**Title:** Soluble corn fiber increases calcium absorption associated with shifts in the gut microbiome: A randomized dose-response trial in free-living pubertal girls

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**Abbreviations:**

BAP – bone alkaline phosphatase

BMC – bone mineral content

BMD – bone mineral density

Ca - calcium

DEXA – dual energy x-ray absorptiometry

EAR – estimated average requirement

FxABS – fractional calcium absorption

NTX/Cre – N-telopeptides of collagen cross links corrected for urinary creatinine

OC – osteocalcin

OUT – operational taxonomic unit

PTH – parathyroid hormone

rRNA – ribosomal ribonucleic acid

SCF – soluble corn fiber

SCFA – short chain fatty acid

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**ABSTRACT**

**Background:** Soluble corn fiber (SCF, 12 g/d fiber) has been shown to increase calcium absorption efficiency, associated with shifts in gut microbiota in adolescent boys and girls participating in a controlled feeding study.

**Objective:** Study goals were to evaluate the dose response of 0, 10, 20 g fiber/d delivered via PROMITOR® SCF 85 (85% fiber) on calcium absorption, biochemical bone properties and the fecal microbiome in free-living adolescents.

**Methods:** Healthy females (n=28; aged 11-14 y), randomized into a 3-phase, double blind, cross-over study, consumed SCF for 4 weeks at each dose (0, 10 and 20 g fiber/d from SCF) alongside their habitual diet followed by 3-day clinical visits and 3-week washout periods. Stable isotope (44Ca and 43Ca) enrichment in pooled urine was measured by Inductively Coupled Plasma Mass Spectrometry. Microbial community composition of feces was assessed by high-throughput sequencing (Illumina) of PCR-amplified *16S* rRNA genes. Mixed model ANOVA and Friedman analysis were used to determine effects of SCF on calcium absorption and compare mean microbial proportions, respectively.

**Results:** Calcium absorption increased significantly with 10 (+13.3 ± 5.3%; p=0.042) and 20 g fiber/d (+12.9 ± 3.6%; p=0.026) from SCF relative to control. Significant differences in fecal microbial community diversity were found after consuming SCF (OTU measures of 601.4±83.5, 634.5±83.8, and 649.6±75.5 for 0, 10 and 20 g fiber/d, respectively; p < 0.05). Proportions of the genus *Parabacteroides* significantly increased with SCF dose (1.1±0.8%, 2.1±1.6% and 3.0±2.0% for 0, 10 and 20 g fiber/d from SCF, respectively; p<0.05). Increases in calcium absorption positively correlated with increases in *Clostridium* (r=0.44, p=0.023) and unclassified Clostridiaceae (r=0.40, p=0.040).

**Conclusions:** SCF, a non-digestible carbohydrate, increased calcium absorption in free-living adolescent females. Two groups of bacteria may be involved; one directly fermenting SCF and the second fermenting SCF metabolites further, thereby promoting increased Ca absorption.

**Clinical Trials Registration:** Clinicaltrials.gov NCT01660503

**KEYWORDS:** adolescent, calcium, bone health, prebiotic, osteoporosis, microbiome, short chain fatty acid

**INTRODUCTION**

Calcium is an essential nutrient for bone mineral deposition and the greatest demand is during the pubertal growth spurt during which approximately 26% of adult bone mass is achieved (1). Daily calcium consumption among adolescents, especially females, falls below recommended intakes (2) thereby increasing the risk of reduced peak bone mass development and ultimately increased risk of osteoporosis and fractures later in life. A strategy for improving calcium nutrition is through enhancing the absorption of any calcium present in the diet with prebiotic dietary fibers, such as non-digestible oligo- and polysaccharides (3). In addition to the numerous health benefits associated with prebiotic consumption, the impact of such bioactive fibers on skeletal health is less well recognized.

Known for its association with improved intestinal health (4,5) and influence on colonic microbiota content (6,7), the corn-derived non-digestible carbohydrate, soluble corn fiber (SCF), has recently been evaluated for its beneficial effects on calcium absorption and bone health. SCF has been found to greatly enhance calcium utilization and bone strength properties in a growing rat model more than other novel fibers (8). We demonstrated in an earlier efficacy study using two randomized 3-wk (0 and 12 g/d fiber) controlled feeding sessions in adolescent boys and girls that SCF increased calcium absorption efficiency by 12% (9). SCF consumption was associated with a greater proportion of microbiota from the phylum Bacteroidetes and the increase in absorption was specifically correlated with microbial genera from phyla Bacteroidetes and Firmicutes known to ferment starch and fiber (9). This was the first study to demonstrate a diet-induced change in gut microbiota associated with a physiological benefit of increased calcium uptake in healthy people.

The ability of SCF to alter gut microbiota and enhance calcium absorption efficiency in free-living adolescent girls on a self-selected diet remain important questions given that the majority of bone mineral accretion is achieved during adolescence thereby presenting an opportunity to maximize peak bone mass and reduce the risk of osteoporosis (10,11). To this end we designed an effectiveness study in adolescent girls, for which the primary objective was to evaluate the dose response (0, 10, 20 g fiber/d) of SCF supplementation (within muffins and drink mixes) on calcium absorption efficiency and biochemical markers of bone turnover in free-living adolescent girls. As secondary endpoints, the dose response effect of SCF on fecal microbial community content, short chain fatty acid (SCFA) production and fecal pH were measured to elucidate potential mechanisms. Specifically, we hypothesized that: 1) increasing intakes of SCF would result in greater fractional calcium absorption; 2) markers of bone formation would be greater with higher SCF intakes; 3) changes in fecal parameters would be suggestive of fermentation mechanisms.

**SUBJECTS AND METHODS**

**Study Participants**

Between the summer of 2012 and winter of 2013, thirty-four Caucasian, female adolescents (11-14 y) were recruited from local schools, neighborhoods and community establishments to participate in this randomized cross-over dose-response study. Eligible participants were identified as healthy adolescents with calcium intakes between the 5th and 95th percentile of usual intake for this age group (550 – 1500 mg/d). Girls were ineligible for participation if they reported taking medication that influences calcium metabolism, had a history of disordered calcium or bone homeostasis, BMI > 90th percentile for age, cigarette or illegal drug use, diagnosis of gastrointestinal diseases (Crohn’s, celiac, inflammatory bowel disease) or diseases affecting the kidneys, were consuming foods/drinks containing prebiotics or probiotics, and/or had a broken bone within the last 6 months. Participants were asked to discontinue consumption of nutritional supplements (vitamins, minerals, etc.) and/or foods containing pre- and probiotics for the entire duration of the study. To assure that participants removed all foods with these bioactive compounds, an extensive list of foods and beverages containing pre- and probiotics was provided to each participant at the time of study enrollment. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Institutional Review Board of Purdue University. Written informed consent was obtained from all subjects and parents/guardians.

**Study Design**

Study participants in this 3-phase, double-blind, cross-over study were assigned 0, 10 and 20 g of fiber from PROMITOR® SCF 85 (provided by Tate & Lyle, Hoffman Estates, IL) in randomized order as these doses of non-digestible fibers were tolerable and effective at improving mineral absorption in similar crossover studies using different prebiotics (12–15). Randomization was performed such that equal numbers of participants would be assigned to each of the three fiber interventions. The SCF ingredient was a fermentable, non-digestible carbohydrate containing a minimum of 85% soluble dietary fiber, less than 2% sugar with a caloric content of 1.2 kcal/g. For 4 weeks, participants consumed half of the daily SCF dose (0, 5 and 10 g fiber/d which is 0, 6.67 and 13.37% PROMITOR® 85) in a muffin and the second half in a fruit-flavored beverage. Control (0 g fiber/d from SCF) beverages contained a maltodextrin placebo while muffins were prepared per the recipe with no placebo. The 4-week consumption period was followed by a 3-day clinical visit (Friday evening through Sunday evening) and a 3-4 week washout phase. During each clinical visit, participants were housed in a local hotel near the Purdue University campus. Participants were provided a controlled basal diet containing 800 mg/d calcium, 15 g/d fiber (from whole grains, fruits and vegetables) and adequate nutrients and calories, as calculated by the Harris-Benedict equation which accounts for resting energy expenditure and activity energy expenditure, in relation to body size.

Brief questionnaires regarding health history, supplement use and sexual maturation (tanner staging) (16) were administered at baseline. Participant race and ethnicity were self-reported. Baseline anthropometric measures were taken; height (cm) was measured using a wall-mounted stadiometer and weight (kg) was measured with a digital scale. Habitual dietary intake was estimated using 6-day diet records which were completed before the start of the study and between each subsequent intervention, during the washout phase. Diet record data were analysed with the Nutrition Data System for Research (software version 2013, Nutrition Coordinating Center University of Minnesota, Minneapolis, MN). Mean macro- and micronutrient intakes were calculated to characterize habitual dietary intakes. During one of the clinical visits, Dual Energy X-Ray Absorptiometry (iDXA, GE Lunar, Madison WI)) scans were taken of the total skeleton, dual hip, and lumbar spine.

For each of the three interventions, baseline and 4-week (end of intervention) fecal samples were collected for high-throughput sequencing of the intestinal microbiome. Participants were provided with fecal collection supplies and instructed to collect the baseline samples at home. These samples were stored on ice and study personnel were notified to pick up the samples the same day. End of intervention (post-4 week supplementation) fecal samples were collected on the Purdue University campus or at the local hotel during each 3-day clinical visit. All samples were stored in a walk-in refrigerator and processed within 48 hours of collection.

**Calcium Absorption**

On the Saturday of each clinical visit, a calcium absorption test was performed using dual stable calcium isotope technique. Urine was collected in 12 hour pools for up to 48 hours and analysed for isotope enrichment, as previously described (17). In brief, modifications to this protocol included the use of 10 mg of orally-consumed 44Ca in milk and 3 mg of intravenously-delivered 43Ca. Blood draws occurred at baseline, 3, 24 and 36 hours post intravenous infusion, of which the first two were 15 ml (taken from intravenous catheter) and the last two were 5 ml samples.

Self-reported compliance was assessed by calendar which was given to the participants to mark when they consumed the muffins and drinks each day. Any products not consumed were returned during the clinical visits, counted and recorded. During each intervention phase, weekly questionnaires were administered to assess gastrointestinal symptoms using a likert scale from zero (no symptoms) to five (severe symptoms). Symptoms assessed included flatulence, bloating, abdominal pain, diarrhea and stomach noises.

**Bone Turnover Markers**

Serum and urine were analysed for markers of bone formation and resorption. Fasting urine samples collected at the beginning of each clinical visit were used to analyse N-telopeptides of collagen cross links adjusted for urinary creatinine (NTX) (Osteomark®, Wampole Laboratories, Princeton NJ), a marker of bone resorption. Fasting serum levels of bone-specific alkaline phosphatase (BAP), osteocalcin (OC) and intact parathyroid hormone (PTH) were measured by enzyme immunoassay (EIA) (Microvue™ Bone Health, Quidel Corporation, San Diego, CA) to evaluate changes in bone formation (BAP, OC) and calcium metabolism (PTH).
**Fecal Processing**

The pH of each fecal sample was assessed by inserting an electrode pH probe (pHSpear, Eutech Instruments, Thermo Fisher Scientific) into three different locations of each stool. Using sterile spatulas, small pieces of the stools were removed and immediately frozen in liquid nitrogen for later analysis of SCFA content (acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate) using a Hewlett-Packard model 6890 gas chromatograph with a flame ionization detector equipped with a 30 m, 0.53 mm ID capillary column, as previously described (18,19). Remaining fecal samples were weighed and twice this weight in ultra-pure (Milli-Q) sterile water was added to each sample. Following homogenization, 5-10 ml of the fecal slurry was stored in 15 ml sterile centrifuge tubes at -20°C and used for all further microbiological analyses.

DNA was extracted and quantified as previously described (9). Extracted DNA was subjected to PCR in two phases using Q5® High Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). The first phase, amplified the 16S rRNA gene using primers specific to the V3-V4 region (forward TAC GGR AGG CAG and reverse CTA CCR GGG TAT CTA ATC C primers) as previously described (20,21). Unincorporated primers and nucleotides were separated from PCR amplicons using Agencourt AMPure XP kit (Becker Coulter, Inc, Brea, CA). The second PCR phase allowed for incorporation of 8-base pair forward and reverse primer tags to allow for sample differentiation after sequencing. The Agencourt AmPure XP Kit (Becker Coulter, Inc, Brea, CA) was used to purify second phase PCR products and purified amplicons were quantified by fluorometry after staining with the PicoGreen DNA Assay Kit.

**Sequence and Phylogenetic Diversity Analysis**

The phylogenetic diversity of bacterial communities was determined after amplicons from each sample were combined in equivalent quantities and sent to the Purdue Genomics facility (West Lafayette, IN) for sequencing by high-throughput, paired-end, MiSeq technology (Illumina, San Diego, CA). After removing primer tags and low quality sequences, paired-end reads were merged and analyzed using the QIIME pipeline (22). Reported results were limited to known genera in the Greengenes database, version 13\_5 (23). These sequences were first pre-filtered with the Greengenes core sequences using a 60% threshold value and operational taxonomic unit (OTU) assignments were made using the uclust method (24). Final representative OTU sequences were obtained after sequence alignment using PyNast (25) to filter out sequences that did not align with the Greengenes core sequences. Taxonomic assignments were made using the RDP classifier at 80% confidence and the Greengenes database. Rarefaction analysis was used to obtain an estimation of sequence coverage of the community. To obtain a rarefied dataset, 10 iterations of randomly choosing 28,800 sequences (lowest number of sequences in a sample) from each dataset was performed then datasets were merged to obtain a set of 28,800 sequences that were representative of each sample. Alpha biodiversity estimations (e.g., Chao1, observed species, PD whole tree indices) were calculated to compare microbiota diversity within subjects under specific SCF interventions. Beta diversity comparisons between communities were made using “Fast UniFrac” analysis of phylogenetic distances (26) as well as non-phylogenetic distance analysis using Euclidean distances (Bray Curtis normalized Manhattan and Binary Euclidean). All alpha and beta diversity measures were made using an equivalent number of taxa (based on lowest number of sequences obtained from a single sample) that were randomly chosen using multiple rarefaction results (10 iterations).

**Statistical Analyses**

Statistical analyses of calcium absorption data were performed using SAS Version 9.2 (SAS Institute, Cary, NC, USA). Mean differences or associations were considered significant when *P* < 0.05.

To determine the effects of SCF on calcium absorption over time, a mixed model Analysis of Variance (proc mixed) was used. The effects of session and sequence of interventions were not significant and were eliminated from the model. Differences of least square means were used to determine differences among doses of SCF. The Bonferroni correction was applied to adjust alpha levels for multiple comparisons and data in text are presented as mean ± SEMs and ranges. Fractional absorption was calculated at the end of each twelve hour time point. Isotope enrichment was summed to calculate absorption over the entire 48 h period. A Pearson correlation was used to evaluate the linear association between the change in BAP and change in fractional calcium absorption; data in text are reported as correlation coefficient (r). Habitual dietary intake and SCF compliance were analysed by ANOVA and data in text are reported as mean ± SDs.

The Wilcoxon rank test was then used to perform pairwise comparisons of samples from the beginning and end of each intervention phase as well as between end samples from each intervention phase (data in text presented as mean ± SDs). Student’s t-test was used to determine significant differences between alpha diversity measures; data in text are presented as mean ± SDs. Bonferroni correction was applied to all statistical tests. Non-parametric permutation multivariate ANOVA (perMANOVA via PAST software, a statistical tool available in the Paleontological Statistics package, version 2.16 (27)), after Bonferroni correction, was used to assess beta diversity (Bray Curtis, binary Euclidean distances) differences. Resultant values were then visualized as clusters in a Principal Coordinate Analysis (PCoA) scatterplot to evaluate how diversity differed at the beginning and end of and with or without SCF interventions. Data are presented graphically.

Spearman’s rank correlations were used to determine associations between the difference in total 48 h fractional Ca absorption with SCF relative to control and the difference in the presence of bacteria genera after each intervention (end minus beginning proportions); correlation coefficients in text were reported as rho. Included in these correlations were only bacterial genera with mean proportions > 0.001 (= 0.1%).

Published means and standard deviations from adolescents for fractional calcium absorption were used to determine the study sample size. A total of 24 children would provide sufficient power (80%) at an alpha level of 0.05 to detect a 5.9% difference in fractional calcium absorption and a standard deviation of 9.6%. A total of 30 girls were enrolled in the study to allow for a 20% attrition rate.

**RESULTS**

 Thirty Caucasian girls that were eligible according to screening criteria were randomized for this study (**Figure 1**). To retain the most data for statistical analyses, data from participants completing two or more phases were included, accounting for a final sample size of 28 participants with fractional calcium absorption data. For the microbial analysis a sample size of 27 was used because one subject did not collect a baseline fecal sample. Anthropometric and physical health characteristics were within healthy ranges (**Table 1**).

 Habitual caloric intake based on data from up to eighteen 24-hour diet records per girl was 1877 ± 446 kcal/d. The mean proportions of calories from carbohydrate, protein and fat were 54%, 14%, and 33% respectively. Overall, this cohort had a habitual calcium intake below the estimated average requirement (EAR; 1100 mg/d) (28) at 817 ± 309 mg/d. Dietary fiber intake was also low with a mean intake of 13.2 ± 5.5 g/d (recommended intake of 14 g/1000 kcal (29)). Product consumption compliance over all three intervention periods was 87.3 ± 11.5%.

 The mean fractional calcium absorption calculated at each of the four 12 h periods, is plotted in **Figure 2**. A linear model was used to calculate the overall mean fractional calcium absorption during the entire 48 h test period. Total calculated fractional calcium absorption for the control, 10 and 20 g fiber/d from SCF intervention was 0.358 ± 0.023, 0.388 ± 0.035 and 0.390 ± 0.030 (mean ± SEMs), respectively. Fractional calcium absorption for each individual varied with the control intervention accounting for a range of 0.219 to 0.568. The percent increase in fractional calcium absorption, over the entire 48 hours, was significant for both the 10 g (13.3 ± 5.3%; mean ± SEM) and 20 g (12.9 ± 3.6%) fiber interventions relative to the control (10 g > 0 g; *P* = 0.042 and 20 g > 0 g, *P* = 0.026, respectively). Biochemical markers of bone turnover were measured in fasting serum and urine taken during the first day of the clinic visit (**Figure 3**). A significant positive correlation (r = 0.31, *P* = 0.03) was observed between the change in BAP, a bone formation marker, and the change from control in fractional calcium absorption.

Participants experienced between 1 and 7 bowel movements during a single weekend clinic visit. Fecal weight increased with SCF in a dose-dependent manner but differences between the interventions were not statistically significant (*P* > 0.05; **Figure 4**). Self-reported gastrointestinal symptoms during all three intervention phases were minimal (Supplemental Fig. 1), although gas and bloating were greater at the highest dose relative to the control (*P* < 0.05). Fecal pH was significantly lower with highest SCF intake than with 0 or 10 g fiber/d intakes (*P* < 0.02; Figure 4). When individual SCFAs were measured in feces, acetate, propionate and butyrate were the most abundant. No significant differences were observed between interventions for any of the individual SCFAs (data not shown; *P* > 0.1).

A total of 12,979,388 high quality merged sequences were obtained using MiSeq Illumina sequencing with a mean of 77,720 ± 28,401 (range: 28,854 - 262,312) sequences per sample. Based on the lowest number of sequences obtained (28,854), all subsequent analyses were rarefied to 28,800 sequences per sample. Comparison of alpha diversity measures for end samples indicated significantly (*P* < 0.05) greater diversity with SCF dosage using the species richness measure Chao1 (1104 ± 126, 1402 ± 276and 1282 ± 192 for 0, 10 and 20 g, respectively) and the observed species OTU measure (601.4 ± 83.5, 634.5 ± 83.8, and 649.6 ± 75.5 for 0, 10 and 20 g, respectively).

Comparisons among communities (beta diversity) using Euclidean distances (Binary Euclidean and Bray Curtis) and Principal Coordinate Analysis (PCoA) indicated that communities at the end of 10 and 20 g fiber/d from SCF interventions grouped separately from the end of the control intervention and all baseline samples as indicated by circled groups in **Figure 5**. Differences between samples with and without SCF were significantly different (perMANOVA, *P* < 0.006) which indicated that the presence or absence of specific taxa were contributing to the observed community differences. These taxa were identified using Wilcoxon rank test with Bonferroni correction comparing proportional means from beginning and end samples. The genera *Parabacteroides*, an unclassified Lachnospiraceae,and reclassified *[Ruminococcus]* differed pre- and post-consumption of 10 g fiber/d from SCF while *Parabacteroides*, an unclassified Lachnospiraceae, *Bacteroides* and *Lachnospira* differed over time on 20 g fiber/d from SCF (**Table 2**). No significant (*P* ≥ 0.05) taxa differences were observed in subjects consuming 0 g fiber/d from SCF.

Furthermore, pairwise comparisons of just the end samples for 0, 10 and 20 g fiber/d from SCF substantiated the differences in communities (**Table 3**). There was a potential dosage effect on the *Parabacteroides* with significant increases as the SCF dose increased. Reclassified *[Ruminococcus]* that had significantly lower proportions at the end of 10 and 20 g fiber/d from SCF compared to control, also differed significantly prior to SCF intervention*.*

Significant (*P* < 0.03) positive correlations were found for *Clostridium* and SBM53 (family Clostridiaceae) with the change in calcium absorption on 20 g fiber/d (relative to control) from SCF intervention (**Table 4**). Negative correlations were observed for changes in calcium absorption on 10 g fiber/d from SCF (*Paraprevotella, Megamonas* and *Sutterella*) and 20 g fiber/d from SCF (*Parabacteroides*, Other Bacteroidales, and Other Clostridiaceae) but there were no common taxa between the two SCF levels tested.

**DISCUSSION**

Fractional calcium absorption was significantly increased in adolescent females consuming 10 and 20 g fiber/d from SCF compared to control. Dose effects were observed for *Parabacteroides* proportions which significantly increased with larger SCF doses and negatively correlated with calcium absorption. Firmicutes members positively correlated with calcium absorption suggesting that the role of the microbiome in fermentation and calcium absorption is complex and not mediated by a single species.

Few studies have evaluated the dose-response effect of prebiotics. Supplementing with 0, 5, 10 or 20 g of inulin (per 100 g of diet) had a dose-response effect on intestinal calcium absorption in 6 week old male Wistar rats (30). Similarly, increases in calcium absorption were observed in 5-36 week old male rats fed diets containing 5 and 10% lactulose (by weight of diet); however, no further increase in absorption was noted with 15% lactulose (31). Among postmenopausal women given 5 and 10 g lactulose for 9 days, dose-dependent increases in fractional calcium absorption were noted (12). Conversely, a dose-response effect was not observed in pre-adolescent females receiving 5 and 10 g/d of galacto-oligosaccharide (17). This is similar to the present study in which 10 and 20 g fiber/d from SCF increased calcium absorption relative to control but no difference was observed between the two interventions. While data for the dose effects of non-digestible carbohydrates on calcium absorption are limited, previous findings suggest a need for continued research to identify effective doses for maximal calcium nutriture.

The increases in calcium absorption (13.3 and 12.9% for 10 and 20 g fiber/d from SCF, respectively) observed in this study are similar to that of a previous investigation of SCF (11.6% with 12 g/d fiber) (9) suggesting the effectiveness of this fiber despite differing study designs. The previous study had a heterogeneous population with highly controlled dietary intake and activity compared to this study which had a homogenous population but uncontrolled diet and activity. It is possible that offsetting variances resulted in similar results in calcium absorption and that a design using a homogeneous population and a controlled diet and activity schedule would result in a larger impact of fiber feeding. In both studies, participants reported minimal gastrointestinal symptoms with consumption of SCF (symptoms no different from control), supporting the feasibility of this prebiotic as an acceptable method of increasing calcium absorption.

The significant correlation observed between BAP (bone formation marker) and absorption measures in this study suggests the potential of SCF consumption for increased bone density. In a similar study in postmenopausal women, consumption of 20 g fiber/d from SCF was associated with increased BAP compared to control (32). Few long-term studies have been conducted to confirm whether increases in calcium absorption translate to increased bone mineral content (BMC) following prebiotic consumption. An adolescent study reported that a similar fiber, inulin-type fructan, significantly increased calcium absorption over a year of daily intake which accounted for an additional skeletal accrual of 11 g of calcium (33). The short duration of our study did not allow for direct measures of skeletal mineral accretion but calculations based on the 800 mg/d calcium consumption during study visits suggest that the 26 mg/d increase in absorption with SCF would equate to an additional calcium absorption of 9.3 g over a year (~1.2% of total bone calcium based on the mean total body BMC of this population), consistent with the gain in skeletal calcium observed by Abrams et al. (33). This increase is comparable to our previous efficacy trial where an 11.6% increase in calcium absorption was observed, and would result in 1.8% greater skeletal accrual if the daily increased calcium absorption translated into increased calcium retention over one year (9).

The higher proportion of *Parabacteroides,* *Bifidobacterium*, unclassified Lachnospiraceae and *Dialister* after SCF addition compared to control suggests that these microbes are involved in SCF fermentation. *Bifidobacterium* has been shown to ferment resistant starch (34) whereas the other taxa have not yet undergone functional tests; however, other studies have reported associations between increases in these taxa and dietary resistant starch (35,36), including *Parabacteroides* in our previous efficacy study (9). Furthermore, *Parabacteroides* belongs to the phylum Bacteroidetes that includes a number of starch fermenting bacteria (37,38). *Bifidobacterium* species are known to metabolize oligosaccharides and are commonly used as probiotics because of their association with health benefits (39). A recent study linked increased gene diversity (analysis of all genes in fecal samples) to increased metabolic markers of health in humans (40). The greater microbial diversity with SCF, as indicated by the significantly greater species richness (Chao1 values and observed species numbers), may suggest a healthier microbiome.

One theory for the underlying mechanism of prebiotic-induced calcium absorption is that microbial production of SCFAs via starch fermentation produces an acidic environment ideal for increasing the solubility and transcellular absorption of mineral ions, such as Ca2+ (41,42). Although a significant increase in SCFAs was not found in this study, pH was significantly reduced on the highest dose. However, a recent direct-to-cecum rat model was used to explore physiological mechanisms for the action of SCFA on calcium absorption; SCFA, rather than through lowering pH which had no effect on calcium absorption efficiency, increased calcium absorption efficiency when inhibitors added to the cecum were prevented from binding to calcium (43). SCFAs are readily absorbed for energy throughout the intestine making it difficult to measure their true luminal concentrations (44). SCFA mechanisms may also be supported by the correlation between taxa in the phylum Firmicutes (*Clostridium* and SBM53) and calcium absorption. *Parabacteroides* proportions were significantly higher with SCF but negatively correlated with Ca absorption on the 20 g fiber/d from SCF intervention. It is possible that cross feeding was occurring in which the Bacteroidetes ferment starches to acetate or lactate and members of the Firmicutes continue fermentation of these substrates to butyrate (45).

Our previous study (9) and those of others (35,46,47) have had difficulty in distinguishing the effect of diet over inherent subject differences because of the naturally high variation in human gut microbiota composition. In our previous study we found that gender and race contributed significantly to differences in the gut microbial communities in human adolescents (48). Some of this natural variation was reduced in this study by using subjects of the same gender, race and age. Technical factors that may have contributed to differences in results of our two studies were the PCR primers, sequencing methods (454 vs. Illumina) and databases used for taxa classification.

Despite having a short study duration, an important finding of this study was the ability to significantly impact calcium absorption by administering intervention in free-living conditions. The inclusion of high-throughput sequencing of fecal microbiota, fecal weight and pH, as well as SCFA content provided important mechanistic data. However, despite support for fermentation with decreased pH, this study may not have been adequately powered to see significant differences in fecal weight and SCFA content. Another potential limitation is the wide variation in sexual maturity of participants in this study, which may have contributed to the large variation in many of the outcome measures.

In summary, the addition of 10 or 20 g fiber/d from PROMITOR™ SCF 85 to the diet of free-living adolescent girls ages 12-15 y over a 30 day period contributed to increased calcium absorption which could be critical for skeletal health at a time when bone growth is rapid. This result is easily translatable to a normal teen population as the study was conducted under free-living conditions. The increase in *Bifidobacterium* and greater species richness are indicators that SCF results in a healthier microbiome. Further work is needed to identify the exact mechanism by which SCF elicits an effect on intestinal microbiota and calcium absorption and the long-term effect of dietary fibers on calcium absorption and bone density and strength.

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 CMWe, BRM, CHN and CMWh designed research; BRM and CMW conducted research; BRM, CHN, GPM, and LDM analyzed data; CMWh, BRM and CHN wrote paper; and CMWe had primary responsibility for final content. All authors read and approved the final content of this manuscript. CMWe serves on the Advisory Board of Pharmavite.

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**Figure Legend**

**Figure 1.** Diagram of recruitment and retention flow throughout this crossover study of adolescent girls.

**Figure 2.** Fractional calcium absorption based on oral and intravenous isotope excretion in 12 hour urine pools collected over 48 hours in healthy girls after consuming 0, 10 and 20 g fiber/d from SCF for 4 weeks each in crossover study. Data are presented as means ± SEMs (*n* = 28) following analysis by ANOVA. Effects of SCF on calcium absorption over time were assessed by mixed model Analysis of Variance including variables for crossover session, intervention sequence, and time. Following Bonferroni correction to adjust for multiple comparisons, no treatment or time differences were observed, *P* ≥ 0.05. SCF, soluble corn fiber.

**Figure 3.** Serum and urine biochemical markers of bone turnover in healthy girls fed 0, 10 and 20 g fiber/d from SCF for 4 weeks each in crossover study. Data are presented as mean ± SEMs (*n* = 28) following analysis by ANOVA; no significant intervention differences were observed *P* ≥ 0.05. Biomarker abbreviations are as follows: BAP, bone alkaline phosphatase; NTX/Cre, N-telopeptides of collagen cross links corrected for urinary creatinine; OC, osteocalcin; PTH, parathyroid hormone. SCF, soluble corn fiber.

**Figure 4.** Fecal weight (A), pH (B) and SCFAs (C) content of feces collected from healthy girls after consuming 0, 10 and 20 g fiber/d from SCF for 4 weeks each in crossover study. Data are presented as mean ± SEMs (*n* = 27) following analysis by ANOVA. Labeled means without a common letter differ, *P* < 0.05. SCF, soluble corn fiber; SCFA, short chain fatty acid.

**Figure 5**. Principal Coordinate Analysis (PCoA) of Jackknife Binary Euclidean distances of community composition coded by samples collected at the beginning and end of three (0, 10 and 20 g fiber/d from SCF) SCF interventions in healthy girls (*n* = 27) for 4 weeks each in crossover study. PC1 and PC2 explained 12.1% and 9.5% of the total multivariate sample variance, respectively. Samples from the 10 and 20 g fiber/d from SCF interventions clustered together (circle labeled “With SCF”) while end samples from the 0 g fiber/d from SCF intervention and all baseline samples clustered separately (circle labeled “No SCF”). B, beginning of intervention; E, end of intervention; PC, principal component; SCF, soluble corn fiber.

**Online Supporting Material**

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**Supplemental Figure 1.** Self-reported gastrointestinal symptoms of healthy adolescent females after consuming 0, 10 and 20 g fiber/d from SCF for 4 weeks. Data are presented as means ± SEMs (*n* = 28); group comparisons were made by a mixed model with treatment and subject id. Participants reported symptoms using a likert scale (scores of 0 and 5 representing no symptoms and severe symptoms, respectively). Labeled means without a common letter differ, *P* < 0.05. SCF, soluble corn fiber.