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INTESTINAL *LACTOBACILLUS REUTERI*: PARTNERS AND BENEFICIAL MICROBES

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INTRODUCTION

According to the Food and Agricultural Organization of the United Nations and the World Health Organization, probiotics are defined as "living microorganisms, which when administered in adequate amounts confer a health benefit on its host" (*FAO/WHO*, 2001). Elie Metchnikoff, who was best known as a laureate for Nobel Prize in Medicine in 1908 for his ground-breaking research in phagocytosis, was one of the first prominent scientists to introduce the concept of probiotics to the general public. He published a seminal report on association between longevity of Bulgarians and their consumption of fermented milk products (*Metchnikoff* and *Mitchell*, 1907). This observation suggested that ingestion of certain microbes could be beneficial for human health. Since then, probiotics had been widely marketed and consumed, mostly as dietary supplements or functional foods without proper validation of their promised beneficial effects. Significant advancements in probiotic research have occurred during the past two decades. Novel beneficial organisms have been identified and characterized. Mechanisms of probiosis include manipulation of intestinal microbial communities, suppression of pathogen, immunomodulation, stimulation of epithelial cell proliferation and differentiation and fortification of the intestinal barrier (Figure 1) (*Thomas* and *Versalovic*, 2010).

The Gram-positive bacterium *Lactobacillus reuteri* is a heterofermentative symbiont indigenous to the gastrointestinal tract of humans and many other vertebrates such as pigs, mice, and rats (*Walter* et al., 2010). A recent evolutionary genomic study revealed a molecular basis of host specificity among *L. reuteri* species, which may be due to physiological and immunological differences between different vertebrates (*Frese* et al., 2011). This species is generally regarded as safe and has never been shown to cause disease in humans (*Britton* and *Versalovic*, 2008). Results from basic science research and clinical trials have demonstrated potential beneficial effects of *L. reuteri* on human health, both in preventive and therapeutic aspects. This review will focus on how *L. reuteri* could affect the physiological processes of the host through intestinal immunomodulation, development and maintenance of the intestinal epithelium, and prevention or treatment of intestinal injury.

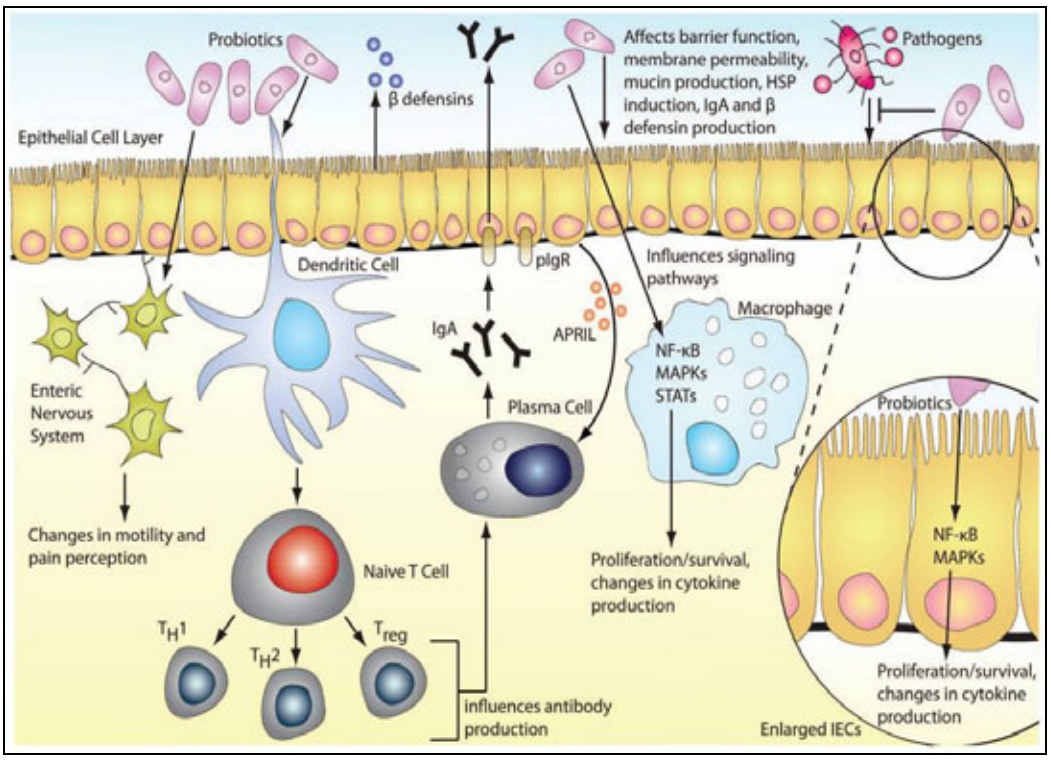

Figure 1: Mechanisms of probiosis in the human gastrointestinal tract. Probiotics affect intestinal functions in several ways. They may manipulate intestinal microbial communities and suppress growth of pathogens by inducing β-defensin and IgA production. Probiotics also enhance the integrity of intestinal barrier by maintaining tight junctions and inducing mucin production. Probiotics can modulate the immune system by mediating cytokine secretion through signaling pathways such as NFκB and MAPKs, which can also affect proliferation and differentiation of immune cells (such as T-cells) or epithelial cells. Moreover, changes in gut motility and pain perception can be altered through modulation of pain receptor expression and secretion of potential neurotransmitter molecules. APRIL, a proliferation-inducing ligand; hsp, heat shock protein; IEC, intestinal epithelial cell; Ig, immunoglobulin; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor-kappaB; pIgR, polymeric immunoglobulin receptor; STAT, signal transducers and activator of transcription; Treg, T regulatory cell. Figure reproduced from *Thomas* and *Versalovic*, 2010.

LACTOBACILLUS REUTERI AND INTESTINAL IMMUNOMODULATION

The human gastrointestinal tract contains approximately 10^{14} commensal bacteria (*Ley* et al., 2006). The intestinal immune system must be able to protect the host from pathogenic microbes, while still maintaining immunological hyporesponsiveness to members of the intestinal microbiome. Disruption of gut homeostasis may result in diseases associated with intestinal inflammation, such as inflammatory bowel disease (IBD), infections and colorectal cancer (*Artis*, 2008; *Karin* et al., 2006).

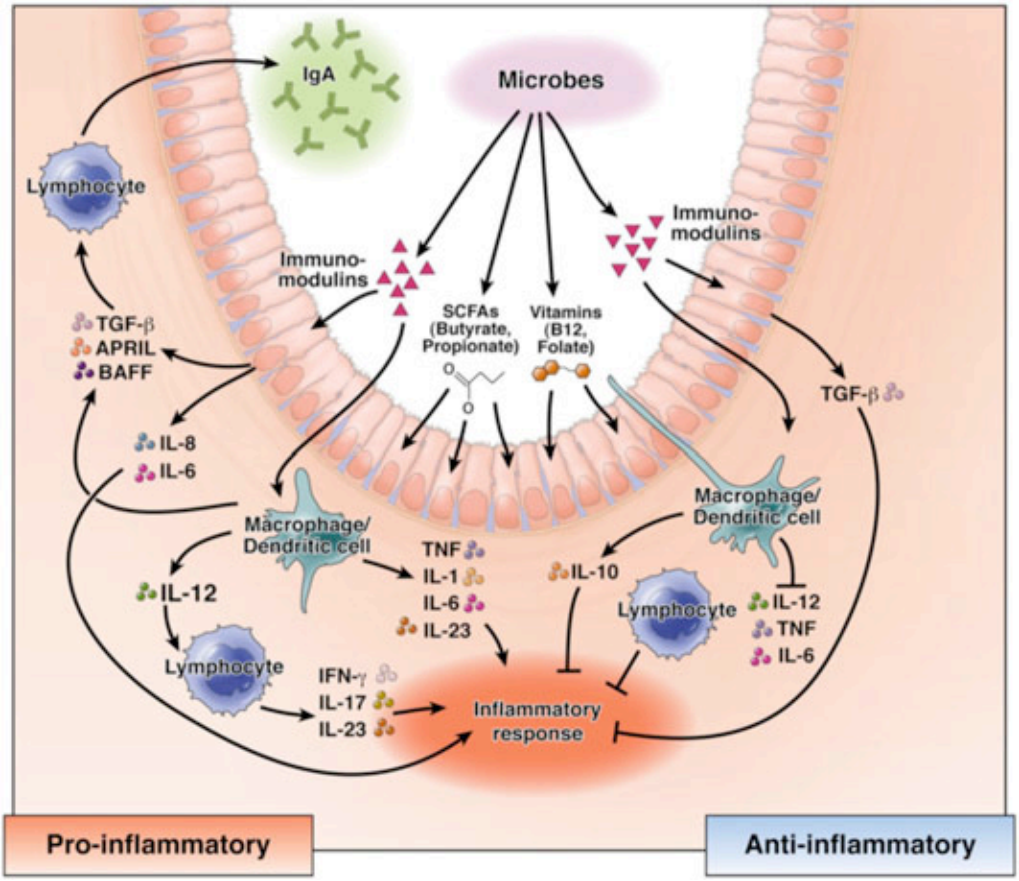

Figure 2: Immunomodulation by beneficial microbes. Probiotics can modulate intestinal immune system by production of secreted soluble factors and metabolites, such as short-chain fatty acids (SCFAs) and vitamins. These factors affect the function of the intestinal epithelium and mucosal immune cells, resulting in production of cytokine and related factors such as a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF). Figure adapted from *Preidis* et al., 2009.

Beneficial microbes in the gastro-intestinal tract have been shown to modulate the intestinal immune system by production of secreted factors and metabolites that affect the growth and function of intestinal epithelial cells and immune cells (Figure 2) (*Preidis* and *Versalovic*, 2009).

L. reuteri regulates the intestinal immune system in several aspects and can be considered an "immunoprobiotic" (*Lin* et al., 2008). Recent *in vitro* and *in vivo* studies have demonstrated its role in host immunomodulation, along with the molecular mechanisms behind it. Interestingly, these activities seem to be highly strain-dependent (*Liu* et al., 2010; *Pena* et al., 2004), and can affect both innate and adaptive immune responses.

Several studies have demonstrated the ability of *L. reuteri* to regulate the activity of immune cells and the production of cytokines from these cells. Heat-killed *L. reuteri* 100-23 induced the production of anti-inflammatory cytokine IL-10 by bone marrow-derived dendritic cells (BMDCs)

(*Livingston* et al., 2009). When these *L. reuteri*-treated cells were incubated with splenic T-cells from ovalbumin T-cell receptor transgenic mice, IL-2 production was reduced and transforming growth factor-β (TGF-β) production increased. Moreover, spleens and mesenteric lymph nodes from *Lactobacillus*-free mice colonized with *L. reuteri* contained more FoxP3-positive cells than that of control mice. These results suggested that besides eliciting intestinal immune responses, *L. reuteri* also regulated the development and recruitment of regulatory T-cells to the gastrointestinal epithelium. *L. reuteri* may regulate intestinal inflammation by controlling recruitment of immune cells in a gnotobiotic neonatal pig model of rotavirus infection. In animals pre-colonized with human-derived *L. reuteri* ATCC 23272 and *L. acidophilus* NCFM, recruitment of monocytes and macrophages to the intestines and spleens was inhibited. This result suggested that colonization by these lactobacilli may reduce rotaviral infection-induced monocyte/macrophage recruitment to the intestine and systemic lymphoid tissue (*Zhang* et al., 2008).

Studies have also suggested that soluble factors from *L. reuteri* could inhibit production of proinflammatory cytokines and inflammatory signal processing in immune cells (*Thomas* and *Versalovic*, 2010). Cell-free culture supernatants from murine-derived *L. reuteri* 6798 were able to inhibit tumour necrosis factor (TNF) production by lipopolysaccharide (LPS)-activated (*Pena* et al., 2004) and *Helicobacter hepaticus*-treated (*Pena* et al., 2005) mouse macrophages. Moreover, culture supernatants from human-derived *L. reuteri* ATCC PTA 6475 demonstrated strain-specific suppression of human TNF production by activated monocytoid cells (THP-1) and primary monocyte-derived macrophages from patients with Crohn's disease. Transcriptional regulation of TNF expression by *L. reuteri* occurred by inhibition of c-Jun-dependent activator protein 1 (AP-1) pathway (*Lin* et al., 2008). Interestingly, *L. reuteri* formed biofilms, and biofilm cultures of *L. reuteri* PTA 6475 also suppressed TNF production by activated THP-1 cells (*Jones* and *Versalovic*, 2009). A recent comparative transcriptomic study identified a number of genes that might play a role in the production of such soluble factors (*Saulnier* et al., 2011a). Further characterization of these genes and their potential roles in immunomodulation is currently on-going.

The *in vivo* effects of *L. reuteri* in the human intestinal immune system was demonstrated in a small clinical investigation, which used *L. reuteri* ATCC 55730 to colonize the gastrointestinal tracts of healthy volunteers and patients with ileostomy (*Valeur* et al., 2004). After supplemented with *L. reuteri*, the numbers of duodenal B-lymphocytes and CD4-positive T-lymphocytes were significantly increased in the ileal epithelium. These observations suggested that *L. reuteri* may be able to regulate both humoral and cell-mediated aspects of the adaptive immune response in humans.

LACTOBACILLUS REUTERI AND THE INTESTINAL EPITHELIUM

Experiments using germfree animals and knockout mice lacking the Toll-like receptor (TLR) signal transduction pathway component MyD88 suggested that gut microbiota could influence the development and differentiation of the intestinal epithelium. Several studies have demonstrated that the intestines of

germfree mice were both morphologically and functionally underdeveloped in multiple aspects (*Smith* et al., 2007). Intestinal microbes also play an important role in maintaining the epithelial lining and integrity of the intestinal barrier. Germfree mice and MyD88-knockout mice had significantly decreased colonic epithelial cell proliferation after colonic mucosal injury compared to that of conventionally housed mice (*Pull* et al., 2005). Colonization of germfree mice with intestinal microbiota resulted in upregulation of genes involved in intestinal barrier fortification (*Hooper* et al., 2001). TLR2 signalling initiated by exposure of intestinal epithelial cells to peptidoglycan from bacteria also enhances the integrity of tight junctions, which are responsible for the maintenance of intestinal barrier integrity (*Chung* and *Kasper*, 2010).

Several *Lactobacillus* strains increased the integrity of the intestinal barrier, which could result in protection from loss of immune tolerance, gastrointestinal infections, irritable bowel syndrome and inflammatory bowel disease (*Lee* and *Bak*, 2011). *L. rhamnosus* GG was able to preserve tight junction architecture and expression of tight junction protein zona occludens-1 (ZO-1) in the presence of pro-inflammatory cytokines such as interferon-gamma (IFN-γ) (*Donato* et al., 2010). Treatment with *L. plantarum* CGMCC 1258 also resulted in amelioration of the loss of colonic paracellular integrity and restoration of expression and distribution of tight junction proteins (*Chen* et al., 2010). A recent study using a dextran sodium sulphate (DSS) colitis mouse model (*Zakostelska* et al., 2011) demonstrated that pre-treatment of animals with *L. casei* DN-114 001 resulted in protection against perturbation of intestinal permeability and barrier integrity, mainly by preserving ZO-1 expression in the mucosa of the terminal ileum and colon.

A recent study from our laboratory demonstrated that *L. reuteri* stimulated intestinal epithelial cell proliferation and differentiation in an outbred neonatal mouse model (*Preidis* et al., 2012a). Further studies are needed to reveal how *L. reuteri* affects the regulation of gene expression in the intestinal epithelium.

LACTOBACILLUS REUTERI AND PREVENTION/TREATMENT OF INTESTINAL INJURY

Several *Lactobacillus* species can facilitate prevention or treatment of intestinal injury caused by infection, excessive inflammation or radiation-induced reactive oxygen radicals. Acute radiation-induced intestinal injury commonly occurs in patients undergoing radiation therapy for malignancies, resulting in malabsorption, bloating, diarrhoea and dehydration (*Ciorba* and *Stenson*, 2009). Several preliminary studies have demonstrated preventative and therapeutic effects of probiotics on such injury in animal models. *L. delbrueckii* subspecies *bulgaricus* B3 was able to increase the villus/crypt ratio and the number of villi per square millimetre in the jejunum of gamma-irradiated mice, along with reduced inflammation and vascularity in all intestinal segments (*Demirer* et al., 2006). A recent study using a similar mouse model pre-treated with *L. rhamnosus* GG showed that probiotic treatment resulted in reduction of weight loss, intestinal cell apoptosis and crypt loss after gamma irradiation. Increased crypt survival was dependent on TLR-2, MyD88 and COX-2 signalling (*Ciorba* et al., 2011).

Crohn's disease (CD) and ulcerative colitis (UC) are two forms of inflammatory bowel disease (IBD) (*Rakoff-Nahoum* and *Bousvaros*, 2010). These debilitating diseases affect the quality of life of the patients worldwide, with highest prevalence rates in Israeli Jewish, North American and European populations (*Menon* et al., 2011). As mentioned in previous sections of the review, probiotics have been suggested to possess anti-inflammatory properties, strengthen the intestinal barrier and alter microbial-mucosal interactions. From these observations, it was hypothesized that probiotics may provide protection against intestinal inflammation (*Guandalini*, 2010). Experimental mouse acute colitis models have been used to study mechanisms of inflammation in the intestine and evaluate the efficacy of novel treatment modalities. Mice can lack the functions encoded by certain genes (such as IL-10, TGF-β, FoxP3) resulting in spontaneous colitis, or intestinal inflammation may be induced via chemicals such as dextran sodium sulphate (DSS), microbial infections (*H. hepaticus*) or hapten-producing compounds such as trinitrobenzene sulphonate (TNBS) or 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) (*Ishiguro* et al., 2010; *Saleh* and *Elson*, 2011). Several probiotic strains have demonstrated beneficial effects in ameliorating intestinal inflammation in these animal models. In a DSS-induced mouse colitis model, treatment with *L. reuteri* BR11 was able to decrease disease activity index (DAI), distal colonic crypt hyperplasia and colitic symptoms compared to that of vehicle-treated mice (*Geier* et al., 2007). Moreover, pre-treatment of rats with a bacterial cocktail containing four strains of rat- and human-derived *L. reuteri* (R2LC, JCM 5869, ATCC PTA 4659 and ATCC 55730) resulted in reduction of mucosal damage and reduction of DAI. Downregulation of the adhesion molecule P-selectin was observed throughout the colon, resulting in decreased leukocyte-endothelial interactions in colonic venules of probiotic-treated animals (*Schreiber* et al., 2009). Interestingly, many other strains of lactobacilli, such as *L. casei* Shirota, *L. paracasei*, *L. plantarum* HY115 and *L. brevis* HY8401, yielded protective effects in similar DSS-induced colitis models as well (*Claes* et al., 2011). In a TNBS-induced rat colitis model, which is helpful in identifying the role of T-lymphocytes in colitis, animals were pre-treated with either *L. fermentum* CECT5716 or *L. reuteri* ATCC 55730 before induction of colitis. Both probiotics were able to demonstrate intestinal anti-inflammatory effects and reduced colonic TNF quantities (*Peran* et al., 2007). Similarly to the DSS-induced colitis model, several different *Lactobacillus* strains such as *L. fermentum* CECT5716, *L. acidophilus* IPL908 and *L. casei* BL23 have shown beneficial effects in alleviating the severity of disease in TNBS-treated animals (*Claes* et al., 2011; *Mane* et al., 2009). Moreover, recent unpublished data from our laboratory using fluorodeoxyglucose (^{18}F)-positron emission tomography (FDG-PET) suggested beneficial effects of *L. reuteri* ATCC PTA 6475 in a TNBS-induced mouse colitis model (Figure 3).

In terms of clinical evidence supporting the therapeutic use of probiotics in IBD, few randomized, placebo-controlled studies have been performed for treatment of CD, yielding largely no significant improvement in disease outcome (*Guandalini*, 2010). However, probiotics seem to perform better in treatment of UC. A recent study in paediatric patients suffering from active distal UC using *L. reuteri* ATCC 55730 administered as an enema showed a

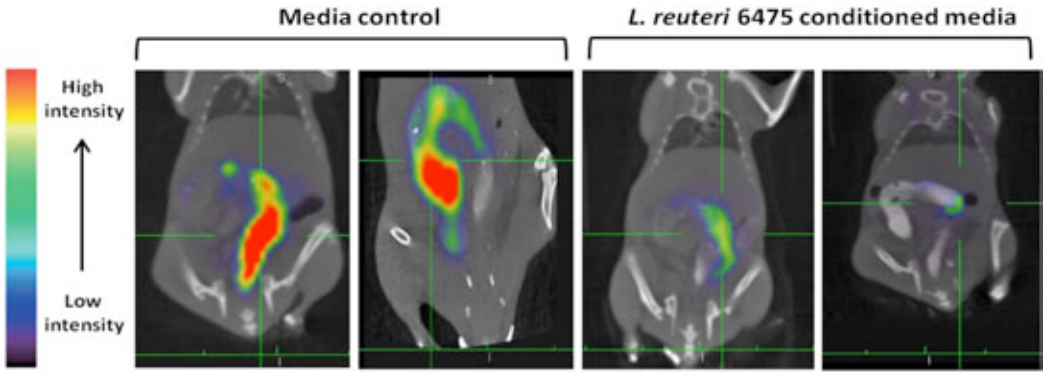

Figure 3: *In vivo* imaging suggested reduction of colitis in Balb/c mice treated with *L. reuteri* ATCC PTA 6475. Mice were treated with media control or conditioned media from *L. reuteri* 6475. Colitis was induced by trinitrobenzene sulfonate (TNBS). Fluorodeoxyglucose (^{18}F)-positron emission tomography (FDG-PET) revealed diminished signal intensity in colons of probiotic-treated mice compared to that of mice treated with a medium only control, suggesting that *L. reuteri* 6475 may have a protective effect against colitis in this animal model.

reduction in disease severity scores (clinically, endoscopically, and histologically observed) and reduced quantities of inflammatory cytokines in rectal tissue (*Oliva* et al., 2011). Since our previously mentioned study has identified *L. reuteri* ATCC PTA 6475 as an anti-inflammatory strain (*Lin* et al., 2008), it would be interesting to see how this strain would perform in a clinical study of similar design.

Bacterial and viral infections can also result in intestinal injury, which includes direct damage to the mucosa and epithelial lining, disruption of the intestinal barrier, and aberration of intestinal immune responses. Different probiotic strains of *Lactobacillus* yielded protective effects against severe intestinal injury in several animal models of gastrointestinal infections. In a study using a rat model of *Shigella dysenteriae* 1 infection, pre-treatment of animals with *L. rhamnosus* or *L. acidophilus* prior to infection resulted in amelioration of the loss of membrane-bound adenosine triphosphatase (ATPase) and tight junction proteins compared to that of vehicle-treated rats (*Moorthy* et al., 2009). The benefit of *Lactobacillus* in gastrointestinal infections was seen in an *Enterobacter sakazakii*-induced necrotizing enterocolitis (NEC) rat pup model. Animals pretreated with *L. bulgaricus* prior to induction of NEC demonstrated reduced nitric oxide production in the intestinal mucosa, a reduction of bacteraemia and improvement in survival compared to that of pups receiving no probiotics (*Hunter* et al., 2009). Beside the preventative effects, the potential therapeutic role of probiotics in recovery from infections was shown in a recent study using a rabbit model of *Staphylococcus aureus* enterocolitis. Young rabbits receiving fermented milk containing live *L. paracasei* after infection had decreased duration of diarrhoea, along with more rapid recovery of intestinal villi and colonic crypts (*Bendali* et al., 2011).

Rotavirus is the main aetiologic agent in acute gastroenteritis in children below one year of age worldwide, and causes more than 600,000 deaths worldwide per year (*Grandy* et al., 2010). A recent study from our laboratory demonstrated the effectiveness of *L. reuteri* DSM 17938 in the treatment

of rotaviral infection in a neonatal mouse gastroenteritis model (*Preidis* et al., 2012b). Results from animal models suggest that treatment with probiotics may serve as a low-cost and effective measure in prevention and treatment of gastrointestinal infections (*Preidis* et al., 2010).

INTESTINAL MICROBIOME AND HUMAN HEALTH: ROLE OF PROBIOTICS IN PREVENTION AND TREATMENT OF DISEASES

The human gastrointestinal tract is sterile *in utero*, but colonization of microbes begins at birth. Shortly after that, it becomes home to more than 10^{14} microbial cells, consisting of more than 1,000 different species which reside mostly in the colon (*Wallace* et al., 2011). The microbial population in each individual becomes relatively stable in terms of richness and diversity in early childhood after the weaning process (*Spor* et al., 2011). The majority of bacteria in the colons of adults are anaerobes, such as *Bacteroides spp.*, *Bifidobacterium spp*., *Clostridium* spp., *Eubacterium spp*. and *Lactobacillus spp.* (*Wallace* et al., 2011). As previously mentioned, the intestinal microbiota plays an important role in the function and integrity of the gastrointestinal tract, maintenance of homeostasis in the immune system, along with the energy metabolism of the host (*Pflughoeft* and *Versalovic*, 2011). Alterations in overall composition of microbial populations, also known as dysbiosis, can result in disruptions of the mutualistic relationships between microbe versus microbe or microbe versus host. These changes may affect the health of the host and result in potentiation of disease (*Frank* et al., 2011). Several reports have demonstrated associations between intestinal dysbiosis and energy harvest or metabolic disorders (*Jumpertz* et al., 2011), which can result in diseases such as metabolic syndrome, obesity and diabetes (*Claus* et al., 2008; *Larsen* et al., 2010; *Pflughoeft* and *Versalovic*, 2011). Alterations in the composition of intestinal microbiome have also been associated with gastrointestinal infections, inflammatory bowel disease, and irritable bowel syndrome (*Pflughoeft* and *Versalovic*, 2011; *Saulnier* et al., 2011b). Several current treatment modalities to manipulate and restore the balance in the richness and diversity of intestinal microbiome are currently being explored (*Sonnenburg* and *Fischbach*, 2011). One of the most studied approaches is the use of probiotics to introduce organisms with beneficial functions into gastrointestinal microbial communities, which may result in protection from or alleviation of diseases. Moreover, probiotics may be able to affect microbial communities by competition for nutritional substances or binding sites, production of growth substrates or inhibitors and modulation of intestinal immune response (*O'Toole* and *Cooney*, 2008). This concept is supported by results from randomized controlled clinical trials that studied beneficial effects of probiotics during the treatment of gastrointestinal diseases [extensively reviewed by *Preidis* and *Versalovic* (2009) and *Thomas* and *Greer* (2010)].

Scientists lack direct evidence regarding the impact of probiotics on the human intestinal microbiome in different human populations. With recent technological innovations in DNA sequencing and advancements in bioinformatics, we have entered the era of metagenomics. The Human Microbiome Project (*Peterson* et al., 2009) has

shaped the way scientists approach research questions related to the human microbiome and how each treatment modality can affect changes in the global composition of microbial communities. Probiotics induce changes in the intestinal microbiota and restore homeostasis in the gastrointestinal tract. However, further studies in humans are needed to explore whether probiotics can make the same impact on the human intestinal microbiome and whether such changes are associated with clinical benefits in the host.

CONCLUSION

A body of evidence has demonstrated beneficial effects of probiotic lactobacilli, including *L. reuteri*, on human health and disease. However, further studies must be done to fully understand the mechanisms of probiosis. Well-designed experiments should be performed in appropriate experimental models (*in vitro* or *in vivo*) and in the human host. Metagenomic, metatranscriptomic and metabolomic approaches should be used to globally examine interactions between probiotics and intestinal microbes and between probiotics and the mammalian host. Once exact mechanisms of probiosis have been identified in detail, efficient and safe probiotics may be engineered or selected as natural strains, resulting in novel preventive and therapeutic interventions for human intestinal disorders.

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