# Galactooligosaccharides Improve Mineral Absorption and Bone Properties in Growing Rats through Gut Fermentation

Connie M. Weaver,[*,†] Berdine R. Martin,[†] Cindy H. Nakatsu,[‡] Arthur P. Armstrong,[‡] Andrea Clavijo,[‡] Linda D. McCabe,[§] George P. McCabe,[§] Sinead Duignan,[†] Margriet H. C. Schoterman,[⊥] and Ellen G. H. M. van den Heuvel[⊗]

Departments of [†]Foods and Nutrition, [‡]Agronomy, and [§]Statistics, Purdue University, West Lafayette, Indiana 47907, United States
[⊥]FrieslandCampina Domo and [⊗]FrieslandCampina Research, Stationsplein 4, Amersfoort, The Netherlands

Ⓢ Supporting Information

**ABSTRACT:** Galactooligosaccharides (GOS), prebiotic nondigestible oligosaccharides derived from lactose, have the potential for improving mineral balance and bone properties. This study examined the dose−response effect of GOS supplementation on calcium and magnesium absorption, mineral retention, bone properties, and gut microbiota in growing rats. Seventy-five 4-week-old male Sprague−Dawley rats were randomized into one of five treatment groups ($n = 15$/group) and fed a diet containing 0, 2, 4, 6, or 8% GOS by weight for 8 weeks. Dietary GOS significantly decreased cecal pH and increased cecal wall weight and content weight in a dose-dependent manner ($p < 0.0001$). Fingerprint patterns of the 16S rRNA gene PCR-DGGE from fecal DNA indicated the variance of bacterial community structure, which was primarily explained by GOS treatments ($p = 0.0001$). Quantitative PCR of the samples revealed an increase in the relative proportion of bifidobacteria with GOS ($p = 0.0001$). Net calcium absorption was increased in a dose−response manner ($p < 0.01$) with GOS supplementation. Dietary GOS also increased ($p < 0.02$) net magnesium absorption, femur [45]Ca uptake, calcium and magnesium retention, and femur and tibia breaking strength. Distal femur total and trabecular volumetric bone mineral density (vBMD) and area and proximal tibia vBMD increased ($p < 0.02$) with GOS supplementation. Trabecular-rich bones, that is, those that rapidly turn over, were most benefited. Regression modeling showed that GOS benefited calcium and magnesium utilization and vBMD through decreased cecal pH, increased cecal wall and content weight, and increased proportion of bifidobacteria.

**KEYWORDS:** galactooligosaccharide (GOS), mineral absorption, bone mineral density, nondigestible oligosaccharides

## ■ INTRODUCTION

One approach to reducing the health care burden of osteoporosis is to improve peak bone mass and strength during growth. Dietary components that may allow for an enhanced absorption of calcium include prebiotic nondigestible oligosaccharides (NDO).[1,2] Prebiotics are nondigestible ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of a limited number of bacteria in the colon and, thus, attempt to improve host health.[3] Galactooligosaccharides (GOS), an example of a prebiotic naturally present in human milk, are composed of chains of galactose and a glucose endpiece, varying in type of linkages and chain length (range 2−8). Commercial GOS are synthesized from lactose using the transglycosylation activity of $\beta$-galactosidase from microorganisms.[4]

Prebiotics are resistant to gastric acid and hydrolysis by mammalian enzymes, lack significant gastrointestinal absorption, undergo fermentation by the intestinal microbiota, and selectively stimulate either the growth or the activity of intestinal bacteria with demonstrable benefit for host health and well-being.[5] One of the mechanisms of action of GOS for a beneficial effect on mineral absorption is thought to be due to their resistance to digestion in the small intestine and the fact that they undergo fermentation in the large intestine, resulting in short-chain fatty acid (SCFA) production.[6,7] The resulting lower cecal pH may increase soluble calcium pools, thereby enhancing calcium absorption from the colon and cecum.[8,9] The role of intestinal bacteria on GOS-induced enhancement of calcium and magnesium apparent absorption was demonstrated by inhibiting bacterial fermentation through neomycin (antibiotic) feeding.[10] Several human studies have shown that GOS can positively influence bacterial communities in the gut.[11−14] However, the direct contribution of the GOS-induced changes in microbiota to mineral absorption has not been studied. Trophic effects of SCFAs on the proliferation of cecal epithelial cells have been reported in both rats[15,16] and humans,[17] allowing for an increase in the surface area available for absorption. A direct uptake of SCFAs and possibly an increased transcription of genes linked to transcellular and paracellular calcium absorption processes may also be involved.[18]

Several animal studies[8−10,19,20] and one human study[21] have shown positive effects of GOS supplementation on calcium absorption. The rat studies were in weanling rats, mimicking human skeletal growth with the exception of one study, in which adult rats were ovariectomized.[8] Apparent calcium absorption increased in rats at levels of 1.5, 5, and 10% GOS by weight. Bone calcium content[8,9,19] and bone ash weight[8,19] were improved by GOS

supplementation. The influence of GOS on gut microbiota has been studied more extensively in humans. Studies in the adults as well as infants demonstrated that consumption of GOS stimulates the growth of bifidobacteria.[11−14]

The aim of this study was to measure the dose−response effect of feeding a diet containing 0, 2, 4, 6, and 8% GOS by weight on cecal pH, cecal wall and content weight, mineral utilization, bone geometry, bone mineral density, bone mineral content, bone breaking parameters, and gut microbiota over an 8 week feeding period in growing rats. We tested the hypotheses through a modeling analysis that increasing GOS supplementation would reduce cecal pH, increase cecal content and wall weights, and favor bifidobacteria, resulting in improved mineral utilization and bone properties.

## ■ MATERIALS AND METHODS

**Animals.** Seventy-five male Sprague−Dawley weanling rats were purchased at 4 weeks of age from Harlan (Indianapolis, IN). The age of 4 weeks was chosen to mimic human skeletal growth in children. They were housed individually in stainless steel cages, in a temperature- and light-controlled environment (12 h from 3:00 a.m. to 3:00 p.m.; temperature, 20 °C; relative humidity, 30−40%). Rats had free access to their respective diets and double-deionized water. All animal housing and procedures were approved by the Purdue Animal Care and Use Committee.

**Study Design.** Rats were placed on an American Institute of Nutrition (AIN) nutritionally adequate diet (AIN 93-G) for 12 days to allow them to adapt to their new environment. Fecal samples were collected for baseline microbial profiling prior to initiating the test diets. Rats were then randomized into one of five groups ($n = 15$/group) so that the average weights of the groups were similar. Groups differed in the percentage GOS by weight to be incorporated into the diet replacing cornstarch: group 1, 0% GOS, control group; group 2, 2% GOS; group 3, 4% GOS; group 4, 6% GOS; and group 5, 8% GOS. The GOS syrup product used (Vivinal GOS 90, a prebiotic ingredient containing about 90% galactooligosaccharides, FrieslandCampina Domo, Amersfoort, The Netherlands) was analyzed for GOS content using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD[22]) to determine the amount of syrup required to achieve a 2% GOS, 4% GOS, 6% GOS, and 8% GOS by weight diet. Energy densities of the diets were calculated using 2 kcal/g GOS syrup, which resulted in the following calculated energy densities in the diets: 3.80 kcal/g (0% GOS), 3.75 kcal/g (2%), 3.7 kcal/g (4%), 3.66 kcal/g (6%), and 3.61 kcal/g (8%). The lactose contents of the final diets were 0.1, 0.3, 0.4, and 0.5%, respectively. The lactose content of the diets was not corrected as these amounts are negligible and not expected to influence gut microbiota. Diet samples were analyzed for calcium content using inductively coupled plasma optical emulsion spectrometry (ICPOES) model Optima 4300 DV (Perkin-Elmer, Shelton, CT). The target calcium concentration of the AIN 93-G diets was 0.5%; by analysis, the average was 0.45%. Adequate calcium levels were used because stimulation of net calcium absorption by GOS was observed in rats fed diets adequate in calcium, but not inadequate dietary calcium.[19]

Rats were split into two sets (two sacrifice groups) to accomplish the many tasks at the end of study. Sampling was across groups to minimize any effects of the slightly different lengths of intervention of 1 week on bone properties. The first set ($n = 8$ per group) was fed test diets for 8 weeks, and the second set ($n = 7$ per group) was fed for 9 weeks. Food intake (adjusted for spillage) was recorded twice weekly, and animal weights were recorded at least weekly to monitor animal growth and for calculation of food efficiency ratios (FERs). FERs were calculated as total change in body weight per gram of food consumed at 8 weeks of intervention. One week before sacrifice, rats were rotated into metabolic cages for 4 days to collect urine and feces in 24 h pools. Mineral balance

and net absorption were determined from these samples on days 2−4 in the metabolic cages as described below.

To determine true fractional absorption by $^{45}$Ca femur uptake, 15 $\mu$Ci of $^{45}$Ca (Perkin-Elmer 940, Boston, MA) as $CaCl_2$ was given to 13 rats per group via a 5 g test meal during the first day in the metabolic cages for determining mineral balance. Intraperitoneal injections containing 5 $\mu$Ci of $^{45}$Ca were administered to two rats per group to mimic 100% calcium absorption. Two rats given the oral tracer were excluded because they consumed <0.5 g of the test meal.

Rats were killed by $CO_2$ overdose. Cecal contents were removed, and cecal pH was measured twice using the Oakton Waterproof Big Display pH Spear (Oakton Eutech Instruments). The cecal wall was then flushed with 0.9% saline, blotted dry with filter paper and weighed, and then stored at −80 °C for future analysis. Femur bones and tibia (loaded) bones were excised, cleaned of muscle tissue, and stored for analysis.

**Bone Analysis.** Bones were chosen to represent non-weight-bearing (femur) and weight-bearing (tibia) bones. Right femurs and tibiae were wrapped in saline-soaked gauze and stored at −20 °C for evaluation of bone mechanical properties. Left femurs and tibiae were placed in 70% ethanol and stored for later imaging analysis.

Right femurs and tibiae were thawed overnight at 4.5 °C. Growth measurements included length and widths of right femurs, measured in millimeters using digimatic calipers (model CD-6″CX, Mitutoyo Corp., % CV = 0.05), and anterior/posterior (Ant/pos) width (% CV = 0.87) and medial/lateral (med/lat) width (% CV = 0.48) at the midshaft of the bone. Bone mineral density (g/cm³) of whole femurs was determined by water displacement (model A6204, Mettler-Toledo) after soaking in physiological saline (0.9% NaCl) for 36 h.

Breaking strength at the midpoint of each femur and tibia was determined by a three-point bending test using a materials testing machine (Alliance RT/5; MTS Systems, Eden Prairie, MN). A preload of 30 N was applied to the midshaft femurs placed ventral side up. Ultimate force (F) and stiffness (S) were determined by load−displacement data using TestWorks software, version 4.06. Broken femurs were dissolved in 3 mL of (70% w/v) nitric acid, diluted with 2% $HNO_3$, and measured by ICPOES to determine bone calcium content.

Imaging by peripheral quantitative computed tomography (pQCT) (XCT Research SA, Stratec Medizintechnic) was performed on femurs and tibiae. Each scan was acquired with a 0.12 mm voxel size, and the scan line was adjusted using the scout view of the software (Stratec, version 5.50 d; Stratec Medizintech). Constant thresholds of 500 and 900 mg/cm₃ were used to segment bone from marrow and trabecular from cortical bone. Total, trabecular, and cortical volumetric bone mineral density (vBMD), BMC and area, cortical thickness, periosteal circumference, and endosteal circumference of distal, midshaft, and proximal (20, 50, and 88% from distal end, respectively) femurs were measured.[23] For tibiae, scans were taken at 1 mm below the proximal condyle and 50 and 12% from the distal end.

**Apparent Absorption and Retention of Minerals.** Mineral retention was determined for calcium and magnesium. Mineral content of diet, urine, and feces from the 3 day balance period was determined by ICP-OES (Optima 4300 DV, Perkin-Elmer). Mineral retention was calculated as intake minus feces minus urine. Net absorption efficiency was calculated as intake minus feces/intake.

**Calcium Absorption by Femur $^{45}$Ca Uptake.** Percent true calcium absorption was determined using the $^{45}$Ca femur uptake method as described by Koo et al.[25] Calcium absorption efficiency was determined using the following equation:[24]

$$\% \text{ calcium absorption} = \frac{(\%\text{dose in femur of oral test group}) \times 100}{(\% \text{ dose in femur of intraperitoneally administered group})}$$

**Fecal Microbial Community Analysis.** *DNA Extraction and PCR.* Microbial analysis was done on a subset of samples consisting of five randomly chosen rats from each group of 15 on feces from 1 day
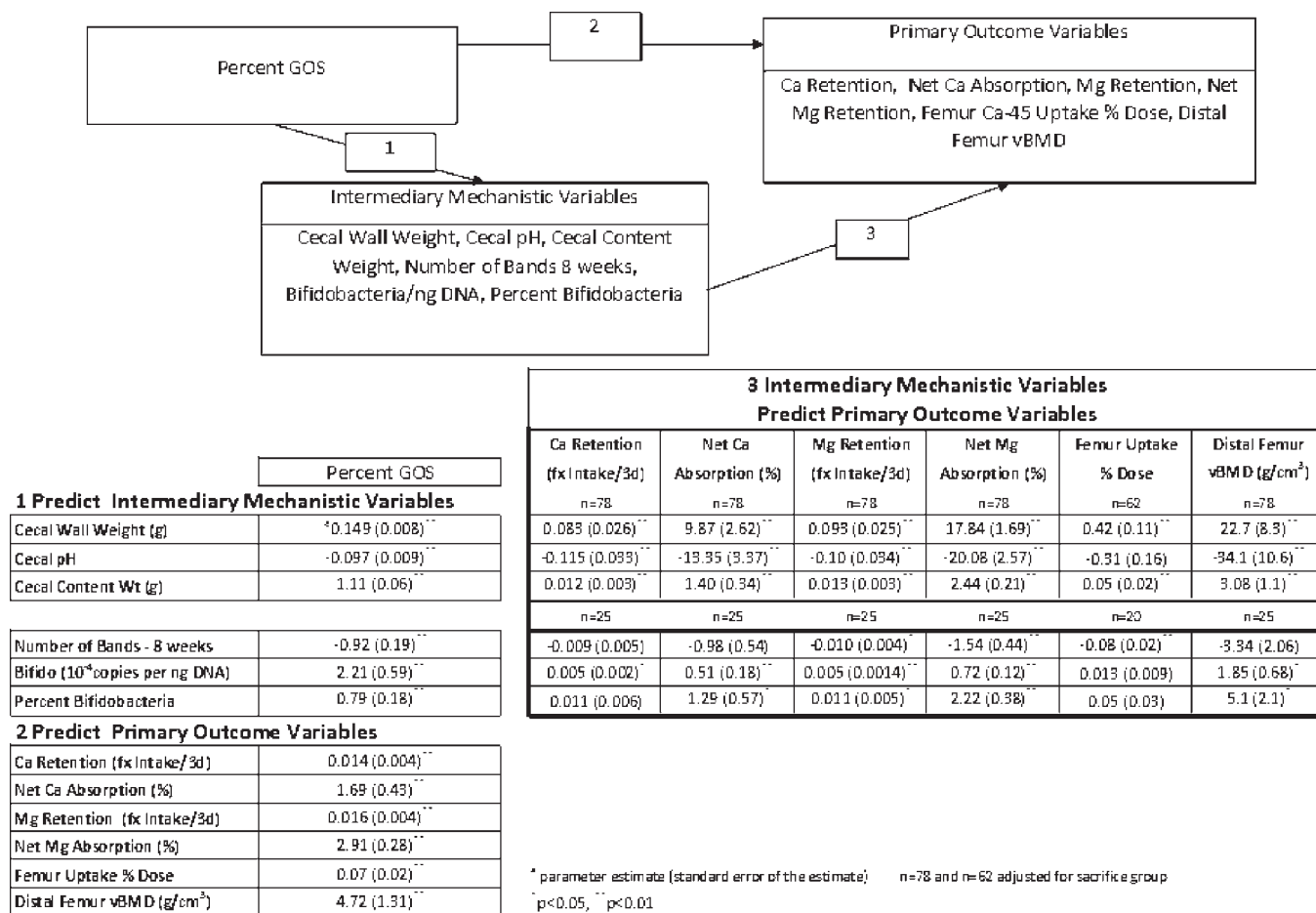
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dx.doi.org/10.1021/jf2009777 |*J. Agric. Food Chem.* 2011, 59, 6501−6510

|  | Percent GOS |
|---|---|
| **1 Predict Intermediary Mechanistic Variables** | |
| Cecal Wall Weight (g) | *0.149 (0.008)** |
| Cecal pH | -0.097 (0.009)** |
| Cecal Content Wt (g) | 1.11 (0.06)** |
|  | |
| Number of Bands - 8 weeks | -0.92 (0.19)** |
| Bifido ($10^4$ copies per ng DNA) | 2.21 (0.59)** |
| Percent Bifidobacteria | 0.79 (0.18)** |
| **2 Predict Primary Outcome Variables** | |
| Ca Retention (fx Intake/3d) | 0.014 (0.004)** |
| Net Ca Absorption (%) | 1.69 (0.43)** |
| Mg Retention (fx Intake/3d) | 0.016 (0.004)** |
| Net Mg Absorption (%) | 2.91 (0.28)** |
| Femur Uptake % Dose | 0.07 (0.02)** |
| Distal Femur vBMD (g/cm³) | 4.72 (1.31)** |

**3 Intermediary Mechanistic Variables Predict Primary Outcome Variables**

| Ca Retention (fx Intake/3d) | Net Ca Absorption (%) | Mg Retention (fx Intake/3d) | Net Mg Absorption (%) | Femur Uptake % Dose | Distal Femur vBMD (g/cm³) |
|---|---|---|---|---|---|
| n=78 | n=78 | n=78 | n=78 | n=62 | n=78 |
| 0.083 (0.026)** | 9.87 (2.62)** | 0.093 (0.025)** | 17.84 (1.69)** | 0.42 (0.11)** | 22.7 (8.3)** |
| -0.115 (0.033)** | -13.35 (3.37)** | -0.10 (0.034)** | -20.08 (2.57)** | -0.31 (0.16)* | -34.1 (10.6)** |
| 0.012 (0.003)** | 1.40 (0.34)** | 0.013 (0.003)** | 2.44 (0.21)** | 0.05 (0.02)** | 3.08 (1.1)** |
| n=25 | n=25 | n=25 | n=25 | n=20 | n=25 |
| -0.009 (0.005) | -0.98 (0.54) | -0.010 (0.004)* | -1.54 (0.44)** | -0.08 (0.02)** | -3.34 (2.06) |
| 0.005 (0.002)* | 0.51 (0.18)** | 0.005 (0.0014)** | 0.72 (0.12)** | 0.013 (0.009) | 1.85 (0.68)** |
| 0.011 (0.006) | 1.29 (0.57)* | 0.011 (0.005)* | 2.22 (0.38)** | 0.05 (0.03) | 5.1 (2.1)* |

* parameter estimate (standard error of the estimate)     n=78 and n=62 adjusted for sacrifice group
* $p < 0.05$, ** $p < 0.01$

**Figure 1.** Model for regression analysis and results of regression analysis testing: 1, influence of GOS on mechanisms that influence skeletal health; 2, dose—response effect of GOS on primary end points; 3, effect of predictors on primary end points. GOS supplementation influenced all intermediary and primary end points. Cecal characteristics and some microbial profile characteristics influenced primary end points.

before dietary treatment was started and from one day during week 8. Feces from each rat for each day were combined into a sterile sampling bag and homogenized using a mortar and pestle. Total DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Irvine, CA) according to the manufacturer's protocol with the exception that 50 mg of homogenized rat feces was used rather than soil.[25] DNA was quantified using a NanoDrop ND-1000 (Thermo Scientific). Microbial communities were analyzed using 16S rRNA gene PCR-DGGE and group-specific PCR primers for the bacteria community (PRBA338F, 5′ **CGC CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG** GAC TCC TAC GGG AGG CAG CAG 3′; the GC-clamp is in boldface and PRUN518R, 5′ ATTA CCG CGG CTG CTGG 3′)[26,27] and bifidobacteria community (Bif164F, 5′-GGG TGG TAA TGC CGG ATG-3′ and Bif662R, 5′-**CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G**-CCA CCG TTA CAC CGG GAA-3′; the GC-clamp is in boldface).[28] On the basis of preliminary empirical experiments optimized PCR conditions for both primer sets were as follows: PCR solutions included primers ($0.5\,\mu M$ of each), PCR buffer ($1\times$) (Promega, Madison, WI) supplemented with $MgCl_2$ (final concentration of 3.5 mM $MgCl_2$), dNTPs ($0.8\,\mu M$), BSA (0.1%), Taq polymerase, and DNA template (5−10 ng). All experiments included PCR solution with no DNA template as negative controls. PCR was performed (PTC thermocycler; MJ Research Inc., Watertown, MA) using an initial denaturation at 94 °C for 5 min followed by 25 cycles of denaturation at 92 °C for 30 s, annealing at 55 °C for 30 s (for bacteria primers) or 62 °C for 30 s (for bifidobacteria primers) and extension at

72 °C for 30 s with a final extension at 72 °C for 15 min. Quality and quantity of PCR products was determined by comparison to size markers (100 bp marker) after separation by agarose gel electrophoresis.

*DGGE.* Equivalent quantities of PCR amplicons were separated by DGGE (DCode Universal Mutation Detection System; Bio-Rad Laboratories, Hercules, CA)[29] using 8% (w/v) polyacrylamide gels (40% acrylamide/bis solution, 37.5:1) in $1\times$ TAE (40 mM Tris, 20 mM acetate, 1.0 mM $Na_2$-EDTA) with denaturing gradients (of 30−50, 35−55, and 45−65%), where a 100% denaturant contained 7 M urea and 40% (v/v) deionized formamide. Three different gradients were used for bacterial community analysis of each sample to optimize band separation to facilitate profile comparisons. To aid between gel comparisons DGGE band migration markers were included on all gels. Markers were composed of PCR products of rRNA genes from organisms that were amplified independently using primers PRBA338F and PRUN518R and then combined in equal quantities to make a ladder that covered the entire range of band separation in all fingerprint profiles. Electrophoresis was initiated at 15 V for 10 min and then increased to 200 V for 4−5 h. Electrophoresis buffer ($1\times$ TAE) was maintained at 60 °C throughout. Gels were then stained using SYBR Green I nucleic acid stain (Cambrex Bio Science, Rockland, ME), visualized on a UV transilluminator, and photographed (UVP BioImaging system, UVP LLC, Upland, CA). The DGGE fingerprint of rRNA gene PCR products provides an approximation of the number of dominant populations and their relative proportion within a given community. Theoretically, each unique band in the fingerprint represents a distinct

**Table 1. Weight Change, Food Intake, and Food Efficiency Ratio in Rats after Feeding of Different Levels of GOS[a]**

| measurement | group 1 (0% GOS) | group 2 (2% GOS) | group 3 (4% GOS) | group 4 (6% GOS) | group 5 (8% GOS) | p value |
|---|---|---|---|---|---|---|
| av daily GOS intake (g) | 0 | 0.38, 0.02 d | 0.77, 0.05 c | 1.18, 0.08 b | 1.65, 0.17 a | **<0.0001** |
| initial wt (g) | 128.3, 4.5 | 128.0, 6.8 | 130.5, 7.8 | 127.8, 10.4 | 126.5, 7.5 | ns |
| final wt[b] (g) | 409.2, 22.9 | 407.2, 27.5 | 414.7, 22.4 | 408.1, 23.9 | 412.9, 25.7 | ns |
| wt change[b] (g) | 280.9, 22.6 | 279.2, 25.1 | 284.3, 21.0 | 280.3, 21.3 | 286.4, 21.0 | ns |
| total food intake[b] (g) | 1280, 122 | 1281, 77 | 1298, 86 | 1317, 77 | 1380, 137 | **0.045** |
| FER | 0.22, 0.02 | 0.22, 0.02 | 0.22, 0.02 | 0.22, 0.02 | 0.21, 0.02 | ns |

[a] Values are expressed as the mean, SD. $n = 15-16$/group. Multiple comparisons were performed using Tukey's procedure. GOS, galactooligosaccharides. Different letters indicate significant differences among groups ($p < 0.05$); ns = not significant. [b] To 8 weeks after initiating intervention.

**Table 2. Cecal pH and Cecal Wall Weight at Sacrifice in Rats after Feeding of Different Levels of GOS[a]**

| measurement | group 1 (0% GOS) | group 2 (2% GOS) | group 3 (4% GOS) | group 4 (6% GOS) | group 5 (8% GOS) | p alue |
|---|---|---|---|---|---|---|
| cecal pH | 7.12, 0.14 a | 6.96, 0.20 ab | 6.75, 0.31 bc | 6.58, 0.27 c | 6.34, 0.24 d | **<0.0001** |
| cecal wall wt (g) | 0.83, 0.11 d | 0.94, 0.19 d | 1.30, 0.22 c | 1.57, 0.24 b | 2.00, 0.18 a | **<0.0001** |
| cecal content wt (g) | 2.51, 0.57 d | 3.64, 0.95 d | 5.50, 1.74 c | 8.71, 2.19 b | 11.11, 1.58 a | **<0.0001** |

[a] Values are expressed as the mean, SD. $n = 15-16$/group. Multiple comparisons were performed using Tukey's procedure. Different letters indicate significant differences among groups ($p < 0.05$).

population. By comparing fingerprints derived from different samples, one can quantify the degree of relatedness across communities.

*Quantitative PCR.* The same primer sets (without the GC clamp) and thermal cycle conditions that were used to perform PCR-DGGE were used to quantify bifidobacteria and total bacteria 16S rRNA gene copy numbers in the rat fecal samples. Each Q-PCR was done in triplicate using iQ SYBR Green Supermix (Bio-Rad) and 1 ng of template DNA. Fluorescence intensity was acquired at 72 °C (above the melting point of primer dimers). To determine the melting temperature and confirm PCR product specificity, a melting curve was acquired after every run by heating amplicons from 4 °C below the annealing temperature ($T_a$) to 97 °C. Data were analyzed using iCycler Optical System Interface software (version 2.3; Bio-Rad). The threshold cycle (Ct) was defined as the cycle number at which a statistically significant increase in fluorescence was detected. Clones of 16S rRNA gene from *Bifidobacterium bifidum* were used as positive controls and to make standard curves for quantification. Gene copy number was calculated from the concentration of the extracted plasmid DNA clone assuming $1.096 \times 10^{-12}$ g/bp. All standard curves were generated from 10-fold serial dilutions of plasmid DNA and were subjected to real-time Q-PCR assay in triplicate. Gene copy numbers were calculated on the basis of total DNA and percentage of bifidobacteria gene copies in total bacteria.

**Statistical Analysis.** *Study Design.* Sample sizes were chosen on the basis of power calculations for percent calcium absorption measured by the femur uptake method using results from a previous study.[30] Assuming a variance of 5% for a treatment effect of 10%, 12 rats per group would yield 87% power at $p < 0.05$. We chose a sample size of 15 rats per group to allow for losses of data points.

*Data Analysis.* Data were analyzed using SAS version v9.2. (SAS Institute, Inc., Cary, NC). Results were expressed as mean values, standard deviation (SD). One-way analysis of variance followed by the Tukey multiple-comparisons procedure was used to compare means of the five diet groups. Regression analysis (Figure 1) was performed to analyze the relationships between variables, controlling for sacrifice group when slight differences in length of intervention period could have affected results. Multiple regression modeling techniques were used to explore the models with several explanatory variables.

*PCR-DGGE Fingerprint Analysis.* Digital gel images were analyzed and PCR-DGGE fingerprint profiles compared using BioNumerics software (version 5.01, Applied Maths, Kortrijk, Belgium). Because three different gradients were run for each sample, the analyzed profiles were composed of three sections, one for each gel that optimized band resolution. Gels were scored for the presence or absence of DNA bands, and all scores were combined using the composite data set function in BioNumerics to make a complete profile. Only bands with intensities of ≥3% were scored as present. DNA banding patterns were compared using Dice Similarity indices, and variation between profiles was assessed by principal component analysis (PCA).

## ■ RESULTS

**Weight Gain, Food Intake, and Food Efficiency Ratio.** There were no significant differences ($p > 0.05$) due to GOS in the average weight gained or average final weight among groups for all rats combined (Table 1). Although the effect of GOS on total food intake was borderline significant ($p = 0.0486$), there were no specific significant group differences by Tukey's comparison test. There were no significant differences in FER among groups.

**Cecal pH and Cecal Wall Weight.** A significant ($p < 0.0001$) and dose-dependent decrease in cecal pH was observed (Table 2). This was accompanied by a significant ($p < 0.0001$) and dose-dependent increase in cecal wall weight and cecal weight.

**Microbial Analysis.** *Bacteria Community.* Comparison of bacteria PCR-DGGE profiles from 25 rats examined before and after treatment ($n = 50$) showed differences in band numbers and community structure with GOS treatment. Band numbers averaged 25.5 in treatment groups at week 1, and after 8 weeks, they averaged 28.2 (0% GOS), 24.0 (2% GOS), 22.8 (4% GOS), 24.4 (6% GOS), and 18.8 (8% GOS). There was no significant difference in band numbers in rat profiles at week 1, but after 8 weeks there was a significant decrease ($p < 0.05$) in band numbers with increasing amounts of GOS consumed. An example of PCR-DGGE profiles of rats on 0 versus 8% GOS diet at week 8 illustrates differences in band numbers and banding patterns (Figure 2).

Pairwise comparisons of the 50 rat PCR-DGGE profiles using Dice Similarity values (data not shown) or major sources of variation using PCA (Figure 2) showed similar trends. Although these two ordination methods are based on different algorithms, they both showed clustering of profiles by GOS
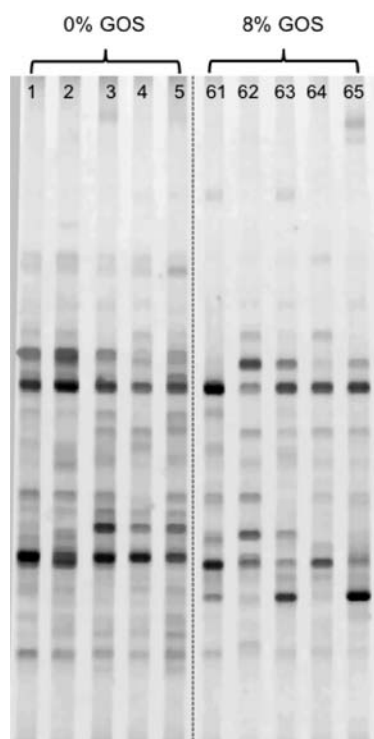
**Figure 2.** Comparison of bacterial community structure in rats at week 8 after being fed 0% (lanes 1−5) and 8% GOS (lanes 61−65) (lanes are labeled by rat designation numbers). Fingerprints were generated by PCR amplification of the V3 region of 16S rRNA gene and separated by denaturing gel electrophoresis DGGE using a gradient range of 32−55% run at 200 V for 4.5 h.

treatment. The major separation of profiles was between initial and week 8 profiles (Figure 2, PCA1). Then the week 8 profiles separated from 0 to 8% GOS (Figure 3, PCA2). The analysis also illustrates that there is some overlap between microbial communities in rats on the 2, 4, and 6% GOS diets as shown in Figure 2.

*Bifidobacteria Community.* PCR using bifidobacteria-specific primers indicated this group was present in all samples before and after dietary treatment. DGGE analysis of amplified PCR products revealed a range of one to four bands in the samples with one band common to the majority of profiles (data not shown). Comparison of baseline and week 8 PCR-DGGE profiles revealed differences in bifidobacteria profiles in 14 of the 25 rats examined, but these changes were not associated with any particular dietary treatment. Because there was no obvious GOS treatment associated change in bifidobacteria community structure, Q-PCR was used to look at quantitative effects of GOS. There were significantly higher absolute numbers of bifidobacteria in the higher GOS treatments compared to the control, but only 8% was significant ($p < 0.0005$, Table 3). Cecal wall weight, intestinal weight, and cecal pH were all correlated with percent bifidobacteria and diversity of microbiota (number of bands at week 8).

**Calcium Absorption and Mineral Retention.** Net calcium and magnesium absorption and retention were greater with GOS intake (Table 4). For magnesium absorption, the effect was significant at 2% GOS and for calcium, at 6% GOS. Increased urinary calcium weakly reflected higher net calcium absorption; the higher calcium absorption led to higher calcium retention in

the rats fed higher GOS diets. Femur $^{45}$Ca uptake was 54, 14 (0% GOS); 56, 20 (2% GOS); 62, 14 (4% GOS); 60, 20 (6% GOS); and 74, 10 (8% GOS) ($p = 0.02$). Urinary potassium and sodium excretions were not affected by GOS.

**Bone Properties.** BMD of the whole femur determined via underwater weighing in all GOS test groups was significantly greater than the control without GOS ($p = <0.0001$), representing an increase in BMD of 2.4% (2% GOS), 1.8% (4% GOS), 3.1% (6% GOS), and 2.9% (8% GOS) relative to the control (Table 5). Parameters of breaking strength, such as ultimate load (F), but not stiffness (S), of the femur measured via three-point bending were significantly ($p = 0.0222$) different among treatment groups. No significant differences ($p > 0.05$) among groups in length and ant/pos or med/lat width of the femur or total calcium content were observed.

pQCT showed several bone parameters improved by GOS feeding (Table 6), especially in distal femurs and proximal tibiae (for other sites, see the Supporting Information). For the distal femurs and the proximal tibiae, total vBMD was greater in 6 and 8% GOS groups than the control and in 4% GOS groups for the distal femur. Distal femur was significantly increased by GOS supplementation. GOS feeding affected trabecular more than cortical vBMD. There were statistically significant group differences in cortical area and thickness and periosteal and endosteal circumference, but GOS groups were not consistently better than the control. Differences were less clear for the midshaft femurs, but cortical vBMD was significantly lower above 2% GOS. Bone size was not generally affected by GOS at the midshaft. At that site, cortical thickness was greater above 6% GOS. For the midshaft tibia, trabecular vBMD decreased in a dose-dependent manner, which reached significance at 8% GOS.

**Relationships and Mechanisms.** Regression modeling was conducted to examine relationships and test potential mechanisms for the effect of GOS on primary mineral balance and bone healthy measures (Figure 1). Dietary GOS significantly increased net calcium and magnesium absorption and retention, femur $^{45}$Ca uptake, and distal femur vBMD. For every 1% increase in GOS added to the diet by weight, net calcium absorption increased by 1.69%, net magnesium absorption by 2.91%, and distal femur by 4.72 g/cm$^3$. Dietary GOS strongly influenced cecal pH, cecal wall weight and content weight, and gut microbiota community structure as well as proportion of bifidobacteria. All of these markers of potential mechanisms generally significantly influenced all primary outcome measures. Not all of the microbial measures significantly predicted outcome variables, but these were taken on only a subset of the sample, that is, five rats per group. For the smaller data set ($n = 25$), the variables for which the final equations included only one explanatory variable were bifiobacteria for calcium retention/calcium intake and net calcium absorption/calcium intake, number of bands for femur uptake percent dose, and urinary sodium/sodium intake for distal femur vBMD. For the large data set ($n = 78$), these were cecal content weight for calcium retention/calcium intake and net calcium absorption/calcium intake, and cecal wall weight for femur uptake percent dose; cecal pH and urinary sodium were both statistically significant at $p < 0.01$ in a model for distal femur vBMC that included both predictors. For the smaller data set ($n = 25$), these were bifidobacteria for calcium retention/calcium intake and net calcium absorption/calcium intake, number of bands for femur uptake percent dose, and urinary sodium/sodium intake for distal femur vBMD.
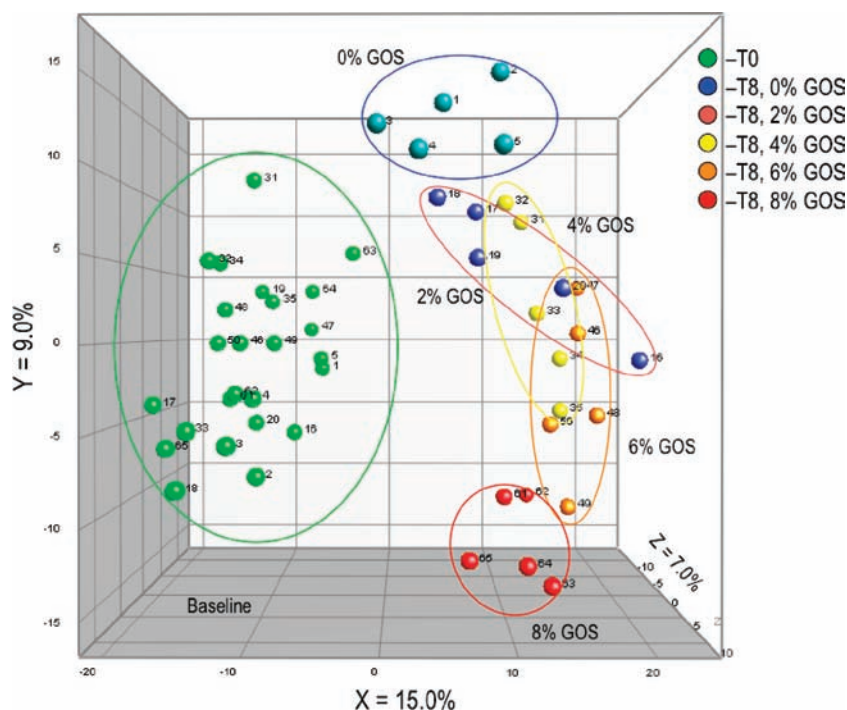
**Figure 3.** Principal component analysis (PCA) of bacterial PCR-DGGE fingerprint profiles of rats before and after 8 weeks of feeding GOS at different levels (0, 2, 4, 6, and 8%). Analysis is based on the presence and absence of bands in pairwise comparison of the 50 rat PCR-DGGE fingerprints. The principal component axes (X, Y, Z) list the percentage of variation explained in each component. The X-axis separates by 0 versus 8 weeks and the Y-axis by GOS concentration. BioNumerics software was used to analyze the combined data from multiple gels run at different gradients.

**Table 3. Bifidobacteria and Total Bacteria 16S rRNA Gene Copy Numbers in Rats after Feeding of Different Levels of GOS[a]**

| measurement | group 1 (0% GOS) | group 2 (2% GOS) | group 3 (4% GOS) | group 4 (6% GOS) | group 5 (8% GOS) | p value |
|---|---|---|---|---|---|---|
| bifidobacteria ($10^{-4}$ copies/ng DNA) | 11.28, 3.08 b | 13.79, 4.16 b | 10.34, 11.39 b | 19.51, 9.49 ab | 30.51, 7.00 a | **0.01** |
| log bifidobacteria | 2.40, 0.26 ab | 2.59, 0.29 ab | 1.65, 1.37 b | 2.88, 0.48 ab | 3.52, 0.29 a | **0.01** |
| total bacteria ($10^{-5}$ copies/ng DNA) | 45.9, 4.46 b | 86.44, 19.24 a | 51.14, 7.89 b | 54.12, 17.86 b | 35.14, 11.96 b | **0.0001** |
| log total bacteria | 3.82, 0.10 bc | 4.44, 0.24 a | 3.93, 0.16 bc | 3.95, 0.29 b | 3.52, 0.29 c | **<0.0001** |
| % bifidobacteria of total bacteria | 2.52, 0.94 b | 1.62, 0.42 b | 1.88, 2.00 b | 3.95, 2.22 b | 9.22, 3.01 a | **<0.0001** |

[a] Values of 16S rRNA gene copy numbers are expressed as the mean, SD. $n = 5$/group. Multiple comparisons were performed using Tukey's procedure. Different letters indicate significant differences among groups ($p < 0.05$); ns = no significant difference between groups ($p > 0.05$). qPCR reaction efficiencies > 90%; $R^2$ of standard curves > 0.996 with slopes ranging from −3.181 to −3.586. GOS, galactooligosaccharides.

## ■ DISCUSSION

This study extends previous studies showing GOS-enhanced calcium absorption and retention and bone calcium content[8−10,19−21,31] with measures of [45]Ca femur uptake, retention of additional minerals, bone geometry and density, and gut microbial communities in growing rats over a range of GOS intakes.

Net mineral absorption from the balance study was significantly higher by GOS feeding. The ability for each percent increase in dietary GOS to enhance calcium absorption by almost 1.7% and that of magnesium by almost 3% is an important contribution of this functional food ingredient. Others have shown increases with GOS consumption at 1.5−10% by weight.[8−10,19,20,31] Perez-Conesa et al.[9,20] also demonstrated in weanling rats a significant increase in calcium absorption at the low level of 1.2% GOS by weight. Perez-Conesa et al.[20] performed three balance studies at 8−10, 18−20, and 28−30 days. GOS enhanced net calcium absorption and retention, but efficiency decreased with age. Our rats were older and fed longer (8−9 weeks) and some adaptation to the prebiotic could have occurred. The possibility of adaptation

of intestinal calcium absorption over time was first reported by Brommage et al.,[32] who showed dietary lactulose enhanced calcium absorption in rats on the first day of the study but not by the seventh day of the study. The authors hypothesized that this adaptive response of intestinal calcium absorption over time occurred by a down-regulation of the active, transcellular route of calcium absorption, which counterbalanced the lactulose-induced increase in passive, paracellular calcium absorption. Brommage et al.[32] proposed that the capacity for the adaptation was limited and suggested that providing higher levels of lactulose in their study may have resulted in continued elevation of intestinal calcium absorption. We showed that after 8 weeks of feeding, GOS effects were apparent at 6% and higher. GOS-enhanced calcium utilization was further demonstrated by [45]Ca femur uptake ($p = 0.02$). Almost 76% of the variation in net calcium absorption could be explained by crypt depth of the distal colon in weanling rats fed GOS.[9] In that study, in contrast to the colon, cecal crypt depth was unaffected by GOS feeding and the pH of the colonic content was related to crypt depth ($r = 0.644$). Crypt depth was not measured

**Table 4. Mineral Balance in Rats after Feeding of Different Levels of GOS[a]**

| measurement | group 1 (0% GOS) | group 2 (2% GOS) | group 3 (4% GOS) | group 4 (6% GOS) | group 5 (8% GOS) | p value |
|---|---|---|---|---|---|---|
| | | | **Calcium** | | | |
| food intake (g/3 days) | 60.0, 5.4 | 58.5, 3.3 | 61.0, 5.3 | 60.5, 4.2 | 63.2, 7.3 | ns |
| calcium intake (mg/3 days) | 265, 24 ab | 254, 14 b | 275, 24 ab | 278, 19 a | 281, 32 a | 0.0105 |
| fecal calcium (mg/3 days) | 198, 23 | 180, 23 | 196, 26 | 179, 23 | 173, 43 | ns |
| urine calcium (mg/3 days) | 2.5, 0.9 c | 2.7, 0.9 c | 4.8, 1.7 b | 6.1, 1.7 b | 8.9, 2.7 a | <0.0001 |
| calcium retention (mg/3 days) | 64, 25 b | 71, 29 ab | 75, 24 ab | 94, 27 ab | 99, 51 a | 0.01 |
| calcium retention (fx intake/3 days) | 0.24, 0.09 b | 0.28, 0.10 ab | 0.27, 0.08 ab | 0.33, 0.09 ab | 0.35, 0.16 a | 0.03 |
| net calcium absorption (%) | 24.8, 8.6 b | 28.7, 10.4 ab | 28.8, 8.2 ab | 35.7, 8.7 a | 38.0, 15.9 a | 0.01 |
| | | | **Magnesium** | | | |
| magnesium intake (mg/3 days) | 30.4, 2.7 ab | 27.9, 1.6 b | 30.2, 2.6 ab | 30.9, 2.1 a | 31.2, 3.6 a | 0.01 |
| fecal magnesium (mg/3 days) | 12.9, 2.1 a | 9.5, 1.8 bc | 11.3, 2.4 ab | 7.7, 1.5 cd | 5.8, 1.6 d | <0.0001 |
| urine magnesium (mg/3 days) | 2.3, 1.7 bc | 1.3, 1.1 c | 4.3, 2.2 ab | 3.5, 2.5 ab | 5.5, 2.1 a | <0.0001 |
| magnesium retention (mg/3 days) | 15.1, 3.5 b | 17.0, 3.1 ab | 14.6, 3.1 b | 19.7, 3.1 a | 20.0, 4.2 a | <0.0001 |
| magnesium retention (fx intake/3 days) | 0.50, 0.10 b | 0.61, 0.09 a | 0.48, 0.07 b | 0.64, 0.10 a | 0.64, 0.09 a | <0.0001 |
| net magnesium absorption (%) | 57.2, 7.2 c | 65.5, 7.3 b | 62.5, 7.0 bc | 75.2, 4.7 a | 81.4, 5.3 a | <0.0001 |

[a] Values represent the mean, SD. Different letters represent significant differences at $p < 0.05$ by Tukey's test; ns = not significant; $n = 16$/group.

**Table 5. Geometric, Bone Density, and Strength Measures of Whole Rat Bones after Feeding of Different Levels of GOS[a]**

| measurement | group 1 (0% GOS) | group 2 (2% GOS) | group 3 (4% GOS) | group 4 (6% GOS) | group 5 (8% GOS) | p value |
|---|---|---|---|---|---|---|
| | | | **Femur** | | | |
| length (mm) | 38.23, 0.62 | 37.97, 0.77 | 37.95, 0.69 | 35.44, 9.12 | 37.93, 0.74 | ns |
| ant/pos width (mm) | 3.63, 0.12 | 3.57, 0.14 | 3.61, 0.12 | 3.44, 0.56 | 3.60, 0.12 | ns |
| med/lateral width (mm) | 4.30, 0.14 | 4.26, 0.14 | 4.29, 0.11 | 4.11, 0.75 | 4.30, 0.19 | ns |
| bone calcium content (mg Ca/bone) | 144, 8 | 143, 8 | 141, 9 | 141, 13 | 142, 10 | ns |
| bone density (g/cm$^3$) | 1.431, 0.030 b | 1.466, 0.020 a | 1.457, 0.023 a | 1.479, 0.026 a | 1.472, 0.023 a | <0.0001 |
| ultimate load (N) | 130.45, 14.01 a | 128.89, 12.39 a | 139.76, 12.64 a | 130.57, 16.05 a | 141.77, 11.31 a | 0.02 |
| stiffness (N/mm) | 51.14, 9.95 | 50.39, 4.21 | 54.21, 6.76 | 54.66, 9.86 | 56.90, 6.70 | ns |
| | | | **Tibia** | | | |
| ultimate load (N) | 98.84, 17.24 ab | 84.89, 23.88 b | 100.68, 7.33 a | 99.66, 12.67 ab | 103.08, 11.39 a | 0.015 |
| stiffness (N/mm) | 37.09, 5.77 b | 38.82, 8.82 ab | 41.86, 6.40 ab | 41.45, 4.92 ab | 43.70, 5.12 a | 0.04 |

[a] Values are expressed as the mean and SD. $n = 15-16$/group. Multiple comparisons were performed using Tukey's procedure. Different letter indicate significant differences among groups ($p < 0.05$); ns = not significant.

in our study, but cecal wall weight was strongly affected by GOS feeding ($p < 0.0001$).

Magnesium absorption and retention were significantly increased by 2% GOS. The effect of magnesium absorption and retention was achieved at lower levels of GOS than for calcium. The calcium absorption response was evident by 6% GOS by weight of the diet. Others have shown improved magnesium and phosphorus absorption and retention by GOS feeding.[20,33] There was no indication that GOS influenced the acid/base balance as neither urinary potassium excretion nor sodium excretion, which is closely related to potassium metabolism,[34] were affected by GOS feeding.

Volumetric bone mineral density of the whole femur by underwater weighing and the of distal femur and proximal tibia by pQCT were positively influenced by GOS ≥2 and 6% of the diet, respectively. The specific bone sites that benefitted most have higher amounts of trabecular bone, that is, distal femur and proximal tibia. In a quadruped, the tibia is a mechanically loaded bone rather than the femur in contrast to bipeds, such as humans. At the midshaft femur, breaking strength was improved by GOS. The increase in cortical area

and thickness at the distal femur and trend for increases in these measures at the proximal tibia further indicated increased strength at these sites. Whole femur calcium content was unaffected by GOS feeding in contrast to others who have reported increases in bone calcium content with GOS feeding.[8,9,19] Whole bone mineral analysis is unable to show bone sites affected as with pQCT.

Regression analysis was used to evaluate the effect of intermediary changes induced by dietary GOS associated with proposed mechanisms of action for benefiting calcium and magnesium utilization and a primary outcome of bone strength, that is, distal femur vBMD. GOS had the greatest dose—response effect on decreasing cecal pH and increasing cecal wall and content weight. This affirms fermentation of this indigestible carbohydrate in the lower bowel. All three of these intermediary changes strongly predicted the GOS-induced increases in distal femur vBMD and mineral balance data. Microbiota also had direct effects but were less strong (lower $R^2$), likely related to the reduced number of samples included in the regression (Figure 1). The microbiota were significantly associated with cecal variables. The cecum was implicated as an important site of GOS-induced increase in calcium

**Table 6. pQCT Measures of Bone Mineral Density and Bone Geometry**[a]

| measurement | group 1 (0% GOS) | group 2 (2% GOS) | group 3 (4% GOS) | group 4 (6% GOS) | group 5 (8% GOS) | p value |
|---|---|---|---|---|---|---|
| | | | **Femur** | | | |
| distal | | | | | | |
| total vBMD $(mg/cm^3)$ | 554.05, 29.51 b | 578.70, 28.35 ab | 550.43, 29.27 b | 590.69, 33.65 a | 594.03, 33.83 a | **0.0001** |
| total area $(mm^2)$ | 19.27, 0.95 a | 18.15, 1.23 a | 19.42, 1.39 a | 18.71, 1.30 a | 19.17, 1.44 a | **0.05** |
| trabecular vBMD $(mg/cm^3)$ | 242.89, 25.83 bc | 239.88, 25.65 c | 246.61, 35.43 abc | 278.64, 39.56 a | 276.48, 41.04 ab | **0.01** |
| trabecular area $(mm^2)$ | 12.72, 0.87 ab | 11.61, 1.03 b | 13.01, 1.50 a | 12.15, 1.11 ab | 12.37, 1.20 ab | **0.02** |
| cortical vBMD $(mg/cm^3)$ | 1142.65, 21.11 | 1164.63, 22.90 | 1142.81, 37.02 | 1142.42, 27.54 | 1147.32, 26.28 | ns |
| cortical area $(mm^2)$ | 7.08, 0.44 ab | 6.97, 0.44 b | 6.97, 0.45 b | 7.27, 0.55 ab | 7.49, 0.61 a | **0.02** |
| cortical thickness (mm) | 0.51, 0.03 bc | 0.52, 0.03 abc | 0.50, 0.03 c | 0.53, 0.04 ab | 0.54, 0.04 a | **0.01** |
| periosteal circumference (mm) | 15.56, 0.39 ab | 15.09, 0.51 b | 15.61, 0.56 a | 15.33, 0.53 ab | 15.51, 0.58 ab | **0.05** |
| endosteal circumference (mm) | 12.37, 0.41 ab | 11.84, 0.53 b | 12.49, 0.62 a | 11.98, 0.55 ab | 12.10, 0.58 ab | **0.01** |
| periosteal circumference (mm) | 12.00, 0.31 | 11.77, 0.30 | 12.01, 0.29 | 11.90, 0.37 | 12.00, 0.33 | ns |
| endosteal circumference (mm) | 7.63, 0.33 a | 7.34, 0.21 b | 7.67, 0.27 a | 7.52, 0.29 ab | 7.60, 0.34 ab | **0.02** |
| | | | **Tibia** | | | |
| proximal | | | | | | |
| total vBMD $(mg/cm^3)$ | 443.51, 33.75 b | 460.17, 25.33 ab | 455.27, 36.19 ab | 481.15, 39.37 a | 482.12, 40.09 a | **0.02** |
| total area $(mm^2)$ | 26.33, 1.39 | 25.86, 2.69 | 26.03, 2.18 | 25.20, 3.21 | 26.16, 3.08 | ns |
| trabecular vBMD $(mg/cm^3)$ | 296.92, 27.31 | 304.64, 46.13 | 291.15, 30.72 | 316.84, 36.83 | 316.13, 46.25 | ns |
| trabecular area $(mm^2)$ | 20.61, 1.63 | 20.01, 3.03 | 20.09, 2.40 | 19.29, 3.31 | 20.03, 3.04 | ns |
| cortical vBMD $(mg/cm^3)$ | 984.54, 28.04 | 989.13, 52.64 | 998.61, 37.90 | 1003.8, 43.67 | 1001.83, 44.12 | ns |
| cortical area $(mm^2)$ | 6.63, 0.59 | 6.72, 0.63 | 6.93, 0.58 | 6.90, 0.72 | 7.33, 0.81 | ns |
| cortical thickness (mm) | 0.39, 0.04 | 0.40, 0.05 | 0.41, 0.04 | 0.42, 0.05 | 0.44, 0.05 | ns |

[a] Values are expressed as the mean and SD. $n = 15-16$/group. Multiple comparisons were performed using Tukey's procedure. Different letters indicate significant differences among groups ($p < 0.05$); ns = no significant difference among groups ($p > 0.05$).

absorption in wealing rats measured 4 h after injection of GOS into the cecum.[8] Also, in agreement with our data, cecal content weight predicted BMD and breaking strength in growing rats fed a variety of novel fibers, presumably due to the larger mineral pool with increased water-holding capacity.[35]

The profiles of gut microbiota were also influenced by dietary GOS and predicted calcium utilization and distal femur vBMD. The fewer numbers of bands in PCR-DGGE profiles of rats fed 8% GOS suggest that the high-GOS diet was selecting a subset of the normal rat gut microbiota. Clustering of fingerprints as seen in the PCA also illustrates the influence of different GOS levels on the gut microbial communities. Regression analysis showed these differences in microbial communities were associated with GOS intake, and correlation analysis showed microbiota and cecal characteristics were related. This is not surprising because changes in variables likely arise from the dietary intake of GOS.

In addition to differences in community structure, we also found increased numbers in bifidobacteria. This group was specifically targeted because other studies have shown by cultivation methods that bifidobacteria numbers increase in response to GOS.[12,20,36] Increases in bifidobacteria are being related to several beneficial effects, such as increases of calcium absorption, although a clear demonstration of these correlations is lacking. In this study, we demonstrated a clear association between an increase in bifidobacteria and an increase in calcium absorption. We found the same response using a cultivation-independent method. Differences in the bacterial PCR-DGGE fingerprints were apparently not directly due to changes in bifidobacteria community structure. However, the recent literature indicates there is a newly described bacterial species, *Anaerostipes caccae*,[37] that may also be involved in GOS fermentation. There are likely other bacterial species contributing to GOS fermentation. With

increasing numbers of studies using molecular genetic techniques, more new species are likely to be identified. A limitation of our study was that microbial analysis was performed in only a subset of the rats.

In conclusion, supplementation of GOS in the diets of growing rats showed beneficial effects on calcium and magnesium absorption and retention, femur $^{45}$Ca uptake, bone strength, and bone mineral density. These effects either directly or indirectly were attributed to changes in cecal pH cecal content and wall weight and number of bifidobacteria. Most benefits to mineral utilization and bone properties from GOS consumption were seen at levels of $\geq 2-6$%. Two percent GOS in a rat's diet by weight corresponds to almost 8 g of GOS/day in a human if determined on the basis of proportional body surface area.[38] Fiber intake recommendations for children are 14 g/1000 kcal,[39] but most children consume well below the recommended levels.[40] Addition of a prebiotic to foods popular with children is a strategy to improve fiber consumption. On the basis of the results of this study, GOS seem to have potential to contribute to improved peak bone mass and strength.

## ■ ASSOCIATED CONTENT

**Ⓢ Supporting Information.** pQCT measures of bone material density and bone geometry. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

**Corresponding Author**
*Postal address: Department of Foods and Nutrition, Purdue University, 700 W. State St., West Lafayette, IN 47907-2059. E-mail: weavercm@purdue.edu.

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dx.doi.org/10.1021/jf2009777 |*J. Agric. Food Chem.* 2011, 59, 6501–6510

## ■ ABBREVIATIONS USED

GOS, galactooligosaccharides; BMD, bone mineral density; BMC, bone mineral content; NDO, nondigestible oligosaccharides; DGGE, denaturing gradient gel electrophoresis.

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