



Safety and Modulatory Effects of Humanized Galacto-Oligosaccharides on the Gut Microbiome

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Complex dietary carbohydrate structures including $\beta(1-4)$ galacto-oligosaccharides (GOS) are resistant to digestion in the upper gastrointestinal (GI) tract and arrive intact to the colon where they benefit the host by selectively stimulating microbial growth. Studies have reported the beneficial impact of GOS (alone or in combination with other prebiotics) by serving as metabolic substrates for modulating the assembly of the infant gut microbiome while reducing GI infections. N-Acetyl-D-lactosamine (LacNAc, Gal β 1,4GlcNAc) is found in breast milk as a free disaccharide. This compound is also found as a component of human milk oligosaccharides (HMOs), which have repeating and variably branched lactose and/or LacNAc units, often attached to sialic acid and fucose monosaccharides. Human glycosyl-hydrolases do not degrade most HMOs, indicating that these structures have evolved as natural prebiotics to drive the proper assembly of the infant healthy gut microbiota. Here, we sought to develop a novel enzymatic method for generating LacNAc-enriched GOS, which we refer to as humanized GOS (hGOS). We showed that the membrane-bound β -hexosyl transferase (rBHT) from *Hamamotoa (Sporobolomyces) singularis* was able to generate GOS and hGOS from lactose and N-Acetyl-glucosamine (GlcNAc). The enzyme catalyzed the regio-selective, repeated addition of galactose from lactose to GlcNAc forming the β -galactosyl linkage at the 4-position of the GlcNAc and at the 1-position of D-galactose generating, in addition to GOS, LacNAc, and Galactosyl-LacNAc trisaccharides which were produced by two sequential transgalactosylations. Humanized GOS is chemically distinct from HMOs, and its effects *in vivo* have yet to be determined. Thus, we evaluated its safety and demonstrated the prebiotic's ability to modulate the gut microbiome in 6-week-old C57BL/6J mice. Longitudinal analysis of gut microbiome composition of stool samples collected from mice fed a diet containing hGOS for 5 weeks showed a transient reduction in alpha diversity. Differences in microbiome community composition mostly

within the *Firmicutes* phylum were observed between hGOS and GOS, compared to control-fed animals. In sum, our study demonstrated the biological synthesis of hGOS, and signaled its safety and ability to modulate the gut microbiome *in vivo*, promoting the growth of beneficial microorganisms, including *Bifidobacterium* and *Akkermansia*.

Keywords: galactooligosaccharide (GOS), N-Acetyl-D-lactosamine (LacNAc), safety, human milk oligosaccharides (HMOs), LacNAc synthesis, mouse models

INTRODUCTION

Gut microbial communities play a critical role in the maintenance of host health (1, 2). Hence, beneficial modulation with probiotics (live microorganisms that when administered in adequate amounts provide a benefit to their hosts) (3) and prebiotics (selectively fermented dietary carbohydrate structures that promote the growth of beneficial microorganisms) (4, 5) is desirable and potentially effective translational therapeutics to treat gastrointestinal (GI) diseases linked to disrupted microbial communities (4, 6–9) [reviewed in (10)]. Synbiotics (combinations of prebiotics and probiotics) are also emerging as a focal point of GI biology research, as each component, individually and synergistically, could provide unique benefits reestablishing community resilience and host physiology (11, 12). In previous studies we evaluated highly pure $\beta(1-4)$ galactooligosaccharides (GOS) formulations produced by the optimized version of the hexosyl-transferase gene from *Hamamotoa* (*Sporobolomyces*) *singularis* heterologously expressed in *Komagataella* (*Pichia*) *pastoris* (13, 14). This enzyme is one of the most promising catalysts in the field of glycobiology due to its high stability, highly desirable enzymatic properties, and the metabolism of its reaction products (GOS) by specific members of the gut microbial community, impacting its composition and function (15, 16). Beneficial members of the gut microbiota, including *Lactobacillus* and *Bifidobacterium*, hydrolyze GOS via β -galactosidases (17). *Lactobacillus rhamnosus* utilize PTS transporters to internalize GOS prior to hydrolysis (17), while other organisms like specific strains of *Bifidobacterium* (*bifidum*) secrete glycosyl hydrolases to break down complex carbohydrates, internalizing the products of hydrolysis (18). Short-chain fatty acids (SCFAs) generated as the result of GOS assimilation include acetate and lactate (17), which community members, including *Roseburia* and *Faecalibacterium*, can transform into butyrate (6, 19).

LacNAc is an essential component of human milk oligosaccharides (HMOs) and has been demonstrated to be a major bifidogenic factor in the 1950s (20–22). Several HMOs contain lactose (Gal β 1-4Glc) at their reducing end, which can be elongated by the addition of β 1-3- or β 1-6-linked lacto-N-biose (Gal β 1-3GlcNAc) or LacNAc (Gal β 1-4GlcNAc). Lactose or the oligosaccharide can be then fucosylated by fucosyltransferases in α 1-2, α 1-3, or α 1-4 linkage and/or sialylated by sialyltransferases in α 2-3 or α 2-6 linkage to yield a variety of terminal structures (23). The study by Yoshida et al.

(24) characterized β -galactosidases of *Bifidobacterium longum* subsp. *infantis* to determine how this organism degrades type-1 (lacto-N-biose) and type-2 (LacNAc) isomers of HMOs. LacNAc has also been recognized as a building block of glycoproteins and glycolipids in the GI tract. These backbones serve to connect the core structure, which is directly linked to a protein or lipid aglycon with terminal sugars [reviewed in (25)]. LacNAc building blocks and terminal sugars also act as an important precursor of several blood group epitopes (Lewis A, Lewis B, sialyl Lewis A), which are involved in biological processes including fertilization (26), mediation of cell adhesion and pathogen adhesion to colonocytes (27–29).

Chemical and enzymatic synthesis processes have been the most frequently evaluated methods for LacNAc production (30). In recent years, glycoside hydrolases (EC 3.2.1.-) and β -galactosidases (EC 2.1.23) with both hydrolytic and transglycosylation activities, have gained special attention for regio- and stereo-selective synthesis of LacNAc oligosaccharides (<https://www.cazypedia.org/index.php/Transglycosylases>) (31–37). Enzymatic biosynthesis is considered the most efficient method for producing LacNAc due to specificity, synthesis in one-step reactions, low-cost substrates, sustainability, and overall low environmental impact, [reviewed in (38)]. Conversely, chemical methods to generate LacNAc require multiple reactive hydroxyl groups and laborious protocols for group protection and deprotection to control the stereo- and regio-specificities (39, 40). Compared to enzymatic synthesis, generation of LacNAc by chemical synthesis has low yields, a cost-competitive disadvantage for industrial production, hindering the use of LacNAc as an additive in food products (30, 41, 42).

In this study, we describe a novel biological synthesis solution to produce N-Acetyl-lactosamine (LacNAc)-enriched GOS (which we refer to as humanized GOS, hGOS) using optimized *Hamamotoa* (*Sporobolomyces*) *singularis* β -hexosyl transferase [rBHT (13, 14)]. The enzyme generates LacNAc-enriched GOS as the product of the reaction between lactose as a galactose donor and N-Acetylglucosamine as acceptor. We first evaluated the efficiency of a *Komagataella* (*Pichia*) *pastoris* cell line carrying membrane-bound β -hexosyl transferase on the generation of GOS and hGOS from lactose and N-Acetyl-glucosamine. Then, conventionally-raised 6-week-old C57BL/6J mice were fed a control diet or modified diets containing GOS or hGOS for 14 days to evaluate its safety and impact on fecal microbial diversity and composition.

MATERIALS AND METHODS

Generation of Dietary Carbohydrate Structures/Prebiotics GOS and hGOS

Membrane-bound β -hexosyl transferase from *Hamamotoa* (*Sporobolomyces*) *singularis* in *Komagataella* (*Pichia*) *pastoris* was produced as previously described (13, 14). The standard transgalactosylation reaction utilizing *Komagataella* (*Pichia*) *pastoris* resting cells (harboring membrane-bound enzyme) was initiated by adding standardized amounts of enzyme (1 U g^{-1} lactose) in 5 mM sodium phosphate buffer (pH 5.0) to a similarly buffered solution containing lactose (200 g $liter^{-1}$) and N-Acetylglucosamine (25 g $liter^{-1}$) at 30°C. Reaction products and substrates were analyzed by high-performance liquid chromatography (HPLC) (Shimadzu Corporation, Kyoto, Japan) under isocratic conditions at 65°C and a 0.5-ml min^{-1} flow rate. The mobile phase was water, and separation was performed by two columns in tandem a Supelco gel Ca++ (Supelco, PA), and HPX-42A (Bio-Rad, CA) columns (300 mm by 7.8 mm) coupled to an SPD-20MA and ELSD-LT II detectors (Shimadzu Corporation, Kyoto, Japan). The column was calibrated using galactosyl-lactose (Carbosynth, Berkshire, United Kingdom), LacNAc, Lactose, N-Acetylglucosamine, Glucose, and Galactose (Sigma-Aldrich, St. Louis, MO). Enzymatic activity was determined using 4-nitrophenyl β -D- glucopyranoside or oNP-Glc as substrate as per the previously described methods (13, 14).

Human Equivalent Dose Calculation

The human equivalent dose (HED) for LacNAc was calculated for the animal study using the methods highlighted by the United States Food and Drug Administration and is based on the approximate body weight of the subject (43). The equation used is as follows:

$$HED \left(\frac{mg}{kg} \right) = Animal \ Dose \left(\frac{mg}{kg} \right) \times \frac{Animal \ K_m}{Human \ K_m}$$

Where the K_m factor is a number based on body surface area. For this study, we used an animal dose of 1,500 mg kg^{-1} , based on ~30 mg LacNAc fed to a ~20 g mouse per day. Additionally, we used a K_m factor of 3 for mice and 16 to represent a 5 kg human infant (43). Using the formula above, the HED for this study represent an equivalent of 281.25 mg LacNAc per kg body weight in infants. While for a 20 kg child ($K_m = 25$), the HED would be 180 mg kg^{-1} .

Animal Housing, Treatment, and Sample Collection

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill (Approved protocol number: 19-084).

A total of 50 6-week-old C57BL/6J mice were co-housed at random in groups of 5–6 animals and fed a defined diet (D17121301; Research Diets INC.) containing no prebiotics to normalize the gut microbiota for 2 weeks. After a 2-week standardization period, fresh stool samples were collected directly from the anus of each animal into a sterile tube. To

avoid cage batch effects, animals were moved into paired housing such that no two animals from the same standardization group were co-housed. Each animal was considered one experimental unit. Upon reassigning housing (Figure 2A), animal pairs were split into three distinct groups, each of which began feeding on either the defined control diet (D17121301) ($n = 17$), GOS diet (D17121302) ($n = 17$) in which 71.8 g of cellulose per kilogram diet was replaced with 71.8 g of pure GOS, or hGOS (D18121401) ($n = 16$) where LacNAc represented a 1% (w/w). Composition of each diet is detailed in **Supplementary Table 1**. Each diet was offered *ad libitum* for 2 weeks prior to stool sample collection. Individual animal mass and dietary consumption were measured daily during the first 14 days of the dietary study to assess animal growth and food consumption (total food consumed in a cage/2 = individual animal food consumption) between diet groups. After day-14 sample collection, half of the animals in each treatment group were removed for a tangential study and all remaining animals (total=24, $n = 8$ per diet) continued to consume their respective diets *ad libitum*, with stool sample collections occurring at three additional time points each ~1-week apart prior to animal sacrifice. At the conclusion of the trial on day 38, each animal was euthanized via CO₂ asphyxiation and cervical dislocation.

Nucleic Acid Isolation

Total DNA was extracted from fecal pellets using the Qiagen ClearMag Extraction system on KingFisher Flex Magnetic Bead processing instrument as described (15). Briefly, stool samples were transferred to a screwcap tube containing 10 mg of sterile acid-washed glass beads (0.1–0.5 mm diameter) and 700 μ l PM1 solution (Qiagen, Valencia, CA). Samples were homogenized for 5-min at 15 Hz in Qiagen Tissue Lyser II (Qiagen). Bead-beaten samples were treated with IRS-PCR inhibitor remover solution (Qiagen) (3:1; lysate:IRS ratio) overnight at 4°C and transferred to KingFisher Deep-well plate containing ClearMag magnetic beads and binding buffer (Qiagen). Sample plates were subsequently processed on KingFisher Flex instrument to isolate and wash DNA. DNA was quantified with Quant-iT™ PicoGreen® dsDNA reagent (Molecular Probes, Thermo Fisher Scientific, Waltham, MA) and stored at –20°C.

16S rRNA Amplicon Sequencing

Total DNA was subject to amplification of the V4 variable region of the 16S rRNA gene using primers 515F and 806R (44) with Illumina adaptors. Amplicons were barcoded using Illumina dual-index barcodes [Index 1(i7) and Index 2(i5)], purified using Agencourt® AMPure® XP reagent (Beckman Coulter, Brea, CA) and quantified with Quant-iT™ PicoGreen™ dsDNA Reagent (Molecular Probes, Thermo Fisher Scientific). Libraries were pooled in equimolar amounts and sequenced on HiSeq2500 instrument (Illumina, San Diego, CA).

Sequencing Data Analysis

Analysis of 16S rRNA amplicon sequencing data was carried out using the QIIME2 pipeline as described (45). Briefly, sequences were grouped into Operational Taxonomic Units (OTUs) using UCLUST (46). OTU sequences were aligned, and phylogenetic

trees were built (47). Before generating the phylogenetic tree, the overall OTU table was collapsed using the “taxa collapse” plugin in QIIME2. The set of representative sequences was then trimmed to include only one representative sequence for each collapsed OTU. The filtered set of representative sequences was then aligned using MAFFT, and a phylogenetic tree was generated from the alignment using RAxML (48, 49). The phylogenetic tree was finally annotated with presence/absence data using iTOL and PhyloToAST (50–52). Alpha and beta diversity metrics were calculated in R 4.0.3 using the phyloseq and vegan packages (53–55). Only data from young animals (6 weeks old) were used in the calculation of diversity metrics. Both the Shannon entropy and inverse Simpson indexes were calculated to ensure an accurate estimation of the true alpha diversity of the samples. Beta diversity was calculated using principal coordinate analysis (PCoA) of the weighted UniFrac distances (56).

Statistical Analysis

Data were evaluated for homogeneity of variance using Levene’s test. Statistical significance of alpha diversity was evaluated using a repeated-measures ANOVA followed by Tukey’s Honest Significant Difference test to separate means. The 95% confidence ellipses for beta diversity plots were calculated in R 4.0.3 using ggplot2 (57). Beta diversity statistical analyses were performed using the PERMANOVA and PERMDISP functions of the vegan package in R 4.0.3. All statistical analysis results for the alpha and beta diversity analyses can be found in **Supplementary Table 2**. The α for all statistical tests was fixed at 0.05.

Availability of Data and Materials

All sequencing data has been submitted to the NCBI repository and can be accessed via the following accession number: PRJNA681811.

RESULTS

rBHT Catalyzed the Repeated Addition of Galactose From Lactose to N-Acetylglucosamine

The reactions catalyzed by the rBHT enzyme were regioselective, forming the β -galactosyl linkage at the 4-position of the GlcNAc and the 1-position of D-galactose, synthesizing various glycoconjugates directly from soluble GlcNAc. The obtained products, in addition to GOS, included Gal- β (1, 4)GlcNAc (LacNAc, **Figure 1A**, panel B) disaccharides and Gal β -(1, 4)Gal β -(1, 4)GlcNAc (Galactosyl-LacNAc, **Figure 1A**, panel C) trisaccharides, which were produced by two sequential transgalactosylations. **Figure 1B** shows the kinetics of the reaction performed during 8 days of incubation using rBHT polypeptides (e.g., whole cells displaying membrane-bound enzyme). The enrichment of GOS with LacNAc at a ratio lactose/N-Acetylglucosamine of 8:1 performed for these experiments (200 g/L lactose and 25 g/L GlcNAc) generated 25 g/L of LacNAc and 100 g/L hGOS after 48 h of incubation. At this time point, the reaction was terminated, and the products of the reaction (hGOS) were freeze-dried.

Animal Health and Diet Consumption

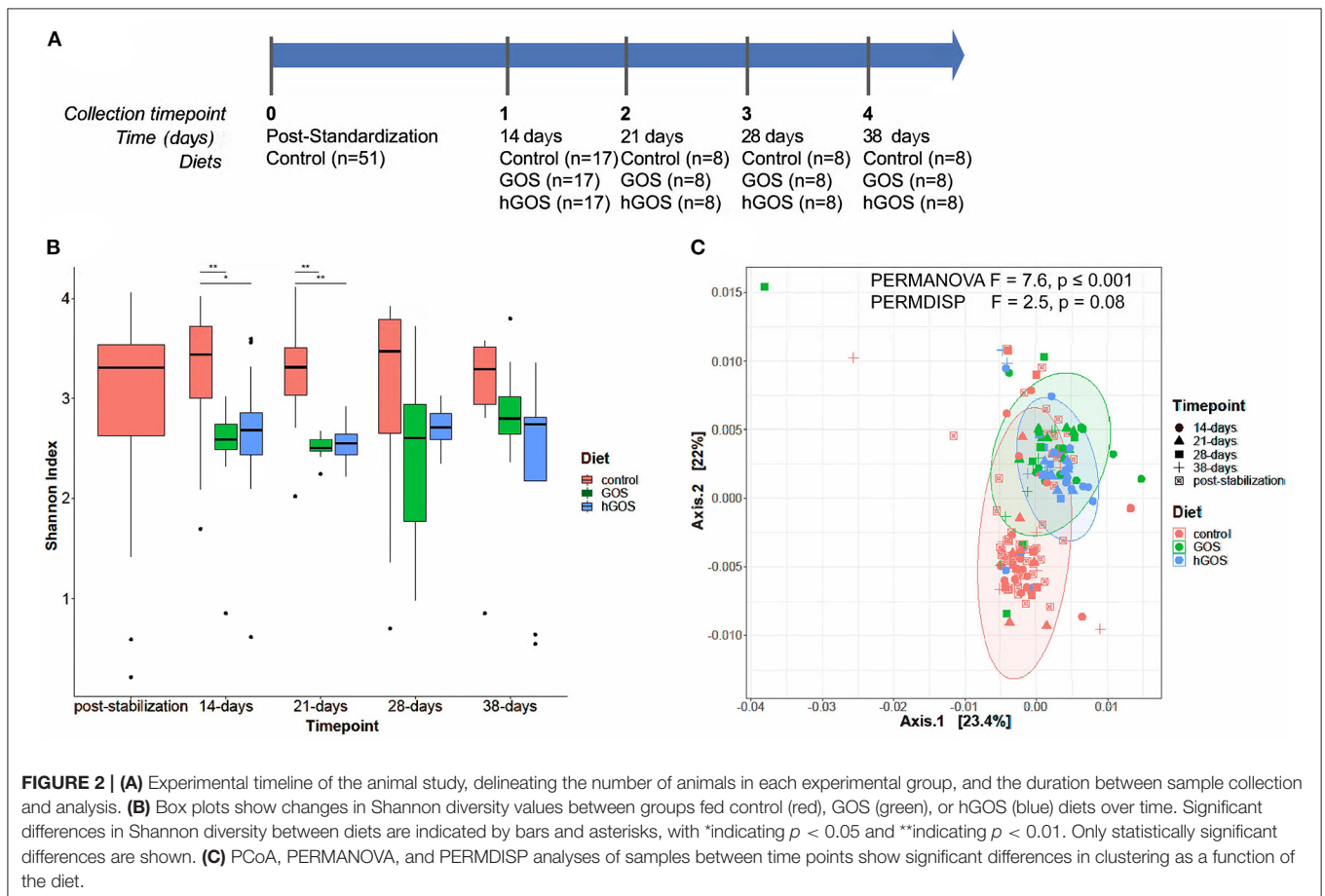
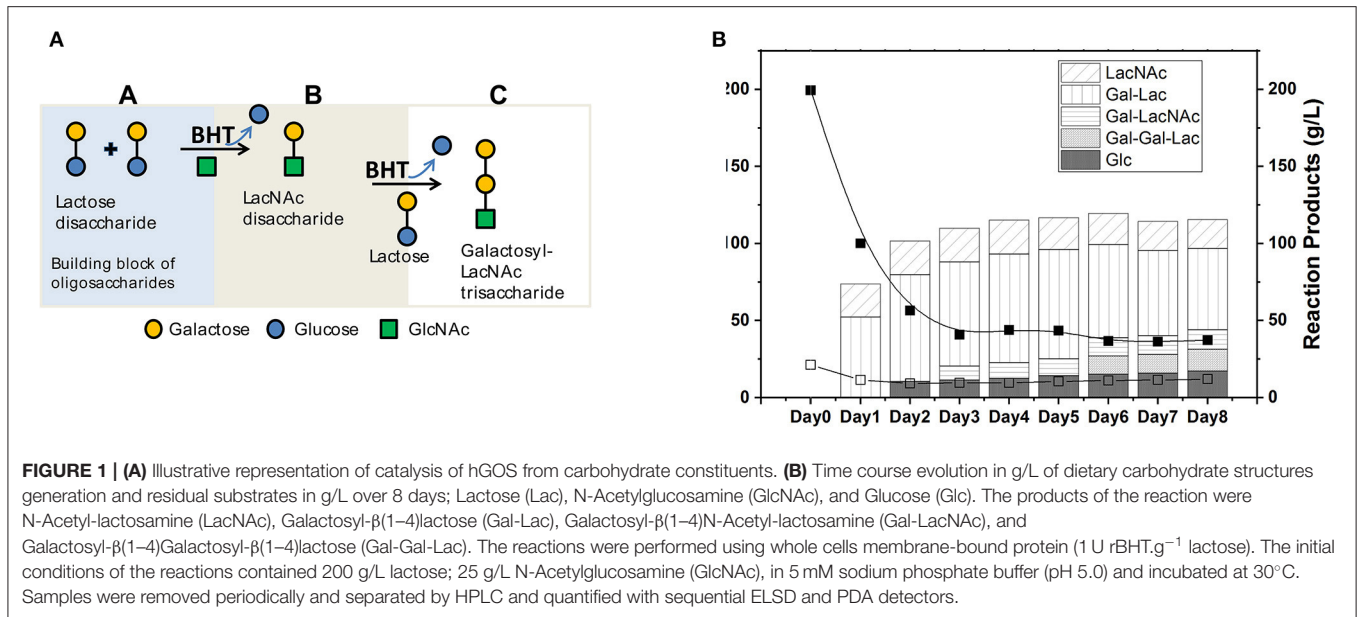
We conducted an animal experiment with conventional 6-week-old C57BL/6J mice fed a control diet, or modified diets containing GOS or hGOS to assess their impact on the gut microbiome (**Figure 2A**). Results showed no impact of GOS or hGOS diets on weight or daily food consumption. Each mouse consumed ~ 3 g of food per day, with no significant differences between the diets (**Supplementary Figure 1**). Therefore, based on the formulation of each prebiotic diet, we calculated that the dose of prebiotic consumed per day by each mouse was 0 mg/day (on control diet), and ~ 190 mg/day of total prebiotic (GOS, hGOS). This estimate translates to ~ 171 mg/day of GOS (GOS diet) and ~ 30 mg/day of LacNAc (hGOS diet) based on prebiotic formulation data.

Modulation of the Gut Microbiota by hGOS

After 2 weeks of feeding on diets containing prebiotics, animals exhibited a significant (repeated measures ANOVA $p < 0.05$) reduction in alpha diversity (**Figure 2B**). Over the length of the study, diversity of prebiotic-fed animals returned to values comparable to the control diet with no statistically significant differences between groups at day 28. PCoA plots revealed distinct clustering of hGOS-fed animals, which displayed a much tighter dispersion pattern compared to control animals (PERMDISP $p = 0.009$), suggesting a higher similarity between communities within hGOS-fed than control-fed animals (**Figure 2C**). Spatial medians were significantly different between groups (PERMANOVA $F = 7.6463$, $p \leq 0.001$).

Taxonomy plots of relative microbial abundance revealed the genus-level variability between animals fed control diets, and those consuming either GOS or hGOS diets over time (**Figure 3A**). The most dramatic changes in the assembled microbial communities were observed between timepoint 0 and 14 days after introducing prebiotic diets. Changes between the communities within prebiotic-fed animals after 14 days were minimal. Analysis of Composition of Microbiome (ANCOM) used to further explore microbial abundance changes within the communities of prebiotic-fed animals across all time points revealed an increased relative abundance of beneficial microorganisms including *Akkermansia*, *Bifidobacterium*, and *Bacteroides*, along with *Allobaculum* in both GOS and hGOS diets. The dietary interventions reduced the relative abundance of *Butyrivococcus*, *Clostridium*, *Turicibacter*, and *Lachnospiraceae* across all time points (**Figures 3A,B, Supplementary Figure 2**).

Figure 4 shows a phylogenetic tree generated using PhyloToAST that includes 117 unique OTUs detected in at least one of the three diets examined. Of the 117 unique OTUs detected, 76 OTUs were detected in all conditions (Control, GOS, hGOS), and 40 were detected in control and GOS but not in hGOS fed mice, while none was detected in control and hGOS but not GOS fed mice. The majority of the OTUs detected in the control and GOS mice, but not in hGOS mice, belonged to the *Firmicutes* phylum. Taxa not detected in the hGOS group compared to control and GOS group within the Phylum *Firmicutes* and Class *Bacilli* included *Lactobacillus reuteri*, *L. zeae*, species of *Enterococcus*, *Brevibacillus*,



Paenibacillus, *Anaerobacillus*, *Virgibacillus*, *Facklamia*, *Unclassified Lactobacillales*, *Bacillales*, *Enterococcaceae*, and *Planococcaceae*. Within the Phylum *Firmicutes* and Class *Clostridia*, the following were not detected in the hGOS group: *Veillonella dispar*, *Ruminococcus flavefaciens*,

Faecalibacterium prausnitzii, species of *Butyrivibrio*, *Pseudobutyrvibrio*, *Lachnospira*, *Oxobacter*, *Roseburia*, *Dialister*, *Veillonella*, *Phascolarctobacterium*, *Anaerotruncus*, *Blautia* and *Unclassified Clostridiales*, *Clostridiaceae*, and *Veillonellaceae*. Only 3 OTUs corresponding to the Phylum

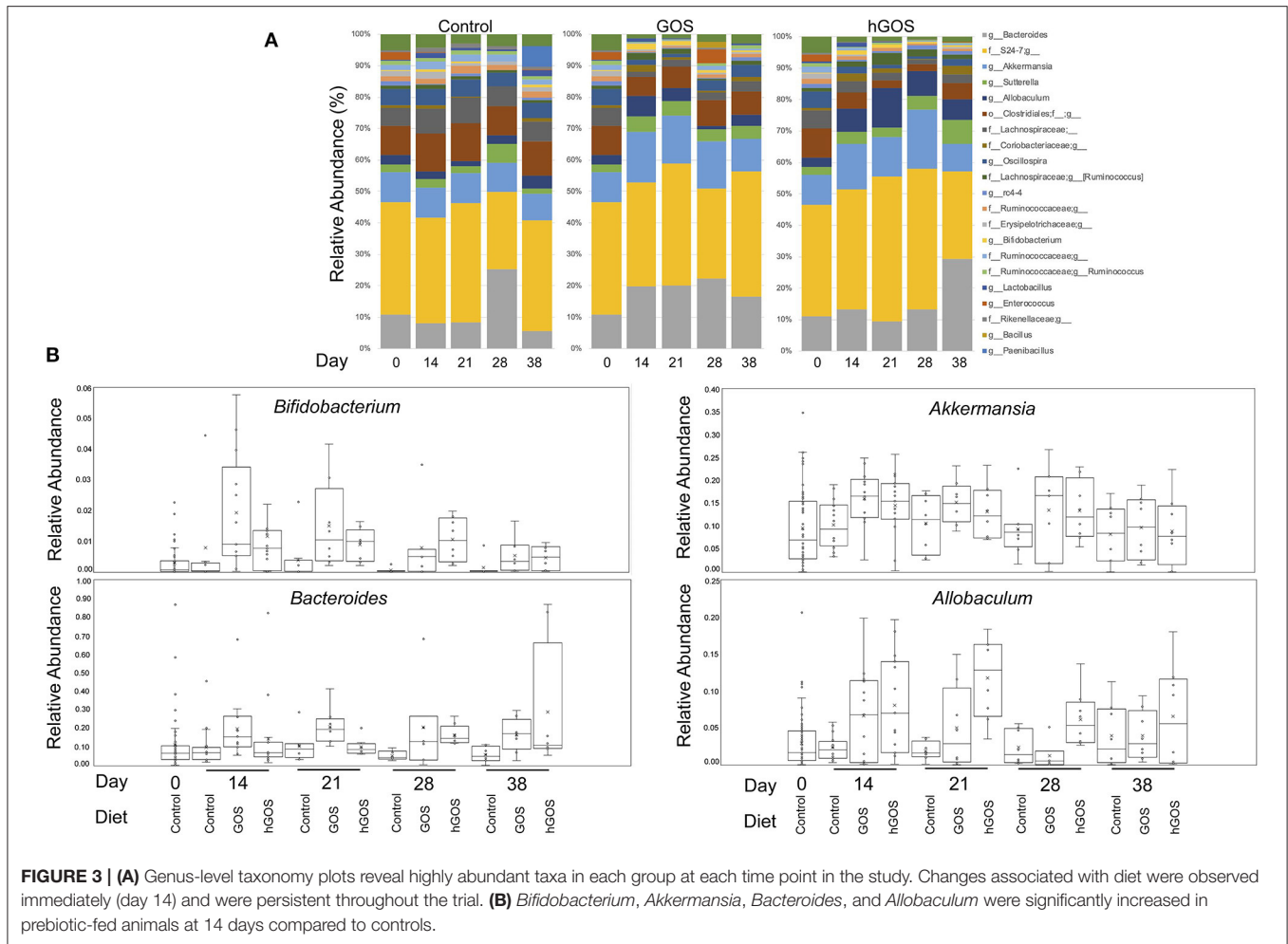


FIGURE 3 | (A) Genus-level taxonomy plots reveal highly abundant taxa in each group at each time point in the study. Changes associated with diet were observed immediately (day 14) and were persistent throughout the trial. **(B)** *Bifidobacterium*, *Akkermansia*, *Bacteroides*, and *Allobaculum* were significantly increased in prebiotic-fed animals at 14 days compared to controls.

Bacteroidetes (*Bacteroides eggerthii*, *B. caccae*, and *B. fragilis*) and 7 Proteobacteria (*Burkholderia bryophila*, *Acinetobacter*, *Bilophila*, *Hydrogenophaga*, *Pseudomonas*, *Halomonas*, and Unclassified *Enterobacteriaceae*) were not detected in the hGOS group. **Supplementary Table 1** presents the mean relative abundance values for each observed OTU in each of the experimental conditions.

DISCUSSION

Prebiotics, including GOS, are selectively fermented by gut microorganisms and promote the growth of beneficial microorganisms when consumed in adequate amounts (16, 17). In this study, we report the biological synthesis of hGOS enriched in LacNAc and determined its lack of adverse effects by determining the impact of feeding on the gut microbiome of healthy 6-week-old C57BL/6J mice in comparison with defined control and GOS-containing diets.

Traditionally, higher values of gut microbiota diversity has been associated with good health (58–60). In our study, feeding of both GOS and hGOS-enriched diets initially reduced diversity, even when constituents of the gut microbial community considered beneficial (*Bifidobacterium*, *Akkermansia*, and species

of *Bacteroides*) increased. Diversity increased at 28 and 38 days, suggesting that sustained hGOS feeding would lead to a diversity comparable to the control group. In addition, we did not observe differences in dietary consumption or weight in prebiotic-fed animals compared to the control group. We have recently reported an initial decreased diversity in 6- and 60-week old GOS-fed C57BL/6J mice after 2 weeks (15), which is in accordance with studies of GOS-supplemented infant formula (61) but contrast with other studies on human adults (6, 62) and young or adult BALB/c mice that showed no changes on diversity due to GOS feeding (63, 64). Considering the biochemical structure of GOS and hGOS and their similarity to HMOs, it makes sense that these prebiotics exert a restrictive selection of microorganisms to only microbes capable of establishing a mutualistic relationship with the host as observed in breastfed infants (65). The restrictive colonization effect leads in babies to the successive establishment of different bacterial groups, from aerotolerant bacteria to progressively stricter anaerobes (66, 67), and could provide in adults and older adults a strategy to beneficially modulate the gut microbiome by the subsequent introduction of microbial network units (68).

Members of the gut microbial community including strains of *Bifidobacterium* and *Lactobacillus* encode galactosidases

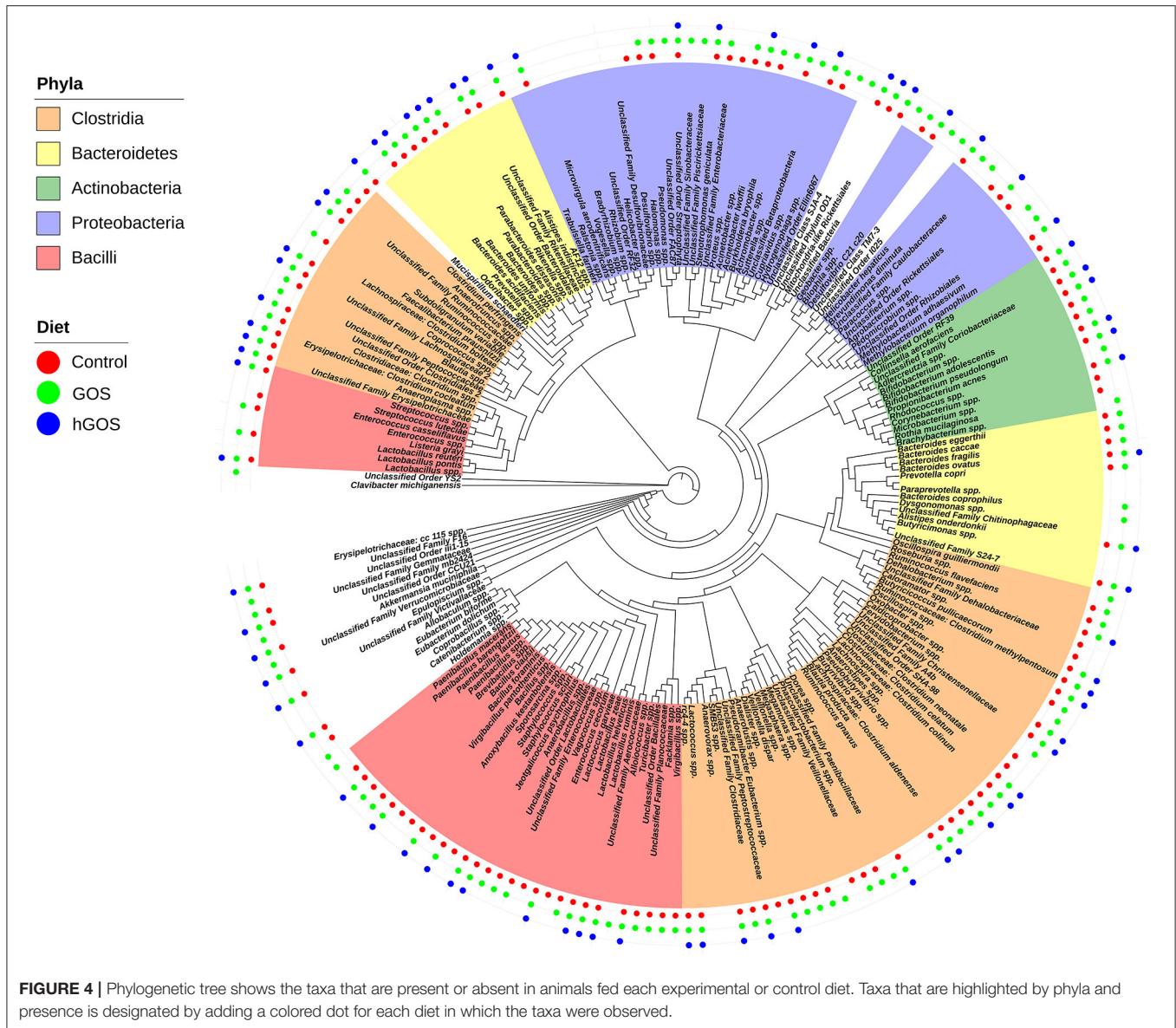


FIGURE 4 | Phylogenetic tree shows the taxa that are present or absent in animals fed each experimental or control diet. Taxa that are highlighted by phyla and presence is designated by adding a colored dot for each diet in which the taxa were observed.

genes that hydrolyze complex carbohydrates including GOS, as demonstrated in our previous and current studies (15–17, 19, 69) generating products which other members of the gut microbiota can further utilize through cross-feeding (19, 70). Due to structural similarities between the dietary carbohydrate structures contained in GOS and hGOS, it can be expected that their hydrolysis will result in similar molecules, including lactate and acetate, which could subsequently be utilized to generate other SCFAs of biological relevance, including butyrate. We anticipated that the additional LacNAc residues in hGOS would provide an additional substrate for bacterial enzymatic systems, allowing for different microorganisms to utilize these compounds compared to GOS. However, our study was not able to detect bacterial groups that used hGOS but not GOS. Further experiments will be required to characterize the gut bacterial metabolism of hGOS. Among other changes

in the gut microbiota, feeding GOS and hGOS increased the abundance of *Akkermansia muciniphila*, a microorganism that predominantly utilizes mucin as its energy source. GOS enrichment of *Akkermansia* is likely a consequence of increased mucin production (15). However, hGOS (containing LacNAc) may be utilized directly by *Akkermansia* due to a similar LacNAc structure found in hGOS and mucin (71). These findings are consistent with our previous animal studies (15, 16); however, animal models have significant limitations due to fundamental differences between human and mouse-originated microorganisms (72). Further studies are currently underway to better assess the impacts of hGOS on human bacterial isolates, with the ultimate goal of developing a prebiotic optimized for human consumption.

Finding the proper dose of a new therapeutic compound is vital not only to ensure safety and efficacy in clinical trials but

is also necessary to ensure the economic feasibility of the new product. For GOS, a low dose (below 2 g per day) may not elicit the desired modulatory effect (6, 73), while an excessively high dose (over 15 grams per day) may induce undesired GI effects (6, 74). Studies have shown the importance of translating the dose of a compound validated in animal models to the HED [reviewed in (75)]. Here, we demonstrated the lack of adverse effects of a HMO mimetic composition, LacNAc-enriched GOS, and its ability to modulate the gut microbiome at a HED of 180 mg kg⁻¹ day⁻¹. The values tested during our experiments are in accordance with the recommended values by the EFSA panel (76) for 2'-O-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT). The tested HED was six times higher than the calculated average amount of LacNAc consumed in a day by a 5 kg infant (1,400 vs. 232 mg day⁻¹), potentially highlighting the lack of adverse effects of LacNAc, even at a higher-than-physiological doses (77, 78).

Breast milk is undoubtedly the optimal source of nutrition for the human infant (79) and, until recently, the HMOs present in mother's milk could not be replicated in enough quantities to add to infant formulas. Five years ago, the study by Marriage et al. (80) showed that weight, length, head circumference growth and uptake of 2'FL, measured in the blood and urine, were similar to those of breastfed babies and today, some infant formulas have already incorporated this HMO. As a major building block of HMOs, the addition of LacNAc to the existing list of prebiotic compounds is of paramount importance for the further development of safe, nutritionally, and immunologically complete formulas. Hence, our study represents the first step in evaluating the safety and efficacy of enzymatically produced hGOS in an animal model of weaned human infants.

DATA AVAILABILITY STATEMENT

All sequencing data has been submitted to NCBI repository and can be accessed via the following accession number: PRJNA681811.

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ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill (Approved protocol number: 19-084).

AUTHOR CONTRIBUTIONS

JA performed and analyzed animal experiments. HW and JR curated and analyzed the amplicon sequencing data presented in the manuscript. SD produced the humanized GOS for the animal experiments. MA-P and JB-B designed the experiments and edited the manuscript. All authors contributed to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnut.2021.640100/full#supplementary-material>

Supplementary Figure 1 | Bodyweight and daily dietary consumption datasets are included to show that animal growth and intake were not inhibited by the introduction of either experimental diet (GOS and hGOS).

Supplementary Figure 2 | Bacterial taxa shown to have a significantly reduced relative abundance in the presence of hGOS.

Supplementary Table 1 | Composition of diets used in the animal study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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