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Original Research Article

Changes in gut microbiota and lactose intolerance symptoms before and after daily lactose supplementation in individuals with the lactase nonpersistent genotype

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A B S T R A C T

Background: Approximately 70%–100% of the Asian adult population is lactase nonpersistent (LNP). The literature shows that many individuals with the LNP-genotype can consume ≤12 g of lactose without experiencing gastrointestinal discomfort. Repetitive consumption of lactose may reduce intolerance symptoms via adaptation of the gut microbiota.

Objective: This study aimed to assess the effects of daily consumption of incremental lactose doses on microbiota composition and function, and intolerance symptoms.

Methods: Twenty-five healthy adults of Asian origin, carrying the LNP-genotype were included in this 12-wk before and after intervention trial. Participants consumed gradually increasing lactose doses from 3 to 6 g to 12 g twice daily, each daily dose of 6 g, 12 g, or 24 g being provided for 4 consecutive weeks. Participants handed-in repeated stool samples and underwent a 25 g lactose challenge hydrogen breath test (HBT) before and after the 12-wk intervention. Daily gastrointestinal symptoms and total symptom scores (TSSs) during the lactose challenge were recorded.

Results: A significant increase from 5.5% ± 7.6% to 10.4% ± 9.6% was observed in *Bifidobacterium* relative abundance after the intervention ($P = 0.009$), accompanied by a 2-fold increase (570 ± 269 U/g; $P < 0.001$) in fecal β-galactosidase activity compared with baseline (272 ± 158 U/g). A 1.5-fold decrease (incremental area under the curve; $P = 0.01$) in expired hydrogen was observed during the second HBT (38 ± 35 ppm·min), compared with the baseline HBT (57 ± 38 ppm·min). There was a nonsignificant decrease in TSS (10.6 ± 8.3 before compared with 8.1 ± 7.2 after intervention; $P = 0.09$). Daily consumption of lactose was well tolerated, with mild to no gastrointestinal complaints reported during the intervention.

Conclusions: Increased levels of *Bifidobacterium* indicate an adaptation of the gut microbiota upon repetitive consumption of incremental doses of lactose, which was well tolerated as demonstrated by reduced expired hydrogen concentrations during the second 25-g lactose HBT. Bifidobacteria metabolize lactose without gas production thereby potentially reducing intestinal gas formation in the gut of individuals with the LNP-genotype. This increased lactose tolerance possibly lifts the necessity to remove nutrient-rich dairy foods completely from the diet.

The trial is registered at the International Clinical Trials Registry Platform: NL9516. The effect of dietary lactose in lactase nonpersistent individuals on gut microbiota.

Keywords: lactose intolerance, lactase nonpersistence, microbiota, bifidobacteria, hydrogen breath test, fecal β-galactosidase activity, gut microbial adaptation, clinical trial

Introduction

Globally, ~70% of the adult population is lactase nonpersistent (LNP), lacking the lactase enzyme required for the digestion of lactose [1,2]. In Asian populations, the percentage ranges even from 70%–100% [3]. During evolution, the lactase-persistent (LP) phenotype emerged (in European and various African, Middle Eastern, and Southern Asian populations) as a result of positive selection because of

Abbreviations: CAZy, carbohydrate-active enzyme; COG, Clusters of Orthologous Group; GI, gastrointestinal; HBT, hydrogen breath test; HRGM, Human Reference Gut Microbiome; iAUC, incremental area under the curve; ITT, intention-to-treat; KEGG, Kyoto Encyclopedia of Genes and Genomes; LNP, lactase nonpersistent; LP, lactase-persistent; MaAsLin, Microbiome Multivariable Associations with Linear Model; MDS, multidimensional scaling; MTHFR, methylenetetrahydrofolate reductase; PP, per protocol; SCFA, short-chain fatty acid.

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a nutritional advantage of the continued ability to consume milk and dairy products, especially in times of famine and when coping with pathogen exposure [4]. Lactase persistence is the most strongly selected monogenetic trait currently known. In LP individuals, lifelong expression of lactase, located in the brush border of the small intestine, enables the hydrolysis of lactose into glucose and galactose, which are subsequently absorbed in the small intestine [1]. Consumption of dairy and dairy-based products by individuals with the LNP-genotype can lead to lactose intolerance symptoms, characterized by gastrointestinal (GI) complaints. When lactose travels through the small intestine, it raises the osmotic pressure that leads to increased water influx that can cause diarrhea. Furthermore, lactose is fermented by gut bacteria, such as *Bacteroides* and *Escherichia*, thereby producing gasses that potentially cause abdominal pain and bloating [5,6].

The threshold of lactose for symptoms to occur is, however, much higher than generally acknowledged. It has been shown that most of the individuals with the LNP-genotype can still consume 12 g (comparable with 250 mL of milk) or more of lactose in a single dose without suffering from any GI discomfort, especially when intake is combined with other foods [7]. This observation can, at least in part, be explained by adaptation of the gut microbiota resulting from repetitive lactose intake. Repetitive lactose consumption supports the growth of lactose-digesting bacteria, such as bifidobacteria. These bacteria possess β-galactosidases that split lactose into glucose and galactose that are further metabolized mainly into short-chain fatty acids (SCFAs). In contrast to other gut bacteria, bifidobacteria do not produce gasses from lactose [5]. Increased populations of bifidobacteria in the gut thus enhances gut fermentation of lactose without gas production that likely results in the reduction of intolerance symptoms otherwise caused by gas production.

Several studies have shown this shift in microbiota composition because of lactose consumption, but most of them used in vitro methods [8]. The few in vivo studies available are incomplete in their design [9–12]. They often lack proper inclusion diagnostics, e.g., LNP genotyping and the avoidance of habitual lactose intake, functional outcomes, such as hydrogen breath test (HBT) or fecal β-galactosidase activity, or they only included simple and targeted microbiota analyses, such as bacterial plate counts. Next to this, the dose of dietary lactose is often not physiologic with high doses $\leq$1 g of lactose per kg bodyweight [12].

Today, >6 billion individuals regularly consume dairy and dairy-based products [13]. Nutritionally, milk and dairy products are an important source of energy, proteins, fats, and nutrients, such as calcium and vitamin B12 [14]. Many individuals with the LNP-genotype avoid consumption of dairy and dairy products to a significant degree, thereby also missing out on the high nutrient density of these foods [15,16]. Especially in regions where affordable nutrition plays an important role, dairy products can vastly contribute to the nutritional health status of the population.

The primary aim of this study was to explore the adaptation of the gut microbiota induced by repetitive consumption of increasing doses of dietary lactose. We furthermore aimed to explore changes in microbial β-galactosidase activity, breath hydrogen concentrations, and total GI symptom score after a 25-g lactose challenge.

Methods

Study design

This single-blinded human intervention study consisted of 3 consecutive 4-wk intervention periods in which study participants consumed increasing doses (3–6–12 g) of lactose twice daily, dissolved in water or tea. The study had a before and after design, in which the "before" measurement served as a control for the "after" measurement within each participant. The participants were blinded for the amount of lactose present in the intervention product as well as the fact that the daily lactose dose was increasing each 4-wk period. Study participants handed-in repeated stool samples and filled out a short daily online questionnaire (LifeData, LLC.) on GI comfort, stool pattern, and study product compliance. GI symptoms bloating, flatulence, and abdominal cramping were scored on a scale of 1 (none) to 4 (severe). The fresh stool samples were transported cooled to the research facility within 24 h after collection. Upon arrival, a fresh portion of the sample was used immediately to assess pH levels and stool consistency according to the Bristol Stool Chart. Simultaneously, aliquots of the sample were prepared and snap-frozen in liquid nitrogen. These frozen aliquots were subsequently stored at −80°C until further analyses. At the start and the end of the 12-wk intervention period, study participants underwent a lactose HBT and filled out a 3-d food diary to assess the baseline diet. Study participants were instructed to keep their habitual lactose-free diet, supported by a research dietician. They were furthermore instructed to not actively lose or gain weight and to stick to their regular exercise routine.

The study was performed from June to December 2021 in the Netherlands. Ethical approval was obtained from the Medical Ethical Committee of Utrecht. The trial is registered at International Clinical Trials Registry Platform (NL9516) and conducted according to the declaration of Helsinki. Digital written informed consent was obtained from each participant before inclusion in the study.

Study participants

Twenty-five adults of Asian ancestry were included in the study. As part of the inclusion criteria for the study, individuals were genotyped for the LNP polymorphism (LCT-13910C>T) to identify homozygotes with the CC genotype. This was accomplished through a cheek swab followed by qPCR analysis using the instructions provided by the manufacturer (ViennaLab Diagnostics GmbH). The LNP-genotype (CC) has been observed at significantly higher frequencies within Asian populations (70%~100%) compared with the Dutch population (5%–15%). Consequently, individuals of Asian ancestry were specifically targeted to facilitate inclusion of individuals with the LNP-genotype in this study. Participants were also required to avoid dietary lactose, prebiotics, and probiotics for $\geq$4 wk before the study. In addition, individuals aged 18–50 y with a BMI between 18.5 and 30 kg/m^2 were eligible. Exclusion criteria for the study included the presence of chronic disease or food allergy, a history of GI surgery, use of medication that could potentially impact the study results (including antibiotics within 6 mo before the study initiation), and individuals experiencing constipation. In addition, participants who were pregnant or breastfeeding, reported excessive alcohol consumption, smoked, or used drugs or nitrous oxide were also excluded.

Intervention product

The daily dose of lactose (beta-lactose 24AN, DFE Pharma) was gradually increased from 6 to 12 to 24 g per 4-wk, divided over 2 sachets per day. One sachet was taken in the morning with breakfast and the other sachet in the evening with dinner. Each sachet contained 12 g comprising 3, 6, or 12 g of lactose topped up with dextrose (Meritose, Tereos Starch & Sweeteners Europe) to ensure blinding of the participants.

DNA isolation and sequencing

The primary outcome of the study was the evaluation of the intervention's impact on microbiota composition and function. DNA extraction from fecal material was performed using the QIAamp

PowerFecal Pro DNA kit (Qiagen) according to the manufacturer's instructions. Subsequently, BaseClear performed shallow shotgun metagenomics sequencing. DNA samples were subjected to Illumina Nextera XT library preparation followed by sequencing on a NovaSeq 6000 with paired-end 150 nt sequencing protocol (L457; NEN-EN-ISO/IEC 17025). FASTQ read sequence files were generated using bcl2fastq version 2.20 (Illumina). Resulting data (5 Gbp paired-end per sample) passed the internal quality assessment criteria of BaseClear.

Processing of sequencing data

The Biomax Informatics AG microbiome pipeline was used to convert raw sequencing data into bacterial counts with in-depth taxonomic classification. Kraken2 software [17,18] was used to map the sequenced reads to the Human Reference Gut Microbiome (HRGM) database [19]. The Bracken software [20] redistributed counts from different taxonomic levels to species level via Bayesian re-estimation. Initially, the shotgun metagenomic sequencing reads were annotated using the National Center for Biotechnology Information (NCBI) database; however, using this database on average 41% (range: 13%–68%) of the sequences could not be classified per sample. This problem was overcome by using the HRGM database [19] for annotation that increased classification of the sequences to an average of 95.2% (range: 91%–98.1%) per sample. This improvement is attributed to a better representation of persons of Asian ancestry, similar to the subjects in our study, in the HRGM database [19]. Functional assessment was performed by BaseClear. To this end, metagenome annotation of the reads against the Carbohydrate-Active enzymes (CAZy), Clusters of Orthologous Groups (COG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases was performed using the Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups mapper.

β-Galactosidase activity

β-Galactosidase activity was measured in frozen fecal samples (stored at −80°C), adapted from He et al. [21]. As a first step, 0.5 g fecal sample was homogenized in 5-mL phosphate-buffered saline (PBS, Merck Life Science) by vortexing (3 min) with solid glass beads (diameter 2 and 4 mm), followed by 2-min centrifugation (300 × *g*, 4°C). Microorganisms in supernatant were lysed with 300 mg 0.1 mm zirconia 6 m/s bead beating (BioSpec Products Inc.,) with a Fastprep-24 (MP Biomedicals) during three 15-s intervals (with a 5-min break on ice) [22]. The supernatant (14,000×*g*, 5 min, 4°C) was used for β-galactosidase activity measurements, according to the method of Van Laere et al. [23]. In short, 75 μL 50 mM phosphate buffer pH 6.0 (N1252, Merck Life Science) and 25 μL supernatant was supplemented with 25 μL 0.1% p-nitrophenyl-b-D-galactopyranoside solution in 50 mM phosphate buffer. The plate was mixed and immediately placed at 40°C with constant absorbance measurement at 405 nm for 1 h in a Tecan Spark spectrophotometer (Tecan Life Sciences).

Fecal β-galactosidase activity was calculated using the slope on the linear part of the data ($R^2 > 0.99$). Activity was expressed as U/g dry weight fecal sample, where 1 unit β-galactosidase activity is defined as 1.0 μmol galactose liberated per minute (at A_{405nm} pH 6.0 and 40°C) with a molar extinction coefficient of 13,700 L/mol×cm^{-1}.

SCFAs

Acetic acid, propionic acid, isobutyric acid, butyric acid, 2-methylbutyric acid, isovaleric acid, valeric acid, and caproic acid (hexanoic acid) were quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS, Metabolon Method TAM135, Metabolome) in fecal samples that were stored frozen at −80°C. Samples of feces were spiked with stable-labeled internal standards, homogenized, and subjected to protein precipitation with an organic solvent. After centrifugation, supernatant was derivatized and analyzed on an Agilent 1290/AB Sciex QTrap 5500 LC-MS/MS system with a C18 reversed phase ultra-High-Performance Liquid Chromatography (UHPLC) column. The MS was operated in negative mode using electrospray ionization. LC-MS/MS raw data were collected and processed using AB SCIEX software Analyst 1.6.3 and SCIEX OS-MQ software version 1.7. The levels were corrected for dry weight into μg/g dry weight fecal sample.

HBT

A lactose HBT was performed based on Szilagyi et al. [11], with some minor adaptations. The day before the HBT, all study participants were requested to keep from excessive exercise and avoid consumption of specific food products (e.g., fruit and fruit juices, onions, leek, garlic, cabbage, beans, and chewing gum) and to consume a standardized low-fiber dinner provided by the research team. Study participants reported to the research unit fasted since 20.00 h, only drinking water and brushing teeth was allowed. The participants stayed fasted during the HBT. Breath hydrogen concentrations (ppm) were measured using a validated handheld device (Gastrolyzer, Bedfont Scientific Ltd.) with a hydrogen chemical sensor. Breath hydrogen concentrations were measured at baseline and at 15-min intervals ≤90 min, and subsequently at 30-min intervals ≤270 min after ingestion of 25 g of lactose. Baseline values were subtracted from readings recorded at each subsequent time interval, to measure the incremental AUC (iAUC). GI symptoms were assessed using a 4-point Likert scale at baseline and at 30-min intervals after lactose ingestion. Bloating, flatulence, and abdominal cramping were graded on a scale of 0 (no symptoms), 1 (mild symptoms), 2 (moderate symptoms), or 3 (severe symptoms). Diarrhea was scored as 0 (none) or 1 (present). Total symptom score was obtained by summing all scores of each of the 10 time points for each symptom; the minimum possible score being 0 and the maximum possible score being 100 ([10 × 3 × 3] + 10 × 1).

Statistical analyses

Power analyses for studies where microbiota composition and function are the primary outcome are difficult because the true effect size is unknown. Other studies, including participants' maldigesting lactose, which have used more simplified methods, but sometimes used less-strict inclusion and exclusion criteria, included 20–24 participants [8–12]. Therefore, 25 participants were recruited for this study. Statistical analyses were performed using R statistics (RStudio, PBC, version 4.0.2). A P value of <0.05 was considered statistically significant. Data and figures show the per protocol (PP) analysis, unless stated otherwise. One study participant dropped out before study completion and was therefore excluded from the intention-to-treat (ITT) population ($n = 24$). One participant had major protocol deviations because of medical reasons, resulting in among others a supplement intake compliance <80%, and was therefore excluded from all PP analyses ($n = 23$). One study participant had a compliance <75% for filling out the daily questionnaires and was therefore excluded from the PP analyses of the daily parameters only ($n = 22$).

Differences over time in the HBT before and after the intervention period were analyzed with linear mixed models. Differences in fecal β-galactosidase activity, SCFA concentrations, and total symptom score during those lactose HBTs, before and after the daily lactose intervention were analyzed using a paired-samples t-test for normally distributed data and a Wilcoxon signed rank test for nonnormally

distributed data. Daily questionnaire data were calculated into weekly averages; for stool consistency, the weekly mode was calculated. Differences in weekly stool frequency, stool consistency, and GI complaints were analyzed with 2-way ANOVA repeated measures, with time (week 1–4 within each intervention period) and daily dose (6–12–24 g) as main and interaction effect. Bonferroni post hoc testing was applied to compare weeks within and between daily doses. Pearson correlation analyses were performed to explore the relation between changes in *Bifidobacterium*, fecal β-galactosidase, and lactose-induced breath hydrogen (iAUC).

Alpha-diversity (Shannon and Simpson) and relative abundance data over all taxonomic hierarchical levels were analyzed for before and after lactose intervention differences using nonparametric Wilcoxon matched-pairs tests. For analysis of β-diversity, Bray–Curtis dissimilarity matrix was used and data were analyzed via PERMANOVA by function adonis2 from the R package vegan, version 2.6-2 [24]. Differences of β-diversity were visualized in a multidimensional scaling (MDS) plot using R package limma. The function removeBatchEffect was applied to correct for interindividual differences in gut microbiota composition between participants.

Correlations between metadata (baseline levels of *Bifidobacterium*, baseline pH, sex, and age) and changes in the top 20 of the most abundant genera were analyzed using Microbiome Multivariable Associations with Linear Models (MaAsLin) by using the maaslin2 Bioconductor R package [25]. This package performs a multivariate regression between taxonomic counts and metadata. MaAsLin was also used for analysis of the correlation between changes in microbiota and the response on the HBT and on symptoms during the HBT. Responders on the HBT were defined as participants with ≥15% reduction of the iAUC and 50% reduction of peak height for the second HBT compared with the baseline HBT. Responders on GI symptoms were defined as subjects with a reduced total symptom score during the second HBT compared with the baseline HBT.

Multiple testing correction for the microbiota analyses was performed by calculating the false discovery rate according to Benjamini–Hochberg with the significance threshold set at $P < 0.05$ [26]. For lactose-digesting genera of the family *Lactobacillaceae* and *Bifidobacterium*, it was hypothesized a priori that the lactose intervention would result in increased abundances [11]; therefore, these genera were tested 1-sided with multiple testing correction only for species within the genus *Bifidobacterium*.

Functional gene α-diversity of COG families, CAZy subfamilies, and KEGG orthologs was calculated using the Inverse Simpson index [27]. The effect of the intervention on functional beta-diversity was analyzed separately for CAZy, COG, and KEGG annotated genes. Maaslin 2 [25] was used to analyze associations between the intervention and abundances of annotated genes.

Results

Baseline characteristics

A total of 25 adults with the LNP-genotype were initially recruited for the study, but 1 participant withdrew before its completion. This resulted in the enrollment of 24 individuals in the ITT analysis, consisting of 16 females and 8 males. A participant flowchart is shown in Figure 1. Baseline characteristics are shown in Table 1. Study participants were on average 29.1 y old (range 22–44 y) with an average BMI of 22.4 kg/m^2 (range 18.5–27.5 kg/m^2).

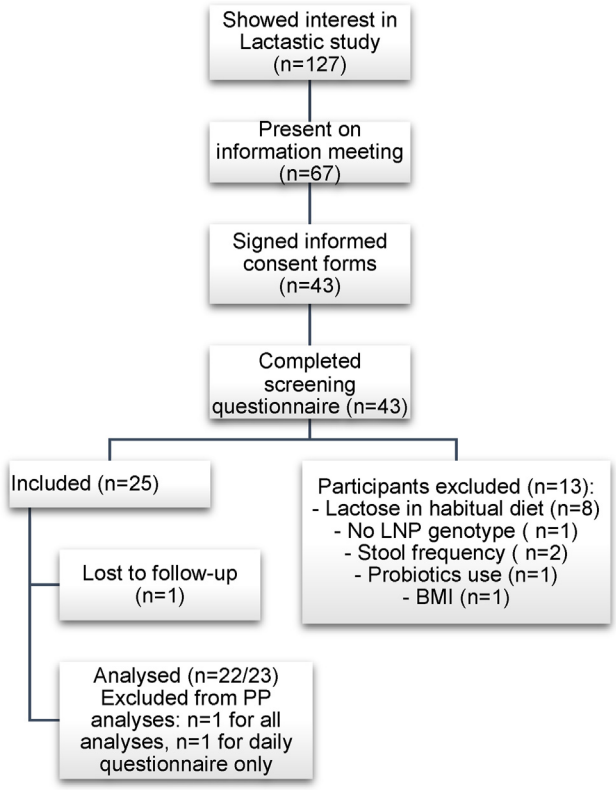

FIGURE 1. Study participant flowchart. The figure shows the flow of study participants from recruitment and screening to final intention-to-treat (ITT) and per protocol (PP) data analyses.

Changes in fecal microbiota composition and functional potential

Bray–Curtis β-diversity analysis of the overall microbiota composition revealed a significant change in microbiota composition after the 12-wk intervention with daily lactose compared with baseline based on PERMANOVA analysis ($P = 0.037$). The shift in microbiota composition is visualized in the MDS plot (Figure 2). The microbiota diversity of participants, as determined by measuring Shannon and Simpson α-diversity, was not influenced by the lactose intervention (Supplemental Table 1).

The relative abundances of genera with an average relative abundance of ≥1% before or after the intervention are shown in Figure 3A. As hypothesized, the average relative abundance of total *Bifidobacterium* significantly increased almost 2-fold from 5.5% ± 7.6% before daily lactose intervention to 10.4% ± 9.6% after the intervention ($P = 0.009$; Figure 3B).

Of the overall 20 most abundant genera, next to *Bifidobacterium*, the relative abundances of *Anaerostipes* and *RC9* significantly increased because of the lactose intervention. After adjusting for multiple testing, the significance was lost.

The relative abundances of different phyla and species are shown in, respectively, Supplemental Figures 1 and 2.

In total 22 different species of *Bifidobacterium* were detected in the samples. Fourteen of these species were significantly increased after intervention (false discovery rate-adjusted $P < 0.05$; Figure 3C; showing individual species with an average relative abundance of ≥0.1%). The 4 most dominant species were *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium bifidum*. *Lactobacillus* was relatively low in abundance (on

TABLE 1
Participant characteristics

Characteristics	Total ($n = 24$)
Age (y)	29.1 ± 5.8
Ancestry	Asian
Sex	Female ($n = 16$), Male ($n = 8$)
BMI (kg/m^2)	22.4 ± 2.7

Age and BMI are mean ± SD, intention-to-treat population—excluding 1 drop-out.

average 0.024% after intervention) and was not significantly stimulated by the lactose intervention. However, the relative abundances of 3 other genera within the same family of Lactobacillaceae (*Schleiferilactobacillus*, *Companilactobacillus*, and *Lapidilactobacillus*) were significantly increased after the lactose intervention.

For none of the covariates (*Bifidobacterium* at baseline, fecal pH at baseline, sex, and age), a correlation was found with the change of the relative abundance of the 20 most abundant genera after correction for multiple testing. Also, subgroup analysis based on change in HBT response and change in total symptom score during HBT before and after the intervention did not reveal any correlation with changes in gut microbiota composition.

Lactose intervention significantly increased α-diversity (Inverse Simpson index) of KEGG orthologs ($P = 0.021$) but not that of COG families and CAZy subfamilies (Supplemental Table 1). Beta-diversity was not significantly influenced by the intervention for COG, CAZy, and KEGG annotated genes.

The abundances (reads per kilobase) of 5 CAZy, 272 COG, and 422 KEGG annotated genes were significantly increased because of the intervention (Supplemental Tables 2–4 show the most significantly influenced genes). Most of the significantly influenced KEGG orthologs are genes involved in energy, amino acid, cofactor, and vitamin metabolism and are related to different ribosomal proteins belonging to both the small and large subunits. On the basis of both KEGG and COG analysis, the abundance of the gene encoding for methylenetetrahydrofolate reductase (MTHFR) was most significantly increased. No significant differences were found for the relative abundance of the β-galactosidase gene but the functional analysis showed a significant increase for several genes coding for enzymes involved in galactose metabolism.

Fecal metabolites and β-galactosidase activity

Acetic acid was the most abundant SCFA in the fecal samples before and after the intervention, see Table 2. No significant differences were found for total SCFA or any of the 8 individual SCFA because of the intervention, with high variability between participants. Repetitive lactose consumption resulted in on average 2-fold increase in fecal β-galactosidase activity compared with baseline ($P < 0.001$), increasing from 272 ± 158 to 570 ± 269 U/g fecal dry weight, see Figure 4. Pearson's correlation analysis showed a moderate positive correlation ($r = 0.47$, $P = 0.02$) between change in *Bifidobacterium* relative abundance and change in fecal β-galactosidase activity per gram fecal dry weight, whereas the change in *Bifidobacterium* relative abundance was moderate inversely correlated ($r = -0.48$, $P = 0.02$) with the change in HBT iAUC.

HBT and intolerance symptoms

Breath hydrogen concentrations after ingestion of a 25-g lactose bolus after the intervention period were significantly reduced compared with baseline, see Figure 5. After the 12-wk intervention, average breath hydrogen iAUC was reduced, with on average 1.5-fold (from 57 ± 38 ppm·min before to 38 ± 35 ppm·min after). There was a nonsignificant decrease ($P = 0.09$) in total symptom score during the HBT after the 12-wk intervention (8.1 ± 7.2 on a 0–100 range) as compared with the baseline HBT (10.6 ± 8.3). GI symptoms (scoring range 1–4) of bloating (average 1.3 ± 0.6), abdominal pain (average 1.1 ± 0.4), and flatulence (average 1.6 ± 0.7), reported daily during the intervention period, were mild (score = 2) to absent (score = 1) with little clinical variation. No significant differences were found for stool frequency and stool consistency during the intervention.

Discussion

In this 12-wk single-blinded intervention trial, repetitive consumption of an incremental daily dose of lactose was associated with changes of gut microbiota composition and activity. Microbiota profiles of fecal samples before and after the lactose intervention were determined using shotgun metagenomics sequencing, allowing accurate annotation of the sequences up to species level. The intervention resulted in a significant shift of the overall microbiota composition and specifically increased *Bifidobacterium* (species). The observed bifidogenic effect of lactose is in accordance with results of other studies with milk [10] and lactose interventions [11]. Also a dietary intervention with a novel galacto-oligosaccharide has shown to affect lactose intolerance symptoms via the gut microbiota [28]. Bifidobacteria are generally considered as beneficial members of the gut microbiota [29–32]. Depletion of bifidobacteria is associated with several metabolic, immune, and intestinal diseases [33–35]. The effect of lactose on

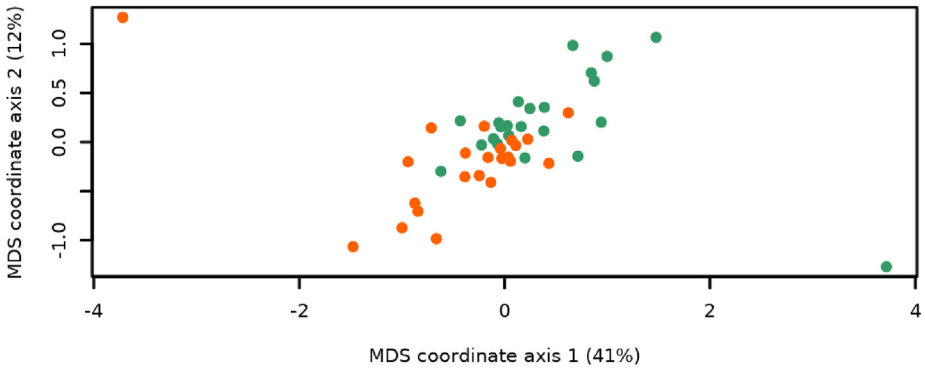

FIGURE 2. Multidimensional scaling plot of the fecal microbial species. Data shown before (●) and after (●) the 12-wk lactose intervention, independent of participant effect. $N = 23$ per time point.

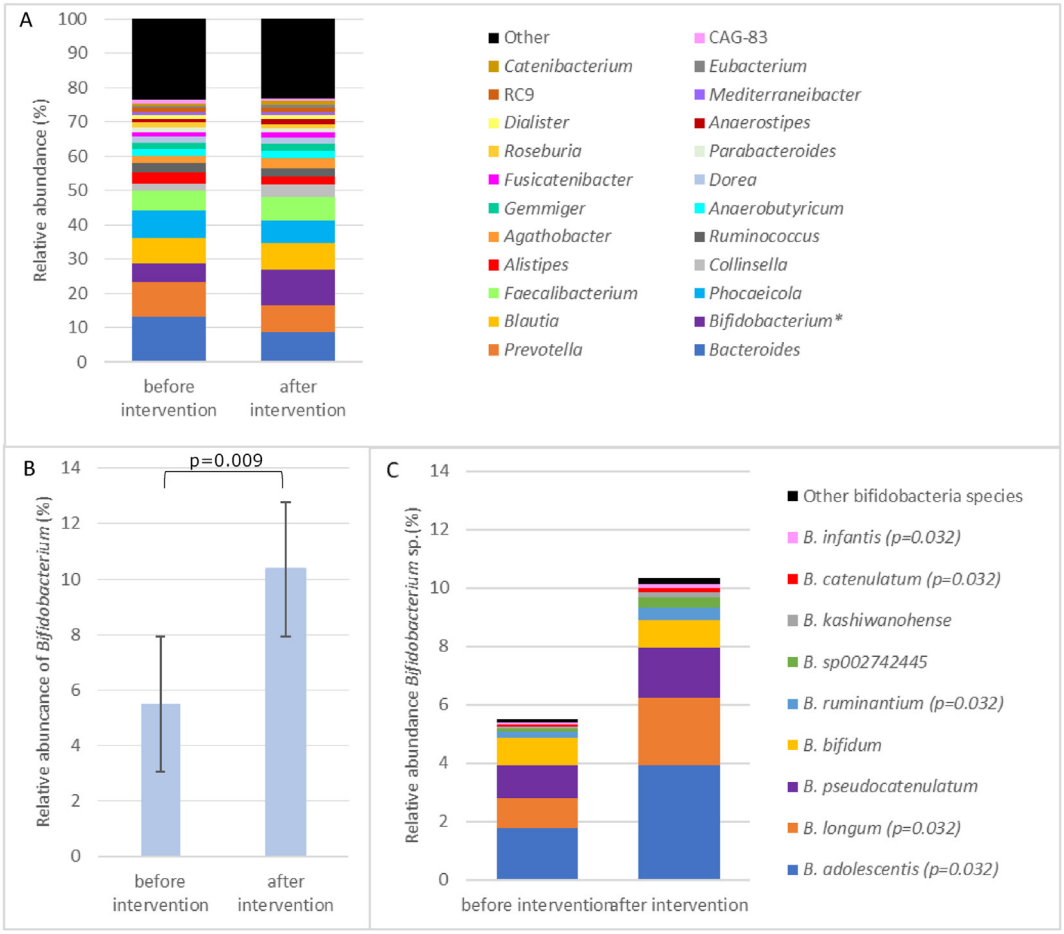

FIGURE 3. Average relative abundance of dominant genera and *Bifidobacterium* species before and after lactose intervention. Panel (A) shows the relative abundances of the most abundant microbial genera in fecal samples of subjects before and after the intervention. The "other" group includes all bacteria with an average relative abundance <1%. Panel (B) shows the relative abundance of *Bifidobacterium,* * $P = 0.009$, and panel (C) shows the relative abundance of the different species within the Bifidobacterium genus before and after 12-wk lactose intervention, *False discovery rate -adjusted P value < 0.05. $N = 23$.

stimulation of bifidobacteria was generic; except for *B. bifidum*, all *Bifidobacterium* species increased in abundance. For 14 of 22 species, this increase was significant, which included the most common adult species *B. adolescentis* and *B. longum* [35].

In line with increased *Bifidobacterium*, fecal β-galactosidase activity doubled after intervention. Bifidobacteria ferment sugars via the bifid shunt that delivers more ATP than fermentation via glycolysis, resulting in a competitive advantage [36]. In contrast to several other common colonic bacteria, such as *Bacteroides* and *Escherichia*, lactate and acetate are the main metabolites of lactose fermentation by bifidobacteria and no hydrogen, carbon dioxide, or methane are produced [5,6]. This is confirmed by the significant decrease in exhaled hydrogen

TABLE 2
Average fecal levels of short-chain fatty acids (SCFAs) before and after the lactose intervention

SCFA (μg/g fecal dry weight)	Before intervention (mean ± SD)	After intervention (mean ± SD)	P value[1]
Acetic acid	15,240 ± 10,685	16,549 ± 7439	0.35
Butyric acid	6284 ± 4183	6283 ± 3561	0.77
Hexanoic acid	316 ± 599	289 ± 458	0.75
Isobutyric acid	596 ± 235	617 ± 202	0.74
Isovaleric acid	623 ± 268	600 ± 208	0.72
2-Methylbutyric acid	444 ± 203	435 ± 165	0.99
Propionic acid	7078 ± 4508	7367 ± 4239	0.61
Valeric acid	1088 ± 806	958 ± 661	0.87
Total SCFA	31,668 ± 18,935	33,098 ± 14,489	0.73

$N = 23$.
[1] Paired t-test, not corrected for multiple testing.

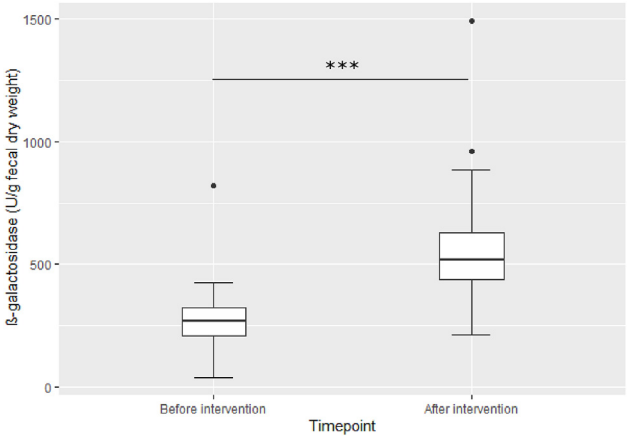

FIGURE 4. Fecal B-galactosidase activity levels before and after the lactose intervention. Paired analysis (Wilcoxon signed rank test) showed a significant increase ($P < 0.0001$) in fecal microbial β-galactosidase activity upon 12-wk lactose consumption. $N = 23$.

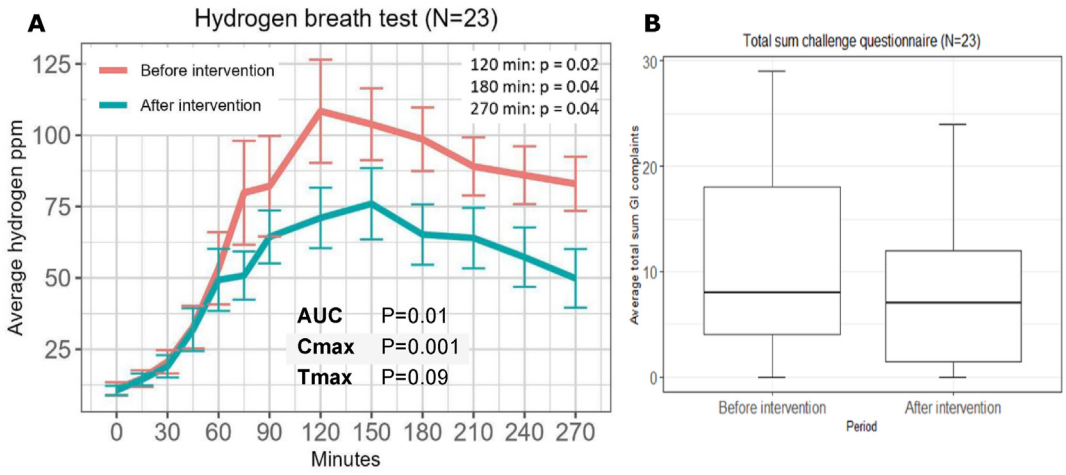

FIGURE 5. Lactose hydrogen breath test before and after the 12-wk lactose intervention. Panel (A) shows the hydrogen concentrations in exhaled breath during 4.5 h after consumption of 25 g of lactose, before (▬) and after (▬) 12 wk of lactose intervention. Paired comparisons (Wilcoxon signed rank test) were performed on parameters derived from the expired hydrogen concentrations: incremental AUC (iAUC), maximum peak height (Cmax) and time-2-max (Tmax). Panel (B) shows the total symptom scores (maximal score range 0–100 points) during both lactose HBTs. LMM, linear mixed model.

during the HBT after intervention. Methane concentrations in exhaled breath were not measured by use of the HBT. Methane could be produced from hydrogen by Archaea, such as *Methanobrevibacter smitthi*; however, no effect of the intervention on Archaea was found (data not shown).

Lactate and acetate serve as substrates for other bacteria in the gut, such as butyrate-producing *Anaerostipes*. The relative abundance of *Anaerostipes* also increased because of the lactose intervention, although not significant after multiple testing correction. Comparable results have been shown with whole milk consumption, which resulted in increased fecal levels of both *Bifidobacterium* and *Anaerostipes* in lactose-maldigesting subjects [10]. It is likely that cross-feeding between *Bifidobacterium* and *Anaerostipes* plays a role in the observed effects, as has been demonstrated before in vitro [37,38]. Butyrate resulting from this cross-feeding is an important energy source for enterocytes, plays a role in improving gut barrier function, and exerts anti-inflammatory activity [39,40]. Fecal levels of butyrate and other SCFAs were unaffected by the current intervention. However, most of the lactose is presumably fermented in the proximal colon and at least part of the resulting SCFAs are efficiently absorbed and do not end up in the feces [41].

Increased levels of bifidobacteria could potentially alleviate symptoms of lactose intolerance because of the absence of gas formation in lactose breakdown by these bacteria. However, this was not picked up by the GI symptom scoring during the current intervention study. In our study, the dietary lactose concentration was gradually increased, as to prevent considerable GI discomfort at the start of the study, before the colon would have had the chance to adapt. The likely advantage of this approach was that none of the study participants dropped out from the study because of lactose intolerance symptoms. On the other hand, because study participants reported absent to mild intolerance symptoms from start to end, there was little room for improvement over time. Such improvement on a day-to-day basis because of adaptation of the colonic microbiota may be expected when we would have started with the highest dose from the start. However, before the 12-wk lactose intervention, the total symptom score during the lactose challenge HBT was already very mild (median score of 8 on a 0–100 scoring range). Therefore, even with an acute 25 g lactose challenge, there was little room for improvement because of adaptation of the colonic microbiota. Applying a higher dose during the HBT, e.g., 50 g, may be necessary to give enough room for improvement between before and after lactose intervention. Alternatively, applying a lactose HBT during the screening phase as to include only individuals with substantial lactose-induced symptoms as well as a positive HBT would have generated more room for improvement. It was difficult to find individuals with the LNP-genotype that were completely avoiding all lactose from their habitual diet. Most were regularly consuming some lactose and were therefore requested to avoid all lactose ≥4 wk before study start. Despite all this, there was a trend toward an even lower total symptom score during the HBT after the 12-wk lactose intervention compared with the baseline HBT. An interesting implication of this result is that it lifts the necessity to remove nutrient-rich dairy foods completely from the diet by individuals with the LNP-genotype because of lactose intolerance symptoms. However, previous studies have shown that after discontinuation of lactose intake, adaptation of the colonic microbiota is lost. This might again lower the lactose threshold for intolerance symptoms when lactose is suddenly reintroduced into the diet [1]. This reinforces the point that regular and continuous consumption of dietary lactose is required to maintain lactose tolerance and avoid potential symptoms after consumption. Alternatively, individuals with the LNP-genotype may consume lactose-free dairy products or dairy with reduced lactose concentrations. These products also deliver all beneficial nutrients of dairy, but generally contain higher concentrations of other sugars, such as glucose. Lactose, in contrast to glucose, has a low glycemic index and a low cariogenic effect [42–44].

Next to the observed bifidogenic effect, intake of lactose by individuals with the LNP-genotype may have other health benefits via an effect on the microbiota, although current knowledge is scarce for the adult population. Analysis of the functional potential of the microbiota suggests an effect of the intervention on the functional gene α- and β-diversity. The abundance of several orthologs was significantly increased because of the intervention. Most of the orthologs are genes involved in energy, amino acid, cofactor, and vitamin metabolism or are related to ribosomal proteins belonging to both the small and large subunits. Their increase is likely a reflection of the increased availability of fermentable sugar in the colon. The most significantly increased gene encoded for MTHFR. This is an essential enzyme for

homocysteine and folate metabolism [45]. Research leads based on the functional analyses may give direction to further research aimed at obtaining more insight in potential other microbiota-mediated health benefits that are a consequence of lactose fermentation.

In conclusion, increased levels of *Bifidobacterium* indicate an adaptation of the colonic microbiota upon repetitive consumption of incremental doses of lactose. Bifidobacteria metabolize lactose without gas production and this potentially reduced intestinal gas formation in the gut of individuals with the LNP-genotype. Repetitive lactose consumption is well tolerated and able to reduce expired hydrogen during a 25-g lactose HBT. These findings indicate that there may not be a need to eliminate nutrient-rich dairy foods from the diet of individuals carrying the LNP-genotype. Our study demonstrates that individuals with the LNP-genotype can well tolerate a daily intake of 2 × 12 g of lactose, equivalent to 2 glasses of milk, when gradually introduced.

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Author contributions

The authors' responsibilities were as follows – LJD, EL, RT, JG: designed research; LJD, MvdB, RA: conducted research; LJD, MvdB, RA, BG, MZ: analyzed data and/or performed statistical analysis; LJD, EL: wrote the paper; and all authors: read and approved the final manuscript.

Conflict of interest

EL, RT, and JG were employees of FrieslandCampina at the time of conceptual development and submission of the manuscript. LJD was employee of FrieslandCampina at the time of conceptual development but switched employment before study execution. The other authors report no conflicts of interest.

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Data availability

Data described in the manuscript and analytic code will not be made available because this was not stated in the ethics application.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2023.12.016.

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