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Human Milk Oligosaccharides Modulate Fecal Microbiota and are Safe for Use in Children with overweight: An RCT

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CONFLICT OF INTEREST AND SOURCE OF FUNDING

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ABSTRACT

Objectives: Human milk oligosaccharides (HMOs) impact the intestinal microbiota by increasing beneficial bacteria in infants and adults and are safe and well tolerated in these age groups. However, effects on intestinal microbiota, safety and digestive tolerance in children have not been assessed. The aims of this trial were to evaluate if HMOs are able to specifically modulate the intestinal microbiota in children, and to assess safety and digestive tolerance. Methods: In this randomized, double-blinded, placebo-controlled trial, 75 children with overweight (including obesity) aged 6-12 years were randomized to receive 2'-fucosyllactose (2'FL), a mix of 2'FL and lacto-N-neotetraose (Mix), or a glucose placebo orally administrated once per day for eight weeks. Results: The relative abundance of bifidobacteria increased significantly after four ($p<0.001$) and eight ($p=0.025$) weeks of intervention in the 2'FL-group and after four weeks ($p=0.033$) in the Mix-group, whereas no change was observed in the placebo group. Compared to placebo, the 2'FL-group had a significant increase in bifidobacteria abundance after four weeks ($p<0.001$) and eight weeks ($p=0.010$) and the Mix-group showed a tendency to increased bifidobacteria abundance after four ($p=0.071$) and eight weeks ($p=0.071$). *Bifidobacterium adolescentis* drove the bifidogenic effect in the two groups. Biochemical markers indicated no safety concerns, and the products did not induce digestive tolerance issues as assessed by Gastrointestinal Symptoms Rating Scale and Bristol Stool Form Scale. Conclusions: Both 2'FL and the Mix beneficially modulate intestinal microbiota by increasing bifidobacteria. Furthermore, supplementation with either 2'FL alone or a Mix is safe and well tolerated in children.

KEYWORDS: Gut microbiota; Bifidobacteria; 2'FL; LNnT

WHAT IS KNOWN

- Human milk oligosaccharides (HMOs) are a heterogeneous mixture of soluble glycans shown to strongly modulate the gut microbiota of breast-fed infants
- Commercially produced HMOs are safe for use, and beneficially modulate gut microbiota, in infants and adults, but the impact in children is not known

WHAT IS NEW

- HMOs induce similar gut microbial changes in children with overweight as seen in infants and adult
- HMOs do not cause safety concerns, nor induce digestive distress in children

INTRODUCTION

Human milk oligosaccharides (HMOs) are a heterogeneous mixture of soluble glycans found in human milk (1, 2). HMOs are not digested in the upper gastrointestinal tract and only 1-2 % are absorbed in infants (3-5). Consequently, most ingested HMOs reach the large intestine where they have multiple roles including acting as selective substrates for specific intestinal bacteria, modulating the immune system, and acting as decoy receptors to prevent epithelial adhesion of pathogens. In particular, HMOs substantially modulate the intestinal microbiota and play a decisive role in the differences in the intestinal microbiota of formula-fed and breastfed infants (6), including favorizing bifidobacteria in the gut of breast-fed infants compared to formula-fed infants (7, 8). The predominance of bifidobacteria is viewed as beneficial for the infant (9-11), as it seems to have a beneficial impact on gut health and immunity throughout childhood (12-14).

The commercially available HMOs are structurally identical to those found in human milk and have previously been evaluated in clinical studies in healthy adults (15-17) and infants (6, 18). These studies showed that HMOs can modulate the intestinal microbiota in both the infant and adult are safe and well tolerated (6, 15, 18). In healthy adults, HMOs increased bifidobacteria, and reduced Firmicutes and Proteobacteria compared to placebo. In infants, HMO supplementation increased *Bifidobacterium* and decreased *Escherichia* and *Peptostreptococcaceae* compared to infants consuming standard formula (6). However, no studies in children have investigated the effect of HMOs on intestinal microbiota, nor safety and digestive tolerance.

The aim of this trial was to determine the impact of the two HMOs 2'-fucosyllactose (2'FL) and lacto-N-neotetraose (LNnT) on intestinal microbiota in children, as well as evaluating safety and digestive tolerance.

MATERIALS AND METHODS

Trial design

This was a prospective, randomized, double-blinded, placebo-controlled clinical trial aiming to evaluate the impact on intestinal microbiota and confirm safety of a daily intake of 4.5g 2'FL alone or a combination of 2'FL and LNnT in a 4:1 mix (Mix) in children. Subjects were randomized using a computer-generated list developed by HB Medical, Horsholm, Denmark. Two complete sets of randomization code envelopes were provided, kept sealed and accessible only to authorized persons until the time of unblinding. All parties were blinded to the allocation, including the investigator, staff, Sponsor, and personnel carrying out the laboratory analyses. After recruitment to the trial and initiating the intervention, an intermediate visit took place after 4 weeks of intervention, and at the end of the intervention. The full intervention lasted for 8 weeks. Subjects were recruited from the Children's Obesity Clinic, accredited European Centre for Obesity Management, Department of Pediatrics, Copenhagen University Hospital Holbaek, Denmark (the Children's Obesity Clinic), but did not suffer from any other known chronic conditions than overweight. Subjects were referred to a multifaceted hospital-based obesity management program designed for the management of overweight in the pediatric population. The program comprised a range of personalized behavioral life-style recommendations, after obtaining a detailed medical history and conducting a questionnaire-based interview aimed at identifying all the daily challenges relevant to overweight (19). This includes e.g. a diet high in dietary fiber, low in sugar, low in fat content, and reduced processed foods intake, where advices are individually specified for each subject (among 10-20 other pieces of advice) and are not based on calorie-counting, nor calorie restriction. The trial was conducted in accordance with the Declaration of Helsinki (20) and approved by the Ethics Committee in Region Zealand, Denmark. The trial was registered at ClinicalTrials.gov with registration number NCT02786160.

Oral assent from the subject and written informed consent from the subjects' legal representatives were obtained for all subjects at the screening visit. After inclusion in the trial, a blood sample for safety markers was collected at baseline and after the end of intervention. The subjects completed the Gastrointestinal Symptom Rating Scale (GSRS) (21), and a clinical examination was completed, at baseline, after 4 weeks of intervention, and after the end of intervention. Additionally, subjects collected fecal samples that were stored in their home freezer until delivery at the hospital within 48 hours prior to the start of the intervention, before the 4 weeks visit, and before the end of intervention visit. For details on clinical examination and handling and analyses of biological samples see supplemental material (supplemental digital content 1, *http://links.lww.com/MPG/C383*).

Inclusion and exclusion criteria

Subjects were eligible for this trial if they were ≥5 and <13 years of age at recruitment and enrolled in the childhood obesity treatment program at the Children's Obesity Clinic with no

other known chronic conditions. Additionally, the children and their legal representatives needed to be fluent in Danish. Subjects were excluded from participation if they had known gastrointestinal diseases, or consumed probiotic supplements or antibiotic drugs three months prior to screening and throughout the trial.

Efficacy and safety measures

The primary endpoint was the change in fecal bifidobacteria in the intervention groups compared to placebo from baseline to the end of intervention. Safety was assessed by clinical evaluation of blood chemistry and an extended panel of blood markers of inflammation (TNF alpha, IL1b, IL6, IL8, IL10, and C-reactive protein), gut barrier integrity (lipopolysaccharide-binding protein, zonulin, and haptoglobin) and metabolism (adiponectin, leptin, resistin, soluble leptin receptor, ApoA1, ApoB100, free fatty acids, and ApoB48), measurement of fecal calprotectin concentration, and by collecting and monitoring adverse events (AEs), defined in accordance with the ICH Harmonised Tripartite Guideline E2A, Step 5 (22), throughout the course of the trial. AEs were rated in terms of severity and the likelihood of it being related to the trial product, and blood markers were assessed for signs of pathology, by a physician blinded to the intervention. Secondary endpoints included HMOs' impact on gastrointestinal symptoms measured by GSRS, which consists of 15 symptoms to be rated with a seven-graded Likert scale score ranging from 1 (no discomfort at all) to 7 (very severe discomfort). GSRS scores were assessed at baseline, after four weeks of intervention, and at the end of intervention.

Trial intervention

The HMOs (2'FL and LNnT) were synthetically produced by Glycom A/S, Denmark and provided as white powder. The placebo product was powdered glucose (Dextropur from Valora Trade Denmark A/S). HB Medical (Hoersholm, Denmark) filled the trial products in single-serve sachets each containing 4.5g of either 2'FL, 2'FL and LNnT in a 4:1 mass ratio, or placebo. The 2'FL/LNnT ratio was selected based on the concentrations of the compounds found in human breastmilk of secretor mother (23, 24). The products were distributed in boxes containing 60 sachets. All products were labelled with identical labels apart from a unique randomization number. Subjects were randomized to one of three intervention arms in a 1:1:1 fashion and were further instructed to consume the content of one sachet daily for 8 weeks.

Statistical methods

This was an exploratory trial, and therefore no formal power calculation was performed. Based on data from a trial in healthy adults (15), a sample size of 25 subjects per group would provide ≥80% power to detect an increase in the abundance of fecal bifidobacteria.

For the fecal microbiota data, Wilcoxon signed-rank tests were used to compare differences in bacterial abundance within groups, whereas Kruskal-Wallis test and Wilcox signed-rank tests were used to compare differences in bacterial abundance between the three groups. The α-diversity of the microbiota community in the fecal samples was assessed by counting the number of operational taxonomical units (OTUs) and calculating the Shannon index; a diversity index that takes into account the number of OTUs of a microbiota community, and

the relative abundance of the OTUs. For the GSRS scores, Wilcoxon signed-rank test was used to test within group change from baseline to end of intervention, and Kruskal-Wallis test was used to test between group differences. As none of the blood safety markers exhibited levels suggesting presence of pathology according to reference values for sex and age groups, which was the case for all groups, no between-group comparison was performed on these markers. One-way ANOVAs were used to test for between-group differences in the extended panel of blood markers at the end of intervention. Data processing was conducted using The 64-bit version of USEARCH 10.0 (Edgar 2013), mothur 1.38 (Schloss et al. 2009), Excel, GraphPad Prism (version 7.05 for Windows, GraphPad Software, La Jolla California USA), and R Studio (R version 3.4.2, The R Foundation for Statistical Computing). A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Trial population

A total of 75 subjects, 6.4 – 12.7 years of age, with overweight or obesity at baseline were included between August 2016 and November 2017 and randomized to receive either a daily dose of 4.5 grams of placebo, 2'FL alone, or the Mix (Figure 1). Baseline demographics and characteristics were comparable between groups (Table 1).

Fecal microbiota

α-diversity

The group receiving the Mix of 2'FL and LNnT had an increase in Shannon index from baseline to week 8 (p=0.004), showing that the Mix impacted the fecal microbiota community by increasing the α-diversity after eight weeks of intervention. The placebo group and the 2'FL group had no statistically significant changes in the number of OTUs or Shannon index for any time interval (p=0.09-1.00).

Changes in bacterial taxa

The 2'FL group had an increase in *Actinobacteria* from baseline to week 4 and week 8, and the Mix group had an increase from baseline to week 4, whereas the impact on the other dominant phyla was minor (Figure 2a). No significant changes were found for the bacterial taxa at genus or order level (taxa with >10 % prevalence) after using a false discovery rate of 0.10 apart from *Bifidobacterium* and Bifidobacteriales, which increased during intervention. Placebo had no impact on the fecal microbiota abundance at phylum level.

For both the 2'FL and the Mix groups, but not the placebo group, a significant increase in *Bifidobacterium* abundance was observed from baseline to week 4 ($p_{2'FL} < 0.001$ and $p_{Mix}=0.033$), while a significant increase was seen only in the 2'FL group from baseline to week 8 (p=0.025) (Figure 2b). No significant differences were observed from week 4 to week 8, indicating that the full bifidogenic effect is reached after four weeks of intervention.

When comparing the change in relative *Bifidobacterium* abundance between groups over time, there was a significant difference between the three groups from baseline to week 4 (p=0.006) and baseline to week 8 (p=0.033) (Figure 2c). Specifically, the 2'FL group showed a significant increase in *Bifidobacterium* abundance compared to the placebo group ($p_{\text{baseline to}}$

$_{\text{week 4}}$=0.001 and $p_{\text{baseline to week 8}}$=0.010), while the Mix group showed a tendency compared to placebo ($p_{\text{baseline to week 4}}$=0.071 and $p_{\text{baseline to week 8}}$=0.071).

Individual *Bifidobacterium* species

The relative abundance of *B. adolescentis* increased significantly in the 2'FL group from baseline to week 4 (p<0.001) and from baseline to week 8 (p=0.01), and in the Mix group from baseline to week 4 (p=0.03). No significant changes for *Bifidobacterium* were observed in the placebo group (supplemental digital content 2, *http://links.lww.com/MPG/C384*).

When performing between-group comparison for the change in *B. adolescentis* abundance over time, there was a significant difference between the three groups ($p_{\text{baseline to week 4}}$=0.004 and $p_{\text{baseline to week 8}}$=0.031). Multiple comparison showed that the 2'FL group had a significant increase in *B. adolescentis* abundance compared to the placebo group ($p_{\text{baseline to week 4}}$=0.002 and $p_{\text{baseline to week 8}}$=0.020), while the Mix group showed a nonsignificant increase in *B. adolescentis* compared to the placebo group from baseline to week 4 (p=0.098).

Safety assessment

Adverse events were generally reported at the scheduled visits and subjects also had the possibility to contact the trial personnel by phone if AEs occurred during the trial period. Overall, 23 (30.7%) of the subjects reported a total of 46 AEs between randomization and end of intervention. Seven, seven, and nine subjects in the placebo group, 2'FL group, and Mix group, respectively reported at least one AE during the intervention. The number of reported AEs were 13, 14, and 19 in the placebo group, the 2'FL group, and the Mix group, respectively. Of the 46 reported AEs, 18 were considered possibly or probably related to the trial product (supplemental digital content 3, *http://links.lww.com/MPG/C385*); 13 of which were mild (five, one, and seven in the placebo group, the 2'FL group, and the Mix group, respectively) and five were moderate in severity. All of the five moderate AEs were reported by one subject who presented with an event with multiple AEs (abdominal cramps, abdominal pain, diarrhea, reflux, and flatulence) occurring simultaneously. No serious AEs were reported. The most frequently reported mild AE possibly or probably related to the trial product was abdominal pain with five reports (two, one, and two reports in the placebo group, the 2'FL group, and the Mix group, respectively) followed by diarrhea with three reports (two reports in the placebo group and one report in the Mix group).

GSRS scores for gastrointestinal symptoms were low at baseline for all three groups and remained low during the intervention (Figure 3). Except from a significant improvement in urgency in the group receiving 2'FL (mean GSRS score decreased from 1.8 at baseline to 1.0 at end of intervention, p=0.013, Wilcoxon signed-rank test), the fluctuations in total GSRS score and scores for individual symptoms did not reach statistically significant levels for the intervention groups. Group effect for rumbling at baseline (p=0.016, Kruskal-Wallis test) and bloating at week 4 (p=0.042, Kruskal-Wallis test) were observed, however, the differences between groups were minor and considered clinically irrelevant.

Blood samples for routine clinical chemistry and hematology were collected at baseline and at end of intervention. None of the markers exhibited levels suggesting pathology at any time point for all three groups (supplemental digital content 4, *http://links.lww.com/MPG/C386*).

The variation between marker values was considered minor and not of clinical relevance. Furthermore, fecal calprotectin was generally low and well below the upper limit for normal values (<50mg/kg) both at baseline and at the end of intervention (supplemental digital content 5, *http://links.lww.com/MPG/C387*).

In addition to routine safety blood markers, an extended panel of markers of inflammation, gut barrier integrity and metabolism were measured. Again, these marker concentrations did not suggest pathology in any of the groups, however appropriate pediatric reference materials for a number of these markers are yet to be established, why clinical interpretation is limited. For inflammatory markers, measurements were comparable in all three groups at baseline and generally unchanged at the end of intervention (supplemental digital content 6, *http://links.lww.com/MPG/C388*). The same was the case with the markers for gut barrier integrity, where measurements were stable across intervention groups and duration of intervention (supplemental digital content 7, *http://links.lww.com/MPG/C389*). Finally, for the metabolism markers, there were no differences between the intervention groups at the end of intervention (supplemental digital content 8, *http://links.lww.com/MPG/C390*).

DISCUSSION

In this randomized, placebo-controlled, double-blinded, 8-week intervention trial, daily intake of 4.5 grams of 2'FL or 2'FL and LNnT in a 4:1 mass ratio modulated the fecal microbiota by increasing the abundance of bifidobacteria. At species level, the relative abundance of *B. adolescentis* was increased from baseline to week 4 and from baseline to week 8 in the 2'FL group, and from baseline to week 4 in the Mix group. Similar results were observed in an adult cohort where *B. adolescentis* also increased after supplementing with 2'FL and LNnT for 2 weeks (15). *B. adolescentis* is one of the dominating *Bifidobacterium* species in the human gut of both children and adults (25). Emerging science has found that supplementation with *B. adolescentis* reduced fat pad weight and increased insulin sensitivity in high fat diet fed Wistar rats (26) and reduced liver damage in mice fed a Western style diet (27). In addition, strains of *B. adolescentis* isolated from the human gut has been identified to produce Gamma aminobutyric acid (GABA) (neurotransmitter), and in the same study, a correlation between *B. adolescentis* load and mental disorders such as depression and anxiety in children was found (28). Furthermore, evidence from a case-control study showed that *Bifidobacterium* was enriched in healthy infants, whereas detrimental bacteria such as *Escherichia/Shigella* was enriched in infants with eczemas (29). Another prospective follow-up study has found that the genus *Bifidobacterium* was higher in number in children who remained normal weight at 7 years than in children developing overweight (30). A recent study has found that children with recorded overweight had significantly less *Bifidobacterium* than normal weight children (31). Hence, an increase of this bacterium in the gut might be important to help improve a child's health. Furthermore, considering both blood markers and reported AEs, the results show that daily intake of 4.5 grams of 2'FL or the Mix is safe in children, and these do not induce gastrointestinal distress. Assessment of safety blood markers showed no clinically relevant deviations after eight weeks of intervention and reported AEs did not raise concerns. The higher frequency of AEs in the Mix group seemed to be driven by one subject reporting an event with multiple symptoms. No serious AEs were

reported. Also, gastrointestinal symptoms remained low during intervention, and the symptom scores were similar between the groups.

To our knowledge, this is the first trial investigating the impact of HMOs on fecal microbiota and safety in children aged 6-12 years. The results are, however, in line with what have previously been seen in infants (6, 18) and adults (15), where supplementation with 2'FL and LNnT resulted in increased abundance of fecal bifidobacteria and were considered to be safe and did not induce digestive distress.

There are several potential explanations for the findings in the current trial. The shift in fecal microbiota can be explained be the prebiotic nature of HMOs. As prebiotic fibers, the HMOs specifically stimulate growth of bifidobacteria (32), a genus generally assumed to be beneficial (9-11). Upon metabolizing HMOs, bifidobacteria produce acetate (33, 34). This short chain fatty acid supports the maintenance of a beneficial environment by lowering colonic pH and acting as a substrate in the production of butyrate (35) which is an important energy source for the colonocytes (36). Furthermore, bifidobacteria do not produce gas when fermenting indigestible carbohydrates and have an indirect inhibiting effect on gas production by competing with gas producing bacteria for food source and thereby potentially inhibiting their growth (37), which may explain why the intervention did not induce gastrointestinal symptoms, such as flatulence or bloating (38).

The current trial also has limitations. Firstly, it was a relatively small trial, resulting in limited statistical power to detect differences between intervention groups and within groups over time. Furthermore, all the included children were all participating in a non-pharmaceutical, non-surgical clinical obesity treatment program, implementing changes in both food habits and physical activity (39), which may have masked potential impact of the trial intervention and, may have been a driver for the observed changes in fecal microbiota.

In conclusion, this trial shows that daily intake of the HMOs 2'FL and LNnT have a microbiota modulating effect, primarily mediated by inducing growth of *B. adolescentis*, in children with overweight. Furthermore, the trial indicates that the HMOs are safe for use in children as the safety biomarkers in blood and feces did not suggest pathology and the extended panel of blood markers did not give rise to concerns of the intervention. Also, adverse events occurred with similar frequency between the three groups, and the interventions did not induce digestive distress.

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FIGURE LEGENDS

Figure 1. Subject flow

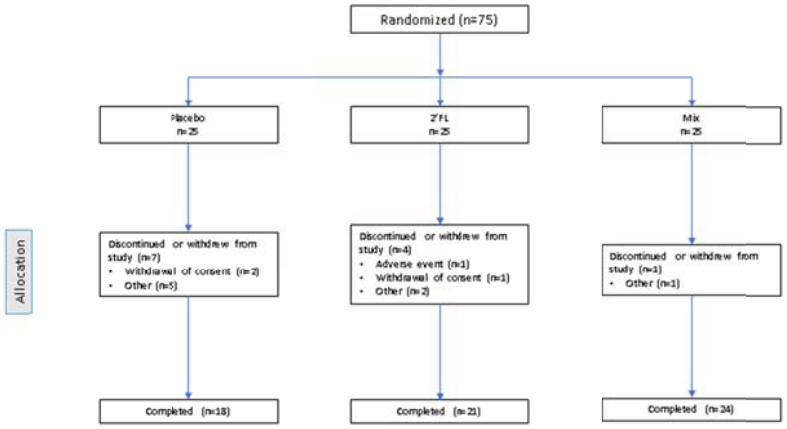

Figure 2a. Box plots of the relative abundance of bacteria at phylum levels in placebo, 2'FL, and Mix over time. Asterisks [p<0.05 (*)] indicate a statistically significant difference, and pound [p<0.10 ($^{\#}$)] indicates a tendency between week 0 compared to 4 or 8 weeks. 2'FL, 2'-Fucosyllactose; Mix, mixture of 2'FL and Lacto-N-neotetraose in a 4:1 mass ratio.

Figure 2b. Box plots of the relative abundance of *Bifidobacterium* by group and time.

Asterisks [p<0.05 (*) and p<0.001 (***)] indicate a statistically significant difference between week 0 and 4 or 8 weeks. 2'FL, 2'-Fucosyllactose; Mix, mixture of 2'FL and Lacto-N-neotetraose in a 4:1 mass ratio.

Figure 2c. Box plots of the change in *Bifidobacterium* abundance by group and time.

Asterisks [p<0.05 (*) and p<0.01 (**)] indicate the statistically significant difference, and pound [p<0.10 ($^{\#}$)] indicates a tendency between week 0 compared to 4 or 8 weeks. 2'FL, 2'-Fucosyllactose; Mix, mixture of 2'FL and Lacto-N-neotetraose in a 4:1 mass ratio.

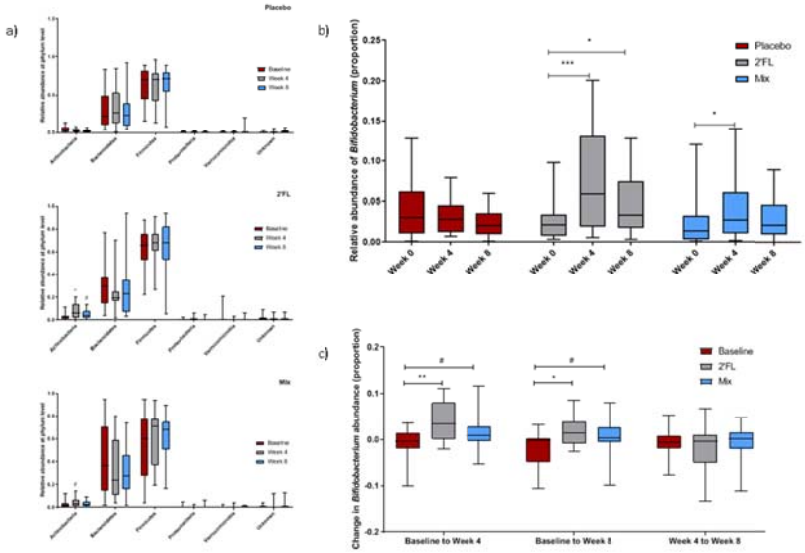

Figure 3. Radar plots of mean GSRS scores for the three groups at each of the visits.

The scale ranges from 1 to 7, where 1 denotes no discomfort and 7 denotes very severe discomfort GSRS, Gastrointestinal Symptom Rating Scale; 2'FL, 2'-Fucosyllactose; Mix, mixture of 2'FL and Lacto-N-neotetraose in a 4:1 mass ratio.

Table 1. Demographics and baseline characteristics of the trial population. Mean ± SD

	Placebo (n=25)	2'FL (n=25)	Mix (n=25)
Sex, n (%) - Girls - Boys	 12 (48%) 13 (52%)	 16 (64%) 9 (36%)	 15 (60%) 10 (40%)
Age (years)	9.0 ± 1.8	9.4 ± 1.6	9.8 ± 1.7
Weight (kg)	49.5 ± 13.0	51.1 ± 12.9	53.6 ± 11.4
Height (cm)	140.8 ± 11.5	142.1 ± 11.6	146.7 ± 12.9
BMI Z-score	3.05 ± 0.84 (n=23)	2.97 ± 0.59 (n=24)	2.88 ± 0.59
SBP Z-score	0.81 ± 0.95	0.84 ± 1.18	0.73 ± 0.84
DBP Z-score	0.42 ± 0.83	0.44 ± 0.67	0.40 ± 0.65

BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; n, number of subjects; 2'FL, 2'-Fucosyllactose; Mix, mixture of 2'FL and Lacto-N- neotetraose in a 4:1 mass ratio; Z-score, standard deviation score; SD, standard deviation