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Efficacy of Galactooliosaccharide (GOS) and/or Rhamnose-Based Synbiotics in Enhancing Ecological Performance of *Lactobacillus reuteri* in the Human Gut and Characterization of Its GOS Metabolic System

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EFFICACY OF GALACTOOLIOSACCHARIDE (GOS) AND/OR RHAMNOSE-BASED SYNBIOTICS IN ENHANCING ECOLOGICAL PERFORMANCE OF LACTOBACILLUS REUTERI IN THE HUMAN GUT AND CHARACTERIZATION OF ITS GOS METABOLIC SYSTEM

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Probiotic *L. reuteri* has potential to produce antimicrobial compounds and secrete immunosuppressive factors. These metabolic attributes could benefit the human host by providing colonization resistance (competitive and metabolic exclusion) against enteropathogens and mitigating inflammation. As metabolically active cells are fundamental to such probiotic properties, synbiotic approaches that supply *L. reuteri* with a source(s) of carbon, energy, and/or external electron acceptor for cell growth in the gut environment could therefore prompt the probiotic to engage in beneficial activities. In this study, the efficacy of GOS and/or rhamnose-based synbiotic approaches in promoting colonization persistence and metabolic activity of *L. reuteri* was evaluated. A single blind, randomized, crossover, placebo-controlled human trial revealed that daily administration of the *L. reuteri* DSM 17938 (5×10^8 cells)/GOS (1g)/rhamnose (1g) synbiotic combination significantly stimulated metabolic activity of the

probiotic strain in the human gut. This positive outcome presumably results from the ability of *L. reuteri* to metabolize GOS as a carbon and energy source, while utilizing rhamnose as an external electron acceptor for redox balance. In contrast, neither GOS (2g) nor rhamnose (2g) alone could exert such stimulatory effect. In addition, after the synbiotic administration was terminated, the extended supplementation of the carbohydrates without *L. reuteri* did not appear to improve the persistence of the probiotic in the gut.

Genetic characterization of GOS metabolic machinery disclosed that GOS metabolism in *L. reuteri* is inducible and is under carbon catabolite repression (CCR). The metabolic system relies on LacS permease and a second transporter to import diverse GOS species into the cytosol. Then, two β-galactosidases, LacA and LacLM, sequentially break down GOS oligosaccharides as well as concertedly hydrolyze GOS disaccharides. The system is regulated by repressor protein LacR and fully activated only in the presence of inducer lactose and in the absence of glucose (i.e., a preferred carbon source). Furthermore, a growth advantage only the wild type strain, but not the GOS metabolic gene-deficient mutant, gained in the GOS-enriched murine gut suggests that the GOS metabolic system be operational in the gut environment.

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Preface

This dissertation consists of four chapters. In Chapter 1, fundamental knowledge on the effects of the gut microbiota on host health is reviewed. In particular, the potential of *L. reuteri* and probiotic bacteria to enhance human health is described. This review primarily focuses on mechanisms and molecules that have been found to contribute to activities of the gut microbiota and probiotic effects.

Chapters 2 and 3 describe findings from two independent research projects. The goal of the first project (Chapter 2) was to evaluate the efficacy of three synbiotic preparations in a human trial. All three synbiotics included *Lactobacillus reuteri* and either prebiotic beta-galactooligosaccharide (GOS), rhamnose, or a combination of both substances. We then measured the ability of these preparations to prolong *L. reuteri* colonization and metabolic activity in the human gut. The results (published in the Journal of Functional Foods, 2014) showed that although the GOS-rhamnose combination had no affect on *L. reuteri* persistence, this treatment did stimulate metabolic activity of the probiotic in the gut.

In Chapter 3, the molecular machinery responsible for GOS metabolism in *L. reuteri* was characterized. By employing comparative genomic analysis, mutant generation, and a series of subsequent phenotypic analyses, we identified transporters, β-galactosidases, and regulatory elements of the GOS metabolic system. A model of GOS metabolism and its regulation was proposed.

Finally, the major findings of this research are summarized in Chapter 4. In addition, directions for future research are proposed. Ultimately, the knowledge gained from these studies provide a basis for formulating effective synbiotic approaches for enhancing gut health.

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Chapter 1

Contributions of Intestinal and Probiotic Microbes to Human Health and Their Galactooligosaccharide, Rhamnose, and 1,2-Propanediol Metabolism: The Literature Review

Part I: Contributions of intestinal and probiotic microbes to human health

Millions of years of shared evolutionary fate have woven a symbiotic relationship between humans and the nearly 100 trillion intestinal bacteria each individual harbors (1, 2). The generally anoxic gut shelters anaerobic microbes from a harmful oxygenated environment, and streams of indigestible food particles and exfoliated epithelial glycans become reliable nutrient sources that sustain growth and persistence and shape the colonic community (3-5). In return, gut microbes excrete metabolites that fuel enterocytes and other human cells, integrate metabolic traits into the host genome to enhances metabolic potential, assist in immune development, and protect the host from enteropathogen invasion and infection.

The relationship between the host and the microbial community that inhabits the intestinal tract is complicated - promoting health or disease (6). Thus, like other ecosystems, when the microbial community is continually perturbed by infection, antibiotic and drug treatments, dietary changes, or inflammation (7, 8), the community structure and function may deviate from healthy and balanced to degrading and imbalanced states. The latter, termed as dysbiosis, has been implicated in the development of a range of disorders.

The evidence for unhealthy outcomes of microbial dysbiosis has led researchers to consider preventive and therapeutic approaches to maintain and regain the healthy status of the gut microbiota (9, 10). Indeed, the notion that human physiology, metabolism, and health were driven in part by indigenous microorganisms was made more than 100 years ago. Elie Metchnikoff envisioned

detrimental outcomes of accumulating toxic flora in the gut and conceived the idea of transforming it into a colony of host-friendly microbes (11). Metchnikoff's approach was based on supplementing the gut with live microorganisms, thereby displacing the toxic flora, and ultimately promoting health and longevity. This concept eventually led to the isolation and development of probiotic bacteria. Defined as "live microorganisms that can provide benefits to human health when administered in adequate amounts, which confer a beneficial health effect on the host" (12), probiotics are now used, as Metchnikoff anticipated, as microbiota-based therapies (9, 10).

Based on human and animal studies, probiotics have shown potential for preventing or treating several disorders or diseases, including antibiotic-associated diseases (13, 14), necrotizing enterocolitis (15), inflammatory bowel disease (16), and metabolic disorders (17, 18). However, the clinical studies have often generated equivocal results, and variations in the response to a probiotic strain is considerably large among individuals (19). This suggests the necessity for a mechanistic understanding of specific probiotic actions in order to enable rational design of probiotic treatments for particular or personalized applications.

Several key traits underlying specific microbial functions have been suggested as criteria for rational selection of probiotics. Below, mechanisms by which the gut microbiota influences 1) host energy balance and metabolism, 2) immune development and homeostasis, and 3) colonization resistance against

enteric pathogens are discussed in parallel with potential roles of probiotics in modulating these functions.

Role of the gut microbiota in energy metabolism and fat storage

The influence of intestinal microbes in energy metabolism was first observed in gnotobiotic animals. Germ-free rats excreted more calories in the feces and needed to consume about 20% more calories to maintain their body weight compared to conventional counterparts (20). It is now well established that intestinal microbes are involved in carbohydrate and lipid metabolism and thereby play a crucial role in host energy balance (21). Intestinal bacteria also increase energy harvesting from undigested dietary carbohydrates and host-derived glycans. They first degrade such complex carbohydrates and then ferment liberated monosaccharides to short-chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate. The SAFAs are absorbed by mammalian cells and used as energy sources (22). In humans on a typical European diet, the gut microbes generally ferment 50 - 60 g of carbohydrates and produce 0.5 - 0.6 mole of SCFAs with a total energy value of 140 - 180 kcal per day (~10% of the maintenance caloric requirement) (23).

In addition to their role as nutrients for colonic cells, SCFAs also appear to have other physiological effects. In particular, they may decrease the risk of developing obesity. Butyrate, propionate, and acetate were showed to protect against diet-induced obesity, while butyrate and propionate were showed to reduce food intake (24). Butyrate also shows capacity to reduce adiposity and

increase insulin sensitivity in obese mice (25), while propionate can also inhibit hepatic lipogenesis by suppressing fatty acid synthase (FAS) expression (26). Furthermore, a recent study showed that SCFAs stimulate fatty acid oxidation in adipose tissue and liver by suppressing peroxisome proliferator-activated receptor-γ (PPARγ) activity (27). These effects are potentially attributed in part to the action of SCFAs as signaling molecules that activate G-protein coupled receptor GPR41 and GPR43 (28) present on enteroendocrine cells (L cells). This triggers L cells to secrete gut hormones, i.e., glucagon-like peptides (GLP1 and GLP2) and peptide YY (PYY), that are known to control energy homeostasis, fat storage, satiety, gut barrier function, and metabolic inflammation (26, 29-31).

Ultimately, alterations in the gut microbiota that reduce SCFA production may contribute to positive energy balance, increased fat storage, and increased gut permeability. The compromised gut barrier could further lead to metabolic endotoxemia that triggers low-grade chronic inflammation and, in turn, metabolic dysregulations in intestinal and peripheral metabolic tissues (e.g. adipose tissue, muscles, liver, pancreas, and brain) (32). As such, the lack of SCFA products may increase risks of developing obesity and the related metabolic disorders such as insulin resistance and nonalcoholic fatty liver disease (21, 33).

In addition to SCFA production, certain members of the gut microbiota, including *Bifidobacterium*, *Lactobacillus,* and *Roseburia* species, are also capable of transforming dietary polyunsaturated fatty acids (PUFA) into bioactive metabolites. In particular, conjugated linoleic acids (CLA) may be produced that have the ability to modulate host energy metabolism and fat storage (34-36). In

mouse adipose tissue, CLA has been found to suppress glucose metabolism, de novo fatty acid synthesis, fatty acid and triglyceride uptake, and pre-adipocyte differentiation, while promoteing fatty acid oxidation and energy expenditure (37). These mechanisms also appear to underlie modulation effects of SCFAs on energy metabolism and fat storage.

Intestinal microbes may also influence energy balance and fat storage through bile acid biotransformation activities. Members of the gut microbiota possess bile salt hydrolases (BSHs) capable of deconjugating bile salts (glycine- or taurine-conjugated bile acids) into free primary bile acids in the intestinal lumen (38, 39). Compared with bile salts, free bile acids (BAs) are less soluble and less efficiently reabsorbed in the ileum to be recirculated to the liver via the enterohepatic cycle and therefore can travel to the colon and eventually be excreted with feces (40, 41). Upon reaching the colon, primary BAs can be further transformed to secondary BAs by 7α-dehydroxylation and 7α/β-epimerization reaction carried out by some colonic bacteria (39). BAs are not only responsible for digestion and absorption of dietary lipids and lipid-soluble vitamins, but also function as signaling molecules that regulate other physiological functions, including their own biosynthesis and detoxification, lipid and glucose metabolism, energy homeostasis, inflammatory responses, and epithelial defense mechanisms (42, 43).

Potential of probiotics to modulate energy metabolism and fat storage

Numerous animal studies have been conducted to assess the ability of potential probiotic candidates to improve obesity and associated diseases. These studies have shown that certain probiotic bacteria can modulate host energy metabolism and fat storage. Specifically, strains of *Lactobacillus* and *Bifidobacterium* reduced body weight, fat deposition, fasting blood glucose, hepatic triglyceride content, and/or serum cholesterol, as well as hepatic and/or adipose tissue inflammation (44-48). Other studies have also reported associations between such effects and up-regulation of fatty acid oxidation and/or anti-inflammatory genes (49-51) as well as suppression of genes or elements involved in fatty acid synthesis (52), glucose uptake (51), or pro-inflammatory responses (53, 54). In *L. reuteri* MM4-1A (ATCC PTA 6475), the anti-obesity effect is attributed to anti-inflammatory properties of the strain that induces IL-10 production and regulatory T cell (T_{reg}) proliferation (55), thereby preventing low-grade chronic inflammation of adipose tissue that promotes the obese state (56).

In addition to *Lactobacillus* and *Biffidobacterium* strains, *Akkermansia muciniphila* is another bacterium that shows promise in modulating host obese and metabolic status. This mucin-degrading bacterium resides in the mucus layer (57), and its decreased abundance has been observed in obese and type 2 diabetic mice and humans. Treatment with live, but not heat-killed, *A. muciniphila* cells increased adipose tissue fatty acid oxidation and mucus layer thickness, while reducing fat mass development and metabolic endotoxemia, which

ultimately reverse high-fat diet-induced obesity and insulin resistance in mice (58).

Probiotics with BSH activity could alter the bile acid pool and therefore have potential to modulate energy and lipid metabolism (40, 41). BSH-mediated modulation of BAs that positively affects host physiology has been clearly demonstrated in a recent controlled experimental study (59). This study showed that colonization of conventional mice with *E. coli* expressing cloned BSH enzymes from *L. salivarius* resulted in a significant reduction in host weight gain, plasma cholesterol, and liver triglycerides as compared to mice colonized with BSH-negative *E. coli* (59). Such physiological effects were accompanied by a decrease in tauroconjugated BAs, including tauro-β-muricholic acid (TβMCA). The latter is a potent antagonist of the host Farnesoid-X-receptor (FXR) that regulates bile acid synthesis through the control of hepatic cholesterol 7α-hydroxylase (CYP7A1) activity (60). This study therefore suggests the feasibility of harnessing BHS-positive probiotics to control obesity, metabolic syndrome, and hypercholesterolemia.

Indeed, the BSH-catalyzing deconjugation of bile salts has long been proposed as a potential mechanism for the hypocholesterolemic effect of probiotics since the lower re-absorbability of unconjugated BAs may stimulate the de novo synthesis of bile acids from cholesterol in the liver. Furthermore, the decreasing solubility of unconjugated bile acids at physiological pH may inhibit cholesterol micelle formation in the intestinal lumen, thereby reducing cholesterol absorption and increasing its excretion in feces (61-64).

The experimental findings described above suggest the potential of harnessing probiotics to modulate host metabolism and therefore improve obesity, metabolic syndrome, and hypercholesterolemia. However, the molecular effectors mediating these beneficial effects have not been identified. There is also a need for translational research to develop probiotic therapies so that effective doses of effector elements will be delivered to the target. Rational selection of probiotics based on effectors known to mediate functional properties of the gut microbiota, for example their ability to deconjugate bile salts, may therefore facilitate the development of probiotics.

Role of the gut microbiota in immune development and homeostasis

One of the most remarkable features of the human immune system is its tolerance toward myriad and diverse gut commensals, while also being able to respond to pathogenic invaders. Such mutualism is initiated as early as the establishment of the immune system when the interaction with the gut microbes is required for its proper development and maturation. Indeed, it is well known that the immune system does not develop normally in germ-free mice (65). Thus, the absence of microbial colonization leads to hypoplastic Peyer's patches containing few germinal centers, low numbers of laminar propria $CD4^+$ T cells and IgA-producing B cells, and underdeveloped lymphoid tissues and isolated lymphoid follicles (ILFs) (65, 66). Bouskra et al. (65) clearly demonstrated that ILFs require Gram negative peptidoglycan for their genesis and subsequent bacterial recognition by Toll-like receptors (TLRs) for their maturation into large

B-cell clusters. Furthermore the absence of ILFs profoundly alters the composition of the gut microbial community. Their study clearly shows the interplay between the host immune system and the intestinal microbes in creating a mutualistic environment.

The absence of particular members of the gut microbiota has been associated with increasing incidence of immune-mediated diseases. For example, *Helicobacter pylori* is thought to have once dominated the gastric niche in most human individuals and is nowadays almost eradicated from Western-born children due to increasing hygienic life-styles. This change in the human microbiota coincides with the rising incidence of allergic and metabolic diseases (67). Indeed, *H. pylori* infection has been showed to prevent asthma in mouse models and augmented regulatory T (T_{reg}) cell proliferation induced by *H. pylori* colonization has been suggested to underlie such protective effect (68). This *H. pylori* scenario emphasizes the essential role of the intestinal colonization with proper microbes in immune development and downstream protection against immune-mediate disorders.

Altered interactions between gut microbes and the mucosal immune system, leading to disintegration of intestinal immune homeostasis is implicated in pathogenesis of inflammatory bowel disease (IBD) (69). This noninfectious intestinal inflammatory disease often develops in human hosts with altered loci implicating impaired immune functions (70, 71). For example, susceptibility to Crohn' s disease has been associated with NOD2 dysfunction that reduces intestinal epithelial production of antimicrobial proteins essential for confining gut

microbes away from the intestinal epithelium and immune cells underneath (72). Hence, the impairment in host-symbiont mutualism in IBD patients is possibly triggered by susceptible loci conspiring with diet and other environmental factors, which facilitates the accumulation of microbial communities capable of driving inflammation (73) as demonstrated by characteristic shifts in composition of the gut microbiota (74) and the lose of anti-inflammatory members such as *Feacalibacterium prausnitzii* (75).

Probiotic potential for modulating immune responses and intestinal epithelial barrier

The ability of probiotics to modulate the immune system and gut barrier function is particularly important in several inflammatory conditions, such as IBD, necrotizing enterocolitis (NEC), and allergic diseases (76). Numerous experimental studies have shown that specific probiotic bacteria can modulate immune and mucosal responses (76, 77). Furthermore, mechanistic studies have identified microbial effectors, along with their cognate immune elements and signaling pathways (78-82). Probiotics can have either pro- or anti-inflammatory properties and elicit immune responses through their cell surface-associated components, bacterial DNA, and/or secreted compounds (81). These effector molecules generally function as microorganism-associated molecular patterns (MAMPs) that interact with pattern recognition receptors (PRRs), including TLRs and nucleotide-binding oligomerization domains (NODs), present in immune cells such as dendritic cells (DCs) and macrophages as well as intestinal epithelial

cells (IECs). Such MAMP-PRR interaction triggers signaling cascades, thereby leading to the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) (83, 84). This activation allows NF-κB and MAPKs to translocate into the cell nucleus and induce gene expression, which thereby mounts cellular responses such as pro- or anti-inflammatory cytokine secretion, mucin and antimicrobial compound production by IEC, and DC maturation and activation. MAMP-activated DCs and their secretory cytokines further govern T cell differentiation and proliferation thereby enabling probiotic recognition to influence adaptive immune responses (78-80, 82, 85).

Among the cell-associated effectors identified in probiotic strains are: (1) peptidoglycan (PG) and peptidoglycan-derived muropeptides (86-88); (2) lipoteichoic acids (LTA) (89, 90); (3) cell wall-associated polysaccharides (CPS) (91-93); (4) flagellin (94); (5) fimbriae/pili (95); (6) surface (S) layer proteins (96); and (7) DNA (97-99). Subtle differences in structure or biochemical properties of these individual cellular components have been suggested as major contributors to strain-specific immunomodulatory attributes of probiotics (100). For example, *Lactobacillus salivarius* Ls33 and *Lactobacillus acidophilus* NCFM possess PG containing the same muropeptide chain, GlcNAc-MurNAc-L-Ala-D-iGln-L-Lys-D-iAsn. However, whereas NCFM releases this muropeptide exclusively during PG degradation, Ls33 also releases an additional muropeptide without D-iAsn. The latter muropeptide is presumably short enough to be taken up by dipeptide/tripeptide transporters and consequently interacts with intracellular NOD2. As a result, only PG purified from Ls33 could stimulate tolerogenic DC

(tol-DC) development and thereby exert an IL-10-mediated protective effect against colitis in a mouse model, while NCFM-isolated PG failed to do so (87, 101).

Another example is the structural variation that affects pro-inflammatory character of LTAs. The difference in the degree of D-alanylation (D-alanine substitutions) on the polyglycerolphosphate chain and polyglycerolphosphate chain length as well as in the acylated glycolipid anchor composition (number of acyl fatty acid chains and saturation) appear to influence LTA-TLR2 or LTA-TLR2/6 interaction, thereby contributing to strain-dependent LTA-mediated immunostimulatory activity of probiotics (89, 102-105). A study into the impact of LTA structure on pro-inflammatory properties of *L. rhamnosus* GG (LGG) has demonstrated that partial removal of acyl fatty acid chains from native LTA structure abolishes capacity of the modified LTA to interact with the PRR couple TLR2/6 and thereby to activate NF-κB in HEK293T cells, while D-Ala removal does not significantly alter LTA-TLR2/6 interaction. However, D-Ala has been showed to be essential for the induction of inflammatory chemokine IL-8 expression in Caco-2 intestinal epithelial cells in which LTA-TLR2/6 signaling seems to require the interaction between the D-Ala substituents and additional coreceptors (104). This study not only emphasizes the role of the glycolipid anchor and D-Ala substituents in immunostimulatory attributes of LTAs, but also suggests that distinct host cells may respond to a certain LTA differently owing to PRRs and cognate coreceptors they express.

In short, the above studies and others (80) have shown that the type and level of a host cell response to a probiotic strain depends on (1) the combinational engagement of distinct MAMPs that interact with their cognate PPRs and associated co-receptors, resulting in finely tuned immune signaling (106), (2) structure, expression, and concentration of MAMPs that could be altered by gut conditions such as acidity, the presence of bile acids, intestinal enzymes, and antimicrobial compounds, and nutrient availability (107), (3) the effect of shielding factors such as exopolysaccharides that could limit MAMP-PRR interaction (100) (4) the accessibility of the host PRRs that can be varied between distinct cell types and limited by the dense mucus layer (108) and the presence of other microbial effector molecules, and (5) host-derived negative regulators of PRR signaling that modulate the downstream signaling pathway at different points (109, 110).

In addition to cell-associated molecules, certain probiotics have potential to elicit host immune and IEC responses through their secreted compounds and metabolites. Such probiotic factors can signal through surface receptors or be taken into the host cells by transporters or endocytosis systems (111) and consequently interact with intracellular regulatory components of diverse signaling pathways in IECs, macrophages, and DCs (78, 112, 113). In IECs, probiotic secreted compounds have been found to modulate cell survival, barrier function, and cytokine secretion. For example, two soluble proteins, Msp1/p75 and p40, secreted by LGG can activate anti-apoptotic protein kinase B (Akt), thereby regulating IEC cell survival and preventing cytokine-induced IEC

apoptosis (114). Furthermore, administration of LGG p40 can mitigate DSS-induced intestinal injury and acute colitis as well as oxazolone-induced T_H2 cytokine-driven chronic colitis in mouse models through the interaction with epidermal growth factor receptor (EGFR), leading to Akt activation (115). In addition to anti-apoptotic effect, activation of EGFR by p40 can also stimulate mucin production in goblet cells and may thereby improve gut barrier function and protect the intestinal epithelium from injury and inflammation (116).

Lactate and acetate produced by probiotic lactic acid bacteria also show capacity to regulate epithelial proliferation. In *L. casei* strain Shirota and *Bifidobacterium brevis* strain Yakult, both organic acids have been recently characterized as effector molecules capable of down-regulating cell cycle regulatory proteins (cyclins) and inducing expression of genes involved in intestinal epithelial cell differentiation (117). Although detailed molecular pathways remains to be elucidated, the potential of probiotic metabolites to modulate intestinal epithelial cell cycle could be useful for the maintenance of functional epithelial barrier during infection of certain pathogens capable of altering such cell cycle (118).

In macrophages, secreted compounds from certain probiotics have potential to suppress proinflammatory tumor necrosis factor (TNF) expression by modulating NF-κB or MAPK signaling (119, 120). For example, soluble factors secreted by *L. reuteri* MM4-1A shows ability to suppress TNFα transcription in monocytes and LPS-activated, Crohn's disease patient-isolated macrophages (120). Such immunosuppressive effect is attributed to the inactivation of MAPK-

activated transcription factor AP-1 that regulates transcription of proinflammatory cytokines (120). This probiotic property may be useful for the treatment of Crohn's disease in which elevated levels of TNF have been commonly detected (121).

Furthermore, *L. reuteri* MM4-1A-secreted factors are able to promote TNFα-induced apoptosis in human myeloid leukemia-derived cells. The secreted factors stabilize the IκBα inhibitor, therefore suppressing NF-κB-dependent expression of mediators of cell survival and proliferation (122). Such probiotic factors also enhance pro-apoptotic MAPK signaling, thereby promoting apoptosis in activated immune cells (122). Such pro-apoptotic effects on activated immune cells may be valuable for colorectal cancer and IBD therapy (122, 123).

Altogether, growing evidence from *in vitro* cell cultures and animal models has substantiated immunomodulatory and epithelial effects of probiotic strains and pinpointed cell-associated and secretory molecules as effectors that interact with receptors or regulatory components of diverse host cell signaling pathways and consequently modulate transcription of response genes involved in cytokine production, cell cycle (maturation, proliferation, differentiation, and apoptosis), and epithelial barrier function. Resultant immunosuppressive effects may reduce the risk of allergic reactions and mitigate inflammation, whereas immunostimulatory and epithelial barrier-strengthening properties could protect the host from pathogen infection. However, such effector molecules are dynamic entities whose expression depends on bacterial growth stages as evidenced by differential modulation of NF-κB signaling components in the human duodenal

mucosa in response to *Lactobacillus plantarum* harvested at different growth phases (124). Moreover, altered expression and modification of effector molecules could proceed during the adaptation of probiotics to the gut environment as implied by the gut-induced transcriptional changes in *L. plantarum* genes involved in extracellular protein and polysaccharide biosynthesis and D-alanylation of LTA (125, 126). Therefore, it is fundamental to consider probiotic effects to be growth-stage dependent. Also of importance is that expression of effector molecules is examined in the gut environment to evaluate the in situ capacity of probiotics.

Contributions of the gut microbiota to colonization resistance against enteropathogens

Colonization resistance is an essential functional property found in well-established ecosystems to protect invasion of new species and overexpansion of a particular member. This ecological attribute is well established within the community of the gut microbes and plays a crucial role in protecting our gut from pathogen colonization and pathobiont overgrowth (127), as evidenced by mounting susceptibility of germ-free and antibiotic-treated mice to enteric pathogen infection, which can be antagonized by re-colonization of the microbial residents (128-132). Efforts to protect their gut environment have been demonstrated among the symbiotic microbes. For example, colonization of Gram-negative symbiont *B. thetaiotaomicron* in germ-free mice induces Paneth cells to express the antimicrobial peptide REGIIIγ that primarily targets

peptidoglycan of Gram-positive bacteria (133). Mechanisms that regulate colonization resistance in the gut have been proposed to fall into two categories, i.e., direct microbe-microbe interaction and immune-mediated colonization resistance (127, 134, 135).

Colonization resistance elicited by direct interactions between the indigenous microbes and invading species involves competitive and metabolic exclusion. Competitive exclusion relies on the ability of the gut symbionts to sequester nutrients and other niches from invaders (136-138). In the gut environment, microbial inhabitants fill available nutritional niches and form complicate cross-feeding networks to circular metabolic substrates within the community (139), thereby actively sequestering nutrients from invading microbes. An example is demonstrated by the pre-colonization of the mouse gut with the combination of three commensal *E. coli* strains that effectively fill the sugar-defined nutritional niche of enterohemorrhagic *E. coli* (EHEC), a leading cause of bloody diarrhea in humans, and consequently prevent the pathogenic strain from colonization (137). Competition for the amino acid proline between indigenous *E. coli* and *E. coli* O157:H7 has also been reported to strongly inhibit growth of the pathogenic strain in a baby-flora-associated mouse model (140). In addition to competition for the same nutrient, occupation of space is also a key of pathogen exclusion. Colonization of indigenous microbes at the mucus layer can exclude mucin-derived binding sites from invaders, thereby restraining the penetration of pathogenic microbes into gut epithelium (141).

The gut microbiota could also inhibit pathogen colonization through metabolic exclusion involving bacteriocin secretion and SCFA production. Members of intestinal microbes are capable of producing bacteriocins with bactericidal activities that can inhibit growth of competing species (142-145). SCFAs are the other metabolites that can mediate colonization resistance by suppressing pathogen growth and expression of virulence factors (146-148). In addition, the gut acidification by SCFAs prevents dissociation of intestinal fatty acids, thereby potentiating their antibacterial properties (149, 150).

The second mechanism of colonization resistance is directed by immunomodulatory activities of the symbionts that enhance host defense against pathogen colonization. The gut symbionts have capacity to stimulate antimicrobial protein production by Paneth cells through NOD2/TLR-bacterial ligand recognition (133, 151, 152). The gut microbiota activation of MyD88-dependant antimicrobial responses from Paneth cells has been found to limit *Salmonella enterica* serova Typhimurium (*S.* Typhimurium) penetration across the host epithelium (151).

The gut symbionts also stimulates host immune defense through their SCFA metabolites. Certain strains of *Bifidobacterium longum* produce acetate that can upregulate host anti-inflammatory and anti-apoptosis gene expression, which reduces translocation of Shiga toxin from the intestine to the blood and therefore prevents *Escherichia coli* O157:H7 infection (153, 154). SCFAs particularly butyrate and propionate have been found to mediate T_{reg} cell proliferation (155, 156) by binding to GPR43 expressed by colonic T_{reg} cells. The

SFCA-GPR43 interaction provides a signal for colonic T_{regs} expansion and, in consequence, augments anti-inflammatory cytokine IL-10 production (157). SCFA signaling through GPR43 present on neutrophils also induces cell apoptosis while limits cell migration and surface expression of pro-inflammatory receptors, thereby potentially aiding in resolving intestinal inflammation by suppressing the damaging effects of neutrophils (158). These SCFA-mediated anti-inflammatory activities have been postulated to promote colonization resistance by counteracting inflammation, induced by pathogen-host interaction, which can surprisingly facilitate pathogen growth (159). This postulation is supported by the finding that major enteric pathogens can utilize by-products of the inflammatory host response, for example nitrate (NO_3^-) derived from nitric oxide (NO) and superoxide radical (O_2^-), for anaerobic respiration (160-163).

Dysbiosis and infectious diseases

A disintegration of the gut microbial community rendering a breakdown of colonization resistance is apparently a key step for enteropathogenic infection. Increasing evidence has demonstrated that pathogenic *Proteobacteria* are capable of triggering host immune responses and then exploiting inflammatory milieu to outcompete the indigenous microbiota and therefore subvert colonization resistance in order to infect the host cell (161, 162, 164, 165). For example, *S.* Typhimurium can convert reactive oxygen species generated during inflammation and endogenous luminal thiosulphate into tetrathionate. The pathogen then uses tetrathionate as a respiratory electron acceptor to gain

growth advantage and consequently overcomes colonization resistance (162). Consistent with these findings, microbial imbalance in which the community structure is shifted toward an increased prevalence of *Proteobacteria* has been observed in individuals with intestinal inflammatory disorders such as inflammatory bowel diseases (IBD) (166-168) and necrotizing enterocolitis in preterm infants (169-171).

In addition to pathogen-inducing inflammation that diminishes colonization resistance, antibiotic treatments that cause collateral damage to sensitive members of the gut microbiota could similarly contribute to the collapse of the microbial defense system and, in consequence, increase susceptibility to enteric infection. Antibiotic-associated disruption of the gut microbiota has been found to trigger infection by various antibiotic resistant pathogens and pathobionts, including *Clostridium difficile* (172), vancomycin-resistant *Enterococcus faecium* (VRE) (131), and Gram-negative bacilli of *Enterobacteriaceae* family (173). The decrease in abundance and activities of sensitive microbial residents allows resistant pathogenic microbes to occupy emptied niches and therefore expand their population and virulence factors, which is a key for their success in invading host cells (127, 134, 159, 174). For example, both *S. typhimurium* and *C. difficile* can exploit a spike in host-derived free sialic acid for their expansion in the antibiotic-treated murine gut in which indigenous sialic acid utilizers are eradicated (175).

Roles of probiotics in promoting colonization resistance

Capacity of probiotics to enhance colonization resistance against pathogen invasion could protect the healthy host from enteropathogenic diseases (176). This probiotic property becomes of the utmost importance when the gut is devoid of the properly established microbiota, for example during the succession of the microbial community in preterm infants or during community restoration after antibiotic treatments (134). Numerous experimental studies have disclosed the potential of probiotic strains to increase colonization resistance through the competition for nutrients (competitive exclusion), secretion of antimicrobial compounds (metabolic exclusion), interference in virulence factor expresssion, and/or stimulation of host immune and mucosal defenses (176, 177).

Colonization resistance mediated by direct competition for nutrients has been primarily demonstrated in probiotic *E. coli* strain Nissle capable of outcompeting *S.* Typhimurium for iron in the inflamed murine gut. Whereas other tested strains of commensal *E. coli* fail to counteract colonization of *S.* Typhimurium, *E. coli* Nissle can employ multiple iron-acquisition systems superior to those of *S.* Typhimurium in overcoming iron sequestration mediated by a siderophore-binding protein highly expressed during host inflammation. Hence, the probiotic minimizes iron availability, thereby suppressing the pathogen colonization (178).

The second mode of direct antagonistic actions involves the secretion of antimicrobial compounds that display bacteriostatic or bactericidal activities against enteropathogens (176). Thus far, a few *in vivo* studies employing infected

mouse models and non-antimicrobial compound-producing mutants for controls have demonstrated the potential of probiotic strains to produce bacteriocin at the site of infection and in adequate amounts to elicit anti-infective effects. For example, *Lactobacillus salivarius* UCC118 capable of producing Abp118 bacteriocin can control *Listeria monocytogenes* infection in mice. This anti-infective effect was abrogated when a Abp118-negaitve mutant was tested or when mice were infected with an *L. monocytogenes* strain expressing the cognate Abp118 immunity protein, thereby confirming that the probiotic elicits the colonization resistance through the *in vivo* production of a bacteriocin (179). Similarly, only *Peddiococcus acidilactici* MM33 able to produce pediocin PA-1 could reduce VRE colonization in the mouse gut, while a non-pediocin-producing mutant showed no anti-VRE effect. This result thereby suggests that the capacity of MM33 to produce pediocin *in vivo* underlie its antagonism against a VRE strain (180).

Another example that showed the anti-infective potential of an antimicrobial compound-producing probiotic strain involves reuterin production in *L. reuteri* MM4-1A. Several human-isolated strains of *L. reuteri* are capable of reducing glycerol to reuterin (3-hydroxypropionaldehyde, 3HPA) that has been showed to exhibit bactericidal activity against various enteropathogens including enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, *Salmonella enterica*, *Shigella sonnei*, and *Vibrio cholera* in a pathogen overlay assay (181). Reuterin appears to exert the bactericidal effect by modifying thiol groups in proteins and small molecules of target microbes, which induces oxidative stress and ultimately leads to cell death (182, 183). A study using three-dimensional organotypic model of

human colonic epithelium has demonstrated that glycerol-stimulated MM4-1A caused a significantly higher reduction of *S.* Typhimurium adhesion and invasion as compared to the *ΔpduC* mutant unable to produce reuterin. This finding thereby suggests reuterin secretion as a mode of *L. reuteri* antagonistic action against *S.* Typhimurium infection (184). However, it remains unclear whether the probiotic is able to produce reuterin from glycerol in the gut.

One further example of metabolite-mediated colonization resistance involves antagonistic activity of potential probiotic *Clostridium scindens* against *C. difficile* expansion. It has been recently showed that *C. scindens* possesses an enzyme 7α-hydroxysteroid dehydrogenase and is able to transform host-derived bile salts into secondary bile acids, which inhibit *C. difficile* growth. As a result, *C. scindens* treatments can restore abundance of secondary bile acids in antibiotic-exposed mice, which thereby confers protection against *C. difficile* colonization (185).

Probiotic strains can also directly interfere the expression and functionality of virulence factors (176). This mode of antagonistic action has also been described in *L. reuteri* strains. *L. reuteri* RC-14, which displayed capability to inhibit *Staphylococcus aureus* infection in a rat surgical implant model (186), has showed potential to secrete small molecules that decreased the expression of a superantigen-like protein (SSL11) in *S. aureus* by repressing the SSL11 promoter activity (187). Similarly, the cell-free supernatant of *L. reuteri* ATCC 55730 cultures has displayed capacity to repress the expression of the locus of enterocyte effacement (LEE)-encoded regulator involved in the

attachment/effacement (A/E) lesion of enterocyte microvilli by E. *coli* O157:H7 (188).

By modifying pathogen virulence factors, certain probiotics have potential to inhibit enteroinvasive pathogens. For example, *Lactobacillus acidophilus* LB can produce a secretory factor that antagonizes intracellular *S.* Typhimurium infection. Treatment with cell-free LB supernatant can reduce the number of apical F-actin rearrangements in infected human enterocyte-like Caco-2/TC-7 cells and therefore decrease transcellular passage of the pathogen (189). The capacity of probiotics to antagonist pathogens that already enter the host cell could become a practical alternative to antibiotics, several of which are only effective against extracellular pathogens (190).

In addition to direct antagonistic effects, probiotics may improve colonization resistance through the stimulation of host immune defenses. One such example is *L. reuteri* that shows capacity to transform dietary tryptophan to indole-3-aldehyde (IAld) that activates the aryl hydrocarbon receptor (AhR) in innate lymphoid cells (ILCs). In turn, ILCs secrete IL-12 that induces antimicrobial responses. As an ultimate result, *L. reuteri* treatment can provide colonization resistance to opportunistic pathogen *Candida albicans* in germ-free mice (191). However, while increased IL-12 abundance appears to antagonist *C. albicans*, it also has potential to promote *S.* Typhimurium infection. Such undesirable outcome results from IL-12-induced expression of antimicrobial protein lipocalin-2 and calprotectin, able to sequester essential metal ions, including iron, zinc, and manganese, from pathogens as well as gut symbionts. *S.* Typhimurium,

however, can overcome the sequestration, while some of its indigenous gut competitors cannot, thereby allowing the pathogen to thrive in such gut milieu (192). Nevertheless, it is possible that the IL-12 inducer like *L. reuteri* can also evade the ion sequestration and therefore protect the IL-12-enriched environment it initiates from pathogenic invaders.

In short, above findings emphasize the necessity of understanding mode of probiotic actions as well as pathogen lifestyles in order to develop competent probiotics that can battle with a target enteropathogen without compromising colonization resistance against other opportunistic invaders.

Part II: Glycan metabolism in gut microbial symbionts and probiotic *L. reuteri*

Activities central to microbial life in any environment are those that generate and conserve energy, maintain redox balance, and acquire carbon and nitrogen skeletons for biosynthesis of macromolecules such as proteins, nucleic acids, polysaccharides, and lipids (139). Members of the gut microbiota have evolved mechanisms to maximize energy and carbon acquisition from available substrates derived from indigestible food particles, host mucin, and microbial metabolites (4), which allows them to multiply at a rate equaling or surpassing peristalsis-driven washout rate and thereby maintain their population in the gut (193). Such mechanisms involve efficient acquisition of lumen substrates, maximization of ATP conservation, and maintenance of redox balance. Below, these mechanisms are discussed relative to metabolism of prebiotic β-

galactooligosaccharides (GOS) and its structurally related human-milk oligosaccharides (HMO). Mechanisms described in certain prototypical gut symbionts are compared with those characterized in probiotics, especially in *L. reuteri* (when available). The insight into lifestyle of gut symbionts and metabolic potential of probiotics could reflect how competent the probiotic is to establish themselves in the gut in relation to the symbiont and how to stimulate metabolism and therefore growth of the probiotic.

Mechanisms for harvesting lumen energy and carbon sources: the GOS paradigm

Glycan metabolism is a principal source of energy and carbon for gut bacteria (4). HMO appear to have no nutritive value to infants due to the absence of appropriate digestive enzymes (194). Nonetheless, they are the third most abundant solid component in human breast milk after lactose and lipids. It is now know that they serve as substrates for a number of colonic bacteria, especially *Bifidobacterium* species, thereby substantially influencing the establishment of colonic microbial community in breast-fed infants (195, 196). Over 130 different HMO species have been identified, most of which are unique to humans and only trace amounts of which are present in bovine milk (197). GOS have been therefore synthesized and supplemented into bovine milk-based formulas to mimic the prebiotic and other biological effects of HMO (198). Commercial GOS typically are the mixture of disaccharides comprising one to two galactose moieties [Gal(β1-3/4/6)Gal/Glc] excluding lactose [Gal(β1-4)Glc] and

oligosaccharides mainly comprising terminal lactose linked to one to six galactose moieties [Gal(β1-3/4/6)]$_{1-6}$Gal(β1-4)Glc] (199, 200).
These structures only slightly resemble the more complex backbones of HMO, which consist of terminal lactose elongated with up to 25 repeats of either lacto-*N*-biose [[**Gal(β1-3)GlcNAc**(β1-3)]$_{1-25}$Gal(β1-4)Glc] or *N*-acetyllactosamine [[**Gal(β1-4)GlcNAc**(β1-3)]$_{1-25}$Gal(β1-4)Glc] (201, 202). Despite these structural differences, their fermentability by *Bifidobacterium* and *Lactobacillus* species of the human gut has been confirmed *in vitro* (203-205). Likewise, their prebiotic ability to selectively promote growth of gut symbionts *in vivo* has also been demonstrated (206-210).

It is worth noting that among GOS fermenters, their ability to growth on GOS does not only vary from genus to genus (204), but also from species to species (211) and even from strain to strain (203, 212). Such phenotypic variability appears to result from distinct metabolic systems that allow some GOS utilizers to gain excess to more varieties of GOS components. For example, among GOS-fermenting human isolates of *Bifidobacterium brevis*, several strains including *B. brevis* UCC2003 isolated from stool can consume most GOS components and reach higher final cell yields than strains incapable of utilizing GOS with a high degree of polymerization. This access to long-chain GOS is facilitated by an extracellular, cell membrane-bound, glycoside hydrolase family 53 (GH53) endogalactanase (GalA) that cleaves GOS oligosaccharides with DP ≥ 4 and thereby allows shortened products to be internalized into the cytosol. In *B. brevis* UCC2003, this extracellular hydrolase works in conjunction with an

ABC transporter (GalCDE), which imports intact and GalA-cleaved GOS components into the cytosol, and an intracellular GH42 β-galactosidase (GalG), which breaks down internalized GOS to monosaccharide moieties (212). These three molecular elements whose coding genes are situated in the same cluster (*galCDEGRA)* are also responsible for the metabolism of plant-derived galactans in *B. brevis* (213). Moreover, GalA endoglycosidase has been described in infant-isolated *B. longum* NCC2705 to have activity on both (β1-4)galactans and (β1-4)GOS (214). These findings therefore suggest that molecular machinery that allows intestinal microbes to utilize GOS indeed have a biological role in metabolizing galactans derived from food plants.

Although the GalA-GalCDE-GalG system is the principal contributor to GOS metabolism in *B. brevis* UCC2003, the strain also possesses the LacS-LacZ and GosDEC-GosG system that aid in GOS metabolism. The former comprises LacS permease and GH2 β-galactosidase LacZ and the letter consists of GosDEC ABC transporter and its associated GH42 β-galactosidase GosG (212). This finding therefore indicates the cooperative actions of multiple transporters and β-galactosidases on GOS metabolism. In *B. lactis* B1-04, GOS induces the expression of two different gene clusters encoding a putative MFS lactose permease-GH2 β-galactosidase and an ABC transporter-GH42 β-galactosidase (215), thereby suggesting that GOS metabolism in this probiotic strain should also rely on the cooperative activities of two metabolic systems. The concerted activities of multiple transporters are in contrast to GOS transport system characterized in probiotic *Lactobacillus acidophilus* NCFM in which LacS

permease is the sole transporter for GOS and lactose (216). In *L. acidophilus*, after LacS imports GOS into the cytosol, two β-galactosidases, i.e., GH42 LacA and GH2 LacLM, appear to be responsible for hydrolyzing internalized GOS to galactose and glucose moieties. However, the relative efficiency of this GOS metabolic system in comparison to the cooperative systems characterized in *Bifidobacterium* species remains to be evaluated.

The findings described above clearly demonstrate that different probiotic bacteria and human gut symbionts could be equipped with distinct molecular tools for metabolizing prebiotic GOS. Importantly, some pathways are more efficient than others. Therefore, some strains are able to harvest and catabolize more varieties of GOS components and thereby have advantages in GOS-enriched environments. Ultimately, the different GOS metabolic capacities between probiotic and human gut symbionts or between different gut symbiotic strains could have a major influence on the microbial composition during synbiotic or prebiotic interventions.

The specific molecular elements involved in GOS metabolism may also have a role in HMO metabolism. For example, in *B. bifidum,* the GH2 β-galactosidase (BbgIII), has activity toward GOS di- and trisaccharides and is highly active toward lactose (217). This system, however, also contributes to the degradation of lacto-*N*-neotetraose [Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc], a major core tetrasaccharide structure of HMO. First, BbgIII liberates Gal and lacto-*N*-triose II [GlcNAc(β1-3)Gal(β1-4)Glc] from the HMO tetrasaccharide. Then, after a β-*N*-acetylhexosaminidase cleaves lacto-*N*-triose II into GlcNAc and

lactose, BbgIII acts again to hydrolyze lactose to galactose and glucose (218). However, GOS metabolic systems alone do not have the capacity to break down intact HMO without the aid of other glycosidases. Thus, fucosidases, *N*-acetylhexosaminidases, and sialidases are necessary to sequentially hydrolyze the diverse glycosidic linkages commonly present in HMO (195).

Extracting energy from the harvest: pathways for energy generation and redox balancing

In nearly anoxic environments, such as the human gut lumen and colon (219) where oxygen is not available for respiration, many microbial inhabitants rely on fermentation to extract energy and carbon from available glycans. The first step involves enzymatic hydrolysis of complex poly- and oligosaccharides into their monosaccharide moieties. Then, saccharolytic microbes typically employ one or a combination of common glycolytic pathways, i.e., Embden-Meyerhof-Parnas (EMP), Entner-Doudoroff (ED), pentose phosphate (PP), and phosphoketolase (PK) to metabolize monosaccharides into the major metabolic intermediate, pyruvate (139, 220). During these metabolic processes, free energy is generated from redox reactions at the expense of NAD^+ as an electron acceptor that is reduced to NADH. In fermentation, released free energy is conserved in form of ATP typically by substrate-level phosphorylation and NADH is re-oxidized to NAD^+ by the reduction of pyruvate to any of a variety of fermentation products, such as organic acids, ethanol, CO_2, and H_2. Reoxidation of NAD^+ is essential to maintain redox balance, given the paucity of terminal electron acceptors (221).

However, some gut bacteria, for example *Bacteroides* species (139), have evolved anaerobic respiration systems employing alternative terminal electron acceptor such as fumarate, SO_4^{2-}, and CO_2. (i.e., rather than O_2). This allows these organisms to dispose electrons from NADH through an electron transport chain, and thereby generates a proton motive force that can drives ATP synthesis by the ATPase. In consequence, more net gain of ATP is achieved and NAD^+ is re-generated.

Alternatively, some gut microbes employ an extracellular electron transfer (EET) system (222) to increase the net energy gain from metabolic processes. For example, one of the most abundant gut bacteria and a strict anaerobe, *Faecalibacterium prausnitzii,* is capable of employing riboflavin- and thiol-mediated EET to shuttle electron from NADH to O_2 present at mucosal surface, therefore converting O_2 toxic to the cell into a terminal electron acceptor and gaining growth advantage from extra ATPs as a result (223, 224).

Given the limited means by which fermentative bacteria can obtain energy from sugars, the ability to generate ATP from the same amount of GOS-derived monosaccharides could therefore dictate species abundance among GOS fermenters. Among well-documented GOS consumers, *Bifidobacterium* species, a prototypical gut symbiont, ferment hexose sugars through a unique glycolytic pathway, termed the “bifid shunt” (225). After hydrolysis of GOS into galactose and glucose, the latter monomer enters bifid shunt directly. The galactose moiety is presumably channeled through the Leloir pathway and converted to glucose-6-phosphate before entering the bifid shunt (212). The bifid shunt allows

bifidobacteria to produce a net gain of 2.5 ATP per glucose fermented (225), which is more than the 2 ATP generated by homofermentative lactobacilli that ferment hexoses through the EMP pathway (220).

In contrast, GOS-fermenting *L. reuteri* is heterofermentative, and obtains only 1 ATP per glucose fermented through phosphoketolase pathway (226, 227). This relatively poor energy yield compared to that of metabolic pathways operating in other GOS utilizers could thereby be a disadvantage for this species in colonizing a GOS-enriched gut. However, this disadvantage can be compensated for by the presence of external electron acceptors that allow the cell to re-oxidize NADH to NAD^+ through an alternative pathway rather than through the reduction of acetyl phosphate to ethanol. Most of the acetyl phosphate can be channeled to produce acetate, thereby yielding up to one additional ATP per glucose fermented. In addition, the external electron acceptors re-oxidize NADH more rapidly than the ethanol pathway. Thus, growth rates can increase by a factor of 2 to 3 (228, 229). Indeed, the ethanol branch of PK pathway has been suggested to be no more than a “salvage route” that permits growth in the absence of an external electron acceptor (230). In *L. reuteri* ATCC 55730, the relatively low ATP level accompanied by slow growth rate and low biomass yield was detected in glucose-growing cells even though a non-limiting concentration of glucose was present. Such energy and growth limitation, however, could be alleviated by the presence of the electron acceptor fructose (227).

In addition to fructose, *L. reuteri* is capable of using glycerol (1,2,3-propanetriol) as an external electron acceptor (231). The existence of glycerol during carbohydrate fermentations results in higher growth rates, increased cell yields, and the shift in end products from predominantly lactate, ethanol, and CO_2 to lactate, acetate, CO_2, and 1,3-propanediol with decreased ethanol yields. The presence of glycerol enables *L. reuteri* to re-oxidize NADH through an alternative route. First, glycerol is dehydrated by a coenzyme B_{12}-dependent glycerol dehydratase to 3-hydroxypropionaldehyde (3-HPA). The latter is then reduced to 1,3-propanediol (1,3-PD) by a 1,3-PD:NAD^+ oxidoreductase (1,3-propanediol dehydrogenase) using electrons donated by NADH (231, 232). With this effective alternate electron acceptor system, high-energy acetyl phosphate can be spared for ATP synthesis mediated by the acetate kinase, thereby allowing the cell to gain extra ATP for growth.

Furthermore, *L. reuteri* strains appear to have a capacity to use 1,2-propanediol (1,2-PD) directly as an energy source and/or as an external electron acceptor to regenerate NAD^+. Sriramulu et al. (233) have demonstrated that *L. reuteri* DSM 20016 possesses a *pdu* (propanediol utilization) operon encoding enzymes for dismutation of 1,2-PD as previously characterized in *S.* Typhimurium (234, 235). This pathway enables the cell to convert 2 moles of 1,2-PD to a propionaldehyde intermediate and then proportionately reduce and oxidize propionaldehyde to *n*-propanol and propionate respectively. These reactions yield approximately equal amounts of *n*-propanol and propionate as end products together with 1 ATP (Figure1). This energetic metabolism thereby

allows *L. reuteri* to employ 1,2-PD as an energy source, which was evidenced by the growth of *L. reuteri* DSM 20016 on 1,2-PD coexisting with a carbon and nitrogen source (233).

In contrast to serving directly as a fermentation substrate, 1,2-PD could also serve an external electron acceptor that allows NADH, generated from the PK pathway, to dispose electrons through the reduction of propionaldehyde intermediate (the reduction arm of the 1,2-PD dismutation). Similar to the glycerol pathway, 1,2-PD can be dehydrated to propionaldehyde by a diol dehydratase. Then propionaldehyde accepts electron from NADH and is therefore reduced to *n*-propanol by the activity of a propanol dehydrogenase. In consequence, NADH is re-oxidized to NAD^+ that can re-enter the glycolytic pathway.

The fate of 1,2-propanediol

1,2-PD could be present in the human gut as an excreted product of anaerobic fermentation of rhamnose (6-deoxy-L-mannose) and fucose (6-deoxy-L-galactose) by *Enterobacteriaceae* (236, 237), *Bacteroides* (238), and *Lactobacillus* species (239). Rhamnose, which is a component of plant cell wall pectic polysaccharides (240) and diverse plant metabolites such as rhamnose-containing flavonoids (241), can be introduced into the gut in form of food plants. Rhamnose can then be released from complex compounds by the activity of rhamnosidases secreted by *Bifidobacterium , Bacteroides* (241-243), and *Lactobacillus* species (244).

Another means by which 1,2-PD can be generated is via metabolism of fucose. Fucose is a common component of HMO (201) and mucin glycans (5) and can be released into the gut lumen by hydrolytic activity of fucosidases. *B. thetaiotaomicron* has been found to be a major contributor to lumen fucose (245, 246). It is capable of regulating epithelial fucosylated glycan synthesis by sensing fucose availability in the gut lumen and inducing the enterocyte production of α1,2-fucosyltransferases when fucose is scarce (247). It also possesses extracellular α-fucosidases that cleave fucosylated glycans and release fucose that its own can use as a carbon and energy source as well as a signaling compound and other intestinal microbes can also exploit (248-250).

Rhamnose and fucose are fermented in *E. coli* through parallel pathways (251). Both pathways converge after each methyl pentose is phosphorylated and then cleaved into the same products, dihydroxyacetone phosphate (DHAP) and L-lactaldehyde. The former enters the EMP pathway, while the latter is reduced to 1,2-PD, which is excreted into environment, resulting in the re-oxidation of NADH to NAD^+. A similar pathway has been described for rhamnose metabolism in *B. thetaiotaomicron* (238) and fucose metabolism in *L. rhamnosus* GG (239).

Given that 1,2-PD could be readily available in the human gut through the route described above, its utilization in *L. reuteri* presumably has evolved to favor intestinal colonization of the species. Without this metabolic capability, *L. reuteri* gains only minimal energy from glycan metabolism, which impedes its growth and therefore decreases its fitness in this competitive environment. Furthermore, 1,2-PD metabolism may enhance *L. reuteri* competiveness in several ways.

First, it may promote competitive exclusion against 1,2-PD-fermenting enteric pathogens such as *S.* Typhimurium (235, 252, 253). Second, metabolism of 1,2-PD may favor accumulation and excretion of glycerol-derived reuterin, a potent antimicrobial compound. The latter could otherwise be further reduced to 1,3-propanediol for NADH re-oxidation (254, 255) in the absence of an alternative electron acceptor such as 1,2-PD. Finally, the metabolic product, propionate, has beneficial immunological and physiological roles in host health (256), which may favor the existence of the species.

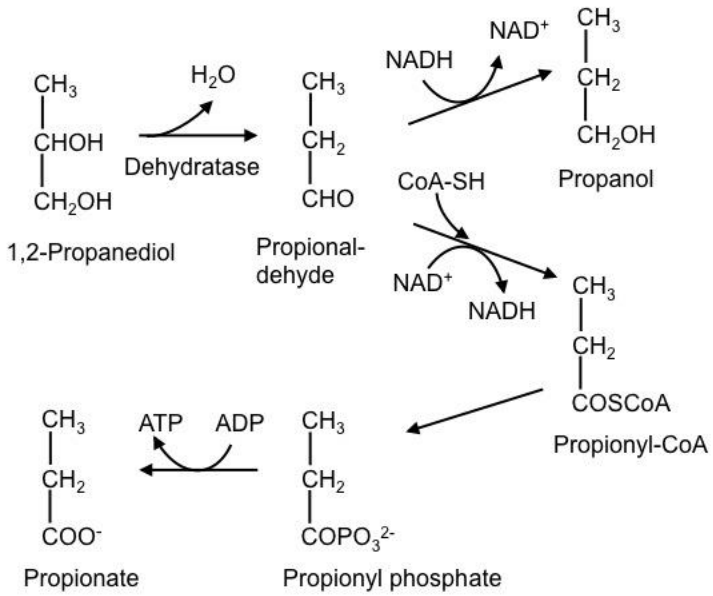

Fig. 1. Proposed pathway of 1,2-propanediol metabolism in *L. reuteri*. (Modified from Sriramulu et al.(233)

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Chapter 2

Quantitative Evaluation of Synbiotic Strategies to Improve Persistence and Metabolic Activity of *Lactobacillus reuteri* DSM 17938 in the Human Gastrointestinal Tract

Quantitative evaluation of synbiotic strategies to improve persistence and metabolic activity of *Lactobacillus reuteri* DSM 17938 in the human gastrointestinal tract

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Abstract

The efficacy of three synbiotic approaches to improve persistence and metabolic activity of the probiotic strain *Lactobacillus reuteri* DSM 17938 in the human gut was determined in a single-blind, randomized, crossover, placebo-controlled trial. Synbiotic preparations consisting of 5 x 10^8 bacterial cells and 2 g of either GOS, rhamnose or the combination of both were given to 15 healthy adults daily for 7 days, followed by 10 days during which only the corresponding carbohydrates were administered. Faecal samples were collected and quantitative real-time PCR was used to determine cell numbers and rRNA copy number. Although *L. reuteri* DSM 17938 was detectable in faecal samples after consumption, the addition of GOS, rhamnose, and the mixture of the both did not increase faecal populations, nor did they enhance persistence after consumption of the probiotic had ended. However, based on rRNA per cell ratios, the combination of GOS and rhamnose significantly stimulated metabolic activity of the *L. reuteri* strain. *In vitro* growth experiments revealed a synergistic effect of GOS and 1,2-propanediol (a product of bacterial fermentation of rhamnose in the gut). In conclusion, the synbiotics used in this study did not enhance establishment and persistence of *L. reuteri*, but did increase metabolic activity.

Introduction

The human gastrointestinal tract is colonized by a complex microbiota that plays a critical role in health and disease predisposition (1, 2). Aberrations of the gut microbiota have been associated with a large number of chronic inflammatory, autoimmune, and metabolic diseases (3, 4), as well as diseases with unknown etiology, such as infant colic (5). These connections provide a rationale for modulating the gut microbiota to redress aberrancies and imbalances linked to human disease (6). Current strategies by which the human gut microbiota can be modulated include probiotics (7, 8), prebiotics (9, 10), and synbiotics (9). The latter are comprised of both probiotic organisms and prebiotic ingredients. According to Kolida and Gibson (11), synbiotics can be either complementary or synergistic. For complementary synbiotics, the prebiotic is chosen based on its ability to stimulate selected members of the gut microbiota, independent of the probiotic. In contrast, for synergistic synbiotics, the prebiotic is selected on the basis of its ability to enhance survival and growth, *in vivo*, of the specific probiotic. Thus, combining probiotics with established health benefits with prebiotics that enhance ecological performance and activity of the specific strains could be a promising strategy to improve health outcomes.

The species *Lactobacillus reuteri* is an inhabitant of the vertebrate gastrointestinal tract and has a long history of being used as a probiotic (12). Probiotic *L. reuteri* have been shown to have strain-specific health benefits in human trials (13), especially in the prevention of diarrhea (14-18), improvement of cholesterol metabolism (19, 20), and the reduction of infantile colic (21-23).

The health benefits are especially well established for *L. reuteri* ATCC55730 (SD2112) (16, 21, 24-27), and its plasmid free derivative DSM 17938 (14, 18, 28-30). Among others, *L. reuteri* DSM 17938 has been shown to reduce the incidence of NEC in neonates (31) and daily crying time in infants suffering from infantile colic (22, 23, 32-34).

L. reuteri ATCC55730 possesses an array of adaptation factors that potentially confer resistance to physiologic stresses (low pH values, bile acids) during the passage through the GI tract (35, 36). Survival of *L. reuteri* 55730 and DSM 17938 in the human GI tract has been demonstrated in several clinical trials (27, 28, 37-40). Transient colonization in the stomach and small intestine of healthy subjects by these strains has also been reported (27). However, colonization in most subjects is only temporal, and even in subjects for whom the strain remained detectable for 4 weeks after consumption, persistence occurred at very low levels (approximately 10^3-10^4 CFU/g feces) (27, 37, 40). This phenomenon is evidently common for other exogenous lactobacilli, which are generally unable to colonize the human GI tract on a permanent basis due to niche exclusion and colonization resistance of the competing microbiota (41).

Although the mechanisms by which *L. reuteri* promotes health are not well understood, it is likely that high levels of metabolically active cells are required. Based on the phenotypic characteristics of *L. reuteri*, several strategies can be envisioned to achieve greater survival, persistence, and activity in the human gut. First, almost all *L. reuteri* strains utilize the prebiotic galactooligosaccharides (GOS) as a growth substrate (42-44), and synbiotics containing GOS would be

expected to enhance, *in vivo,* activity of GOS-fermenting strains. In addition, although *L. reuteri* does not utilize rhamnose, it has been reported to use 1,2-propandiol, a product of rhamnose fermentation by a number of enteric bacteria such as *Escherichia coli* (45) and *Bacteroides thetaiotaomicron* (46), as an energy source (47). Thus, there is a rationale for an addition of rhamnose to *L. reuteri*-containing synbiotics to support *in vivo* growth and activity of *L. reuteri* strains.

The aim of this study was to evaluate the potential of synbiotic formulations containing GOS, rhamnose, and a combination of the two to prolong persistence and promote metabolic activity of *L. reuteri* DSM 17938 in the human GI tract. The effectiveness of this approach was evaluated in a human crossover, placebo-controlled trial. The study revealed that although the prebiotics did not enhance persistence, the combination of GOS and rhamnose had a significant impact on rRNA levels, suggesting an increased metabolic activity of the *L. reuteri* cells.

Materials and methods

Synbiotic and prebiotic preparations. The synbiotic preparations contained freeze-dried powders of the probiotic *Lactobacillus reuteri* DSM 17938 (BioGaia, Sweden) and the prebiotic substrates or placebo (provided by BioGaia, Sweden). All were packaged in daily doses of 5×10^8 bacterial cells and 2 gram of the carbohydrate powders in airtight aluminum foil pouches. The four synbiotic treatments were: (1) *L. reuteri* DSM 17938 with GOS (Purimune GOS powder

containing 90-92% GOS, GTC Nutrition, USA); (2) *L. reuteri* DSM 17938 with rhamnose (L-(+)-rhamnose monohydrate, 99%, Symrise Bioactives, Germany); (3) *L. reuteri* DSM 17938 with GOS (1 g) and rhamnose (1 g); and (4) placebo control containing *L. reuteri* DSM 17938 and maltodextrin (Maldex G120, Syral, France). Prebiotic-only treatments consumed during the test of persistence period (see below) were also prepared in the same manner to deliver 2 g/d. All study products were kept refrigerated at all times up to the point of consumption.

Human trial. The study was performed as a single blind, randomized, crossover and placebo-controlled trial with fifteen healthy individuals. Sample size was estimated based on data from Frese et al. (2012) (48) using the GPower software(49). The study was conducted assuming a power (1-β) of 0.80 and α = 0.05).

The subjects (7 male and 8 female) were recruited on the University of Nebraska-Lincoln campus and were between 20 to 35 years of age. None had been on antibiotics within three months before or during the study, and probiotic foods were not permitted during the study. The subjects were instructed to dissolve the synbiotic/prebiotic powders in a glass of cold water and then consume them immediately in one setting. Subjects were randomly assigned to different successions of the treatments without knowledge of the specific treatment contents. Each treatment period (28 days) consisted of an 11-day run-in/washout period in which a baseline fecal sample was collected at day 11, followed by a 7-day test period in which the synbiotic was administered. At the

last day of this period, a fecal sample was collected, which served as day 0 for the Test of Persistence (TOP) period. During the 10-day TOP period, the prebiotic-only treatment was continued and fecal samples were collected at day 2, 4, 7 and 10. The experimental design is shown in Figure 1. The study was approved by the Institutional Review Board of the University of Nebraska (IRB number: 20111012067EP approved on 10/07/2011).

Analysis of gastrointestinal symptoms. Gastrointestinal symptoms were evaluated over the course of the study using the Gastrointestinal Symptom Rating Scale (GSRS) questionnaire comprised of 14 questions to assess reflux syndrome (heartburn and acid regurgitation), abdominal pain (abdominal pain, sucking sensations in the stomach and nausea) indigestion syndrome (borborygmus, abdominal distension, eructation and increased flatus), diarrhea (loose stools and urgent need for defecation) and constipation (passage of stools, hard stools and feeling of incomplete evacuation). Subjects were requested to rate each symptom on a four-point scale where 0 represents no symptoms. The GSRS questionnaire was filled out at the beginning of each treatment before synbiotic administration (Baseline), after 7 days of daily synbiotic consumption (Day 0 for TOP the persistence test) and after 10 days of prebiotic consumption (Day 10 of persistence test).

Processing of fecal samples. Fecal samples were processed within one hour of defecation. For DNA isolation, a ten-fold dilution of each sample in sterile phosphate-buffered saline (PBS), pH 7.0 was prepared and then subjected to a low-speed centrifugation at 180 x *g* for 5 min (Centrifuge 5810 R with Rotor A-4-62, Eppendorf, Hamburg, Germany). The collected supernatant was centrifuged again at 300 x *g* for 5 min to separate faecal materials. The remaining fecal materials and bacterial cells were then pelleted from the supernatant at 14,000 x *g* for 5 min (Marathon 16KM Microcentrifuge, Fisher Scientific, USA). The pellet was frozen at -80^{o}C for later DNA extraction. For RNA isolation, the fecal sample was immediately mixed with RNAprotect bacterial reagent (Qiagen, Valencia, CA) at a ratio of 1 g feces: 5 ml RNAprotect. After a 5-minute incubation at room temperature, the mixture was centrifuged twice at low speeds, followed by a high-speed centrifugation as described above for DNA isolation. The pellet was stored at -80^{o}C until used for RNA isolation.

DNA and RNA isolation. After washing twice with PBS buffer, pH 7.0 and once with sterile water, cell pellets were resuspended in 100 µl of lysis buffer, and DNA was isolated from the cell suspensions as described by Walter and coworkers (50) with the modification that the DNA solution after cell lysis was extracted three times instead of once with phenol-chloroform-isoamyl alcohol. Total RNA was isolated after the cell pellet was washed once with PBS buffer and resuspended in 100 µl of lysis buffer (30 mM Tris-HCl; 1 mM EDTA, pH 8.0; 15 mg/ml lysozyme; 10 U/ml mutanolysin; and 100 µg/ml Proteinase K). After

incubation at 25°C for 10 min, 350 µl of Buffer RLT (RNeasy Mini Kit, Qiagen) containing 10 µl/ml β-mercaptoethanol was added to the cell lysis. The mixture was then extracted once with 900 µl of acid phenol (phenol: chloroform: isoamyl alcohol [25:24:1], pH 4.3). After incubation at room temperature for 5 min and centrifugation at 14,000 x *g*, 4°C for 5 min, a 300-µl aliquot of the aqueous phase was separated and then extracted with 900 µl of chloroform-isoamyl alcohol (24:1). The aqueous phase was collected following centrifugation at 14,000 x *g*, 4°C for 5 min, and 200-µl aliquot was mixed with 700 µl Buffer RLT and 500 µl of ethanol. The mixture was transferred to a RNeasy Mini spin column. RNA cleanup and on-column DNase digestion using RNase-Free DNase Set (Qiagen) were then performed as described in the protocol of the RNeasy Mini Kit (Qiagen, Hilden, Germany). The purified RNA was subsequently treated with the TURBO DNA-*free*™ kit according to the manufacture's protocol (Applied Biosystems/Ambion, Austin, TX) to remove trace amounts of contaminated DNA. The DNA-free RNA was quantified using Qubit® RNA BR Assay Kit (Invitrogen, Carlsbad, CA) and RNA integrity was observed on a 1% agarose gel.

Reverse transcription. The purified RNA was reverse transcribed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). A 20-µl reaction mix containing 4 µl of 5X VILO™ Reaction Mix, 2 µl of 10X SuperScript® Enzyme Mix, and 4 µl (up to 2.5 µg) of the RNA extract was incubated for 10 minutes at 25°C followed by 30 minutes at 50°C. At the final step, the reaction was terminated by heating to 85°C for 5 minutes. The synthesized cDNA was

subsequently used for real-time PCR quantification of 16S rRNA in fecal samples.

Determination of absolute cell numbers of *L. reuteri* DSM 17938 in fecal sample. Quantitative real-time PCR was used to determine absolute cell numbers of *L. reuteri* DSM 17938 in fecal samples. Strain specific PCR primers 1694f (5′-TTAAGGATGCAAACCCGAAC-3′) and 1694r (5′-CCTTGTCACCTGGAACCACT-3′) were used to detect a chromosome-located gene encoding a strain-specific surface protein (51). SYBR Green-based qPCR analysis was performed using a Mastercycler Realplex2 (Eppendorf, Hamburg, Germany). A PCR reaction mix (25 µl) consisting of 12.5 µl of 2X QuantiFast SYBR PCR Master mix (Qiagen, Valencia, CA), 1 µM of each primer and 1 µl of template DNA was amplified with the following program: 5 min at 95°C, 40 cycles with 10 s at 95°C and 30 s at 62°C. Melting curve analysis was performed thereafter, consisting of a denaturation step of 10 s at 95°C, a following step of 30 s at 62°C, an increase from 62°C-95°C over a 20-min period, and a final step of 10 s at 95°C. Standard curves were generated from DNA extracted from spiked feces containing known cell numbers of *L. reuteri* DSM 17938.

Determination of metabolic activity of *L. reuteri* in fecal samples. To test if probiotic substrates can increase the metabolic activity of *L. reuteri* in the human GI tract, numbers of 16S rRNA molecules were determined based on the premise that the amount of rRNA is indicative of protein synthesis activities essential for

survival and growth of the bacterial cells (52) That is, the higher the number of 16S rRNA copies the cell has, the more metabolically active the organism is. The copy number of 16s rRNA was determined using reverse-transcription quantitative PCR with *L. reuteri* specific, 16S rRNA-targeted forward primer 5′-GTACGCACTGGCCCAA-3′ and reverse primer 5′-ACCGCAGGTCCATCCCAG-3′. Primer specificity was checked with NCBI databases using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and validated using DNA and cDNA of reference strains and baseline faecal samples. The cDNA template was amplified with the following program: 5 min at 95°C, 40 cycles with 10 s at 95°C and 30 s at 65°C. Thereafter melting curve analysis consisted of a denaturation step of 10 s at 95°C, a next step of 30S at 65°C an increase from 65-95°C over a 20-min period, and a final step of 10 s at 95°C was performed. The number of *L. reuteri* 16S rRNA cDNA molecules was quantified using standard curves generated with known DNA amounts of 16S rRNA PCR amplicons. Values were normalized by dividing rRNA amounts by *L. reuteri* cell numbers determined by the strain specific real-time PCR as described above.

***In vitro* fermentation of probiotic substrates.** The ability of *L. reuteri* DSM 17938 to utilize GOS, rhamnose and maltodextrin as growth substrates was determined by measuring growth in MRS in which glucose was replaced by the corresponding carbohydrates. An overnight MRS culture of *L. reuteri* DSM 17938 was transferred to modified MRS (mMRS) broth supplemented with 2% w/v of GOS, rhamnose, the mixture of GOS and rhamnose, and maltodextrin

(from the same batch used in the human trial). The basal mMRS consisted of (per liter) 5 g proteose peptone, 5 g beef extract, 2.5 g yeast extract, 1 g Tween 80, 2.0 g ammonium citrate dibasic, 5.0 g CH_3COONa, 2.0 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, and 0.05 g $MnSO_4$. The cell cultures were incubated at 37°C and the growth was monitored using a spectrophotometer (Biomate3, Thermo Electron Coporation, Madison, WI).

***In vitro* fermentation of 1,2-propanediol.** Utilization of 1,2-PD alone and in the presence of GOS was determined for *L. reuteri* DSM 17938. Cells grown overnight in MRS broth were harvested and washed with phosphate buffer saline pH 7.0 and then resuspended in the same volume of modified MRS (MOD-MRS) without glucose containing (per liter) 5 g Bacto-peptone, 4 g beef extract, 2 g yeast extract, 0.5 ml Tween 80, 1.0 g K_2HPO_4, 3.0 g $NaH_2PO_4.H_2O$, 0.6 g CH_3COONa, 0.3 g $MgSO_4.7H_2O$, and 0.04 g $MnSO_4.H_2O$ (53). The cell suspension was then used to inoculate (1%) fresh MOD-MRS supplemented with 50 mM 1,2-PD and/or 1% w/v GOS. Growth at 37°C under anaerobic conditions (5% CO_2, 5% H_2, and 90% N_2) was monitored by spectrophotometry.

TLC analysis of GOS consumption. *L. reuteri* was grown in mMRS supplemented with 1% GOS (Purimune) at 37°C. After incubation for 24 h, the spent fermentation media was separated from bacterial cells by centrifugation, heated at 95°C for 5 min to inactivate glycosylhydrolase activity, and filter sterilized through 0.22 μm membranes. Then, 5 μl aliquots were spotted onto

high-performance TLC plates (Dynamic Adsorbents, Inc., Atlanta, GA). Control (sterile GOS-supplemented mMRS) samples were also applied. The TLC plate was developed twice with a mixture of n-butanol/ acetic acid, and water at a 2:1:1 ratio. The TLC plate was dried and sprayed with 0.5% α-naphthol and 5% H_2SO_4 in ethanol, and heated at 150^{o}C for 10 min.

Statistical analysis. One-way ANOVA with repeated measures was used to determine the significant difference in absolute cell numbers of *L. reuteri* DSM 17938 between four synbiotic/prebiotic treatments. Wilcoxon matched pair test was used to test for differences in 16S rRNA copies/cell between the treatments and the placebo control.

Results

Utilization of probiotic substrates by *L. reuteri* DSM 17938. The ability of *L. reuteri* DSM 17938 to utilize the carbohydrates used in the human trial as growth substrates was tested in vitro. As shown in Figure 2, the strain grew on GOS but was unable to ferment rhamnose (an indirect substrate) or maltodextrin (a placebo). The result also showed that the mixture of GOS and rhamnose did not provide a growth advantage to the strain compared to GOS alone. TLC analysis revealed that *L. reuteri* DSM 17938 was able to utilize different species of GOS oligosaccharides ranging from DP 2 to DP 6 (Figure 3).

Human trial and tolerance of synbiotic supplements. To evaluate the potential of the different synbiotic approaches to support *L. reuteri* DSM 17938 in the human GI tract, we performed a human crossover, placebo-controlled trial study in which subjects received *L. reuteri* DSM 17938 with either GOS, rhamnose, a mixture of the two substrates, or a placebo (maltodextrin). All fifteen subjects completed the trial without any significant increase in GSRS scores of any gastrointestinal symptom during any of the treatments (data not shown) with all mean scores range from 0 to 1. This result indicates no adverse effect of the treatments on gastrointestinal symptoms.

Determination of absolute cell numbers of *L. reuteri* in fecal samples. As shown in Figure 4A, administration of a daily dose of 5 x 10^8 cells of *L. reuteri* DSM 17938 for 7 days resulted in the strain becoming detectable at 10^8 cells/gram on average at the last day of synbiotic consumption. After the discontinuous of probiotic consumption, cell numbers in fecal samples decreased 10 fold at day 2 and below the detection limit (10^5 cells/gram) in the majority of subjects at day 4 and thereafter (Figure 14A and B). One-way ANOVA analyses showed no significant differences in fecal DSM 17938 populations between the control and prebiotic treatments, indicating that the synbiotic approaches did neither improve establishment nor persistence of the probiotic strain.

Determination of metabolic activity of *L. reuteri* in human fecal samples. *L. reuteri* specific rRNA templates were quantified in fecal samples by qRT-PCR with species-specific, RNA targeted primers, during baseline and at day 0 of the test of persistence. The amount of 16S rRNA templates per cell was determined based on the premise that metabolic activity of the cell is proportional to the number of rRNA molecules per cell. This analysis revealed higher rRNA per cell ratios at day 0 during the treatment with a combination of GOS and rhamnose compared to the control treatment (Figure 5C). Most subjects (11 out of 14) showed an increase ranging from two-fold to 800-fold compared to the control. By contrast, no significant change in *L. reuteri* metabolic activity was found with GOS or rhamnose alone (Figure 5A and B). No *L. reuteri* rRNA was detected during the baseline, indicating that only the probiotic strain was detected using the species-specific PCR approach employed here.

Utilization of 1,2-propanediol by *L. reuteri* DSM 17938. Based on the observed increase in metabolic activity in response to the combination of GOS and rhamnose *in vivo*, we next tested whether GOS and 1,2-propandiol, a potential direct substrate derived from rhamnose fermentation by enteric bacteria, exert a synergistic effect on growth of *L. reuteri* DSM 17938. The *in vitro* fermentation results revealed that the strain was unable to utilize 1,2-PD as a single growth substrate when grown in MOD-MRS (Figure 6). However, a substantial synergetic effect on growth was observed when 1,2-PD was provided

in combination with GOS as demonstrated by a higher growth rate and a significant increase in final cell yield (Figure 6).

Discussion

According to Kolida and Gibson (11), rational selection of synbiotic combinations should be based, in part, on the ability of the prebiotic to “improve the survivability and implantation of the probiotic”. However, it is still unknown if synbiotic strategies can be formulated to achieve this goal as almost all studies that assessed the efficacy of synbiotic preparations to prolong persistence of probiotics did not compare the ecological performance of the probiotic strain in the presence and absence of the prebiotic (54-56).

In this study, we formulated substrate-directed synbiotic strategies with the goal to enhance the persistence and to stimulate metabolic activity of the probiotic *L. reuteri* DSM 17938 in the human gut, and tested the formulations in a human crossover trial. We hypothesized that GOS and/or rhamnose could serve as carbon and energy sources for the growth and metabolic activity of *L. reuteri* DSM 17938 and improve persistence of this strain in the gut. However, our study revealed that the synbiotic approaches did not increase implantation and persistence of the probiotic strain. Our findings are therefore consistent with those of Alander and coworkers (57), who reported no significant differences in the prevalence or numbers of *Bifidobacterium lactis* Bb-12 between the GOS+Bb-12 and Bb-12 group.

Similarly, Tannock et al. (58) also reported that 2.5 g doses of GOS did not cause changes in the microbiota of healthy human subjects when measured by selective culture or nucleic acid-based analysis. In contrast, these authors did report that changes in specific members of the microbiota could be detected using RNA-based methods. Specifically, RNA-amplified denaturing gradient gel electrophoresis (DGGE) profiles were altered in the majority of subjects who had consumed these relatively low doses of GOS.

For our study, GOS was specifically selected for use in the synbiotic combinations due to the ability of *L. reuteri* DSM 17938 to efficiently grow on this substrate *in vitro* (Figure 2). It also had the metabolic capacity to consume oligomers ranging from 2 to 6 monomers (Figure 3). While the *in vivo* availability of GOS constituents in the gut has not been determined, consumption of a wide range of GOS species could provide the strain a competitive advantage over GOS-fermenting residents that have a more narrow substrate preference (59, 60). Despite these *in vitro* results, the GOS-based synbiotic did not enhance persistence of *L. reuteri* DSM 17938 in the GI tract of the adult subjects. Nor was metabolic activity increased in the GOS group compared to the placebo control containing *L. reuteri* plus maltodextrin (Figure 4 and 5A). This neutral impact may be due in part to the low dosage of 2 g GOS powder per day (approximately 1.82 g GOS) that was used in this study. Indeed, results from a previous prebiotic human feeding trial indicated that 5 grams of GOS were necessary to induce a detectable bifidogenic effect (61). However, we used the lower dose in this synbiotic study due to the expectation that co-delivery of GOS and *L. reuteri*

would increase substrate availability to the cell. We also expected that GOS combined with rhamnose would exert a synergistic effect on growth and metabolic activity of the strain.

In the human gut, rhamnose can be fermented by other enteric microbes, generating 1,2-propanediol (46, 62). The 1,2-propanediol can be utilized by human-derived *L. reuteri* DSM 20016 via the dismutation pathway facilitated by *pdu* operon-encoding enzymes, generating one ATP at the expense of NAD^+ with no metabolites diverted into central metabolic pathways (47). Based on these findings, 1,2-PD was suggested to be an energy source for *L. reuteri* (47). However, the pathway is also suggested to regenerate the NAD^+ by conversion of the intermediate propionaldehyde to propanol thus reflecting the role of 1,2-PD as an electron acceptor. Interestingly, *L. reuteri* DSM 17938, which possesses an almost identical *pdu* operon, was unable to utilize 1,2-PD as the sole growth substrate (Figure 6). However, when grown in the presence of GOS, 1,2-PD led to a higher growth rate and significant increase in total cell mass (Figure 6), indicating that the substance can act as an electron acceptor in the presence of fermentable sugar. The role of 1,2-PD as an external electron acceptor is supported by a recent observation in which a favored NAD^+-regenerating flux toward propanol production was detected in *L. reuteri* DSM 20016 during glucose fermentation (63). The influence of external electron acceptors, such as fructose and glycerol, on stimulating growth during sugar metabolism has been already well described in several *L. reuteri* strains (64, 65).

In this human trial, rhamnose was supplemented alone and in combination

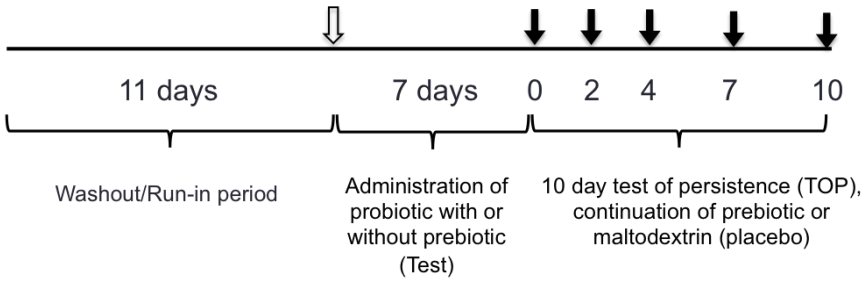
with GOS. As a single substrate, rhamnose had no effect on persistence or metabolic activity (Figure 4 and 5B). Although this result may be due to the low dose used in this study, it is also possible that providing *L. reuteri* with rhamnose as an additional energy source might not be sufficient to facilitate growth, in the absence of additional carbon and nitrogen sources. In contrast, when combined with a carbon source like GOS, the synbiotic preparation contributed to the stimulation of metabolic activity of *L. reuteri* DSM 17938 in most subjects (Figure 5C). This result is consistent with the synergistic effect of the 1,2-PD-GOS mixture observed for *in vitro* growth experiments.

Conclusion

We show here that the well-tolerated 2-gram dosages of direct (GOS) and indirect (rhamnose) growth substrate showed no efficacy in enhancing implantation and persistence of the probiotic strain *L. reuteri* DSM 17938. However, the combination of GOS and rhamnose may increase metabolic activity of the strain in the human gut, as reflected by higher 16S rRNA/cell ratios in fecal samples. This finding is relevant as, if the beneficial effects of *L. reuteri* DSM 17938 require the bacteria to be metabolically active in the gut, enhanced health benefits could be achieved by the GOS/rhamnose approach. Interestingly, Tannock et al. (58) also suggested that while GOS consumption may not result in a numerical increase in bifidobacteria (or other GOS-responding bacteria), GOS may still cause an increase in metabolic activity, as we observed in this study. Clearly, the findings merit further investigations on dose-effect of GOS/rhamnose

on ecological performance of DSM 17938, and the impact of the synbiotic into future human trials that access health outcomes.

A



B

Treatment	Test	TOP
Placebo control	5 x 10^8 CFU/d *L. reuteri* + 2 g/d maltodextrin	2 g/d maltodextrin
GOS	5 x 10^8 CFU/d *L. reuteri* + 2 g/d GOS	2 g/d GOS
rhamnose	5 x 10^8 CFU/d *L. reuteri* + 2 g/d rhamnose	2 g/d rhamnose
GOS/ rhamnose	5 x 10^8 CFU/d *L. reuteri* + 1 g/d GOS &1 g/d rhamnose	1 g/d GOS+1 g/d rhamnose

Fig. 1. (A) Set-up of the synbiotic treatment; White arrow, the sampling point of baseline samples; black arrows, the sampling points during the test of persistence (TOP). (B) Synbiotic and prebiotic preparations used in four treatments. Each of 15 subjects received all four treatments in a random order.

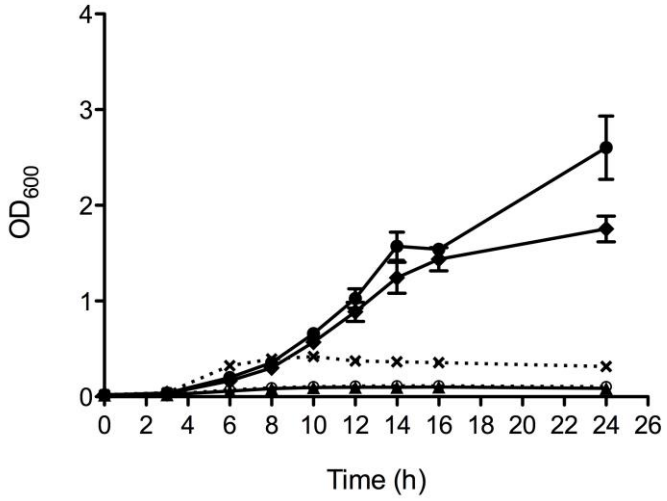


Fig. 2. Growth of *L. reuteri* DSM 17938 on mMRS supplemented with 2% GOS (●); 2% rhamnose (▲); 2% mixture of GOS and rhamnose (◆); 2% maltodextrin (×); and unsupplemented (○). Results are expressed as means ± SD obtained from three independent replicates.

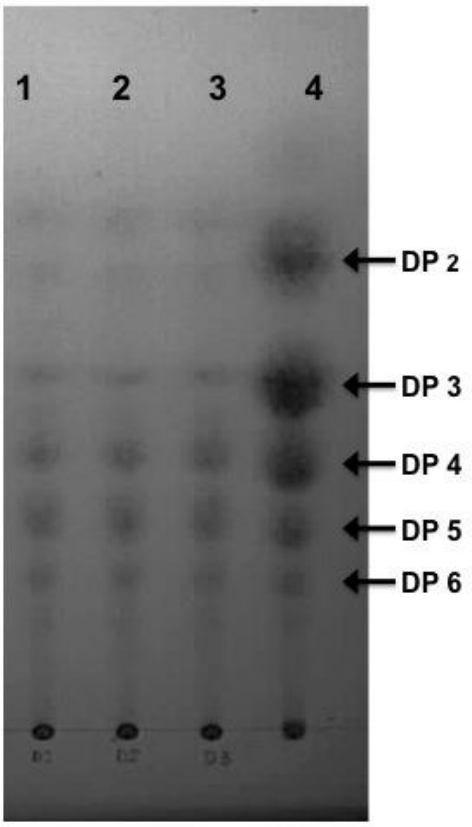


Fig. 3. TLC analysis of GOS consumption by *L. reuteri* DSM 17938. Spent media collected at 24 hour of GOS fermentation (lane 1-3) and a control mMRS + 1% GOS (lane 4) incubated and prepared under the same conditions were analyzed on a TLC plate.

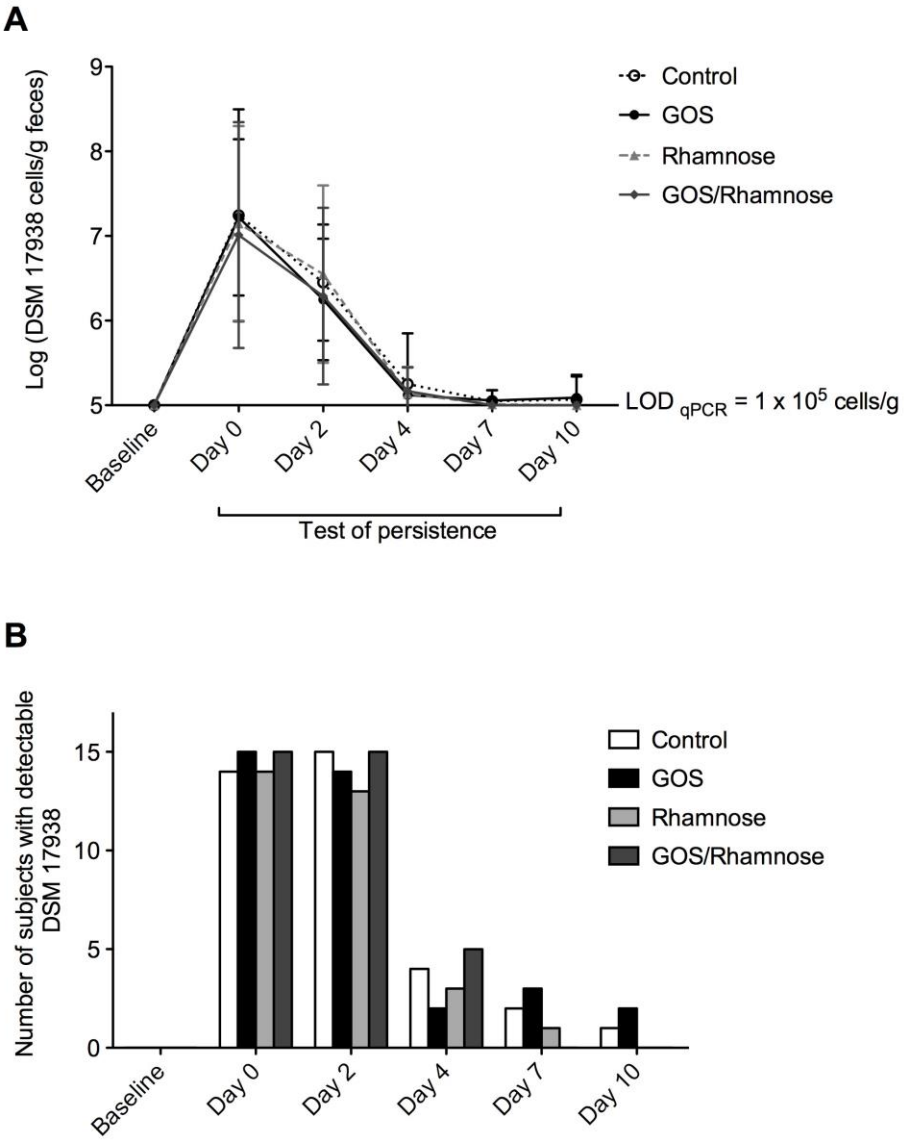


Fig. 4. (A) Changes in mean numbers of fecal *L. reuteri* DSM 17938 cells before and over the test of persistence, determined by qPCR and present as the average $\log_{10}$ cells per gram feces ± standard deviation. Undetectable measurements were transformed to 1 x10^5 cells/ g feces and included in One-way ANOVA analysis. (B) Prevalence of DSM 17938 among subjects with detectable cell levels (> 1 x10^5 cells/ g feces).

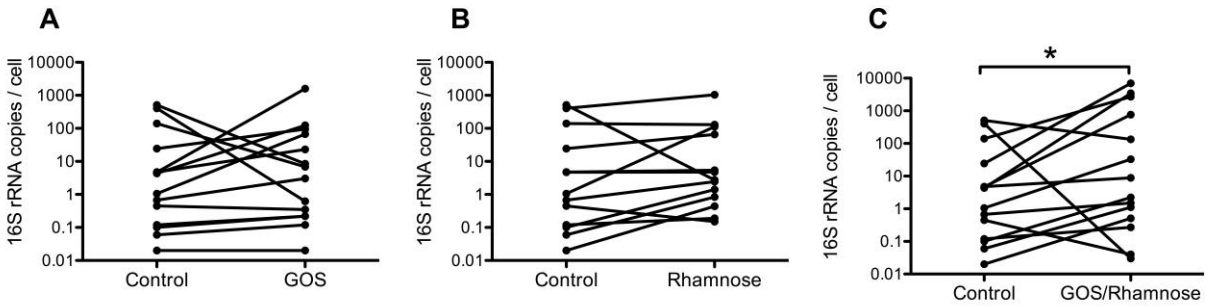


Fig. 5. Changes in *L. reuteri* metabolic activity as determined by reuteri 16S rRNA/ DSM 17938 cell ratios after 7-consecutive-day consumption of *L. reuteri* DSM17938 with GOS (A), DSM17938 with rhamnose (B) and DSM17938 with GOS and rhamnose (C) in comparison to the control (DSM 17938 without prebiotic) (*$p < 0.05$; Wilcoxon matched pair test).

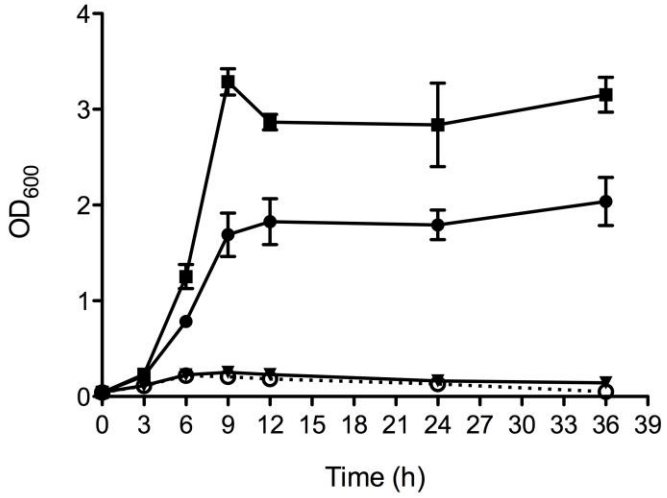


Fig. 6. Growth of *L. reuteri* DSM 17938 on MOD-MRS supplemented with 50 mM 1,2-propandiol (▼); 1% GOS (●); 50 mM 1,2-propandiol and 1% GOS (■); and unsupplemented (○). Results are expressed as means ± SD obtained from three independent replicates.

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Chapter 3

Characterization of Galactooligosaccharide Metabolism in *Lactobacillus reuteri*

Characterization of Galactooligosaccharide Metabolism in *Lactobacillus reuteri*

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Abstract

The ability of probiotic *Lactobacillus reuteri* to produce antimicrobial compounds and secrete immunosuppressive factors has been associated with its metabolic exclusion against enteropathogen infection and anti-inflammatory properties. In this regard, supplementing *L. reuteri* with galactooligosacchride (GOS) to serve as a carbon and energy source during the gut transit could enhance survivability and metabolic activity and consequently prompt the probiotic to engage in beneficial activities. The optimization of GOS metabolism in *L. reuteri* cells requires the knowledge of GOS metabolic machinery and its regulation. Here we characterized such molecular elements and disclosed that GOS metabolism in *L. reuteri* is inducible and under the influence of carbon catabolite repression. The metabolic system relies on LacS permease and a second transporter to import diverse GOS species into the cytosol where two β-galactosidases, GH42 LacA and GH2 LacLM, sequentially break down GOS oligosaccharides as well as concertedly hydrolyze GOS disaccharides. The system is regulated by repressor protein LacR and fully activated only in the presence of inducer lactose and in the absence of glucose. Furthermore, such metabolic system appears to be operational in the gut environment as evidenced by a growth advantage only the wild type strain, but not the GOS metabolic gene-deficient mutant, gained in the GOS-enriched murine gut. The application of these findings in the preparation of GOS-based synbiotics may favor the establishment of *L. reuteri* in the human gut and thereby potentiate its probiotic properties.

Introduction

Interconnection between humans and intestinal microbes has been woven through millennia of coevolution (1, 2). From birth till death, life-long microbial partners profoundly influence our physiology, metabolism, immune function, and defense mechanism against pathogenic organisms. Instantaneously after birth, the first microbial settlers we inherit from the maternal microbiota and/or acquire from surrounding environment rapidly occupy empty niches in the gut (3, 4). Following the interaction with new species and host selective pressures, founder microorganisms diversify and ultimately turn to a dynamic complex microbial community (5, 6), which during our infancy, protects us from detrimental pathogen invasion, assists in intestinal maturation (7), and fosters the development of immune system (8, 9).

At adulthood, our individual gut becomes home to a relatively more structurally stable microbial community of approximately 10^{14} microorganisms (10) categorized into at least 160 bacterial species (11). These symbiotic microbes harvest energy and biosynthesis building blocks from streams of diverse indigestible food particles and host-derived glycans (12, 13). In return, the symbionts fuel our intestinal epithelial cells and peripheral tissues with their predominant fermentation products, short chain fatty acids (SCFAs) (14, 15), which also exert trophic effects on intestinal epithelium (16, 17), reduce colonic epithelial permeability (18), and regulate gut motility and ion absorption (19). Furthermore, SCFAs can interact with G-protein-coupled receptors (GPRs) and inhibit histone-deacetylase (HDAC) activity, thereby enabling gut symbionts to

modulate a wide array of host biological responses, including anti-inflammation, antitumorigenic activities, lipogenesis, and satiety (20-22). In addition to carbohydrate metabolism, the intestinal microbes actively engage in diverse host metabolic pathways, including bile acids, choline, xenobiotic, and drug metabolism (23, 24), and therefore can serve as a major contributor of biologically active metabolites that have a vital role in our health and disease (25).

The gut symbionts are also capable of stimulating antimicrobial protein production by intestinal epithelial cells (26), IgA secretion by B cells (27), and pro-inflammatory T_H17 cell proliferation (28, 29) as well as promoting the development of naïve T cells into anti-inflammatory T_{reg} cells with their antigenic signals (30) and SCFA metabolites (31-33). These immunomodulatory activities set the fundamental role of the symbiotic microbes in our immune homeostasis by engendering the defense system to be tolerant of symbiotic antigens yet responsive to pathogenic invaders (34, 35).

Furthermore, metabolic and immunomodulatory activities of the gut microbial community lay the foundation for direct and immune-mediated colonization resistance against invading species (36-40). Competition for similar metabolic niches (41-43), secretion of bacteriocins (44-47), and modification of the gut into unfavorable environments for pathogen growth and virulence gene expression (48-51) all are antagonistic mechanisms microbial residents directly initiate to protect their gut niches from invading species. Furthermore, their

cellular and metabolic signals can recruit essential host pro- and anti-inflammatory responses to reinforce such protective system (37).

Intricate connection between humans and microbial partners entitles status of the microbial community to drive our health into either healthy or diseased states (52). The gut microbial community that services us with balance energy, activated drugs, and detoxified xenobiotics as well as immune and intestinal homeostasis can shape us into healthy hosts. However, when the community is disturbed to the point that it loses structural stability and, in consequence, cannot properly provide such vital services, this dysbiosis can put us at risk of a wide array of gastrointestinal and extraintestinal diseases (53). Loss in colonization resistance within a collapsing gut microbiota predisposes human hosts to enteropathogenic infection by various pathogens (54-58) and pathobionts such as *Clostridium difficile* (59) and vancomycin-resistant *Enterococcus faecium* (60). Lack of symbiont-mediated immune development in relation to the loss of particular microbial residents such as *Helicobacter pylori* increases risk for allergic diseases (61, 62). Disintegration of host-symbiont mutualism and immune homeostasis is implicated in the pathogenesis of noninfectious intestinal inflammatory disorders such as Crohn's disease and ulcerative colitis (63, 64). Alterations in the gut microbiota that promote gut permeability and metabolic endotoxemia can trigger low-grade chronic inflammation in intestinal and peripheral metabolic tissues (e.g. adipose, muscles, liver, pancreas, and brain) that contributes to the development of

obesity and associated metabolic disorders such as insulin resistance and nonalcoholic fatty liver disease (65-67).

Growing evidence for unhealthy outcomes of the microbial imbalance urges therapeutic approaches to regain its equilibrium states, leading to the emergence of microbiota-targeted therapies as attempts to redress microbial communities from degrading to healthy states or maintain intestinal homeostasis to prevents dysbiosis from emerging in the first place (68, 69). Probiotic is a class of microbe-based therapies that harnesses cellular and metabolic properties of benign microorganisms to promote colonization resistance against the expansion of pathogenic species within the gut microbiota (70-72) as well as modulate host immune responses essential for maintaining gut homeostasis (73, 74). Thus far, probiotics, in a strain-specific manner, have showed promise in treatment of various diseases, including antibiotic-associated diarrhea (75), necrotizing enterocolitis in preterm infants (76), IBD (77, 78), metabolic disorders (79), and nonalcoholic fatty liver disease (67).

In order to support the gut microbiota in maintaining colonization resistance and eliciting essential host immune responses, probiotics need to stay metabolically active to exert competitive exclusion and efficiently produce immune-stimulating metabolites. To establish themselves in the gut, probiotics have to face two major challenges. First, they have to surmount various gut physical barriers and defense mechanisms such as peristalsis, low acidity, bile salts, and antimicrobial peptides as well as host inflammatory immune responses (71). Second, they have to compete with earlier colonizers for nutrients sufficient

for driving metabolic activities, which is apparently a formidable hurdle as both indigenous and pathogenic microbes seem to allow only a minimal, if any, nutrient leak into the gut environment; indigenous inhabitants usually fill available nutritional niches and establish interspecies cross-feeding webs that actively sequester their nutritional metabolites from foreign species (13, 80) and pathogenic settlers have evolved superior mechanisms that allow them to efficiently scavenge limited intestinal resources for growth (58, 81, 82).

A synbiotic concept of closely supply probiotics with fermentable prebiotics to selectively support growth and activity of probiotic components in the gut (83) is therefore a rational approach to cope with nutrient starvation. However, its proof of efficacy in promoting survivability and activity of target probiotics in the gut remains scarce and controversial (84-87) and its translational research is facing a fundamental challenge of how to stimulate probiotics to utilize supplemented prebiotics during their passage through the gastrointestinal tract. These highlight the need for the evidence for the *in vivo* capacity of probiotics to metabolize prebiotics under suboptimal growth conditions to propel the synbiotic concept forward as well as the need for the better understanding of molecular mechanisms underlying prebiotic metabolism, especially how such metabolic processes are regulated, which would be valuable for the formulation of synbiotic preparations that ensure the maximal expression of metabolic machinery for rapid and effective utilization of prebiotic substrates upon their arrival in the gut.

In this study, we sought to identify metabolic and regulatory elements responsible for β-galactooligosaccharide (βGOS) metabolism in probiotic

Lactobacillus reuteri MM4-1A and validate the capability of the strain to utilize βGOS in the gut, which would lay the foundation for synbiotic development to improve therapeutic effects of this probiotic species. Probiotic strains of *L. reuteri* have showed promise in the prophylactic therapy against infantile colic (88-91), NEC in preterm infants (92), and antibiotic-associated diarrhea (93) as well as the treatment of children diarrhea (94, 95), ulcerative colitis (96), and hypercholesterolemia (97) . Although exact mechanisms underlying such beneficial effects remain obscure, the probiotic *L. reuteri* strains inherit a number of traits that could promote colonization resistance against enteropathogen infection and suppress host inflammatory responses. Such traits include the production of biologically active molecules (i.e. antimicrobial compound reuterin (98-100), acetate (101), and propionate (102), the expression of mucus-binding proteins that bind receptor sites on the mucus layer (103), the capacity to induce T_{reg} responses (104, 105), and the ability to suppress pro-inflammatory cytokines through their secretory histamine (106, 107).

The *in vitro* capacity of the probiotic *L. reuteri* strains to ferment βGOS was observed elsewhere (108), but its underlying molecular mechanism has never been experimentally disclosed nonetheless. In this study, we first analyzed genomes of βGOS-fermenting strains in comparison to a non-βGOS-fermenting strain of *L. reuteri* to identify genes potentially involved in βGOS metabolism. We then generated single- and double-gene-deficient mutants and studied their phenotypes afterwards. Different phenotypic analyses allowed us to characterize transporters, β-galactosidases, a regulatory element, and an inducer of the

βGOS metabolism in *L. reuteri* as well as assemble them into a coherent metabolic model. Furthermore, colonization of germ-free mouse gut with a mixture of the wide type and a mutant deficient in βGOS metabolism enabled us to confirm the capability of *L. reuteri* to utilize βGOS in the gut environment.

Materials and methods

***Lactobacillus reuteri* cultures.** *L. reuteri* MM4-1A was obtained from BioGaia AB, Sweden. The strain and its GOS-metabolic-gene-deficient mutants generated in this study were routinely prepared from frozen stock cultures. Stock cultures were streaked onto De Mann, Rogose, and Sharp (MRS, Difco Laboratories) agar plates and incubated at 37°C for 36-48 h under anaerobic conditions consisting of 5% CO_2, 5% H_2, and 90% N_2. Single colonies isolated on the plates were transferred into MRS broth. Cultures were anaerobically cultivated at 37°C for 16-24 h and then subcultured into fresh MRS at 1%. After 12-hour incubation, cell inoculums were ready for the following experiments.

Beta-galactooligosaccharides (GOS) and fractionated GOS components. Purimune GOS provided by GTC Nutrition, USA (now Ingredion, Inc., Westchester, Illinois), was used for phenotypic assays. The GOS powder is comprised of 90-92% GOS with varied degree of polymerization, 7-10% lactose, 0-1% glucose, and 0-0.5% galactose (109). The detailed description of the GOS powder was included in Supplementary Table S1.

Di- and oligosaccharide components of the GOS powder was fractionated and then used for β-galactosidase activity assay and gene expression analysis. Different GOS fractions were separated by size exclusion chromatography performed on a Sephadex G-10 (Sigma-Aldrich) column. For each separation, 5 ml of 30% (w/v) GOS solution was applied to the column (96 x 2.5 cm), and fractions were eluted with Nanopure water at a flow rate of 0.16 ml/min. After the void volume (195 ml) was eluted, 1-ml fractions were collected and immediately monitored for the presence of carbohydrate using a refractometer (Reichert Rhino BRIX30). Approximately 70 fractions were collected from each run. Saccharide compositions were subsequently identified by thin-layer chromatography (TLC) on HPTLC silica gel 60 plates (Merck KGaA, Darmstadt, Germany) developed twice using a solvent mixture containing 50% n-butanol, 25% acetic acid, and 25% water. After the second run, TLC plates were dried at room temperature, sprayed with 0.5% α-naphthol and 5% H_2SO_4 in ethanol, dried again, and then heated at 150°C until separated spots became visible. Fractions containing the desired DP were pooled and then freeze-dried. Freeze-dried products were re-analyzed again by TLC to confirm that purified components were obtained. The TLC analysis of the fractionated products is shown in Supplementary Figure S1.

Genomic analysis for annotated genes potentially involved in GOS metabolism in *L. reuteri*. Genome sequences of four human-derived, GOS-fermenting *L. reuteri* strains, including *L. reuteri* MM4-1A (ATCC PTA 6475), *L. reuteri* ATCC 55730 (SD2112), *L. reuteri* F275 (JCM 1112, DSM 20016), and *L. reuteri* MM2-3 (ATCC PTA 4659) as well as one swine-derived, poor-GOS-fermenting *L. reuteri* ATCC 53608 were compared and searched for genes functionally annotated as β-galactosidases using the Integral Microbial Genome (IMG) Platform (http://img.jgi.doe.gov/) (110). DNA loci surrounding identified β-galactosidase-encoding genes were further examined for neighboring genes annotated with functions related to carbohydrate metabolism. Gene clusters potentially involved in GOS metabolism were then analyzed for the presence of promoter and regulatory elements. Promoter prediction was performed with BPROM (online analysis tool, SoftBerry, Inc., Mount Kisco, NY). A catabolite-responsive element (CRE) was identified using the program DNA-pattern at Regulatory Sequences Analysis Tools (RSAT) website (http://rsat.ulb.ac.be) (111) with a query sequence, WTGNAANCGNWNNCW (112).

Generation of mutants deficient in GOS metabolic genes. Genes predicted to be responsible for GOS metabolism in *L. reuteri* were subjected to targeted point mutations generated by oligonucleotide-mediated recombineering using a protocol described by van Pijkeren and Britton (113). Briefly, recombineering 85-mer oligonucleotides were designed to resemble nucleotide sequences of target genes except for five non-homologous bases at the center where a stop codon

was incorporated. The oligonucleotides were electroporated into competent *L. reuteri* MM4-1A cells expressing RecT ssDNA-binding protein that promotes annealing of oligonucleotides and complementary sequences on the host chromosome. Following the electroporation and cultivation of transformed cells, colonies growing on MRS agar plates were screened for desired mutants by Mismatch Amplification Mutation Assay (MAMA)-PCR (113, 114) using specific primers homologous to mutated gene sequences, but containing five mismatches at the 3′ end to wild-type sequences. Five mutants with a nonsense mutation in a single gene and one with a double-gene mutation were generated in this study. Recombineering oligonucleotides harboring stop codons, targeted gene sequences, and primers for MAMA PCR are listed in Table 1.

Phenotypic confirmation in GOS-metabolic-gene-deficient mutants

Growth on different carbon sources. The ability of the mutants and the wild type to utilize GOS was determined by measuring growth in modified MRS (mMRS) broth supplemented with GOS or other carbon sources. The mMRS medium was devoid of glucose and contained only half amounts of complex ingredients present in standard MRS to minimize the carbon-source content. The basal mMRS consisted of (per litre) 5 g proteose peptone, 5 g beef extract, 2.5 g yeast extract, 1 g Tween 80, 2.0 g ammonium citrate dibasic, 5.0 g CH_3COONa, 2.0 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, and 0.05 g $MnSO_4$. Twelve-hour cultures prepared as described above were used as the source of the inoculum. These cultures were then inoculated at 1% (v/v) into pre-warmed mMRS broth

supplemented with either 1% GOS (GOS-mMRS), 1% lactose (Lac-mMRS), 1% glucose (Glc-mMRS), or a mixture of 0.076% lactose, 0.008% glucose, and 0.003% galactose. The latter represented the approximate amount of contaminating sugars present in Purimune GOS powder. After inoculation, cell cultures were incubated at 37°C under an anaerobic atmosphere. Growth on different carbon sources was determined by optical density measurement at 600 nm (Biomate3, Thermo Electron Corporation, Madison, WI).

GOS utilization. GOS utilization by the *ΔlacS* mutant and the wild type MM4-1A was determined by TLC analysis of spent fermentation media. Log-phase cells were prepared by inoculating 12-hour inoculums into pre-warmed MRS at 1% and anaerobically incubating cell cultures at 37°C for 5 h or until obtaining OD_{600} of 1.5-2.5. Log-phase cells were harvested by centrifugation at 3,220 x *g* for 10 min (Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany), washed twice with PBS buffer (pH 7.4), and resuspended in PBS buffer to obtain a cell concentration at the OD_{600} of 10. Three milliliters of cell suspensions were inoculated into 27 ml of pre-warmed GOS-mMRS so that an initial cell concentration of approximately 10^9 cells/ml (OD_{600} of 1) was obtained. A high cell concentration was used to ensure that the number of the mutant cells was high enough for cell activity to be observed. After an anaerobic incubation at 37°C for 0, 2, 4, 6, 9, 12, and 24h, spent fermentation media were separated from cell cultures by centrifugation and filter sterilized through 0.22 μm membranes. Five-microliter aliquots of spent media were spotted onto HPTLC silica gel plates

(Dynamic Adsorbents, Inc., Atlanta, GA) that were subsequently developed and visualized as described previously.

β-galactosidase activity. *In vitro* hydrolysis was performed using cell-free extracts of the *ΔlacA* and *ΔlacM* mutant to determine the hydrolytic activity of the LacA and LacLM β-galactosidase on di- and oligosaccharide components of GOS as well as lactose. GOS-grown cells of the *ΔlacA* and *ΔlacM* mutant were prepared by inoculating 12-hour inoculums at 1% into pre-warmed GOS-mMRS. After cell cultures were anaerobically incubated at 37°C for 9 h, log-phase cells were harvested by centrifugation at 3,220 x *g*, 4°C for 10 min and washed twice with ice-cold sodium phosphate buffer (PB), pH 6.5. Cell pellets were resuspended in PB with 10% w/v glycerol and 1 mM dithiothreitol (DTT) to obtain a cell concentration at OD_{600} of 10. One-milliliter aliquots of cell suspensions were transferred into ice-cold 2-ml microtubes containing 400 mg of 0.1 mm glass beads (Zirconia/Silica, BioSpec Products, Inc., Bartlesville, OK). Cells were then disrupted with a bead beater (Mini-Beadbeater, BioSpec Products, Inc., Bartlesville, OK) at a maximum speed for three 1 min intervals, each separated by 1 min on ice. Cell-free extracts were separated from cell debris by centrifugation at 14,000 x *g*, 4°C for 10 min. The protein content of cell-free extracts was measured using Qubit Protein Assay Kit (Life Technologies, Grand Island, NY) and adjusted to 0.5 mg protein/ml. Enzyme assay reaction mixtures (200 μl in total) consisted of 20 μl of either 50 mg/ml GOS components or 10 mg/ml lactose, 160 μl PB pH 6.5, and 20 μl of cell-free extracts with 0.5 mg/ml

protein content. Reaction mixtures were incubated in an incubator shaker (Thermomixer R, Eppendorf AG, Hamburg, Germany) at 37°C, 200 rpm for 2, 4, 6, and12 h and then immediately heated at 95°C for 5 min to terminate the reactions. Five- microliter aliquots of reaction mixes were spotted onto TLC plates and analyzed as described previously.

Gene expression analysis

Expression levels of GOS metabolic genes in response to different carbohydrates were quantified in the wild type and the *⊿lacR* mutant to observe the inducibility of GOS gene clusters, identify inducers, and confirm the regulatory role of the LacR protein.

Induction of gene expression. Mid-log-phase cells growing in MRS medium were harvested, washed twice with PBS buffer (pH 7.4), and resuspended in PBS buffer to obtain a cell concentration at OD_{600} of 10. One-milliliter aliquots of cell suspensions containing approximately 10^{10} cells were transferred to 8 ml of pre-warmed basal mMRS (80% water content). Cell cultures were anaerobically incubated at 37°C for 3 h to allow cells to consume all carbon sources present in basal mMRS. After 3 hours, 1 ml sterile water or 1 ml of 10% solutions of either GOS disaccharides, GOS oligosaccharides, lactose, glucose, mellibiose, or lactose (10%) plus glucose (1%) was added to each cell culture. The cultures were allowed to incubate for 30 min and then 1-ml aliquots were mixed with 3 ml of RNAprotect reagent (Qiagen, Valencia, CA) to stabilize RNA.

RNA isolation and purification. After 10-minute incubation in RNAprotect, cells were harvested by centrifugation at 3,220 x *g* for 10 min, washed once with RNase-free PBS buffer (pH 7.4), and suspended in 100 µl of RNase-free lysis buffer (30 mM Tris-HCl; 1 mM EDTA, pH 8.0; 15 mg/ml lysozyme; 10 U/ml mutanolysin; and 100 µg/ml Proteinase K). After incubation at 25°C for 10 min, 350 µl of Buffer RLT (RNeasy Mini Kit, Qiagen) containing 10 µl/ml β-mercaptoethanol was added to the cell lysis. The mixture was then transferred into a 2-ml ice-cold microtube containing 100 mg of acid-washed 30 µm glass beads (Sigma-Aldrich Co., St. Louis, MO). Enzymatically-lysed cells were further disrupted in a bead beater at a maximum speed for 2 min. Homogenized mixtures were then extracted once with 900 µl of acid phenol (phenol: chloroform: isoamyl alcohol [25:24:1], pH 4.3). After incubation at room temperature for 5 min and centrifugation at 14,000 x *g*, 4°C for 5 min (Centrifuge 5424 R, Eppendorf AG, Hamburg, Germany), 300-µl aliquots of the aqueous phase were extracted with 900 µl of chloroform-isoamyl alcohol (24:1) and then 200-µl aliquots were mixed with 700 µl Buffer RLT and 500 µl of ethanol. Mixtures were transferred to RNeasy Mini spin columns. RNA cleanup and on-column DNase digestion using RNase-Free DNase set (Qiagen, Hilden, Germany) were then carried out as described in the manual of RNeasy Mini Kit (Qiagen, MD). Purified RNA was subsequently treated with the TURBO DNA-*free*™ kit according to the manufacture's protocol (Applied Biosystems/Ambion, Austin, TX) to remove trace amounts of contaminated DNA.

DNA contamination in RNA extracts was assessed by real-time PCR quantification of the 16S rRNA gene using *L. reuteri*-specific forward primer 5′-GTACGCACTGGCCCAA-3′ and reverse primer 5′-ACCGCAGGTCCATCCCAG-3′. Twenty five-microliter reaction mixes consisting of 12.5 µl of 2X QuantiFast SYBR PCR Master mix (Qiagen, Valencia, CA), 0.5 µM of each primer, and 1 µl of RNA extracts were amplified with the following program: 5 min of initial heat activation at 95°C followed by 40 cycles of 10 s denaturation at 95°C and 30 s combined annealing/extension at 63°C. Melting curve analysis, carried out thereafter, consisted of 10 s at 95°C, 30 s at 63°C, a step of temperature increase from 63° to 95°C over a 20-minute period, and a final step of 10 s at 95°C. Following the DNA contamination check, 5-µl aliquots of DNA-free RNA extracts were used for total RNA quantification using Qubit® RNA HS Assay kit (Invitrogen, Carlsbad, CA) and RNA integrity was examined on 1% agarose gels.

Reverse transcription and real-time PCR analysis of target gene transcripts. Purified RNA was reverse transcribed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Twenty-microliter reaction mixes containing 4 µl of 5X VILO™ Reaction Mix, 2 µl of 10X SuperScript® Enzyme Mix, and 5 µl (up to 2.5 µg) of RNA extracts were incubated for 10 minutes at 25°C, followed by 30 minutes at 50°C. At the final step, the reaction was terminated by heating to 85°C for 5 minutes.

Synthesized cDNA was used for subsequent relative real-time PCR quantification of GOS-metabolic-gene transcripts. Real-time PCR reaction mixes and the PCR program were set as described previously in the DNA contamination assay except that 1 µl of cDNA products were used as templates rather than RNA extracts. Target and reference genes and their specific primers used in this transcriptional analysis were listed in Supplementary Table S2. Expression levels of target genes in response to different carbon sources were present as fold changes relative to the expression level detected in cells incubated in basal mMRS without an additional carbohydrate. The relative changes in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (115) and *recA* as the reference gene. Expression of the *recA* gene, encoding recombination protein RecA was previously observed and found to be considerably stable in all of the experimental conditions studied.

In vitro **co-culture of the wild type and mutant depleted in GOS metabolic machinery**

In vitro co-culture. An *in vitro* co-culture of *L. reuteri* MM4-1A and the *ΔlacSΔlacM* mutant with impaired ability to utilize GOS was performed to test for the impact of GOS metabolism on the growth advantage of GOS-fermenting strains. Wild-type and mutant cells were harvested from 12-hour inocula as descripted previously. After centrifugation at 3,220 x *g* for 10 min, cell pellets were washed twice with PBS buffer (pH 7.4) and resuspended in PBS buffer to

obtain a cell concentration at OD_{600} of 1. The cell suspensions of the two strains were mixed together at a 1:1 ratio before the cell mixtures were inoculated at 1% into either 1%GOS-mMRS or 1%Glc-mMRS. Cultures were incubated anaerobically at 37°C and then subcultured into fresh media at 1% every 12 h until approximately 28 generations of the wild type on 1%GOS-mMRS was obtained. One-milliliter aliquots of mix cell cultures were collected at 0, 12, 24, 36, and 48 h for future real-time PCR quantification of each cell type.

DNA isolation. Cells were centrifuged, washed twice with 1 ml ice-cold PBS buffer (pH 7.4), and once with ice-cold water, and resuspended in 100 µl of lysis buffer consisting of 20 mg/ml lysozyme, 10 U/µl mutanolysin, and 0.1 mg/ml RNase A in STE buffer [6.7% Sucrose, 50 mM Tris-Cl (pH 8.0), and 10 mM EDTA (pH 8)]. These suspensions were incubated at 37°C for 1 h and then 6 µl of 20% SDS and 5 µl of 15 mg/ml Proteinase K were added into cell lysates. The lysates were incubated at 60°C for additional 30 min, cooled on ice, and diluted with 400 µl of 10 mM Tris-Cl, pH 8.0. Next, cell lysates were extracted three times with 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1). Following the phenol extraction, 250-µl aliquots of aqueous phase were collected and extracted three more times with 500 µl of chloroform/isoamyl alcohol (24:1). Then, DNA was precipitated by mixing 50 µl aliquots of aqueous phase with 375 µl (2.5 volumes) of absolute ethanol and 15 µl (0.1 volume) of 3M sodium acetate and incubating the mixture at -20°C for at least 1 h. DNA pellets were collected by centrifugation at 14,000 x *g*, 4 °C for 20 min, washed once with 1 ml of ice-cold

70% ethanol, and spun down using the same centrifugation condition. After DNA pellets had been air-dried at room temperature, DNA was resuspended in 100 µl of 10 mM Tris-Cl, pH 8.0.

Real-time PCR quantification of wild-type and mutant cells. The population of the wild type and the *ΔlacSΔlacM* mutant in co-cultures was quantified by real-time PCR using primers listed in Supplementary Table S2. Reaction mixes were prepared as described previously in the DNA contamination assay but with 1 µl of DNA isolates as templates instead of RNA extracts. A similar PCR program with a lower annealing/extension temperature was used in this analysis. The program consisted of 5 min of initial heat activation at 95°C followed by 40 cycles of 10 s denaturation at 95°C and 30 s combined annealing/extension at 62°C. Melting curve analysis program consisted of 10 s at 95°C, 30 s at 62°C, a step of temperature increase from 62° to 95°C over a 20-minute period, and a final step of 10 s at 95°C. Standard curves prepared from pure cultures of each strain were used for the absolute quantification.

GOS utilization in the murine gut. To assess ability of *L. reuteri* to utilize GOS in the gut environment, *in vivo* experiments were conducted in germ-free C3H mice. The experimental design is depicted in supplementary Figure S2. Twenty-seven six-week-old female mice were randomly assigned into either a GOS-feeding or control group. Mice were caged in small groups of two to three and fed

with a purified ingredient no-sucrose diet (D12450K DIO series diets, Research Diets Inc., New Brunswick, NJ). The special diet was used in this study to minimize amounts of alternative carbon sources *L. reuteri* strains could possibly utilize in addition to or instead of GOS. While 13 control mice were supplied with sterile Nanopure water, 14 mice in the GOS-feeding group were supplied with sterile GOS solution (30 mg/ml) as the drinking water. GOS feeding was begun 24 hours prior to the inoculation of *L. reuteri* strains to allow GOS to enrich the gut and the amount of GOS consumed was monitored daily.

A mixture of wild type and *ΔlacSΔlacM* cell suspension was prepared immediately before the inoculation. Cells were harvested from 14-hour MRS cultures ($OD_{600} \approx 3$) by centrifugation at 600 x *g* for 10 min and resuspended in PBS buffer, pH 7.4, to obtain a cell concentration at OD_{600} of 1.5 (ca. 5×10^9 CFU/ml). The cell suspension of the wild type and the *ΔlacSΔlacM* mutant were mixed together at a 1:1 ratio. A 100-µl aliquot of the resulting mixture was then orally gavaged to each mouse.

Fecal samples were collected at 12, 24, and 48 h after the. Samples were diluted 10-fold with PBS buffer, pH 7.4 and homogenized by vortexing. One-milliliter or less of fecal suspensions was transferred into 2 ml safe-lock tubes containing 300 mg of glass beads. Fecal pellets were centrifuged at 10,000 x *g* for 5 min, washed twice with 1 ml ice-cold PBS buffer, and once with 1 ml ice-cold water. Washed fecal pellets were mixed with 500 µl of the same lysis buffer described in the *in vitro* co-culture study and incubated at 37°C for 1 hour. Following this step, 30 µl of 20% SDS and 25 µl of 15 mg/ml Proteinase K were

added into cell lysates After incubated at 60°C for 30 min and cooled down on ice for 1 min, cell lysates were mixed with 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1). Cell lysates were further disrupted in a bead beater at a maximum speed for 2 min. Cell homogenates were extracted with 500 µl of phenol/chloroform/isoamyl alcohol two more times and then with 500 µl of chloroform/isoamyl alcohol (24:1) three times. Next, 450-µl aliquots of aqueous phase were mixed with 2.5 volumes of ice-cold ethanol and 0.1 volume of 3M sodium acetate. DNA was allowed to precipitate at -20ºC for at least 1 h before spun down by centrifugation at 14,000 x *g* for 20 min. DNA pellets were washed with 1 ml of ice-cold 70% ethanol, spun down, air-dried, and subsequently resuspended in 100 µl of Tris-Cl buffer, pH 8.0. One-microliter aliquots of DNA isolates were used for absolute real-time PCR quantification of the wild-type and mutant population using the same protocol as described in *in vivo* co-culture study.

Results

***In silico* identification of potential GOS metabolic genes in *L. reuteri* genomes.** Genome analysis based on IMG database revealed that all four human-derived, GOS-fermenting strains harbored a β-galactosidase-encoding gene, *lacA,* which was absent from the genome of the poor-GOS-fermenting strain ATCC 53680 isolated from swine intestine (Table 2). The *lacA* gene encoding an intracellular β-galactosidase [EC:3.2.1.23] assigned to the glycoside hydrolase family 42 (GH42) was situated in the same cluster as the *lacR* and *lacS* (Figure 1), both of which were also missing from the poor-GOS-fermenting strain. The *lacR* and *lacS* genes were annotated in the *L. reuteri* genomes as LacI family transcriptional regulator LacR/lactose operon repressor and GPH family glycoside-pentoside-hexuronide:cation symporter/lactose permease respectively.

In addition to the *lacRSA* gene cluster, the other DNA locus harboring *lacL* and *lacM* gene*,* encoding large and small subunit of a heterodimer GH2-family β-galactosidase was detected in all studied strains with more than 95% identity in amino acid sequences (Table 2). Although present in the poor-GOS-fermenting strain, their functional β-galactosidase activity toward lactose has been previously evidenced in *L. reuteri* strains (116, 117) and their expression in *L. acidophilus* was found to be inducible by GOS (118). Hence the l*acL* and *lacM* were also considered potential GOS metabolic genes in this study. Organization of the two gene clusters potentially involved in GOS metabolism in *L. reuteri* was depicted in Figure 1.

Generation of single- and double-gene-deficient mutants. The functional roles of individual genes in GOS metabolism was determined by generating single- and double-gene-deficient mutants, and then assessing the phenotypes of these mutant strains. Mutants were generated by ssDNA recombineering that introduced a stop codon into the coding region proximate to the start codon, thereby prematurely terminating translation of functional encoded proteins. The presence of an in-frame stop codon in the targeted gene locus was confirmed by DNA sequencing. Five mutants, i.e. *ΔlacS, ΔlacA, ΔlacL, ΔlacM* and *ΔlacR*, harboring a single disrupted gene together with the *ΔlacSΔlacM* with double mutation were generated in this study. All resultant mutants showed no difference in their growth on glucose-mMRS compared to the wild type (Figure 2C), suggesting the absence of pleiotropic effect of the nonsense mutation.

Role of functional proteins in GOS metabolism. To confirm the role of the annotated transporter, β-galactosidases and transcriptional regulator in GOS metabolism, the ability of the *ΔlacS, ΔlacA, ΔlacL, ΔlacM, ΔlacR* and *ΔlacSΔlacM* mutants to utilize GOS were examined. Compared to the wild type MM4-1A, all mutants showed diminished growth on GOS-mMRS, indicating the essential role of operational encoded proteins in GOS metabolism. However, none of the individual gene disruptions completely abolished GOS fermentation (Figure 2A), suggesting that (1) LacS was not a sole transporter for GOS uptake and (2) hydrolytic activity of two β-galactosidases, LacA and LacLM, was not interchangeable.

Phenotypes of the mutants on lactose supported the possibility that a second transporter may be present. The *ΔlacS* mutant grew on lactose at a noticeably slower rate, but eventually reached the same final cell yield as the wild type (Figure 2B). This second lactose transporter may also have affinity for GOS components with DP of 2 [β-D-gal-(1,3)-D-glc; β-D-gal-(1,6)-D-glc; and β-D-gal-(1,4)-D-gal], and therefore enables the *ΔlacS* mutant to partially grow on GOS.

This hypothesis was supported by TLC analysis of GOS components remaining in spent mMRS medium containing GOS as a sole carbon source. The analysis revealed that GOS with DP of 2 was largely consumed by the *ΔlacS* mutant, while the components with DP ≥ 3 remained almost the same after 24-hour fermentation (Figure 3). This result confirmed the presence of the second transporter that has strong affinity for the disaccharide components of GOS.

Based on genome analysis, the second transporter is potentially encoded by a gene [HMPREF0536_1595] also annotated as GPH family glycoside-pentoside-hexuronide:cation symporter. This gene is present in all studied *L. reuteri* strains, including ATCC 53608, and its product and LacS are similar in size (650 vs 640aa) but share only 38% identity in amino acid sequences.

Not only did growth of the mutants on GOS and lactose suggest the cooperation of LacS permease and the second transporter in the GOS uptake system, it also indicated distinct capabilities of two β-galactosidases to hydrolyze GOS. This was demonstrated by the difference in growth profiles on GOS between Δ*lacL/ΔlacM* and Δ*lacA* mutant (Figure 2A). A clear difference in the activity of these two enzymes was observed when the mutants were growing on

lactose (Figure 2B). Whereas the Δ*lacL* and *ΔlacM* mutant completely lost their ability to grow on lactose, the Δ*lacA* grew almost as well as the wild type. This result indicated that the GH2 β-galactosidase, LacLM, is essential for lactose [Gal(β1-4)Glc] degradation, while the GH42 β-galactosidase, LacA, is not. It also raised the question of whether the degradation of other disaccharides present in Purimune GOS, including Gal(β1-3)Glc; Gal(β1-6)Glc; and Gal(β1-4)Gal, also relies primarily on the hydrolytic activity of the LacLM β-galactosidase.

To address this question, the activity of intracellular LacA and LacLM β-galactosidase on the disaccharide components of GOS as well as lactose was determined using cell-free extracts separated from the GOS-grown cells of the *ΔlacM* and Δ*lacA* mutants. TLC analysis of lactose hydrolysates clearly showed that unlike the *ΔlacA*-cell-free extract which was as capable of hydrolyzing lactose as was the wild type, the *ΔlacM* cell-free extract devoid of the functional LacLM β-galactosidase completely lacked the ability to do so (Figure 4A). This TLC analysis confirmed that only LacLM, but not LacA, has cleavage specificity for the β(1-4) linkage between galactose and glucose and suggested that the cleavage of terminal lactose from GOS with DP ≥ 3 be potentially achieved by the activity of LacLM.

Despite losing the capacity to hydrolyze lactose, the *ΔlacM*-cell-free extract still demonstrated an ability to degrade some other forms of GOS disaccharides and consequently release free glucose and galactose into the reaction mix (Figure 4B, lane 13-16). This result suggested that LacA which remains intact in the *ΔlacM* mutant be capable of hydrolyzing either Gal(β1-3)Glc, Gal(β1-6)Glc, or

both, even though it cannot cleave the β(1-4) linkage of lactose [Gal(β1-4)Glc]. Furthermore, the absence of functional LacA in the *ΔlacA*-cell-free extract noticeably diminished hydrolytic activity on the disaccharide fraction (Figure 4B, lane 9-12), emphasizing the indispensable role of the enzyme in the degradation of GOS disaccharides.

Further *in vitro* hydrolysis was conducted using a pool of fractionated GOS primarily containing trisaccharides [Gal(β1-3, 4, or 6)Gal(β1-4)Glc] and tretrasaccharides [Gal(β1-6)Gal(β1-4)Gal(β1-4)Glc]. This fraction of GOS represents the substrate with a Gal-Gal linkage(s) and lactose terminus. The TLC analysis showed that, although disaccharide products were detected in the reaction mix of the *ΔlacA*-cell-free extract, tri- and tetrasaccharide portion remained almost the same throughout the 12 hours of enzymatic reaction (Figure 4C, lane 9-12). This result indicated that LacLM had only minimal hydrolytic activity on GOS with DP ≥ 3. In comparison to the *ΔlacA*-cell-free extract, both tri- and tetrasaccharides in particular were remained to a lesser extent in the reaction mix of the *ΔlacM*-cell-free extract (Figure 4C, lane 13-16), indicating the higher activity of LacA on such GOS components. Moreover, the accumulation of lactose as a hydrolysis product suggested that LacA cleave tri- and tetrasaccharides at Gal-Gal β-linkages and consequently release a lactose terminus, which was resistant to LacA hydrolytic activity, into the reaction mix.

Regulation of GOS metabolic genes. The presence of the *lacR* gene functionally annotated as a LacI family transcriptional regulator/ lactose operon repressor in the upstream region of GOS metabolic genes, *lacS* and *lacA*, (Figure 2) suggesting the regulatory role of LacR. Sequence analysis of the LacR protein sequence for conserved domains using NCBI CD-Search interface (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (119) revealed that LacR harbored a helix-turn-helix (HTH) DNA binding domain and a ligand-binding domain. This raised a hypothesis that the LacR repressor may negatively control the transcription of the metabolic genes by binding to an operator site situated upstream of *lacSA*, and therefore blocking the activity of RNA polymerase. It also suggested that the LacR repressor should be released from the operation site in the presence of an inducer, which binds to the ligand-binding domain and consequently inactivates LacR by altering its conformation. Furthermore, DNA sequence analysis predicted the presence of a promoter region preceding a putative catabolite responsive element (CRE) site, which potentially serves as the operator site of the LacR repressor (120), in the upstream region of *lacSA* and downstream of *lacR* (Figure 1). Such organization of the lacRSA gene locus suggests that GOS metabolism is subject to negative regulation.

To experimentally determine whether the expression of *lacSA* is under the negative regulation, firstly, gene expression analysis in the wild-type *L. reuteri* MM4-1A was performed to assess the inducibility of the *lacSA* gene cluster as well as to identify an inducer(s). Secondly, the constitutive expression of the metabolic gene was determined in the *ΔlacR* mutant to confirm the function of

LacR as a repressor protein. In the wild type, the level of the *lacS* transcript was measured after the mMRS-grown cell had been stimulated by either 1% GOS components, 1% lactose, 1% melibiose [Gal(α1-6)Glc, an αGOS], 1% glucose, 1% lactose plus 0.1% glucose, or no additional carbohydrate. The transcription analysis (Figure 5A) showed that in comparison to the basal level of the *lacS* transcript detected in mMRS-grown cell with no additional carbohydrate added, *lacS* was up-regulated approximately 20-fold exclusively by lactose stimulation, while the transcription at only the basal level was detected in response to other carbohydrates, including GOS disaccharides and components with DP ≥ 3. This finding indicated that the expression of *lacS* and *lacA* is inducible and lactose, but not any other forms of GOS or αGOS, is the inducer that regulates the transcription of such GOS metabolic genes. Furthermore, the transcriptional analysis in the wild type also revealed that metabolism of GOS in *L. reuteri* is controlled by carbon catabolite repression (CCR), as demonstrated by the repression of the *lacS* transcription when glucose coexisted with lactose (Figure 5A).

Unlike the controlled expression in the wild type, no significant difference in the level of *lacS* transcript was detected from the *ΔlacR* mutant in response to different carbohydrates (Figure 5B). The *lacS* gene was expressed at similar levels 13-fold, on average, higher than the basal transcriptional level found in the wild type upon the exposure to the inducer lactose, the CCR repressor glucose, melibiose, and even without sugar. The absence of a functional LacR resulted in a constitutive mutant, confirming the function of LacR as a repressor protein that

controls expression of GOS metabolic genes.

***In vivo* capacity to utilize GOS in the gut.** To assess the ability of *L. reuteri* to metabolize GOS during its transit through the gut, we exploited the *ΔlacSΔlacM* mutant, whose key GOS metabolic machinery was inactivated. The mutant and wild type *L. reuteri* MM4-1A were first co-cultured *in vitro* to determine if GOS metabolism conferred a growth advantage for the wild-type strain. Both strains were co-inoculated in 1% GOS-mMRS and continually subcultured into the fresh medium until the 28th generation of the wild type was achieved. Quantitative real-time PCR analysis using strain-specific primers revealed that the wild type out-competed the mutant and quickly became the dominant strain. Indeed, the mutant was consistently diluted to near extinction at the end of the experiment (Figure 6A). By contrast, no such growth advantage was observed when these strains were co-cultured in 1% glucose-mMRS (Figure 6B). This result established that GOS metabolism confers a significant growth advantage to the wild type in a favorable growth condition rich in GOS.

Next, we examined the *in vivo* capacity of the wild type to utilize GOS in the gut of gnotobiotic mice. We predicted that if the wild type consumes GOS, its population in the GOS-enriched gut should be higher than that in mice fed a control diet (i.e., without GOS), whereas the population of the mutant, which cannot take advantage of GOS, should be similar. In addition, a purified ingredient diet was used to minimize the amount of fermentable sugars that could serve as an alternative carbon source and consequently could obscure the

impact of GOS. The fecal population of both strains was quantified by real-time PCR. As predicted, the wild type colonized at higher levels in the presence of GOS, while no significant difference between populations of the mutant was observed (Figure 7). This result showed that the wild type *L. reuteri* was capable of utilizing GOS in the murine gut environment.

Discussion

Probiotic microroganisms can improve host health by one of several mechanisms. They can contribute to colonization resistance and modulate immune activities through the production of biologically active metabolites, such as antimicrobial compounds and SCFAs (121-123). Probiotic bacteria may also compete directly with enteropathogens for nutritional and physical niches (72, 73, 124). These modes of action generally require metabolically active cells to exert the effects. However, nutrient sequestration by indigenous microbes could greatly deplete nutrient availability in the gut (13, 80, 81), thereby restraining probiotic organisms from engaging in beneficial activities. Theoretically, supplying probiotics with fermentable prebiotics in the form of synergistic synbiotics could promote survivability and enhance metabolic activity (83). Accordingly, development of appropriate synbiotic preparations requires knowledge of the molecular mechanisms responsible for metabolism of the prebiotic to ensure that the prebiotic substrates can support growth of the probiotic strain, *in vivo*, and ultimately enhance host health.

In this study, we identified the transport and metabolic genes and pathways used by *L. reuteri* to utilize the prebiotic, GOS. We first compared the genome of four human-isolated strains exhibiting strong GOS-fermenting capacity with that of a swine isolate deficient in GOS utilization. We also identified and compared genes encoding for putative β-galactosidases within these genomes. Five potential genes were subsequently identified (Table 2) and mutants harboring in-frame nonsense mutations in those genes were generated.

Phenotypic analyses of the resulting mutants confirmed that the LacS permease, GH42 β-galactosidase (LacA), and GH2 heterodimeric β-galactosidase (LacLM) were necessary for GOS metabolism in *L. reuteri.* The growth of the *ΔlacS* mutant on GOS, although impaired, was not completely abolished (Figure 2A), thereby indicating that the LacS permease was not the sole GOS transporter in *L. reuteri.* Moreover, the absence of disaccharide species in the spent fermentation media of the mutant (Figure 3) suggested that a second transporter may import GOS disaccharides (including lactose). Indeed, genome analysis revealed the presence of another *lacS* gene [HMPREF0536_1595] predicted to encode GPH-family LacS permease and share 38% amino acid homology with the *lacS* gene characterized in this study.

Cooperative action between the β-galactosidases, LacA and LacLM was also observed. The inability of the *ΔlacM* mutant to grow on lactose (Figure 2B) indicated that the LacLM β-galactosidase had activity toward the Galβ(1 → 4)Glc galactosidic linkage, including the terminal lactose of GOS oligosaccharides. The accumulation of lactose detected in the *ΔlacM*-cell-free extracts on GOS tri- and

tetrasaccharides (Figure 4C, lane 13-16) further supports the hydrolytic activity of LacLM at the terminal lactose. In contrast, LacA does not possess lactose-hydrolyzing activity, which was demonstrated by similar growth on lactose between the *ΔlacA* mutant and the wild type (Figure 2B) as well as the degradation of lactose in the cell free extract devoid of LacA (Figure 4A, lane 8-11). However, intact LacA present in the *ΔlacM*-cell-free extract allowed hydrolysis of GOS tri- and tetrasaccharides and the liberation of galactose and lactose moieties (Figure 4C, lane 13-16), whereas its absence reduced these hydrolytic activities (Figure 4C, lane 9-12). These results thereby suggest the activity of LacA toward Gal(β1→ 3, 4, and/or 6)Gal galactosidic linkages constituting GOS di- and oligosaccharides used in this study (Table S1). Furthermore, the liberation of glucose moieties from GOS disaccharides in the presence of LacA when LacLM was absent (Figure 4B, lane 13-16) and the reduced degradation of some disaccharide species in the absence of LacA (Figure 4B, lane 9-12) suggest potential LacA activity toward Galβ(1 →3 and/or 6)Glc as well as Gal(β1→ 4)Gal linkages.

These observations indicate the cooperative action between LacA and LacLM in hydrolyzing different GOS disaccharides and different β-galactosidic linkages constituting GOS oligosaccharides. Based on the observation that β-galactosidases are exoglycosidases that cleave β-galactosidic linkages at the terminal β-galactosyl residue [E•Gal-R ↔ E•Gal + R] (125), GOS oligosaccharides should be cleaved first by LacA at their terminal β-galactosyl residue, eventually yielding free galactose and terminal lactose moieties. LacLM

with lactase activity then hydrolyzes liberated terminal lactose into galactose and glucose.

The distinct hydrolytic activities of *L. reuteri* LacLM and LacA are congruent with different enzymatic properties between GH2 and GH42 β-galactosidases (EC 3.2.1.23) previously reported. The GH2 β-galactosidases commonly have lactose as their natural substrate (125) and enzymes in this family, including the LacLM and LacZ type, have been frequently characterized in lactic acid bacteria associated with dairy fermentation (126) and in bifidobacteria of human intestinal origin (127). Evidence for GH2 enzyme collaboratively acting on oligosaccharides is present in *Bifidobacterium bifidum* whose extracellular GH2 β-galactosidase (BbgIII) appears to cooperate with a β-N-acetylhexosaminidase (BbhI) in sequential degradation of lacto-*N*-neotetraose [Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc] backbone of human milk oligosaccharides. It has been suggested that, at first, BbgIII, which is active on *N*-acetyllactosamine [Gal(β1-4)GlcNAc] and lactose (128, 129), liberates galactose and lacto-*N*-triose II [GlcNAc(β1-3)Gal(β1-4)Glc]. The enzyme BbhI then hydrolyzes lacto-*N*-triose II into GlcNAc and lactose that is subsequently cleaved by BbgIII into galactose and glucose (129).

In contrast, the GH42 β-galactosidases typically have weak affinity toward lactose and prefer to act on other galactose-containing glycosides (130-132). In *Bifidobacterium adolescentis* GH42 β-galactosidase is highly active toward Gal(β1-4)Gal and Gal(β1-4)Gal-containing oligosaccharides derived from potato galactan (130, 133).

The cooperative action between GH2 LacLM and GH42 LacA in hydrolyzing GOS characterized here in *L. reuteri* is in agreement with other studies. In *B. bifidum* NCIMB41171, the β-galactosidases from these two families appear to preferentially break down different galactosidic linkages and thereby complementarily hydrolyze GOS (128, 132). The *B. bifidum* GH42 BbgΠ is highly active toward Gal(β1-6)Gal and shows capacity to hydrolyze GOS tri- and tetrasaccharides. On the other hand, three GH2 β-galactosidases including two intracellular BbgI and BbgIV and one extracellular BbgIII prefer lactose as a substrate (128, 132).

Gopal et al. (134) were the first to associate the lactose permease/β-galactosidase system, one of the major mechanisms for lactose metabolism in lactic acid bacteria (126), with the ability of lactobacilli strains to utilize GOS. Specifically, they observed that all identified GOS-fermenting strains possess a β-galactosidase. The involvement of the system was also supported by Andersen et al. (118) who employed microarray transcriptome analysis to study GOS metabolism in *Lactobacillus acidophilus* NCFM. They subsequently identified LacS permease and two β-galactosidases, LacA and LacLM, as being responsible for GOS metabolism. In particular, the LacS permease was shown to be the sole transporter for GOS uptake in *L. acidophilus* NCFM. This permease shares 71% amino acid identity with the *L. reuteri* LacS permease. However, in *L. reuteri*, the LacS permease is not the only transporter for GOS uptake. Instead, it cooperates with a second transporter in accumulating GOS disaccharides and lactose as described previously

Further support for the key role of LacS, LacA, and LacLM was reported for *Lactobacillus ruminis* (135). In this bacterium, two operons encode for systems involved in β-galactoside utilization. One includes a *lacIYZ* predicted to encode a LacI-family transcriptional regulator, GPH-family lactose permease, and GH42 β-galactosidase (LacA). A second operon, *lacYZ,* encodes for a putative lactose permease and GH2 β-galactosidase (LacZ). Both of these operons are present only in the GOS-fermenting strain but not in the strain incapable of utilizing GOS and lactose, suggesting their fundamental role in GOS metabolism (135).

In *L. reuteri*, the poor GOS-fermenting strain (ATCC 53608) does not possess the *lacRSA* but still maintains *lacLM* in its genome (Table 2). Not surprisingly, the absence of the former operon limits the transport and hydrolysis of most GOS components, as well as prevents terminal lactose from serving as a substrate of LacLM. These limitations can apparently explain the substantial decrease in GOS-fermenting capacity in the *lacRSA* deficient strain. Gene expression analysis revealed that *L. reuteri* typically expresses a basal level of LacS and LacA (data not showed) and that the *lacSA* operon is inducible (Figure 5A). It is important to note that only 'lactose' but none of GOS di- and oligosaccharides can act as an inducer that relieves repression of mRNA synthesis (Figure 5A). Furthermore, the transcriptional analysis in the *ΔlacR* mutant confirms the function of LacR as a repressor protein whose inactivation completely abolished the repression of the *lacSA* transcription (Figure 5B).

Based on these findings and the presence of a putative operator and promoter site within the *lacRSA* locus (Figure 1), we argue that *L. reuteri* regulates GOS metabolism through the LacR-mediated negative control of metabolic gene transcription (136). Accordingly, in the absence of the inducer lactose, the repressor protein LacR binds to the operator region (*cre* site, *cis*-acting sequence) situated between the promoter region and the *lacS* start codon (Figure 1), thereby preventing the RNA polymerase from initiating DNA transcription. The transcription of GOS metabolic genes occurs only when the inducer lactose binds to LacR and therefore inactivates its DNA-binding capacity.

Gene expression analysis also indicated that GOS metabolism is under carbon catabolite repression (CCR) (137). The presence of glucose prevented the expression of GOS metabolic genes, even though lactose was concomitantly present (Figure 5A). Nevertheless, glucose could not repress gene transcription in the absence of the functional repressor protein LacR (Figure 5B), suggesting that *L. reuteri* elicits CCR through inducer exclusion (137, 138). We postulate that the presence of a preferred carbon source (i.e., glucose) inhibits LacS permease from transporting the inducer lactose, thereby preventing the activation of *lacSA* transcription and therefore GOS metabolism. Inhibition of LacS activity is presumably elicited by phosphorylation-dependent control of an Enzyme IIA-homologous domain present at the C-terminus of LacS, adjacent to the N-terminal carrier domain. Regulation of non-PTS transport systems via PTS-mediated phosphorylation of a IIA domain is well established in lactic acid bacteria (139). In *Streptococcus thermophilus,* for example, a C-terminal IIA-

homologous domain of lactose permease is phosphorylated by the PTS pathway. The phosphorylated IIA domain interacts with the lactose carrier domain, activating the permease for lactose transport (140, 141).

Interestingly, in *S. thermophilus*, the action of β-galactosidase co-expressed with the lactose permease is essential for the control of transport activity and metabolic gene expression. The *S. thermophilus* β-galactosidase hydrolyzes lactose and supplies galactose to the permease for the exchange of external lactose (141). As for the *L. reuteri* β-galactosidase, LacA, its inability to hydrolyze lactose may have evolved to facilitate the transcriptional regulation of GOS metabolic genes, *lacSA*, which require lactose to relieve the action of the repressor protein LacR. Finally, we noted that *L. reuteri* typically expresses two key GOS metabolic proteins, LacS permease and LacA β-galactosidase, at a basal level. This enables the organism to respond rapidly to the availability of GOS.

The phenotypic and gene expression analyses described above allow us to assemble a coherent model for GOS metabolism in *L. reuteri* (Figure 8). Accordingly, GOS is taken up into the cytosol by the activity of two different transporters, i.e., the LacS permease and a second transporter predicted to be another GPH-family symporter. The LacS permease has broad substrate specificity and is responsible for the transport of various GOS components, especially with a DP of 2 - 4, as well as lactose. In contrast, the second transporter appears to have affinity mainly for lactose and GOS disaccharides. In the cytosol, LacA sequentially cleaves GOS oligosaccharides from the terminal

β-galactosyl residue until all galactose moieties and terminal lactose are liberated. The LacLM subsequently breaks down the terminal lactose into glucose and galactose. Both LacA and LacLM also cooperatively hydrolyze internalized GOS disaccharides by potentially targeting different β-galactosidic linkages.

This metabolic activity is regulated at the level of LacS transport activity and at the level of LacS and LacA expression. The GOS metabolic machinery is fully active only in the presence of lactose and in the absence of glucose or other preferred carbon sources (Figure 8A). In this condition, LacS is presumably activated by PTS-mediated phosphorylation of its IIA-like domain triggered by a high level of PEP. As a result, LacS actively transports GOS and lactose into the cytosol where imported or GOS-liberated lactose binds to LacR repressor and thereby induces the *lacSA* transcription. When glucose is present (Figure 8B), LacS transport activity is inhibited presumably by elevated conversion of PEP into pyruvate and ATP in fast growing cells, which thereby represses LacS-IIA phosphorylation. Accordingly, lactose is excluded from the LacR repressor and *lacSA* transcription is repressed.

Despite having characterized the molecular basis for GOS metabolism in *L. reuteri,* whether or not this machinery is operational *in vivo* (i.e., the gut) has not been established. We therefore addressed this question using a gnotobiotic mouse model. In the model, the presence of GOS significantly stimulated the growth of the wild-type strain, while no such effect was observed in the *ΔlacSΔlacM* mutant deficient in GOS-fermenting capacity (Figure 7A and B). This

result thereby indicates the ability of *L. reuteri* to utilize GOS in the gut. However, the wild type did not quickly displace the mutant in the presence of GOS (Figure 7C) as it did when co-cultured with the mutant in GOS-containing media (Figure 6). Although a purified mouse feed was used to minimize the amounts of alternative fermentable carbohydrates, it seems likely that there were sufficient carbohydrates present in this diet to minimize any effect the GOS mutation may have had. Interestingly, when mice were fed with a standard chow diet (LabDiet 5K67), the wild type appeared to outcompete the mutant regardless of GOS (Supplementary Figure S3), suggesting specificity of the GOS-metabolic system for other substrates. Such substrates may be beta-galactans that could be derived from plant ingredients (142) and be the potential targets of the GH42 β-galactosidase (130).

The ability of probiotic strains to metabolize GOS (or other prebiotics) in the gut environment is an important criterion for development of rational, synergistic synbiotics (83). However, to successfully complete against other intestinal microbes (which may also utilize GOS (143, 144)), the targeted probiotic must express uptake systems and catabolic enzymes necessary for GOS metabolism. Based on the results obtained in this study, expression of these systems requires particular conditions, namely the presence of lactose and an absence of glucose. Hence, translational research aimed at using metabolically active cells of *L. reuteri* to promote host health should also consider how the *L. reuteri*-GOS mixture is prepared, so that GOS metabolism, *in vivo*, is optimized.

Table 1. Recombineering oligonucleotides and MAMA-PCR primers used to generate mutants deficient in potential GOS metabolic genes

Mutant	Oligonucleotide (5′-3′)
ΔlacR	Recombineering: (230) AGTTGTTCTTTACCACTTACCCGATACAATAGAGCAAGAT**gctat**CGGATTGGTTGCTGCTGA GAGTTTTTCTTGTGGTTGTTCC (146) MAMA-PCR primers: Fwd: AGCAACCAATCCG**atagc**/ Rev: ACACCAACACTTAGCGTAT
ΔlacS	Recombineering: (178) TCAACCCAATTAGTTTATTAGCAACCGTTTTACTTAGTCC**ctact**ACATTCCGCTTGTGACAA AGATAATAAAGTAAGTACTCAT (94) MAMA-PCR primers: Fwd: CACAAGCGGAATGT**agtag**/ Rev: CACAGATAACAGCCAATGC
ΔlacA	Recombineering: (167) TTTCCCTCCTGCGGTTCAAGCAAAGCCCATGAAAAAACAT**actag**GTGGCGGAATTTATATCT GCCTGTTTAAATACTTTAATAT (83) MAMA-PCR primers: Fwd: TATAAATTCCGCCAC**ctagt**/ Rev: GCACAACAATCTCATTCCA
ΔlacL	Recombineering: (291) CAAAGTGTTAATGTAATTATTTTGGGCGTAATTATTTAGT**agcta**CTCACTAGGCACTTCGAT TGTTCCAAAACTACTACTATCA (207) MAMA-PCR primers: Fwd: AAGTGCCTAGTGAG**tagct**/ Rev: CGAGCGTATTACCATCATTG
ΔlacM	Recombineering: (228) CGCTGCTAACCACTGAGCAGACCGAATATTAAAGCCACTT**tacta**GTCATTATCAGTGGTTGC CCGCCAAAACGTCGGCATTGGT (144) MAMA-PCR primers: Fwd: GCAACCACTGATAATGAC**tag**/ Rev: GGAACGAGATACTTAGTAACC

- Recombineering oligosaccharides are identical to antisense strands except for five mismatches showed in bold lowercase bases. Underlined bases denote introducing stop codons (TAG), and figures in parentheses indicate annealing sites relative to the first base of the start codons of the target genes.
- MAMA-PCR forward primers are homologous to mutated sequences, but harbor three to five mismatches (in bold lowercase) with wild-type sequences. Arrows illustrates the annealing sites of the forward primers.

Table 2. Similarity among protein sequences encoded by GOS metabolic genes identified in different *L. reuteri* genomes

Locus tag[a] HMPREF0536_	Gene symbol[b]	Annotated gene product	*L. reuteri* strains				
			GOS-fermenting				Poor-GOS-fermenting
			MM4-1A	F275	MM2-3	ATCC 55730	ATCC 53608
659	*lacR*	LacI family transcriptional regulator LacR	100%[c]	100%	100%	95%	absent
660	*lacS*	Glycoside-pentoside-hexuronide:cation symporter	100%	100%	100%	96%	absent
661	*lacA*	GH42 β-galactosidase	100%	100%	100%	95%	absent
317	*lacL*	GH2 β-galactosidase large subunit	100%	100%	100%	98%	98%
316	*lacM*	GH2 β-galactosidase small subunit	100%	100%	100%	96%	95%

[a] Locus tag as assigned in *L. reuteri* MM4-1A genome

[b] Given symbols: *lacR*, *lacS*, and *lacA* as referred to in Andersen et al. (2011); *lacL* and *lacM* as assigned in the Integrated Microbial Genomes (IMG) system

[c] %Similarity in comparison to MM4-1A query sequences

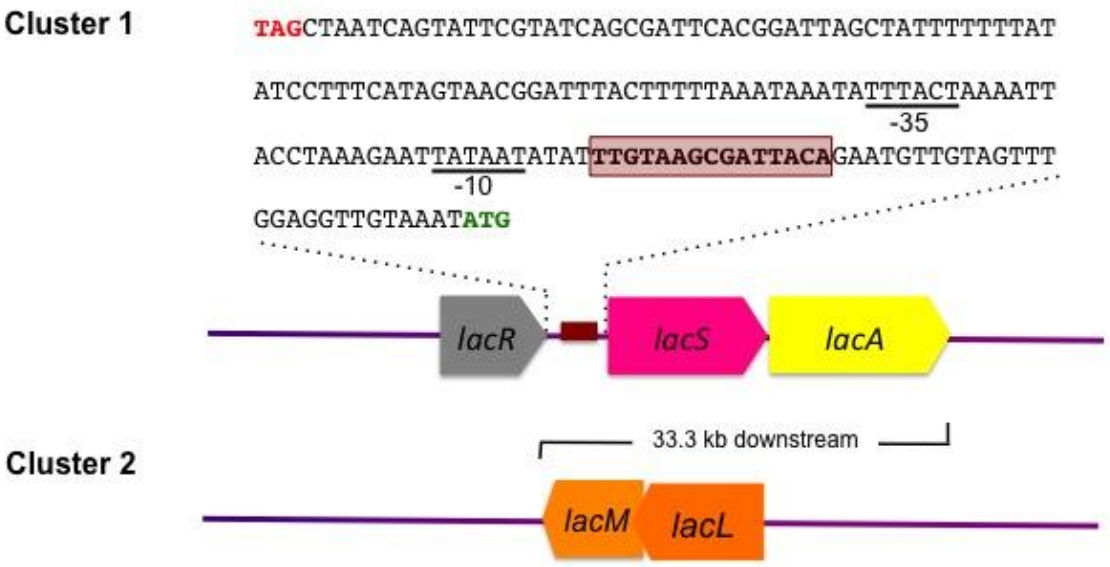

Fig. 1. Organization of two gene clusters potentially responsible for GOS metabolism in *L. reuteri*. The predicted CRE/operator sequence is boxed in red and the predicted -35 and -10 sequence of the σ^{70}-promotor are underlined. The stop codon of *lacR* and the start codon of *lacS* are colored red and green respectively. The *lacLM* gene is located 33.3 kb downstream of the *lacRSA* cluster in *L. reuteri* MM4-1A genome.

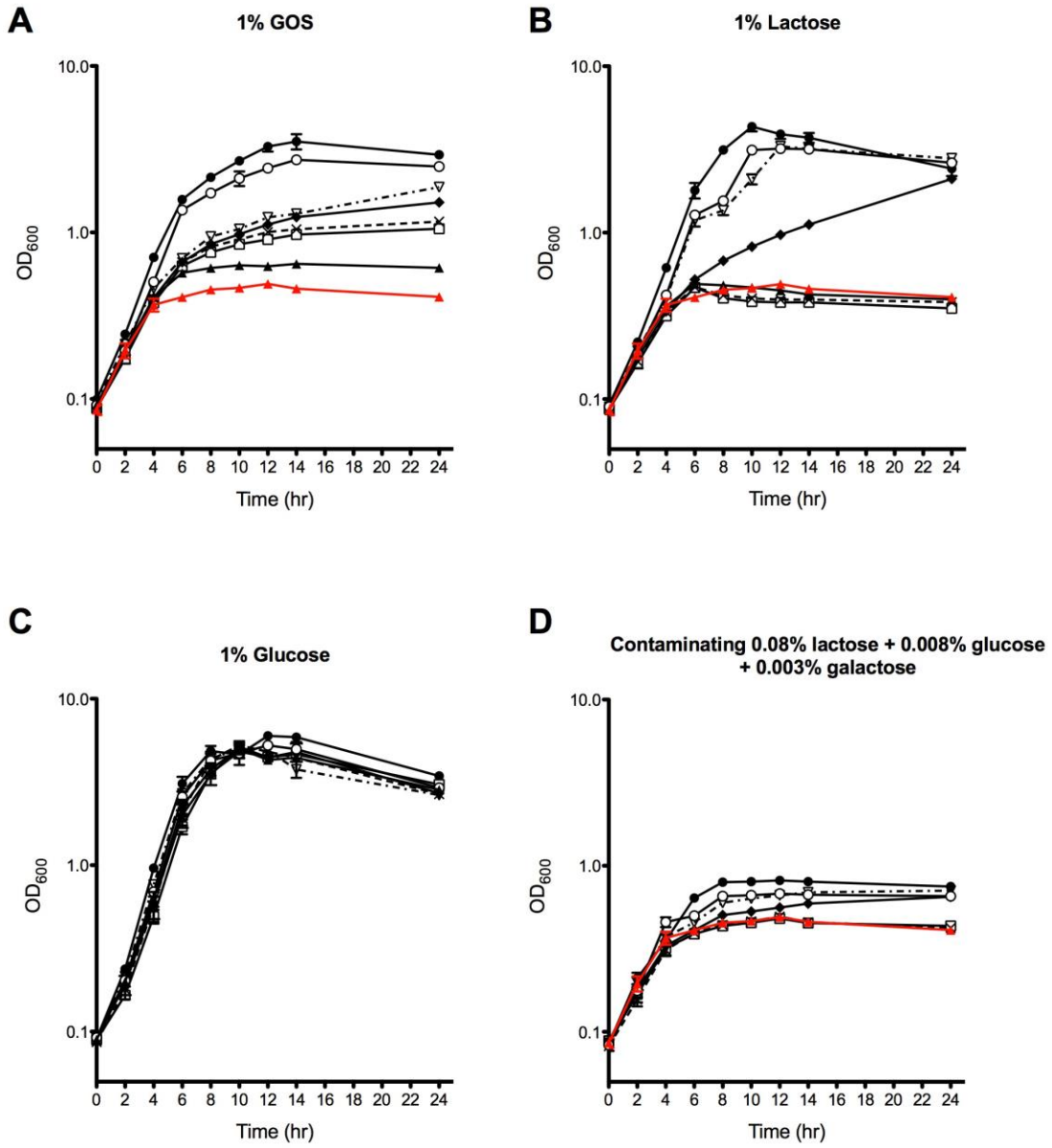

Fig. 2. Growth of wild-type *L. reuteri* MM4-1A (●), *ΔlacR* (○), *ΔlacS* (◆), *ΔlacA* (▽), *ΔlacL* (×), *ΔlacM* (□), and *ΔlacSΔlacM* (▲) on mMRS supplemented with (A) 1% GOS ; (B) 1% lactose; (C) 1% glucose; and (D) GOS-contaminating sugars. The red line represents the minimal growth observed when the *ΔlacSΔlacM* mutant was grown on contaminating sugar-mMRS. Results are expressed as means ± SD obtained from three independent replicates.

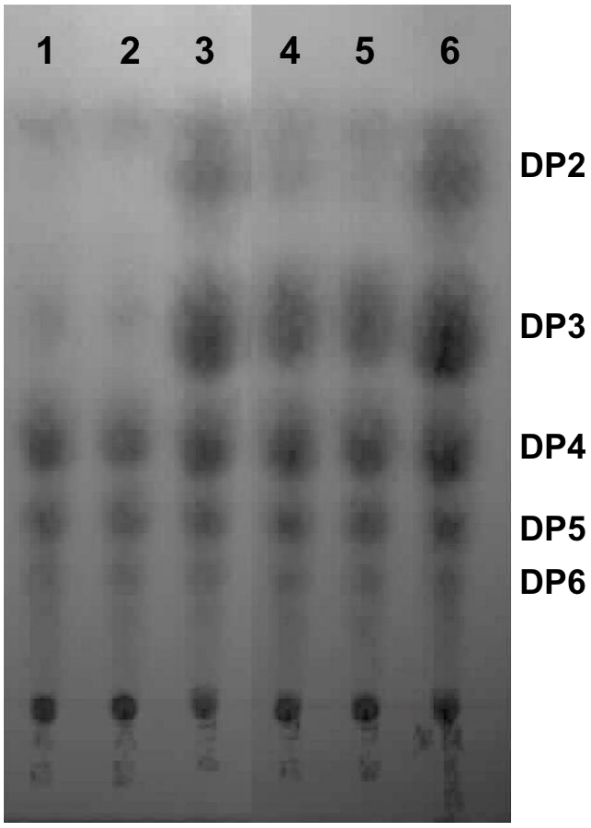

Fig. 3. TLC analysis of GOS consumption by wild-type *L. reuteri* MM4-1A and the Δ*lacS* mutant. Lanes 1 and 2: spent medium of the wild type at 12 and 24 h; lanes 3 and 6: standard 1% GOS-mMRS medium; and lanes 4 and 5: spent medium of the mutant at 12 and 24 h. DP: degree of polymerization

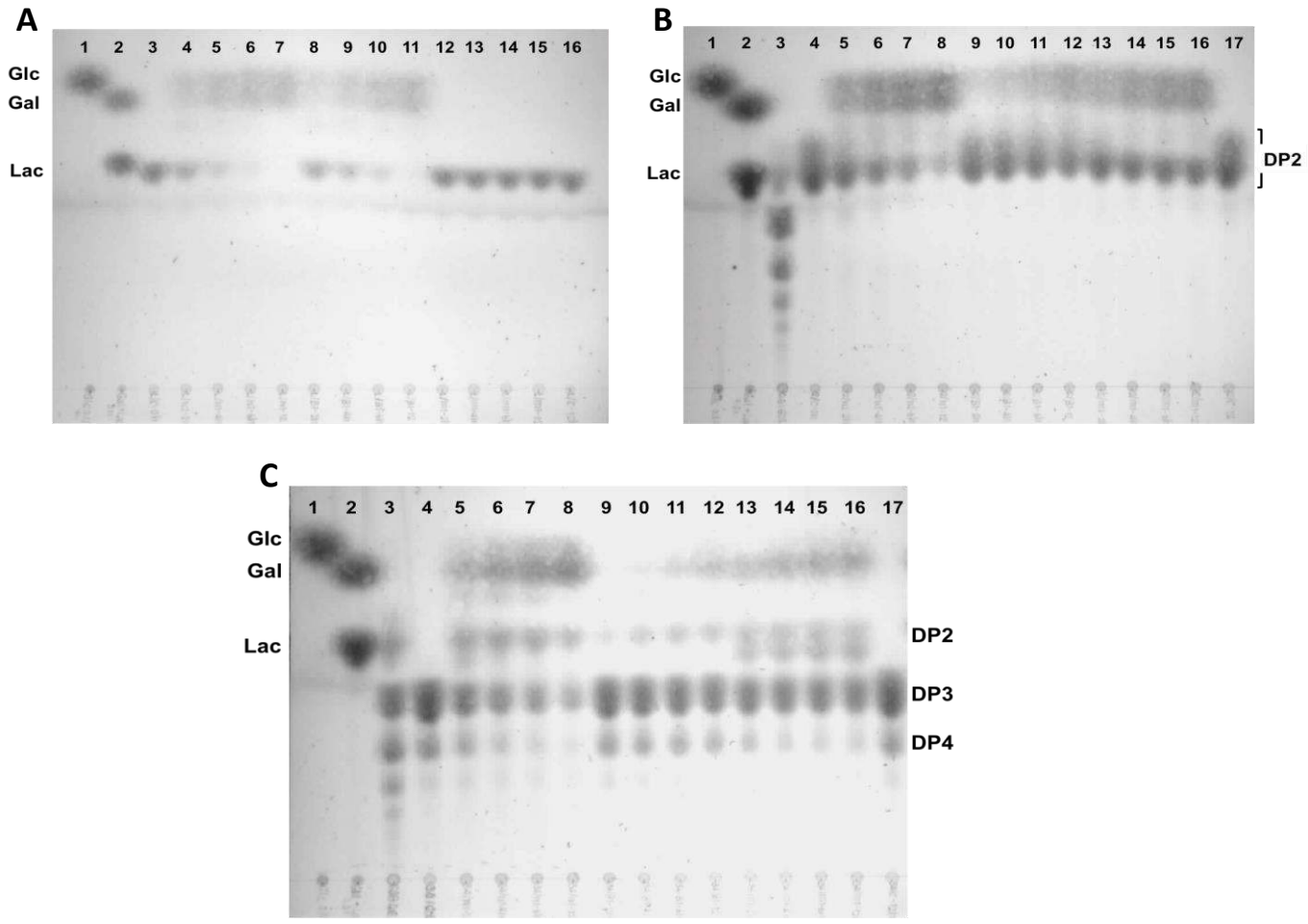

Fig. 4. TLC analysis to determine β-galactosidase activities of cell-free extracts (CFEs) on different β-galactosides. (A) Activity on lactose. Lane 1: glucose; lane 2: lactose and galactose; lanes 3 and 16: lactose in the reaction mix; lanes 4-7: wt-CFEs at 2, 4, 6, 12h; lanes 8-11: *ΔlacA*-CFEs at 2, 4, 6, 12 h; and lanes 12-15: *ΔlacM*-CFEs at 2, 4, 6, 12 h. (B) Activity on GOS disaccharides. Lane 1: glucose; lane 2: lactose and galactose; lane 3: GOS; lanes 4 and 17: disaccharides in the reaction mix; lanes 5-8: wt-CFEs at 2, 4, 6, 12h; lanes 9-12: *ΔlacA*-CFEs at 2, 4, 6, 12 h; and lanes 13-16: *ΔlacM*-CFEs at 2, 4, 6, 12 h. (C) Activity on GOS tri- and tetrasaccharides. Lane 1: glucose; lane 2: lactose and galactose; lane 3: GOS; lanes 4 and 17: oligosaccharides in the reaction mix; lanes 5-8: wt-CFEs at 2, 4, 6, 12h; lanes 9-12: *ΔlacA*-CFEs at 2, 4, 6, 12 h; and lanes 13-16: *ΔlacM*-CFEs at 2, 4, 6, 12 h.

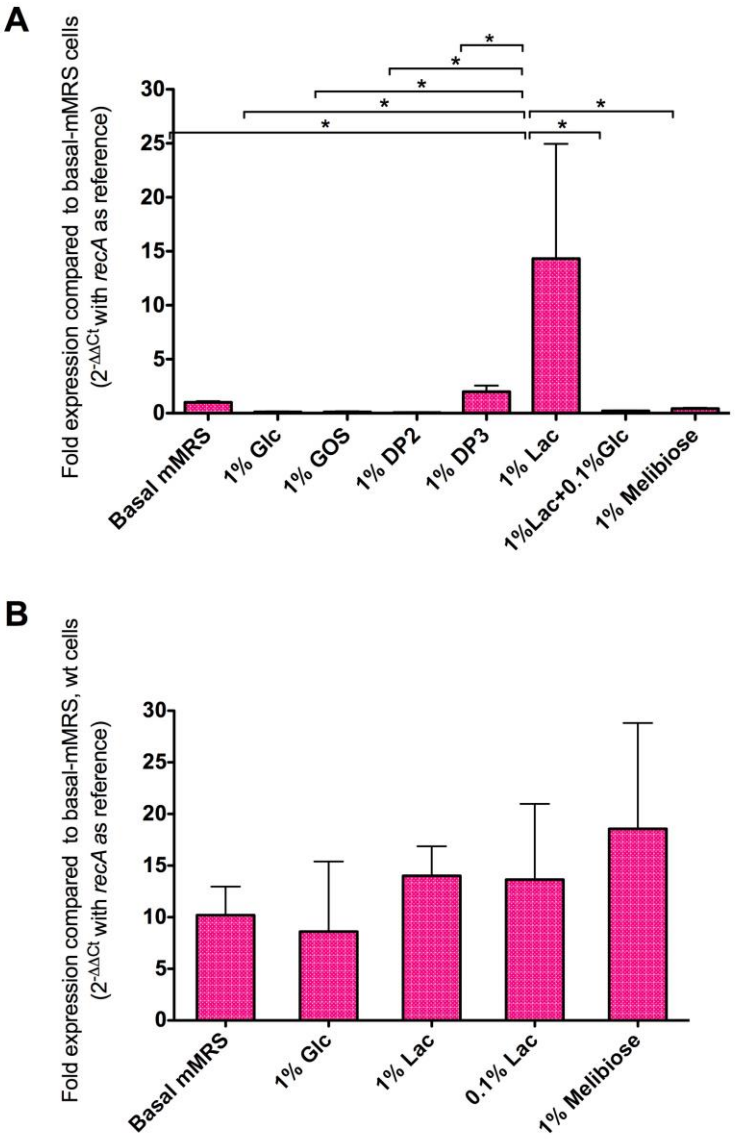

Fig. 5. Relative real-time PCR quantitative analysis of the *lacS* gene transcript in response to different carbon-sources. (A) The *lacS* expression in wild-type *L. reuteri* MM4-1A and (B) in the Δ*lacR* mutant. Glc: glucose, Lac: lactose, DP2: GOS disaccharides, DP3,4: GOS tri- and tetrasaccharides. Results are expressed as means ± SD obtained from three independent replicates. Asterisks denote significant differences ($p < 0.05$) analyzed by Repeated Measures ANOVA with Tukey's post test.

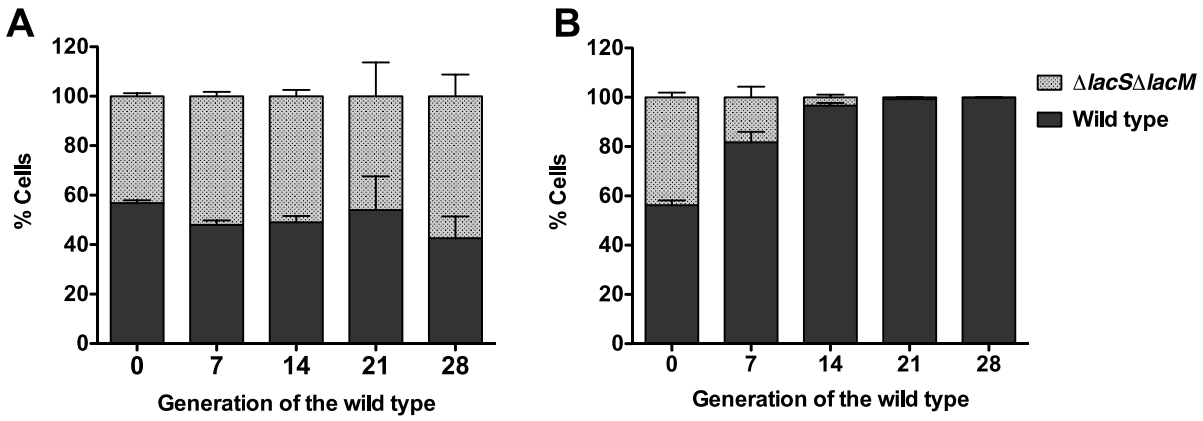

Fig. 6. Proportions of the total *L. reuteri* population comprised of wild-type *L. reuteri* MM4-1A and the *ΔlacSΔlacM* mutant during the *in vitro* co-culture in (A) 1%Glc-mMRS and (B) 1% GOS-mMRS.

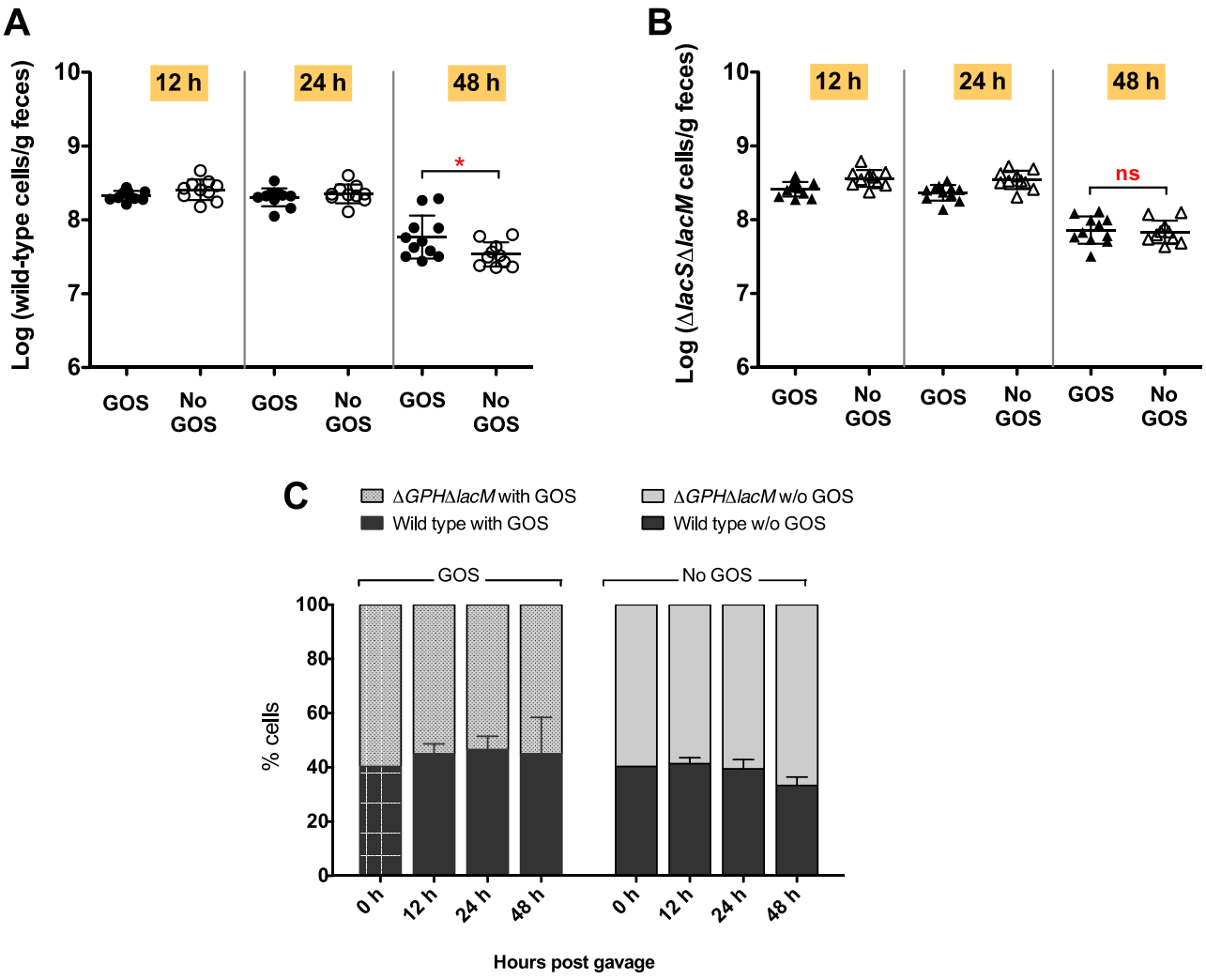

Fig. 7. Changes in fecal populations of wild-type *L. reuteri* MM4-1A and the *ΔlacSΔlacM* mutant, co-inoculated into the ex-germ-free C3H mouse gut. (A) Wild-type populations in GOS-fed mice (●) and in control mice (○). (B) Mutant populations in GOS -fed mice (▲) and in control mice (△). An asterisk denotes significant differences ($p < 0.05$) analyzed by one-way ANOVA with Tukey's post test. Proportion of the total *L. reuteri* comprised of wild-type MM4-1A and the *ΔlacSΔlacM* mutant in the mouse gut in the presence (C: left panel) or absence of GOS (C: right panel).

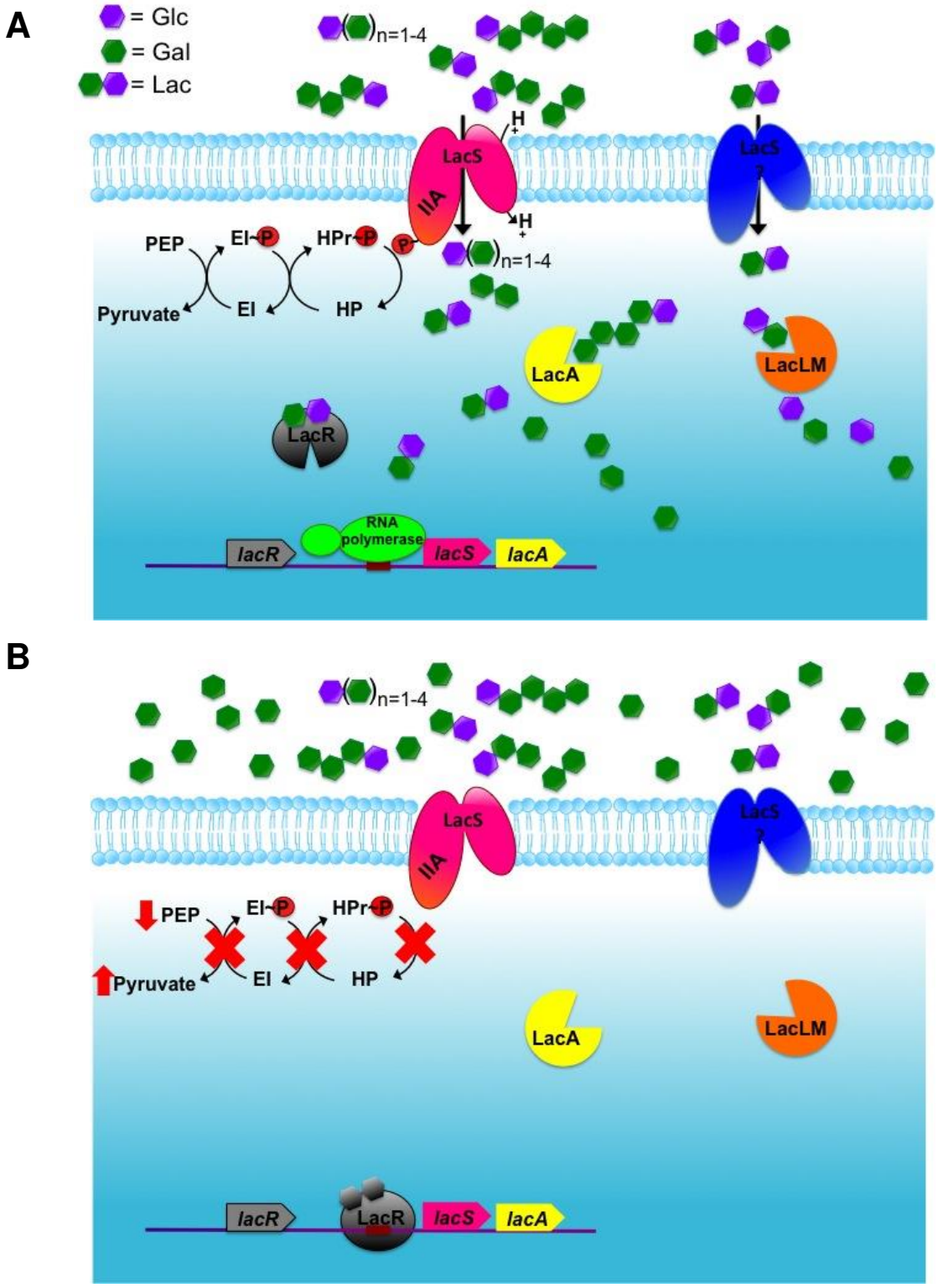

Fig. 8. The proposed model for GOS metabolism in *L. reuteri*. (A) In the presence of lactose and the absence of glucose and (B) in the presence of glucose.

Table S1. Compositions of Purimune GOS powder (109)

Composition		Content (%)
Monosaccharides	Glucose	0.0-1.0
	Galactose	0.0-0.5
Disaccharides	Lactose [Gal(β1-4)Glc]	7.0-10.0
	Gal(β1-3)Glc, Gal(β1-4)Gal	7.0-9.0
	Gal(β1-6)Glc (allolactose)	9.0-12.0
Trisaccharides	Gal(β1-4)Gal(β1-4)Glc	16.0-20.0
	Gal(β1-6)Gal(β1-4)Glc	8.0-13.0
	Gal(β1-3)Gal(β1-4)Glc	14.0-19.0
Tetrasaccharides and higher oligomers	Gal[(β1-6)Gal(β1-4)]$_n$Gal(β1-4)Glc	25.0-29.0

Table S2. Real-time PCR primers used in the study

Experiment	Primer (5′- 3′)	Target
Gene expression analysis	Fwd: TTTCTCGCGCTTCGTTTTGC Rev: TCCTGCAAACATTCCGCTTG	*lacS*
	Fwd: TCCGCCATTCAAACGTTGTG Rev: TTGCCCAATACGTTCGCTAC	*recA* (reference gene)
	Fwd: GTACGCACTGGCCCAA Rev: ACCGCAGGTCCATCCCAG	16S rRNA gene, 16S rRNA
In vitro co-culture and mouse experiment	Fwd: CACAAGCGGAATGTTTGCA Rev: CGGGTCTTCGTATTATCAACAA	Wild-type *lacS* in *L. reuteri* MM4-1A
	Fwd: CACAAGCGGAATGTAGTAG Rev: CGGGTCTTCGTATTATCAAC	Mutated *lacS* in the *ΔlacSΔlacM* mutant

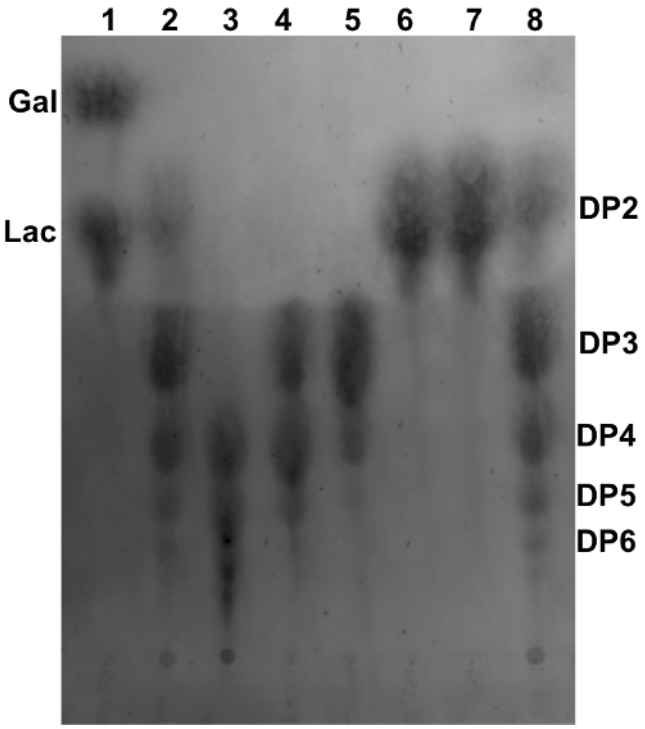

Fig. S1. TLC analysis of freeze-dried GOS fractions separated from Purimune GOS by Sephadex G-10 size exclusion chromatography. Lane 1: lactose and galactose; lanes 2 and 8: standard Purimune GOS; lane 3: fraction with the degree of polymerization (DP) ≥ 4; lane 4: fraction with DP 3-5; lane 5: tri- and tetrasaccharide fraction; lanes 6 and 7: disaccharide fraction.

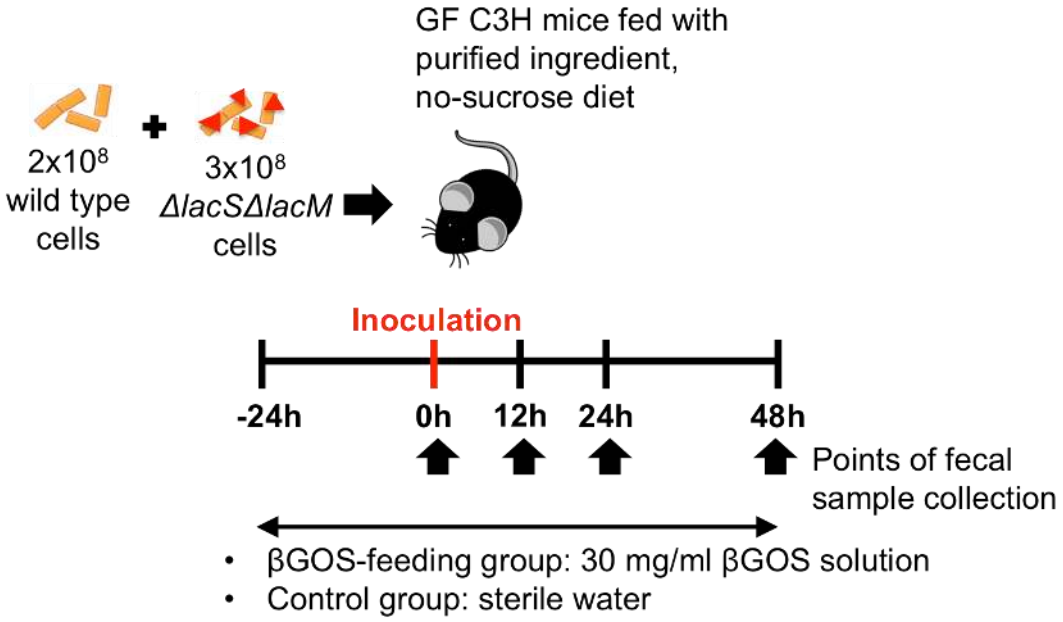

Fig. S2. The experimental design for the *in vivo* determination of capability of *L. reuteri* to utilize GOS in the gut

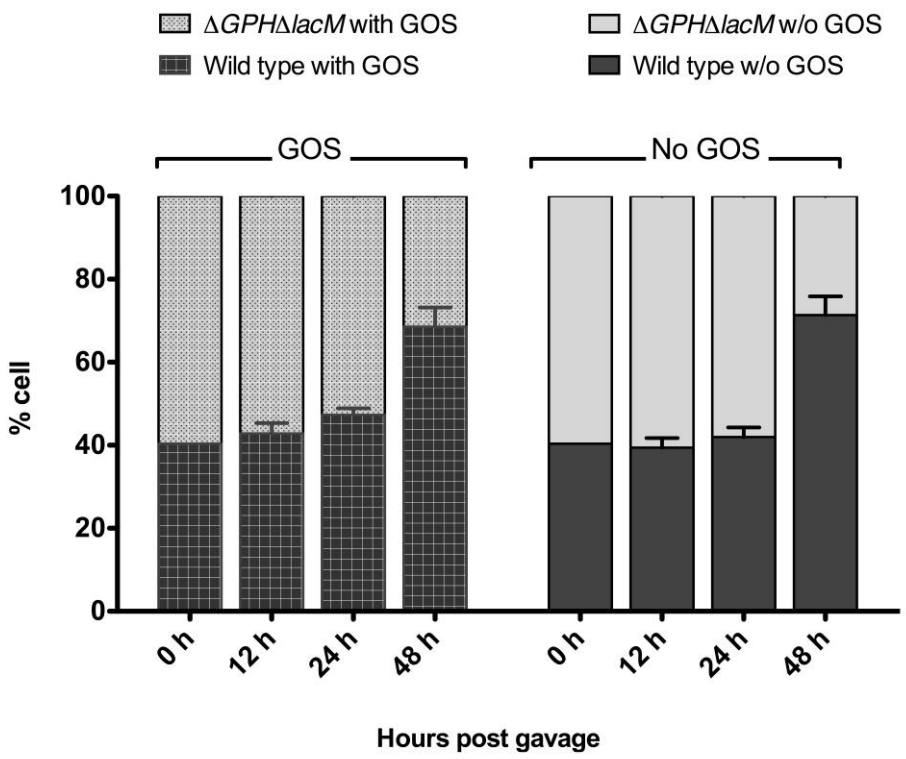

Fig. S3. Proportions of the total fecal *L. reuteri* population comprised of wild-type *L. reuteri* MM4-1A and the *ΔlacSΔlacM* mutant after both strains were co-inoculated into germ-free C3H mouse fed with a standard chow diet with (left panel) or without GOS supplementation (right panel).

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Chapter 4

Conclusion

This study describes the efficacy of GOS and/or rhamnose-based synbiotic approaches aiming to enhance colonization persistence and metabolic activity of probiotic *L. reuteri* in the human gastrointestinal tract. It also describes the metabolic system equipping *L. reuteri* with the ability to ferment prebiotic GOS and regulate such metabolism. Key findings obtained are as the followings.

- In a single blind, randomized, crossover, placebo-controlled human study, the addition of GOS, rhamnose, and the mixture of the both substances did not increase fecal *L. reuteri* populations, nor did they enhance its persistence after consumption of the probiotic had ended.
- Although both *L. reuteri*/GOS (2 g/d) and *L. reuteri/*rhamnose (2 g/d) synbiotic failed to stimulate *L. reuteri* metabolic activity, daily administration of *L. reuteri* DSM 17938 (5×10^8 cells) and the mixture of GOS (1g) and rhamnose (1g) significantly increased metabolic activity of the probiotic in the human gut.
- Correspondingly, GOS/rhamnose-derived 1,2-propanediol (1,2-PD) synergistically enhanced growth of *L. reuteri in vitro*, suggesting the ability of 1,2-PD to complement GOS metabolism as an external electron acceptor or additional energy source.
- The metabolic machinery enabling GOS metabolism in *L. reuteri* was characterized and a coherent model of GOS metabolism and regulation has been assembled.
- According to the model in *L. reuteri* MM4-1A, the metabolic system relies on LacS permease and a second transporter to import diverse GOS species into

the cytosol where two β-galactosidases, LacA and LacLM sequentially break down GOS oligosaccharides as well as concertedly hydrolyze GOS disaccharides.

- Studies on system regulation revealed that GOS metabolism in *L. reuteri* is regulated by repressor protein LacR and subject to carbon catabolite repression (CCR).
- The metabolic system was fully induced only in the presence of inducer lactose and in the absence of glucose (i.e., a preferred carbon source), whereas none of GOS di- and oligosaccharide components showed capacity to induce the GOS metabolic gene transcription.
- This GOS metabolic system appears to be operational in the gut as evidenced by a growth advantage only the wild type strain, but not the GOS metabolic gene-deficient mutant, gain in the GOS-enriched murine gut.
- Interestingly, the wild type strain could outcompete the GOS metabolic gene-deficient mutant when mice were fed with a standard chow diet, irrespective of the presence or absence of GOS, suggesting the specificity of the GOS metabolic system for other carbohydrates.

The findings on the potential of the GOS/rhamnose combination to stimulate *L. reuteri* metabolic activity and the inducibility of the GOS metabolic system could be valuable for the development of an effective synbiotic approach. The use of the lactose-activated *L. reuteri* cells/GOS/rhamnose combination may stimulate cell growth and activity by maximizing cell conversion of supplemented

carbohydrates into energy and carbon skeletons. This synbiotic approach therefore merits further investigation. Furthermore, the additional substrate(s) of the GOS metabolic system is worth characterizing. Such characterization may lead to the discovery of prebiotics that effectively stimulate probiotic activities of *L. reuteri*. Such study may also disclose a true role of the GOS metabolic system in sustaining the existence of this autochthonous bacterium in the human gut.