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Characterization of the Role of Host and Dietary Factors in the Establishment of Bacteria in the Gastrointestinal Tract

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Characterization of the Role of Host and Dietary Factors in the Establishment of Bacteria in the Gastrointestinal Tract

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Probiotic bacteria and synbiotics are used as therapeutic and prophylactic agents. The majority of probiotic and synbiotic applications contain bacterial strains that are allochthonous to the human gastrointestinal (GI) tract. Accordingly, many bacterial strains do not survive digestion, or are not capable of persisting and competing the resident gut microbiota, and are therefore washed out of the GI tract shortly after the treatment is discontinued. This might reduce the health effects of these treatments. Therefore, research is needed to address the ecological challenges that probiotic strains encounter in the GI tract in order to develop probiotic regimens. Determining which ecological factors are limiting the colonization of bacteria remains a challenge. To gain insight into the complex interplay between host and microbe, we chose *Lactobacillus reuteri* and its rodent host as a model to investigate which genes of *L. reuteri* contribute to tolerance towards host gastric acid secretion. We established the urease cluster as the predominant factor in mediating resistance to gastric acid, and a mutation of this cluster resulted in substantially decreased population levels of *L. reuteri* in mice.

Secondly, we established a method to select for synergistic synbiotic combinations. Based on *in vivo* selection (IVS), autochthonous putative probiotic strains are enriched in the GI tract of subjects by the continued consumption of a prebiotic. We

used IVS to select a strain of *Bifidobacterium adolescentis* that became enriched in a human feeding trail with galactooligosaccharides (GOS). Here we have shown that the synbiotic combination of *Bifidobacterium adolescentis* IVS-1 and GOS significantly enriched for the putative probiotic component in rats. IVS-1 became the most dominant operational taxonomic unit in the GI tract, outcompeting the resident *Bifidobacterium* species. Similarly, we tested this synbiotic in a human trial with obese adults. In this random, placebo-controlled parallel arm study, the synbiotic combination of IVS-1 and GOS led to establishment of IVS-1 in significantly higher numbers in the GI tract than a commercial synbiotic.

Together, the studies presented in this dissertation allowed new insights into the colonization factors of a true GI symbiont, which could contribute to the development of improved probiotics, and provided novel insight into a rational selection of probiotics and synbiotics.

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PREFACE

This dissertation is comprised of five chapters. Chapter 1 focuses on the current literature on synbiotic applications, with an emphasis on clinical studies. In particular, the claimed health benefits of synbiotic applications and the implications of recent studies on future design of synbiotics to promote gastrointestinal health are addressed. Chapter 2 describes our published research on the ecological role of genes that mediate acid resistance in *Lactobacillus reuteri* during colonization of the rodent gastrointestinal tract (Krumbeck et al. 2015). In Chapter 3 our published work on the novel concept of *in vivo* selection is introduced, which can be applied to identify bacterial strains that possess enhanced ecological performance in synbiotic applications (Krumbeck et al. 2015). Chapter 4 describes the results of a human clinical trial that applied a synbiotic combination that has been selected by *in vivo* selection. Finally, Chapter 5 provides a conclusion that summarizes the findings provided in this thesis.

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Chapter 1

Recent developments on formulating synbiotics to improve gastrointestinal health.

1.1 Purpose of review

Research on combining probiotics and prebiotics as synbiotics to enhance human and animal health has accelerated in the past ten years. Included are many clinical trials that have assessed a wide variety of synbiotic formulations. In this review, we summarize those recent clinical studies as well as other research and commercial applications of synbiotics. In particular, we address the claimed health benefits of synbiotic applications and the implications of recent studies on future design of synbiotics to promote gastrointestinal health.

1.2 Introduction

The impact of the gastrointestinal (GI) microbiota on the health of humans and animals is now one of the most studied fields in biology and medicine. New discoveries made during the past 20 years have dramatically changed the way that clinicians and researchers associate food and diet with health and disease. While the microbiota that resides in the gut has long been considered as important to health, the methods and techniques necessary to gain an appropriate appreciation of this complex microbial ecosystem have just been developed within the last decade. In addition, how this microbiota interacts with the host and how the composition and activity of a healthy state

microbiota is distinguished from an unhealthy or dysbiotic state had also been experimentally difficult questions to address. While modern techniques now allow the recognition of a dysbiotic microbial state, the identification of cause and effect between a dysbiotic microbiota and a disease phenotype remains challenge.

1.3 Functional importance of the colonic microbiota

The human gastrointestinal tract is colonized by approximately 10^{14} microbial cells, with the majority (10^{11} – 10^{12} per gram) residing in the colon and less than 10^{2} - 10^{3} per gram in the stomach and small intestine (1, 2). This microbiota serves several critical physiological functions. It protects the host from invasive pathogenic microorganisms by competing with them for nutrients and niches, as well as by resistance against infections (3–5). Commensal organisms may also produce a variety of antimicrobial substances, including bacteriocins and other antagonistic peptides and small molecules (6, 7). In addition, the microbiota aids in the development of the adaptive and innate immune system, produces essential vitamins, amino-acids and other metabolites, and facilitates utilization of nutrients, especially polymeric carbohydrates (8). Finally, the microbiota contributes caloric energy to the host. Assuming a typical European diet is consumed, the gut microbiota can potentially yield as much as 140-180 kcal a day via fermentation of the 50-60 g of carbohydrates that escaped host metabolism (9).

The extent and rate of carbohydrate digestion and utilization in humans depends primarily on anatomical location. Initially, complex carbohydrates are hydrolyzed in the mouth via amylases, and starch and glycogen are further hydrolyzed and broken down into sugars, which are absorbed in the stomach. In the large intestine, indigestible substrates,

including various dietary fibers and carbohydrates that were not absorbed in the small intestine, are hydrolyzed and fermented by bacteria. The proximal part of the large intestine is responsible for most of the absorption of the short chain fatty acids (mainly acetate, butyrate and propionate) that are produced by the colonic bacteria from fiber fermentation at a rate of approximately 0.5- 0.6 mole per day (1, 9, 10), depending on the microbiota composition, the nature of the fermentable carbohydrate, and the dietary intake (6).

Short chain fatty acids (SCFA) have several beneficial effects on host health (11, 12). As the most important and preferred energy source for colonocytes (13), SCFA promote epithelial integrity (11). Additionally, SCFA affect the thickness of the mucus layer, support epithelial cell survival, and regulate expression of tight junction proteins (6, 14). Disruption of gut integrity has been attributed to serious intestinal diseases, including celiac disease, inflammatory bowel disease, and colorectal cancer (12, 15, 16). The local and systemic immunomodulatory properties of SCFA include the suppression of NF-κB activity (17–19) and support of increased infiltration of immune cells into the lamina propria. In addition, SCFA have anti-inflammatory properties by modulating immune cell chemotaxis, reactive oxygen species, and cytokine release (11). SCFA also regulate colonic mobility and blood flow and can influence colon pH, which has a direct impact on the uptake and absorption of nutrients and electrolytes (20).

Butyrate formation by the colonic microbiota is of particular interest since this compound has been shown to have multiple biological effects. Butyrate has anti-inflammatory properties, inhibits IL-12 and up regulates IL-10 in monocytes (19, 21). In addition, butyrate has signaling capacities via G-protein coupled receptors (14, 16, 22, 23)

and increases levels of anorectic hormones like PYY and GLP-1, that contribute to energy metabolism and appetite control (6). Butyrate also induces apoptosis of neutrophils (24), and has anticancer activity in several human cell lines (25–27).

The colon offers an especially favorable environment for anaerobic microbes, with high quantities of nutrients that escaped host digestion, a thick mucus layer secreted by a higher number of Goblet cells, reduced intestinal motility, and a favorable pH (28). Since the majority (approximately 70%) of the gut microbiota reside in the large intestine, these organisms may have a profound effect on energy storage, host metabolism, and intestinal health (1). While the microbiota provides many beneficial effects on the host, the composition of an individual's microbiota may also predispose that individual for certain intestinal as well as systemic diseases, including obesity and diabetes. Importantly, the microbiota is not only shaped by host genetic factors and endogenous factors (gastric acid and bile), but also by dietary components that favor specific taxa or groups of bacteria in the colon by promoting their growth or activity. Therefore, establishment of bacteria that are associated with a stable and healthy microbiota may be facilitated by dietary strategies. However, short-term dietary strategies have shown that overall the gut microbiota is exceptionally stable and resilient, since most of the alterations induced to the gut microbiota by dietary treatments are only temporary and the pre-treatment conditions are re-established once the treatment is discontinued (29–31). Still, the dietary approach is now one of the most promising methods for correcting bacterial dysbiosis and restoring homeostasis. In particular, foods and supplements containing prebiotics, or combined with probiotics as a synbiotic, have considerable potential for promoting gut health.

1.4 Addressing intestinal health with pro-, pre- and synbiotics

Among the first dietary products used as intestinal therapeutic agents were probiotics. Indeed, what we now call "probiotics" have been produced and consumed for more than 100 years (32, 33), long before the term was actually defined. Probiotics are currently defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO 2001-2014). There are hundreds of probiotic strains and products in the marketplace, and many clinicians recommend probiotics to patients for a variety of conditions, including antibiotic-associated diarrhea, management of acute gastroenteritis, general gastrointestinal disorders, treatment of mild ulcerative colitis, and for improved lactose digestion (34). While consumers have a general understanding of probiotics (35), the definition itself has been controversial among researchers and regulators (34). It has been criticized, for example, as being too broad or not being broad enough (34). Leaving the discussion about the definition itself aside, it is interesting to note that in Europe, and the U.S., no health claims for probiotic products have been approved by regulators. In contrast, Canada has accepted a limited number of claims about the nature of probiotic microorganisms (36), as has Japan (37). In Australia and New Zealand, products can be labelled as probiotics, but this may change as new legislation was passed in 2013 (effective in 2016) that regulates nutrition content and health claims on food labels and in advertisements (38).

Despite the hesitation of regulators to confer health claims for probiotics, clinical evidence continues to emerge suggesting that probiotics can be effective for a range of conditions, including constipation, irritable bowel syndrome, and lactose intolerance (6, 39). Systematic and meta-analyses have shown that probiotics may aid the treatment of

antibiotic associated diarrhea (40), the prevention of necrotizing enterocolitis in preterm neonates (41), the induction of remission and maintenance of IBD (42), the prevention and control of hyperglycemia (43), improve levels of total cholesterol HDL and TNF-α in patients of non-alcoholic fatty liver disease (44), and reduce glucose, insulin, and HOMA-IR in diabetes patients (45). In addition, the effectiveness of probiotics for preventing or reducing severity of infectious and antibiotic-associated diarrhea and respiratory tract infections has also been reported (6).

In contrast to the century-old history of probiotics, the prebiotic concept was more recently formally introduced in 1995 by Gibson and Roberfroid. Defined originally as "a nondigestable food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (46), the current criteria for prebiotics is now the subject of considerable debate (47, 48). While the general requirements of a prebiotic have been retained in the most recently proposed definition, some key elements of the definition, including specificity and selectively, have been questioned (48). The Canadian Food Inspection Agency (CFIA) has now established its own definition for prebiotics, but neither the U.S. Food and Drug Administration (FDA) nor Europe's European Food Safety Authority (EFSA) have established a definition.

Prebiotics are comprised of simpler molecules such as inulin, fructo-oligosaccharides, galactooligosaccharides, isomaltooligosaccharides, and mannan oligosaccharides, or more complex compounds such as pectins, resistant starches, arabinoxylan or human milk oligosaccharides (49). Depending on the nature of the prebiotic, these substrates may support the growth of certain members of the gut

microbiota, such as bifidobacteria, lactobacilli, or ruminococcus (50). Prebiotics are thought to aid their health benefits through several different mechanisms. These mechanisms include the induction of compositional or metabolic changes to the resident microorganisms, by stimulating the activity and/or growth of health-promoting bacteria, and by the production of SCFA and other end products that reduce the local pH, induce the production of immunomodulatory cytokines, and stimulate mucin production (51). Substrates like FOS and GOS may also have fermentation independent health effects, such as adherence inhibition of pathogens (52).

Systematic meta-analyses showed that, in some cases, a prebiotic treatment reduces fasting insulin levels (53), can aid the treatment of infectious diseases (54) and diarrhea (55), and restore bowel function (56). There are also reports that prebiotics may contribute to abdominal pain, diarrhea, and increased production of gas depending on the doses, nature of the prebiotic, and the susceptibility of the host (6).

1.5 Synbiotic concepts

When Gibson and Roberfroid first articulated the prebiotic concept more than twenty years ago, they envisioned that prebiotics and probiotics could be combined as synbiotics. Later, Kolida and Gibson (2011) described the two general ways synbiotics could enhance the effects of their parts. Complementary synbiotics are those that contain probiotics and prebiotics chosen independently of one another, with each responsible for a particular effect or health benefit. Accordingly, the best case scenario for such a synbiotic would be that each constituent, i.e., the probiotic and prebiotic, would have a beneficial effect and that the effects would be additive. For example, the prebiotic would stimulate

resident strains of bifidobacteria (presumably stains that improved intestinal health), while the probiotic would be established independently, providing an additional health benefit. In this complementary approach, the prebiotic component is not necessarily preferentially fermented by the probiotic strain and could theoretically support other members of the gastrointestinal microbiota. The probiotic strain would gain no ecological advantage by being combined with the prebiotic, and indeed, may not be capable of fermenting the substrate at all.

When the prebiotic is introduced together with a probiotic that cannot ferment the substrate, the outcome may be highly unpredictable and would likely depend on the composition of an individual's gut microbiota. Already it is apparent from human clinical studies that a bifidogenic response or other changes in the microbiota following prebiotic supplementation occur in some subjects but not in others. The nature of the responder/non-responder phenotype (i.e., what makes a responder a responder) remains the subject of considerable interest. Davis et al. suggested that specific bacterial strains capable of fermenting the prebiotic or competing well in the colon might be absent in the non-responder population (29), but that has not been established yet. The inability of members of the gut microbiota to compete in the highly competitive gastrointestinal environment could also affect the responder status of subjects. Indeed, Davis et al. showed that even a high abundance of taxa that would be expected to ferment a given prebiotic substrate was not a reliable predictor of whether or not the prebiotic was fermented (29). Salonen et al. have shown that obese male individuals on a resistant starch diet could be divided into responders and non-responders based on the shifts in the composition of their gut microbiota. In this case a high microbial diversity correlated with a low dietary responsiveness (57). Similarly Martinez et al. have reported a microbial responder and

non-responder phenotype in normal weight human subjects who had consumed resistant starches (58). Kovatcheva et al. divided their study cohort in responder versus non-responders based on the metabolic response to a dietary fiber treatment (59). A subsequent analysis of the gut microbiota of both groups showed that the *Prevotella* and *Bacteroides* ratio was significantly higher in the responder group. Due to this responder and non-responder phenomenon, a prediction of whether or not a subject will have a health beneficial effect by a dietary treatment is difficult to establish. The response to the treatment depends not only on the functional and taxonomic composition of the gut microbiota but possibly also on host factors. These factors include the amount of digestive enzymes provided by the host, the food transit time, and other potential environmental constraints, which could limit the increase of certain bacterial numbers, even if the growth substrate is provided (58). While these host factors may be limiting the success of dietary interventions to a certain degree, synergistic synbiotics may provide the functional and taxonomic microorganisms that are not present in non-responders. In contrast to complementary synbiotics, synergistic synbiotics consist of a prebiotic substrate that specifically supports the growth of a cognate probiotic strain in the gastrointestinal ecosystem (32). Assuming the target strain reaches the colon, this approach potentially addresses the responder/non-responder problem by providing the strain and its growth substrate *in situ*. The presence of an autochthonous member of the gut microbiota capable of fermenting the prebiotic prior to the treatment is not necessary. However, the synergistic synbiotics are not limited to addressing the responder/ non-responder phenomenon. One important limitation of many probiotic and synbiotic applications is the low ecological success of the probiotic strain (60). In order to become established in the GI tract, the probiotic must not only secure nutrients and other growth factors, but must also outcompete

the resident microbiota for these resources. By providing the probiotic partner organism with a new resource opportunity, in this case a selectively fermentable prebiotic, the probiotic strain's competitive fitness can be significantly increased and its persistence enhanced (32).

A literature search has shown that most of the synbiotic combinations used in clinical studies and reported in the literature have not been synergistically supporting the probiotic strain. Indeed, only a few studies have provided evidence that a prebiotic supports its probiotic counterpart *in vivo* (Table 1.1), and only one such study was conducted with human subjects. There are a variety of reasons why it is difficult to achieve synergism between a probiotic and a prebiotic *in vivo*. Most importantly, the synbiotics used in these studies have not been rationally designed, and have instead been formulated on rather arbitrary bases, such as shelf life, cost, and industrial performance (60), on the basis of availability, cost, or other marketing reasons. So often probiotic strains do not utilize the respective prebiotic. Even when *in vitro* or *in situ* screenings of synbiotic combinations are applied, these techniques do not account for the ecological factors that will affect the probiotic strain *in vivo*, nor do they account for how other autochthonous members of the gut microbiota may benefit from the prebiotic substrate (29, 61, 62). It can be challenging to identify a prebiotic that will specifically and selectively enhance the probiotic strain of interest. New strategies to develop synergistic synbiotic combinations now include *in vivo* selected synbiotic combinations or Multi-taxon Insertion Sequencing, which have been recently discussed (49).

Table 1.1. Synergistic synbiotics reported in the literature

Authors/ Year	Probiotic component	Prebiotic component	Study subjects	Increase of probiotic abundance	*P* value	Health outcome
Tanaka et al., 1983 (63)	*Bifidobacterium breve* 4006	Transgalactosylated oligosaccharide (TOS)	Healthy adults	Pro: 9-10.2 log/g feces; Syn: 10-10.5 log/g feces§	0.05	Not measured
Wang et al., 1999 (64)	*Bifidobacterium* Lafti^{TM} 8B.	Amylomaize	BALB/c mice	Pro: 4.3% recovery rate; Syn: 27.92% recovery rate in feces	0.05	Not measured
Femia et al., 2002 (65)	*Lactobacillus rhamnosus* LGG + *Bifidobacterium lactis* Bb12	Oligofructose enriched inulin	Male F344 rats	LGG: Pro: $4.8 \pm 3.4 \cdot 10^5$; Syn: $21.1 \pm 18 \cdot 10^5$ CFU/g of feces; Bb12: Pro: $6.1 \pm 8.1 \cdot 10^5$; Syn: $8.4 \pm 12 \cdot 10^5$ CFU/g of feces	Not given	Anti-tumoric activity in azoxymethane induced cancer
Ogawa et al., 2005, 2006 (66, 67)	*Lactobacillus casei* subsp. *casei* JCM 1134^T (Lcc)	Dextran	BALB/c mice	Pro: $1 \cdot 10^4$ CFU/mg of feces; Syn: $1.4 \cdot 10^6$ CFU/mg of feces§	0.01	Significantly elevated natural killer cell activity in spleen mononuclear cells
Krumbeck et al., 2015 (60)	*Bifidobacterium adolescentis* IVS-1	Galactooligosaccharide (GOS)	Male Sprague-Dawley rats	Pro: 7.9 ± 0.1 log10 cells/ g colon content; Syn: 9.47 ± 0.2 log10 cells/ g colon content	0.0001	None

Pro: Probiotic; Syn: Synbiotic

§: Absolute microbial numbers are not given in the original publication and are estimated by the author by careful evaluation of graphs in the original publication

That there are few reports describing the use of synergistic synbiotics in clinical trials is somewhat surprising considering the many publications on synbiotics. Just in 2015 alone, more than 90 publications on synbiotics were published (Figure 1.1). Most of these studies included lactobacilli and bifidobacteria as the probiotic component, and various oligosaccharides, inulin or dietary fibers as the prebiotic component (68).

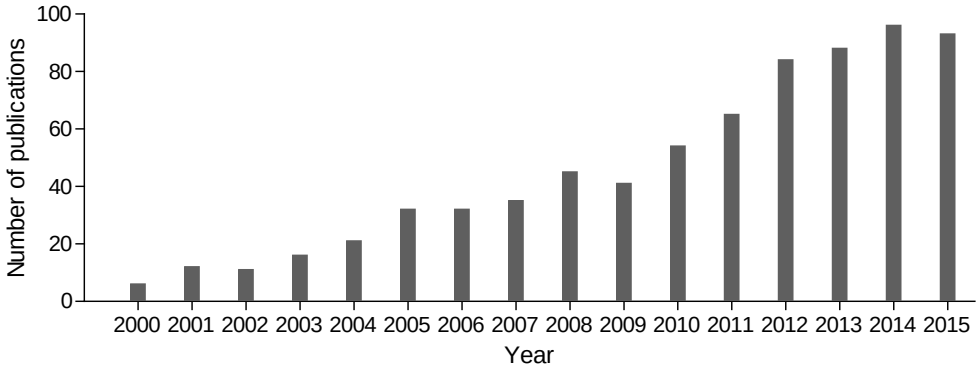

Fig. 1.1. Number of publications on the topic "synbiotic" over the past 15 years.

1.6 Synbiotics and their outcome on human health in clinical studies

The reported literature on synbiotics includes studies from a wide-range of subject cohorts. Research subjects have included humans of all ages, as well as companion animals (mainly dogs and cats), and food production animals, such as chicken, cows, pigs, cattle, rabbits and fish (69–77). Rodent animal models have also been widely used (60, 78–80). In this review, we focus on human clinical studies and the health claims made for synbiotic combinations to improve human health.

It is important to note that despite the many health claims made for synbiotic combinations in the literature and in the commercial market (Figure 1.2), no claims have actually been approved by regulatory agencies in the U.S. and Europe.

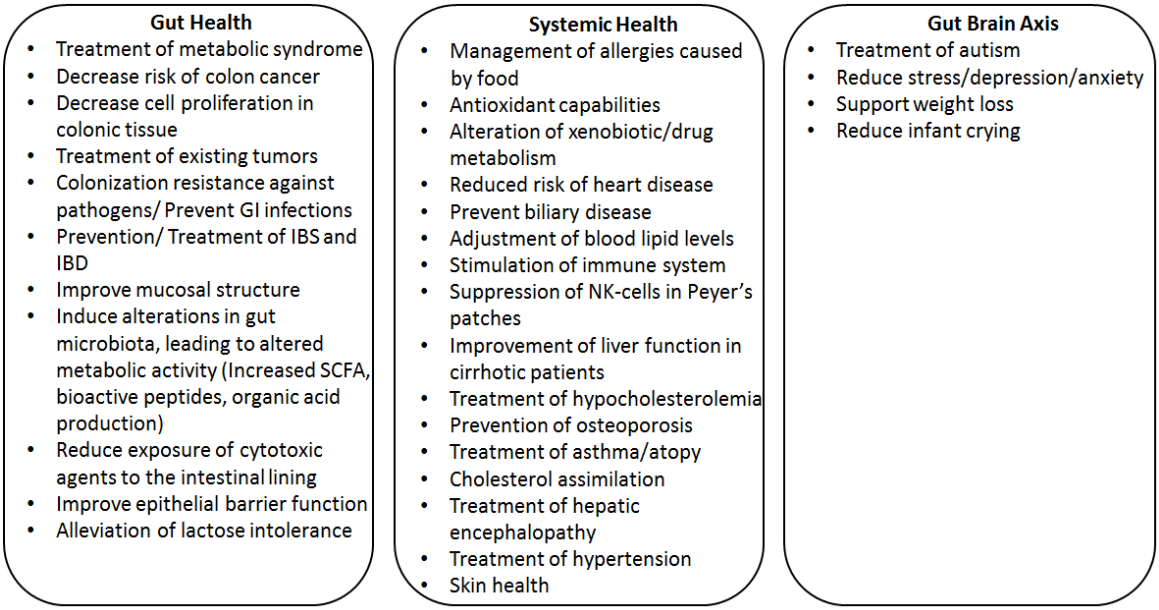

Fig. 1.2. Health claims made for synbiotics in human populations.

Nonetheless, several meta-analyses and systematic reviews suggest that synbiotic treatments may provide beneficial health effects (Table 1.2, and 1.3).

Table 1.2. Overview of published meta-analyses on synbiotic treatments (adapted from Krumbeck et al., 2016 and updated).

Author/ Year	Disease phenotype	Studies and subjects included	*P* value	Type of synbiotic	Study subjects	Outcome
Shukla et al., 2011 (68)	Hepatic Encephalopathy (HE)	1 trial (n=55)	0.004	Probiotic: PP, LM, LPSP, LP2; Prebiotic: BG, I, P, RS	HE patients	Syn. use reduced the risk of no improvement of Minimal HE.
		1 trial (n=60)		Probiotic: BL; Prebiotic: FOS, vitamins B1, B2, B6, B12		
Ford et al., 2014 (81)	IBS and chronic idiopathic constipation	2 trials (n=198)	0.09	Probiotic: BL, BB, LR, LA, LB,ST , LC; Prebiotic: FOS	IBS patients	No reduced symptoms.
		2 trials (n=160)	0.003	Probiotic: BL2, LP, LR, LA; Prebiotic: FOS		Beneficial for chronic idiopathic constipation treatment.
Kinross et al., 2013 (82)	Clinical outcome after elective surgery	8 trials (n=361)	0.002	Probiotic: LC, LP2, LP, LM, LA, LB, BL2, ST, PP, BB, EF, CB, BM, LS, BB2, LL; Prebiotic: OAF, OF, BG, I, P, RS, GOS	Patients undergoing elective surgery	The incidence of postoperative sepsis was reduced by syn.
		4 trials (n=135)	0.03	Probiotic: PP, LM, LP2; Prebiotic: BG, I, P, RS		Syn. reduced the length of postoperative antibiotic use.
		2 or 3 trails each (n between 198 and 260)	>0.05	Probiotic: LC, LP2, LP, LM, LA, LB, BL2, ST, PP, BB, EF, CB, BM, LS, BB2, LL; Prebiotic: OAF, OF, BG, I, P, RS, GOS		No significant changes observed for prevention of pneumonia, wound infection, urinary tract infection, mortality and length of hospital stay.
Beserra et al., 2015 (53)	Glycaemia, insulin concentrations and lipid parameters	2 trials (n=364)	0.04	Probiotic: BL, LC, LR, ST, BB, LA, LB; Prebiotic: FOS	Adults with overweight or obesity	Reduced plasma fasting insulin concentrations.
		3 trials (n=260)	<0.05	Probiotic: LS2, BL, LA, BB; Prebiotic: I, FOS		Reduced plasma triglyceride concentrations.
		2, 3 or 4 trails each (n between 49 and 104)	Not given	Probiotics: LC, LR, ST, BB, LA, BL, LB, LS2; Prebiotic: I, FOS		No significant changes were observed for total cholesterol, LDL-c, HDL-c and fasting glucose.

Table 1.2 continued

Mugambi et al., 2012 (77)	Growth and stool frequency	2 trials (n=227) 2 trials (n=122)	0.29 0.006	Probiotic: BL, LR, LP; Prebiotic: GOS, ScFOS Probiotic: BL; Prebiotic: GOS, FOS	Infants	Syn. failed to improve growth rate, but significantly improved stool frequency.
Chang et al., 2016 (83)	Atopic dermatitis (AD)	6 trials (n=369)	0.03 0.048	Probiotic: LR,BL2, LA, BB, LC, ST, BI, LB, LS; Prebiotic: FOS, lcFOS, GOS, scGOS, starch	Children	Syn. support the treatment of atopic dermatitis, particularly mixed strains of bacteria are used. Syn. support the treatment of atopic dermatitis in children older than 1 year.
		2 trials (n=1320)	0.26	Probiotic: BL, BB, LR, PF; Prebiotic: GOS, ScFOS		Syn. do not support prevention of AD.
Sawas et al., 2015 (84)	Prevention of infections after liver transplant	4 trials (n=246)	<0.001	Probiotic: BB, BL2, LP2, PP, LPSP, LM, LA, LC, LR, LB2; Prebiotic: GOS, fiber	Adult patients receiving a liver transplant	Syn. reduced infection rate of urinary tract and intra-abdominal infections. Syn. reduced hospital stay and duration of antibiotic use.
Yang et al., 2016 (85)	Prevention of infections after GI surgery	16 trials (n=1,370)	Not given	Probiotic: LC, ST, BB, LA, BL2, LB, LP2, PP, LM, LP, LS2, BM, CB; Prebiotic: FOS, GOS, OAF, OF, BG, I, P, RS	Patients undergoing GI surgery	Subgroup analysis of synbiotic trials showed no health benefits due to synbiotics

Probiotic type: BB= *Bifidobacterium breve*; BB2= *Bifidobacterium bifidum*; BI= *Bifidobacterium infantis*; BL= *Bifidobacterium longum*; BL2= *Bifidobacterium lactis*; BM= *Bacillus mesentericus*; CB= *Clostridium butyricum*; EF= *Enterococcus faecium*; LA= *Lactobacillus acidophilus*; LA2= *Lactobacillus affinolactis*; LB= *Lactobacillus bulgaricus*;LB2= *Lactobacillus brevis*; LC= *Lactobacillus casei*; LL= *Lactococcus lactis*; LM= *Leuconostoc mesenteroides*; LP= *Lactobacillus paracasei*; LP2= *Lactobacillus plantarum*; LPA= *Lactobacillus paracasei*; LPSP= *Lactobacillus paracasei* subsp. *paracasei*; LR= *Lactobacillus rhamnosus*; LS= *Lactobacillus salivarius*; LS2= *Lactobacillus sporogenes*; PF= *Propionibacterium freudenreichii*; PP= *Pediococcus pentosaceus*; ST= *Streptococcus thermophilus*

Prebiotic type: BG= β-glucan; GOS= galacto-oligosaccharides; I= inulin; P= pectin; OF= oligofructose; OAF= oat fiber; RS= resistant starch; FOS= fructo-oligosaccharides; Sc= short chain; Syn= Synbiotic

Table 1.3. Overview of systematic reviews on synbiotic treatments.

Author/ Year	Disease phenotype	Studies and subjects included	*P* value	Type of synbiotic	Study subjects	Outcome
Ghouri et al., 2014 and Saez-Lara et al., 2015 (86, 87)	Ulcerative colitis (UC) maintenance	1 trial (n=120) &	0.03	Probiotic: BL; Prebiotic: Psyllium		IBDQ score: improved quality of life.
	Ulcerative colitis induction and maintenance	1 trial (n=41) &	0.05	Probiotic: BB; Prebiotic: GOS	UC patients	Improvement of endoscopic grading compared to standard therapy group.
	Ulcerative colitis induction	1 trial (n=18) *#&	0.06	Probiotic: BL; Prebiotic: Synergy I (I+OF mix)		Sigmoidoscopy score not improved.
			0.05			Inflammatory markers improved.
	Management of Crohn's Disease (CD)	1 trial (n=35) &	0.02	Probiotic: BL; Prebiotic: Synergy I (I+OF mix)	CD patients	Improved clinical response compared to placebo.
		1 trial (n=24) * &	>0.05	Synbiotic 2000: Probiotic: LA2, PP, LP2, LPSP; Prebiotic: BG, I, P, RS		No improvement in endoscopic, clinical and laboratory parameters.
Saez-Lara et al., 2015 (87)	Ulcerative colitis (UC)	1 trial (n=10)	Not given	Synbiotic therapy; Probiotic: BB, BL, LC; Prebiotic: Psyllium	Patients with active UC	Synbiotic was safe and effective.

*: also discussed in Hedin et al., 2007 (88); #: also discussed in Zigra et al., 2007 (89);

IBDQ: Inflammatory bowel disease questionnaire

Probiotic type: BB= *Bifidobacterium breve*; BL= *Bifidobacterium longum*; LA2= *Lactobacillus affinolactis*; LP2= *Lactobacillus plantarum*; LPSP= *Lactobacillus paracasei* subsp. *paracasei*; PP= *Pediococcus pentosaceus.*

Prebiotic type: BG= β-glucan; GOS= galacto-oligosaccharides; I= inulin; P= pectin; OF= oligofructose; RS= resistant starch.

However, comparisons between different trials is rather difficult, since studies often vary between the specific probiotics and prebiotics used, their respective doses, the duration of the study, the targeted population, expected and measured effects of treatment. Even the funding source has been suggested to influence outcomes (90). Of particular concern for synbiotic trials is when investigators did not determine the treatment effects

independently. In such an approach it is not possible to establish that improvement of a clinical endpoint in the synbiotic treatment group was indeed more beneficial than just the pro- or prebiotic treatments alone. Therefore the synbiotic concept cannot be validated in that case. The majority of clinical trials have chosen such an approach. A literature research of synbiotic trials published within the last 15 years (see below), showed that out of 26 trials presented here, only one provided a prebiotic only, probiotic only, and synbiotic only group (91), and only one trial provided an additional placebo control group (92).

Out of the 26 trials presented here, only one performed a genus specific analysis of the microbiota of the subjects (93), and only three of the trials used a species specific analysis for the applied probiotic (92, 94, 95). All other studies did not conduct any microbial analyses to confirm the survival or activity of the probiotic component at a strain-specific level or even at higher taxonomical levels. Finally, another common limitation of these studies is the lack of experimental power, which may result in overstating or underestimating the actual health benefits of the applied synbiotic. This phenomenon of disadvantages of synbiotic meta-analyses has been previously recognized and criticized for probiotic meta-analyses as well (96).

In general, most of the meta-analyses have focused on the disease phenotype rather than on the exact nature of the treatment. Consequently, these analyses often do not differentiate between prebiotic, probiotic or synbiotic trials, with very few trials specifically using synbiotics. However, systematic meta-analyses specifically analyzing synbiotics are often impossible due the limited number of trials. Other meta-analyses do not distinguish between a probiotic or synbiotic treatment and combine those trials into one

analysis, which is not appropriate for assessing the potential health benefits of a synbiotic treatment (Table 1.4).

Table 1.4. Overview of Meta-analyses synbiotic treatments that combined pro- and synbiotic trials into one analyses.

Authors/Year	Disease phenotype	Overall outcome
Pitsouni et al., 2009 (97)	Patients undergoing abdominal surgery	Pro-/ synbiotic treatment may reduce postoperative infections after abdominal surgery.
Rossi et al., 2012 (98)	Patients with chronic kidney disease	Limited but supportive evidence for the effectiveness of pre- and probiotics on reducing uremic toxins. No conclusion about synbiotics.
Zhang et al., 2010 (99)	Patients with acute pancreatitis	Pre-, pro- or synbiotics treatment shows no statistically significant benefit. Safety and efficacy: Use pre- pro- or synbiotics with caution in critically ill patients and patients with severe acute pancreatitis.
Watkinson et al., 2007 (101)	Patients admitted to adult intensive units	There is currently a lack of evidence to support the use of pre- pro- or synbiotics.
He et al., 2013 (102)	Patients undergoing colorectal resection for cancer	Pro-/synbiotics administration had a positive effect on the incidence of diarrhea (P = 0.001), the incidence of symptomatic intestinal obstructions (P =0.008), the incidence of operative total infections (P =0.0010), and pneumonia infection (P = 0.04).
		Pro-/synbiotics administration increased numbers of *Lactobacillus* (P < 0.00001), and decreased the counts of Enterobacteriaceae.
Dang et al., 2013 (103)	Prevention of eczema	Pro- and synbiotic treatment may reduce incidence of infant eczema. Prebiotics alone have no effect.
Lytvyn et al., 2015 (104)	Prevention of postoperative infections following abdominal surgery in adults	Probiotics/synbiotics reduce the risk of surgical site infections compared to placebo or standard of care and potentially benefit for urinary tract infections with no increased risk of adverse events, and no occurrence of serious adverse events reported as related to study product.
Petrof et al., 2012 (105)	Critically ill patients, including burn, multiple trauma, pancreatis, diarrhea patients and general intensive care unit patients	Clinical trials suggest that probiotics patients may reduce overall infection rates in critically ill patients.
Arumugam et al., 2016 (106)	Decrease of postoperative sepsis GI surgical patients	Pro-/synbiotics significantly reduced risk of postoperative sepsis by 38% (P = <0.0001)

Review of selected clinical trials on synbiotic treatments

As introduced earlier, the study design is crucial when synbiotic treatments are being assessed for their health beneficial effects. To justify the application of a synbiotic treatment instead of a probiotic or prebiotic only treatment, appropriate controls must be used to assess if the synbiotic treatment acts synergistically. Thus, controls must include a placebo, the probiotic treatment alone, the prebiotic treatment alone, and the synbiotic combination. Only this study design allows investigators to assess whether or not a synbiotic treatment is more effective than the probiotic and prebiotic treatments alone and whether or not synergy is given. In addition, the survival and/or metabolic activity of the probiotic component should be quantified in a strain-specific manner to ensure survival and establishment of the probiotic. However, this requires a significantly higher number of subjects, and only a small number of trials fulfill these criteria.

In the next sections, recent human trials that applied synbiotics to treat specific clinical disorders are reviewed. These include trials on metabolic syndrome, inflammatory bowel disease, diarrhea, colon cancer, and inflammatory bowel syndrome

Metabolic syndrome

Metabolic syndrome refers to a group of metabolic disorders that collectively contribute to heart and other health problems. Risk factors include central obesity, impaired glucose tolerance, dyslipidemia and hypertension (107, 108). This syndrome is associated with obesity, type II diabetes, heart disease, and cancer (109).

Several synbiotic formulations have been used in clinical trials. Eslamparast et al. conducted a prospective, randomized, double-blind, and placebo-controlled study analyzing the effect of 250 mg fructooligosaccharide (FOS) and a probiotic cocktail of seven different strains (*Lactobacillus casei, Lactobacillus rhamnosus, Streptococcus thermophilus, Bifidobacterium breve, Lactobacillus acidophilus, Bifidobacterium longum,* and *Lactobacillus bulgaricus*) on 38 subjects suffering from metabolic syndrome (110). Subjects were instructed to consume the supplement or placebo (maltodextrin) twice daily for 28 weeks, and were instructed to follow strict dietary recommendations, lower their energy intake, and increase their physical activity. At the end of the study, individuals in the synbiotic treatment had significantly improved levels of insulin resistance, fasting blood sugar, triacylglyceride, and serum high-density lipoprotein levels compared to the placebo treatment. No difference was observed in body mass index (BMI), low-density lipoprotein levels, anthropometric parameters, and energy intake/expenditure. The authors concluded that the synbiotic treatment can increase the efficacy of a dietary therapy in the management of metabolic syndrome and insulin resistance. While the design and analysis of this study were adequate for the study goals, an analysis of the gut microbiota was not included. The authors stated that "because previous studies had shown beneficial effects of VLS#3 and *Lactobacillus longum* and fructooligosaccharide, demonstrating their beneficial effects on intestinal microbiota, this synbiotic capsule was chosen for the present study as it contained all these strains in addition to others." However, the mentioned "*Lactobacillus longum"* is not a strain that has ever been described in the literature before and is most likely either a *B. longum* or a different *Lactobacillus* strain. Additionally, the authors claim that VLS#3 was chosen for its previously reported health benefits. But in the study conducted here, the applied synbiotic mixture did not contain all the strains

present in VLS#3 and left, seemingly randomly, two strains, *B. infantis* and *L. delbrueckii*, out. Therefore no comparison to other studies using VLS#3 can be made. Leaving these issues aside, no comparison was made between a pro- and prebiotic treatment alone, thus not confirming that the synbiotic treatment was more effective than the probiotic or prebiotic treatment alone.

Lactobacilli, bifidobacteria and FOS were combined as synbiotic treatments for gastric bypass patients. The synbiotic treatment contained *Lactobacillus paracasei* LPC-37, *Lactobacillus rhamnosus* HN001, *Lactobacillus acidophilus* NCFM, and *Bifidobacterium lactis* HN019 (each at 10^9 CFU) and 6 g of FOS (111). Patients were divided into a placebo, prebiotic, and synbiotic treatment group. The supplement was taken for 15 days after which a significant weight loss was seen in the prebiotic group. The BMI was also lower in the prebiotic and placebo group than in the synbiotic group. Other blood and inflammatory markers were not different among the groups, and the fecal microbiota was not addressed. Effects of SCFA production induced by FOS treatment were only indirectly measured by assessing plasma cytokines, and no effect was detected. Although clinical benefits for each of the tested strains are reported by the authors, there was no rationale given by the authors for combining them as a synbiotic. Also, the low number of bypass patients (n = 3 per group) makes the interpretation of this data rather difficult and further investigation will be needed.

The effect of a synbiotic consisting of *Lactobacillus sporogenes* (2.7×10^8 CFU) and 1.08 g of inulin was tested in 62 type II diabetic patients in a randomized double-blinded cross-over controlled clinical trial (112). The study lasted three weeks, and patients were instructed to consume the treatments three times a day. The synbiotic was compared

to a placebo, and results suggested the synbiotic led to significantly decreased serum insulin levels ($P = 0.03$) and serum high-sensitivity C-reactive protein levels ($P = 0.01$). Increases in plasma total glutathione ($P < 0.001$) and serum uric acid levels ($P = 0.04$) were also reported. However, no effects on cholesterol levels were observed, and the fecal microbiota of the patients were not investigated. The same research group tested this synbiotic in combination with beta-carotene, and a reduced amount of *L. sporogenes* and inulin in 102 diabetic patients (113). In this case cholesterol levels were affected significantly in addition to insulin compared to a control. Pro- or prebiotic-alone treatments were not included, making it impossible to determine if the observed effects were due to the synbiotic or the individual synbiotic components. Inulin has been previously shown to have beneficial effects in the treatment of diabetes (114–116). Finally, as noted previously (117), "*Lactobacillus sporogenes*" is not a validly named species and the applied species here is more likely a *Bacillus coagulens*. However, without a classification analysis, the identity of the strain used in this study cannot be established.

The same synbiotic combination of *Lactobacillus sporogenes* (40×10^8 CFU, consumed three times a day) and inulin (2.8 g, consumed three times a day) was tested in 81 diabetic patients (118). The synbiotic, probiotic, or placebo was delivered in form of bread. After eight weeks of treatment, plasma nitric oxide was increased ($P = < 0.0001$) and malondialdehyde levels were significantly reduced ($P = 0.001$) compared to both placebo and probiotic only treatments. Since a prebiotic only treatment only was not included it remains unclear if this effect could have been achieved by inulin only. The survival of the probiotic strain during the bread making process or storage was not reported, nor any analysis of the gut microbiota. The actual dose of *L. sporogenes* per day therefore remains speculative.

A synbiotic cocktail containing *Lactobacillus casei, Lactobacillus rhamnosus, Streptococcus thermophilus, Bifidobacterium breve, Lactobacillus acidophilus, Bifidobacterium longum, Lactobacillus bulgaricus* (2 x 10^8 CFU total), and FOS (unknown amount) was tested for its potential to support a weight loss regime (119). Forty-six patients of metabolic syndrome consumed the synbiotic or placebo (maltodextrin) for 12 weeks twice a day. All patients experienced significant weight loss, and the synbiotic treatment reduced the systolic blood pressure ($P < 0.05$). However, 90% of the subjects in the synbiotic group used medication to reduce blood pressure compared to 75% in the placebo group before and throughout the duration of the study. While this difference was not significant, and could have affected the outcome. The fecal microbiota was not examined and the nature of the study design precludes any conclusions about the efficacy of the prebiotic or the probiotic strains applied here.

A study conducted with patients suffering from type II diabetes claimed a health benefit of a synbiotic combination compared to a placebo (120). However, the value of this study is limited by the fact that no further details of the nature of either the synbiotic or placebo treatment was given.

Inflammatory bowel disease

Ulcerative colitis (UC) and Crohn's disease (CD), the two types of inflammatory bowel diseases (IBD), are chronic inflammatory pathologies of the gastrointestinal tract. Both conditions occur in individuals who are genetically susceptible and exposed to environmental risk factors (121). Even though the etiology of IBD has been extensively studied, the disease pathogenesis is not fully known, nor is there a cure (122). The

characteristics of the inflammation are different, with CD being scattered throughout the GIT, typically involving the distal small intestine and colon with transmural inflammation and occasionally associated with granulomas, whereas in UC the inflammation is usually confined to the mucosa of the colon (123, 124). Both UC and CD are characterized by a relapsing and remitting course leading to a very significant reduction in life quality during the disease (125).

Several synbiotic formulations have been used in clinical studies to treat IBD. In Furrie et al., Synergy 1 (6 g of inulin/oligofructose) and 2 x 10^{11} CFU *Bifidobacterium longum* were administered to UC patients (93). One of the strengths of this study was that the probiotic strain was isolated from a healthy human subject and had been assessed for its aerotolerance, acid tolerance, resistance to bile salt, and adherence to epithelial cells. Its ability to use the prebiotic substrate as an energy source was also established *in vitro*. The organism was further shown to alter the cytokine expression in a HT29 epithelial cell line and reduce proinflammatory cytokine levels *in vitro*. For the clinical study, 18 patients were divided into synbiotic and placebo groups, each receiving the respective treatments twice daily for four weeks. The synbiotic treatment led to reduced inflammation and regeneration of epithelial tissue compared to the placebo group, reduced mRNA levels of human beta defensins, and lowered levels of tumor necrosis factor α and interleukin 1 α. Although survival of the probiotic strain was not measured in a strain-specific manner, bifidobacteria-specific rRNA levels were increased 42-fold in the synbiotic group compared to approximately 5-fold in the placebo group. Unfortunately, this study did not investigate the effects of the probiotic independently.

Another *B. longum* synbiotic clinical trial was performed by Fujimori et al. In this study, a *B. longum* strain (2 x 10^9 CFU) was combined with eight grams of psyllium as the prebiotic component. Subjects were UC outpatients (n = 120) (91). This trial did include probiotic and prebiotic only treatment groups in the study design to allow for comparisons. While most tested blood markers showed no differences among the three treatments, C-reactive protein was significantly decreased (P = 0.04) and the total protein level in the blood samples increased (P = 0.03) in the synbiotic group. Hemoglobin and hematocrit only increased in the probiotic group (P = 0.04). Total inflammatory bowel disease questionnaire scores showed significant improvement only for the synbiotic group. The investigators concluded that the synbiotic treatment led to a greater life-quality than the pre- and probiotic treatments alone. However, this study did not investigate the mechanisms responsible for this improvement nor was the survival of the probiotic determined. Therefore no conclusion about the nature of the synbiotic can be drawn, i.e. if the synbiotic acted synergistic or complementary.

In another clinical study, the effect of a *Bifidobacterium breve*-GOS synbiotic on subjects with mild to moderate UC was assessed (94). Synbiotics contained *B. breve* (10^9 CFU, Yakult) and 5.5 g of galactooligosaccharide. Forty-one patients were treated with a placebo or the synbiotic for one year. End-points included endoscopic scores and myeloperoxidase levels in lavage solutions; both were significantly lower in the synbiotic treated group. An analysis of the fecal microbiota by plate counting was also performed for subjects in the synbiotic group before and after the treatment. Of all assessed microbes, only *Bacteroidaceae* were significantly decreased after the synbiotic treatment. The abundance of *Bifidobacterium* remained the same, and *B. breve* was only detected after the treatment but not before (5.75 ± 1.65 $\log_{10}$ CFU/g feces). Therefore, it can be concluded

that *B. breve* survived the passage through the GI tract. Whether or not the applied prebiotic was supporting the probiotic could not be determined from this study. Interestingly, here no bifidogenic effect was observed due to galactooligosaccharide treatment. This is contrary to results previously reported (126).

A combination of 6 g of Synergy 1 (inulin/oligofructose) and 2 x 10^{11} CFU *Bifidobacterium longum* showed improvements in Crohn's disease in patients that continued using their conventional CD medication (95). Compared to a placebo, the synbiotic significantly reduced TNF-α gene expression (P = 0.041), disease activity indexes (P = 0.02), and histological scores (P = 0.018) after six months of treatment. The microbiota of tissue biopsies was analyzed in both species- and genus-specific manner. Interestingly, 8 out of 13 patients had increased numbers of *Bifidobacterium longum* and bifidobacteria at the three month time point compared to the baseline in the synbiotic group, increasing to 11 patients after six months. The nature of this responder/non-responder phenomenon was not addressed.

Chermesh et al. investigated the potential of Synbiotic 2000 to prevent postoperative recurrence of CD (127). This formulation contained 10^{10} CFU *Pediococcus pentoseceus*, 10^{10} CFU *L. raffinolactis*, 10^{10} CFU *L. paracasei* subsp. *paracasei* 19, and 10^{10} CFU *L. plantarum* 2362 and as fermentable fibers 2.5 g of β-glucans, 2.5 g of inulin, 2.5 g of pectin, and 2.5 g of resistant starch. The frequency of the treatment was not stated. Of 30 enrolled patients, nine patients completed the study, which lasted 24 months. Synbiotic 2000 had no effect compared to the placebo on endoscopic or clinical relapse, nor the postoperative occurrence of CD. However, it significantly improved weight increase and normalization of hemoglobin levels at the three month follow up time point.

No analysis of the gut microbiota was done, nor were reasons given for the selection of the synbiotic combination. Of the prebiotic components none were correlated to any health benefits by the authors. Only fructooligosaccharides were mentioned as beneficial in general. Unfortunately, this study had a small sample size, lacked a pro- or prebiotic control arm, and used a seemingly arbitrarily selection process to determine the synbiotic combination.

The plant fiber, psyllium, was combined as a synbiotic with bifidobacteria and lactobacilli, and used to treat CD patients (128). The synbiotic contained 9.9 g of psyllium and 3×10^{10} CFU *Bifidobacterium breve*, 3×10^{10} CFU of *Lactobacillus casei*, and 1.5×10^{10} CFU of *Bifidobacterium longum.* Ten active CD patients were enrolled in this 13 month trial. The trial was not placebo controlled and subjects were allowed to discontinue using psyllium during the trial if abdominal bloating occurred. All subjects were allowed to reduce the pro- and/or prebiotic treatment on their own will, thereby individualizing the treatments. Subjects also received aminosalicylates and prednisolone at varying doses. All subjects continued taking the probiotic treatment, but their doses varied between 12 and 73 $\times 10^{10}$ CFU. Four subjects discontinued the prebiotic treatment, and for the remaining six patients the doses varied between 3.3 and 9.9 g per day. Based on the clinical outcome, the authors divided the subjects into complete responder, partial responder, and non-responders. Eight complete responders lowered their Crohn's Disease activity index scores by more than 70 points and six achieved remission. Patients who discontinued the synbiotic treatment and those following it through the whole duration of the study were found in the responder as well as the non-responder group. Therefore, no correlation between a synbiotic treatment and a health improvement could be determined.

Rossi et al. conducted a observational study applying SynGut^{TM} (*Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W22, *Lactobacillus plantarum* W21, *Lactococcus lactis* W19, and inulin) to 96 IBS patients for two months (129). While this study had no control group, no pro- or prebiotic group, and no standardized scoring system, it reported an improvement of IBS symptoms in 74% of the participants.

Diarrhea

Diarrheal diseases are often caused by infectious agents, which lead to lose, or liquid, bowel movements with increased frequency, water content, and volume. Worldwide, diarrhea is the leading cause of hospitalizations, morbidity, and mortality (130).

The potential of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Enterococcus faecium* (2.5×10^9 CFU total), combined with 625 mg of fructooligosaccharide as a synbiotic, was tested in children with acute diarrhea (130). Treatment with oral rehydration salts (ORS) and intravenous therapy was also provided. Compared to a control group (receiving only ORS and/or intravenous therapy), the synbiotic combination significantly shortened the duration of diarrhea ($P < 0.0001$) and shortened the hospital stay ($P = 0.002$). The gut microbiota of these children was not analyzed.

In a similar study, children with acute rotavirus diarrhea were treated with *Lactobacillus* sp., *Streptococcus* sp., *Bifidobacterium* sp. (1×10^9 CFU total) and 990 mg of FOS (131). A standard fluid therapy and nutritional support were provided. A total of

35 children were enrolled in the synbiotic group and were compared to a placebo group. The duration of diarrhea was significantly shorter in the synbiotic group ($P < 0.0001$), and for half of the patients receiving a synbiotic treatment, intestinal mucosal healing was reported 50 hours after the synbiotic administration. The gut microbiota was not analyzed. No further descriptions were given to explain why half of the group responded to the treatment.

An arabinogalactan and xylooligosaccharide mixture was used to formulate a synbiotic that also included *Lactobacillus paracasei* B21060 (2.5×10^9 CFU). The prebiotics were present at 500 mg and 700 mg, respectively. Subjects included 55 children with acute diarrhea, who also received ORS treatment. The synbiotic showed a significantly higher resolution rate ($P = 0.005$) than the placebo group after the first 72 hours (132). This study allowed for additional treatments (e.g., diosmectite, domperidone or racecadotril) given by the parents after the first 72 hours, which may have influenced the total duration of diarrhea. No analysis of the fecal microbiota was performed. The additional treatments may have influenced the efficacy of the synbiotic treatment.

A combination of *Streptococcus thermophilus*, *Lactobacillus bulgaricus* (9.7×10^8 CFU), *Bifidobacterium animalis* subspecies *lactis* (Bb-12) (5×10^9 CFU), and 1g of inulin was assessed for its potential to prevent diarrhea, vomiting and other infections in young children (133). One hundred and forty-nine children participated in this placebo controlled double-blind study. After 16 weeks of treatment, synbiotic treated children had significantly fewer days of fewer, but significantly more days with watery stools ($P < 0.05$). No analysis of the microbiota was performed and it remains unknown which components of the treatment might have caused this effect.

To assess the potential to prevent and/or shorten the occurrence of traveler's diarrhea, 196 healthy adults received a synbiotic combination named Agri-King Synbiotic (AKSB). This preparation contained fructooligosaccharide, 4.5 x 10^9 CFU *Enterococcus faecium* SF68 and 5 x 10^9 CFU *Saccharomyces cerevisiae* CNCM I 4444 consumed twice daily (134). This paper does not state the amount of fructooligosaccharide present in the AKSB capsules. A literature research suggests that this preparation contains 115 mg of fructooligosaccharide per capsule (135). A phase I study had shown that AKSB was safe and also that both strains were washed out within seven days after the treatment was discontinued. Study subjects traveling to Asia, Africa, South and Central America were instructed to consume the synbiotic or placebo treatment one to two times daily, and to continue the treatment if diarrhea should occur. Approximately half of the study cohort experienced traveler's diarrhea, but no benefit of the synbiotic treatment was detected. To justify the combination *of E. faecium* and *S. cerevisiae* the authors stated that *E. faecium* was capable of competing with other Gram-negative bacteria. However, the authors do not show evidence for this here and instead refer to another paper that analyzed this phenomenon in stored meat samples and not for the gut microbiota (136). No further comments were made regarding *S. cerevisiae* or fructooligosaccharide.

Colon cancer

Colorectal cancer is the third most common form of cancer and has a very high mortality. In addition to genetic factors, environmental factors including radiation, chemical carcinogens, and diet contribute to tumorigenesis in the colon (137). Current treatments are associated with a high risk of complications and a low success rate.

Investigators have suggested that by maintaining a healthy weight, diet, and physical activity, up to one third of colon cancers may be prevented (138). Numerous pro-, pre-, and synbiotic studies using rodent models suggest that these treatments may have preventive and therapeutic properties. However, human studies are difficult to perform and therefore rare.

Rafter et al. and Roller et al. assessed a combination of *Lactobacillus rhamnosus* GG (1×10^{10} CFU), *Bifidobacterium lactis* Bb12 (1×10^{10} CFU) and 12 or 10 g of Synergy 1 on colon cancer and polypectomized patients in two similar phase II anti-cancer studies (139, 140). The synbiotic treatment was compared to a placebo in a 12 week trial. Fecal water obtained from the cancer patients did not improve barrier function in Caco-2 cells, but did increase production of interferon γ. For polypectomized patients, several benefits were observed among the synbiotic group, including significant decreased DNA damage in colonic mucosa, reduced proliferation, and decreased secretion of IL-2. The fecal water improved barrier function in Caco-2 cells and significantly reduced necrosis in HCT116 cells. The investigators also assessed survival of each of the probiotic strains in an independent study with healthy human subjects who consumed rifampicin resistant mutants of each strain (139). Rifampicin resistance was used to identify the probiotics in a strain specific manner in the fecal samples. A full recovery of both strains in healthy subjects was reported. For the study patients, a fecal analysis was not performed at the strain level, but at the genus level. The number of *Lactobacillus* and *Bifidobacterium* consistently increased in the synbiotic group for both cancer and polypectomized patients over the 12 week trial, while *Clostridium* numbers decreased. Since a probiotic only treatment was not applied, a synergy between the probiotic strains and Synergy 1 could not be confirmed based on this data. Roller et al. did not analyze the fecal microbiota, but

referred to another study that reported that only 10 % of the consumed amount of LGG and Bb12 survived the GI tract in the same synbiotic treatment (141). Neither study analyzed the probiotic and prebiotic components alone.

In a four week cross-over trial the effect of the synbiotic combination of 12.5 g of resistant starch and 5 x 10^9 CFU *Bifidobacterium lactis* was investigated on twenty healthy subjects (92). Even though these patients were healthy, the effect of the dietary treatments on markers of early colorectal carcinogenesis was assessed. A placebo, a prebiotic only, and a probiotic only arm were also included. Full analyses of the fecal microbiota were conducted using DGGE and quantitative real-time PCR to assess levels of *Bifidobacterium lactis.* The DGGE banding patterns showed that the synbiotic treatment introduced significantly more changes to the gut microbiota than the placebo or the pro- or prebiotic treatments alone. Interestingly the probiotic treatment led to higher numbers of *B. lactis* than the synbiotic treatment (8.8 x 10^7 versus 5.4 x 10^7 *B. lactis*/g feces). Therefore a synergistic relationship between this strain of *B. lactis* and the applied resistant starch is not likely. No differences were detected for the SCFA profile, fecal ammonia or pH, serum inflammatory markers, or epithelial variables among the treatments. This study demonstrates how even significant changes introduced to the gut microbiota by a dietary intervention do not necessarily lead to a change in disease associated phenotypes.

Irritable bowel syndrome

Irritable bowel syndrome (IBS) is an intestinal disorder characterized by abdominal pain, bloating, diarrhea, alternate constipation, distention, or a combination of these symptoms. The cause of this illness has not been established, but visceral hypersensitivity,

genetics, the gut microbiota, constant low-grade inflammation, and environment are contributing factors (81). Approximately 11 % of the world's population may be affected by IBS, with higher occurrences among women and younger individuals (142, 143). Physiological interventions, dietary manipulations, pharmacologic agents, and modulation of the gut microbiota are part of current treatments for IBS (144).

In one double-blinded, randomized and placebo-controlled study, a synbiotic mixture of 5×10^9 *Lactobacillus plantarum*, 2×10^9 *Lactobacillus casei* subp. *rhamnosus*, 2×10^9 *Lactobacillus gasseri*,1×10^9 *Bifidobacterium infantis*, 1×10^9 *Bifidobacterium longum*, 1×10^9 *Lactobacillus acidophilus*, 1×10^9 *Lactobacillus salivarus*, 1×10^9 *Lactobacillus sporogenes*, and 5×10^9 *Streptococcus thermophilus* in combination with 2.2 g Synergy 1 (inulin/oligofructose) was tested for its potential to reduce symptoms of IBS (145). Sixty-four patients were enrolled and treated for four weeks. No overall satisfactory relief was achieved with the synbiotic treatment. However the synbiotic did improve quality of life scores, and the severity of flatulence was significantly decreased. Interestingly, the authors provided a rationale for selecting this particular synbiotic combination. Namely, the product is readily available, has a history of safe use, and there was only one other study using single-strain synbiotic mixture for the treatment of IBS. Nonetheless, effect on the gut microbiota was not studied.

A recent study examined the effect of *Lactobacillus acidophilus* (1.8×10^7 CFU/g), *Bifidobacterium animalis* subsp. *lactis* Bb-12 (2.5×10^7 CFU/g), and Beneo dietary fibers (2%) on the quality of life and IBS symptoms of 76 constipation-predominant IBS patients (146). The synbiotic was delivered twice daily in 180 g of fermented milk for four weeks.

Several markers of IBS symptoms improved after four weeks, but there was no difference between the synbiotic and the placebo (fermented milk).

Bittner et al. tested the efficacy of the synbiotic Prescript-Assit™ in a two week randomized, placebo-controlled study, followed by a two week open label treatment and a follow up 60 weeks later (147, 148). Prescript-Assit™ is a combination of 29 soil-based microorganisms, including several *Anthrobacter*, *Bacillus*, *Brevibacterium*, *Pseudomonas*, and *Streptomyces* strains. The prebiotic components are not well defined, except that one of them is leonardite. A total of 25 patients completed the first two week study, and 22 completed the 60 week follow up. The authors concluded that Prescript-Assit™ was capable of reducing short-term and long-term symptoms of IBS in the study cohort. These symptoms included general ill feeling/nausea, indigestion/flatulence, and colitis. Since the synbiotic composition was not clearly defined, an assessment of the synergy of this synbiotic cannot be made. The fecal microbiota of the patients was not analyzed and therefore no conclusions regarding the mode of action of this synbiotic can be inferred. Interestingly, the applied organisms are mostly found in soil are not considered members of the human gut microbiota. Whether or not these organisms are capable of reaching the colon or interacting with the autochthonous microbiota remains unknown. Unfortunately, a rationale for choosing soil organisms in a human trial is not given.

The efficacy of Flortec, a synbiotic combination containing 5 x 10^9 CFU *Lactobacillus paracasei* B21060, xylo-oligosaccharides, glutamine, and arabinogalactone, was tested in a parallel-arm, double blind study in patients of IBS (149). Patients were instructed to consume the synbiotic or prebiotic treatment twice a day for twelve weeks and were asked to report GI symptoms on a daily basis. No placebo control or probiotic

only arm was included. A total of 167 IBS patients were enrolled in this study, however, 55 discontinued the treatment prematurely. The main reason to withdraw was a perceived lack of benefit (33 % of the cases). For the remaining patients, no differences were observed between the prebiotic and the probiotic treatment groups. Compared to the baseline, both treatments led to a decrease in IBS scores after one week. There was, however, a significant difference among the number of patients that had at least one bowel movement per day with 70 % in the synbiotic group and 35 % in the prebiotic group. An analysis to assess how a responder differed from a non-responder was not conducted. Subsequently, 47 patients who had a diarrhea predominance were analyzed as a subgroup. The number of bowel movements and IBS score significantly decreased due to the synbiotic treatment compared to the baseline and the prebiotic group. In this study, patients were allowed to use a "rescue treatment". Approximately 8 % of the study subjects used such a treatment, which was not closer defined then "medications effecting gastrointestinal motility and/or perception". These additional treatments were not taken into account during the assessment of the syn- and prebiotic treatments. Collectively, the absence of both a placebo control arm and an analysis of the gut microbiota makes it difficult to establish the effectiveness of the synbiotic.

Dughera et al. conducted an open-label, uncontrolled, and multi-center study in ten Italian gastroenterological centers (150). The applied synbiotic was Zir Fos®, containing 5 x 10^9 CFU of *Bifidobacterium longum* W11 and 2.5 g of Fos-Actilight, a short-chain fructooligosaccharide. A total of 129 patients with constipation-predominant IBS were enrolled and received the treatment for three months. Measured symptoms included abdominal pain, bloating, well-being, stool shape, stool frequency, concomitant treatments

and comorbidity. The results showed that the synbiotic treatment significantly reduced abdominal pain and bloating, and induced a higher stool frequency ($P < 0.0001$).

This same synbiotic (i.e., *Bifidobacterium longum* W11 and Fos-Actilight) was tested on 636 patients with constipation-variant IBS (151). A clear statement about duration of the treatment was not given, only that the treatment lasted for at least 36 days. The dose was the same as in the study above (150), and the treatment effects were evaluated at the end of the study and at a one month follow up visit. The results are similar to those reported by Dughera et al., in that the treatment resulted in significantly decreased bloating and abdominal pain ($P < 0.0001$) and increased stool frequency significantly. Most (~84%) of the patients reported improved symptoms at the end of the treatment, but at the follow-up evaluation the number decreased to 63 %.

In neither trial were the fecal microbiota analyzed nor were control groups with probiotic, prebiotic, or placebo treatment included. Based on these studies no conclusion can be drawn about a synergy between the probiotic and prebiotic.

The ideal human trial to assess health benefits of a synbiotic treatment

The development and selection of successful synbiotic combinations is a very complex issue (32). As noted above and previously recommended by Kolida and Gibson (32), synbiotic trials have to be carefully designed and controlled in order to demonstrate the additive effect of each component, and to assess the minimum effective dose of each component of the synbiotic in order to achieve the desired health benefit, while avoiding side effects. As demonstrated here and previously (32), most studies did not provide the

necessary controls to confirm an additive or synergistic effect of the synbiotic. Importantly, the rationale for how the synbiotic had been formulated is rarely stated. In contrast, an ideal clinical trial would include synergistic synbiotics that had been shown to survive passage through the GI tract and also had an ability to become established in the GI environment. Considering ecological criteria is also important when formulating synbiotic combinations, including demonstrating that the probiotic strain is capable of metabolizing the given prebiotic under competitive conditions. Changes introduced to the gut microbiota should also be assessed to determine if cross feeding or other ecological events had occurred, e.g. niche competition, niche partitioning, or niche exclusion with the resident microbiota. It is also critical to validate that the probiotic had been enriched using strain-specific probes or primers in quantitative PCR assays. The experimental design should include treatments consisting of each component of the synbiotic. Once the characteristics of the pro- and prebiotic are established independently as well as in combinations and an additive effect of the components has been demonstrated, randomized, controlled and double-blinded human trials should be conducted, with prebiotic, probiotic, and synbiotic treatments and a placebo control. Survival of the test strains and changes in the composition of the gut microbiota should be assessed, in addition to measuring the health or clinical biomarkers of interest. The study cohort needs to be sufficiently large to assure adequate power for the statistical analysis. Ideally, the synbiotic should be compared to another a similar synbiotic containing a different strain.

1.7 Commercial synbiotics: recent developments and future prospects

The functional foods market, that includes gastrointestinal health products, is estimated to be worth more than US $ 43 billion. Currently, the US, Europe, and Japan represent 90 % of the global functional food market (152). Within the US market, probiotic products had an estimated revenue of US $ 3.4 billion in 2013, and already in 2014 sales of probiotic products were the fasted growing of all supplements with a 22 % increase and US $ 10 billion revenue (153). It is therefore the most popular functional food ingredient after minerals and vitamins (154). The market revenue for prebiotics is estimated at US $ 334 million, and synbiotics at 69 million. These numbers are expected to increase with the new markets in the Middle East, China, India and New Zealand (35).

Despite the substantial market opportunities for these products, researchers, clinicians, and regulatory agencies continue to emphasize several important issues. In particular, demonstrating that products are safe and effective remains a top priority. Manufacturers are especially motivated to develop appropriate health claim strategies (155). This situation is complicated by different health claim regulations that vary from one country to another and the degree of evidence required to support a health claim (156). Currently, neither EFSA nor the FDA have approved any health claims made for pre-, pro- or synbiotic combinations. Moreover, probiotic and synbiotic products must be distinguished between a pharmaceutical product and a food product (157). The FDA guidelines state that if any agent, including probiotics, is ingested for the purpose of curing, mitigating, treating, diagnosing or preventing disease, it is classified as a “drug” and must undergo the regulatory process similar to any new pharmaceutical. This can be a burdensome process for probiotic/synbiotic foods, especially since the majority of food

products are not produced in pharma grade facilities and the FDA may require phase I safety studies for new synbiotic products. In Europe the European Nutrition and Health Claims regulations intend to: (i) ensure that claims are "clear, accurate and based on scientific evidence"; and (ii) prohibit foods that bear "claims that could mislead consumers". Ultimately, high-quality human intervention studies are necessary to support any health claims of a product (156).

Another obstacle for the food industry is the lack of consumer understanding of these products. Although consumers apparently understand probiotics, at least in general, they are less familiar with prebiotics (or confuse it with probiotics), and even fewer understand synbiotics (35, 47). Therefore some products are not even marketed as synbiotics, but rather as probiotics. Nonetheless many synbiotic products are on the market. To date, most synbiotic-containing foods claim to improve general gut health or the body's natural defense mechanisms by supporting the immune system or lowering blood cholesterol.

Synbiotic products are most commonly presented to consumers as powders or cultured dairy products like yogurts or smoothies. Prebiotics can be used in most food applications, but the environmental sensitivity of probiotics limits their practical use in non-refrigerated foods, since the survivability of the probiotic strains dictates which synbiotics can be developed (35). However, new microencapsulation technologies that protect the bacteria against otherwise detrimental processing treatments could lead to a variety of new synbiotic products, including desserts, candy, juices, cheeses, or chocolate (158–162). Interestingly many pre- and synbiotic products contain rather small amounts of the prebiotic component (on a per serving basis), which may be too low to induce a

health effect. Low doses are used, in part, to avoid adverse gastrointestinal complaints (163), but perhaps also for cost reasons.

Interestingly, despite the new products introduced into the marketplace and questions concerning safety and efficacy, synbiotic foods have a long history of save consumption. As reviewed by Ashwani et al., many indigenous synbiotic foods can be found around the world, including Central America, India, Eastern Europe, China and Africa (164). Most of these synbiotic preparations are fermented beverages, either with defined starter cultures, or by "spontaneous fermentation" (165). However, the uncertainty of the actual microbial composition likely results in inconsistent final food products, unsuitable for industrial sale. Nonetheless, these indigenous synbiotic foods have market potential if produced on an industrial scale and with appropriate quality standards (164).

1.8 Remaining questions and specific aims

Disturbances of the microbial composition in the gastrointestinal tract have been associated with deterioration of host health and functions. These developments may either be directly induced by the gut microbiota, via an altered metabolite synthesis, or via the host immune system (6). While the microbiota composition in the human gastrointestinal tract is remarkably stable, it can be successfully modulated by certain synbiotic treatments. These treatments could offer great advantages to human health when selected on a rational basis. However, there are currently many potential limitations that hinder the development of synergistic synbiotic formulations. There is a need to validate the potential health benefits of synbiotics in carefully controlled human clinical trials. Additionally the future development of new synbiotic combinations should focus on the development of

synergistic synbiotics that prioritize the ecological properties and requirements of the probiotic strain.

The work presented in this thesis aims to answer three important questions, which are relevant for our understanding of how the gut microbiota is shaped and how we can establish successful concepts for the modulation of the gut microbiota.

The first objective of this thesis was to gain insight into the complex interplay between the host and intestinal microbes. For this approach we chose *Lactobacillus reuteri*, which is a commonly used probiotic strain. *L. reuteri* has been shown to be a true symbiont in rodents and densely colonizes the forestomach of mice (166, 167). The aim of this study presented in Chapter 2 was to systematically determine which genes of *L. reuteri* 100-23 contribute to tolerance towards host gastric acid secretion. A better understanding of microbial colonization factors in their host contributes to our understanding of ecological requirements for novel probiotic strains. If these and other findings are truly understood and applied to the rational selection of synbiotic strains, the next generation of synbiotic combinations may have a greater ecological advantage and could be more competitive than current synbiotic combinations on the market.

The second objective of this thesis was to test if rationally selected synbiotic applications have a higher potential to establish probiotic strains in the gastrointestinal tract. To answer this question, Chapter 3 analyses the potential of a novel technique to select synergistic synbiotic combinations. In this study we characterized the potential of an *in vivo* selected combination of *Bifidobacterium adolescentis* and GOS in a rat model.

After the ecological advantages of *in vivo* selected synbiotics had successfully been established, the third objective was to test the potential of this synbiotic combination in

human subjects in Chapter 4 in comparison to a commercial synbiotic. The rationally, *in vivo* selected synbiotic was established in the GI tract of the subjects in significantly higher numbers then the commercial synbiotic.

Together, the studies performed for this thesis present a comprehensive examination of the role of the stomach and dietary factors, such as probiotics, prebiotics and synbiotics, on the establishment of bacteria in the gastrointestinal tract.

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Chapter 2

Characterization of the ecological role of genes mediating acid resistance in *Lactobacillus reuteri* during colonization of the gastrointestinal tract.

Preface

This chapter has been previously published: Characterization of the ecological role of genes mediating acid resistance in *Lactobacillus reuteri* during colonization of the gastrointestinal tract. Janina A. Krumbeck, Nathan L. Marsteller, Steven A. Frese, Daniel A. Peterson, Amanda E. Ramer-Tait, Robert W. Hutkins, and Jens Walter. *Environmetal Microbiology* (2015). doi:10.1111/1462-2920.1310

2.1 Summary

Rodent-derived strains of *Lactobacillus reuteri* densely colonize the forestomach of mice and possess several genes whose predicted functions constitute adaptations towards an acidic environment. The objective of this study was to systematically determine which genes of *L. reuteri* 100-23 contribute to tolerance towards host gastric acid secretion. Genes predicted to be involved in acid resistance were inactivated, and their contribution to survival under acidic conditions was confirmed in model gastric juice. Fitness of five mutants that showed impaired *in vitro* acid resistance were then compared through competition experiments in ex-germ-free mice that were either treated with omeprazole, a

proton-pump inhibitor that suppresses acid secretion in the stomach, or left untreated. This analysis revealed that the urease cluster was the predominant factor in mediating resistance to gastric acid production. Population levels of the mutant, which were substantially decreased in untreated mice, were almost completely restored through omeprazole, demonstrating that urease production in *L. reuteri* is mainly devoted to overcome gastric acid. The findings provide novel information on the mechanisms by which *L. reuteri* colonizes its gastric niche and demonstrate that in silico gene predictions and *in vitro* tests have limitations for predicting the ecological functions of colonization factors in bacterial symbionts.

2.2 Introduction

A complex and diverse collection of microorganisms colonizes the gastrointestinal (GI) tract of mammals, affecting the health and immune status of the host. Among other functions, these microbial communities enhance energy absorption from ingested food, contribute to the development of their host's immune system, and provide colonization resistance against pathogens (Sekirov *et al*., 2010). As a result of co-evolution, the bacteria that reside in the mammalian gut have developed a high degree of ecological fitness and specialization (Oh *et al.*, 2010; Frese *et al*., 2011; O'Callaghan and O'Toole, 2013). Given the importance of the gut microbiota to the health of its host, there is currently much interest in formulating strategies that modulate its composition. However, remodeling this complex ecosystem requires an understanding of the mechanisms by which specific gut microbes colonize the GI tract and the factors that distinguish resident autochthonous members of the microbiota from allochthonous ones (Walter, 2008).

Among the bacteria that are autochthonous to several mammalian species is *Lactobacillus reuteri* (Walter, 2008). *L. reuteri* forms high populations in the rodent stomach that are maintained throughout the life of the animal. Colonization is achieved, in part, by the ability of the organism to adhere to the surface of the non-secretory epithelium present in the forestomach, resulting in formation of a biofilm-like structure (Walter *et al*., 2007; Frese *et al*., 2013). The ability of *L. reuteri* strains to form these biofilms is strictly dependent on their host origin, with only rodent isolates being capable of forming biofilms (Frese *et al.*, 2013). This translates to a higher ecological fitness of rodent strains when colonizing the mouse gastrointestinal tract (Frese *et al*., 2011). *L. reuteri* is therefore an example of a bacterium that maintains a tight, host-specific relationship with its mammalian host (Oh *et al.*, 2010), and hence serves as a model to study ecologically important traits that facilitate host-microbe symbiosis in mammals at the molecular level (Frese *et al*., 2011; Tannock *et al.*, 2005).

A combination of comparative genomic and transcriptomic analyses have been used to identify genes that were overexpressed during gut colonization and contributed to host specificity and biofilm formation in rodent strains of *L. reuteri* (Frese *et al*., 2011, 2013; Schwab *et al*., 2014; Wilson *et al*., 2014). Several of these genes (Table 2.1) are predicted to be involved in acid resistance, reflecting the acidic pH in the gastric niche, which varies depending on food loading and emptying (McConnell *et al*., 2008) from pH 4 and 5.7 in the lumen (Ward and Coates, 1987), and between pH 3.5 and 4 in the forestomach (Ward and Coates, 1987; Gärtner, 2001; Teixeira *et al*., 2014). In particular, the presence of the urease gene cluster is mostly specific to rodent strains and its expression was induced during colonization of the mouse gut, without contributing to biofilm formation (Frese *et al*., 2011, 2013). Wilson and colleagues confirmed its induction *in vivo* and showed that the cluster contributed to ecological performance in *Lactobacillus*-free mice (Wilson *et al.*, 2014). In addition, genes encoding glutamate decarboxylase and glutaminase were also found to be overexpressed during stomach colonization (Schwab *et al*., 2014; Wilson *et al*., 2014). The *dlt* operon, which contributes to acid resistance through the incorporation of D-alanine

esters into cell wall-associated teichoic acids, is essential for *L. reuteri* colonization of the gastrointestinal tract (Walter *et al*., 2007). Several other acid resistance mechanisms (glutamate decarboxylase, glutaminase and arginine deaminase) support growth of *L. reuteri* during sour dough fermentation (Su *et al*., 2011; Teixeira *et al*., 2014).

Although it is established that gastric acid constitutes a potent barrier to bacterial pathogens (Tennant *et al*., 2008), little is known about how lactobacilli autochthonous to the stomach overcome this environmental filter. Of the genes predicted to be involved in acid resistance, only the urease cluster and the *dlt* operon have been studied in colonization experiments in mice (Walter *et al*., 2007; Wilson *et al*., 2014). However, the mechanisms by which these factors facilitate colonization have not been determined, and other functions, independent of acidity, could explain the importance of these factors *in vivo*. In addition, it is unknown which of the other acid resistance factors present in *L. reuteri* contribute to acid resistance during stomach colonization. The goal of this study was therefore to determine the ecological significance of acid-resistance genes present in *L. reuteri* 100-23 during gut colonization, and to systematically determine to what degree they contribute to tolerance to host gastric acid secretion. To achieve this, we compared the ecological fitness of mutants in ex-germ-free mice treated with omeprazole, a proton-pump inhibitor that raises the pH of the stomach from approximately pH 3 to 5 (depending on food loading) to approximately 6.8 to 7.0 (Betton *et al*., 1988), with mice that were left untreated.

2.3 Results

2.3.1 Selection of genes of *L. reuteri* 100-23 predicted to be involved in acid resistance

Genes selected for this study are listed in Table 2.1 and included: (i) the gene encoding for the α-subunit of the urease enzyme (*ureC*). This gene cluster, which hydrolyses urea to ammonia, which increases the pH (Cotter and Hill, 2003), is mainly found in rodent strains of *L. reuteri* but is absent in other isolates and is overexpressed

during gut colonization (Frese *et al*., 2011; 2013; Wilson *et al*., 2014); (ii) arginine deiminase (Adi), which increases acid resistance by intracellular consumption of protons and ammonia production (Arena *et al*., 2002; Cotter and Hill, 2003; Rollan *et al*., 2003; Vrancken *et al*., 2009; Teixeira *et al*., 2014); (iii) the glutamate decarboxylase (GadB), which is specific to *L. reuteri* strains isolated from rodents (Frese *et al*., 2011) and induced *in vivo* (Wilson *et al*., 2014), and further implicated in acid resistance during growth in sourdoughs (Su *et al*., 2011; Teixeira *et al*., 2014); (iv) the cystathionine γ-lyase (Cgl), which catalyses several reactions transforming compounds such as L-cystine, L-cystathionine, L-homoserine, or L-cysteine (De Angelis *et al*., 2002; Wang, 2002). L-cysteine is degraded into pyruvate, hydrogen sulfide and ammonia, the latter of which could increase the pH (Wang, 2002); (v) the *dltA* gene, which is involved in D-alanyl esterification of teichoic acids associated with cell walls, is an important colonization factor of *L. reuteri* (Walter *et al*., 2007) associated with *in vitro* acid resistance in *L. reuteri* (Walter *et al*., 2007) and other organisms (Boyd *et al*., 2000; Kristian *et al*., 2005); and (vi) homologues of a two-component regulatory system consisting of a histidine sensor kinase (*lisK*, lr69622) and response regulator (*lisR*, lr69623), which has been previously shown to be involved in acid response regulation in *Listeria monocytogenes* and *Lactobacillus acidophilus* (Kallipolitis and Ingmer, 2001; Cotter and Hill, 2003; Azcarate-Peril *et al*., 2005). At the protein level, the LisR and LisK homologues have 76 % and 47 % similarity to the proteins in *L. acidophilus*, whereas they show less than 32 % similarity to other two-component systems (cemAKR, bfrKRT, and lr70529/lr70530) described for *L. reuteri* 100-23 (Frese *et a*l., 2011; Su and Gänzle, 2014). As a negative control, a mutant with an inactivated high molecular mass surface protein (*lsp* mutant) was included in our studies,

as this adhesin contributes to ecological performance *in vivo* but is not predicted to be involved in acid resistance (Walter *et al*., 2005).

Table 2.1. Genes selected for functional characterization

Gene	Protein	Description	Putative Function	Reason for Study
*lr*70114	UreC	Urease enzyme, α subunit	Acid resistance	Host specific (Frese *et al*., 2011; Wilson *et al*., 2014), upregulated in biofilms (Frese *et al*., 2013) and *in vivo* (Frese *et al*., 2011; Schwab *et al*., 2014; Wilson *et al*., 2014), and involved in acid resistance and critical for ecological success (Kakimoto *et al*., 1990; Cotter and Hill, 2003; Wilson *et al*., 2014).
*Ir*71325	GadB	Glutamate decarboxylase	Acid resistance	Upregulated in biofilm in *L. reuteri* 100-23 (Wilson *et al*., 2014) and involved in acid resistance (Su et al., 2011; Teixeira et al., 2014).
*Ir*69360	Cgl	Cystathionine γ-lyase	Reactive oxygen resistance (Lo *et al*., 2009)	Upregulated in biofilm in *L. reuteri* 100-23 (Frese *et al*., 2013) and in acid-adapted *B. longum* biotype *longum* (Sánchez *et al*., 2007); pathway produces ammonia (Lo *et al*., 2009), which may have buffering capacity.
*Ir*71377	Adi	Arginine deiminase	Acid resistance	Upregulated in the stomach when compared to the cecum in conventional mice (Schwab *et al*., 2014) and involved in acid resistance in *L. reuteri* 100-23 (Teixeira *et al*., 2014).
*Ir*69622	LisK	Histidine sensor kinase of two-component regulatory system	Two-component regulatory system	Involved in acid response regulation in *Listeria monocytogenes* (Kallipolitis and Ingmer, 2001; Cotter and Hill, 2003) and *Lactobacillus acidophilus* (Azcarate-Peril *et al*., 2005).
*Ir*69623	LisR	Response regulator of two-component regulatory system	Two-component regulatory system	Involved in acid response regulation in *Listeria monocytogenes* (Kallipolitis and Ingmer, 2001; Cotter and Hill, 2003) and *Lactobacillus acidophilus* (Azcarate-Peril *et al*., 2005).
*Ir*1649-*Ir*1652	DltA	D-alanylation of lipoteichoic acids	Acid resistance, biofilm formation	Involved in acid resistance in several organisms (Boyd *et al*., 2000; Kristian *et al*., 2005; Lebeer *et al*., 2008) and strongly contributes to ecological performance in *L. reuteri* 100-23 during gut colonization (Walter *et al*., 2007).

2.3.2 *In vitro* characterization of putative acid resistance genes

Isogenic mutants (Table 2.2) of each gene were generated by insertional mutagenesis and compared with the wild- type for survival in simulated gastric juice at pH 1.5 and 2 (Fig. 2.1A–F). Depending on the gene tested, the gastric fluid was supplemented with the substrate necessary for the particular pathway. The analysis revealed that the *ureC*, *adi*, *cgl*, *gadB* and *dlt* mutants were all impaired in their ability to tolerate acidic pH. For the *ureC*, *Cgl*, *gadB* and *dltA* mutants, the inhibitory effect of acidic conditions appeared to be similar at pH 1.5 and 2. Exceptions were the *adi* mutant, which was more impaired at pH 2 (Fig. 2.1B), and the *dlt* mutant, which was considerably more impaired in its survival at pH 1.5 than at pH 2 (Fig. 2.1E). The omission of urea, arginine, glutamic acid or cysteine reduced the survival rates of the wild-type to those of the respective mutants (grown with the substrates), showing that acid resistance is facilitated by these substrates. The two-component system with similarity to LisRK did not contribute to acid resistance (Fig. 2.1F). As expected, the *lsp* mutant was not impaired in survival in gastric juice (data not shown).

Table 2.2. Strains used in this study

Lactobacillus reuteri 100-23	Isolate of rat gastrointestinal tract	Wesney and Tannock (1979)
Lactobacillus reuteri 100-23c	Plasmid-cured derivate of strain 100-23	McCOnnell and colleagues (1991)
Lactobacillus reuteri 100-23 *ure* C mutant	Urease α-subunit inactivated	Frese and colleagues (2013)
Lactobacillus reuteri 100-23 *lsp* mutant	Large surface protein inactivated	Walter and colleagues (2005)
Lactobacillus reuteri 100-23 *cgl* mutant	Cystathionine γ-lyase inactivated	Frese and colleagues (2013)
Lactobacillus reuteri 100-23 *gadB* mutant	Glutamate decarboxylase inactivated	This study
Lactobacillus reuteri 100-23c *lisR* mutant	Response regulator of two-component regulatory system involved in acid resistance in *Listeria monocytogenes* and *Lactobacillus acidophilus*	This study
Lactobacillus reuteri 100-23c *lisK* mutant	Histidine sensor kinase of two-component regulatory system involved in acid resistance in *Listeria monocytogenes* and *Lactobacillus acidophilus*	This study
Lactobacillus reuteri 100-23c *dlt* mutant	D-alanylation of lipoteichoic acids in the bacterial cell wall inactivated	Walter and colleagues (2007)
Lactobacillus reuteri 100-23c *adi* mutant	Arginine deiminase inactivated	This study
Escherichia coli EC1000	Contains copy of pVW01 *repA* gene	Russell and Klaenhammer (2001)

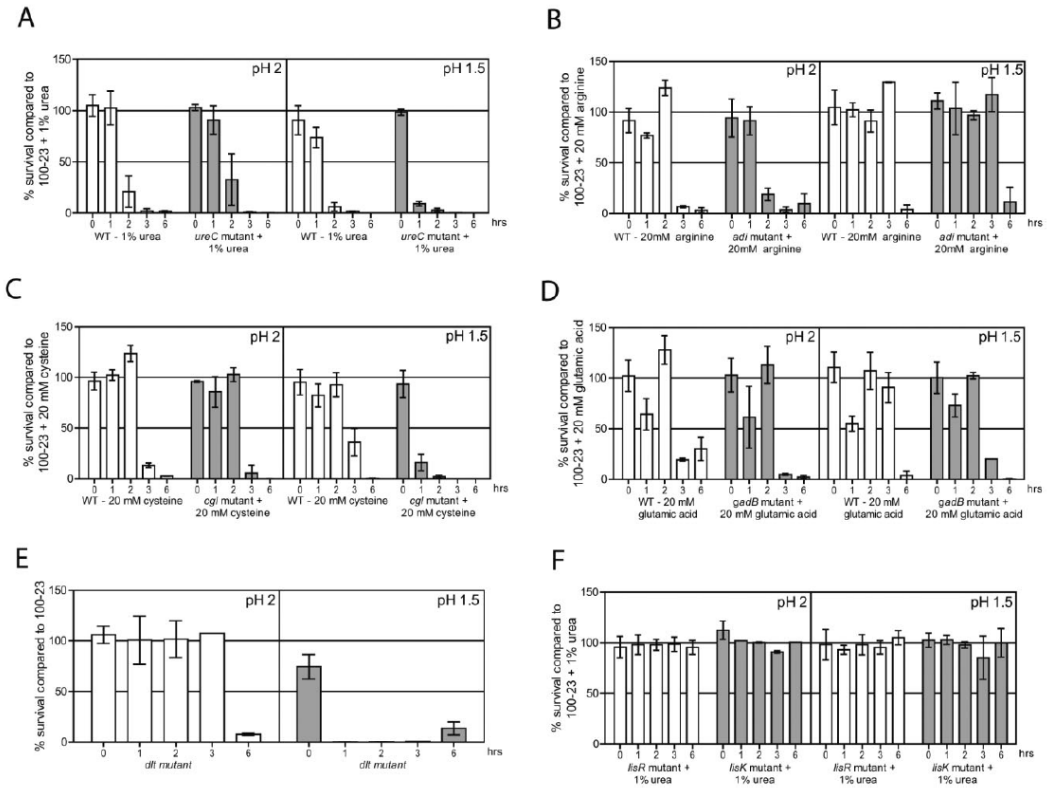

Fig. 2.1. Determination of the capacity of genes (Table 2.1) to confer survival under acidic conditions. The viability of strains was determined after incubation in artificial gastric fluid at pH 2 and 1.5 for 6 h at 37°C. Survival of (I) wild-type strains incubated without the substrate of the respective enzyme and (II) mutant strains incubated with the respective substrate are shown relative (%) to that of the wild-type strain incubated with the respective substrate. (A) *ureC*; (B) *adi*; (C) *cgl*; (D) *gadB*; (E) *dltA*; and (F) *lisR* and *lisK*. Because there is no added substrate for the *dltA*, *lisR* and *lisK* genes, only the survival of the mutants was compared with the wild-type. Data are shown as means with standard deviations of triplicate independent experiments (biological replicates).

2.3.3 Importance of acid resistance genes during colonization of the mouse GI tract

The ecological importance of the five genes found to contribute to acid resistance *in vitro* (see above) was subsequently tested via competition experiments of mutant and wild-type strains in germ-free mice that were treated with omeprazole or left untreated. Therefore, for genes that contribute to acid resistance *in vivo*, omeprazole would lead to an increase in competitive fitness of the mutants. Controls received either a sham treatment [containing only the dimethylsulphoxide (DMSO), polyethylene glycol and water used to dissolve the omeprazole] or no treatment (bacteria only). A schematic summary of the experimental design is depicted in Fig. 2.2A.

The analysis revealed that the inactivation of *ureC* had a large impact on the tolerance of *L. reuteri* 100-23c towards host gastric acid secretion (Fig. 2.2B). Without the neutralizing effect of omeprazole, the *ureC* mutant represented around 0.1% of the *L. reuteri* population in the gut after 8 days of colonization. Omeprazole treatment restored the population of the *ureC* mutant to 29.8% ± 11 of the total lactobacilli population detected in the forestomach and 50.2% ± 15 in the cecum. The omeprazole solvent, polyethylene glycol, has weak buffering capacity, which likely is responsible for the increase of mutant abundance in mice on the sham treatment (Fig. 2.2B).

The *adi* mutant was only slightly impaired *in vivo*. When in direct competition with the wild-type strain, the mutant represented 26.9% ± 13 of the total lactobacilli in the forestomach and 35.7% ± 17 in the cecum. No significant differences were observed for omeprazole or sham treatment (Fig. 2.2C). The inactivation of *gadB* also only led to a slight impairment *in vivo* (Fig. 2.2D), with the mutant comprising 19.5% ± 11 and 13.0% ± 10 of the total lactobacilli population in forestomach and in the cecum, respectively.

Omeprazole treatment had no detectable effect in the forestomach (23.5% ± 14), but significantly enhanced survival in the cecum (31.3% ± 25).

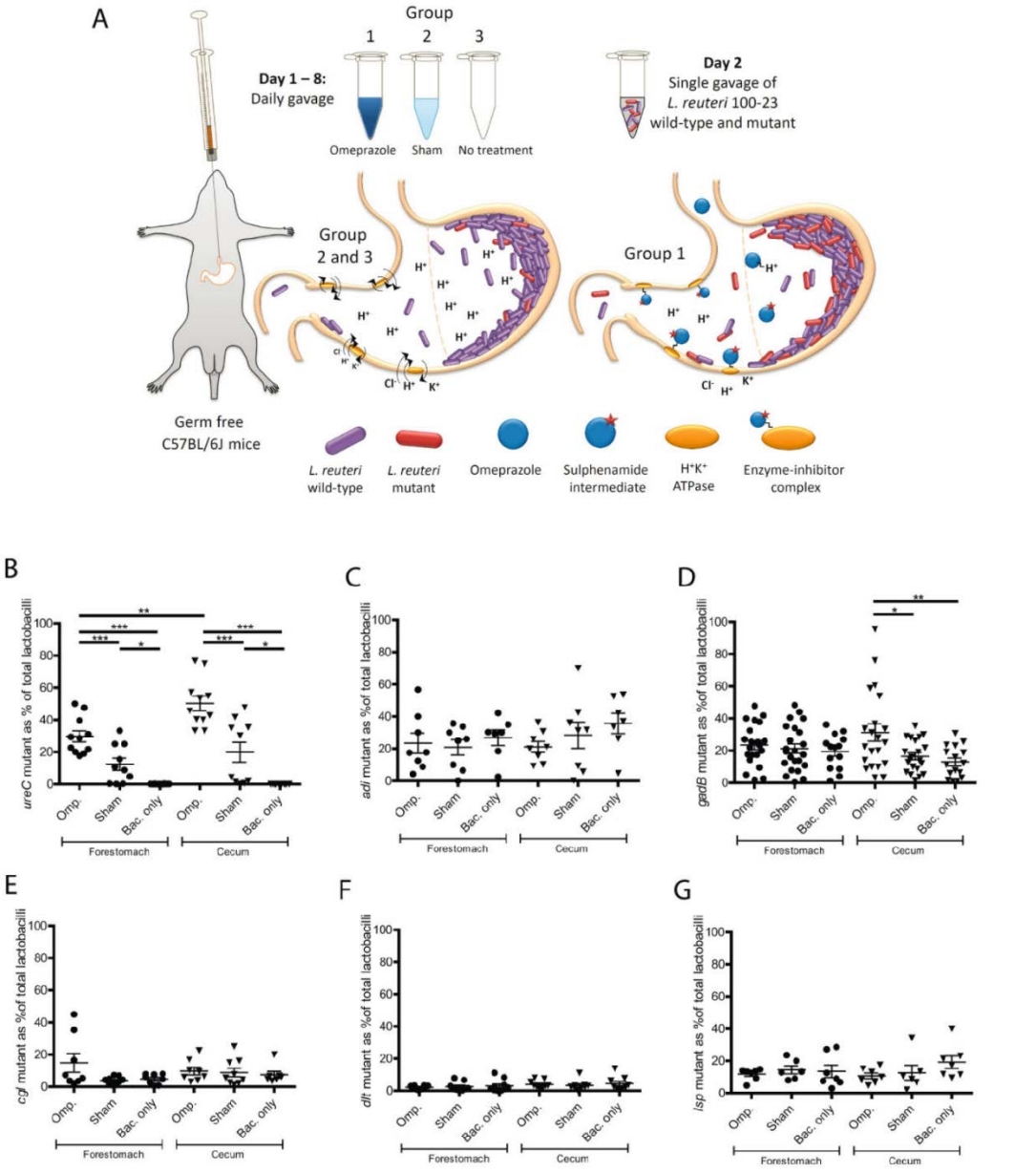

Fig. 2.2. Mouse competition experiment with mutant and wild-type strains in ex-germ-free C57BL/6J mice treated with omeprazole, sham or no treatment. A. Conceptual summary of the experimental design for mouse experiments. Mice were divided into three groups. Group 1 mice were treated daily with omeprazole, whereas group 2 mice were gavaged

with the polyethylene glycol, water and DMSO sham. Group 3 mice served as the control animals and received no treatment. All treatments were administered for 8 days. On day two, all mice received a single gavage with a 1:1 mixture of wild-type and mutant. The proportions of total lactobacilli composed of each mutant in the forestomach and cecum of mice co-inoculated with wild-type and mutant strains were shown. B–G. *In vivo* competition experiment between wild-type and *ureC* mutant (B); wild-type and *adi* mutant (C); wild-type and *gadB* mutant (D); wild- type and *cgl* mutant (E); wild-type and *dltA* mutant (F); wild-type and *lsp* mutant (G). Data are shown as means with standard errors of the mean. Significance of $P \leq 0.05$ is denoted by a single asterisk (*), $P \leq 0.01$ as two asterisks (**), and $P \leq 0.001$ by three asterisks (***). Circles and triangles represent the forestomach and cecum, respectively, of a single mouse.

The *cgl* mutant showed a high degree of impairment when competing with the wild-type in both the forestomach (4.8% ± 3) and the cecum (7.5% ± 6), demonstrating that this gene is ecologically relevant *in vivo* (Fig. 2.2E). Similar findings were obtained for the *dlt* mutant, which was highly impaired (Fig. 2.2F), an observation consistent with previous findings in *Lactobacillus*-free mice (Walter *et al*., 2007). In both mutants, omeprazole did not influence the ecological performance. Thus, it appears that both *cgl* and *dltA* encode for ecologically relevant colonization factors that are not involved in providing resistance to gastric acid secretion.

The *lsp* mutant lacks a putative adhesin that is not involved in acid resistance. We included this mutant to determine unspecific effects of omeprazole on the competiveness of mutant strains in general. As shown previously (Walter *et al*., 2005), the *lsp* mutant was impaired *in vivo*, forming around 10% of the population. However, as expected, no difference between the three treatments was observed (Fig. 2.2G).

Altogether, these experiments demonstrate that the urease gene cluster is the only factor that mediates resistance against gastric acid secretion in *L. reuteri* 100-23 during stomach colonization, and that no other acid resistance factor was able to compensate for its loss under the given experimental and dietary conditions.

2.3.4 Urease activity is regulated by pH

The *in vivo* competition experiments demonstrated the importance of the urease gene cluster as an acid-related colonization factor for *L. reuteri* 100-23 in the rodent forestomach. To characterize the regulation of this cluster, the wild-type strain was grown

in mMRS broth supplemented with 1% urea and growth and urease activity was monitored for 24 h (Fig. 2.3A). The addition of urea caused a slightly decreased growth rate, but led to a rapid increase in pH after 12 h of incubation. The final pH after 24 h was 7.4 with urea supplementation, compared with pH 3.9 without urea in the media ($P < 0.0001$). This alkalization of the supernatant was not observed for the *ureC* mutant (data not shown). There was no detectable urease activity in the first 8 h. However, urease activity became detectable after 10 h when the pH approached pH 4 (Fig. 2.3A).

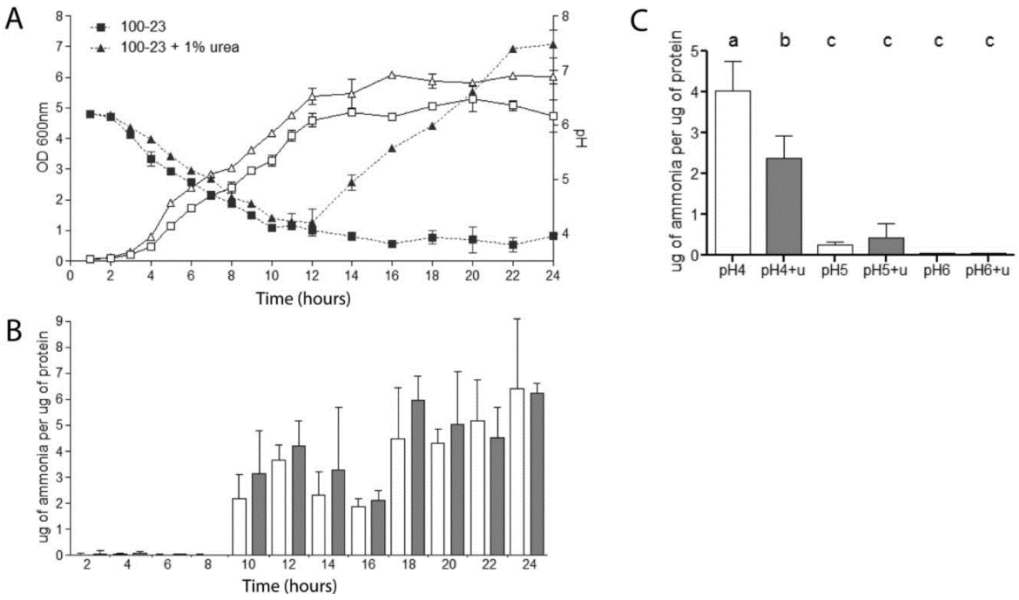

Fig. 2.3. A. Bacterial growth (OD 600) of *L. reuteri* 100-23 (continuous line, empty symbols, left ordinate axis) and the pH of the media (dotted line, full symbols, right ordinate axis) with (triangle symbol) and without urea (square symbol). B. Urease activity over time in cell lysates of wild-type strain 100-23 grown with and without 1% urea in mMRS media, gray and white bars respectively. C. Urease activity in cell lysates of wild-type strain 100-23 in induction experiment. mMRS media adjusted to pH 4, 5 or 6 with and

without 1% urea supplementation, gray and white bars respectively. n = 3, means and standard deviations are shown. Treatments with different letters (a, b, c) are significantly different from one another ($P \leq 0.05$).

Urea supplementation had no significant effect on the urease activity at any time point. These findings suggest that urease activity in *L. reuteri* was not induced by the substrate but by rather acidic conditions. To confirm that induction of urease activity in *L. reuteri* 100-23 occurs via acidity and not urea, cells were grown for 6 h in mMRS, centrifuged and re-suspended in fresh mMRS media adjusted to pH 4, 5 or 6. Cells were incubated for another 2 h before urease activity was measured. Cells after 6 h of growth were used for these experiments as *L. reuteri* did not show any urease activity until 10 h of growth (Fig. 2.3A), allowing the determination of conditions that induce urease activity. This experiment demonstrated that urease activity was induced at pH 4, unaffected at pH 5 and not detectable at pH 6 ($P < 0.01$). The presence of urea did not enhance urease activity. Transcript analysis revealed that expression of *ureC* was 124 times higher at pH 4 compared with pH 6 independently of the presence of urea. These findings demonstrate that the urease activity in *L. reuteri* is regulated on the transcriptional level.

Two-component systems are commonly used by lactic acid bacteria for environmental sensing and signal transduction and are often involved in the acid stress response (Cotter and Hill, 2003). *Lactobacillus reuteri* 100-23 possess homologues to the LisRK system, which has been implicated in mediating acid resistance in *L. monocytogenes* (Cotter et al., 1999) and *L. acidophilus* (Azcarate-Peril et al., 2005). To test if the *lisRK* genes are involved in regulating urease activity, we compared culture supernatant pH of

the *lisK* and *lisR* mutants during growth in the presence of urea. The *lisR* mutant was also tested in the same pH induction experiment described above. Neither the *lisK* nor the *lisR* mutation had an effect on the buffering capacity during growth in the presence of urea, and urease activity was still induced by low pH in the *lisR* mutant in the pH induction experiment (data not shown). Hence, it was concluded that this two-component regulatory system is not involved in the regulation of the urease gene cluster, which is consistent with the finding that the *lisR* and *lisK* mutants were not impaired in simulated gastric juice (Fig. 2.1K). Therefore, it is currently unknown how *L. reuteri* senses acidic pH and induces gene expression of the urease cluster.

2.4 Discussion

The rodent stomach consists of two parts: forestomach and corpus. The forestomach represents about two thirds of the total stomach volume and is lined by a squamous stratified epithelium. The corpus is lined by a glandular and secretory epithelium covered by a mucus layer (Gärtner, 2001) and harbours the H^+ /K^+ proton-pumps responsible for the low pH in the stomach (Fig. 2.2A). *Lactobacillus reuteri* colonizes the forestomach epithelium, but it is also found throughout the digestive tract, including the cecum, where pH values are closer to neutral. However, the spatial patterns of *L. reuteri* populations throughout the mouse digestive tract suggest that cells in the cecum are likely allochthonous to this site and originated from cells colonizing the stomach (Walter, 2008). This notion was supported by the findings of this study; cecal mutant proportions always mirrored those of the forestomach, independently of gene function (Fig. 2.2B–G). This agrees with previous findings concerning the *dlt* (Walter *et al*., 2007), *gtfA*,

inu, (Walter *et al*., 2008), *lsp*, *msrB* (Walter *et al*., 2005) and *ftf* (Sims *et al*., 2011) mutants. The forestomach of mice is therefore the primary habitat of *L. reuteri*, which makes acid resistance a key factor for successful colonization.

Accordingly, several pathways and factors have been identified and functionally characterized to contribute to acid resistance in *L. reuteri* (Fig. 2.4). However, our experiments in omeprazole-treated mice identified the urease gene cluster as the predominant factor necessary for *L. reuteri* 100-23 to tolerate host gastric acidic secretion (Figs 2.2B and 2.4A). Inactivation of the *ureC* gene resulted in the lowest levels of colonization (around 0.1%) of all mutants tested here. This finding, consistent with observations in *Lactobacillus*-free mice (Wilson *et al*., 2014), demonstrated the paramount ecological importance of the urease cluster. Restoration of mutant proportions to around 30% and to 50% with omeprazole in the forestomach and cecum, respectively, indicated that host acid secretion is the main ecological factor decreasing mutant levels, and that urease production of *L. reuteri* is almost completely devoted towards resistance to host gastric acid production. Furthermore, the percentage of mutant strains was significantly lower in the forestomach in omeprazole treated mice compared with the cecum. One could speculate that *ureC* has a residual function in the forestomach that is unrelated to host acid production. Instead, *ureC* may contribute to resistance against the build-up of acidic metabolic end-products in the biofilm generated through bacterial fermentation. Overall, our findings show that host acid secretion exerts a substantial selective pressure on the *L. reuteri* population, even in the non-secretory forestomach, and that urease production serves as an adaptive phenotype to overcome this pressure.

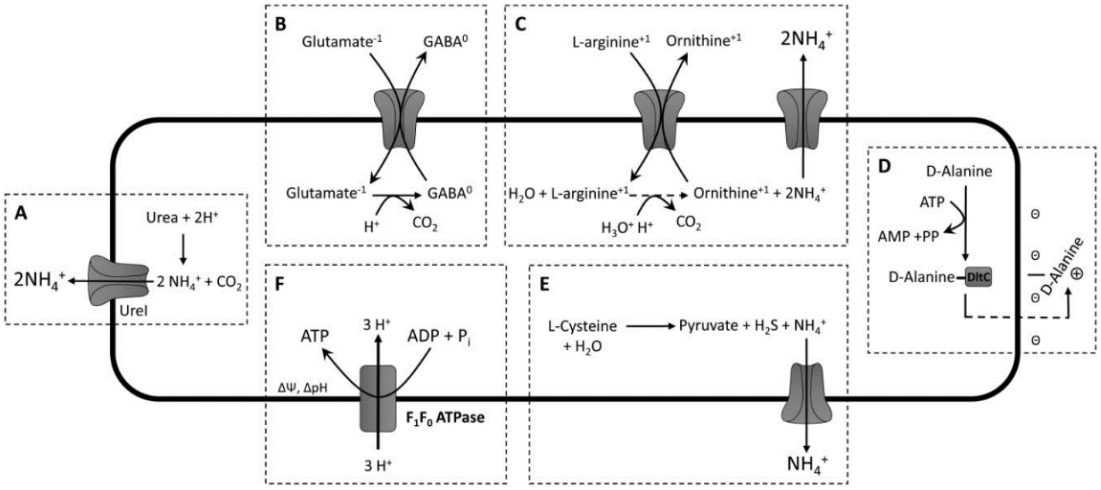

Fig. 2.4. Overview of metabolic pathways of genes assessed in this study. A. The urease gene converts urea to ammonia and CO_2. Ammonia is exported from the cytoplasm using the UreI transporter, thus buffering the cell from its surrounding environment. B. Glutamate is imported into the cell by an antiport system and converted to GABA using the glutamate decarboxylase pathway while generating a $\Delta\Psi$ and ΔpH. H^+ consumption raises the intracellular pH. Adapted from Su and colleagues (2011) and Price and colleagues (2012). C. L-arginine is imported using an L-arginine–ornithine antiporter and converted into citrulline and ammonia by the arginine deiminase enzyme. Citrulline is further catalysed to ornithine and ammonia, while consuming H^+. Ammonia is exported from the cytoplasm potentially using the UreI transporter. D. D-Alanine is coupled to a DltC carrier protein, exported across the cytoplasmic cell membrane and used for esterification of teichoic acids associated with the cell wall. This esterification results in a positive charge of the cell wall. Adapted and simplified from Peschel and colleagues (1999). E. Cysteine is converted to ammonia, hydrogen sulfide and pyruvate by the cystathionine γ-lyase. Ammonia is exported from the cytoplasm using the potentially via the UreI transporter, thus buffering the cell from its surrounding environment. F. F_1 -F_0 -

ATPase-producing ATP using a ΔΨ and ΔpH, which is generated, in part, by the glutamate decarboxylase pathway. Dashed arrows indicate that more than one step is involved in the pathway.

Although the other four genes (*gadB*, *cgl*, *adi* and *dltA*) evaluated here also contributed to both survival in the in vitro gastric model and ecological performance in mice, our findings indicate that they do not contribute to tolerance of host gastric acid secretion during forestomach colonization. Mutants for two of the genes, *gadB* and *adi*, were only marginally impaired, comprising >20% of the population in competition experiments. The *gadB* gene has been identified as the most important mechanism of acid resistance in *Escherichia coli* (Feehily and Karatzas, 2013) and was previously shown to contribute to acid resistance (Teixeira *et al*., 2014) and ecological performance of *L. reuteri* 100-23 during growth in sourdough (Su *et al*., 2011). In our experiments, omeprazole treatment did lead to small but significantly higher levels of the *gadB* mutant in the cecum, suggesting that the gene contributed to acid survival during transit into the cecum but not the forestomach. These findings may be explained by GadB of *L. reuteri* being active primarily at pH 2.5 (Teixeira *et al*., 2014), a feature that may confer a survival benefit when the stomach lumen becomes very acidic. Expression of *gadB* is required for the conversion of glutamate to CO_2 and g-aminobutyric acid (GABA) (Su *et al*., 2011); this function is independent of its role in acid resistance. When glutamate is exchanged with GABA by an antiporter- system, a ΔΨ and ΔpH are generated. This proton motive force generated by GadB provides a mechanism for conserving ATP that would otherwise be required to fuel the F_1 F_0 ATPase (Su *et al*., 2011) (Fig. 2.4B and F). The loss of the proton motive

force may also explain the impairment of this mutant during transit because less energy may be available for the cells to launch a stress response towards the acidic conditions in the stomach lumen.

The arginine deiminase pathway is widely distributed among bacteria (Senouci-Rezkallah *et al*., 2011), triggered in *L. reuteri* CRL 1098 by low pH (Rollan *et al*., 2003), and over-expressed in the *Lactobacillus* population colonizing the stomach of conventional mice when compared with the cecum (Schwab *et al.,* 2014). This pathway consumes intracellular protons and raises the cytoplasmatic pH when converting L-arginine and H_2O to ammonia and citrulline, which is further catalyzed to ornithine, ammonia and CO_2 (Konings, 2002; Teixeira *et al*., 2014) (Fig. 2.4C). *In vivo*, however, the *adi* mutant was only marginally impaired, and the gene did not confer resistance against host acid secretion.

In contrast to the *gadB* and *adi* mutants, the ecological performance of the *dlt* and *cgl* mutants was substantially impaired *in vivo*. Contrary to the consistent involvement of the *dltA* gene in acid resistance of *L. reuteri* and other organisms *in vitro* (Boyd *et al*., 2000; Kristian *et al*., 2005; Walter *et al.*, 2007; Lebeer *et al*., 2008), host acid production was not the factor that reduced mutant populations in mice. An alternative function of the *dlt* operon is to increase resistance to cationic antimicrobial peptides by generating a positive net charge of the cell surface (Kristian *et al*., 2005; Walter *et al*., 2007). This positive charge leads to a decreased binding of positively charged antimicrobial peptides, e.g. defensins, which may result in increased cell lysis and impaired ecological performance *in vivo* (Walter *et al*., 2007) (Fig. 2.4D). A recent study showed that a reduction of the negative cell surface charge through Lipid A dephosphorylation mediates resistance to

antimicrobial peptides in the Gram-negative Bacteroides thetaiotaomicron (Cullen *et al*., 2015). The *dlt* operon could have a similar function in *L. reuteri*.

Although bacterial cystathionine γ-lyases have not been associated with acid resistance, the *cgl* mutant of *L. reuteri* 100-23c was impaired in the *in vitro* acid resistance assays (Fig. 2.1C). Among other reactions, these enzymes catalyze the transformation of L-cysteine and water to hydrogen sulfide, pyruvate and $NH4^+$ (Wang, 2002; Lo *et al*., 2009) (Fig. 2.4E). Although expression of the *cgl* gene is upregulated in *L. reuteri* 100-23 growing in biofilms *in vitro* (Frese *et al*., 2013) and in acid-adapted *B. longum* subsp. *longum* (Sánchez *et al*., 2007), our mouse experiments did not support a role for *cgl* in overcoming gastric acid secretion. In *L. reuteri* BR11, this pathway was shown to improve oxidative stress defense and is required for thiol production (Lo *et al.*, 2009); it could be important during forestomach colonization. This study establishes the *cgl* gene as an important colonization factor of *L. reuteri* 100-23, but further research is needed to elucidate the mechanism by which this gene contributes to gut colonization.

Together with our previous phylogenetic and comparative genomic studies on *L. reuteri* (Oh *et al*., 2010; Frese *et al.*, 2011), this work provides novel insight into the ecology and evolution of a vertebrate gut symbiont, and the mechanisms by which a host-specific lifestyle can emerge. Urease is commonly used by bacteria from different phyla to tolerate stomach acidity (e.g. in Helicobacter pylori) and in some pathogens (e.g. Clostridium perfringens and Yersinina enterocolitica), urease is considered a virulence factor that facilitates survival during gastric transit (Mora and Arioli, 2014). *Lactobacillus reuteri* has acquired the urease cluster, which is extremely rare in the genus *Lactobacillus* (Zheng *et al*., 2015), by horizontal gene transfer (Frese *et al*., 2011). The cluster has then

been stably maintained within rodent lineages of the species (Walter *et al.*, 2011). The findings presented here now provide an explanation for the conservation of this trait among rodent strains – it constitutes an essential colonization factor that provides a key adaptation to the gastric niche in rodents. During the evolutionary process, it appears that *L. reuteri* has tailored transcriptional regulation of the cluster towards the environmental conditions of the murine stomach. Transcriptional expression of the urease cluster is strictly regulated by pH (Fig. 2.3), allowing the organism to respond to the variation in gastric pH and only produce urease when the habitat becomes too acidic. Urea, in contrast, is always present as it enters the stomach by diffusion and through the saliva (Burne and Chen, 2000), and it was therefore not required for *L. reuteri* to evolve a mechanism of substrate induction. Substrate availability through the rodent host is also a likely reason why urease formation evolved to become more important than GadB and Adi, as the latters' substrates (glutamate and arginine) must be provided in the diet where supply is not reliable. Urea hydrolysis is therefore a key facet of host adaptation (and potentially even co-evolution) in the *L. reuteri*–rodent symbiosis, and the absence of the phenotype in most non-rodent strains (Walter *et al.*, 2011) is likely an important reason for their low ecological performance in the mouse GI tract (Oh *et al.*, 2010; Frese *et al.*, 2011).

In conclusion, the findings obtained during this study demonstrated that urease production is essential and sufficient for *L. reuteri* 100-23 to cope with host gastric acid secretion. Other genes, such as *adi*, *clg*, *dltA* and *gadB*, and genes encoding for glutaminase [which were overexpressed in acid resistance tests *in vitro* (Teixeira *et al.*, 2014) and in the forestomach (Wilson *et al.*, 2014) but were not studied here due to the presence of several copies in the genome] might contribute to resistance against acidic bacterial metabolic end-

products, or may become more important in a different dietary context. However, in the experiments conducted here, none of these genes was able to complement the loss of *ureC* in mediating resistance to host gastric acid secretion, which appears to exert a major selective pressure. This study provides a better understanding of the phenotypic adaptations of vertebrate gut symbionts that contribute to both a highly successful lifestyle and specialization towards a particular host. Most importantly, it demonstrates that gene annotations and *in vitro* tests have limitations to predict the exact ecological functions of colonization factors of bacterial gut symbionts.

2.5 Experimental procedures ethics statement

All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of Nebraska (Project ID 731).

2.5.1 Bacterial strains and media used in the study

All strains of *L. reuteri* and *E. coli* are listed in Table 2. Lactobacilli were grown anaerobically at 37°C in de Man, Rogosa and Sharpe (MRS) medium medium (Difco™; Le point-de-Claix, France) supplemented with 10 g l^{-1} maltose and 5 g l^{-1} fructose (referred to as mMRS). For gene inactivation in *L. reuteri* 100-23c (plasmid-free derivative of strain 100-23), *E. coli* EC1000 was used as a cloning vector and grown aerobically in Luria–Bertani media (Difco™; Sparks, MD, USA) at 37°C. Erythromycin (200 µgml^{-1} for *E. coli*, 5 µgml−1 for lactobacilli), kanamycin (40 µgml^{-1} for *E. coli*) and chloramphenicol (7.5 µgml^{-1} for lactobacilli) were used for the propagation of recombinant strains.

2.5.2 Determination of genes predicted to be involved in acid resistance of *L. reuteri* 100-23

Several different approaches were used to select genes of interest for this study. First, we identified genes that were specific to rodent strains of *L. reuteri* (Frese *et al*., 2011) and predicted to be involved in acid resistance. Second, putative acid resistance genes that were upregulated *in vivo* compared with *in vitro* cultures were identified (Frese *et al.*, 2013; Wilson *et al*., 2014). Third, genes coding for metabolic path- ways that produce ammonia (Lo *et al*., 2009) and two- component systems involved in acid resistance in other bacteria were also considered (Kallipolitis and Ingmer, 2001; Cotter and Hill, 2003). One additional criterion for the selection of genes was that the gene had to be a single copy gene to generate the knock-out mutants according to the method described by Walter and colleagues (2005).

2.5.3 Derivation of mutants

Genes of interest were inactivated by insertional mutagenesis by site-specific integration of the plasmid pORI28 into the target sites in the *L. reuteri* 100-23c genome (Walter *et al*., 2005). Internal regions of the genes of interest were amplified using the primers in Table S1 for each mutant. Each knockout mutation was confirmed by polymerase chain reaction (PCR) using primers flanking the target region of each gene. Strains were routinely maintained in mMRS medium containing 5 $\mu g ml^{-1}$ of erythromycin, unless the mutant was used for the *in vitro* acid survival assay. Growth curves showed no growth impairments in any of the mutants (data not shown).

2.5.4 *In vitro* acid survival assay

To simulate the acidic conditions in the mouse stomach, an artificial gastric fluid developed by Cotter and colleagues (2001) was used. The experiment was performed with wild- type *L. reuteri* 100-23c and all mutants; bacterial survival was monitored over time. To evaluate acid resistance, lactobacilli were grown in mMRS (pH 6.5) for 12–16 h, harvested by centrifugation and washed in PBS. Pre-warmed gastric fluid was adjusted to pH 1.5 and 2 with HCl, and inoculated with approximately 108 cells ml^{-1}. Samples were incubated at 37°C and quantified by serial plating after 0, 1, 2, 3 and 6 h. To assess the importance of the *ureC*, *adi*, *gadB* and *LisK/R* genes, assays were performed in gastric fluid with and without 1% urea, 20 mM arginine, 20 mM glutamic acid or 20 mM cysteine respectively. The role of *lsp* and *dlt* genes was assessed without supplementation of the gastric fluid. To gain an insight on the effect of gene inactivation on survival and to allow for a better comparison between experiments, cell numbers of the wild-type strain plus supplement was set to 100%. Based on that value the cell numbers of wild-type without the respective substrate and mutant strains with the substrate was expressed as percent of those obtained with the wild-type incubated with the substrate. Experiments were done in triplicate of biological replicates.

2.5.5 Determination of genes' role in *in vivo* acid resistance

Germ-free C57BL/6J mice (males and females) were bred and reared in flexible film isolators and maintained under gnotobiotic conditions at the University of Nebraska-Lincoln and were randomly assigned to one of three treatment groups. Mice in group 1

received a daily oral gavage of 400 μmol of omeprazole kg^{-1} (6-methoxy-2-[(4-methoxy-3,5- dimethylpyridin-2-yl)methanesulfinyl]-1H-1,3-benzodiazole; Sigma) for 8 days (Tennant et al., 2008). Omeprazole was dissolved in 50 μl of a DMSO-polyethylene glycol solution (90% DMSO, 4.5% polyethylene glycol and 5.5% water) and was filter sterilized (Zavros et al., 2002). Mice in group 2 were orally gavaged daily with the DMSO–polyethylene glycol vehicle and otherwise treated the same way as group 1 animals. Mice in group 3 did not receive any treatment. On day two, each mouse was inoculated with 10^6 cells in a 1:1 ratio of 100-23c wild-type and a mutant strains in a single oral gavage. The inoculum was also plated on mMRS plates with and without erythromycin (5 $\mu g ml^{-1}$) to confirm equal representation of the two strains. Mice had access to food and water ad libitum. After 8 days, mice were euthanized and forestomach and cecum contents were serially diluted and plated on mMRS with and without erythromycin (5 $\mu g ml^{-1}$) to determine the ratio of the wild-type and mutant strains in the samples. A total of 6–11 mice per each group (omeprazole, sham, control) were used per experiment. The experiment was repeated twice with the *gadB* mutant because the first experiment showed a trend towards a higher survival rate due to omeprazole treatment compared with the sham in the forestomach. However, this tendency was not confirmed (Fig. 2.2D). It should be noted that polyethylene glycol possesses weak buffering capacity, which may therefore impact acid exposure to the lactobacilli. Therefore, the amount of solution was kept as low as possible (50 μl total). For all analysed gene clusters, it was assumed that the corresponding substrates, i.e. glutamic acid, arginine, urea etc., were present in the forestomach as they are supplied by the diet, or in the case of urea, enter the stomach by diffusion (Burne and Chen, 2000).

2.5.6 Determination of pH regulation of urease activity

Lactobacillus reuteri 100-23c was grown for 6 h in mMRS at 37°C, centrifuged and re-suspended in fresh mMRS media adjusted to pH 4, 5 or 6 with HCl (before sterile filtration). Cells were incubated for another 2 h at 37°C, and subsequently 10 ml of *L. reuteri* 100-23c culture was centrifuged for 5 min at 15 000 × g and stored in 10% glycerol at −20°C until determination of urease activity.

2.5.7 Measurement of urease activity

Cell solutions were thawed on ice, washed twice with citrate buffer (pH 4) and disrupted with 0.3 g sterile silica beads (0.5 mm) at maximum speed in a cell mill (Mini-Beadbeater Biospec product) for three 1 min intervals. Tubes were cooled on ice for 2 min between intervals to prevent overheating. Samples were centrifuged at 10 000 × g for 2 min. Supernatant was collected and stored at −20°C until the assays were performed. Urease activity in the supernatant was determined by conversion of urea to ammonia, as described previously (Chaney and Marbach, 1962). Citrate buffer (pH 4) containing 167 mM urea was mixed in equal volumes with cell supernatant and incubated at 30°C for 30 min. Ammonia was quantified by the Berthelot reaction (Chaney and Marbach, 1962). To determine protein, cell pellets were washed twice with 10 mM Tris (pH 8) and disrupted as described above. Protein concentration was determined according to Lowry and colleagues (1951). Urease activity is expressed as microgram of ammonia formed per microgram of protein.

2.5.8 RNA extraction from *L. reuteri* cell cultures

Lactobacillus reuteri 100-23 was grown for 6 h in mMRS media at 37°C, and cells were collected by centrifugation for 10 min at 3214 × g and re-suspended in fresh mMRS media adjusted to pH 4, 5 or 6 with HCl. Cells were incubated for another 30 min at 37°C, and subsequently mixed with RNAprotect bacterial reagent (Qiagen, Valencia, CA, USA) at a ratio of 1 to 5. The solution was incubated for 5 min at room temperature, centrifuged and stored at −80°C until used for RNA isolation. Total RNA was isolated after the cell pellet was washed with RNase-free PBS buffer and re-suspended in 100 µl of lysis buffer (30 mM Tris–HCl; 1 mM EDTA, pH 8.0; 15 mg ml^{-1} lysozyme; 10 U ml−1 mutanolysin; and 100 µgml^{-1} Proteinase K). Samples were treated as previously described (Rattanaprasert et al., 2014) and subsequently transferred to an RNeasy Mini spin column (Qiagen, Hilden, Germany). Mixtures were centrifuged for 15 s at 14 000 × g and the eluate discarded. 350 µl of Buffer RW1 was added and centrifuged as before. There was 80 µl of DNase I incubation mix applied to the RNeasy column and incubated at room temperature for 15 min. And, 350 µl of RW1 buffer was added and centrifuged as described above. The flow-through was discarded, 500 µl of Buffer RPE added and centrifuged. 500 µl of Buffer RPE was added again and centrifuged for 2 min at 14 000 × g. RNeasy column was placed in a new 2.0 ml collection tube and centrifuged for 1 min at 14 000 × g. RNeasy column was placed in a new 1.5 ml collection tube and RNA eluted with 50 µl of RNase-free water. Samples were centrifuged for 1 min at 12 000 × g. According to the manufacturer's protocol (Applied Biosystems/Ambion, Austin, TX, USA) the purified RNAwas subsequently treated with the TURBO DNA-free kit. RNA was quantified using the Qubit® RNABRAssay kit (Invitrogen, Carlsbad, CA, USA), and

RNA integrity was validated on a 1% agarose gel. The absence of DNA contamination was confirmed by real-time PCR.

2.5.9 Determination of gene expression by quantitative reverse transcription PCR (qRT-PCR)

The purified RNA was reverse transcribed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions with minor modifications as described by Frese and colleagues (2013). qRT PCR was performed using an Eppendorf Mastercycler Realplex2 machine (Eppendorf AG, Hamburg, Germany) and Quanti-Fast SYBR Green PCR kits (Qiagen, Valencia, CA, USA). The ureC and glyceraldehyde 3-phosphate dehydrogenase primers (Table S1) were previously validated using serial 10-fold dilutions of pooled cDNA to determine specificity and efficiency (Frese *et al.*, 2013). For each 25 µl qRT-PCR reaction, 12.5 µl of 2x Quantifast SYBR Green Mastermix, 1 µl of cDNA and 10 µMol of each primer were used. The DNA was denatured at 95°C for 5 min and followed by 40 two-step cycles of 10 s at 95°C, then 30 s at 60°C. Each PCR product was validated on an agarose gel and by inspection of their melting curves. Gene transcripts of the urease α-subunit were quantified relative to the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene, and relative quantification was performed using the method by Pfaffl (2001).

2.5.10 Statistical analysis

Data are expressed as means ± standard deviations unless otherwise stated. Statistical analyses were carried out using GRAPHPAD PRISM 5 (GraphPad Software, California, USA). If only two groups were compared, Student's t-tests were per- formed. ANOVA and Tukey's post-tests were used for multiple comparisons. Significance of $P \leq 0.05$ is denoted by a single asterisk (*), $P \leq 0.01$ as two asterisks (**), and $P \leq 0.001$ by three asterisks (***).

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Chapter 3

In vivo selection to identify bacterial strains with enhanced ecological performance in synbiotic applications.

Preface

This chapter has been previously published: *In Vivo* Selection to identify bacterial strains with enhanced ecological performance in synbiotic applications. Krumbeck, Janina A., María X. Maldonado-Gomez, Inés Martínez, Steven A. Frese, Thomas E. Burkey, Karuna Rasineni, Amanda E. Ramer-Tait, Edward N. Harris, Robert W. Hutkins, and Jens Walter. *In vivo* selection to identify bacterial strains with enhanced ecological performance in synbiotic applications. *Applied and Environmental Microbiology* 81, no. 7 (2015): 2455-2465

3.1 Abstract

One strategy for enhancing the establishment of probiotic bacteria in the human intestinal tract is via the parallel administration of a prebiotic, which is referred to as a synbiotic. Here we present a novel method that allows a rational selection of putative probiotic strains to be used in synbiotic applications: *in vivo* selection (IVS). This method consists of isolating candidate probiotic strains from fecal samples following enrichment with the respective prebiotic. To test the potential of IVS, we isolated bifidobacteria from human subjects who consumed increasing doses of galactooligosaccharides (GOS) for 9 weeks. A retrospective analysis of the fecal microbiota of one subject revealed an 8-fold enrichment in *Bifidobacterium adolescentis* strain IVS-1 during GOS administration. The functionality of GOS to support the establishment of IVS-1 in the gastrointestinal tract was then evaluated in rats administered the bacterial strain alone, the prebiotic alone, or the synbiotic combination. Strain-specific quantitative real-time PCR showed that the addition of GOS increased *B. adolescentis* IVS-1 abundance in the distal intestine by nearly 2 logs compared to rats receiving only the probiotic. Illumina 16S rRNA sequencing not only confirmed the increased establishment of IVS-1 in the intestine but also revealed that the strain was able to outcompete the resident *Bifidobacterium* population when provided with GOS. In conclusion, this study demonstrated that IVS can be used to successfully formulate a synergistic synbiotic that can substantially enhance the establishment and competitiveness of a putative probiotic strain in the gastrointestinal tract.

3.2 Introduction

The mechanistic role of the gastrointestinal (GI) microbiota and its metabolites in maintaining human health has been well demonstrated (1–3). Gut microbes provide several important benefits for their host, including provision of nutrients, development and maturation of the immune system, and protection against pathogens via colonization resistance (4). However, the gut microbiota may also contribute to obesity, inflammatory and autoimmune diseases, and other chronic disease states (5–7). Such diseases are often associated with compositional alterations in the fecal microbiota, a condition referred to as "dysbiosis" (8). Given that the presence of specific types of bacteria and their relative abundance within the gut are considered to affect host health, there is much interest in devising strategies that modulate gut microbiota composition and potentially redress disease related dysbiotic patterns (9).

Dietary approaches currently available to modulate the gut microbiota include prebiotics (10–12), fermentable fibers (13, 14), probiotics (or live biotherapeutics) (15), and synbiotics, which are a combination of a probiotic and a prebiotic (11, 16). According to Kolida and Gibson (16), synbiotics can be either complementary or synergistic. Complementary synbiotics consist of a probiotic and a prebiotic selected to independently confer benefits to the host. In contrast, synergistic synbiotics are comprised of a prebiotic chosen specifically for the selected probiotic to stimulate its growth, activity, and survival in the gastrointestinal tract (16).

Synergistic synbiotics therefore hold the potential to improve the establishment of a specific bacterial strain when introduced into the gastrointestinal tract. Unfortunately, successful synergistic synbiotic combinations are not well established in the literature

despite a large number of studies. To our knowledge, only two reports describe a synbiotic combination in which the prebiotic significantly enhanced the stability, persistence, or metabolic activity of a specific probiotic strain *in vivo* (17–19). As noted by Kolida and Gibson (16), this low success rate may be explained by the selection of most synbiotic combinations on an arbitrary basis, including shelf life, industrial performance, availability, and cost. Indeed, few synbiotic preparations are formulated based on a rational selection of both the prebiotic and the probiotic (12, 16), such as via *in vitro* or *in vivo* screens assessing the ability of the probiotic to utilize the prebiotic (17–21). Even if synbiotic formulations were based on these criteria, synergism between the probiotic strain and the prebiotic was rarely observed in human and animal trials (22–24). These observations suggest that the probiotic strains were unable to utilize the selected prebiotic to expand their populations under the prevailing ecological conditions in the gastrointestinal tract. We therefore propose that synergistic synbiotics are likely to be more successful if selection of the probiotic organism is based on ecological criteria.

In this report, we introduce the concept of *in vivo* selection (IVS) to identify putative probiotic strains with enhanced ecological performance when used in synbiotic applications. The concept consists of isolating putative probiotic strains from fecal or intestinal samples after enriching for them with dietary administration of the prebiotic. We reasoned that such strains would likely be able to successfully utilize the prebiotic *in vivo* within the constraints of the competitive gastrointestinal environment. To test IVS, we isolated bifidobacteria from fecal samples of human individuals who had consumed the prebiotic galactooligosaccharide (GOS) during a previous human trial (25). A combination of approaches was used to select a candidate probiotic strain (*Bifidobacterium adolescentis*

strain IVS-1) enriched by GOS *in vivo*. We then tested the synergistic potential of this strain and GOS when administered as a synbiotic combination in a rat model of high- fat-diet-induced nonalcoholic fatty liver disease (NAFLD). A NAFLD model without severe inflammatory disease was chosen, as inflammation would potentially confound the ecological analysis due to its effects on gut microbiota composition. Although no direct physiological benefits were observed in the rats, the results from the gut microbiota analysis demonstrated that IVS can be used to select a synergistic synbiotic combination that substantially increases the ecological performance of the bacterial strain *in vivo*.

3.3 Materials and methods

3.3.1 Isolation of *in vivo*-enriched bifidobacteria from humans.

In a previous study (25), fecal samples were collected from subjects who consumed cumulative doses of GOS (0, 2.5, 5, and 10 g per day for 3 weeks each). Throughout the study, fresh fecal samples were collected and immediately plated onto Rogosa LS agar to enumerate bifidobacteria. Bacterial counts were used to identify GOS responders (i.e., individuals who experienced significant increases in numbers of bifidobacteria), and colonies were picked during the period in which 10 g GOS day^{-1} was consumed. Colonies were purified by successive liquid and plate cultures, and stock cultures were prepared and stored at -80°C. A total of 28 individual colonies (2 to 3 per subject) were propagated. To classify isolates, DNA was extracted by using the phenol-chloroform extraction method (26), and the 16S rRNA gene was amplified by using the 8F and 1391R universal primers. The amplification product was purified (QIAquick PCR purification kit; Qiagen Inc., MD) and sequenced by a commercial provider (Eurofins MWG Operon, Huntsville, AL).

Identity was determined by comparing sequences to sequences in the GenBank database; species were assigned based on the best match.

3.3.2 *In vitro* growth on GOS.

Each isolate was screened for its ability to use GOS as a growth substrate in an MRS broth culture. Growth experiments were performed with basal MRS broth containing 2% (wt/vol) glucose or GOS (Purimune; GTCNutrition, Golden, CO). The latter contained 92% GOS, with residual carbohydrates being mainly lactose. Control cultures were therefore also grown on basal MRS broth supplemented with the same amount of lactose as that present in the commercial GOS (giving a final concentration of 0.16% lactose). Cultures were incubated anaerobically at 37°C, and growth was determined by optical density measurement at 600 nm. Strains that grew on GOS to cell densities similar to those on glucose were considered GOS fermenters.

3.3.3 Strain-specific primer design and validation.

The genome of *B. adolescentis* IVS-1 was sequenced to draft status by using a standard shotgun library prep kit on a Roche GS FLX sequencer at the former Core for Applied Genomics and Ecology (CAGE) (University of Nebraska, Lincoln, NE). Sequencing resulted in 65,460 reads that were assembled *de novo* by using the gsAssembler (Newbler) module of the GS-FLX Off- Instrument software suite. This resulted in draft sequences of 148 contigs with ~15-fold coverage.

Unique genes in *B. adolescentis* IVS-1 were identified by comparing the annotated genome with other available *B. adolescentis* genomes in the JGI database (using the Phylogenetic Profiler for Single Genes tool in IMG). From this analysis, the clustered regularly interspaced short palindromic repeat (CRISPR)-associated helicase Cas3 was selected as the target gene, and a putative primer pair was designed by using Primer 3 software (27). Candidate primers were evaluated for hairpin and dimer formation by using Netprimer (Premier Biosoft International, Palo Alto, CA). The selected forward (F) primer TTGCTTTTGCTCTGGAACATAC and reverse (R) primer GTAATGAGGTAATACTGCGTCC were validated in silico by performing a BLAST search against the NCBI database. These primers were also validated experimentally by quantitative real-time PCR (qRT- PCR) using DNA from 10 different *Bifidobacterium* strains related to strain IVS-1 (each having >96% identity at the 16S rRNA gene level). These strains included *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium adolescentis* L2-32, *Bifidobacterium longum* subsp. *longum* ATCC 15707, *Bifidobacterium longum* DJO10A, *Bifidobacterium longum* ATCC 15697, *Bifidobacterium longum* subsp. *longum* F8, *Bifidobacterium longum* subsp. *longum* JDM301, *Bifidobacterium* sp. strain 113, *Bifidobacterium* sp. strain 12_1_47BFAA, and *Bifidobacterium* sp. strain HMLN14. Furthermore, to test if primers could select against fecal bacterial communities in both humans and rats, DNA from 23 human fecal samples and 10 Sprague-Dawley rat fecal samples from an independent study were tested. Human fecal materials analyzed included the baseline samples (i.e., before GOS supplementation) from 18 subjects from a previous study by Davis *et al.* (25) as well as five other human fecal samples from an independent study.

3.3.4 Quantitative real-time PCR.

qRT-PCR was performed by using a Mastercycler Realplex2 instrument (Eppendorf AG, Hamburg, Germany). Each PCR was performed with 25-µl volumes using real-time master mix containing SYBR(5 Prime Inc., Gaithersburg, MD) and either genus-specific primers for *Bifidobacterium*, F primer TCGCGTC(C/T)G GTGTGAAAG and R primer CACATCCAGC(A/G)TCCAC (25, 26), or the strain-specific primers for *B. adolescentis* IVS-1 (described above), each at a concentration of 0.8 µM. Annealing temperatures of 58°C and 61°C were used for the genus- and strain-specific PCRs, respectively. Standard curves for absolute quantification of bacterial cell numbers were prepared by using cultures of *B. adolescentis* IVS-1 grown overnight (14 h), as described previously (25, 26).

3.3.5 Administration of the probiotic, prebiotic, and synbiotic to rats.

A freeze-dried powder of *Bifidobacterium adolescentis* IVS-1 was produced by a contract manufacturer (Culture Systems, Mishawaka, IN). The powder contained 5×10^{10} CFU g^{-1} and was stable during the entire course of the study. For delivery to the rats, the powder was suspended in drinking water (double-distilled water) to reach a concentration of 3×10^{7} cells ml^{-1}. GOS was diluted in water at a concentration of 0.033 g ml^{-1}, and the synbiotic was prepared by mixing both IVS-1 and GOS in the abovementioned concentrations. All preparations were prepared fresh daily in drinking water for the duration of the experiment. Cell viability and stability were validated by plating samples on MRS medium at different time points. This analysis revealed that IVS-1 was highly

stable in drinking water, with levels dropping <1 log over 24 h. The addition of GOS did not influence the viability of the probiotic in drinking water (data not shown).

3.3.6 Rat study design.

Synergism of the synbiotic preparation was tested in a rat model of NAFLD (28). Four-week-old male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) and acclimated for five days prior to study initiation. All animals were housed in pairs in individually vented cages mounted on a rack with positive airflow. The room environment was maintained at 20°C to 21°C with a 12-h light-dark cycle. Prior to the start of the study, all rats received a standard rat chow and autoclaved, double-distilled water ad libitum during the five day acclimation period. All animal procedures were approved by University of Nebraska—Lincoln IACUC.

Rats were randomly assigned to one of five treatments, with three to six rats per group. Groups one through four were fed a high-fat diet (60%kcal from fat) (AIN-58G9 TestDiet) (see Table 3.S1 in the supplemental material), while group five received a standard diet (12% fat) (AIN-58G7 TestDiet) for eight weeks. After four weeks of feeding, groups were assigned to one of the following supplement treatments. Rats in groups one and five received no additional treatment. Group two rats received drinking water supplemented with 3.3% GOS to give ~1 g of GOS day^{-1} rat^{-1}. Group three rats were given drinking water supplemented with ~1 x 10^9 CFU of *B. adolescentis* IVS-1 day^{-1} rat^{-1}. Group four rats received both the GOS and IVS-1 (synbiotic mixture), at the same doses as those given to groups two and three. All treatments were prepared fresh daily and

administered for four weeks. The daily water intake per rat was significantly different among groups and was used to calculate the absolute doses of probiotic cells per day (P = 0.001) (see Table 3.S2 in the supplemental material). Rats fed the probiotic drank significantly more water (41.9 ± 8.6 ml) than did rats fed the synbiotic (35.4 x 4.5 ml), resulting in a significantly higher dose of IVS-1 in the probiotic group (1.26 x 109 CFU versus 1.06 x 109 CFU; P = 0.0001). GOS consumption was not significantly different between the prebiotic- and synbiotic-fed groups (P = 0.2063) (see Table 3.S2 in the supplemental material).

Body weights were determined weekly throughout the study. All rats were necropsied after eight weeks of study. Blood, cecum, colon content, liver, and epididymal fat pads were collected, and the cecum and colon content were immediately frozen in liquid nitrogen and stored at -80°C until further use.

3.3.7 Evaluation of host physiological parameters in rats.

Liver lipid extraction was performed according to methods described previously by Folch and colleagues (29). Aliquots of lipid extract were saponified to quantify triglycerides (TGs) by using the TG diagnostic kit (Thermo dimethyl adipimidate kit; Thermo Electron Clinical Chemistry, Louisville, CO). Data are reported as μg TGmg^{-1} (wet weight) liver tissue. To evaluate liver damage, plasma alanine aminotransferase (ALT) and alkaline phosphatase (ALP) enzyme levels were measured, which are indicators of hepatocyte damage/leakage and cholangiocyte stress, respectively (30, 31). Blood was collected into heparinized tubes at necropsy, and ALT and ALP levels were quantified by

using a Mammalian Liver Profile rotor in a VetScan VS2 analyzer (Abaxis, Union City, CA). Levels of tumor necrosis factor alpha (TNF-α) and monocyte chemoattractant protein 1 (MCP-1) were quantified as measures of systemic inflammation by using a Milliplex rat magnetic bead multiplex assay (Merck Millipore, Billerica, MA) according to the manufacturer's protocol.

3.3.8 Illumina 16S RNA sequencing and sequence analysis.

Colonic and cecal contents were flash-frozen in liquid nitrogen at necropsy, and DNA was extracted as described previously (26), with one modification: the lysis buffer contained 20mM Tris-HCl (pH 8), 2mM EDTA, 1.2% Triton X-100 (pH 8.0), and 20 mg ml^{-1} Lysozyme (MP Biomedicals, Solon, OH). Amplicon sequencing of colonic contents was performed by the University of Minnesota Genomics Center, and all samples were sequenced together in the same run. First, theV5-V6region of the 16SrRNA gene was amplified with primer pair 784F (5'-RGGATTAGATACCC-3') and 1064R (5'-CGACRRCCATGCANCACCT-3') in a 25µl PCR mixture containing 5 µl of template DNA, 5µl of 2x HotStarTaqPCRmaster mix, a final concentration of primers of 500 nM, and 0.025 U $µl^{-1}$ HotStarTaq polymerase (Qiagen Inc.). Amplification reactions included an initial denaturation step at 95°C for 5 min followed by 20 to 25 cycles of denaturation (50 s at 94°C), annealing (30 s at 40°C), and elongation (30 s at 72°C). Next, samples were diluted 1:100 in water for input into library tailing PCR. The PCR was analogous to the one conducted for initial amplification except for a *Taq* polymerase concentration of 0.25 U $µl^{-1}$, and the PCR conditions consisted of an initial denaturation step at 95°C for 5 min

followed by 10 to 15 cycles of denaturation (50 s at 94°C), annealing (30 s at 40°C), and elongation (1 min at 72°C).

PCR products were quantified by using the Quant-iT PicoGreen double-stranded DNA (dsDNA) assay kit (Life Technologies). A subset of the amplicon libraries was spot checked on a Bioanalyzer High-Sensitivity DNA chip (Agilent Technologies, Santa Clara, CA) for correct amplicon size. Next, samples were normalized to 2 nM and pooled. The total volume of the libraries was reduced by the use of a SpeedVac, and amplicons were size selected at 420 bp ± 20% by using the Caliper XT system (PerkinElmer, Waltham, MA). Afterwards, library pools were cleaned with 1.8 x AMPureXP beads (Beckman Coulter, Brea, CA) and eluted in water. The amount of DNA in the final pool was quantified with PicoGreen and normalized to 2 nM for input into the Illumina MiSeq platform (v3 kit) to produce 300-bp paired-end sequencing products. Clustering was done at 10 pM with a 5% spike of PhiX. The generated sequences were quality filtered with Illumina software at the University of Minnesota Genomics Center. Twenty-two of 24 samples met all quality control criteria and were used for the microbial community analysis.

3.3.9 Microbial community analysis.

Reads were trimmed to 240 bp with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and paired- end reads were merged with the merge-illumina-pairs application (https: //github.com/meren/illumina-utils/) (P value of 0.03, enforced Q30 check, perfect matching to primers, and no ambiguous nucleotides al-

lowed). Files exceeding 30,000 reads were subsampled to this number in Mothur v.1.31.162 to standardize the sequencing depth across samples. Subsequently, USEARCH v7.0.100163 was used to generate operational taxonomic units (OTUs) with a 98% similarity cutoff. OTU generation included the removal of putative chimeras identified against the Gold reference database, in addition to the chimera removal inherent to the OTU clustering step in UPARSE. After quality control and chimera removal, samples contained an average of 25,718 ± 941 sequences. The resulting sequences were also taxonomically characterized from phylum to genus levels with Ribosomal Database Project (RDP) Classifier with the MultiClassifier v1.1 tool. All phylotypes were computed as percent proportions based on the total number of sequences in each sample.

3.3.10 Statistical analysis.

Results are expressed as means ± standard deviations (SD) unless otherwise stated. To analyze bacterial composition, diversity differences, and host physiological parameters, one-way analyses of variance (ANOVA) with repeated measures in combination with Tukey's post hoc tests were applied. To achieve normality for data that were not normally distributed, values were subjected to $\log_{10}$ transformations. If only two groups were compared, Student's t tests were performed. Spearman's correlations were used to assess correlations between bacterial groups. To account for type I errors, the false discovery rate was used. A P value of < 0.05 and correlation coefficient (r) values of > 0.60 (in absolute values) were considered significant. Analyses of variance and false discovery rate control were performed by using SAS/STAT (SAS Institute Inc., Cary, NC, USA), while

correlations were determined by using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

3.3.11 Nucleotide sequence accession number.

The genome sequence of B. adolescentis IVS-1 has been deposited in the DDBJ/EMBL/GenBank data- base under accession number JRNZ01000000.

3.4 Results

3.4.1 *In vivo* selection of *B. adolescentis* IVS-1.

In a previous study (25, 32), we reported a significant and remarkably specific enrichment of *Bifidobacterium* populations in human subjects during dietary supplementation with GOS (as demonstrated by 454 sequencing, genus-specific qRT-PCR, and quantitative culture), which is in agreement with data from other GOS feeding studies (33–38). Cultural enumeration of fecal samples during the human trial allowed us to identify individuals in which bifidobacteria were enriched by GOS and from whom strains likely to utilize GOS *in vivo* could be selected. This novel strategy for selection and recovery of autochthonous strains enriched by a prebiotic is referred to as *in vivo* selection (IVS) (Fig. 3.1A). Using the IVS approach, a total of 28 presumptive bifidobacterial colonies from 11 subjects were isolated and classified by sequencing of the 16S rRNA genes. Eight isolates were classified as *Bifidobacterium adolescentis*, eight were classified as *Bifidobacterium longum*, three were classified as *Bifidobacterium pseudocatenulatum*, and one was classified as *Bifidobacterium bifidum*. Of the remaining isolates, four

belonged to the *Coriobacterium* genus, one could be classified only to the family level (*Lachnospiraceae*), and three could not be sequenced due to insufficient growth. All strains resulting in pure cultures were also screened for their ability to ferment GOS during *in vitro* growth, and 13 were classified as GOS fermenters, 12 were classified as non-fermenters, and three could not be propagated to be tested (data not shown). Out of the 13 strains able to ferment GOS, five were classified as *B. longum*, five were classified as *B. adolescentis*, one was classified as *B. bifidum*, one was classified as *B. pseudocatenulatum*, and another one was classified as *Lachnospiraceae*. None of the isolated *Coriobacterium* strains were classified as fermenters.

Based on the culture data, 454 sequencing (32), and the GOS fermentation tests, we selected one strain and designated it IVS-1. This strain originated from a subject who showed a strong bifidogenic response to GOS (Fig. 3.1B). Based on 16S rRNA sequencing, IVS-1 had 98.4% identity (100% query coverage and an E value of zero) with the 16S rRNA gene of *B. adolescentis* ATCC15703T and was therefore allotted to this species. However, the strain belongs to a distinct phylogenetic cluster (*Bifidobacterium* species II cluster) detectable by using the V1-V3 region of the 16S rRNA gene (32). This cluster was significantly enriched by GOS in all subjects, including the individual from whom IVS-1 was isolated (Fig. 3.1C). The ability of *B. adolescentis* IVS-1 to utilize GOS was demonstrated by growth in MRS broth containing 2% GOS (see Fig. 3.S1 in the supplemental material). The established metabolic benefits of the species *B. adolescentis* serve as another rationale for the selection of IVS-1 for future applications (39, 40).

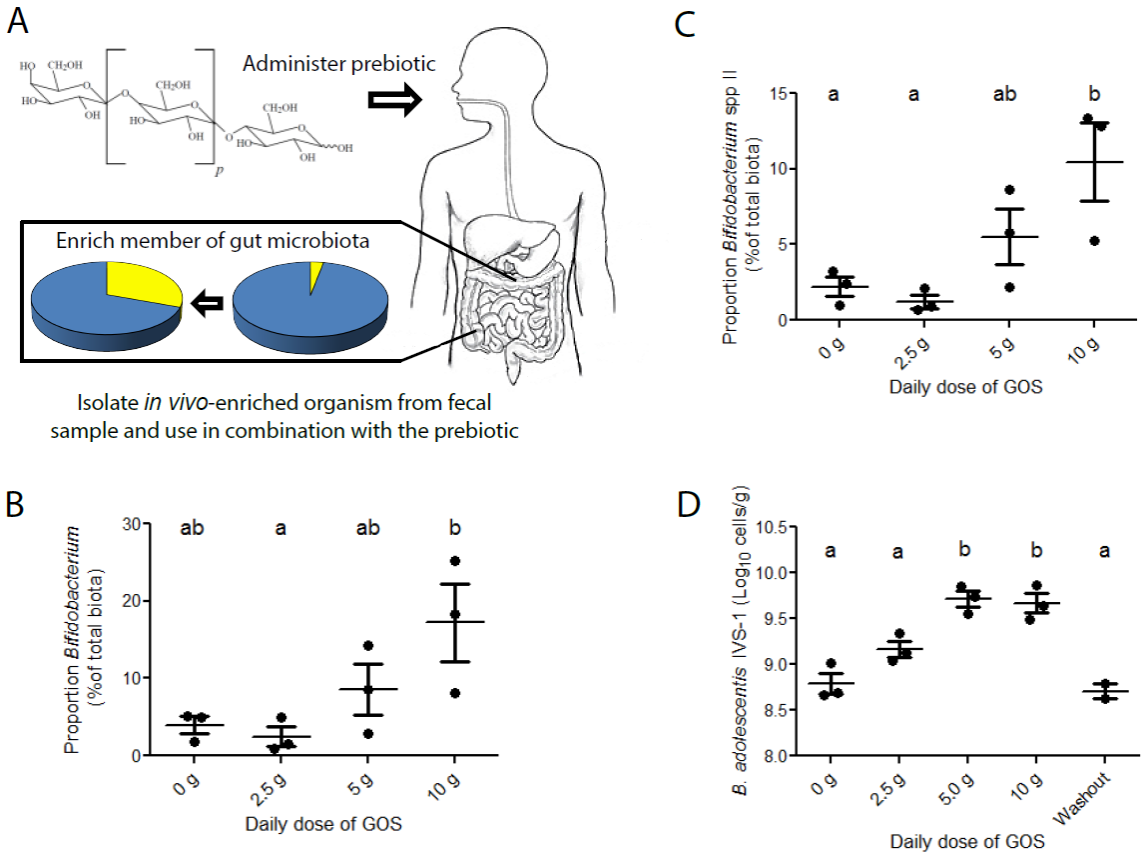

Fig. 3.1. *In vivo* selection to identify putative probiotic strains to be used in synbiotic applications. A. Concept of *in vivo* selection. B. Proportion of fecal bifidobacteria in a human individual consuming GOS (included in chews) in four increasing doses (0, 2.5, 5, and 10 g) during a human feeding trial (25), as determined by 454 pyrosequencing of 16S rRNA tags. C. Proportion of *Bifidobacterium* lineage species II in the same individual, as determined by pyrosequencing. D. Cell numbers of *B. adolescentis* IVS-1 in the same individual, as quantified by strain-specific qRT-PCR.

To verify that *B. adolescentis* strain IVS-1 was specifically enriched by GOS *in vivo*, we devised a strain-specific qRT-PCR approach with primers based on the genome sequence of IVS-1. Primer specificity was validated against ten closely related

Bifidobacterium strains, fecal DNA from all subjects included in the human feeding trial (25) and five additional human individuals, and ten fecal samples from Sprague-Dawley rats from an independent experiment. A detectable PCR product was obtained only with DNA from *B. adolescentis* IVS-1 and the fecal sample from which the strain was isolated. This finding indicated that the primers were highly strain specific and that strain IVS-1 was present only in the human subject from whom it was isolated.

The strain-specific qRT-PCR system was then used to quantify the abundance of IVS-1 in fecal samples from this subject during the GOS feeding study. This analysis revealed that IVS-1 levels were increased 8-fold during both the 5-g and 10-g GOS dose periods compared to the 0-g period ($P < 0.001$) (Fig. 3.1D), before returning to baseline levels immediately after GOS consumption ended. Collectively, these results demonstrated the utility of IVS to select a bacterial strain enriched in the human gastrointestinal tract through dietary administration of a prebiotic.

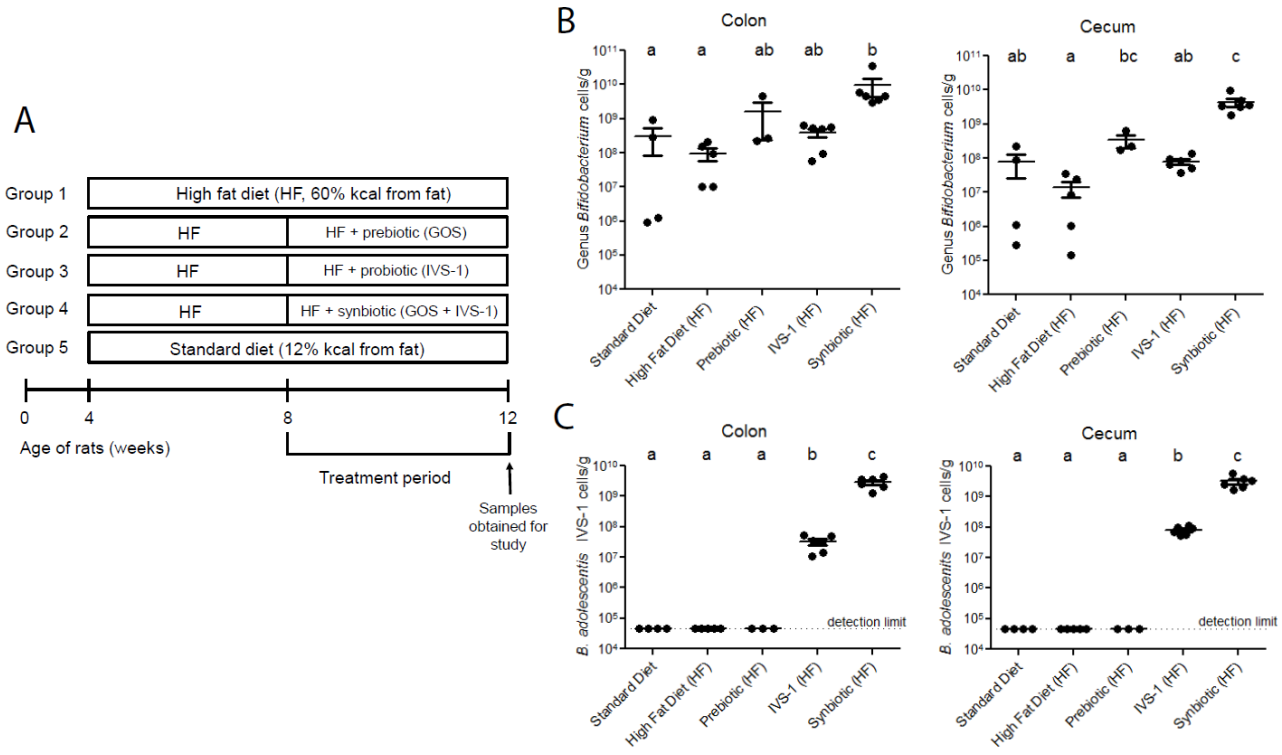

Fig. 3.2. Test of a synbiotic combination of *B. adolescentis* IVS-1 and GOS in a high-fat-diet rat model. A. Experimental design of the rat study. Rats were fed either a standard diet or a high-fat diet for 8 weeks, supplemented with or without a probiotic (IVS-1), a prebiotic (GOS), or a synbiotic (IVS-1 plus GOS) for the last 4 weeks. B. Quantification of absolute cell numbers of bifidobacteria in colonic and cecal contents by genus-specific qRT-PCR. C. Strain-specific qRT-PCR was used to quantify absolute numbers of *B. adolescentis* IVS-1 in colonic and cecal contents.

3.4.2 Test of the synbiotic combination using rats on a high-fat diet.

We systematically tested synergism between strain IVS-1 and GOS when used as a synbiotic in rats fed a high-fat diet (Fig. 3.2A). Decreases in numbers of bifidobacteria are often observed during high-fat-diet feeding (41–43). To determine if our synbiotic

strategy could redress this decrease, we employed a rat model of high-fat-diet-induced NAFLD where rats develop steatosis (fatty liver) but do not show an increase in body weight, develop liver inflammation, or progress to nonalcoholic steatohepatitis (NASH) (28). In our study, all high-fat-diet-fed rats developed steatosis (i.e., liver triglyceride levels of > 50 μg mg^{-1} of tissue) and had slightly increased plasma ALP levels compared to rats fed a standard diet (see Table 3.S2 in the supplemental material). Dietary supplements significantly influenced triglyceride liver contents; however, high-fat-diet-fed rats did not develop the histopathological liver inflammation characteristic of NASH (data not shown) and did not have increased plasma ALT levels (see Table 3.S2 in the supplemental material). Plasma tumor necrosis factor alpha (TNF-α) and monocyte chemoattractant protein 1 (MCP-1) levels were not significantly elevated in the high-fat-fed rats compared to the controls (see Table 3.S2 in the supplemental material), indicating a lack of systemic inflammation. Together, these data indicated that all rats receiving a high-fat diet developed NAFLD but not severe inflammatory disease that would confound the evaluation of synbiotic synergy and gut microbial ecology.

3.4.3 Experiments in rats demonstrate strong synergism between IVS-1 and GOS.

To test the functionality of the prebiotic to support the establishment of *B. adolescentis* IVS-1 in the rat intestine, rats fed a high-fat diet were administered either IVS-1 alone, GOS alone, or the synbiotic combination; all findings were compared to results for the high-fat controls (Fig. 3.2A). Consistent with data from previous studies (41, 42), high-fat feeding decreased the abundance of bifidobacteria in both the colon and cecum of the rats, although this reduction did not reach statistical significance (Fig. 3.2B and Table

3.1). Genus-specific qRT-PCR analysis revealed that the prebiotic, but not IVS-1, significantly increased the total number of bifidobacteria in the cecum (Fig. 3.2B). These findings indicate that the introduction of IVS-1 alone did not increase *Bifidobacterium* abundance above baseline levels (~10^8 cells/g), whereas the prebiotic substrate was able to support the resident population. Compared to IVS-1 and GOS alone, the combination of the two dramatically increased the total number of bifidobacteria in the cecum ($P < 0.01$ between synbiotic and prebiotic treatments; $P < 0.001$ between synbiotic and probiotic treatments) (Fig. 3.2B).

Strain-specific qRT-PCR analysis of *B. adolescentis* IVS-1 clearly demonstrated a synergistic effect of IVS-1 and GOS in the colon and in the cecum. Even though rats receiving IVS-1 alone consumed significantly more IVS-1 on a daily basis than did rats given the synbiotic due to increased drinking water consumption ($P < 0.0001$) (see Table 3.S2 in the supplemental material), the synbiotic led to an almost 2-log increase in the level of IVS-1 in the colon and cecum (9.47 ± 0.2 $\log_{10}$ cells g^{-1} and 9.43 ± 0.2 $\log_{10}$ cells g^{-1}, respectively) compared with the probiotic treatment (7.9 ± 0.1 and 7.44 ± 0.3 $\log_{10}$ cells g^{-1} in the cecum and colon, respectively) ($P < 0.0001$) (Fig. 3.2C). No IVS-1 was detected in rats fed the standard diet, the high-fat diet, or the prebiotic alone.

3.4.4. 16S rRNA sequencing confirms synergism between IVS-1 and GOS *in vivo*.

We analyzed the 16S rRNA tags obtained via Illumina sequencing to gain a community-wide perspective on treatment effects on the resident gut microbiota. The ability of probiotic and synbiotic treatments to establish IVS-1 in rats was assessed based

on the abundance of an operational taxonomic unit (OTU) representing the species *B. adolescentis* (OTU_2). This species was undetectable in rats that did not receive the probiotic treatment but constituted 3.4% of the microbiota in rats fed IVS-1 (Fig. 3.3A and Table 3.1). This finding indicates that the *B. adolescentis* population observed in rats was due solely to the administration of IVS-1. This finding was expected, as this species is not a member of the normal rat microbiota. Sequences representing *B. adolescentis* were enriched to 37.0% in rats receiving the synbiotic treatment, indicating a significant enhancement of the probiotic (in terms of abundance) due to the addition of the prebiotic ($P = 0.0159$). Without GOS, IVS-1 was only the eighth most abundant OTU in the rats' colonic microbiota, while it became the most abundant OTU when given together with GOS, having an abundance almost four times higher than that of the second most abundant OTU (a *Blautia* species, at 9.7%) (Fig. 3.3A). This finding demonstrated that IVS-1 could be introduced as the dominant member of the rat gut microbiota when GOS was also provided.

Table 3.1: Proportions of bacterial taxa significantly influenced by dietary treatments

Taxonomic group	Mean % bacterial abundance ± SD[c]					
	Standard diet	Control High-Fat Diet (HF)	Prebiotic (HF)	Probiotic (HF)	Synbiotic (HF)	ANOVA *P*- value
Phylum						
Firmicutes	87.6±5 A	88.8±9 A	76.9±8 A	87.8±5 A	59.3±7 B	<0.0001
Actinobacteria	8.9±6 AB	3.6±2 A	19.6±8 BC	7.6±4 A	39.1±7 C	<0.0001
Family						
Clostridiaceae	3.9±6	0.5±1	0.8±1	5.4±6 A	0.2±0 B	0.0061
Incertae Sedis XIV	3.7±6	7.5±10	11.0±15	1.1±2 A	17.3±12 B	0.0342
Streptococcaceae	12.7±5	21.3±5 A	9.3±1	8.9±6 B	6.6±2 B	0.0045
Erysipelotrichaceae	16.8±11	21.3±17	9.2±1 A	26.5±10 B	8.3±3 A	0.0226
Bifidobacteriaceae	5.9±7 A	1.3±1 A	17.0±9	4.1±2 A	37.8±7 B	0.0017
Coriobacteriaceae	0.6±0	0.3±0	1.0±1	1.9±3 A	0.2±0 B	0.0263
Rikenellaceae	0.9±1 A	0.1±0	0.3±0	0.1±0	0.0±0 B	0.0181
Genus						
Clostridium	3.9±6	0.5±1 A	0.8±1	5.3±6 B	0.2±0 A	0.0122
Blautia	3.4±6	7.4±10	11.0±15	0.9±1 A	17.2±12 B	0.0431
Holdemania	0.1±0	1.0±2 A	0.9±0	0.0±0 B	0.0±0 B	0.0117
Bifidobacterium	5.9±7 A	1.3±1 A	17.0±9	4.1±2 A	37.8±7 B	0.0017
Lactococcus	12.4±4	21.0±5 A	9.1±1	8.7±6 B	6.3±2 B	0.0045
Alistipes	0.9±1 A	0.1±0	0.3±0	0.1±0	0.0±0 B	0.0181
OTUs [a]						
OTU_2 (*B. adolescentis*, 99%)	0.0±0 A	0.0±0 A	0.0±0 A	3.4±2 BC	37.0±7 BD	<0.0001
OTU_1 (*L. lactis*, 100%)	12.4±4	21.0±5 A	9.1±1	8.6±6 B	6.3±2 B	0.0045
OTU_626 (*Lachnospiraceae*[b])	0.4±1 A	0.1±0 A	0.0±0 B	ND	0.1±0	0.0002
OTU_7 (*Turicibacter* sanguinis, 97%)	3.7±3	2.3±3	2.3±2	9.1±7 A	0.5±1 B	0.0279
OTU_14 (*Blautia*[b])	0.0±0 A	0.1±0 AB	1.5±1 BC	0.0±0 AB	9.7±6 CF	0.0003
OTU_33 (*L. intestinalis*, 99%)	ND	0.0±0 A	ND	1.1±2 B	0.0±0 A	0.0022
OTU_9 (*Clostridium* sp.[b])	3.8±6	0.5±1 A	0.7±1	5.3±6 B	0.2±0 A	0.0128
OTU_6 (*B. pseudolongum*, 97%)	5.8±7	0.9±1	16.6±8 A	0.6±1	0.0±0 B	0.0293
OTU_44 (*C. cocleatum*, 99%)	ND	1.0±1 A	ND	0.0±0 B	ND	0.0121

[a] Percent homologies to the closest type strain in the database are shown in parentheses. If the strain could not be assigned to a type strain (<97% homology), RDP Classifier was used to determine the most likely genus, and the RDP Classifier value is shown (80% cutoff).

[b] OTU without closely related type strain (<97% homology) classified with RDP Classifier. [c] Values with different uppercase letters are significantly different from each other. HF, high fat; ND, not detected.

3.4.5 Community-wide characterization of effects on gut microbiota.

GOS treatment alone promoted a remarkably specific bifidogenic response, leading to an increase in the abundance of only one OTU related to *Bifidobacterium pseudolongum* (OTU_6) (Table 3.1 and Fig. 3.3A). These findings confirm the highly specific bifidogenic response of GOS, which was previously demonstrated in humans (32).

Although IVS-1 treatment alone did not significantly increase the abundance of the genus *Bifidobacterium*, it induced a significant increase in the abundance of *Bifidobacterium adolescentis* at the species level (Table 3.1). Of note, several unexpected changes were also detected, such as enrichment of the family *Clostridiaceae*, the genus *Clostridium*, and an OTU within this genus (OTU_9). Furthermore, the abundance of an OTU related to *Lactobacillus intestinalis* (OTU_33) increased, while that of an OTU related to *Lactococcus lactis* (OTU_1) decreased (Table 3.1).

Synbiotic treatment significantly increased the proportion of *Actinobacteria* ($P < 0.0001$), the family *Bifidobacteriaceae* ($P < 0.0017$), and the genus *Bifidobacterium* ($P < 0.0017$) (Table 3.1). These shifts were almost completely equivalent to shifts of OTU_2, showing that the above-described alterations at higher taxonomic levels were due to the enrichment of IVS-1. The establishment of IVS-1 was associated with an increase in the abundances of the genus *Blautia* and one OTU within this genus (OTU_14). In addition, there was a reduction in the abundances of the phylum *Firmicutes* ($P < 0.0001$) and families within this phylum, including *Clostridiaceae*, *Streptococcaceae*, and *Erysipelotrichaceae*. The abundances of the genera *Clostridium* and *Lactococcus* and OTUs within these genera were also decreased (Table 3.1).

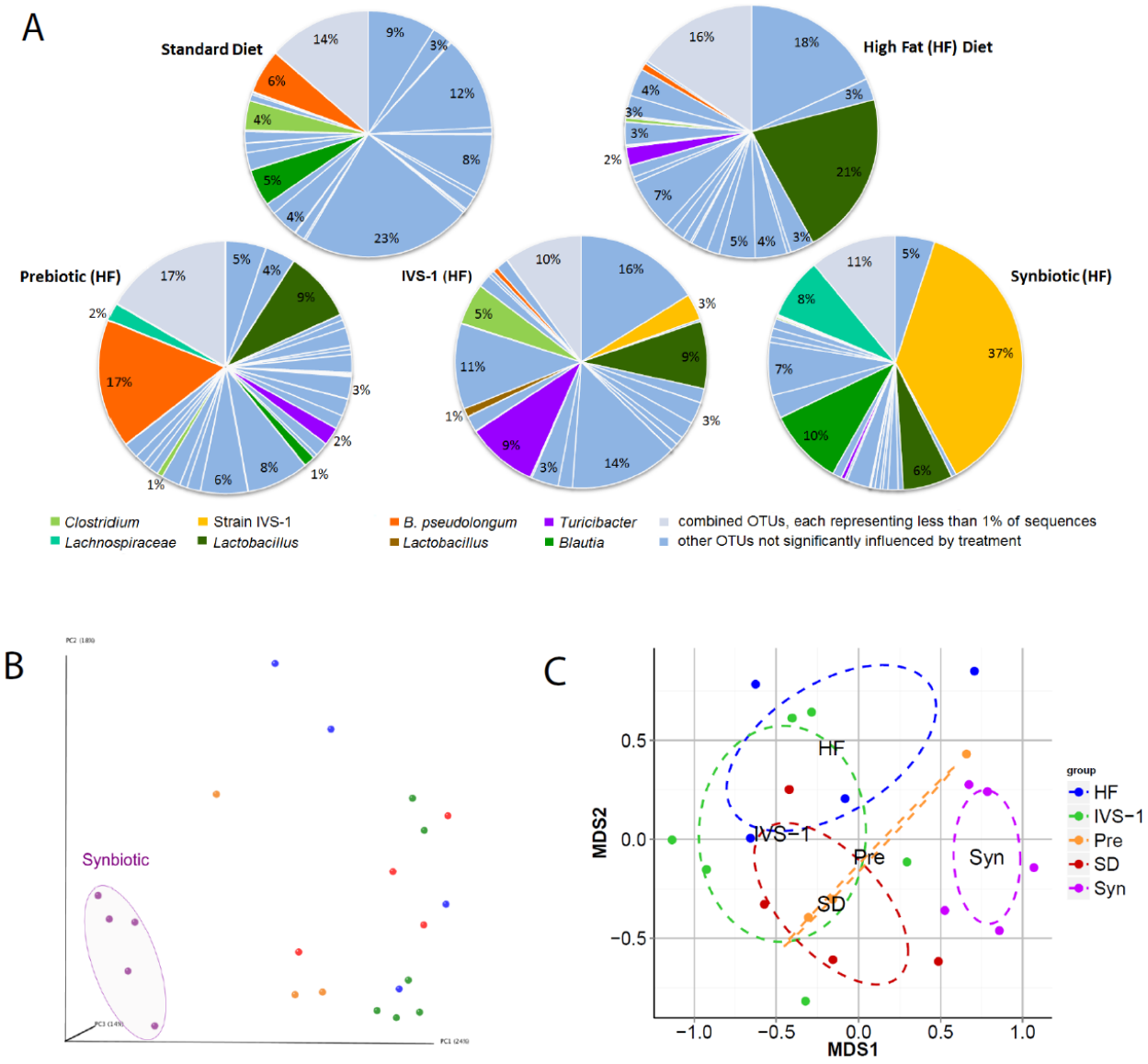

Fig. 3.3. Characterization of the rat colonic microbiota composition by Illumina sequencing of 16S rRNA tags. A. Analysis of colonic microbiota at the OTU level. OTUs representing at least 1% of total sequences are shown individually, while OTUs representing <1% are grouped. OTUs in colors other than light blue were significantly influenced by the dietary treatment. B. Principal coordinate analysis (Bray-Curtis distance) of beta diversity. C. NMDS plot of beta diversity based on Bray-Curtis distance. SD, standard diet.

To assess both the alpha and beta diversities of the community in the colon, different diversity indexes were calculated from the data. Specifically, Shannon's index and the number of observed OTUs were used to determine the alpha diversity, and principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) plots based on Bray-Curtis distance were used to visualize the similarity between samples for each treatment.

On average, 135.41 ± 34.4 OTUs per sample were identified. Alpha diversity based on Shannon's index was not significantly influenced by the treatment; however, there was a tendency for reduced diversity in the synbiotic group. This was caused by a slight reduction in community evenness, likely due to the expansion of a single species (*B. adolescentis*) (data not shown). Two independent approaches were used to analyze the beta diversity of the microbiota communities among treatments. PCoA and NMDS, based on Bray-Curtis distances of beta diversity, revealed that communities from rats fed the synbiotic clustered separately from the microbiomes of rats fed all the other treatments, which clustered together (Fig. 3.3B and C). This finding demonstrated that only the synbiotic treatment caused a global shift in microbiota structure.

3.4.6 Systematic analyses of associations between members of the gut microbiota.

To identify potential interactions between IVS-1 and members of the gut microbiota, and among other bacterial members, we performed correlation analyses on all taxon combinations in the data set. Correlations were performed by using bacterial abundance data from all treatments. Strong negative correlations between the family

Bifidobacteriaceae and the family *Clostridiaceae* (Fig. 3.4A), the genera *Bifidobacterium* and *Lactococcus* (Fig. 3.4B), and the genera *Bifidobacterium* and *Akkermansia* (Fig. 3.4C) were observed. In addition, strain IVS-1 levels (OTU_2) showed a negative correlation with *Lactococcus lactis* (Fig.3.4D) and a very tight negative association with resident *B. pseudolongum* (r = -0.64; *P* = 0.0004) (Fig. 3.4E). These negative associations suggest direct or indirect competition between these bacterial taxa. Positive associations between both *Bifidobacteriaceae* and *Bifidobacterium* and the genus *Blautia* were detected, suggesting a synergistic relationship, which may be supported by the addition of GOS (Table 3.1 and Fig. 3.4F).

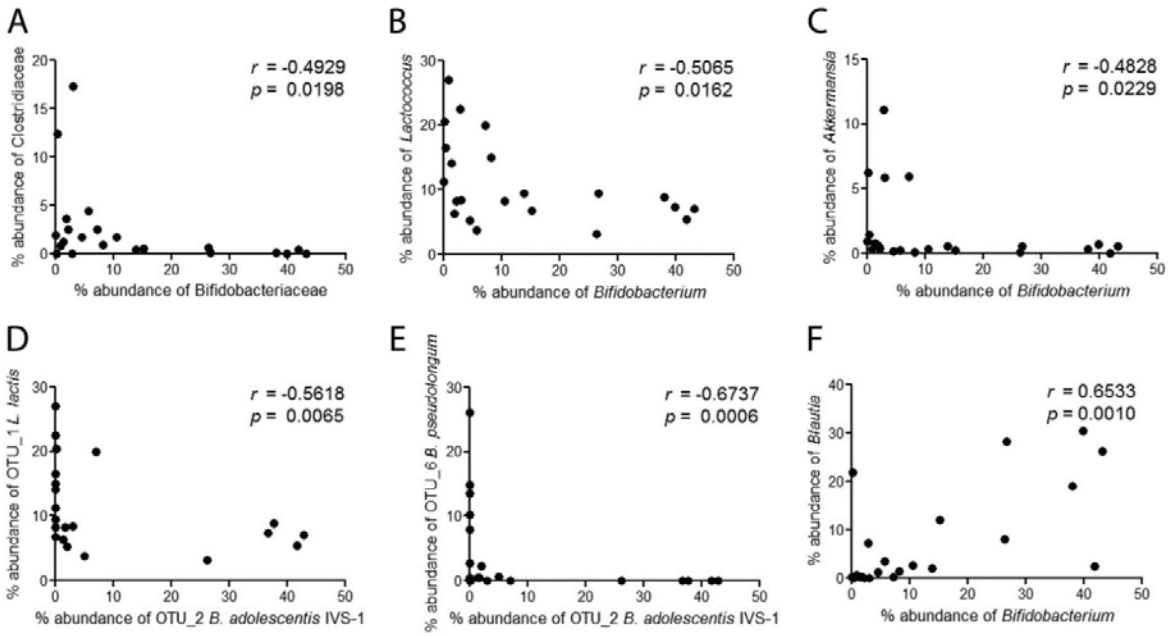

Fig. 3.4. Correlation analysis of colonic taxa present in rats fed a high-fat diet supplemented with or without a probiotic (IVS-1), a prebiotic (GOS), or a synbiotic (IVS-1 plus GOS) or a standard diet. Bacterial quantities are expressed as percent abundances of total bacteria as determined by 16S rRNA sequencing. Spearman's correlations between *Bifidobacteriaceae* and *Clostridiaceae* (A), *Bifidobacterium* and *Lactococcus* (B), *Bifidobacterium* and *Akkermansia* (C), *Bifidobacterium adolescentis* IVS-1 and *Lactococcus lactis* (D), *Bifidobacterium adolescentis* IVS-1 and *Bifidobacterium pseudolongum* (E), and *Bifidobacterium* and *Blautia* (F) were determined.

3.5 Discussion

Synergistic synbiotics are a promising concept to modulate the composition of the gut microbiota and promote the establishment of probiotic organisms in the gut (16). Despite this potential, however, there are few *in vivo* human or animal studies providing evidence that prebiotics can be used to support specific probiotic strains. Unfortunately, most synbiotic studies, including work in rats (44–49), mice (50–52), pigs (53–57), chickens (58, 59), and humans (60), did not employ strain-specific detection methods and therefore did not provide information on the potential synergism between pre- and probiotics. Of the *in vivo* studies that did discriminate the probiotic strain, most still did not demonstrate that *in vivo* performance could be enhanced by a prebiotic. This accounts for experiments using synbiotic formulations in humans (61), rats (62, 63), and other animal models (64). These findings suggest that, with few exceptions (17–19), probiotic strains are unable to compete against the resident gut microbiota, which is inherently resistant to outside colonizers (65), even when an exogenous growth substrate in the form of a prebiotic is provided.

Several reasons may explain the low success rates of synergistic synbiotics when evaluated *in vivo*, even for combinations in which the probiotic strain is able to utilize the prebiotic substrate *in vitro*. First, to become established in the gut, the probiotic strain must be able to occupy an ecological niche. This means that strains must not only outcompete the resident microbiota for the prebiotic substrate but also secure other nutrients that might be growth limiting (such as amino acids, lipids, vitamins, minerals, and nucleotides, etc.). In addition, probiotic strains must tolerate the prevailing environmental conditions in the digestive tract (including pH, bile acids, IgA, and defensins). Ultimately, *in vitro* tests are

unable to predict the ability of a probiotic to benefit from a prebiotic substrate within the constraints of the competitive gastrointestinal environment. In contrast, the IVS approach described here overcomes many limitations of *in vitro* tests used to formulate synbiotics because it provides a basis for identifying bacterial strains that are able to utilize the prebiotic substrate under the same ecological conditions in which they are intended to function.

In this study, we employed IVS and selected a synbiotic combination that was tested in a rat model of NAFLD. Although the synbiotic did not influence host phenotypes, it was highly efficient at enhancing population levels of the probiotic strain, making it the most dominant OTU in the gut (Fig. 3.3A and Table 3.1). These findings provide a proof of concept for the potential of *in vivo* selection to identify synbiotic combinations that are, in ecological terms, highly synergistic. In addition to enhancing the abundance of strain IVS-1, the synbiotic used here also redressed the high-fat-diet-induced reduction in the level of bifidobacteria detected in rats that is often reported in the literature (41–43). Therefore, although no metabolic benefits were seen in the rat model used in our study, the synbiotic may be beneficial in other scenarios, as bifidobacteria are considered health-promoting organisms (6, 26, 66–68).

The community-wide analysis provided evidence that synergism between GOS and strain IVS-1 increased the competitive fitness of the strain in the rat intestinal tract. *B. pseudolongum*, which is a natural member of the rat GI tract (69), was detected in relative abundances of 5.8 % and 0.9 % in rats fed the standard and high-fat diets, respectively. Although the probiotic treatment did not affect levels of *B. pseudolongum*, the prebiotic treatment increased the abundance of this species to 16.6 %, indicating that *B.*

pseudolongum utilized GOS *in vivo*. However, the parallel addition of strain IVS-1 with GOS completely excluded *B. pseudolongum*, and a strong negative correlation between this species and IVS-1 was observed (r = -0.67; *P* = 0.0006) (Fig. 3.4E). These findings indicate that IVS-1 not only had a higher affinity for GOS *in vivo* than the resident *Bifidobacterium* species but also utilized GOS to increase its competitiveness and effectively outcompete a closely related resident species. This finding is consistent with the niche exclusion model, which states that the organism most efficient at using limited nutrients outcompetes its competitors for the same niche (70). Strong inverse correlations between bifidobacteria and *Clostridiaceae*, *Lactococcus*, and *Akkermansia* (Fig. 3.4A to C) were also observed. It is likely that these associations are also due to niche competition and are potentially enhanced by GOS administration. Bifidobacteria produce short-chain fatty acids that are inhibitory to other bacteria either by lowering the pH or via direct antimicrobial effects (e.g., acetic acid) (71). In summary, these findings demonstrate that the competitive fitness of strain IVS-1 was increased by GOS, which supports the conclusion that IVS can select synbiotic combinations with extremely high synergism. To what degree the increased competitive interactions between IVS-1 and the resident microbiota impact host health is difficult to predict and likely context dependent, but they clearly should be considered in future studies.

Correlation analyses revealed only one positive association among members of the rat microbiota, between the bifidobacteria (at the family and genus levels) and the genus *Blautia*. The abundance of OTU_14, an uncultured *Blautia* strain, was also significantly increased by GOS and in the synbiotic treatment (Table 3.1). The positive correlation between *Bifidobacterium* and *Blautia* (Fig. 3.4F) indicates a synergistic effect between the

two taxa. The significant increase in the abundance of *Blautia* in the synbiotic treatments further suggests a syntrophic interaction between IVS-1 and *Blautia*, as GOS is consumed mainly by bifidobacteria (72), and the genus *Blautia* is not reported to utilize GOS. In contrast, the genus *Blautia* contains bacteria that are hydrogenotrophic acetogens, which utilize H_2 and CO_2 as energy sources (73). Although bifidobacteria do not produce these gases, cross-feeding between bifidobacteria and butyrate-producing colon bacteria can result in H_2 and CO_2 production (74), which might explain the positive correlations between *Bifidobacterium* and *Blautia*. However, additional experiments are necessary to establish the mechanism by which GOS can enhance the populations of *Blautia* in the gut and the positive associations between this genus and IVS-1.

In this study, we have shown how IVS can be used to formulate a highly synergistic synbiotic that can substantially enhance population levels and the competitiveness of a putative probiotic strain in the gastrointestinal tract and establish it as the dominant member of the gut microbiota in a conventional animal model. To our knowledge, this has not yet been reported in the probiotic literature. The process of IVS is broadly applicable and can easily be extended to other host species, body sites, prebiotic substrates (or dietary fibers), or target organisms. For example, it may be possible to use IVS to enhance other putative health-promoting genera such as *Akkermansia*, which has been shown to respond to prebiotics *in vivo* (75). While we selected *B. adolescentis* IVS-1 during a human trial that did not determine the physiological effect of GOS, IVS might be especially powerful when combined with a human clinical trial that determines the beneficial effect of a prebiotic on the host as the primary selection criterion. Therefore, to develop synbiotics for specific health applications, the IVS concept should be extended to select bacterial strains that not

only responded to the prebiotic but whose expansion correlated with beneficial physiological effects for the host. Such an approach would have the potential to identify health-promoting strains whose metabolic activity *in vivo* could be increased through a prebiotic. This might also result in synbiotic applications with greater health effects than those of the prebiotic alone, especially in the subset of humans who do not respond to the prebiotic (14, 32). A human study testing the synbiotic combination identified here (and comparing it with a synbiotic that includes a *Bifidobacterium* strain that can ferment GOS but was not selected by IVS) is currently in progress. Clearly, the application of IVS is likely to enhance the ecological performance of probiotic strains or live biotherapeutics within the habitats in which they are thought to function, and the technology could be readily applied in the design of microbiota-modulating therapies.

3.6 Supplemental material

Table 3.S1. Composition of standard and high-fat diets.

Nutritional profile	High-Fat Diet	Standard Diet
Protein [%]	24.2	17.6
Fat [%]	34.7	5.2
Fiber (max) [%]	5.5	3.9
Carbohydrates [%]	27.8	68.3
Energy (kcal/g)	Kcal / %	Kcal / %
From Protein	0.969 / 18.6	0.705 / 18.3
From Fat (ether extract)	3.122 / 59.9	0.464 / 12.1
From Carbohydrates	1.113 / 21.4	2.733 / 71.0

Table 3.S2. Body weight, relative epididymal fat pad weight, consumed drinking water, consumed IVS-1 and GOS, and host physiological markers.

	Standard Diet	High-Fat Diet	High-Fat Diet Prebiotic	High-Fat Diet Probiotic	High-Fat Diet Synbiotic	*P* - value ANOVA
Average body weight [g rat^{-1}]	475 ±19	449 ±46	499 ±81	496 ±62	502 ±51	0.5446
Average relative epididymal fat pad weight [% rat^{-1}]	0.84 ±0.2	1.04 ±0.4	1.12 ±0.3	1.01 ±0.2	1.08 ±0.5	0.8267
Average water consumption [ml rat^{-1} day^{-1}]	29.3 ±4.1^{b}	35.6 ±4.5^{a}	36.3 ±4.9^{a}	41.9 ±8.6^{c}	35.4 ±4.5^{a}	<0.0001
Average IVS-1 consumption [CFU rat^{-1} day^{-1}]	NA	NA	NA	1.26 x $10^{9\,a}$	1.06 x $10^{9\,b}$	0.0001$^{\#}$
Average GOS consumption [g rat^{-1} day^{-1}]	NA	NA	1.20	NA	1.17	0.2063$^{\#}$
Triglyceride content [µg TG mg^{-1} tissue] *	17.8 ±4.3^{a}	70.7 ±1.4^{b}	62.7 ±1.2^{bc}	53.5 ±0.8^{c}	92.4 ±2.4^{d}	<0.0001
Alkanine Phosphatase (ALP) [units $liter^{-1}$]	227 ±83	399 ±55	464 ±127	458 ±167	460 ±120	0.0646
Alanine Transferase (ALT) [units $liter^{-1}$]	34 ±13	42 ±11	36 ±5	37±7	41 ±4	0.6418
TNF-α [pg ml^{-1}]	14.2 ±2.3	14.1 ±2.6	16.2 ±3.2	16.7 ±7.9	13.8 ±1.4	0.4718
MCP-1 [pg ml^{-1}]	224 ±25	228 ±37	239 ±21	248 ±63	243 ±36	0.6345

* as a threshold for steatosis was a liver triglyceride levels greater than 50 µg/mg of tissue
NA: not applicable
$^{\#}$ Student's *t*-test was applied

Values with different letters are significantly different from each other

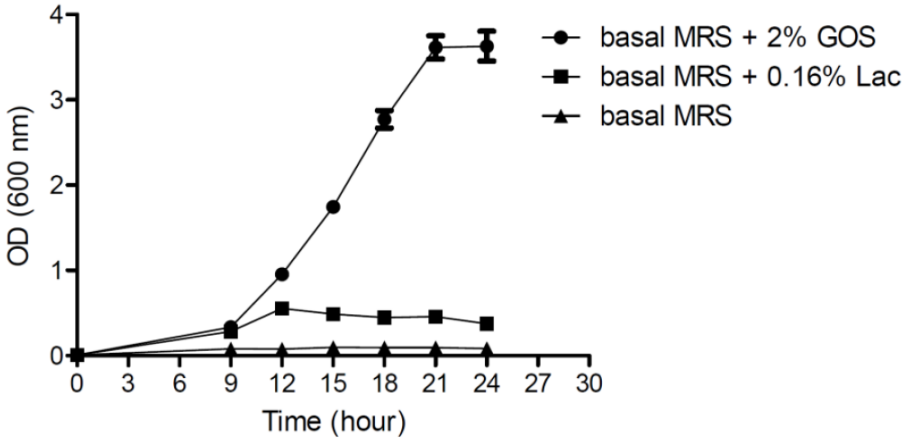

Figure 3.S1. Growth of Bifidobacterium adolescentis IVS-1 in basal MRS supplemented with GOS, 0.16% lactose (residual sugar present in the commercial GOS), or basal MRS without carbohydrates.

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Chapter 4

Functional characterization of a rationally selected synbiotic application in obese adults.

4.1 Abstract

One strategy for enhancing the establishment of probiotic bacteria in the human intestinal tract is via the parallel administration of a prebiotic. Such combinations are referred to as synbiotics. We have developed a rationally formulated synbiotic combination based on *in vivo* selection (IVS). This approach employed ecological criteria to select a highly synergistic synbiotic combination, specifically, *Bifidobacterium adolescentis* strain IVS-1 and galactooligosaccharides (GOS). This synbiotic was previously shown to be synergistic in a rat model (1), and we have now tested the ability of this synbiotic to improve the abundance of bifidobacteria, and specifically of the probiotic strain, in obese human subjects. The study was designed as a randomized, placebo controlled, parallel arm clinical trial. When the rationally selected synbiotic was compared to a commercial synbiotic (*Bifidobacterium lactis* Bb12 and GOS), the synergistic synbiotic combination led to significantly higher levels of the probiotic strain in the gastrointestinal tract of the subjects than the control. In conclusion, this study demonstrated that IVS can be used to successfully formulate a synbiotic that can enhance the establishment and competitiveness of a putative probiotic strain in the human gastrointestinal tract.

4.2 Introduction

The contribution of the gut microbiome to human and animal health is now well established (2–4). Indeed, there are substantial efforts aimed at designing dietary strategies that modify the composition of the gut microbiota with the goal of preventing disease and promoting health (5, 6). In particular, probiotic bacteria and prebiotic fibers, as well as in a combined form as synbiotics, have been tested in clinical trials to treat a range of conditions, including IBS (7, 8), IBD (9–12), lactose intolerance (13, 14), and other gastrointestinal (GI) diseases and disorders (15–18).

In addition to GI diseases, the development of several systemic conditions, including metabolic endotoxemia and metabolic syndrome, are also associated with a gut dysbiosis that could potentially be redressed through dietary modulations. Evidence from human and animal studies suggests that a constant low grade inflammation of the GI lining may precede or initiate the development of metabolic disorders (19). The origin of this inflammation may be caused by alterations in the composition of the gut microbiota, which is directly involved in controlling the host's gut barrier function, and increases systemic exposure to pro-inflammatory free fatty acids (20, 21). While the exact process of decreased barrier function and increased permeability due to the gut microbiota remains unclear, data from animal studies suggest that several mechanisms are included. For example, changes in the abundance of certain members of the gut microbiota lead to changes in the production and availability of short chain fatty acids, which are absorbed by the gut epithelial cells. Additionally, the gut microbiota affects the distribution of tight junction proteins, such as ZO-1 and Occludin, and influences the endocannabinoid system tone, leading to a higher expression of anandamide and cannabinoid receptor 1 (22, 23). These factors lead to increased intestinal permeability and subsequently to an increased

translocation of microbe-derived lipopolysaccharides (LPS) into the bloodstream. This induces metabolic endotoxemia, which eventually leads to metabolic syndrome (22, 24, 25).

Several studies have established that *Bifidobacterium* spp. have beneficial effects in the pathology associated with impaired barrier function by reducing gut permeability and improving epithelial cell barrier function (6, 24, 26–29). Specifically, *Bifidobacterium adolescentis* administration to rats ameliorated insulin sensitivity, white fat accumulation, and liver weight (30), and significantly lowered rates of bacterial translocation (27). This species was also shown to attenuate the formation of reactive oxygen species, activate nuclear factor κB (NFκB), and reduced markers of inflammation in the rodent liver (31). Similarly, administration of *Bifidobacterium longum* reduced GI inflammation and metabolic syndrome, and reduced levels of LPS and interleukin beta in a rat model (32). Another species, *Bifidobacterium breve* reduced triacylglycerol content, decreased serum TNF-α levels, and restored serum LPS levels to levels that were observed in control rats (33). It also suppressed accumulation of epididymal fat pad and body weight, improved fasting levels of glucose and insulin, and improved total cholesterol values in a mouse model (34). Finally, supplementation of newborn mice with *Bifidobacterium infantis* and *Bifidobacterium bifidum* significantly lowered intestinal endotoxin levels compared to control mice (35), and *B. infantis* normalized gut permeability and decreased colonic IFN-γ secretion in IL-10-deficient mice (26).

Collectively, these reports suggest that dietary strategies that support both the size and physiologic activity of *Bifidobacterium* populations in the human GI tract could be effective for a range of metabolic disorders. Ordinarily, the abundance of these bacteria in adults is relatively low (< 3%) (36), and negatively correlates with high-fat and low fiber

diets, which are common in Westernized societies (24, 37). This low abundance of bifidobacteria may be addressed by dietary treatments such as probiotics, prebiotics, or synbiotics. The consumption of prebiotic carbohydrates, such as galactooligosaccharide (GOS) and other fibers, have been shown to increase autochthonous bifidobacteria in infants (38, 39) and adults (40–42). However, not all subjects respond to prebiotic interventions, even at high doses (36, 43, 44), and the subjects may not possess a particular *Bifidobacterium* strain of interest. One strategy to enrich for bifidobacteria, even in non-responders, would be to administer the prebiotic together with a *Bifidobacterium* strain that is capable of metabolizing the prebiotic component *in vivo*. Such pairings are referred to as synergistic synbiotics (45).

However, the rational selection of these synbiotic strains is critical as the potential of the probiotic strains to become established *in vivo* is significantly limited due to colonization resistance conferred by the resident or commensal microbial population, the host, and other abiotic factors. Allochthonous strains may, for example, lack adaptive traits necessary to become competitive and physiologically active in the GI environment. Their ability to compete with the autochthonous microbiota in the GI tract is also compromised by niche exclusion, colonization resistance, nutrient availability, the host's immune system, and the prevailing environmental conditions in the digestive tract (46). These abiotic and biotic ecological factors have a major influence on the ability of allochthonous organisms (including most probiotics) to reach, and then become established in the human GI tract, even if only transiently. Indeed, studies have shown that bacterial strains that are allochthonous to the GI tract are washed out shortly after administration is discontinued, and pre-treatment conditions are quickly re-established (36, 47–50). By selecting bacterial strains that are autochthonous, adapted to the host GI environment, the colonization

resistance towards the strains based on these abiotic and biotic factors may be attenuated. Moreover, incorporating strains autochthonous to the adult GI tract, such as *B. adolescentis*, as a synbiotic might also be expected to enhance their probiotic function and colonization success.

We recently described a novel method for the selection of an autochthonous strain of *B. adolescentis* that was enriched *in vivo* by GOS (1). When this strain, *B. adolescentis* IVS-1, was fed to rats, its abundance reached 3.4 % of the total bacterial gut microbiota. However, when combined with GOS as a synbiotic, abundance of strain IVS-1 increased to more than 35 %. To determine if this rationally selected synbiotic would also show enhanced colonization in humans, we tested it in a parallel arm placebo controlled clinical trial with obese adults. Our goal was to assess the potential of this *in vivo* selected strain, combined with GOS as a synbiotic to establish bifidobacteria and redress metabolic aberrancies, with gut permeability as the primary endpoint. We compared establishment with a widely used commercial strain, *B. animalis* subsp. *lactis* Bb12, which has been used in synbiotic applications with GOS (48, 51, 52). Each strain was provided as individual treatments as well as combined with GOS as synbiotics. We compared the ability of the *in vivo* selected rationally designed synbiotic to the commercial synbiotic to alter the gut microbiota in obese individuals, and tested if GOS supported the colonization of the probiotic strains. This report focusses on the impact of the dietary treatments on the gut microbiota, while an assessment of the ability of each synbiotic to improve intestinal permeability and endotoxemia is currently ongoing.

4.3 Methods:

Subjects. This study was designed as a randomized, placebo-controlled, parallel-arm clinical trial conducted at Rush University Medical Center (RUMC). Women and men between 18 and 60 years with a BMI of 30.0 - 40.0 kg/m^2 were recruited. Subjects were permitted to have elevated liver enzymes due to fatty liver and metabolic syndrome, but were otherwise considered as healthy. Exclusion criteria included (1) prior intestinal resection, (2) patient history of GI diseases except for hiatal hernia, GERD, hemorrhoids, (3) severe renal disease defined by creatinine more than twice normal, (4) markedly abnormal liver function defined by ALT/AST over 4 times normal levels or elevated bilirubin (5) antibiotic use within the last 12 weeks prior to enrollment, (6) lean or overweight (BMI < 30 kg/m^2), (7) intolerant to aspirin, (8) regular use of aspirin, (9) excessive alcohol intake (>2 drinks for men, 1 drink for women daily), (10) presence of chronic metabolic disease (cardiovascular disease, insulin requiring diabetes or uncontrolled diabetes, cancer, etc.), (11) a plan to have a major change in dietary habit during the following 6 months, (12) consumption of probiotics, prebiotics or synbiotics without an appropriate 4 week washout period, (13) lactose intolerance or malabsorption, (14) subjects younger than 18 or older than 60, (15) unwillingness to consent to the study.

Study design. A total of 105 subjects were recruited and randomly assigned to one of six treatment groups (Table 4.1). The randomization was controlled for age and race. Three visits were required for each subject (Figure 4.1). At Visit 1, potential subjects were screened for eligibility and provided with a form for a 3-day diet record, all supplies for

stool collection, and instructions for specimen handling and for completing these tasks before the next visit.

Table 4.1. Treatment groups

Treatment group	Treatment
Group A	Lactose control
Group B	1 x 10^9 CFU *B. adolescentis* IVS-1
Group C	1 x 10^9 CFU *B. animalis* subsp. *lactis* Bb12
Group D	1 x 10^9 CFU *B. adolescentis* IVS-1 + GOS
Group E	1 x 10^9 CFU *B. animalis* subsp. *lactis* Bb12 + GOS
Group F	Galactooligosaccharide (GOS)

Subjects were instructed to store stool samples in Styrofoam coolers with freezer packs until delivery to the hospital. The samples were not allowed to be older than 24 hours if stored at -20°C, and not older than 5 hours if stored at room temperature. At visit 2, study subjects provided the completed food record and the baseline stool sample. Subjects were provided with one of the six treatments and consumed their randomly assigned supplement daily for three weeks as instructed. At the end of three weeks, subjects returned to the clinic to provide stool, as previously described. At the visit after 3 weeks of supplementation, the subject provided a stool sample, 3-day food records, and completed GI symptom questionnaires regarding adverse events. The latter included a standardized survey that rates bowel movement, stool consistency, discomfort, flatulence, abdominal pain, and bloating on a scale from 1 (best) to 10 (worst) (44). Weight, height, waist

circumference, and blood pressure were measured, and BMI was calculated at each visit. Blood pressure was measured using an automated cuff with the average of three assessments used for statistical comparisons. Four weeks after the last treatment was consumed subjects provided a wash-out stool sample. Subjects gave a written informed consent before the study procedure.

Eleven subjects (two in group A, four in group B, two in group C, one in group D, and two in group E) did not follow all protocol requirements and were subsequently excluded from the per protocol analysis presented here. These subjects were included in an intend to treat (ITT) analysis, which can be found in the supplements.

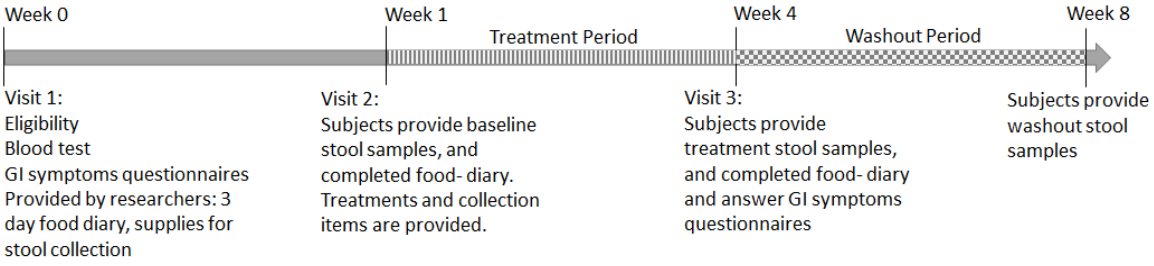


Fig. 4.1. Time line for synbiotic trial.

Dietary treatments. The prebiotic, GOS, was obtained from Friesland Foods (sold under the trade name, Vivinal®). This product contained 72.5 % of GOS, 22.8 % lactose, and 4.7 % mono-sugars (galactose and glucose). It was previously established that a dose of 5 g per day of GOS was sufficient to induce a bifidogenic response (40). Therefore the total amount of GOS powder was raised to 6.9 g to achieve a 5 g GOS treatment. The material was packaged in sachets in the Food Processing Product Development Lab (UNL). An

additional 0.1 g of lactose was added to achieve the same weight as the other preparations. The sachet material was impermeable to oxygen and moisture.

The two organisms that were used in this feeding study were *Bifidobacterium adolescentis* IVS-1 and *Bifidobacterium animalis* subsp. *lactis* Bb-12. The latter was obtained from Chrs. Hansen as a high cell density powder. Strain IVS-1 was produced from a contract manufacturer (Danwell Technology, Garden Grove, CA). The probiotic powder was stored for up to six month at -18°C and showed stable CFU numbers (data not shown). Probiotic mixtures were portioned into sachets, each contained 0.1 g of cell powder (10^{10} CFU/g). In addition, 6.9 g of lactose were added as a carrier/control, for a total dose of 7.0 g. Synbiotics contained 6.9 g of Vivinal® and 0.1 g of probiotic (either *B. adolescentis* IVS-1 or *B. animalis* Bb12), for a total dose of 7.0 g. Placebo samples contained 7.0 g of lactose. Subjects were provided with enough samples for the entire length of the study and were instructed to consume each dose on a daily basis, either mixed with food or liquid, but no tab water. The subjects were instructed to store samples in a cold (-18 °C) environment.

Analysis of fecal microbiota. Fecal samples were aliquoted and stored at -80°C until further analysis. The DNA was extracted as previously described (29). Amplicon sequencing was performed at the University of Minnesota Genomics Center. All samples were amplified and sequenced in a single run. The V5-V6 region of the 16S rRNA gene was amplified as previously described (1). Quality filtering performed by the University of Minnesota Genomics Center showed that more than 96 % of the samples met all quality control criteria. All reads were trimmed to 240 base pairs using the FASTX-Toolkit. The

reads were quality controlled, merged, OTU clustered, and taxonomically assigned, as previously described (1). If a sample exceeded 37,000 reads it was subsampled using Mothur v.1.31.162 to standardize the sequencing depth across samples. After processing samples contained an average of 22,012.7 ± 6,623 sequences.

Quantitative real-time PCR (qPCR). qPCR was performed by using a Mastercycler Realplex2 instrument (Eppendorf AG, Hamburg, Germany). Each PCR was performed with 25-µl volumes using real-time master mix containing SYBR (5 Prime Inc., Gaithersburg,MD) and either genus-specific primers for *Bifidobacterium* (40), or the strain-specific primers for *B. adolescentis* IVS-1 (1), as described previously. For strain specific detection of *B. animalis* subsp. *lactis* Bb12 the PCR mixture contained 25 µl of PCR reaction mix (QuantiFast® Probe PCR Kit, QIAGEN, Hilden, Germany), 0.3 µmol of each primer (BAL-23S-F 5'-CAGGTGGTCTGGTAGAGTATACCG-3' and BAL-23S-R 5'-ACGGCGACTTGCGTCTTG-3'), 0.25 µmol of probe (BAL-23S-P 5'-FAM-CGCCCACGACCCGCAAG-TAMRA-3'), and 5 µl DNA as previously described (53). The target of these primers and probe is the elongation factor Tu (tuf) gene of Bb12. The specificity of the primers and probe for Bb12 was validated experimentally by qPCR using DNA from 11 different *Bifidobacterium* strains related to strain Bb12. These strains included *Bifidobacterium adolescentis* IVS-1, *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium adolescentis* L2-32, *Bifidobacterium longum* subsp. *longum* ATCC 15707, *Bifidobacterium longum* DJO10A, *Bifidobacterium longum* ATCC 15697, *Bifidobacterium longum* subsp. *longum* F8, *Bifidobacterium longum* subsp. *longum* JDM301, *Bifidobacterium* sp. strain 113, *Bifidobacterium* sp. strain 12_1_47BFAA, and

Bifidobacterium sp. strain HMLN14. Furthermore, to test if primers could strain specifically select against fecal bacterial communities in humans, baseline DNA samples from subjects in groups C (Bb12 + lactose) and group E (Bb12 + GOS), and 10 randomly selected samples from other subjects, were tested.

Standard curves for absolute quantification of bacterial cell numbers were prepared by using cultures of *B. animalis* subsp. *lactis* Bb12 and *B. adolescentis* IVS-1 grown overnight (14 h).

Statistical analysis. All data present in the main body of the text was analyzed based on a per protocol analysis. Subjects that were recruited but violated the protocol in any way, for example took antibiotics, stored the treatments at room temperature, etc., were excluded from the analysis. These subjects were included in an intend to treat (ITT) analysis, which can be found in the supplements. Data is presented as mean ± SEM for variables that can be considered normally distributed (or median and range for variables not normally distributed). Group means were compared by ANOVA and post-hoc tests except when data was not normally distributed, in which case nonparametric analyses of medians was done using the Kruskal-Wallis test. Correlation analysis were done using the Spearman's correlation test for parametric analysis. Chi-square tests or Fisher's Exact Tests were used for incidence data. $P < 0.05$ is considered statistical significant. If only two groups were compared, Student's *t* tests were performed. For analysis of the gut microbiota the data was normalized by $\log_{10}$ transformation. To account for type I errors, a false discovery rate was used. A P value of < 0.05 and correlation coefficient (r) values of > 0.50 (in absolute values) were considered significant. Genera above 0.5 % and OTUs above 1 % abundance on average were considered for correlations. Correlation graphs were generated for

parameters that showed significant correlations and were visually inspected. If the removal of one single data-point caused the association to become non-significant, the data point was considered an outlier and removed. All analyses used SPSS (Chicago, IL), GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA), R studio (R Core Team, 2014), or SAS/STAT (SAS Institute Inc., Cary, NC, USA).

4.4 Results

4.4.1 Subject demographics and clinical characteristics

The subject cohort for this study was primarily female (71 %), middle-aged, non-Hispanic or Latino, and African American (Table 4.2). All subjects were obese, with participants in group D (IVS-1 + GOS) classifying as extreme obese, in group E (Bb12 + GOS) and F (GOS) as Class II obese, and in group A (Lactose), B (IVS-1+ lactose), and C (Bb12 + lactose) as class I obese. Subjects in group D had a significantly higher body mass index (BMI) than subjects in group B ($P = 0.049$). The average waist circumference was 40 inches.

4.4.2 Test of synergy of GOS in addition to IVS-1 or Bb12.

Our first goal was to determine if the ability of two strains of bifidobacteria to become established in the GI tract of obese adults would be enhanced by the addition of GOS. The strains included *B. adolescentis* IVS-1 that had previously been isolated from a GOS-enriched subject, and *B. animalis* subsp. *lactis* Bb12, a widely used commercial probiotic. Both strains were capable of fermenting GOS *in vitro* (1, 54). Treatment groups

included subjects who consumed each of the two test strains alone (groups B and C), subjects who consumed the synbiotics (i.e., test strains combined with GOS; groups D and E), a GOS-only group (F), and a placebo (lactose) group (A). Strain-specific qPCRs were used to quantify bacterial cell levels for those groups that received either one of the test strains alone or as synbiotics using the corresponding strain specific primers for IVS-1 and Bb12. Because *Bifidobacterium adolescentis* is a common species in the adult GI tract, it was necessary to establish that the IVS-1 primers were specific for this strain.

Results confirmed that no signal was detected in any baseline sample in subjects of group B (IVS-1 alone) or D (IVS-1 + GOS) (Figure 4.2A), or in the baseline of another additional 20 subjects that were randomly selected (data not shown). Therefore, it was concluded that any IVS-1 that was detected by qPCR after the treatment was given to the subjects, was indeed the probiotic strain selected for this study.

A similar approach was used to test the specificity of the Bb12 primers and probe. The primers and probe had been previously designed (53) and are the standard used by Chr. Hansen to identify their probiotic product. The strain Bb12 is a commonly used probiotic in dairy products. It was detected in three subjects before the treatment was started. Analysis of food diaries of these subjects, however, did not identify any indication of Bb12 product consumption.

Table 4.2. Demographic and metabolic characteristics of study subjects[1]

	Total Sample (n=94)	Group A Control (n=17)	Group B IVS-1 (n=18)	Group C Bb12 (n=164)	Group D IVS-1+GOS (n=16)	Group E Bb12+GOS (n=17)	Group F GOS (n=16)
Demographic Characteristics							
Gender							
Female, N (%)	71 (75.5)	13 (76.5)	9 (64.3)	12 (75.0)	11 (78.6)	12 (75.0)	12 (75.0)
Age, years, mean ± SD	44.3±11.2	44.3±11.2	44.3±11.2	44.3±11.2	44.3±11.2	44.3±11.2	45.9±9.6
Race, n (%)							
Hispanic/ Latino	9 (9.6)	0 (0.0)	2 (14.3)	2 (14.3)	2 (12.5)	2 (11.8)	1 (6.3)
Non-Hispanic/ Latino	85 (90.4)	17 (100.0)	12 (85.7)	12 (85.7)	14 (87.5)	15 (88.2)	15 (93.8)
Ethnicity, n (%)							
White	31 (33.0)	5 (29.4)	8 (57.1)	4 (28.6)	3 (18.8)	6 (35.3)	5 (31.3)
African American	58 (61.7)	10 (58.8)	6 (42.9)	7 (50.0)	13 (81.3)	11 (64.7)	11 (68.8)
Other[2]	5 (5.3)	2 (11.8)	0 (0.0)	3 (21.4)	0 (0.0)	0 (0.0)	0 (0.0)
Education, n (%)							
≤ 12 years	20 (21.3)	3 (17.6)	1 (7.1)	2 (14.3)	6 (37.5)	2 (11.8)	6 (37.5)
> 12 years	74 (78.7)	14 (82.4)	13 (92.9)	12 (85.7)	10 (62.5)	15 (88.2)	10 (62.5)
Clinical Characteristics[3]							
Body weight, kg[4]	100.0 (25.7)	96.8 (17.7)	94.8 (14.6)	98.5 (32.2)	118.0 (36.7)	112.8 (31.8)	102.3 (18.3)
BMI, kg/m^2[,5]	36.7 (8.5)	34.0 (4.5)	33.9 (6.2) A	35.5 (10.3)	41.6 (12.4) B	40.5 (7.1)	36.8 (5.6)
Waist circum-ference, inches	45.0 (7.3)	44.0 (11.0)	43.5 (4.4)	43.0 (9.9)	47.8 (12.3)	47.0 (8.7)	45.0 (3.2)

[1]Sample based on those that were randomized to treatment and completed the post-treatment visit (Visit 4)

[2]White ethnicity includes 2 Hispanic and 1 Middle Eastern participant; Black/African American ethnicity incudes 1 mixed ethnicity participant

[3]All clinical characteristics are listed as median (IQR)

[4]Significant difference based on Kruskal-Wallis, but no differences based on post-hoc pairwise comparisons after adjustment for multiple comparisons.

[5]Different letters indicate significant differences between treatment groups

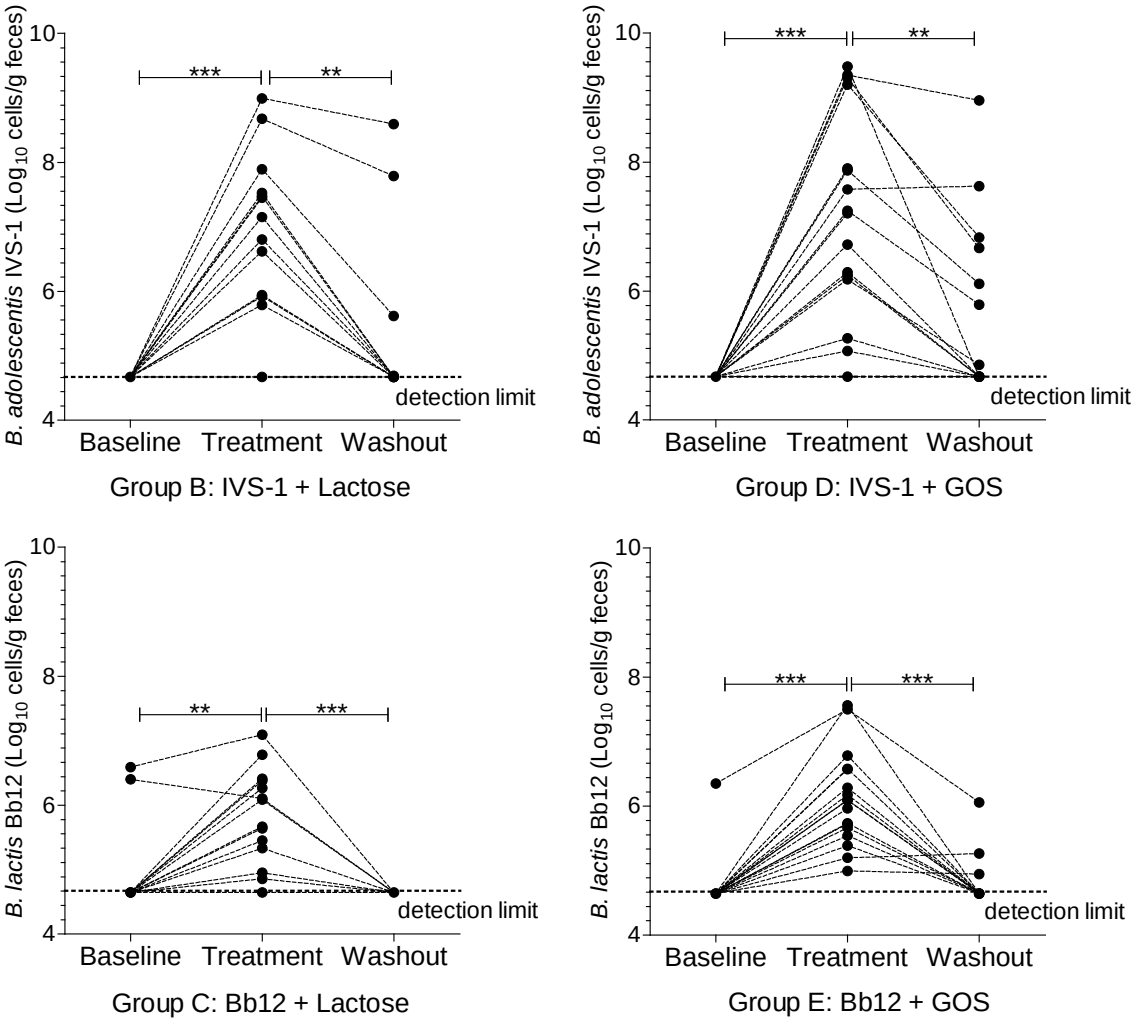
The results showed that compared to the baseline and washout in the fecal samples, both strains reached significant increases in total numbers ($P < 0.001$ in group B (IVS-1 + lactose), D (IVS-1 + GOS), and E (Bb12 + GOS); $P < 0.01$ in group C (Bb12 + lactose)), in the presence as well as absence of GOS (Figure 4.2A). In group B (IVS-1 alone), an average of 6.99 ± 1.2 $\log_{10}$ of IVS-1 cells g^{-1} was detected and in group D, receiving the synbiotic IVS-1 plus GOS, cell levels reached 7.22 ± 1.6 $\log_{10}$ of IVS-1 cells g^{-1}. Group C received the commercial strain Bb12, which was detected at absolute numbers of 5.83 ± 0.7 $\log_{10}$ of Bb12 cells g^{-1} and in group E (Bb12 + GOS) Bb12 numbers reached 6.11 ± 0.7 $\log_{10}$ of Bb12 cells g^{-1}.

The numbers of the two probiotic strains for each treatment at each time point were then compared directly (Figure 4.2B). IVS-1 colonized the GI tract at a significantly higher number than the commercial probiotic Bb12 in the probiotic only treatments ($P = 0.0056$). Although GOS enriched for both strains, Bb12 and IVS-1, relative to the test strains alone, in both cases this trend was not significant ($P = 0.7382$ and $P = 0.3034$, respectively). In contrast, when comparing the two synbiotics, GOS significantly increased the number of IVS-1 by more than one log compared to the commercial Bb12 synbiotic (7.22 ± 1.6 $\log_{10}$ and 6.11 ± 0.7 $\log_{10}$ respectively, $P = 0.0195$). This result demonstrates an ecological advantage of IVS-1 over Bb12 and a limited effect of GOS supplementation on either strain. After a four week washout period, Bb12 could not be detected in group C subjects, and only in three subjects at a very low number in the synbiotic group (group E). One of these subjects already had Bb12 present in the baseline sample. In the IVS-1 + GOS synbiotic group D, IVS-1 persisted in six subjects during the washout period, and the number of IVS-1 was significantly higher than the number of Bb12 in group E ($P = 0.0057$).

In addition to strain-specific quantification, the absolute numbers of bifidobacteria were measured for all six groups at the three time points by genus-specific quantitative real time PCR (Figure 4.3A). Even though both *Bifidobacterium* strains were significantly enriched as determined by strain-specific qPCR, neither IVS-1 nor Bb12 supplementation increased the total number of bifidobacteria. Only groups D (IVS-1 + GOS) and F (GOS) showed a significant increase of bifidobacteria due to the treatments ($P = 0.0203$ and $P = 0.0191$, respectively).

The *Bifidobacterium* numbers at the baseline varied greatly within each group, ranging from the detection limit ($\log_{10}$ 4.67 cells g^{-1} feces) to $\log_{10}$ 10.42 cells g^{-1} feces in group F (GOS). Therefore, the change in cell number was calculated for each subject (Figure 4.3B). The highest increases in *Bifidobacterium* numbers were for group F (GOS) with 1.30 ± 1.7 $\log_{10}$, followed by group B (IVS-1 only) with 1.22 ± 1.4 $\log_{10}$. Interestingly, the lactose group also had an increase in bifidobacteria (0.51 ± 0.9 $\log_{10}$). However, none of these differences reached statistical significance.

A



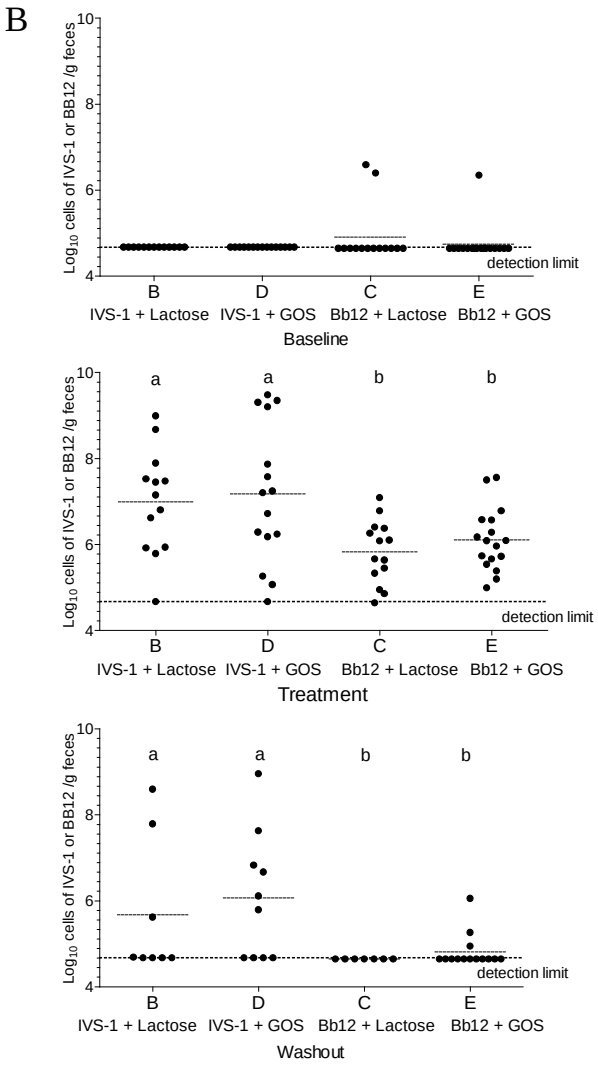
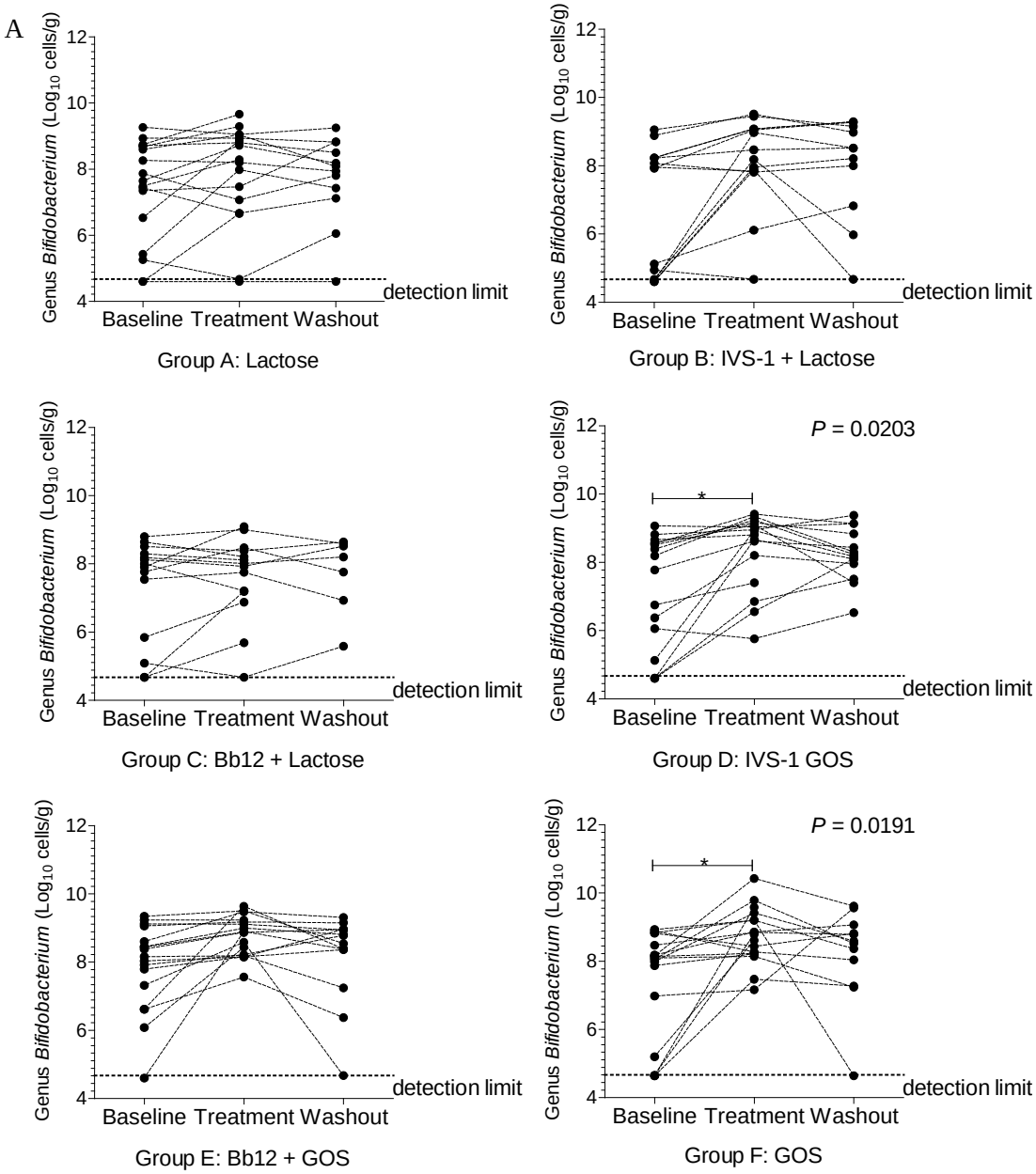


Fig. 4.2. A. Test of *in vivo* selected synergistic synbiotic application compared to a commercial synbiotic. Quantification of absolute cell numbers of bifidobacteria in fecal samples by qPCR using strain-specific primers for strains *B. adolescentis* IVS-1 and *B. lactis* Bb12. Shown are probiotic and synbiotic treatment groups at baseline, treatment and washout time points. Significance of $P \leq 0.05$ is denoted by a single asterisk (*), $P \leq 0.01$ as two asterisks (**), and $P \leq 0.001$ by three asterisks (***). B. Direct comparison of abundances of *B. adolescentis* IVS-1 and *B. lactis* Bb12 at each time point. Different letters indicate significant differences between groups ($P \leq 0.05$).

A



B

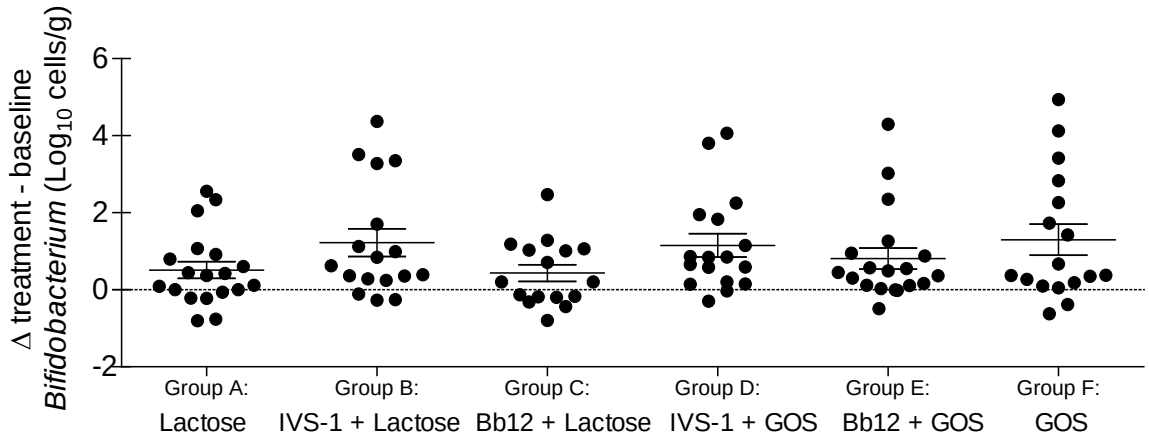


Fig. 4.3. A. Quantification of absolute cell numbers of bifidobacteria in fecal samples by genus-specific qPCR. B. Change in abundance of bifidobacteria for each subject due to treatment consumption.

4.4.3 Community-wide characterization of effects on gut microbiota confirms ecological advantage of GOS and IVS-1 compared to Bb12 and GOS.

Community-wide changes that were introduced by the dietary treatments to the resident gut microbiota were assessed by sequencing 16S rRNA tags. Interestingly, only one phylum was significantly influenced by the dietary treatments as *Actinobacteria* was significantly higher in subjects treated with IVS-1 + lactose (group B, $P = 0.0181$, Table 4.2, Figure 4.S1), and the genus *Bifidobacterium* in groups B (IVS-1 + lactose) and C (Bb12 + lactose). Apart from this, only at the OTU level were significant changes introduced to the gut microbiota. However, the abundance of these OTUs was extremely low in most cases, and high variations in the sample populations were also observed. Accordingly, the alpha and beta diversity of each group and between groups was not significantly different (data not shown).

The ability of the test strains (alone and as synbiotics) to become established in the GI tract was based on the abundance of operational taxonomic units (OTU) representing the species *B. adolescentis* (OTU_1) and *B. lactis* subsp. *animalis* (OTU_167) (Table 4.3). *B. adolescentis* is an autochthonous species in the human GI tract and was detected at low average abundances in the GI tract of the subjects at baseline (2.27 ± 4.7 %). In contrast, *B. animalis* subsp. *lactis* was detected in baseline samples for only seven subjects. In four of these subjects the abundance of OTU_167 was below 0.01 %. The other three subjects are identical to the ones identified in the qPCR analysis (Figure 4.2).

Based on the sequencing analysis, OTU_1 was significantly enriched by the probiotic-alone and synbiotic treatments to 3.7 and 7.3 %, respectively (Table 4.2). In groups D (IVS-1 + GOS), E (Bb12 + GOS), and F (GOS) OTU_1 *B. adolescentis* became

the most abundant OTU, representing an average of 7.3 %, 7.2 %, and 6.7 % of the microbiota, respectively, and the second most abundant in group B (3.4 %). This increase was clearly based on the presence of IVS-1 and/or GOS in the treatments, as the abundance of OTU_1 *B. adolescentis* was only 0.01 % in group A (Lactose) and 1.2 % in group C (Bb12 + lactose). A comparison of the abundance of OTU_1 between group B (IVS-1 + lactose), C (IVS-1 + GOS), and group F (GOS) showed no significant difference ($P > 0.1$). There was a significant increase in the relative abundance of OTU_167 *B. animalis* between baseline and treatment in group C (Bb12 + lactose) and E (Bb12 + GOS) (0.4 % and 0.1 %, respectively).

4.4.4 Systematic analyses of members of the gut microbiota.

To identify potential interactions between the test strains and other members of the gut microbiota, we performed correlation analyses at the treatment time point. As a cutoff, the genus had to have an average abundance of at least 0.5 %, and the OTU an average abundance of at least 1 %, with the exception of OTU_167 (*B. animalis)* which was included even though its average abundance was below 1 %. No significant correlations between the genus *Bifidobacterium*, or any *Bifidobacterium* OTUs, and other genera or OTU could be identified once outliers were removed. However, several other significant correlations between other members of the gut microbiota were observed (Figure 4.S2 and 4.S3).

Table 4.3. Proportions of bacterial taxa significantly influenced by dietary treatments

		Mean % bacterial abundance ± SD [b]			
Treatments	**Taxonomic Group**	**Baseline**	**Treatment**	**Washout**	***P* value**
Group A Lactose control	**OTUs [a]**				
	OTU_2050 (*Ruminococcus2* sp.)	0.01±0.0 AB	0.01±0.0 A	0.02±0.0 B	0.0139
Group B IVS-1 Probiotic	**Phylum**				
	Actinobacteria	9.16±5.5 A	15.44±6.2 B	14.39±5.9 AB	0.0181
	Genus				
	Bifidobacterium	7.85±7.8 A	14.66±7.2 B	13.43A±6.9 B	0.0378
	OTUs [a]				
	OTU_2353 (*Dorea* sp.))	0.01±0.0 A	0.02±0.0 AB	0.02±0.0 B	0.0068
	OTU_1 (*B. adolescentis*)	1.30±4.4 A	3.69±5.2 B	3.56±4.7 B	0.0010
Group C Bb12 Probiotic	**Genus**				
	Bifidobacterium	9.53±5.3 A	10.18±8.3 B	9.96±6.1 AB	0.0378
	OTUs [a]				
	OTU_167 (*B. animalis* subsp. *lactis*)	0.04±0.1 AB	0.04±0.1 A	0.00±0.0 B	0.0328
Group D IVS-1 GOS	**OTUs [a]**				
	OTU_469 (*Anaerotruncus* sp.)	0.03±0.1 A	0.00±0.0 B	0.01±0.0 AB	0.0188
	OTU_102: (*Lachnospiracea incertae sedis* sp.)	0.35±0.5 A	0.01±0.2 B	0.24±0.6 AB	0.0444
	OTU_1 (*B. adolescentis*)	2.48±4.5 A	7.34±7.2 B	4.75±5.1 B	0.0343
Group E Bb12 GOS	**OTUs [a]**				
	OTU_167 (*B. animalis* subsp. *lactis*)	0.00±0.0 A	0.12±0.2 B	0.01±0.0 A	0.0043
	OTU_1800 (unclass. *Lachnospiraceae* sp.)	0.00±0.0 AB	0.01±0.0 A	0.00±0.0 B	0.0241
Group F GOS	**OTUs [a]**				
	OTU_643 (*Bacteroides* sp.)	0.02±0.0 A	0.00±0.0 B	0.00±0.0 AB	0.0372
	OTU_319 (*Butyricimonas* sp.)	0.00±0.0 AB	0.00±0.0 A	0.05±0.1 B	0.0382

[a] If the strain could not be assigned to a type strain (< 97 % homology), RDP Classifier was used to determine the most likely genus (80 % cutoff). [b] Values with different uppercase letters are significantly different from each other.

The ratio of *Prevotella* to *Bacteroides* has been previously suggested to be strongly associated with diet, especially diets rich in animal fat (*Bacteroides*) versus carbohydrates (*Prevotella*) (55). Other studies have reported a significant change in the ratio between *Prevotella* and *Bacteroides* due to dietary treatments (56), but an analysis of the *Prevotella* and *Bacteroides* ratio in this study showed no significant difference within a treatment group, or when groups were compared (Figure 4.S4). Furthermore, the abundance of butyrate producing genera such as *Faecalibacterium*, *Eubacterium*, *Roseburia*,

Lachnobacterium, and *Ruminococcus* was not significantly influenced by any of the treatments, nor was their combined abundance changed by any of the treatments (data not shown).

4.4.5 Systematic analyses of subjects that showed IVS-1 persistence after treatment termination.

We observed that for nine subjects IVS-1 persisted during the four week washout period. Three subjects were from group B and six were from group D (IVS-1 alone and IVS-1 + GOS, respectively). The microbiota of these nine persisters was compared with the microbiota of all the other subjects in groups B and D (referred to as non-persisters). The aim of this analysis was to determine if the persister status could be predicted before the treatment had begun (i.e., from the baseline samples), based on the composition of the gut microbiota. In order to identify genera and OTUs that affected persistence of IVS-1 in the GI tract of these two groups, a Random Forest classification was performed. This analysis shows the importance that the relative abundances of different taxa have in predicting persistence. Output of this analysis is a "value of mean decrease in accuracy". The higher the value of mean decrease in accuracy of the taxa, the stronger the prediction of persistence of IVS-1. All genera and OTUs with a value of mean decrease in accuracy of at least one are reported here (Figure 4.4). The random forest analysis identified *Slackia* as the most important predictor of persistence. When the abundance of *Slackia* was compared between persisters and non-persisters, *Slackia* abundance was higher in non-persisters with a ratio of almost 200. However, despite the high ratio, the absolute abundance of *Slackia* was only 0.23 % in non-persisters and almost absent in persisters.

Other members of the *Coriobacteriales* order are also predictive for the ability of IVS-1 to persist in the subjects, including *Asaccharobacter*, *Collinsella*, and *Olsenella*. Except for *Asaccharobacter*, all of these *Coriobacteriales* were present at a higher abundance in non-persisters than in persisters. Three members of the *Clostridiales* order, *Ruminococcus*, *Eubacterium* and *Mogibacterium*, one member of the *Bacteroides* order, *Prevotella*, and one member of the *Erysipelotrichales* order, *Holdemania*, also appeared to have the greatest impact on IVS-1 persistence. At the OTU level, eight OTUs had a value of mean decrease in accuracy of at least one; six belonged to the order *Clostridiales*, one to *Coriobacteriales*, and one to *Lactobacillales*. The prediction value of the OTUs for persistence appears to be strain dependent. For example, OTU_21 *Blautia* sp. and OTU_98 *Blautia* sp. were present in higher abundance in persisters then in non-persisters, while OTU_103 *Blautia* sp. had a significantly higher abundance in non-persisters ($P = 0.0353$). Three *Lachnospiraceae* OTUs were also identified. OTU_61 *Lachnospiraceae incertea sedis* and OTU_2093 *Lachnospiraceae incertea sedis* share 96 % identity and are both in higher abundance in persisters than in non-persisters ($P = 0.0365$ and $P = 0.0805$, respectively). OTU_76, an unclassified *Lachnospiraceae* sp., on the other hand was present in significantly higher numbers in non-persisters ($P = 0.0177$) and shared 91 % and 90 % identity with OTU_61 and OTU_2093, respectively. OTU_1 *B. adolescentis* was only present in two of the persister subjects at the baseline and was not detected by the Random Forest analysis (< -1.0).

Even though no *Bifidobacterium* species had a Random Forest importance value above 1, three *Bifidobacterium* OTUs had values above zero and were further analyzed (Figure 4.6A). Those three OTUs consisted of OTU_2055 *B. pseudocatenulatum* (99% identity, e-value $6 \cdot 10^{-120}$, 100% query cover), OTU_2202 *B. pseudocatenulatum* (98%

identity, e-value $2 \cdot 10^{-119}$, 100% query cover), and OTU_7 *B. ruminantium*/*adolescentis* (100 % identity, e-value $4 \cdot 10^{-126}$, 100% query cover in each case according to NCBI). OTU_7 had a 144-fold higher relative abundance in non-persisters than in persisters on average ($P = 0.0454$), even though it was not present in all non-persisters at the baseline. OTU_7 is very closely related to IVS-1 (98 % identity) and possibly the same species (Figure 4.6B). OTU_2055 *B. pseudocatenulatum* and OTU_2202 *B. pseudocatenulatum*, were higher in persisters by a ratio of 24 and 5.5, respectively, but their abundance was not significantly different between the groups.

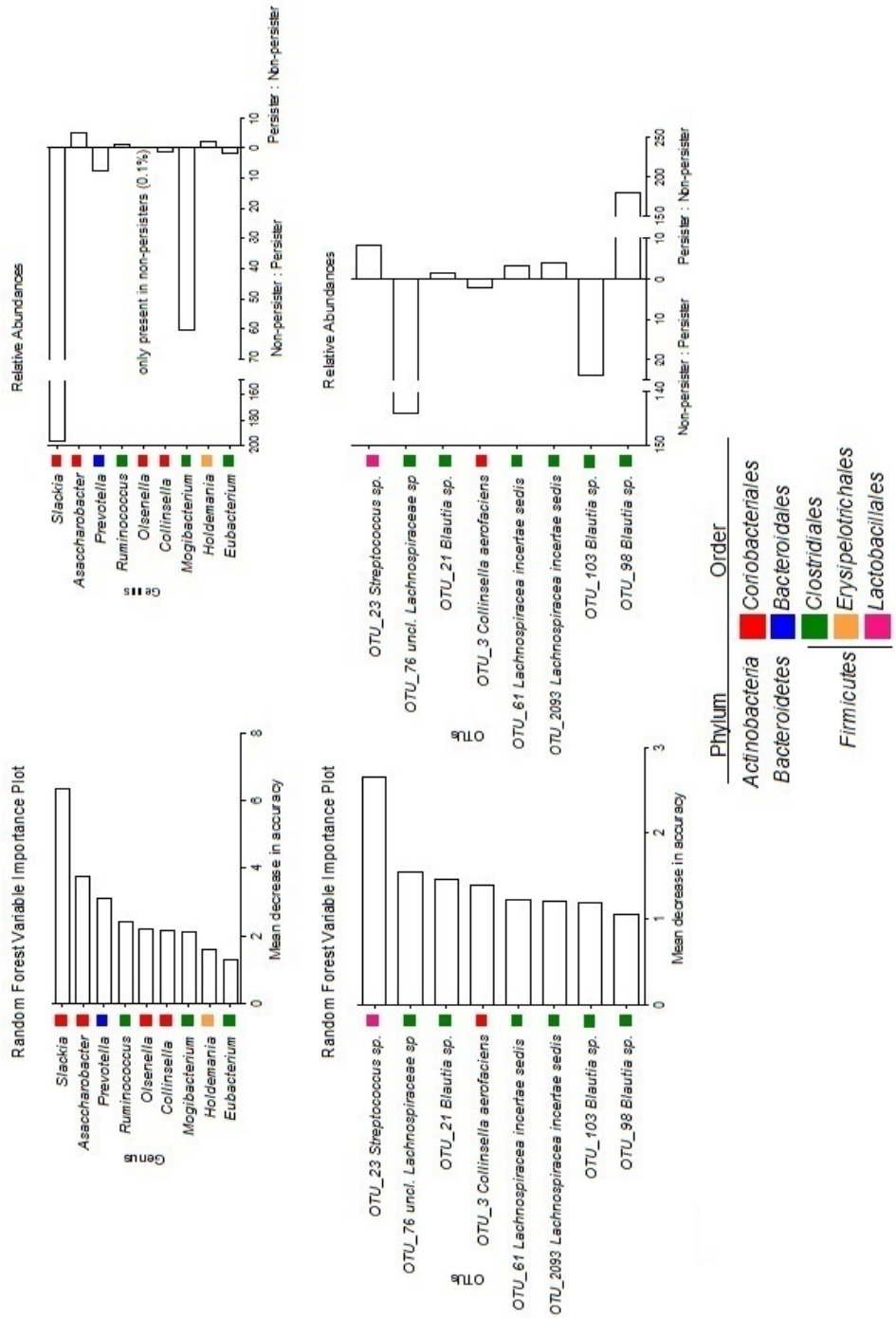


Fig. 4.4. Prediction analysis of IVS-1 persisters and non-persisters. Random Forest variable importance plots and relative abundance of genera (A) and OTUs (B) with a variable importance of at least 1 in mean decrease in accuracy. The ratio of the given taxa between responders and non-responders is shown on the right. Taxa are color coded by order.

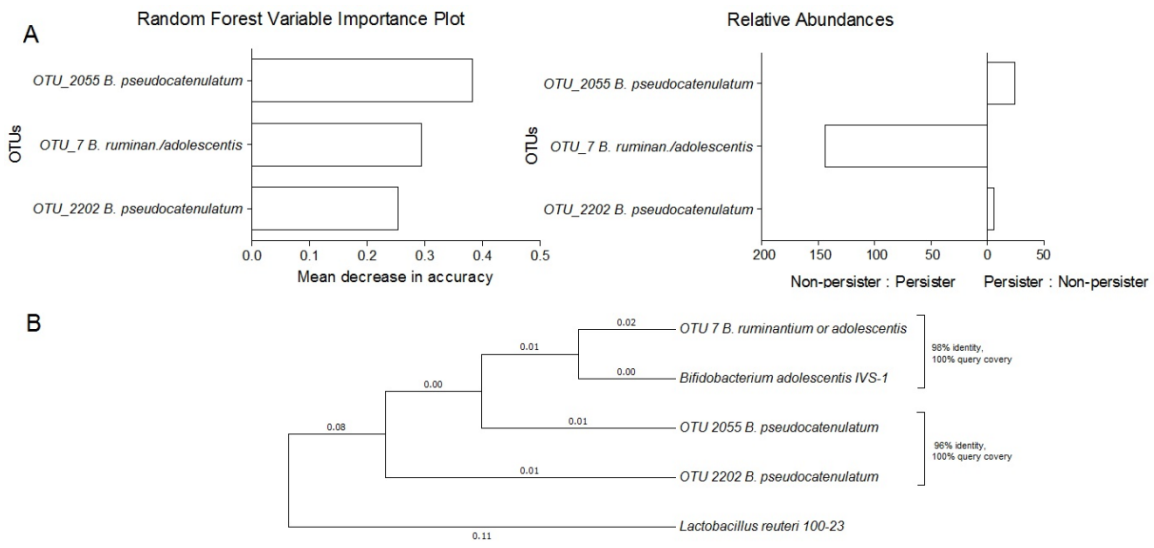


Fig. 4.5. A. Prediction analysis of IVS-1 persisters and non-persisters. Random Forest variable importance plots and relative abundance of *Bifidobacterium* OTUs with a variable importance of at least zero in mean decrease in accuracy. B. The ratio of the given *Bifidobacterium* OTUs between responders and non-responders.

While different markers for alpha diversity such as Shannon index, Simpson index, and the number of observed species was not significantly different among persisters and non-persisters in the baseline sample (Figure 4.S5), the beta diversity of the samples tended to separate (Figure 4.6). However, this trend was not significant (P = 0.2637)

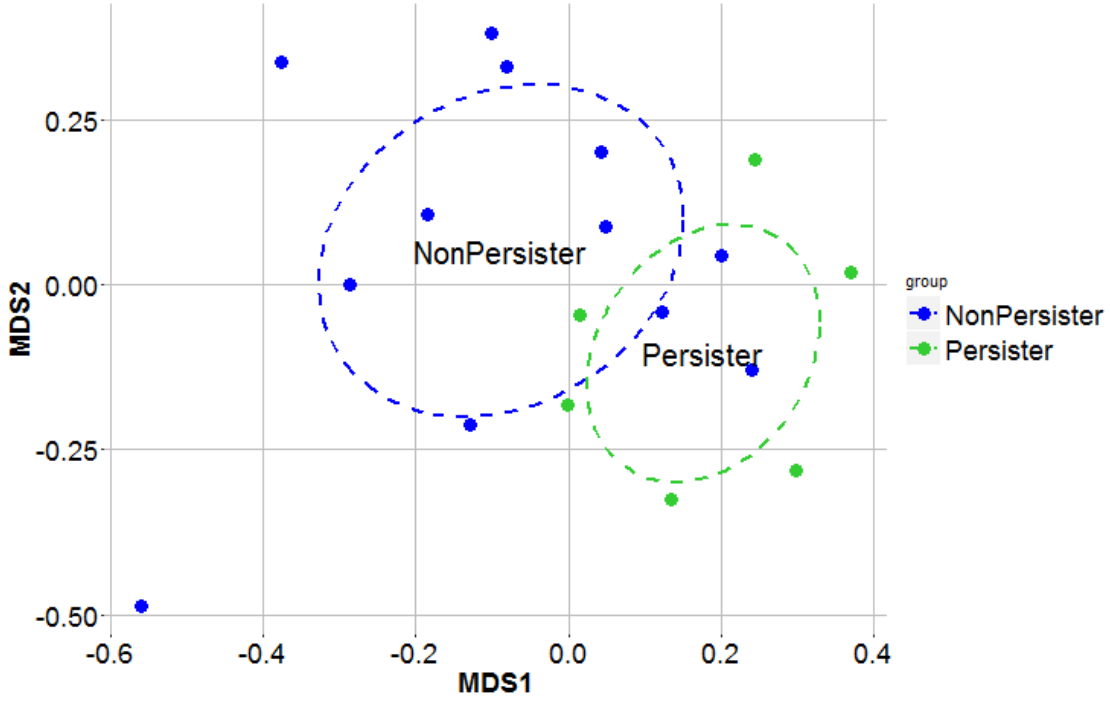


Fig. 4.6. NMDS plot of beta diversity analysis based on Bray-Curtis distance of baseline samples between IVS-1 persisters and non-persisters.

4.4.6. Analysis of anthropometric markers and gastrointestinal symptoms

Anthropometric markers were analyzed at the baseline and after three weeks of treatment, and the change (as percent) is reported in Table 4.4. There was no change detected in anthropometrics between groups.

Table 4.4. Percent change in anthropometrics in participants compared to the baseline[1]

	Total Sample (n=94)	Group A Lactose (n=17)	Group B IVS-1 (n=14)	Group C Bb12 (n=14)	Group D IVS-1 + GOS (n=16)	Group E Bb12 + GOS (n=17)	Group F GOS (n=16)
Anthropometrics							
Body weight, kg	0.4 (2.9)	0.4 (5.0)	-0.1 (2.9)	0.6 (2.4)	-0.3 (3.4)	-0.1 (3.6)	1.3 (3.6)
BMI, kg/m^2	0.4 (2.9)	0.4 (5.0)	-0.1 (2.9)	0.6 (2.4)	-0.3 (3.4)	-0.1 (3.6)	1.3 (3.6)
Waist circum-ference, inches	0.0 (4.6)	-1.3 (3.8)	0.0 (6.9)	0.2 (5.2)	0.0 (5.5)	1.8 (3.0)	0.0 (5.1)

[1]Per-protocol sample based on those that were randomized to treatment and were considered compliant to the treatment

[2]All clinical characteristics are listed as median (IQR)

A structured 34-item questionnaire with a Likert scale from 0 (no symptoms) to 10 (very severe symptoms) was used to assess gastrointestinal symptoms at baseline and any changes in these symptoms with supplementation. At baseline, subjects reported no symptoms to 28 of 34 (82.4 %) GI symptoms included on the questionnaire; this number of symptoms increased to 85.3 % (29 of 34 items) at the end of the treatment. The most common symptoms at baseline were bloating, passing gas, hard stools, and watery stools, with 60.6 %, 85.1 %, 46.8 %, and 43.6 % overall indicating presence of these symptoms, respectively. The median symptom score for each of these four symptoms both before and after treatment are listed in Table 4.5. Overall, low median scores indicated that most subjects either had minimal GI symptoms or low severity of that symptom. No significant differences existed between median symptom score for the six groups at baseline. At the end of treatment, those in the GOS group had significantly more hard stools than the Bb12 + GOS group (Kruskal-Wallis; $P = 0.024$). Passing gas increased from a median of 2.0 to 5.0 with lactose supplementation, but this was not significantly different (Mann-Whitney,

P = 0.15). The severity of passing gas significantly reduced from 4.0 to 1.0 in the Bb12 + GOS group (*P* = 0.040), and severity of hard stools increased from 1.0 to 3.5 in the GOS group (Wilcoxon Signed Ranks Test, *P* = 0.030).

Table 4.5. Gastrointestinal symptoms by supplementation group[1,2,3]

	Total Sample (n=92)	Group A Lactose (n=16)	Group B IVS-1 (n=14)	Group C Bb12 (n=14)	Group D IVS-1 + GOS (n=15)	Group E Bb12 + GOS (n=17)	Group F GOS (n=16)
Baseline [2]							
Bloating	2.0 (4.8)	2.0 (4.8)	1.0 (3.3)	2.0 (4.0)	2.0 (5.0)	4.0 (6.0)	2.5 (7.8)
Passing Gas	3.0 (5.0)	2.5 (4.8)	2.5 (3.5)	2.5 (4.5)	3.0 (5.0)	4.0 (3.5) *	2.5 (5.8)
Hard Stools	1.0 (2.8)	0.5 (3.0)	1.0 (2.3)	0.0 (2.3)	0.0 (3.0)	1.0 (3.5)	1.0 (3.8) *
Watery Stools	1.0 (4.8)	1.0 (3.8)	1.0 (3.5)	1.5 (3.5)	2.0 (5.0)	1.0 (4.5)	2.0 (5.8)
Treatment End [3,4]							
Bloating	1.0 (4.0)	0.0 (4.0)	1.5 (4.0)	1.0 (2.0)	1.0 (3.0)	2.0 (4.0)	2.5 (5.0)
Passing Gas	3.0 (5.0)	5.0 (4.8)	3.0 (2.0)	3.0 (6.3)	2.0 (3.0)	1.0 (5.0) *	3.0 (5.8)
Hard Stools	1.0 (3.0)	0.0 (3.0)	1.0 (1.3)	1.0 (3.3)	1.0 (3.0)	0.0 (1.0) A	3.5 (4.8) B *
Watery Stools	1.0 (3.0)	1.0 (2.0)	1.0 (3.3)	0.5 (1.5)	1.0 (4.0)	1.0 (2.5)	2.5 (4.8)

[1]Per-protocol sample based on those that were randomized to treatment and were considered compliant to the treatment

[2]Change in gastrointestinal symptoms are listed as median (IQR). Only the most common symptoms experienced are listed

[4]Different letters indicate a significant difference in distribution between groups within symptom; identical symbol indicate differences in symptoms before and after treatment within a treatment group

4.5 Discussion

There is much interest in the health promoting capabilities of bifidobacteria in the human GI tract. Synbiotics have advantages in promoting bifidobacteria as they could result in improved establishment of a specific *Bifidobacterium* strains in the human gut when compared to the probiotic alone, and increase bifidobacteria in individuals that do not possess them or that do not respond to probiotics alone. However, if synbiotics are in fact more successful than their parts has not adequately been studied in humans. Here we

systematically assessed the ability of two *Bifidobacterium* strains, administered alone and combined with GOS as synbiotics, to become established in the gastrointestinal tract of obese adults. In addition, community sequencing was used to identify other changes introduced to the gastrointestinal microbiota by the dietary treatments.

The first objective of this study was to test if the prebiotic carbohydrate GOS included in the two synbiotic preparations supported the establishment of the test probiotic strains in the human gut. Both strains were significantly enriched during the treatment period compared to baseline and washout levels, whether consumed alone or as synbiotics according to the qPCR analysis (Figure 4.2). The absolute numbers of IVS-1 were significantly higher than those detected for Bb12, independently of the presence of GOS. This suggests that the autochthonous strain IVS-1 has an ecological advantage over the allochthonous commercial strain Bb12. This is an important finding as it emphasizes the necessity to consider ecological requirements of the probiotic strains when synbiotic combinations are formulated. Bb12 and IVS-1 are both capable of utilizing GOS *in vitro* (1, 54), however enhanced Bb12 colonization of the human GI tract is not supported by GOS as demonstrated here, and as also previously reported (48–50). There was a tendency of IVS-1 being specifically enriched by GOS. However, this synergistic effect between IVS-1 and GOS did not reach significance in this study cohort. Interestingly, the synbiotic of IVS-1 and GOS led to significantly higher numbers of IVS-1 than the commercial synbiotic of Bb12 and GOS. This indicates that IVS can be used to formulate a synergistic synbiotic that can enhance population levels and the competitiveness of a putative probiotic strain in the gastrointestinal tract compared to a commercial synbiotic. Indeed, this is only the second report of a prebiotic specifically enriching for a putative probiotic strain in humans (57).

Secondly, we compared the ability of the two strains and their respective synbiotic combinations to alter the composition of the gut microbiota in obese individuals. Our analyses showed that the probiotic, prebiotic, and synbiotic treatments altered the gut microbiota of the study subjects to a very limited extent (Table 4.3). Only in treatment group B (IVS-1 + lactose) the phylum *Actinobacteria* was significantly higher in the treatment sample than at the baseline, and the genus *Bifidobacterium* was significantly higher in groups B (IVS-1 + lactose) and C (Bb12 + lactose) compared to the baseline based on 16S rRNA sequencing. One limitation of this sequencing approach is that it returns the relative abundance of taxa, instead of the absolute numbers present in the GI tract. Therefore a qPCR approach was done, which is more quantitative. This analysis showed that the absolute number of bifidobacteria was significantly increased by the IVS-1 + GOS synbiotic (group D) and GOS alone (group F) (Figure 4.3). This increase in numbers and abundance of bifidobacteria due to GOS feeding has been previously shown (1, 36). Interestingly, however, the treatment of Bb12 and GOS did not significantly enrich for bifidobacteria. This may be caused by a large variance in the subject cohort's microbiota, as there was no other member of the gut microbiota identified that was enriched by this treatment and could have outcompeted the bifidobacteria for GOS. While both strains, IVS-1 and Bb12, were significantly enriched by the probiotic dietary treatments in group B (IVS-1 + lactose) and C (Bb12 + lactose) (Figure 4.2), but the absolute number of bifidobacteria was not increased in these groups (Figure 4.3).

According to the 16S rRNA sequencing analysis, in all other cases only OTUs were significantly influenced in their abundances by the treatments (Table 4.2). This overall resilience of the gut microbiota to the treatments was characterized by a great variability between the subjects. For example, the bifidogenic response to GOS treatment varied

between an increase of almost 5 logs in one subject and a decrease of 0.6 logs in a different individual. This responder and non-responder phenomenon was previously described for GOS treatment in healthy adult subjects (40), and was also observed in this study, despite the presence of the added probiotic strain. The stability of the gut microbiota was also confirmed by the lack of change in the alpha and beta diversities, no change in the abundance of butyrate producers, and a consistency of the *Prevotella* and *Bacteroides* ratio. Few significant correlations were found between members of the gut microbiota, and none between bifidobacteria and any other taxa.

Overall these analyses showed that a strain of a core species of the human gut microbiota, *B. adolescentis*, can be established in almost all of the subjects by probiotic and synbiotic consumption, and at significantly higher numbers than an allochthonous strain. However, this establishment had little effect on the resident community in the GI tract.

Strain-specific qPCR analysis at the four week washout time point showed that IVS-1 did not only reach higher colonization levels compared to Bb12, but IVS-1 was also significantly more persistent than Bb12 (Figure 4.2 B). This finding supports the concept of *in vivo* selection to select for ecologically more competitive probiotic strains. The autochthonous strain IVS-1 had a significant advantage to become established in the GI tract and avoid niche exclusion from the resident microbiota compared to the allochthonous strain Bb12.

Interestingly, IVS-1 persisted in twice as many subjects when consumed as a synbiotic with GOS compared to IVS consumption alone. It has to be taken into consideration, however, that the number of subjects that showed persistence was very low,

only three in group B (IVS-1 + lactose) and six in group D (IVS-1 + GOS), and the washout sample was taken four weeks after the last consumption of the treatment. This study was not designed to specifically test persistence and a strain detection at only four weeks after the end of the treatment does not allow any conclusion about long-term persistence of IVS-1. In this study the addition of GOS did not significantly support the persistence of IVS-1, but because of the small sample size a final conclusion about the synergistic effect of GOS and IVS-1 in regard to persistence cannot be drawn. A future study may consider to extend the consumption of GOS after IVS-1 consumption has been terminated in order to establish if GOS specifically supports IVS-1 colonization and competitive fitness.

In order to get insight into the ecological niche that IVS-1 may be occupying in the GI tract of persisters, the composition of the gut microbiota was compared between persisters and non-persisters in order to determine if a persistence of IVS-1 could be predicted before the beginning of the treatment. By Random Forest analysis, mostly members of the *Coriobacteriaceae* family were identified (Figure 4.4). *Coriobacteriaceae* are frequently found in patients suffering from overweight (58) or inflammatory bowel diseases, but there is no corresponding quantitative or functional data available yet (59). *Slackia* was identified as the most important genus predictor of persistence (Figure 4.4). The genus *Slackia* is part of the family *Coriobacteriaceae* and contains five species (60–63). *In vitro* analyses suggest that none of these species are capable of utilizing GOS (62, 63). Additionally, *Slackia* has been characterized as asaccharolytic (as well as *Eubacterium* and *Mogibacterium*) (64). Therefore it is unlikely that *Slackia* would have been competing with IVS-1 for GOS. This genus is known as a commensal of the mammalian microbiota (59) and two of its members, *S. isoflavoniconvertens* and *S. equolifaciens.* are known equol producers (63). Whether or not the *Slackia* detected here

produces equol remains unknown. Overall *Slackia* is present at very low abundance in non-persisters (0.2 %) but almost absent in persisters.

Other members of the *Coriobacteriaceae* family, *Asaccharobacter*, *Collinsella*, and *Olsenella*, were also predictors of persistence. OTU_3 *Collinsella aerofaciens* and the genus *Collinsella* were both present at approximately 3 % abundance in persisters. Therefore the genus *Collinsella* mostly likely consists only of OTU_3 *Collinsella aerofaciens* in this case. This species is considered to be a member of the core human gut microbiome (65). Interestingly, the identification of *Collinsella*, *C. aerofaciens*, and *Coriobacteriaceae* has been consistent in comparison to another study (Maldonado-Gomez et al., unpublished). In that case, these taxa were predictive of long-term persistence for *B. longum* in healthy human subjects. In this study no significant correlation between IVS-1 or bifidobacteria and any of these taxa could be identified that could explain this phenomenon. For now it remains speculative which effect the presence of *Coriobacteriaceae* has on the persistence of IVS-1.

Three *Blautia* OTUs were identified by Random Forest. Two of the three *Blautia* OTUs were in higher abundance in persisters than in non-persisters. *Blautia* has not been reported to utilize GOS, but a strong positive correlation and potentially cross-feeding between *Blautia* and *Bifidobacterium* was previously reported (1). On the other side, OTU_23, a *Streptococcus* sp., was also identified to be predictive of IVS-1 persistence. It has been previously shown that this species is capable of utilizing complex carbohydrates like GOS (66), so this species could have been competing with IVS-1. However, this OTU was in significantly higher abundance in persisters than in non-persisters (P = 0.0266). Again, the number of persister subjects was very low for this analysis and, unfortunately,

for now it cannot be explained how the taxa identified by Random Forest shape the GI niche that can be occupied by IVS-1.

Interestingly, three *Bifidobacterium* OTUs were identified as predictors of IVS-1 colonization (Figure 4.5). While the mean decrease in accuracy values were quite low and therefore these results have to be considered with caution, two OTUs that potentially contributed to IVS-1 colonization were identified (both *B. pseudocatenulatum*), and one that was possibly preventing it (OTU_7). While the identity of OTU_7 could not be clearly established, OTU_7 was a very closely related strain to IVS-1 with 98 % identity. Therefore, these two strains may have very similar ecological niche preferences and requirements. Based on niche exclusion theory (67) it could be possible that OTU_7 was occupying a niche in the GI tract that could have been occupied by IVS-1 as well, but not by both strains at the same time. IVS-1 was not capable of outcompeting the closely related resident strain in this case.

4.6 Conclusion

This study has provided novel insights into the complex interactions between the gut microbiota and dietary regimens consisting of prebiotics, probiotics, and synbiotics. The potential of synbiotic applications on human health has been previously established (45). However, many synbiotic formulas lack synergistic activity in that the probiotic is not enriched by the prebiotic. Thus, the probiotic strain is established in the GI tract not more effectively had it been introduced by itself. While this lack of success may be due to the arbitrary selection of the synbiotic components, we have previously shown that *in vivo* selection can overcome the ecological limitations imposed on the probiotic strains in the

GI tract (1). By specifically enriching a *B. adolescentis* strain, IVS-1, with its cognate prebiotic substrate, we have previously validated this synergistic synbiotic concept in rats (1). We have now demonstrated the potential of *in vivo* selected probiotics and synbiotics in a human trial as well. IVS-1 was successfully established in the GI tract of human subjects in significantly higher numbers than a commercial probiotic, *B. animalis* subsp. *lactis* Bb12, and also when the two synbiotic combinations were compared. IVS-1, as a strain of a core species of the human gut microbiota, was established in all but one subject, and OTU_1 *B. adolescentis* became the dominant member of the gut microbiota. This study has clearly established that *in vivo* selection can identify autochthonous probiotic strains that are highly competitive in the GI environment when introduced as probiotics or synbiotics. While there was a trend for GOS specifically and synergistically enriching for IVS-1 in this study, this trend did not reach significance.

This study provided important proof of concept that a rational selection of synbiotic combinations based on the ecological requirements of the probiotic strain can significantly enhance the colonization, and persistence of probiotic strains.

4.7 References

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4.8 Supplements

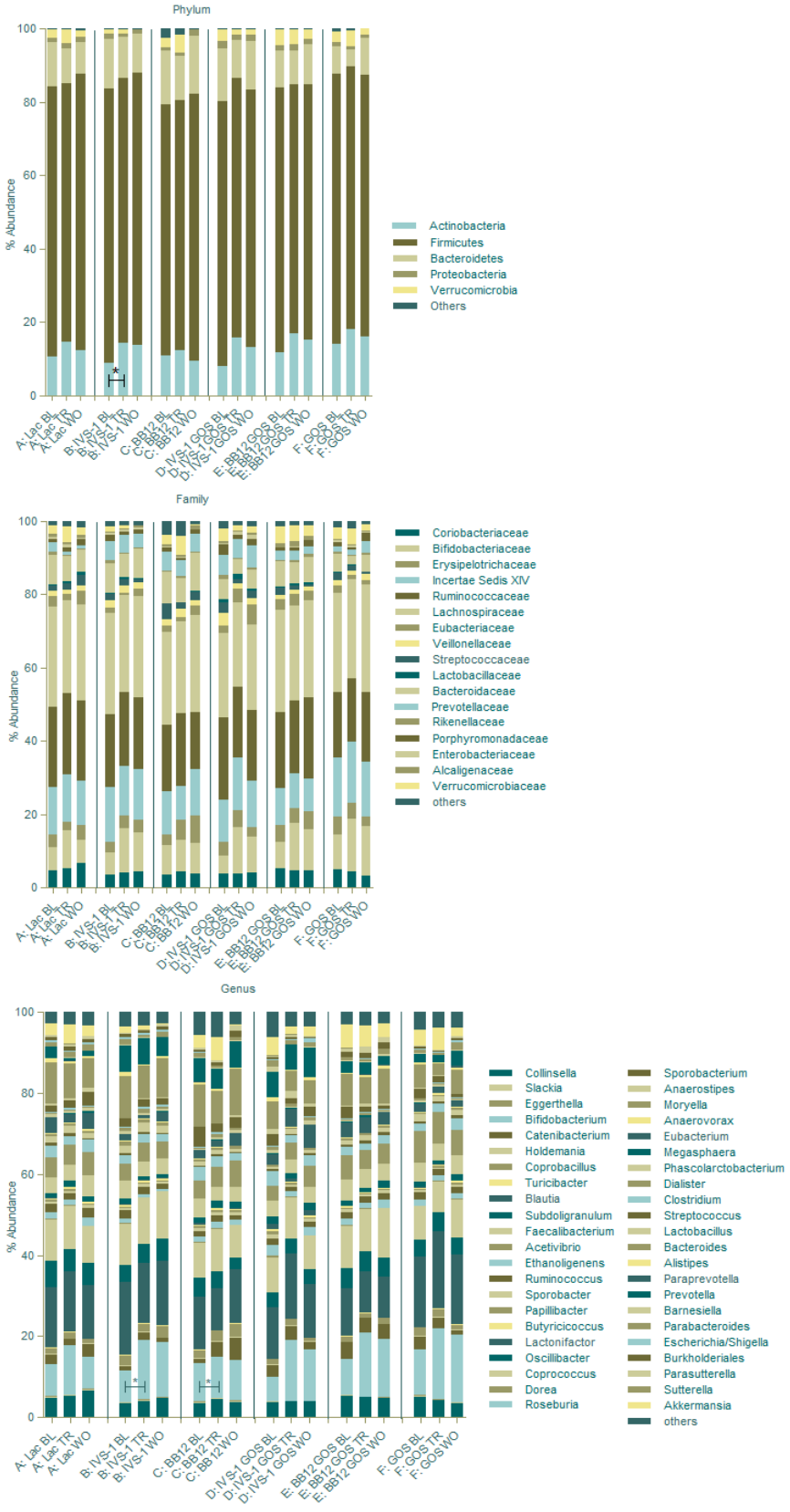

Fig. 4.S1. Average abundances of taxa in fecal samples of subjects consuming dietary treatments.

Bacterial quantities are expressed as percent abundances of total bacteria as determined by 16S rRNA sequencing. Significance of $P \leq 0.05$ is denoted by a single asterisk (*).

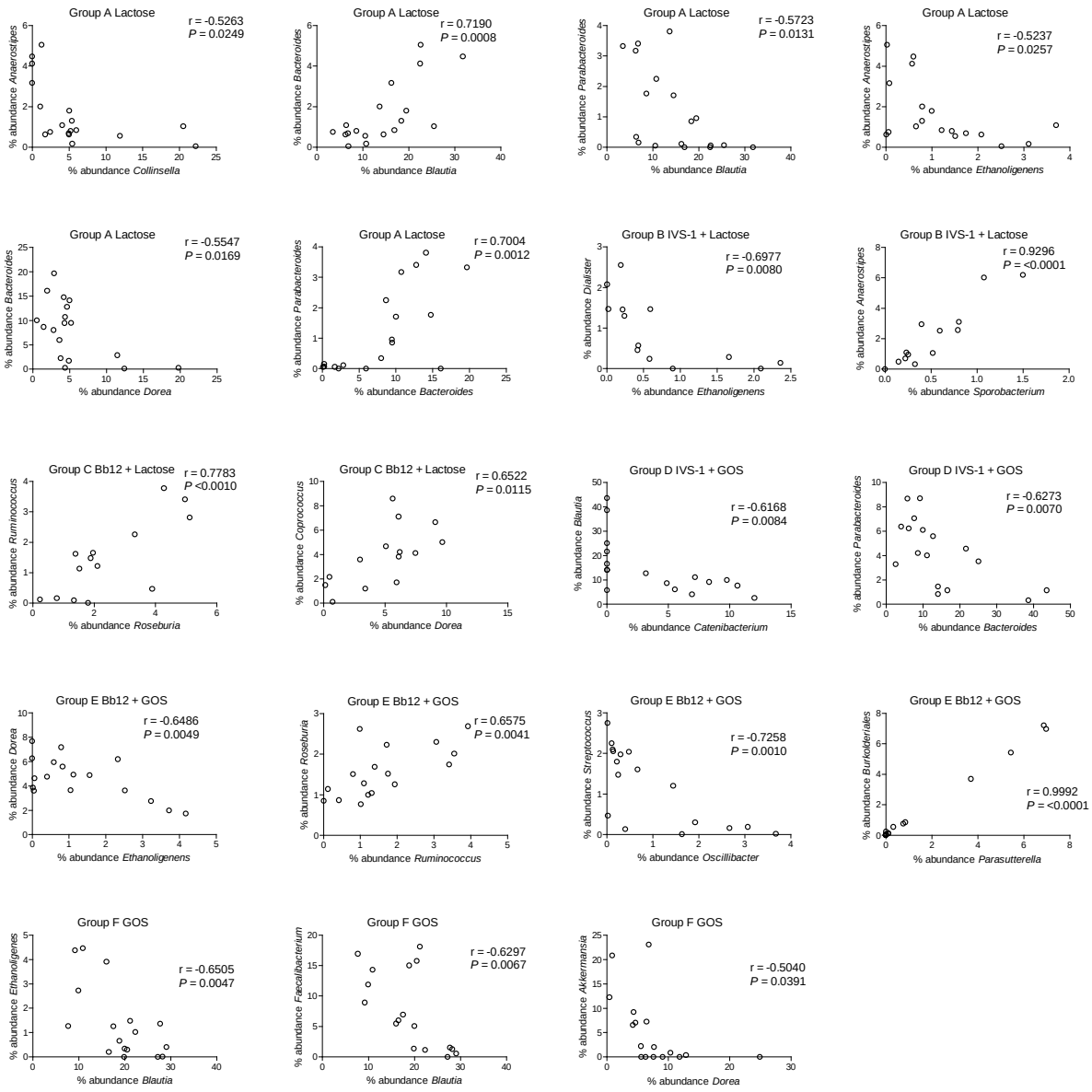

Fig. 4.S2. Correlation analysis of fecal genera present with at least 0.5% abundance in subjects consuming dietary treatments. Bacterial quantities are expressed as percent abundances of total bacteria as determined by 16S rRNA sequencing.

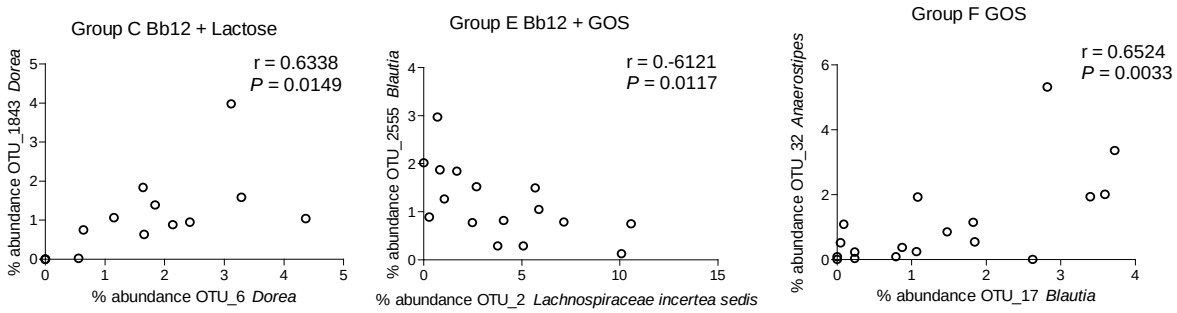

Fig. 4.S3. Correlation analysis of fecal OTUs present with at least 1% abundance in subjects consuming dietary treatments. Bacterial quantities are expressed as percent abundances of total bacteria as determined by 16S rRNA sequencing.

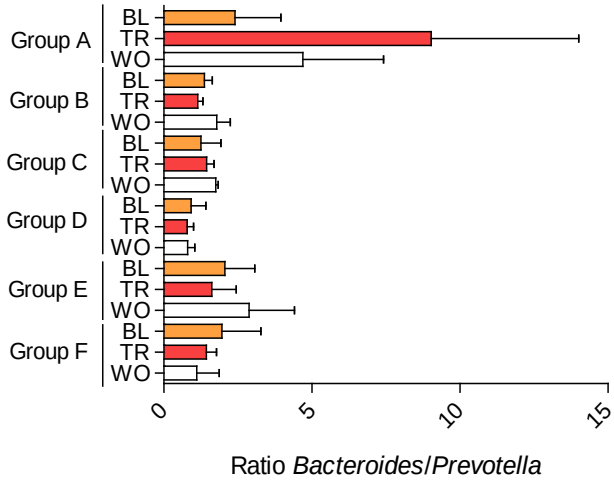

Fig. 4.S4. Ratio of *Bacteroides* and *Prevetolla* taxa present in fecal samples of subjects consuming dietary treatments.

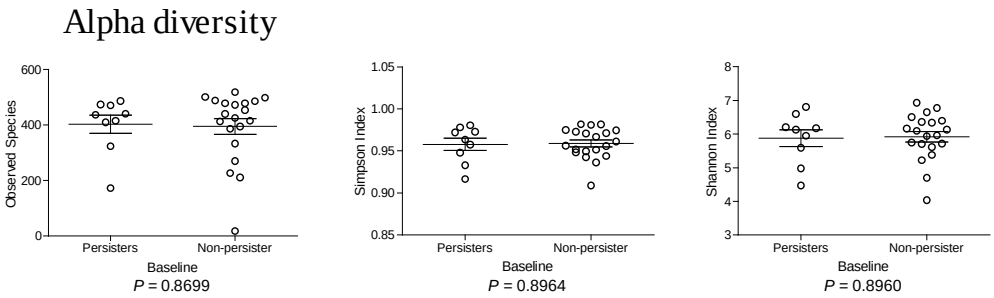

Fig. 4.S5. Alpha diversity comparison between persisters and non-persisters.

ITT analysis

Table 4.S1. Proportions of bacterial taxa significantly influenced by dietary treatments based on intend to treat analysis.

		Mean % bacterial abundance ± SD [b]			
Treatment		**Baseline**	**Treatment**	**Washout**	***P* value**
	Taxonomic group				
Group A Lactose control	**OTUs [a]**				
	OTU_2047 (*Blautia* sp.)	0.01±0.0 A	0.01±0.0 B	0.02±0.0 AB	0.0270
	OTU_2511 (*Blautia* sp.)	0.89±1.2 A	1.06±1.6 B	0.91±1.1 AB	0.0278
Group B IVS-1 Probiotic	**OTUs [a]**				
	OTU_1 (*B. adolescentis*)	1.32±3.9 A	3.66±4.9 B	3.25±4.2 B	0.0130
	OTU_2353 (*Dorea* sp.)	0.01±0.0 A	0.02±0.0 B	0.01±0.0 AB	0.0243
Group C Bb12 Probiotic	**OTUs [a]**				
	OTU_2003 (unclass. *Lachnospiraceae*)	0.00±0.0 A	0.08±0.2 AB	0.04±0.1 B	0.0375
Group D IVS-1 GOS	**Family**				
	Bifidobacteriaceae	4.67± 4.1 A	11.70±6.6 B	9.02±8.7 AB	0.0260
	OTUs [a]				
	OTU_1 (*B. adolescentis*)	2.31±4.4 A	6.92±7.0 B	4.43±5.0 AB	0.0106
	OTU_469 (*Anaerotruncus* sp.)	0.03±0.1 A	0.00±0.0 B	0.01±0.0 AB	0.0391
Group E Bb12 GOS	**OTUs [a]**				
	OTU_156 (*Clostridium XI* sp.)	0.24±0.3 A	0.07±0.2 B	0.20±0.5 AB	0.0214
Group F GOS	**OTUs [a]**				
	OTU_643 (*Bacteroides* sp.)	0.02±0.0 A	0.00±0.0 B	0.00±0.0 AB	0.0372
	OTU_319 (*Butyricimonas* sp.)	0.00±0.0 AB	0.00±0.0 A	0.05±0.1 B	0.0382

[a] If the strain could not be assigned to a type strain (<97% homology), RDP Classifier was used to determine the most likely genus, and the RDP Classifier value is shown (80% cutoff).

[b] Values with different uppercase letters are significantly different from each other.

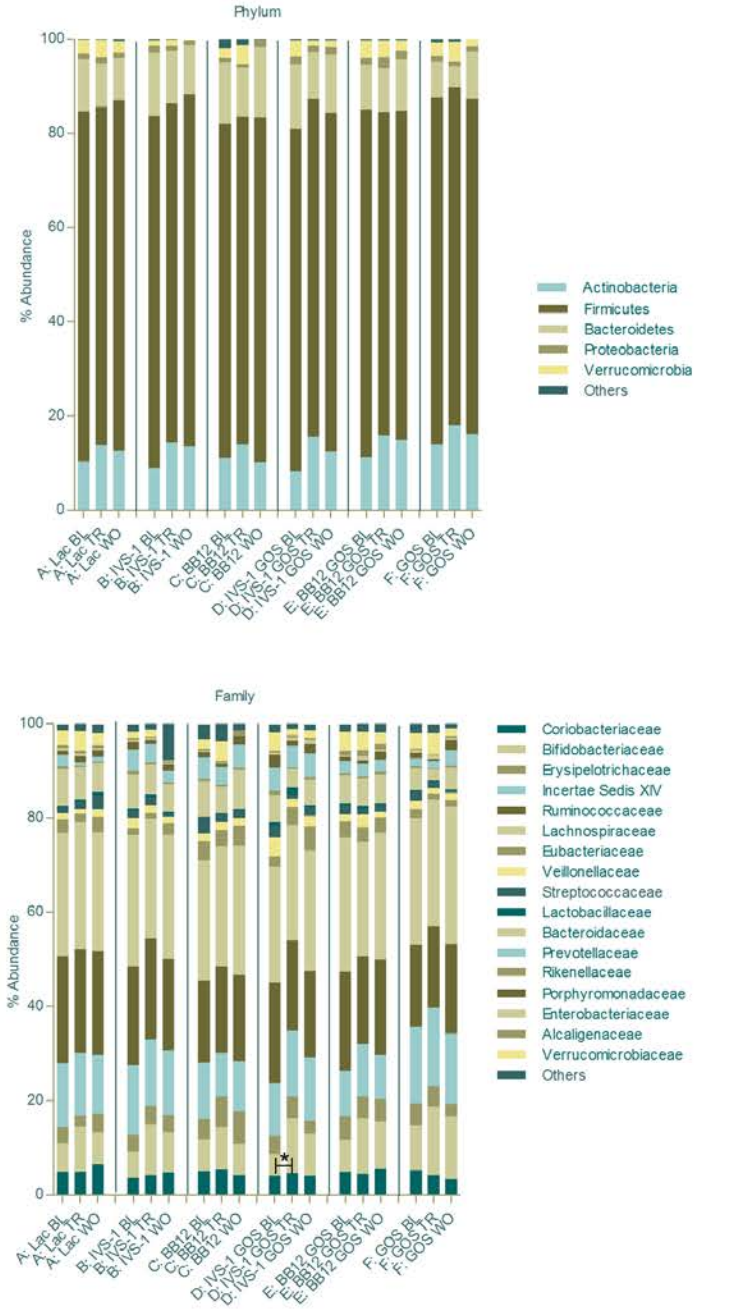

Fig. 4.S6. Average abundances of taxa in fecal samples of subjects consuming dietary treatments based on intend to treat analysis. Bacterial quantities are expressed as percent abundances of total bacteria as determined by 16S rRNA sequencing.

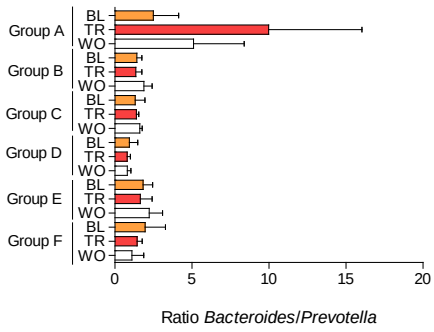

Fig. 4.S7. Ratio of *Bacteroides* and *Prevetolla* taxa present in fecal samples of subjects consuming dietary treatments based on intend to treat analysis.

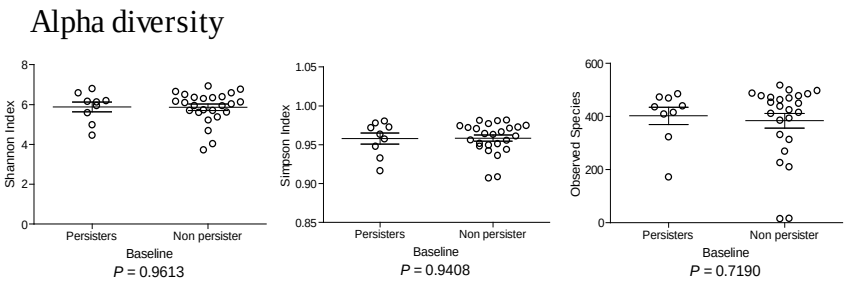

Fig. 4.S8. Alpha diversity comparison between persisters and non persisters based on intend to treat analysis.

Chapter 5

Conclusions and future direction.

5.1 Conclusions

The importance of gut microbiota on human health has been well demonstrated over the past two decades. New analytical methods, as well as bioinformatics tools have led to many new insights into this complex ecosystem. Food and diet have been shown to strongly associate with health and disease, and it has become increasingly recognized that human and animal health is profoundly affected by the specific types and proportions of microorganisms that inhabit the intestinal tract. There is now much interest in formulating dietary strategies to support a health associated gut microbiota. However, there is a considerable knowledge gap on how diet shapes the bacterial populations, which bacteria should be preferably enriched for to support host health, how to formulate dietary treatments in order to be most effective, and which ecological requirements have to be met in order to introduce new members into the gut microbiota.

One strategy to establish microbial members that confer health benefits to the host in the gut microbiota is with the application of probiotics, prebiotics, and synbiotics. As reviewed in Chapter 1, there is much clinical and commercial interest in the development of novel synbiotics. While the health claims made for synbiotic applications are currently outpacing the research, there is little known about the mechanisms by which probiotics and synbiotics become established in the GI tract. The development of novel synbiotics based

on ecological requirements of the probiotic strains to survive and colonize the GI tract may enhance the beneficial health effects already observed for some of the synbiotic applications. However, a better understanding of the gastrointestinal niche, with its very specific biotic and abiotic factors, and the interaction of the resident microbiota is fundamental in order to develop novel probiotics and synbiotics.

The studies presented here first address ecological colonization factors that enable a truly symbiotic model organism, *L. reuteri*, to densely colonize its rodent host in Chapter 2, and secondly introduce and establish a novel method to select for putative probiotic strains and synbiotic combinations *in vivo* in Chapters 3 and 4.

Chapter 2 provided a better understanding of the phenotypic adaptations of a vertebrate gut symbiont, *L. reuteri*, that contribute to both specialization towards a particular host and a highly successful lifestyle. The analysis of this probiotic model organism provided new insights into the ecological requirements and challenges that probiotic strains face in the GI environment, specifically in the stomach. This study's objective was to systematically determine which genes of *L. reuteri* 100-23 contribute to tolerance towards host gastric acid secretion. There were three main findings of this study: (i) the urease cluster was the predominant factor in mediating resistance to gastric acid production; (ii) gene annotations and *in vitro* tests have limitations to predict the exact ecological functions of colonization factors of bacterial gut symbionts; and (iii) novel information was revealed on the mechanisms by which *L. reuteri* colonizes its gastric niche. Ultimately, the basic molecular research described in this chapter broadens our understanding of GI niches and the ecological challenges probiotic strains have to tolerate.

Chapters 3 and 4 focus on the effect of probiotic, prebiotic, and synbiotic applications on the microbial community in the GI tract. Relatively little is known about the effects of synbiotic treatments on the gut microbiota. In order to address this knowledge gap, a functional analysis of specific microbes to colonize the GI tract was done. In Chapter 3, we introduced *in vivo* selection (IVS) as a technique to select synergistic synbiotic combinations. In this approach putative probiotic strains are enriched in subjects by extended consumption of a prebiotic substrate, in this case GOS. A rat study was then conducted to assess the impact of the selected synergistic synbiotic in comparison to prebiotic and probiotic feeding alone. The main finding in this rat model of non-alcoholic fatty liver disease (NAFLD) was that even though the synbiotic did not influence host phenotypes, it was highly efficient at enhancing population levels of the probiotic strain, and making it the most dominant operational taxonomic unit in the GI tract of the rats. This was a novel finding that has not been reported in the probiotic literature. Our findings indicated that *B. adolescentis* IVS-1 not only had a higher affinity for GOS *in vivo* than the resident *Bifidobacterium* species but also utilized GOS to increase its competitiveness and effectively outcompete a closely related resident species. This study showed that IVS-1 and GOS were acting as a truly synergistic synbiotic in rats.

To test the potential of IVS-1 as a probiotic and as a synbiotic application (i.e., IVS-1 and GOS specifically) in human subjects, we conducted a clinical trial assessing the impact of the probiotic, prebiotic, and synbiotic treatments on the microbial community in the GI tract of human subjects suffering from obesity. As described in Chapter 4, we compared the rationally selected synbiotic to a commercial synbiotic that applied an allochthonous probiotic component (*B. animalis* subsp. *lactis* Bb12). This study aimed to

answer two important ecological questions: (i) does the *in vivo* selected autochthonous bacterial strain IVS-1 have an ecological advantage compared to the allochthonous bacterial strain Bb12 in the GI tract of human subjects; and (ii) does the supplementation of each strain with the prebiotic GOS support the colonization of the respective strain *in vivo*. Even though both strains were bifidobacteria, there was a significant difference in the colonization rate. IVS-1 colonized the GI tract in significantly higher numbers than Bb12 and even became most dominant operational taxonomic unit in the subjects. This suggested that the autochthonous strain IVS-1 had an ecological advantage over the allochthonous commercial strain Bb12. This finding supported our hypothesis that IVS can be used to select a bacterial strain that can substantially enhance population levels and the competitiveness of a putative probiotic strain in the gastrointestinal tract compared to a commercial probiotic. This was an important finding as it underlined the necessity to consider ecological requirements of the probiotic strains when synbiotic combinations are formulated. A clear synergistic effect between IVS-1 and GOS was previously established in rats in Chapter 3. However, in the human study the synergistic effect between IVS-1 and GOS did not reach significance.

In summary, the findings in Chapters 3 and 4 demonstrated that the competitive fitness of strain IVS-1 was increased by GOS, which supported the conclusion that *in vivo* selection can be a valuable technique to screen for synbiotic combinations with high synergism *in vivo*.

5.2 Future direction

Our group is currently working on the metabolic data of the human trial described in Chapter 4. This additional data will allow us to correlate health symptoms and improvements with the gut microbiota of the study subjects. This analysis will give us important insight into to the health impacts of the prebiotic, probiotic and synbiotic treatment in comparison to a commercial synbiotic application.

The results presented in this thesis have clearly demonstrated that the application of IVS is likely to enhance the ecological performance of probiotic strains or live biotherapeutics within the habitats in which they are thought to function. The technology could be readily applied in the design of microbiota-modulating therapies, including novel and rational synbiotics.