

Special Article: Nutrition Diet

A Novel Synbiotic Blend of Galactooligosaccharide (GOS) and a Two-Strain Probiotic Acts Synergistically to Increase Lactate and Short-Chain Fatty Acid Production in a Short-Term Ex Vivo Colon Fermentation Model

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Received: January 30, 2024

Accepted: March 04, 2024

Published: March 11, 2024

Abstract

Synbiotics are mixtures of prebiotics and probiotics that improve health and, when combined, can have superior benefits compared to either component alone. Ex vivo short-term colonic simulations were used to evaluate the synbiotic potential of the prebiotic Bimuno® GOS (galactooligosaccharides) and the probiotic Probi Defendum® (*L. plantarum* HEAL9 and *Lactocaseibacillus paracasei* 8700:2). Test conditions included: blank, GOS, the control probiotic Probi Digestis® (*Lactiplantibacillus plantarum* 299v), Probi Defendum®, Probi Digestis® + GOS, and Probi Defendum® + GOS. Stool samples from five healthy donors were used. GOS supplementation, alone or combined with either probiotic, increased gas pressure, acetate production, propionate production (numeric, non-significant), butyrate production (numeric, non-significant), and lactate production. Additionally, biomass was increased and the microbial community composition shifted, most notably demonstrated by an increase in bifidobacteria. In contrast with Probi Digestis®, Probi Defendum® was able to utilize Bimuno® GOS for growth, which highlights the substrate specificity. Probi Defendum® + GOS resulted in an increased lactogenic effect and a donor dependent increase in butyrate production relative to GOS alone, revealing a synergistic effect in ex vivo short-term colonic simulations.

Keywords: Galactooligosaccharide; *Lactocaseibacillus paracasei*; *Lactiplantibacillus plantarum*; Prebiotic; Probiotic; Synbiotic

Abbreviations: AUC: Area Under the Curve; CFU: Colony-Forming Unit; GOS: Galacto-Oligosaccharide; LD: Linear Discriminant; LOQ: Limit of Quantification; MRS: Man-Rogosa-Sharpe; OD: Optical Density; PBS: Phosphate-Buffered Saline; RPM: Rotations Per Minute; SCFA: Short-Chain Fatty Acid; LEfSe: Linear Discriminant Analysis Effect Size; LDA: Linear Discriminant Analysis

Introduction

Both prebiotics and probiotics are known for their ability to provide health benefits. Prebiotics are defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” [1]. These dietary fibers resist digestion and absorption in the small intestine but are fermented by bacteria that reside in the large intestine. A major byproduct of prebiotic digestion is Short-Chain Fatty Acids (SCFAs), which are known to have several health benefits [2,3]. SCFAs are associated with a reduction in intestinal inflammation and an increase in the integrity of the intestinal epithelial barrier [4] and are reported to be involved in immune system function and the regulation of inflammatory responses [5]. Galactooligosaccharides (GOS) are

a well-studied class of prebiotics that are known for their ability to strongly stimulate bifidobacteria expansion, and to a lesser extent, the growth of Bacteroidetes and lactobacilli in the gut [6-12]. Bifidobacteria are considered highly beneficial, largely owing to their ability to produce SCFAs, which, as noted, support intestinal epithelial barrier function and immune regulation [13-16]. The prebiotic supplement, Bimuno®, contains GOS produced from the activity of galactosyltransferases from *Bifidobacterium bifidum* NCIMB 41170 in the presence of lactose [17]. This GOS has demonstrated a variety of prebiotic effects, including the ability to reduce the incidence and duration of traveler’s diarrhea [18,19], reduce colonization of *Salmonella*

enterica serovar *typhimurium* [20], increase SCFA and lactic acid production [10,12,21], stimulate the growth of bifidobacteria and lactobacilli [6,7,10,12,21-23], to exert immunomodulatory effects [7,10], to reduce levels of unfavorable metabolites (ammonium and branched SCFAs) [12], and to reduce gastrointestinal symptoms, including flatulence, bloating, and abdominal pain [22,23].

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit to the host” [24]. They modify the intestinal microbiome composition, have immunomodulatory effects, are able to suppress pathogens, and stimulate the proliferation and differentiation of epithelial cells, which improves the intestinal epithelial barrier [25,26]. Probi Defendum® is a probiotic mixture consisting of *Lactiplantibacillus plantarum* HEAL9 and *Lactocaseibacillus paracasei* 8700:2. In studies of *L. plantarum* HEAL9, supplementation is associated with improved cognition in people who are experiencing moderate stress [27] and a reduction of inflammatory markers associated with acute stress [28]. *L. paracasei* 8700:2 has been shown to have antagonistic activity against *Salmonella enterica* subsp. *enterica* and *Helicobacter pylori* in vitro [29]. Additionally, a clinical study reported that *L. paracasei* 8700:2 supplementation was associated with enhanced endothelial function in participants with metabolic syndrome [30]. General health benefits reported for the *Lactobacillus casei* group, of which *L. paracasei* is a member, include enhanced brain function, enhanced intestinal barrier function, pathogen resistance, immune modulation, and anti-cancer activity [31]. When supplemented together, these probiotics have been shown to reduce the severity of the common cold in children and adults, to reduce the risk of acquiring the common cold [32-35], and to modulate the peripheral immune response in children with celiac disease [36]. Probi Digestis®, a *Lactobacillus*-based probiotic that consists of *L. plantarum* 299v, stands out as the most extensively documented of its species. It was therefore used as a probiotic control in our study. Indeed, clinical evidence on this strain shows its positive impact on gastrointestinal health and iron absorption, and the strain has been reported to affect gut microbiota composition, to inhibit pathogens, and to have immunomodulatory effects [37].

Synbiotics are mixtures of prebiotics and probiotics that improve health and, when used in combination, can have superior benefits compared to either component alone [38]. When choosing synbiotic pairings, consideration should be given to the positive effects of the prebiotic on the probiotic, ideally pairing a prebiotic that is able to improve the survival of the probiotic and stimulate its proliferation in the gastrointestinal tract. This study was conducted to evaluate the synergistic synbiotic potential of Probi Defendum® + GOS when compared with its individual components, and with another synbiotic (Probi Digestis® + GOS) to demonstrate substrate specificity. Ex vivo short-term colonic simulations were used to evaluate changes in microbial community activity and composition, following supplementation with GOS (Bimuno®), Probi Defendum®, Probi Digestis®, Probi Defendum® + GOS, or Probi Digestis® + GOS.

Materials and Methods

Fecal Samples

Stool samples were collected from five healthy adult donors (no history of antibiotic use within four months prior to stool collection, no history of chronic diseases). The fecal material was processed to 7.5% (w/v) suspensions using PBS un-

der anaerobic conditions and mixed with a cryoprotectant [39], then aliquoted, flash frozen, and stored at -80°C in an anaerobic atmosphere.

GOS Dialysis

To simulate absorption processes during small intestinal passage, Bimuno® GOS (provided by Clasado Biosciences Ltd., Reading, UK) was dialyzed as previously described [12]. Briefly, stock solutions of GOS were prepared in water (35 g/L), added to dialysis membranes (0.5 kDa pore size), and dialyzed in a solution of NaHCO₃ (3.75 g/L, pH 7.0) for 24 h to remove monosaccharides and disaccharides.

Short-Term Colonic Incubations

At the start of the experiment, individual colonic reactors were filled with 56 mL nutritional medium (nutritional medium PD01; ProDigest, Gent, Belgium). Next, a single dose (7 mL) of dialyzed GOS and/or probiotic agent was added to respective reactors, resulting in a GOS 3.5 g/L concentration (assuming no absorption), and 1×10⁷ CFU/mL of probiotic agent. Finally, 7 mL of an individual fecal inoculum suspension was added, bringing the total reactor volume to 70 mL. The following six test conditions were used: (a) blank (nutritional medium only), (b) dialyzed GOS (Bimuno® GOS; 3.5 g/L), (c) *L. plantarum* 299v (Probi Digestis®; 1×10⁷ CFU/mL), (d) *L. plantarum* 299v (1×10⁷ CFU/mL) + dialyzed GOS (3.5 g/L) (synbiotic), (e) *L. plantarum* HEAL9 (5×10⁶ CFU/mL) + *L. paracasei* 8700:2 (5×10⁶ CFU/mL) (Probi Defendum®), and (f) *L. plantarum* HEAL9 (5×10⁶ CFU/mL) + *L. paracasei* 8700:2 (5×10⁶ CFU/mL) + dialyzed GOS (3.5 g/L) (synbiotic). Probi Digestis® and Probi Defendum® were provided by Probi AB (Lund, Sweden). Each condition was run in two technical replicates.

Microbial Metabolic Activity Analysis

Change in pH, gas pressure, SCFAs, branched SCFA, lactate, and ammonium were measured at 0 h, 6 h, 24 h, and 48 h. A Senseline F410 pH meter (ProSense, Oosterhout, The Netherlands) was used to measure changes in pH and a hand-held pressure indicator (CPH6200; Wika, Echt, The Netherlands) was used to measure gas pressure at the indicated timepoints. Acetate, propionate, and butyrate, and the branched SCFAs, isobutyrate, isovalerate, and isocaproate, were measured according to the methods of De Weirde et al. [40]. Lactate levels were assessed according to the manufacturer's instructions using an enzymatic assay kit from R-Biopharm (Darmstadt, Germany). The method of Tzollas et al. was used to evaluate ammonium levels [41].

Microbial Community Analysis

Samples collected at 24 h were subjected to shallow shotgun sequencing to assess microbial community composition. The Illumina Nextera XT library preparation kit, with protocol modifications, was used to prepare DNA libraries. The library was quantified with Qubit (ThermoFisher, Waltham, MA, USA). An Illumina HiSeq platform 2×150 bp was used for library sequencing. The methods of Ottensen et al. [42], Ponnusamy et al. [43], Hasan et al. [44], and Lax et al. [45] were used to analyze un-assembled sequencing reads for multi-kingdom microbiome analysis and quantification of relative abundances. Briefly, we used curated genome databases together with a high-performance data-mining algorithm to rapidly disambiguate hundreds of millions of metagenomic sequence reads into the discrete microorganisms engendering the sequences. A BD FACSVerse

Cell Analyzer (BD Biosciences, Franklin Lakes, NJ, USA) was used to determine total bacterial cell counts (high flow rate setting; 200 thresholds on the SYTO channel). The relative abundances of each population in a sample were multiplied with the total cell count obtained with flow cytometry, allowing for the conversion of proportional values obtained using shotgun sequencing to absolute quantities [46].

Growth in GOS

The effect of Bimuno® GOS as a carbohydrate source for the growth of *L. plantarum* HEAL9 and *L. paracasei* 8700:2 either individually or in combination, and *L. plantarum* 299v was assessed. Each probiotic strain was inoculated onto a standard MRS agar plate and incubated anaerobically for 2–3 days at 37°C. Bacteria from 1–2 colonies were then transferred to complete MRS broth with 2% glucose as a carbohydrate source and incubated overnight at 37°C until exponential growth was reached. Next, aliquots were centrifuged (6000 RPM, 3 min) and washed twice using an equal volume of MRS broth without glucose, and then diluted to equivalent OD600. Finally, 2 µL of one individual strain (monocultures) or 1 µL each of both strains (co-cultures, *L. plantarum* HEAL9 and *L. paracasei* 8700:2) was added to each well of a sterile 96-well plate containing 198 µL fresh MRS broth (without glucose) with or without 0.5% dialyzed GOS as a carbohydrate source. Plates were incubated at 37°C overnight in a plate reader and periodic OD600 measurements preceded by 5s shaking at 100 rpm were collected to generate growth curves.

Statistical Methods

Between group comparisons of supplementation effects for microbial metabolic activity analysis endpoints across all donors were made using paired two-sided t-tests. Averages of technical replicates per donor were used as input values, with one input value per donor. The following comparisons were made: GOS alone versus blank (prebiotic effect), Probi Digestis® or Probi Defendum® alone versus blank (probiotic effect), Probi Digestis® + GOS or Probi Defendum® + GOS versus GOS alone (synbiotic effect) and versus blank, and Probi Digestis® + GOS versus Probi Defendum® + GOS (differences between two synbiotics).

Alpha diversity was analyzed using four common indices: observed taxa (species richness), Chao1 (species richness), Shannon (species richness and evenness), and Simpson (species richness and evenness, giving more weight to common or dominant species). Beta diversity was used to determine whether supplementation affected overall community composition. This assessment was made using Discriminant Analysis of Principal Components, which joins two analysis methods to assess effects on population structure. Sequence data were transformed using principal component analysis and clusters were subsequently identified with discriminant analysis, which aims to maximize among-group variation and minimize within-group variation.

Differential abundance analysis was conducted using two statistical methods, treeclimbR and linear discriminant analysis effect size (LEfSe). For both analysis methods, relative abundance data obtained by total sum scaling was used. For treeclimbR analysis [47], bacterial enrichments exceeding a fold change of 4 (corresponding to $\log_2 2$) as compared to the reference condition were considered biologically significant; a p-value of <0.05 (corresponding to $-\log_{10} 0.05=1.3$ on the y-axis) was considered statistically significant, i.e., bacterial enrich-

ments with a $-\log(p\text{-value}) > 1.3$ were considered statistically significant. For LEfSe, the algorithm couples statistical significance with biological consistency and effect size estimation to provide in-depth insight to the biological relevance and magnitude of bacterial enrichments [48]. p-values ≤ 0.05 by the Kruskal-Wallis and Wilcoxon tests were considered statistically significant and LDA scores ≥ 2.0 or < -2.0 were generally considered biologically relevant.

Results and Discussion

Microbial Metabolic Activity

The pH in the reactors reflected colonic pH in vivo, confirming that the colonic simulations were conducted under optimal conditions to support the growth of a wide diversity of gut microbial community members (Figure 1a). The greatest initial drop in pH was observed with GOS alone, Probi Digestis® + GOS, and Probi Defendum® + GOS. For gas pressure, the blank and probiotic alone conditions demonstrated similar profiles, while the GOS, Probi Digestis® + GOS, and Probi Defendum® + GOS conditions had a significant increase in gas pressure compared with blank (Figure 1b). There was no significant difference in gas production between the two synbiotic conditions (Probi Digestis® + GOS vs Probi Defendum® + GOS).

There was significantly more acetate production with GOS alone and with the two synbiotic test conditions compared with blank, but not with the probiotic alone conditions. Acetate production was also not significantly different between GOS alone and either of the two synbiotic conditions (Figure 2a). Propi-

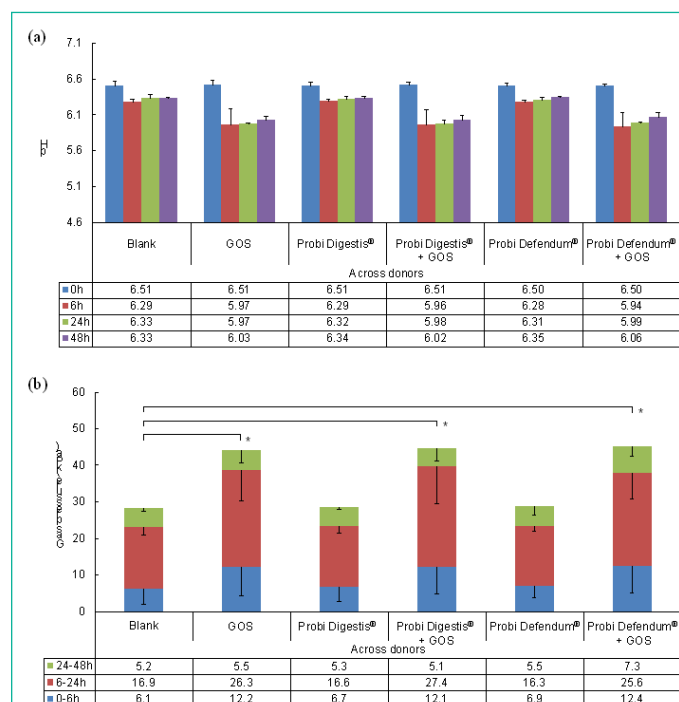


Figure 1: Overall microbial community activity (acidification and gas production) shown as (a) pH and (b) gas pressure. Measurements were collected in triplicate. Data for average values were derived using data from five healthy donors. Error bars represent standard deviation. Two-sided t-tests were used to determine significant differences between each supplemented condition versus blank, between the two synbiotic supplement conditions (Probi Digestis® + GOS and Probi Defendum® + GOS) versus GOS alone, and between Probi Digestis® + GOS versus Probi Defendum® + GOS. * indicates significant differences between the supplemented versus blank test condition when accounting for the entire 48 h period. $p < 0.05$ was considered statistically significant. GOS = Bimuno® galactooligosaccharides.

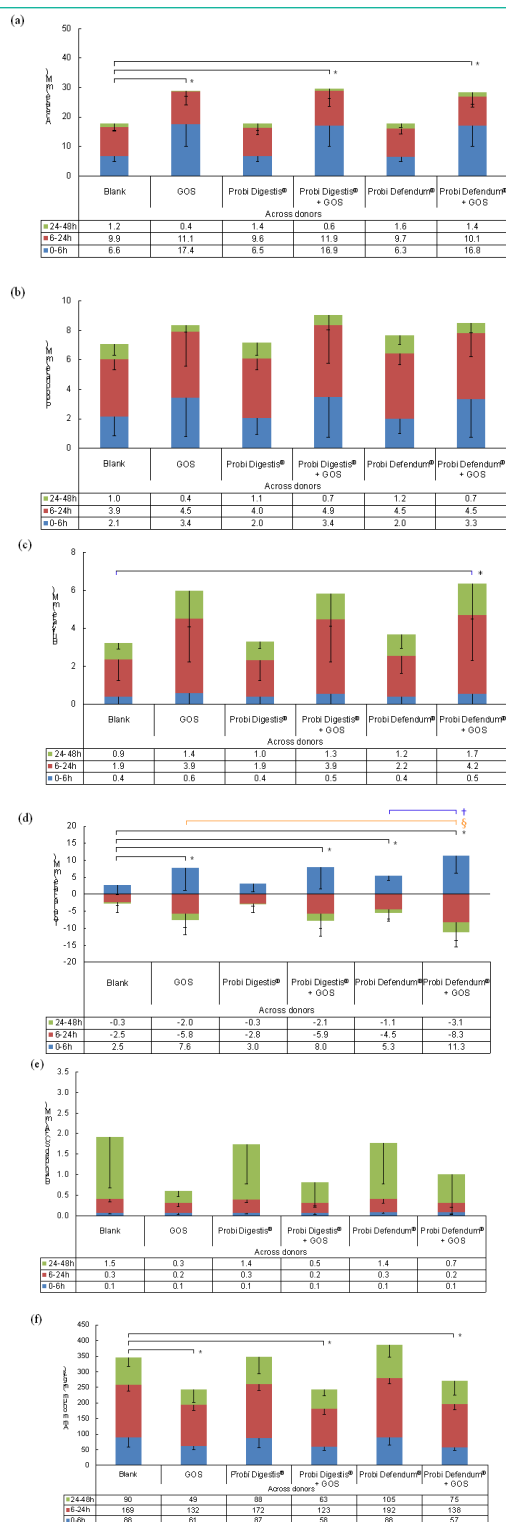


Figure 2: Microbial metabolic activity (a) acetate, (b) propionate, (c) butyrate, (d) lactate, (e) branched SCFA, and (f) ammonium. Data for average values were derived using data from five healthy donors. Error bars represent standard deviation. Paired two-sided t-tests were used to determine significant differences between each supplemented condition versus blank, between the two synbiotic supplement conditions (Probi Digestis® + GOS and Probi Defendum® + GOS) versus GOS alone, and between Probi Digestis® + GOS versus Probi Defendum® + GOS. * indicates significant differences between the supplemented versus blank test condition, § represents significant differences between Probi Digestis® + GOS or Probi Defendum® + GOS compared with GOS alone, and † represents a significant difference between Probi Defendum® + GOS compared with Probi Digestis® + GOS when accounting for the entire 48 h period (acetate, propionate, butyrate, branched SCFA, and ammonium) or the period between 0 and 6 h (lactate). $p < 0.05$ was considered statistically significant. GOS = Bimuno® galactooligosaccharides.

onate production was also similar between blank and the two probiotics alone, while production was numerically, but not significantly greater with GOS alone compared with blank (Figure 2b). Similar numeric increases in propionate were seen for both synbiotic conditions (compared with GOS alone). As with acetate and propionate, the butyrate profiles for the two probiotics alone were similar to that of blank (Figure 2c). There were numeric, but not significant increases in butyrate production with GOS alone and Probi Digestis® + GOS versus blank, and significantly more butyrate production with Probi Defendum® + GOS compared with blank. GOS stimulated butyrate production in four of five donors, with an average increase of 3.5 mM (+91%) across these four donors. The butyrate production profiles for Probi Digestis® and Probi Defendum® were similar to blank. Co-supplementation of either probiotic + GOS did not significantly alter butyrate levels versus GOS alone, but mild stimulatory effects were observed at the individual level for Probi Defendum® + GOS in two donors, with an average increase in butyrate of +1.4 mM (+26%). GOS alone, Probi Digestis® + GOS, Probi Defendum® alone, and Probi Defendum® + GOS stimulated a significant increase in lactate production versus blank during the first six hours of fermentation (Figure 2d). Production of lactate with Probi Digestis® alone was however, similar to blank during this time. A synergistic synbiotic effect was observed for Probi Defendum® + GOS, as lactate production was significantly higher compared with GOS alone. Lactate production was also significantly higher for the synbiotic Probi Defendum® + GOS compared with Probi Digestis® + GOS.

Supplementation with GOS alone or either of the two synbiotics resulted in a non-significant reduction of branched SCFAs relative to blank (Figure 2e) and a significant reduction in ammonium versus blank (Figure 2f). In contrast, levels of both branched SCFAs and ammonium with either probiotic alone were similar to blank.

Microbial Community Composition

The microbial community composition for each individual donor at the start of the study (prior to supplementation) primarily consisted of members of the Firmicutes, Bacteroidetes, and Actinobacteria phyla (data not shown). Bacterial biomass was significantly increased with GOS supplementation versus blank (Figure 3). There was also an increased biomass with probiotic alone compared with blank, which was significant with Probi Defendum® but not with Probi Digestis®. Biomass was numerically increased with Probi Digestis® + GOS and significantly increased with Probi Defendum® + GOS versus blank. Biomass was similar for GOS alone or GOS + either probiotic (range, $4.26\text{--}4.54 \times 10^9$ total cells/mL).

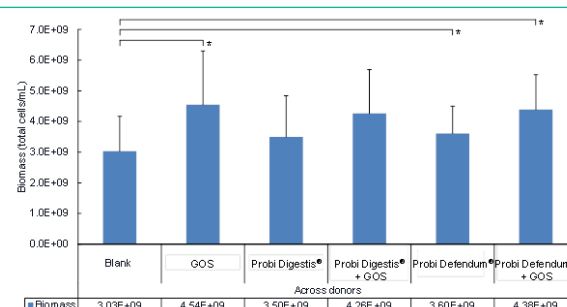


Figure 3: Total bacterial biomass (total cells/mL) at 24 h after the start of incubation. Data for average values were derived using data from five healthy donors. Error bars represent standard deviation. Two-sided t-tests were used to determine significant differences between each supplemented condition versus blank. * indicates significant differences between the supplemented versus blank test condition. $p < 0.05$ was considered statistically significant. GOS = Bimuno® galactooligosaccharides.

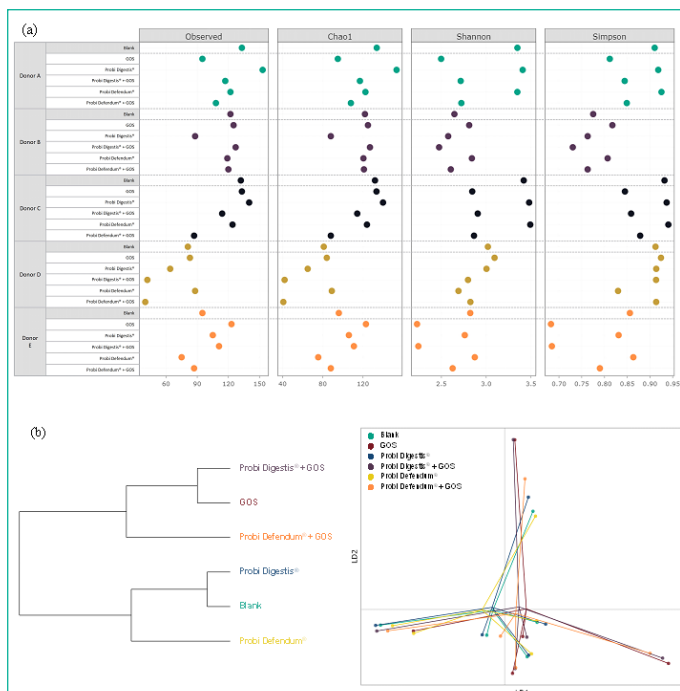


Figure 4: (a) Alpha diversity, represented by the observed, Chao1, Shannon, and Simpson diversity indices, and (b) beta diversity are shown for each donor at 24 h after the start of incubation. Alpha diversity analysis is based on relative abundance data (total sum scaling); each color represents a different donor and each dot represents the average across technical replicates (n=2). Beta diversity is represented by hierarchical clustering showing dissimilarities in community composition (sum of the horizontal lines separating two conditions is a measure for dissimilarity in their community compositions; panel B, left side) and a scatter plot (panel B, right side). Each color represents a different treatment and each dot represents a different donor; calculated from relative abundance data averaged across technical replicates (n=2). GOS = Bimuno® galactooligosaccharides; LD = linear discriminant.

Changes in alpha diversity for each of the donors are shown in Figure 4a. The observed and Chao1 indices showed that, on average, there was no major impact of GOS on species richness. Probi Digestis® had an inconsistent effect on species richness across donors, while a moderate decrease was observed for most donors with Probi Defendum®. The overall tendency for synbiotic supplementation was a decrease in species richness compared with blank. The Simpson and Shannon indices revealed that GOS alone and GOS in combination with either probiotic had a tendency to decrease species evenness in most donors, while species evenness was largely unaffected by either probiotic alone. Beta diversity analyses demonstrated that GOS supplementation, with or without probiotics, altered the community composition (Figure 4b). The effect of GOS with or without either probiotic was greater than with probiotic supplementation alone. Supplementation with Probi Defendum® had a stronger impact on the microbial community composition than with Probi Digestis®. This trend was similar when either probiotic was combined with GOS.

Bacterial enrichments following supplementation with GOS alone versus blank were analyzed using treeclimbR and LEfSe analysis. treeclimbR analysis across donors revealed a significant enrichment of *Bifidobacterium longum* with GOS treatment relative to blank (Figure 5), which was also detected with LEfSe analysis (data not shown). treeclimbR analysis also showed an enrichment of the *Anaerobutyricum* genus and of several bacterial species, including *Bifidobacterium adolescentis*, *Bifidobacterium ruminantium*, *Eubacterium ramulus*, *Slackia*

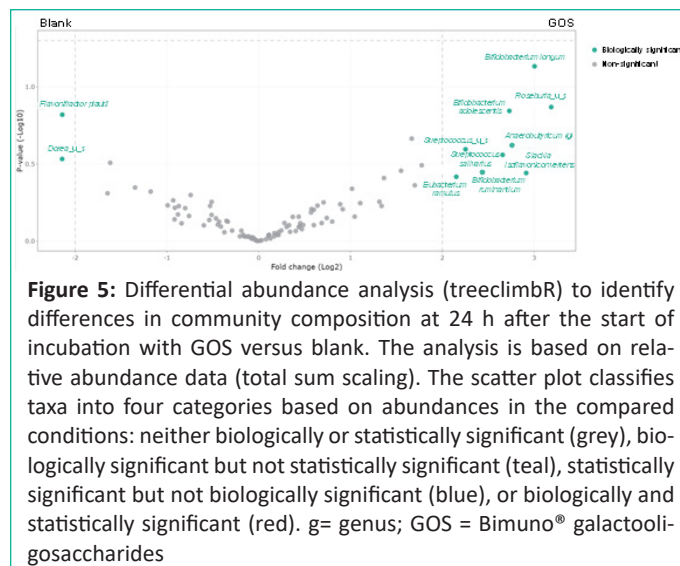


Figure 5: Differential abundance analysis (treeclimbR) to identify differences in community composition at 24 h after the start of incubation with GOS versus blank. The analysis is based on relative abundance data (total sum scaling). The scatter plot classifies taxa into four categories based on abundances in the compared conditions: neither biologically or statistically significant (grey), biologically significant but not statistically significant (teal), statistically significant but not biologically significant (blue), or biologically and statistically significant (red). g = genus; GOS = Bimuno® galactooligosaccharides

isoflavoniconvertens, *Streptococcus salivarius*, and an uncultured *Streptococcus* and *Roseburia* spp (Figure 5). These enrichments reached the threshold for biological relevance, with an average fold change >4, but not statistical significance.

The engraftment of the probiotic strains in Probi Digestis® and Probi Defendum® at 24 h was evaluated. The abundance of *L. paracasei* was elevated with both Probi Defendum® alone and Probi Defendum® + GOS relative to the other conditions (Figure 6a), whereas *L. plantarum* was not detected with Probi Defendum® supplementation (Figure 6b). The abundances of *L. paracasei* were higher than the concentration supplemented (5×10^6 CFU/mL) (Figure 6c). *L. plantarum* was elevated with Probi Digestis®, both alone and with GOS (Figure 6b); however, the abundance was lower than the concentration supplemented (1×10^7 CFU/mL) (Figure 6c). To confirm the ability of the probiotic strains to utilize GOS, they were grown in MRS broth media with and without dialyzed GOS as the carbohydrate source. *L. plantarum* HEAL9 alone, *L. paracasei* 8700:2 alone, and the combination of *L. plantarum* HEAL9 and *L. paracasei* 8700:2 (Probi Defendum® strains) was able to grow in GOS (Figure 7a, 7b, 7c, respectively). However, *L. plantarum* 299v (Probi Digestis® strain) was not able to utilize GOS for growth (Figure 7d), in agreement with the results of the colonic simulation.

treeclimbR and LEfSe analysis were also utilized to compare the effects of the two probiotic supplements, Probi Digestis® and Probi Defendum® to blank and each probiotic + GOS to GOS alone. In the treeclimbR analysis, there were no statistically or biologically significant enrichments in the microbial community other than *L. plantarum* with Probi Digestis® supplementation

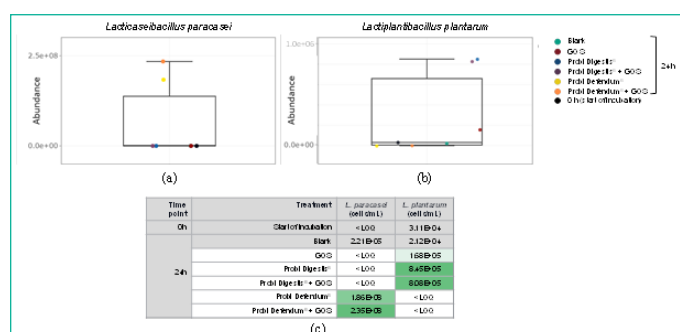


Figure 6: Abundances of (a) *Lactocaseibacillus paracasei* (b) *Lactiplantibacillus plantarum*, and (c) an overview of bacterial abundances for all test conditions at 24 h after start of incubation. Data for average values were derived using data from five healthy donors. GOS = Bimuno® galactooligosaccharides; LOQ = limit of quantification.

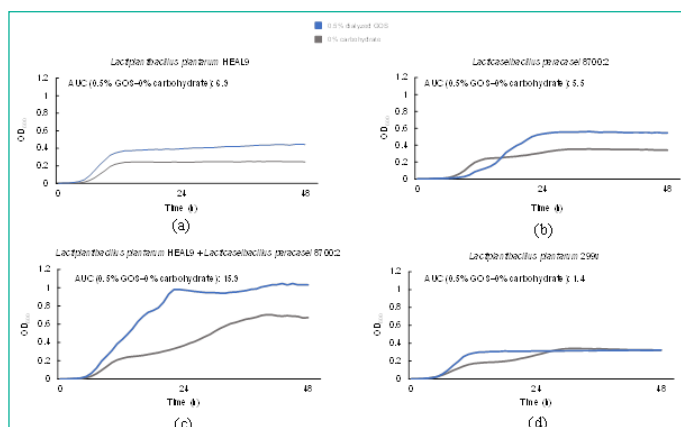


Figure 7: Average growth curves for (a) *Lactiplantibacillus plantarum* HEAL9, (b) *Lacticaseibacillus paracasei* 8700:2, (c) *Lactiplantibacillus plantarum* HEAL9 + *Lacticaseibacillus paracasei* 8700:2, and (d) *Lactiplantibacillus plantarum* 299v to determine growth with dialyzed GOS. Measurements were collected in duplicate. Probiotic strains were added to wells of sterile 96 well plates containing MRS broth without a carbohydrate source or with 0.5% dialyzed GOS. Plates were incubated at 37°C overnight on a plate reader and OD600 measurements were taken periodically to generate growth curves. AUC = area under the curve; GOS = Bimuno® galactooligosaccharides

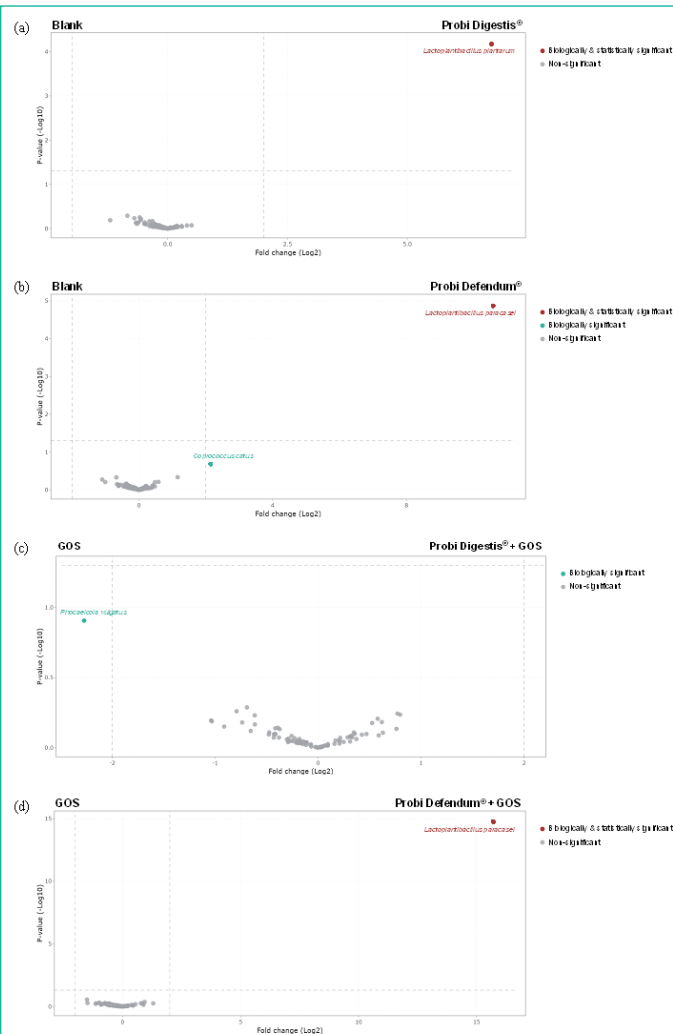


Figure 8: Differential abundance analysis (treeclimbR) to identify differences in community composition at 24 h after the start of incubation with (a) Probi Digestis® versus blank, (b) Probi Defendum® versus blank, (c) Probi Digestis® + GOS versus GOS, and (d) Probi Defendum® + GOS versus GOS. The analysis is based on relative abundance data (total sum scaling). The scatter plot classifies taxa into four categories based on abundances in the compared conditions: neither biologically or statistically significant (grey), biologically significant but not statistically significant (teal), statistically significant but not biologically significant (blue), or biologically and statistically significant (red). GOS = Bimuno® galactooligosaccharides.

(Figure 8a). There was a biologically and statistically significant enrichment of *L. paracasei* and a biologically significant enrichment of *Coprococcus catus* with Probi Defendum® supplementation compared with blank (Figure 8b). When comparing the effect of Probi Digestis® + GOS with GOS alone, there was a biologically significant enrichment of *Phocaicola vulgatus* with GOS alone (Figure 8c). The comparison between Probi Defendum® + GOS and GOS alone showed a biologically and statistically significant enrichment of *L. paracasei* with Probi Defendum® + GOS supplementation (Figure 8d). LEfSe analysis of the impact of probiotic supplementation only showed differences related to probiotic strains (data not shown).

Discussion

Using short-term colonic incubations, we evaluated the synbiotic potential of Probi Defendum® with a specific GOS. Evaluations included overall microbial fermentation, microbial metabolic activity, and microbial community composition of colonic bacteria isolated from five healthy adult donors. Overall, the metabolic and metagenomic profile of the Probi Defendum® + GOS synbiotic was comparable with that of GOS alone; however, this synbiotic had a greater lactogenic effect than GOS alone, especially for donors characterized with low lactate production. Butyrate production was also increased, which was likely an effect of the enhanced availability of lactate during the first 6 hours. This enhanced lactogenic effect also differentiated the Probi Defendum® + GOS synbiotic from the Probi Digestis® + GOS synbiotic.

The greatest decreases in pH and increases in gas pressure were observed with GOS, Probi Digestis® + GOS, and Probi Defendum® + GOS, indicating that fermentative activity was higher with these conditions compared with blank or supplementation with either probiotic alone. This activity was driven by GOS, as there was no difference between the two synbiotic conditions and GOS alone. The observation that gas production was similar with GOS supplementation with or without probiotic co-supplementation indicates that administration of the synbiotic formulations to individuals would be tolerable, as GOS intake by humans is tolerable and has been shown to reduce bloating, flatulence, and abdominal pain in participants who suffer these gastrointestinal symptoms [49].

The study findings indicate that GOS was primarily responsible for the increased acetate and butyrate production, given that increases in acetate and butyrate over blank were not observed with either probiotic alone. Although there was also an increase in acetate and butyrate production with both synbiotics relative to blank, the increase was similar to that observed with GOS alone. Butyrate production was significantly greater with the synbiotic Probi Defendum® + GOS compared with blank, and was numerically increased as compared to GOS. Increased SCFA production is a well-known feature of GOS and was confirmed in this study [12,21,50,51]. GOS, and to a lesser extent, Probi Defendum®, stimulated lactate production. Co-supplementation of GOS with Probi Defendum® resulted in significantly higher lactate production than GOS alone, demonstrating an improved lactogenic effect and supporting a synbiotic synergistic effect for this product. This was especially apparent for donors characterized by low lactate production, suggesting that these donors' inability to produce lactate from GOS was implanted with this probiotic. The greater lactogenic effect can likely be attributed to the probiotic strain *L. paracasei*, as the *L. plantarum* probiotics in both the Probi Digestis® and Probi Defendum® formulations were unable to compete

with the intestinal microbiome for common nutrients, as evidenced by the metagenomic findings. Given that lactate can be converted to butyrate through cross-feeding, a lactogenic effect is considered beneficial. Indeed, the increase in butyrate production with Probi Defendum® + GOS compared with GOS alone is likely attributable to the strong lactogenic effect of this synbiotic. This is a relevant finding, as butyrate is important for gut membrane health [52-55].

The production of branched SCFAs and ammonium are the result of proteolytic microbial activity. In line with previous reports of GOS fermentation, our study demonstrated a reduction in branched SCFA and ammonium for all GOS-containing test conditions [12,50]. Given that the decreases in branched SCFA and ammonium were similar for GOS alone and both Probi Digestis® + GOS and Probi Defendum® + GOS, the reduction in proteolytic fermentation was driven primarily by GOS. Toxic compounds are produced during proteolytic fermentation; therefore, a reduction in this process, as evidenced by the reduction in branched SCFA and ammonium production, is considered beneficial to human health [56].

Microbial community analysis was conducted 24 h after supplementation since metabolite production largely occurred within 24 h, suggesting that this timepoint was suitable to capture the relevant community shifts. The overall tendency for GOS alone or probiotic + GOS supplementation to decrease species richness versus blank suggests that GOS was primarily fermented by a select group of bacteria, rather than promoting the growth of a diverse array of microbial species. Bacterial biomass increases appeared to be mainly dependent on GOS, as the increase in biomass versus blank was greatest with GOS alone, and reactor biomass following supplementation with GOS alone was similar to that observed with either Probi Digestis® + GOS or Probi Defendum® + GOS. The increase in biomass could be attributed to the enrichment of *B. longum*, and, to a lesser degree, *B. adolescentis* and *B. ruminantium*. The enrichment of *Bifidobacterium* spp confirms the findings of several clinical trials that have reported the enrichment of bifidobacteria with GOS supplementation [6,7]. The decrease in species richness and/or evenness observed with GOS supplementation alone and in synbiotic combination suggest that GOS favors the growth of a select group of bacteria, shifting the microbial community composition as described above. GOS had the biggest impact on microbial community composition and the impact of Probi Defendum® was stronger than that of Probi Digestis®.

We selected a background medium low in other substrates to observe a specific effect of the added GOS on fermentation by the probiotic strains. In conditions with only probiotics, there were virtually no fermentable substrates available. Therefore, the lack of effect on microbial metabolite production when the probiotics were supplemented without any carbohydrate source (i.e., without GOS) was expected. Further, addition of probiotics alone had no significant impact on the gut microbial community composition. The metagenomic data demonstrated that neither *L. plantarum* HEAL9 nor *L. plantarum* 299v, present in Probi Defendum® and Probi Digestis®, respectively, proliferated well in the presence of the donor gut microbiome. In contrast, *L. paracasei* 8700:2 grew well, correlating with the lactogenic effects observed with Probi Defendum® supplementation.

Both *L. plantarum* HEAL9 and *L. paracasei* 8700:2 were able to grow with GOS as a carbohydrate source, but *L. plantarum* 299v was not. The growth of *L. plantarum* HEAL9 and *L. paracasei* 8700:2 appeared to be enhanced when combined com-

pared with either probiotic strain alone, likely due to increase of *L. paracasei* 8700:2 as observed in the colonic simulations, where only the growth of *L. paracasei* 8700:2 was enhanced. The growth of these probiotic strains could still be different in vivo, where other carbohydrate substrates are available. An in vitro study demonstrated varied prebiotic carbohydrate utilization by *L. plantarum* strains, including HEAL9 and 299v [57]. Although *L. plantarum* 299v was not able to utilize Bimuno® GOS for growth, this probiotic is able to utilize other prebiotic compounds, including another GOS [58,59], and has well established probiotic effects in humans (reviewed in [37]). The inability of *L. plantarum* 299v to utilize Bimuno® GOS, coupled with the lack of synergistic effect when co-supplemented with Bimuno® GOS highlight the importance of appropriate coupling of prebiotics and probiotics when a synbiotic effect is desired.

Given that the colonic microbiota of five donors were used in this study to address interindividual differences, and that prebiotic effects of easily fermentable fibers (i.e., fibers characterized by low molecular complexity, like GOS) are typically characterized by low interindividual variation, we expect that the effects observed for all donors combined will translate to the general population. In contrast, other substances, such as polyphenols, can be highly donor specific, and would require a larger donor number to be representative for a large population. Although the technology used is validated with in vivo - in vitro correlation [60], this study was limited in that ex vivo findings do not always translate to in vivo effects. Therefore, the synergistic synbiotic effects of co-supplementation of Probi Defendum® and GOS should be validated in clinical trials.

Conclusions

Overall, the known prebiotic effects of GOS, including the stimulation of increased SCFA and lactate production, reduction of proteolytic fermentation, and enrichment of beneficial bifidobacteria were confirmed in this short-term colonic simulation study, and synbiotic effects, most notably an increased lactogenic effect, were identified for the combination of Probi Defendum® + GOS. This effect was most pronounced in donors characterized by an inability to produce lactate, likely because this role was taken up by *L. paracasei* 8700:2 in the synbiotic formulation.

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