteins. The TLRs are the immune sensors of PAMPs and/or DAMPs initiating a signaling cascade leading to the activation of pro-inflammatory genes. Notably, each TLR has selectivity for specific PAMPs or DAMPs and can also differ from their proper localization (Alisi et al., 2011). In the liver, TLRs are expressed in many different cell types including Kupffer cells, hepatocytes, and HSCs (Schwabe et al., 2006) being the TLR4 the specific receptor for the bacterial endotoxin LPS, which is the key inducer of pro-inflammatory cytokines (as TNFα, IL-6, IL-8, IL-12, etc.) through the activation of the transcription factors NF-kB (nuclear factor kappa B), AP-1 (activating protein 1) and also LITAF (LPS-induced TNFα factor) in the liver (Alisi et al., 2011).

The gut colonization initiates at birth establishing a dynamic repertoire of gut microbiota during life that can be altered in several way giving rise to different inflammatory conditions possibly leading to more severe disturbances such as NAFLD. Many effort have been made to characterize which bacterial composition is more healthy to preserve the host from metabolic disorders and many progresses have been obtained in animal model on the study of the role of gut microbiota and accumulating evidence from human studies are available, although the examination along the time of the gastrointestinal bacteria composition is more difficult to assess. It has been extensively demonstrated that the proportion of intestinal microbiota is dependent on diet regimen. Interestingly, both in obese human and in mice the amount of the phyla Bacteroidetes and Firmicutes, which represent more than 90% of the totality of the gut microbiota, are altered (the first decreased and the second increased) (Eckburg et al., 2005). It has also been shown that a different balance of *Bifidobacterium* spp. and faecal *Staphylococcus aureus* results from the comparison between normal weight children and children which, at long last, can develop into overweight or obese subjects, suggesting a possible preceding condition that can foretell the future obesity occurrence (Kalliomäki et al., 2008). Further, the fat consumption can produce a wide amount of lipoprotein-containing chylomicrons which can guide the translocation of LPS toward other organs comprised the liver (Vreugdenhil et al., 2003). Interestingly, in a study in human choline-depleted diet it was described a correlation among the balance of *Gammaproteobacteria/Erysipelotrichi* classes and the occurrence of fatty liver (Spencer et al., 2011). Besides, in a murine models fed high-fat diet, the *Bifidobacterium* spp. administration determined an amelioration of the metabolic panel and a decrease of pro-inflammatory

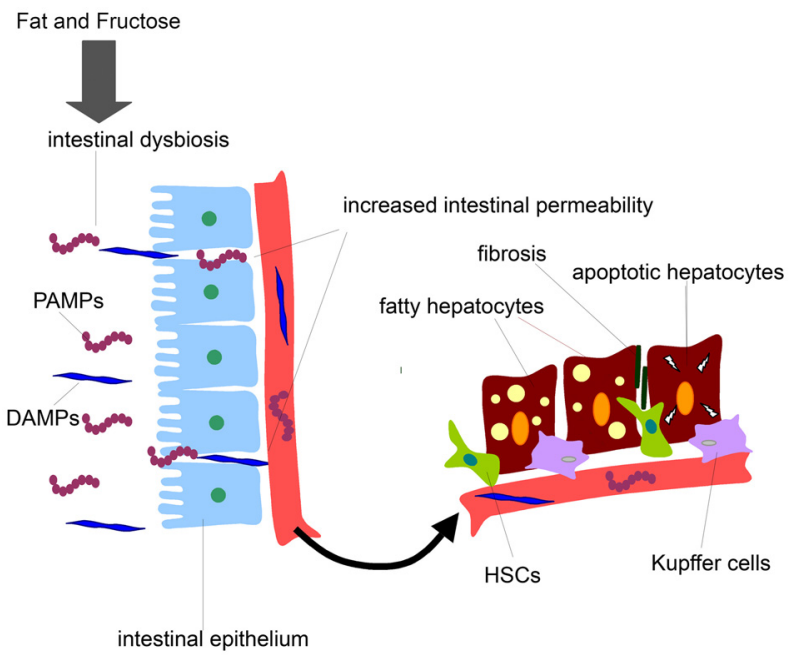

FIGURE 1 | Gut-derived product effects on liver cells during NAFLD development. Diets enriched in fat and fructose may disrupt intestinal barrier, increase intestinal permeability to gut-derived products, and release of PAMPs and DAMPs into the systemic circulation. PAMPs and DAMPs may reach liver cells promoting firstly fat accumulation in hepatocytes and secondly fibrosis and liver cell damage.

cytokines. Interestingly, the cytokine levels were augmented in parallel with LPS amount and inversely to *Bifidobacterium* spp. totality (Cani et al., 2007a,b). Confirming the important correlation among gut microbiota alteration and diet, various studies have reported that an high-fat diet increases the circulating LPS level amplifying the expansion of bacteria releasing LPS (Cani et al., 2007a,b). Moreover, an high-fat diet can cause a suppression of *Eubacterium rectale*, *Clostridium coccoides,* and *Bifidobacterium* spp (Cani and Delzenne, 2009). On the light of this evidence many probiotics have been tested in order to restore a balance in the altered gut microflora. *Lactobacillus* and *Bifidobacterium* spp are the most used having diversified beneficial results in the metabolic derangements such amelioration of dysplidemia, reduction of both total, LDL and VLDL cholesterol, decrease of triglycerides which overall are diversified effects depending on the bacterial species (Xiao et al., 2003; Tannock, 2004; Larkin et al., 2009). Noteworthy, the alteration of "healthy microbiota" is only one side of the complex interactions of the gut-liver axis. The disbiosys is a fraction of the causes leading to metabolic and gastrointestinal disturbances. All along, it is emerging a number of studies correlating the reciprocal interactions occurring among bacteria, viruses, eukaryotes, and in turn their communication with the host immune system. Indeed, the sequencing project Human Microbiome Project and the Earth Microbiome Project would be valid instruments to reach a wider and comprehensive overall view in order to better understand the interdependence between the host microbiota and the numerous disorders and diseases to attempt cutting edge therapeutic strategies (Clemente et al., 2012).

Appropriate dietetic regimens and physical exercise may improve simple steatosis, even though this lifestyle approach is unable to recover NAFLD-associated liver damage. In fact, it is widely recognized that lifestyle modifications combined with a multi-targeted therapeutic approach against specific triggering factors could be more effective than mono-therapeutic approach in at least paediatric NAFLD (Alisi and Nobili, 2011). Unfortunately, various inadequate pharmacological therapies (e.g., insulin-sensitizers, antioxidants, and cytoprotective agents) have been developed over recent years in the attempt of modifying one or more of the major factors involved in NAFLD pathogenesis. Therefore, modifications of gut microbiota may be one of the possible objectives of an efficient multi-target therapy. This option is supported by several investigations in animal models studies suggesting that gut microbiota manipulation with probiotics reduces intestinal inflammation and improves the epithelial barrier function (Iacono et al., 2011). Furthermore, Loguercio et al. (2005) demonstrated that a chronic therapy with a probiotic (VSL#3) in patients affected by several types of chronic liver diseases, including NAFLD may reduce liver damage and improve serum levels of various biomarkers. Interestingly, a pilot study, involving 20 obese children with hypertransaminasemia and bright liver at ultrasound, found that 8 weeks of treatment with probiotic *Lactobacillus rhamnosus* strain GG reduced transaminase levels and antipeptidoglycan-polysaccharide antibodies, a surrogate test for SIBO evaluation (Vajro et al., 2011).

In conclusion, as several observations suggest a potential role of microbiota in NAFLD development, we believe that probiotics effects on gut flora associated to their excellent tolerability may represent promising therapeutic agents to revert NASH-related liver damage.

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Gut microbiota and developmental programming of the brain: from evidence in behavioral endophenotypes to novel perspective in obesity

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GUT MICROBIOTA AND DEVELOPMENTAL ORIGIN OF THE HEALTH AND DISEASE

Onset of obesity has been anticipated at earlier ages and prevalence of pediatric obesity has dramatically increased worldwide over the past decades (Han et al., 2010). While epidemic obesity is mainly attributable to westernized lifestyle with excessive consumption of refined carbohydrates and fats and reduced physical activity, onset of obesity in children has been, in part, attributed to the fetus' exposure to disadvantageous conditions (i.e., hormonal and nutritional cues) during the intrauterine life which can exert a profound impact on the organism's later development, structure, and function. This phenomenon, which extends to peri- and post-natal periods, is known as "developmental programming of health and disease" (Hochberg et al., 2011).

With the enormous effort that microbiologists are investing in trying to understand the contribution of gut microbiota to human health and disease, a pivotal role of the gut microbiota is emerging as an environmental cue which influences the developmental programming. Most of the evidence has so far been mostly collected with regard to psychopathological "endophenotypes" (i.e., the set of behavioral/physiological symptoms which result into more stable phenotypes of complex traits characterized by low level genetic variability). Epidemiological studies in humans have indicated associations between common neurodevelopmental endophenotypes, such as autism, schizophrenia, depression, and anxiety, with microbial pathogen infections during the perinatal period (Mittal et al., 2008; Finegold et al., 2010). Epidemiological findings have been supported by experimental evidences in germ-free (GF) mice (mice born and raised in a sterile environment and that are devoid of an enteric bacteria) which demonstrated that exposure to microbial pathogens during vulnerable periods result in behavioral abnormalities, including anxiety-like behavior, impaired cognitive function (Sullivan et al., 2006; Goehler et al., 2008), and more elevated home-cage activity counts than conventionalized animals (Bäckhed et al., 2007).

PARALLELING NEURODEVELOPMENTAL AND OBESE ENDOPHENOTYPES. THE BRAIN–GUT–MICROBIOTA AXIS

Both neurodevelopmental and obese endophenotypes seem to have their root during the intrauterine life. Early development of gut microbiota may impact the programming of obesity as it does for neurodevelopmental traits.

As observed in mouse models and patients affected by behavioral abnormalities, specific phyla, classes, or species of bacteria, or bacterial metabolic activities characterize also the gut microbiota of obese individuals (Manco et al., 2010). Gut dysbiosis in obesity has been strongly associated with increased ability to harvest energy from the diet, to influence the expression of host genes in particular those regulating lipid and glucose metabolisms at peripheral sites, to promote systemic low-grade inflammation and insulin resistance (Manco et al., 2010). It is, hence, tempting to speculate that gut dysbiosis of individuals prone to obesity may establish in the perinatal life and drive developmental programming of later obesity.

The common soil for development and adult self-sustain of both neurodevelopmental disorders and obesity is the continuous cross-talk between the gut and the brain. The complex bidirectional communication system between gut and brain is vital for maintaining conditions of homeostasis (in these cases, stable behavior, and energy balance, respectively). Through this top-down and bottom-up perspective of information flow, signals from the brain can influence the motor, sensory, and secretory modalities of the gastro-intestinal tract and conversely, visceral messages from the gastro-intestinal trait can influence brain function (Mayer, 2011; O'Mahony et al., 2011). The humans' internal ecosystem ("human microbiome") seems to intrude and modify significantly this bidirectional communication starting very early in the life so much to led some to suggest that the brain–gut axis may be more accurately termed "the brain–gut–microbiota axis" (Rhee et al., 2009). Therefore, the human microbiome may enrich and complicate the control system of energy balance which is already one of the most highly integrated and complex functions of the body; not surprisingly, given its importance. At the level of the central nervous system, information from the periphery is integrated and allows initiating appropriate behavioral, humoral, and neural outputs which are often common to both endophenotypes.

Recognition of the interaction between gut microbiota and central nervous system may shed new light to explain epidemic obesity (Forsythe et al., 2010; Bercik et al., 2011; Cryan and O'Mahony, 2011) by explain some of its trans-generational transmission.

GUT COLONIZATION, PROGRAMMING OF THE HYPOTHALAMIC-PITUITARY-ADRENAL, AND STRESS RESPONSE

Microbial colonization of mammals is an evolution-driven process that modulates many host physiological functions, many of which are associated with nutrient intake. Colonization of the infant gut commences at birth when delivery exposes to a complex microflora. The infant's microbiota expresses unquestionably a maternal fingerprint, but soon after the birth, the newborn organism is rapidly, and densely populated with complex forms of indigenous microbes (Manco et al., 2010; Putignani et al., 2010).

Gut colonization has been found to exert an effect on the development of the hypothalamic-pituitary-adrenal (HPA) axis and on the acute response to stress conditions (Sudo et al., 2004). Dysregulation of the HPA axis and impaired stress response are common to different behavioral endophenotypes such as depression, anxiety, but also visceral obesity. Indeed, impaired stress response influences several metabolic pathways, including some involved in the control of satiation, body growth gluconeogenesis, insulin resistance, and insulin secretion (Chrousos, 2000). GF mice exhibit a less anxious phenotype in the elevated plus maze, a well validated model of anxiolytic action (Sudo et al., 2004; Neufeld et al., 2011) and an exaggerated release of corticosterone and adrenocorticotrophin hormone (ACTH) compared to the specific pathogen free (SPF) animals in response to a mild restraint stress induced (Sudo et al., 2004). Administration of exogenous glucocorticoids (the equivalent of corticosterone in mice) is known to reduce synaptophysin expression in the fetal brain of non-human primates (Antonow-Schlorke et al., 2003). Therefore, excessive release of steroids during vulnerable periods of the life can be one of the mechanisms by which gut microbiota modulates HPA neuroplasticity and may, hence, enhance the risk to develop obesity later in adulthood.

In experiments comparing the stress response of GF and SPF, a decrease in the brain derived neurotrophic factor (BDNF) was reported (Sudo et al., 2004). BDNF is a key neurotrophin involved in neuronal growth and survival, which regulates the growth and differentiation of new neurons and synapses; it is involved in the regulation of multiple aspects of cognitive and emotional behaviors (Zola et al., 2000). BDNF is involved in the regulation of appetite and control of energy metabolism (Rothman et al., 2012). There was also a decreased expression of the *N*-methyl-D-aspartate (NMDA) receptor subunit 2a (NR2a) in the cortex and hippocampus of GF animals compared to SPF controls (Sudo et al., 2004). More recently, Neufeld et al. (2011) reported, in contrary, an up-regulation of the BDNF expression in the dentate gyrus of the hippocampus of GF animals. The researchers found also a decrease in the NR2B subunit of the NMDA receptor in the amygdala of GF animals.

In obesity, which is often characterized by impaired hippocampal synaptic plasticity and cognitive abilities such as learning and memory, significant decreases in NR2A and NR2B subunit expressions have been observed in the hippocampus of obese animals and their expressions seem to significantly increase in the obese rats following 60% calorie restriction (Yilmaz et al., 2011).

MODULATION OF MICROBIOTA AND BRAIN REPROGRAMMING

The response to stress stimuli observed in the GF mice is partially reversed by re-colonization with fecal matter from SPF animals and fully restored by mono-association with *Bacillus infantis* in a time dependent manner (Sudo et al., 2004).

Studies investigating the effect of administration of probiotics (i.e., live microorganisms which when administered in adequate amounts confer a health benefit on the host) support a role for the microbiota in anxiety-like behaviors but with divergent effects depending on the strain.

For example, administration of *Lactobacillus helveticus* R0052 and *Bacillus longum* R0175 taken in combination, induced anxiety-like activity in rats (Messaoudi et al., 2011), while chronic treatment with *Lactobacillus rhamnous* (JB-1) over 28 days produced animals with lower levels of stress induced corticosterone release and reduced depressive behaviors in the forced swim test in addition to a less anxious phenotype in the elevated plus maze. Animals treated by *L. rhamnosus* also showed alterations of gamma-aminobutyric acid (GABA) B1b mRNA in the brain with increased expression in the hippocampus, amygdala, and locus coeruleus as reduced GABAAα2 mRNA expression in prefrontal cortex and amygdala and increased GABAAα2 in the hippocampus. Interestingly, the authors demonstrated that vagotomized mice did not display the neurochemical and behavioral effects caused by the *L. rhamnosus*, thus implicating the vagus nerve in the direct communication between the bacteria and the brain (Bravo et al., 2011). As to commonalities between pathogenesis of behavioral disorders and obesity, there are two remarks. The increased expression of GABAAα2 gene in the brainstem, hippocampus, and amygdala is described in the Prader–Willi syndrome, a form of genetic obesity, characterized by compulsive food seeking behaviors (Scoles et al., 2011). As to the nerve vague, it plays a pivotal role in the gut-brain axis control of food intake (Sam et al., 2012). However, the anti-anxiety effect of the *L. rhamnosus* (*Lr* JB-1) via the vagus nerve and the central GABA system observed in healthy mice by Bravo et al. (2011), confirmed previous results by Bercik et al. (2011) who, however, observed that this effect is independent of the vagus nerve.

GUT MICROBIOTA AND RELEASE OF BRAIN TRANSMITTERS

Gut microbiota influences the release of some of the major brain transmitters which act in the gut-brain axis and modulate food intake and energy balance (Gruninger et al., 2007), i.e., short chain fatty acids (SCFAs), Peptide YY (PYY), tryptophan, serotonin, endocannabinoid ligands, and ghrelin. For instance, the interaction between SCFAs produced by the gut bacteria, and Gpr41 increases circulating levels of PYY, a potent orexigenic agent (Samuel et al., 2008). Conventionalized GF mice present with a 2.8-fold increase in plasma serotonin levels respect to control animals (Wikoff et al., 2009). Administration of *B. infantis* 35624 to Sprague-Dawley rats, for example, has been shown to induce an elevation in plasma tryptophan levels, a precursor to serotonin (Desbonnet et al., 2008). Diet supplementation with prebiotic fiber has been associated with alterations in the expression or content of various gut hormones linked to the regulation of energy balance, notably increasing the satiety hormone PYY and reducing the expression of the orexigenic peptide ghrelin (Delzenne et al., 2005).

CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, as learned by experiments on acute response to stress in GF and SPF mice, gut microbiota initiates a signaling soon after birth at a time when the newborn mice become exposed to gut microbiota.

Alternatively, it is tempting to speculate that exposure to gut microbiota metabolites, generated by the flora of the pregnant mother, can influence brain development during embryogenesis. Hence the influence of the maternal gut microbiota may contribute to the trans-generational transmission of endophenotypes characterized by dysfunctional HPA, primarily neurodevelopmental and obese endophenotypes. So, investigation of the impact of gut microbiota on the developmental programming of obesity and modulation of adult gut microbiota ("re-programming") by different microbial strains represent research priorities. Such investigation will reveal the full therapeutic potential of nurturing gut bacteria starting since the perinatal life to prevent obesity and associated comorbidities and to reduce the burden of obesity by modulating not only energy harvesting, but influencing feeding behaviors and energy expenditure. Mechanisms of action which deserve investigations include release and turnover of neurotransmitters, altered parasympathetic activity and expression profiles of canonical signaling pathways. Thus, probiotic, prebiotic, or antimicrobial administration and the evaluation of food-related behaviors and metabolic outputs, in healthy and obese humans, are worthwhile pursuits in order to reduce the burden of epidemic obesity.

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Integration of datasets from different analytical techniques to assess the impact of nutrition on human metabolome

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Bacteria colonizing the human intestinal tract exhibit a high phylogenetic diversity that reflects their immense metabolic potentials. The catalytic activity of gut microbes has an important impact on gastrointestinal (GI) functions and host health. The microbial conversion of carbohydrates and other food components leads to the formation of a large number of compounds that affect the host metabolome and have beneficial or adverse effects on human health. Metabolomics is a metabolic-biology system approach focused on the metabolic responses understanding of living systems to physio-pathological stimuli by using multivariate statistical data on human body fluids obtained by different instrumental techniques. A metabolomic approach based on an analytical platform could be able to separate, detect, characterize and quantify a wide range of metabolites and its metabolic pathways. This approach has been recently applied to study the metabolic changes triggered in the gut microbiota by specific diet components and diet variations, specific diseases, probiotic and synbiotic food intake. This review describes the metabolomic data obtained by analyzing human fluids by using different techniques and particularly Gas Chromatography Mass Spectrometry Solid-phase Micro Extraction (GC-MS/SPME), Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy and Fourier Transform Infrared (FTIR) Spectroscopy. This instrumental approach has a good potential in the identification and detection of specific food intake and diseases biomarkers.

Keywords: metabolites, instrumental methods, gut microbiota, diet, biomarkers

INTRODUCTION

The human intestine is home of some 100 trillion of microorganisms of at least hundreds species. The density of bacterial cells in the colon has been estimated at 10^{11}–10^{12} per ml, which makes it one of the most densely populated known microbial habitats (Eckburg et al., 2005; Ley et al., 2006; Vitali et al., 2010). This microbial ecosystem serves numerous important functions for the human host, including protection against pathogens, nutrient processing, stimulation of angiogenesis, modulation of intestinal immune response, and regulation of host fat storage (Palmer et al., 2007). The composition of the adult gastrointestinal (GI) microbiota has been intensely studied, using both classical microbiology cultivation and, more recently, culture-independent, small subunit (SSU) ribosomal DNA (rDNA) sequence-based methods (Palmer et al., 2006, 2007; Huse et al., 2008; Vitali et al., 2010).

The prevention and treatment of gut-related diseases will strongly depend on understanding the mechanisms involved in the complex processes of digestion. However, the interactions between the diet, microbiota and host are largely unknown (Quigley, 2011; Del Chierico et al., 2012; Nicholson et al., 2012; Payne et al., 2012).

This review is addressed to evaluate the potential of different analytical approaches to identify and detect the molecules present in body fluids, such as blood, urine, feces, which can be considered originated by food digestion, diet/gut microbiota, and host/microbiota interactions as well as disease markers.

DIET EFFECT ON HUMAN GUT METABOLOME

Foods contain thousands of compounds which, upon digestion and metabolism, give rise to complex physiological reactions and possible changes in the intestinal microbiota profile resulting in a plethora of metabolites present in body fluids such as blood, urine, feces, and saliva. The number and diversity of chemical compounds in foods is amazing. It has been estimated that an omnivore diet exposes humans to more than 15,000 components, 8000 out of which are non-nutrients such as dietary fiber, antioxidants, prebiotics, and probiotics (Wishart, 2008).

An individual food system such as milk contains more than 200 different oligosaccharides, and the edible plants metabolome consist of more than 10,000 detectable compounds and 800 non-nutrient phytochemicals (Wishart, 2008). These molecules and their accumulation in body fluids, and in particular in urine, can

be regarded as a repository of metabolites in which any nutrient or non-nutrient that is not needed or present in excess is found. Several food-specific biomarkers, e.g., related to wine, black tea, coffee, fruit, and vegetables, have been identified in urine and blood samples (Spencer et al., 2008; Wishart, 2008). Other compounds occurring in different body fluids, namely blood, can be regarded as biomarkers of physiological response to foods. In particular, they are products of lipid peroxidation such as 8-isoprostaglandin F2alpha, and/or oxidative stress markers such as malonaldehyde or glutathione.

Some of the diet components have a potentially direct quantitative and qualitative impact on the microbial species characterizing the gut microbiota. Their metabolic output can harbor physiologically active compounds for the human host which in turn provides a stable environment for proliferation. On the other hand the host has evolved and can use bacterial fermentation products as an energy source for the epithelial cells. Some of the fermentation products, e.g., short chain fatty acids (SCFAs), can positively affect the biochemical and physiological processes at colon level and support some important biological functions (Wong et al., 2006). However, the influence of the bacterial population composition on inter-individual differences in metabolites production and colon health status is poorly understood, except for the well-known relationships between some specific metabolites and metabolic diseases (Le Gall et al., 2011).

Metabolic profiling has a wide potential for understanding the complex interactions between components of the gut microbiota, and elucidate the cause/effects relationships associated with specific nutritional choices and the related shifts in the microbiota composition.

Particularly challenging is the metabolic signatures identification of many phenotypes and their linkage with nutritional choices. In fact, the whole set of metabolites which can be detected in body fluids, and which characterizes the metabolic phenotype, named "metabotype" (Waldram et al., 2009), of an individual, is affected by various intrinsic and extrinsic factors including environment, drugs, diets, lifestyle, and genetics. It should be also considered that the content of non-nutrients in diets is higher than that of nutrients. Such non-nutrients can exert a significant effect on metabolomic profiles thus resulting in several compounds that can be used as biomarkers to trace the food origin.

HOST-MICROBIOME METABOLIC INTERACTIONS AND CELL-CELL COMMUNICATION

Mammalian–microbial symbiosis can play a role in the etiology and development of several diseases, e.g., insulin resistance, Crohn's disease (Marchesi et al., 2007; Kinross et al., 2011), irritable bowel syndrome (IBS) (Martin et al., 2006; Sartor, 2008), food allergies, gastritis and peptic ulcers, obesity, cardiovascular disease, and GI cancers (Martin et al., 2011). Activities of gut microbiota can be highly specific, and it has been reported that the establishment of Bifidobacteria is important for the development of the immune system and management of gut functions (Ouwehand, 2007). As the microbiome strongly interacts with the host to determine the metabotype, which influences outcomes of drug interventions, the knowledge of these interactions can provide personalized healthcare solutions (Martin et al., 2011). Also the diet has a key role in the gut microbiota modulation and shaping and, as a consequence, the different foods or their ingredients play a crucial role in the microbes selection and in a metabolic signaling network construction.

The host and its gut microbiota coproduce a large array of small molecules during the conversion of food and xenobiotics (compounds of non-host origin that enter the gut with the diet or are produced by microbiota), many of which play critical roles in shuttling information between host cells and the host's microbial symbionts. This chemical dialog includes signaling via low molecular weight metabolites, peptides, and proteins or may take place indirectly through immune-mediated pathways (Nicholson et al., 2012). The metabolites production by microbes may influence host health status and their detection in different body fluids can be considered as dysbiosis or disease biomarkers (Holmes et al., 2011; Nicholson et al., 2012) (**Figure 1**).

Different regions of the human GI tract vary in terms of composition of the indigenous microbiota (Gordon, 2012). For each compartment of the GI tract, a chemical dialog exists among different microbial species, e.g., direct substrate provision in the microbial food web, quorum sensing, contact-dependent signaling, and potentially gastro-transmitters (Ryan et al., 2009) as well as between microbial symbionts and host cells (Li et al., 2008).

Metabolites are the products and by-products of the many intricate biosynthetic and catabolic pathways existing in all living systems. Biological systems that exist at steady state maintain approximately constant concentrations of metabolic intermediates (Martin et al., 2011).

Fecal extracts are the most common material which can help to decipher the complex metabolic interplay between mammals and their intestinal ecosystems, and metabolic profiles-related to SCFAs, amino acids, organic acids, nucleosides and nucleotides, polyamines, phenolic compounds, bile acids, and lipid components can yield information on a range of gut diseases (Martin et al., 2011).

As described by Grider and Piland (2007), SCFAs stimulate intestinal transit, and at physiological concentrations they induce an 8–10-fold increase in serotonin release in an *in vitro* colonic mucosal system. Moreover, Musso et al. (2011) showed that SCFAs are clearly one of the most important gut microbiota products and affect a range of host processes including energy utilization, host-microbe signaling, and control of colonic pH with consequent effects on microbiota composition, gut motility, and epithelial cells proliferation.

The monitoring of the fecal metabolome also may unravel diagnostic information for inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis (UC), *Hirschsprung's* disease, celiac disease, allergy etc (Martin et al., 2011). Metabolomics has many potential applications. It supports functional genomics studies, systems biology, pharmacology, toxicology, and nutrigenomics. An approach called discovery metabolite profiling (DMP) is used to analyze all metabolites generated by a particular enzyme, providing a link between proteome and metabolome.

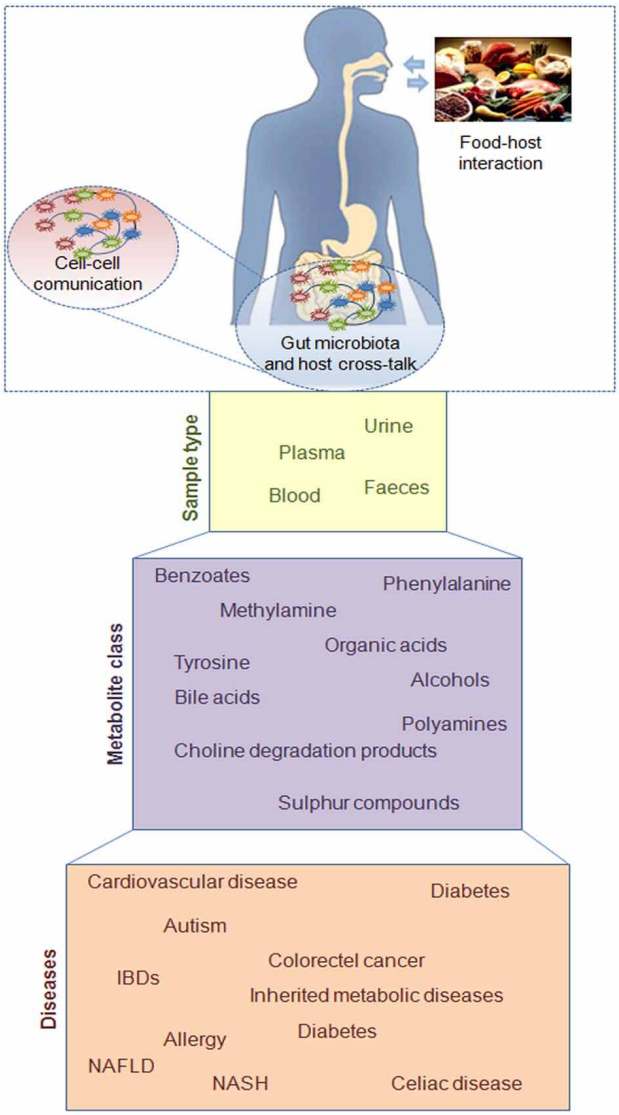

FIGURE 1 | Schematic representation of diet, microbes, and host interaction at gut level. The chemical dialogue via low molecular weight metabolites, peptides, and proteins between cell-cell and host-microbes leads to the metabolite production in different body fluids which could be considered as disease biomarkers.

ANALYTICAL TECHNIQUES TO DETECT METABOLITES

Metabonomics represents a well-recognized metabolic system approach that involves the study of multivariate metabolic responses of complex cellular organisms to different stimuli (Nicholson et al., 1999).

Ideally, it could be based on this sequence: samples collection (e.g., urine, blood, or some other body fluids), scanning them in a machine and finding a profile of tens or hundreds of chemicals that can predict whether an individual is on the road to a disease, or likely to experience side-effects from a particular drug (Pearson, 2007). However, this vision presents some drawbacks. The first complication is that one person's profile of metabolites is likely to be dramatically different from another's, and each may fluctuate markedly depending on different aspects, for example the lifestyle, the diseases, the diet, the nutrition etc (Pearson, 2007).

The detection and identification of hundreds metabolites can offer deep insights on the influence of lifestyle and dietary factors in relation to specific diseases. The recent rapid development of a range of analytical platforms, including gas chromatography (GC), liquid chromatography (LC), high pressure LC (HPLC), ultra pressure LC (UPLC) coupled to mass spectrometry (MS), capillary electrophoresis (CE) coupled to MS, Fourier Transform Infrared (FTIR) spectroscopy, and nuclear magnetic resonance (NMR) [e.g., proton (^{1}H)-NMR] spectroscopy, can enable to separate, detect, characterize, and quantify such metabolites and related metabolic pathways (Zhang et al., 2011). Metabolomics focuses on the complex interactions of system components and highlights the whole system rather than the individual parts, providing a distinct perspective on cellular homeostasis (Liu et al., 2010). Even if nowadays NMR, GC-MS, LC-MS are the prevalent techniques used, none of them is a perfect technique that can meet the requirements of metabolomics for measuring all metabolites. MS-based metabolomics offers high selectivity and sensitivity for the identification and quantification of metabolites, and its combination with advanced and high-throughput separation techniques can reduce the complexity of metabolite separation (Zhang et al., 2011) (**Table 1**).

NMR-based metabolomics is able to provide a "holistic view" of the metabolites under certain conditions, and thus is well-suited and advantageous for metabolomic studies (Wu et al., 2010). In particular, it is becoming a useful tool in the study of body fluids and for a non-invasive detection of metabolites and into diagnosis of the diet effects on gut microbiota or significant public health problems (Martin et al., 2012).

Usually, the measurement of the gut microbial metabolism is confined to fecal samples, which is typically limited because of the elevated colonic absorption of bacterial metabolites. The Proton Nuclear Magnetic Resonance (^{1}H-NMR) monitoring of the human fecal metabolome also may unravel diagnostic information for IBD including UC (Marchesi et al., 2007). Saric et al. (2008) evaluated the similarity and dissimilarity across different mammals namely humans, mice, and rats by using ^{1}H-NMR analysis. The authors reported how human fecal extracts showed greater inter-individual variation than in rodents, reflecting the natural genetic and environmental diversity in human population.

A major source of intestinal metabolites is both host and microbial processing of dietary nutrients. As reported by Martin et al. (2010) the ^{1}H-NMR analysis of feces revealed that the supplementation with probiotics significantly affects the host microbiota interaction. Probiotic supplementation of "humanized" mice, inoculated with a model of human baby microbiota, was associated with metabolic changes in the protein metabolism of *Lactobacillus paracasei* in particular with specific aminoacidic pattern.

Table 1 | Common analytical techniques used in metabolomics.

Analytical method	Advantages	Disadvantages	Comments
NMR	• Rapid analysis	• Low sensitivity	• **Chemical consideration:** gives detailed strucutural information, particularly using 2-D-NMR of isolated metabolites
	• High resolution	• Convoluted spectra	• **Chemical bias:** these methods have little chemical bias and can be used directly on the sample
	• No derivatization method	• More than one peak per component	• **Speed:** few minutes to hours. Depends on the strength of the magnet, sensitivity can be improved by magic angle spinning
	• Non-destructive	• Libraries of limited use due to complex matrix	
GC-MS	• Sensitive	• Slow	• **Chemical consideration:** on its own will not generally lead to metabolite identification. However, coupled with MS and NMR is very powerful for analyte identification
	• Robust	• Often requires derivaization	• **Chemical bias:** solvent extraction bias: non-polar vs. polar analytes. Need for chemical derivatization
	• Large linear range	• Many analytes thermally-unstable or too large for analysis	• **Speed:** very useful for separation, but typically take 10–30 min
	• Large commercial and public libraries		
LC-MS	• No derivatization required (usually)	• Slow	• **Chemical consideration:** on its own will not generally lead to metabolite identification. However, coupled with MS and NMR is very powerful for analyte identification
	• Many modes of separation available	• Limited commercial libraries	• **Chemical bias:** solvent bias means it is usually more applicable to polar compounds
			• **Speed:** very useful for separation, but typically take 10–30 min
	• Large sample capacity		
FT-IR	• Rapid analysis	• Extremely convoluted spectra	• **Chemical consideration:** provide limited structural information, but useful for identification of functional groups
	• Complete fingerprint of sample chemical composition	• More than one peak per component	• **Chemical bias:** these methods have little chemical bias and can be used directly on the sample
		• Metabolite identification nearly impossible	• **Speed:** 10–60 s
	• No derivatization needed	• Requires samples drying	

Dumas et al. (2006) have recently applied ^{1}H-NMR technique to characterize the intergenome interactions in mice with synbiotic gut microbiota as well as to monitor the gut-microbial metabolite variation in rats and to study the intricate relationships between gut microbiota and host co-metabotype associated with dietary-induced changes. Ndagijimana et al. (2009) studied, by means of the ^{1}H-NMR analysis of healthy human subjects faeces, the effect of the supplementation with a synbiotic food based on *Lactobacillus acidophilus*, *Bifidobactrium longum*, and Fructooligosaccharides. These authors reported that the number and the extent of metabolites in faecal slurries were strongly affected by the synbiotic food consumption and gave rise to characteristic metabolic signatures. Reproducibility of ^{1}H-NMR metabolic profiles generated from water and methanol extracts from human stools were assessed by Jacobs et al. (2008). On the other hand ^{1}H-NMR is one of the preferred platforms also for urine and plasma analysis (Ala-Korpela, 2008). The first published study, in which a metabolomics approach on urine was described, used the ^{1}H-NMR technique to monitor the effect of the inclusion of soy in the diet (Solanky et al., 2003). Urine samples have also been used to investigate responses to ingestion of chamomile tea, or other foods such as coffee, wine, and tea, evidencing that hippurate and glycine are important discriminatory metabolites (Ito et al., 2005; Wang et al., 2005).

The ability to predict the occurrence of exercise-induced ischemia in patients with suspected cardiovascular disease was investigated by ^{1}H-NMR blood analysis. Barba et al. (2008) demonstrated that lactate, glucose, lipids, and long-CFAs are

the main metabolites involved. Xanthine and ascorbate were proposed as possible markers of plaque formation in an artherosclerotic mouse model (Leo and Darrow, 2009) and lipoprotein subclasses can now be analyzed by a commercial ^{1}H-NMR -based protocol (Jeyarajah et al., 2006; Ala-Korpela, 2007).

Even if the information provided using ^{1}H-NMR is highly valuable, it is still limited due to the low resolution and sensitivity which enables the annotation and quantification of only a limited number of low molecular weight molecules (Jansson et al., 2009).

GC-MS-based metabolomics requires a high-throughput technology to handle a large volume of samples and accurate peak identification through the standard retention times and mass spectra. GC-MS has been widely used for metabolomics and can provide efficient and reproducible analysis (Zhang et al., 2011). In fact, it is also possible to obtain, simultaneously profiles of several hundred compounds including organic acids, most amino acids, sugars, sugar alcohols, aromatic amines, and FAs. GC-MS Solid-Phase Microextraction (SPME) based analysis can be considered as a very effective method for rapidly qualitatively and quantitatively analyze faecal samples most of which have not been previously reported (Garner et al., 2007). In particular, GC-MS/SPME represents a novel method to study metabolic profiles of biological samples. This approach has been used to compare neonates and adult feces (De Lacy Costello et al., 2008) and to identify volatile markers of GI diseases (Garner et al., 2007). The main metabolites identified by GC-MS/SPME belong to sulphur compounds, nitrogen compounds such as pyridine, and its derivatives, pyrazines, indoles, aldehydes, ketones, esters, alcohols, phenols, organic acids, hydrocarbons, and stress molecules such as furans and furanones. Vitali et al. (2010) investigated the impact of a synbiotic food on a human gut microbial ecology and metabolic profiles by using this technique. While no significant changes in the structure of the gut microbiota of healthy subjects was observed, the synbiotic food intake generated significant changes in some gut metabolic activities. The Canonical discriminant Analysis of Principal coordinates (CAP) of the fecal metabolic profiles showed a separation of subjects depending on synbiotic food intake. Recently, the GC-MS/SPME analysis of human feces has been also proposed as a tool to evaluate the *in vitro* effect of prebiotics and probiotics on the human microbiota (Vitali et al., 2012).

Zheng et al. (2011) applied an untargeted GC-MS-based metabonomics approach to profile bacterial metabolites in normal Wistar rats administrated with a broad spectrum β-lactam antibiotic imipenem/cilastatin sodium. In depth, metabolic phenotyping allowed the identification of 202 urinary and 223 fecal metabolites, many of which not previously reported (e.g., oligopeptides and carbohydrates), significantly related to a functional metagenome (Zheng et al., 2011). Moreover, Maccaferri et al. (2010) investigated the impact of rifaximin administration on microbial metabolic profiles by using GC-MS/SPME. At the same time this technique was also used in combination with the ^{1}H-NMR technique to investigate the metabolome of 19 celiac disease children under gluten-free diet (treated celiac disease, T-CD) and 15 healthy children (HC). The metabolome of T-CD and HC children was studied using fecal and urine samples. With this approach the authors showed that the levels of volatile organic compounds and free amino acids in fecal and/or urine samples were markedly due to affected by CD (Di Cagno et al., 2011). Francavilla et al. (2012) used GC-MS/SPME to study the influence of lactose on the composition of the gut microbiota and metabolome of infants presenting with cow's milk allergy.

Moreover, MS and HPLC techniques are commonly used for compounds' characterization at structural level. In the field of metabolomics, both MS and HPLC are often combined to characterize unknown endogenous or exogenous from a complex biological matrix. LC is probably the most versatile separation method, as it allows separation of compounds of a wide range of polarity with little effort in sample preparation (compared to GC-MS) (Moco et al., 2007). Indeed, large-scale metabolomic technologies based on LC-MS are increasingly gaining attention for their use in human disease diagnosis (Courant et al., 2009).

In addition, GC-MS and LC-MS have been integrated to provide the comprehensive metabolic signature of the malnutrition in rat model and to discover differential metabolites (Wu et al., 2010).

Recently, the combination of UPLC with MS has covered a large number of polar metabolites enlarging the number of detectable analytes.

Like NMR, vibrational spectroscopies such as RAMAN and FTIR are comparable in sensibility, but the latter allows high-throughput screening and biological samples classification, and equally fits the "omics philosophy" of providing unbiased, whole-system measurements (Kell, 2004). In particular, the use of FTIR spectroscopy to monitor biochemical changes in living cells has gained considerable importance in the last decade. In fact, this technique presents the possibility to simultaneously identify various cellular biochemical targets, both *in vivo* and *in vitro* conditions, exploiting the differential infrared radiation absorption of each metabolites at specific wave number (Salman et al., 2004). Molecules and systems of biological relevance that can be detected by FTIR spectroscopy including molecules such as lipids and fatty acids, proteins, peptides, carbohydrates, nucleic acids as well as biomembranes, animal tissues, microbial cells plants, and clinical samples (Dole et al., 2011). This technique has been employed to characterize isolated biological molecules, particularly proteins, and lipids. However, more recently it has been used, with the aid of sophisticated sampling techniques such as infrared imaging, in the diagnosis of many diseases such as cervical cancer, Parkinson's disease, Alzheimer's disease, kidney stone and arthritis (Dole et al., 2011). In fact, FTIR spectroscopy can effectively provide chemical variation information about the structure and the composition of biological material at molecular level. Li et al. (2012) reported that colitis and colon cancer can be successfully identified and discern by analyzing colon biopsies through FTIR spectroscopy and chemometrics. Also, Argov et al. (2004) used FTIR microspectroscopy to distinguish IBDs from colitis-associated colon carcinomas, when pathological symptoms are similar. In particular, the study of differences in specific regions of the infrared spectra allowed the identification of the discriminating molecules, e.g., the phosphate

content and RNA/DNA ratio, as different in IBD, cancer, or normal tissues. Furthermore, FTIR spectroscopy has also been used by Bright et al. (2011) to determine the efficacy of plasma homocysteine levels on vitamin supplementation in diet in hyperlipidemic patients. Human plasma samples from healthy and chronic lymphocytic leukemia patients have been analyzed by FTIR spectroscopy by Erukhimovitch et al. (2006), leading to identify of specific spectral peaks as associated with biomarkers of the disease. Cluster analysis of the selected spectra provided excellent classifications correlated completely with clinical data, showing, not with standing. Although these results are preliminary, but promising, rapid, effective, and economic technique which can assist in the disease diagnosis.

However, the technique selection depends on the kind of followed approach: (1) HPLC, GC-MS, LC-MS are employed in metabolite target analysis, e.g., determination and quantification of known metabolites or products of particular biochemical pathways; (2) HPLC-MS, CE-MS, LC-NMR, are used in metabolite profiling studies, e.g., larger, defined set of compounds survey and lipid analysis; (3) NMR, Direct Infusion electrospray Ionization-MS (DIMS), Laser Desorption Ionization-MS (LDI-MS), Matrix Suppressed LDI-MS (MSLDI-MS), FTIR, and Raman spectroscopy are used in metabolic fingerprinting, e.g., generation and comparison of sample metabolic profiles to identify differences (Shulaev, 2006) (**Figure 2**).

Furthermore, the chromatography-MS systems are considered the most favorable for the detection of large numbers of metabolites in metabolomics coupling the chromatographic metabolite separation with the sensitivity of MS detection. GC-Time of Flight (TOF)-MS, two dimensional GC coupled to TOF MS (GCxGC-TOF-MS), HPLC-MS, the analytically superior UPLC-MS and CE-MS have all been employed in mammalian metabolomic studies, either in a metabolic profiling or targeted analysis approach (Dunn et al., 2008). The GC-MS or GC-MS/SPME have been widely used in metabolomic and metabonomic studies, especially the second approach is useful because it does not require a difficult or strongly pre-analytical work flow. Garner et al. (2009) demonstrated the possibility to discriminate with GC-MS/SPME between the volatile organic compounds profile in fecal samples from preterm infants developing necrotizing enterocolitis (NEC) compared with non-NEC controls. Recent studies have suggested that the gut microbiota is involved in numerous important biochemical functions for the host, in healthy and pathological conditions (Del Chierico et al., 2012).

The extraction of valuable conclusions from the analysis of metabolomic data is important to perform the analytical measurements; in fact, there is a variety of methods that allow the instrument raw data transformation analyzed by the use of different software which provide a list of metabolites. Some parameters, such as biological variation present among individuals, sampling, sample preparation, and analytical measurement, influence the reproducibility of results, and these should be monitored as much as possible by measuring replicates, both analytical and biological. In principle, biological variance should surpass all analytical variance (Moco et al., 2007). Retention-time shifts are common in GC and more severely, LC, but only occasionally in NMR and FTIR spectra. In NMR spectra, non-reproducibility seems to be strictly related to sample preparation and hardly ever due to instrumental incoherence. Nevertheless, even in strictly controlled conditions, signal shifts may persist. For this reason, the use of signal-alignment software [e.g., MetAlign (De Vos et al., 2007), XCMS (Smith et al., 2006), and MZmine (Katajamaa et al., 2006)] has become a routine procedure for comparing chromatograms or spectra in MS applications, while HiRes is suitable in NMR (Zhao et al., 2006), transforming raw data into workable informative datasets.

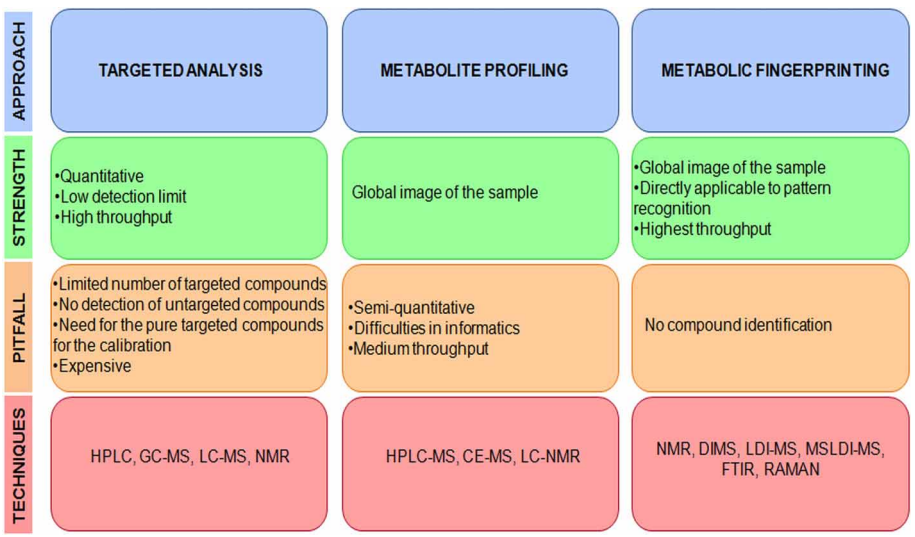

FIGURE 2 | Different approaches and respective techniques: pitfalls and strengths. The employed technique depends on the followed approach: targeted analysis, metabolite profiling, and metabolic fingerprinting.

HOW TO MANAGE AND INTEGRATE ALL DATASETS?

It is difficult to correlate specific markers with risk for disease, nutritional state or diet choices. Instead, by examining the system as a whole and exploring multiple pathways simultaneously, a greater indication of a healthy or pathological state can be acquired. Hence, this new approach, characterized by a high-throughput determination of hundreds of metabolites, leads to a torrent of data, in fact, over the years, there have been constant upgrade in the hardware and the software of these technologies to meet the demands for robustness, practicality, applicability, and efficiency of the analyses. Therefore, powerful analytical strategies in combination with advanced multivariate statistical tools are required to have a look at this new "black box" and to extract maximum relevant information and knowledge about the complex biological system (Bictash et al., 2010).

The use of single or combined analytical techniques or different kind of samples inevitably leads to face the big problem of how to manage and represent the thousands of data collected.

One of the expectations of the system biologist is that these datasets can be integrated to give a holistic picture of the state of the system, e.g., development, ageing, health, or disease, which provides insights enable a more biology fundamental understanding via unveiling network connections at molecular level (Richards et al., 2010).

Appropriate experimental design, sample numbers and statistical analyses are required to ensure generation of accurate and valid hypotheses and biological conclusions (Tseng and Wong, 2005). Powerful multivariate analyses, either supervised or unsupervised, are required to interrogate the data and define structure-related to biological system similarities or differences (Thalamuthu et al., 2006).

The first step for simplification of all data, but not in an arbitrary way, could be the use of Heat maps, where the relative intensity value of one peak is replaced by a small colored block (Zhou et al., 2011). Initially, it has been developed for microarray studies, but it is already used in some metabolomic works (Spitale et al., 2012). Rajaram and Oono (2010) proposed a new analysis, the so-called NeatMap as an alternative to the traditional Heat map, this offers a variety of novel plots (in 2 and 3 dimensions) to be used in conjunction with principal component analysis (PCA) and multi-dimensional scaling (MDS). Although, the NeatMap has been used so far only in genomics, it could be exploited in metabolomics as well. The second step could be the use of multivariate statistical analysis methods that can interpret different datasets. According to Richards et al. (2010) there are three possible integration strategies: (1) inter-omic, or the integration of data obtained from different -omic platforms (metabonomics, genomics, proteomics, and transcriptomics); (2) inter-platform, or the integration of data from different spectroscopic platforms (NMR, GC-MS, LC-MS etc.,); (3) inter-samples, or the integration of data obtained from different human samples (plasma, urine, tissue, and faeces) (**Figure 3**).

The most diffused multivariate methods are the PCA and the Partial Least Squares (PLS) (Holmes et al., 2011). In particular, PCA is widely used for multivariate of NMR or GC-MS analysis profiling data, it is based on modeling the natural variance within a dataset, which held to identify the underlying metabolite variables that contribute to that variance (Biais et al., 2009). PLS and

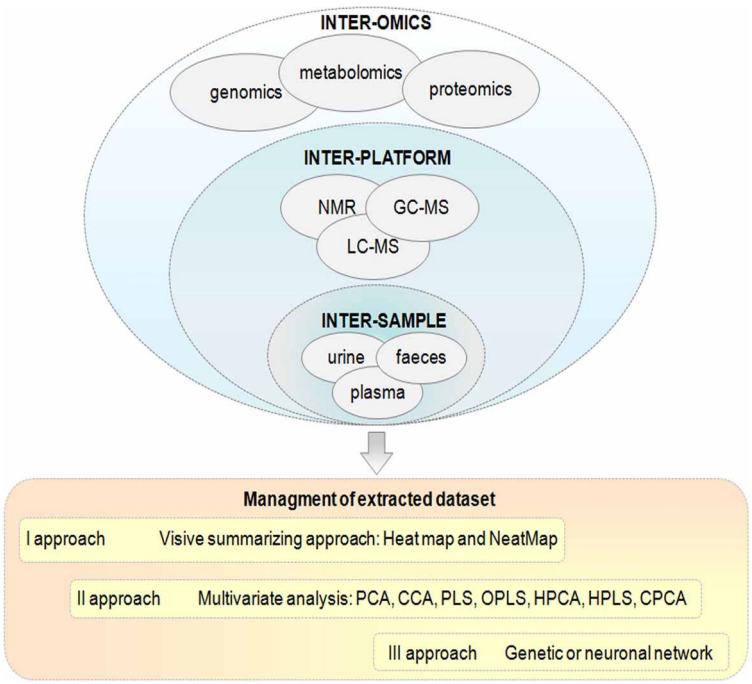

FIGURE 3 | Schematic representation of statistical data integration methods in the area of inter-omic, inter-platform, and inter-sample integration. The management of different dataset derived from the metabolomic approaches are integrated by the most diffuse multivariate methods.

its derivative orthogonal PLS (OPLS) have been also used for NMR profiling data. In comparison with PLS, OPLS produces models which are more clear and therefore easier to interpret, leading to a better class-resolution in a discriminant problem (Stella et al., 2006). However, when PCA and PLS are used for the interpretation of several different, but potentially connected, datasets (called "blocks"), the loading plots are usually complex due to the co-variation in the spectrum, and therefore difficult to correlate to the corresponding score plot (Janné et al., 2001). For this reason a multiblock technique must be used. The common trend of the different blocks is revealed in the "super scores" plot, where the distribution of the samples of each individual block are shown in their respective "block scores," and similarly to classical PCA, the contribution of variables to the trend shown in the blocks scores plot is shown in their "block loadings" plot (Biais et al., 2009).

The hierarchical multiblock segmentation techniques (HPCA, HPLS) are based on new variables created from the original data by blocking the spectra into sub-spectra, and then projecting the sub-spectra by PCA. These new variables are then used in the coming PCA or PLS calculations, reducing the random and non-wanted signals from e.g., light scatter, but still conserving all systematic information in the signals. This technique gives the greatest advantage of easier interpretation of the correlation between scores and loadings (Janné et al., 2001).

Another kind of multiblock PCA used is the Consensus PCA (CPCA), it can focus the data analysis on the relation between the specified metabolites and the remaining metabolites, searches for trends that explain as much as of the variation as possible (Biais et al., 2009). CPCA was introduced as a method for comparing several blocks of descriptor variables measured on the same objects and its difference whit respect to HPCA is in the data normalization (Westerhuis et al., 1998).

Also, the multivariate analysis, such as the Canonical Correlation Analysis (CCA) and the regularized CCA are widely used to integrate datasets obtained from different source materials (Yamamoto et al., 2008). However, when the "normal" variance is greater than that explained by the process of interest, the most sophisticated genetic (Johnson et al., 2001) or neural network (Ahmed et al., 2009) based algorithms, may identify the underlying variables of significance which are not associated with the greatest variance (the "normal" condition). In these cases, such algorithm-based models should not be overfit (Biais et al., 2009).

As the holistic approach to biological systems comprehension continually evolves, the techniques used to analyze and integrate the datasets must be steadily updated to manage the problems occurring during techniques application. Hence, system biologists have a key role in the inter-pretation of the meaning of the results obtained by the datasets management.

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