A Calcium-Rich Multimineral Intervention to Modulate Colonic Microbial Communities and Metabolomic Profiles in Humans: Results from a 90-Day Trial

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ABSTRACT

Aquamin is a calcium-, magnesium-, and multiple trace element–rich natural product with colon polyp prevention efficacy based on preclinical studies. The goal of this study was to determine the effects of Aquamin on colonic microbial community and attendant metabolomic profile. Thirty healthy human participants were enrolled in a 90-day trial in which Aquamin (delivering 800 mg of calcium per day) was compared with calcium alone or placebo. Before and after the intervention, colonic biopsies and stool specimens were obtained. All 30 participants completed the study without serious adverse event or change in liver and renal function markers. Compared with pretreatment values, intervention with Aquamin led to a reduction in total bacterial DNA ($P = 0.0001$) and a shift in the microbial community measured by thetaYC (θ_{YC}; $P = 0.0087$). Treatment with calcium also produced a decline in total bacteria, but smaller than seen with Aquamin, whereas no reduction was observed with placebo in the colon. In parallel with microbial changes, a reduction in total bile acid levels ($P = 0.0375$) and a slight increase in the level of the short-chain fatty acid (SCFA) acetate in stool specimens ($P < 0.0001$) from Aquamin-treated participants were noted. No change in bile acids or SCFAs was observed with calcium or placebo. We conclude that Aquamin is safe and tolerable in healthy human participants and may produce beneficial alterations in the colonic microbial community and the attendant metabolomic profile. Because the number of participants was small, the findings should be considered preliminary.

Introduction

Epidemiologic studies have shown an inverse relationship between calcium intake and colon cancer incidence (1). Experimental studies in animals have substantiated antitumor efficacy in the colon (2), and *in vitro* studies have provided mechanistic insight into how calcium influences epithelial cell proliferation and differentiation (3). In spite of these data, interventional trials with calcium have had only modest success in reducing colon polyp formation, with some trials demonstrating a reduction in incidence (4, 5), while others showing essentially no protection (6, 7) or an increase in incidence of colon polyps with a more-aggressive sessile-serrated phenotype (8). This complicated picture supports the idea that adequate or optimal calcium intake throughout life may be beneficial, but in a realistic dietary setting, the efficacy of supplementation by calcium alone may be limited by dietary complexity. Potentially adverse effects at higher dose levels further complicate the picture (9).

Recent evidence suggests that the antineoplastic activity of calcium supplementation may be enhanced by concomitant inclusion of additional trace minerals along with calcium. This has been demonstrated in our own studies using Aquamin, a calcium- and magnesium-rich multimineral product obtained from mineralized red marine algae. In two long-term (15–18 months) studies in mice, Aquamin more effectively suppressed colon polyp formation than calcium alone (10, 11). Further, Aquamin was more effective than calcium alone at suppressing proliferation and inducing differentiation in human colon carcinoma cells in monolayer culture (12, 13) and human adenoma–derived colonoids (14). Thus, Aquamin (as a source of calcium along with magnesium and additional trace minerals) may, ultimately, prove to be a more effective dietary colon polyp chemopreventive agent than calcium alone.

Exact mechanisms by which calcium alone or multimineral supplementation exerts antineoplastic activity are currently unknown. In addition to direct antiproliferative and prodifferentiating effects on colonic epithelium, beneficial activity may be mediated indirectly through effects on the gut microbial

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population and/or changes in gut microbial metabolic activity. The bacterial community in the gastrointestinal tract plays several important roles that contribute to health. A "healthy" microbiome is important for food digestion and production of important metabolites. In addition, a healthy microbial community helps maintain the tissue barrier, regulates the host immune response, and provides protection against pathogen overgrowth (15). Dysbiosis, especially in the colon, can lead to barrier breakdown and initiate a chronic inflammatory response. Dysbiosis has been directly linked to inflammatory bowel diseases and indirectly to the formation of premalignant colon polyps (15–17). In previous studies in mice, dietary calcium supplementation has been shown to cause a shift in the gut microbial community in comparison with control (18, 19). In another study, Aquamin itself induced gut microbial changes (20).

Metabolic changes may also be important. At high concentrations, bile acids are membrane-active agents and can be cytotoxic (21). In addition, certain bacterially derived secondary bile acids have carcinogenic properties (22). Alterations in the gut microbial population can affect bile acid profiles (23). In our own murine study (19), calcium supplementation altered the gut microbiome and reduced the level of total bile acids and certain microbially derived secondary bile acids. In addition to bile acids, gut microbes also produce short-chain fatty acids (SCFA; e.g., acetate, propionate, and butyrate) in the colon. SCFAs have demonstrable protective effect against colonic inflammation and carcinogenesis (24). Thus, a shift in the gut microbial community may affect colon health through alterations in bacterially derived metabolites.

Whether similar microbial/metabolic changes might also be seen in human participants is not known. To test the feasibility of using dietary Aquamin as an interventional strategy in humans, we conducted a 90-day, FDA-approved pilot study, comparing daily supplementation with Aquamin with calcium alone and placebo in healthy human participants. The main purpose of the study was to assess Aquamin's effect on gut microbial community structure and bile acid, SFCA, and eicosanoid profiles at endpoint in comparison with baseline values in participants from all three groups. Elucidating the microbial and metabolic changes resulting from intervention is a first step toward identifying potential mechanisms of action with Aquamin. Identifying measurable alterations in microbial and metabolomic parameters and determining effect-size with intervention will also be helpful for determining the necessary sample size going forward in a larger-scale clinical trial. As part of the study, we also obtained information on tolerability and safety of Aquamin. Initial results from the current study are reported herein.

Materials and Methods

Aquamin and control interventions

Aquamin is a calcium- and magnesium-rich natural product obtained from the skeletal remains of red marine algae of the *Lithothamnion* genus. In addition to calcium and magnesium,

Aquamin contains detectable levels of 72 additional trace minerals including trace elements from the lanthanide family (essentially all of the minerals accumulated by the algae from seawater). Aquamin is sold as a food supplement (GRAS 000028; Marigot Ltd.) and is used in various products for human consumption in Europe, Asia, Australia, and North America. A single batch of Aquamin-Food Grade was used for this study. Mineral composition was established via an independent laboratory (Advanced Laboratories). Supplementary Table S1 provides a complete list of elements detected in Aquamin and their relative amounts. Aquamin has been used in past clinical studies in human subjects with no serious safety or tolerability issues reported (25–27). Calcium carbonate was used for active comparison, and maltodextrin was used as a placebo.

Study participants

This study was a pilot, double-blind, parallel assignment, randomized clinical interventional trial in which 30 participants were included. Participants were male or nonpregnant female, in general good health, but having "an increased risk for colon cancer" based on (i) a personal history of colorectal polyp, early stage (stage I or II) colon cancer treated by surgical removal without recommendation for adjuvant therapy, or stage III colon cancer treated with surgery > 5 years prior or (ii) a first-degree relative diagnosed under the age of 60 with colorectal cancer. Exclusion criteria included history of kidney disease or kidney stones, Crohn's disease or ulcerative colitis, gastrointestinal hemorrhagic disorders, or coagulopathy, hereditary nonpolyposis coli, or familial adenomatous polyposis. The participants were recruited through the Michigan Medicine web portal and by posting flyers in the hospital.

This clinical interventional trial was conducted with FDA approval of Aquamin as an Investigational New Drug (IND#118194) and with oversight by the Institutional Review Board at the University of Michigan Medical School (IRBMED)—IRB#HUM00076276. The study was registered as an interventional clinical trial with details at Clinical-trials.gov (study identifier NCT02647671). All participants provided written informed consent prior to inclusion. This phase I trial involving human participants was carried out in accordance with recognized ethical guidelines, for example, Declaration of Helsinki, International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS), the Belmont Report, and the U.S. Common Rule.

Study design

Figure 1 summarizes the study design of this trial in a flowchart. Briefly, at screening participants were given the NIH Diet History Questionnaire II (DHQ II), a food frequency questionnaire which includes portion size and dietary supplement questions as a way to evaluate baseline calcium levels in the past year (28). Participants were also asked about use of dietary supplements, antibiotics, and nonsteroidal anti-inflammatory drugs. No participants were on antibiotics at or

Figure 1.
Study design. Study flow diagram highlighting enrollment, group randomization, intervention allocation, study duration, and study sample collection plan.
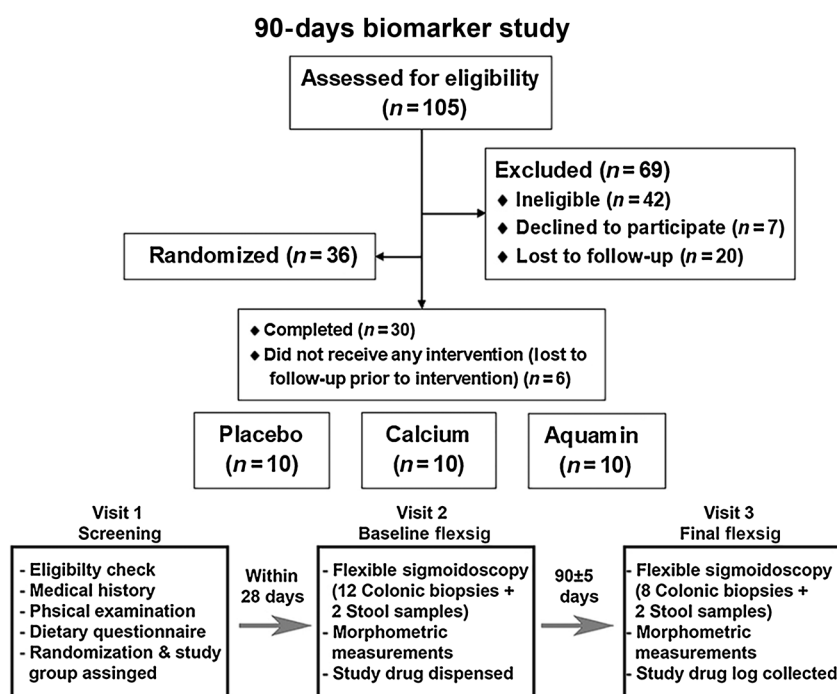
in close proximity to the time of screening. Individuals ingesting supplements containing calcium and/or vitamin D were required to undergo a 2-week "wash out" period prior to starting and to not use these supplements during the study participation.

Thirty participants underwent baseline flexible sigmoidoscopy (unprepped; i.e., without bowel cleansing procedure). Twelve 2.5 mm colonic biopsies were obtained along with two stool specimens from within the sigmoid colon (20 cm above the anus). Tissue and stool samples were saved in 10% formalin, cryopreserved for cultures or snap frozen in liquid nitrogen, and saved at −80°C. Blood was drawn for the complete metabolic panel including liver function/liver injury markers (total albumin, bilirubin, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase) and renal function tests (blood urea nitrogen and creatinine).

After baseline sigmoidoscopy, participants were randomized to one of three groups. Ten participants were treated daily for 90 days with Aquamin providing 800 mg of calcium per day. Ten participants received 800 mg of calcium carbonate daily, and ten participants received maltodextrin as placebo. During the interventional period, participants were contacted by study coordinators on a monthly basis to assess study progress/ adherence to the study protocol and to identify unwanted side effects. Compliance was assessed by capsule log entries and by counting unused capsules returned at the end of the study.

At the end of the 90-day intervention period (90 ± 5 days), participants again underwent unprepped flexible sigmoidoscopy and eight colonic biopsies along with two stool specimens were collected and stored as at baseline. Blood was also taken for the same serum markers as at baseline. For each of the two

timepoints, one biopsy and one stool specimen from each participant were utilized for microbiome analysis, and one biopsy and one stool specimen were utilized for metabolomic analysis. The remaining tissue samples were retained for backup or were used for histologic, IHC, and proteomic analyses. The results of the latter analyses will be reported separately.

Microbial analysis

DNA was isolated from colon and stool samples using the Qiagen MagAttract PowerMicrobiome DNA/RNA kit. Total bacterial DNA levels were estimated using qPCR with broad-range primers BSF8 (AGAGTTTGATCCTGGCTCAG) and BSR357 (CTGCTGCCTYCCGTA), targeting bacterial 16S rRNA genes (29). Each 10 µL qPCR reaction contained 5 µL PowerUp SYBR Green Master Mix (Applied Biosystems by Thermo Fisher Scientific), 4 µL sample DNA (1:40 dilution), and 0.4 µmol/L of primers. The following cycles were run on a Light Cycler 96 (Roche): 1x(2 minutes at 50°C, 10 minutes at 95°C), 40x(15 seconds at 95°C, 1 minute at 60°C). Each sample was run in duplicate.

Microbial community profiles were generated by Illumina MiSeq sequencing of the V4 region of 16S rRNA–encoding genes after amplifying the extracted colon tissue and stool DNA of all the participants as described previously (30). Samples were amplified, normalized, and sequenced on the MiSeq, and analysis was performed using the MiSeq SOP (31) for the software mothur (v.1.39.0 and v.1.39.5) as described in our earlier study (19). In case of low bacterial biomass, 3 µL of DNA were amplified by touchdown PCR method [1x(2 minutes at 95°C), 20x(20 seconds at 95°C, 15 seconds at annealing

temperature (starts at 60°C, decreases 0.3°C/cycle), 5 minutes at 72°C), 20x(20 seconds at 95°C, 15 seconds at 55°C, 5 minutes at 72°C), 1x(10 minutes at 72°C)]. Thirteen samples, including 8 of 10 postinterventional Aquamin colon samples, required touchdown PCR (with 40 amplification cycles vs. standard PCR with 30 cycles) to achieve sufficient sample amplification for sequencing. After processing, sequences were binned into operational taxonomic units (OTU) based on 3% difference in sequence using the OptiClust method (32). Pre- and post-comparisons between groups included differences in community structure using OTU-based Yue and Clayton distance metric (θ_{YC}; ref. 33). θ_{YC} distances were visualized using principal coordinates analysis (PCoA). θ_{YC} is a beta diversity metric. Specific OTUs driving community differences were identified by linear discriminant analysis effect size (LEfSe; ref. 34), as was done in our previous study (19). LEfSe utilizes both statistical significance and effect size (linear discriminant analysis score, or LDA) to determine the features (OTUs, in this case) that are differentially abundant between groups (i.e., pre- and postdifferences). FDRs of the significant LEfSe data were corrected using the *p.adjust()* function with the Benjamini–Hochberg algorithm employing *R*, and stated as *q* values. Alpha diversity was assessed by change in the Shannon diversity index from baseline. To evaluate Aquamin-associated microbial community differences in major gut phyla, we sorted the 1,000 most abundant OTUs in each of the baseline and final visit samples into phyla and compared intergroup differences in each of these phyla at endpoint. The DNA isolation and 16S rRNA gene sequencing were done by the University of Michigan Microbial Systems Molecular Biology Laboratory. Sequence data generated in this project are accessible within the NCBI Sequence Read Archive database (BioProject accession number: PRJNA575562).

Metabolomic analysis

Bile acids, SCFAs, and eicosanoid composition were quantified in colon biopsies and stool specimens from a randomly chosen subset of participants ($n = 6$ per group) at each time point (pre- and postintervention). Bile acids were quantified using LC-MS in a two-step solvent extraction (35) as performed in the Regional Comprehensive Metabolomics Resource Cores (RCMRC) at the University of Michigan. Supernatants were combined, dried, and resuspended for LC-MS separation by reverse-phase liquid chromatography (RPLC) and measured by multiple reaction monitoring (MRM). Sample identification was performed by comparison of retention times and mass with an in-house library of bile acids. SCFA analysis was performed by the RCMRC by electron ionization-gas chromatography–mass spectrometry as described recently (36). Compound identification was performed against a library of known SCFAs. Colon tissue was also utilized to analyze a profile of eicosanoids. Eicosanoids were extracted and concentrated using solid phase extraction in colon biopsies. The eluent was dried and resuspended for LC-MS separation by RPLC and measurements by MRM methods (37). Stool specimens were not analyzed for eicosanoids because these metabolites are not bacterial pro-

ducts. All analytes were reported as pmol/mg, after normalization to the sample weight. Metabolomics data acquired in this study are available on the Metabolomics Workbench (Project ID: PR000822; doi: 10.21228/M8F69D) as part of National Metabolomics Data Repository.

Statistical analysis

Pre (baseline) versus post (endpoint) comparisons were conducted for each microbial or metabolomic endpoint for individual participants in each of the three treatment groups (placebo, calcium, Aquamin). Group means and SDs were then calculated for baseline and endpoint measures. Intergroup differences for normally distributed continuous data were made by ANOVA followed by pairwise group comparisons with Bonferroni corrections for multiple comparisons to generate an adjusted *P* value. The two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli was applied to correct for multiple comparisons by controlling the FDR. Microbial community distances were compared by analysis of molecular variance (AMOVA) within the program mothur (v.1.39.0 and v.1.39.5; ref. 38). For continuous data that failed normality testing, intergroup comparisons were made by Kruskal–Wallis ANOVA followed by Mann—Whitney–Wilcoxon pairwise comparison. Statistical analyses were performed using GraphPad Prism (version8) and *R* computing software (*R* version 3.5.3 and RStudio Version 1.1.463). Pearson correlation coefficients were applied to compute correlations among colon and stool samples for θ_{YC} metric. A value of <0.05 was considered significant for both *P* and *q* values for all the analyses. Due to the small cohort size, analyses were not adjusted to any baseline sociodemographic or clinical characteristics or dietary calcium intake.

Results

Participant characteristics

Thirty-six participants were randomly enrolled. Six subjects were lost to follow-up prior to intervention, and a total of 30 participants (10 per arm) completed the study (**Fig. 1**). This included of 22 female and 8 male participants. Participants were randomized to study arms without regard to age or gender (placebo: 4 males and 6 females; calcium: 2 males and 8 females; Aquamin: 2 males and 8 females). Ages ranged from 20 to 66 years. Compliance (capsule intake) was estimated to be 96% across the three groups. Demographic characteristics are presented in Supplementary Table S2. Based on responses to DHQ II, the average dietary calcium intake (mg/day) values for the three groups at baseline were estimated to be: placebo = 817 ± 245; calcium = 964 ± 412; and Aquamin = 919 ± 545 (no significant differences found).

Safety and tolerability

Self-reported adverse events over the course of study are shown in **Table 1**. All adverse events were minor (i.e., headache, gastrointestinal symptoms) and did not preclude any individual from completing the study. The number of individuals

Table 1. Adverse events reported by the study subjects.

Events	Placebo	Calcium	Aquamin
Number of subjects participated	10	10	10
Number of subjects reported events	3	6	3
Total number of adverse events	**5**	**15**	**7**
Upper respiratory events (Flu-like symptoms)	1	1	0
Flu with fever (upper respiratory)	1	0	0
Skin rash	0	0	1
Headache	1	1	0
Gastrointestinal events	2	13	6
Nausea	0	1	0
Constipation	1	1	3
Diarrhea	0	4	1
Flatulence (& bloating)	1	2	0
Borborygmi	0	1	0
Belching	0	1	0
Abdominal pain	0	0	2
Blood in stool	0	3[a]	0

Note: Adverse events (health issues) reported by subjects over the course of the study. No significant differences were among three groups.
[a]One subject reported three separate incidents of blood in stool over the period of 90 days.

reporting adverse events in the Aquamin group was the same as in the placebo group (3 of 10), whereas 6 of 10 individuals in the calcium group reported one or more events. No serious adverse events (defined as necessitating cessation of study participation or medical intervention/hospitalization) occurred. Serum metabolic markers (liver function/liver injury and renal function) are presented in **Table 2**. No significant change in any individual marker was observed over the course of the study. Likewise, there was no difference among the treatment groups in a panel of metabolic markers and no change in serum calcium levels before and after intervention (**Table 2**).

Effects on bacterial DNA and microbial communities in colon biopsy and stool specimens

qPCR was used to assess bacterial 16S rRNA gene levels in colon biopsy and stool specimens as a way to estimate total bacterial DNA. **Figure 2** demonstrates that in both colon biopsy and stool specimens, posttreatment Aquamin samples had higher cycle quantification (Cq) values, indicating lower amounts of total bacterial DNA than at baseline (Colon: $P = 0.0001/q < 0.0001$; Stool: $P < 0.0001/q < 0.0001$). There was also a decrease in total bacterial DNA in specimens from calcium-treated participants (Colon: $P = 0.032/q = 0.0021$; Stool: $P < 0.0001/q < 0.0001$), although, on average, the decrease was not as great as that seen with Aquamin. Placebo specimens showed no (average) decline in DNA content at endpoint.

Alterations in gut microbial communities

To determine if Aquamin altered the composition of the gut microbiota in the participants, we analyzed bacterial 16S rRNA gene sequences from colon and stool specimens to explore pre-postinterventional differences. After sequence processing and exclusion of 2 samples for low sequence counts (i.e., one placebo stool and one calcium stool), a total of 7,081,672 sequences from 118 samples [median: 59,233 ± 23,618 (SD) sequences/sample; range 2,338–11,4235 sequences/sample] were included in this analysis. When we analyzed the sequence data by samples types for both colon biopsy and stool specimens, there was a total of 3,051,764 sequences from 60 colon samples [median: 49,853 ± 17,198 (SD) sequences/sample; range 12,481–102,310 sequences/sample]. The total number of sequences from 58 stool samples was 4,029,908 [median: 76,797 ± 25,547 (SD) sequences/sample; range: 2,338–11,4235 sequences/sample].

Shifts in gut microbial community composition were assessed by calculating θ_{YC} distances between the pre- and

Table 2. Serum markers of safety and tolerability (Serum metabolic panel).

Group	Total protein (6.0–8.3 g/dl)	Albumin (3.5–4.9 g/dl)	AST (8–30 IU/L)	ALT (≤35 IU/L)	ALKP (30–116 IU/L)	Bilirubin (0.2–1.2 mg/dl)	Glucose (<100 mg/dl)
Placebo pre	7.2 ± 0.4	4.4 ± 0.2	25.6 ± 3.7	28.7 ± 8.3	75.8 ± 9.0	0.46 ± 0.2	91.4 ± 5.3
Placebo post	7.3 ± 0.3	4.5 ± 0.2	26.3 ± 5.5	31.9 ± 7.1	77.9 ± 15.3	0.54 ± 0.2	91.7 ± 6.4
Calcium pre	7.1 ± 0.7	4.4 ± 0.4	28.1 ± 10.1	25.9 ± 12.3	78.1 ± 20.5	0.59 ± 0.6	114.2 ± 58.7
Calcium post	7.2 ± 0.6	4.5 ± 0.4	28.7 ± 9.6	28.8 ± 11.5	80.5 ± 21.0	0.60 ± 0.2	102.0 ± 28.7
Aquamin pre	7.1 ± 0.4	4.5 ± 0.1	24.8 ± 6.8	23.4 ± 11.2	73.7 ± 18.4	0.57 ± 0.2	97.0 ± 22.3
Aquamin post	7.3 ± 0.4	4.6 ± 0.2	30.9 ± 10.9	32.5 ± 19.6	74.4 ± 19.7	0.61 ± 0.4	98.3 ± 22.7

Group	BUN (10–20 mg/dl)	Creatinine (0.6–1.2 mg/dl)	Carbon dioxide (23–30 mmol/L)	Sodium (136–145 mmol/L)	Potassium (3.5–5.2 mmol/L)	Chloride (96–106 mmol/L)	Calcium (8.6–10.3 mg/dl)
Placebo pre	16.9 ± 2.6	0.8 ± 0.1	28.4 ± 1.8	139.9 ± 2.0	4.4 ± 0.3	104.5 ± 1.4	9.5 ± 0.2
Placebo post	17.2 ± 4.5	0.8 ± 0.2	28.5 ± 3.0	140.1 ± 2.1	4.3 ± 0.3	105.2 ± 3.2	9.7 ± 0.3
Calcium pre	14.6 ± 5.1	0.8 ± 0.1	28.2 ± 3.5	140.9 ± 2.0	4.3 ± 0.6	105.8 ± 2.2	9.7 ± 0.5
Calcium post	14.0 ± 5.2	0.8 ± 0.1	28.6 ± 3.7	140.2 ± 1.5	4.2 ± 0.6	104.5 ± 3.5	9.8 ± 0.7
Aquamin pre	12.8 ± 2.8	0.7 ± 0.2	28.1 ± 2.7	140.5 ± 2.4	4.5 ± 0.3	104.6 ± 2.6	9.6 ± 0.4
Aquamin post	12.1 ± 4.1	0.7 ± 0.2	27.8 ± 1.9	140.5 ± 3.5	4.4 ± 0.3	105.2 ± 3.1	9.8 ± 0.4

Note: A comprehensive metabolic panel was done on participant's serum before and after the intervention.
No significant differences found among groups and within a group from the baseline.
Abbreviations: AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALKP: Alkaline phosphatase; BUN: Blood Urea Nitrogen.
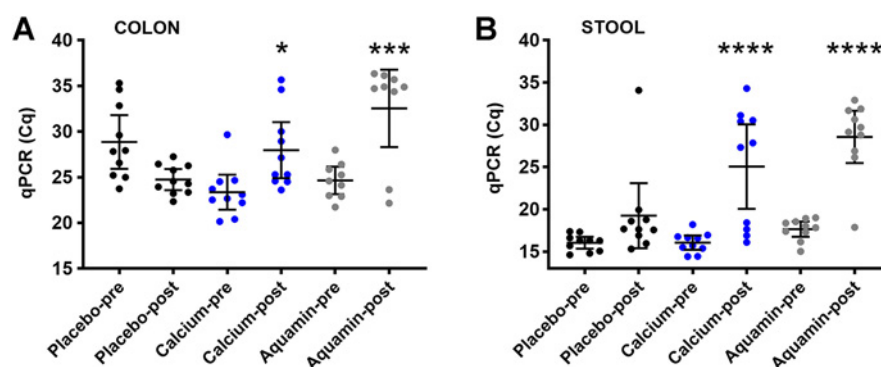
Figure 2.
Decrease in bacterial DNA with Aquamin. qPCR for bacterial DNA in colon (**A**) and stool (**B**) samples. The average cycle quantification (Cq) value from duplicate qPCR runs was plotted except for one post-Aquamin colon sample which failed to reach the amplification threshold in one duplicate and was therefore represented by a single Cq value (34.4) rather than the average. Another post-Aquamin colon sample failed to reach the amplification threshold in both duplicates and was, therefore, not plotted or included in the statistical analysis. A decrease in the total bacterial DNA is indicated by a higher Cq value (longer time to reach amplification threshold). There was no difference in pre- vs. postsupplementation for the placebo group. *, $P < 0.05$; ***, $P < 0.001$; and ****, $P < 0.0001$ compared with the corresponding pretreatment value.

postsupplementation communities (beta diversity) for each individual. Individual pre- postdistance values, grouped by intervention, are shown in **Fig. 3A** and **B**. Colonic microbial communities demonstrated a bigger within-participant shift in the Aquamin-supplemented group than was observed for either the placebo or calcium-supplemented group. The difference between Aquamin and calcium in the colon biopsy specimens reached the level of statistical significance ($P = 0.0087/q = 0.0061$). Both figure insets show that the majority of participants that received Aquamin (8 of 10 in colon and 7 of 10 in stool) were above the median value for within-participant

bacterial community change from pre- to postsupplementation for all samples. In colon biopsies, when samples were pooled as shown in the **Fig. 3A** inset, θ_{YC} distance was significantly increased in Aquamin subjects ($n = 10$) as compared with both calcium and placebo subjects ($n = 20$; $P = 0.019$). **Figure 3C** demonstrates a strong correlation between the two specimen types (colon biopsy and stool) for within-participant microbial community shifts (θ_{YC} distances; $P = 0.0041$). The correlation data, thus, suggest that stool specimens could be used as an estimate of the bacterial community in the colonic mucosa. It should be noted, however, that the stool specimens used here
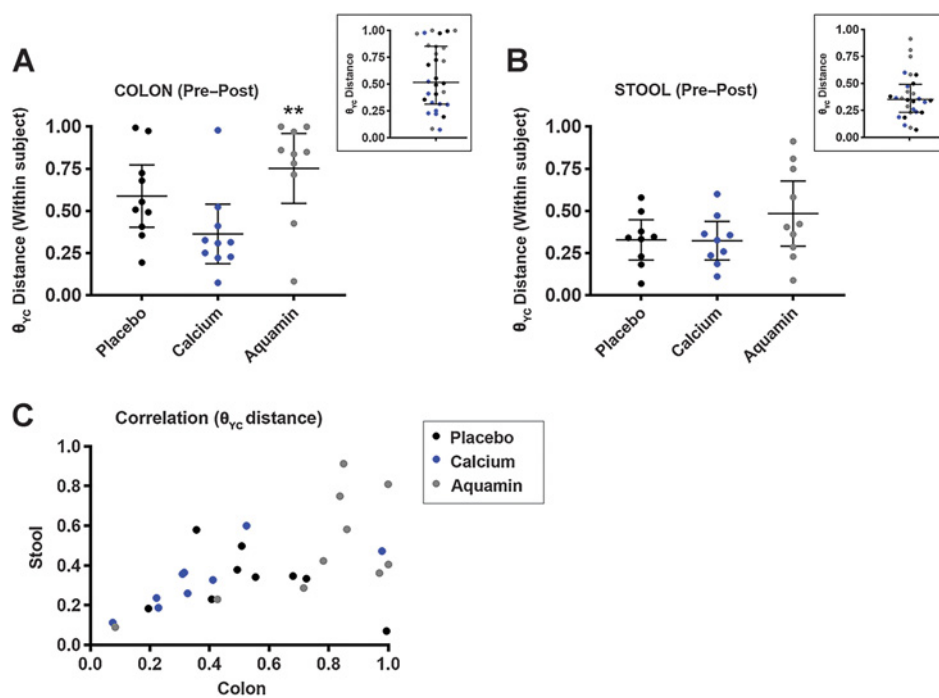
Figure 3.
θ_{YC} distances for comparison of pre- vs. postsupplementation values in gut bacterial community composition for colon (**A**) and stool (**B**) samples. Higher values reflect greater differences. **, $P = 0.0087$ relative to calcium. The insets show that the majority of the Aquamin (colon and stool) samples were above the median value for all samples. The majority of placebo and calcium samples were at or below the combined median value. **C,** Correlation between θ_{YC} in colon biopsy and stool specimens based on pre- vs. postsupplementation values ($P = 0.0041$).

were obtained during flexible sigmoidoscopy and were not shed specimens. In addition, as part of this analysis, intrasubject versus intersubject variability was determined. θ_{YC} distances between the pre- and postintervention samples for each of the 30 individuals were compared with the θ_{YC} distances between each preintervention sample and all other samples. As seen in Supplementary Fig. S1, intrasubject differences were smaller than intersubject differences in both colon and stool samples ($P \leq 0.0001$).

Figure 4A–D presents PCoA of θ_{YC} distances between samples for all three treatment groups in both colon and stool specimens. At baseline, there were no significant differences in the colon bacterial communities (Fig. 4A), but following the 90-day intervention period, microbial communities in the colon biopsies from participants treated with Aquamin segregated from those in the placebo or calcium groups (Aquamin vs. placebo: AMOVA $P = 0.005$ and Aquamin vs. calcium: AMOVA $P = 0.009$; Fig. 4A). Further, colonic microbial communities from the posttreatment Aquamin biopsy samples segregated from their own baseline communities (AMOVA, $P = 0.022$; Fig. 4B), whereas there was no difference in pre- versus postsupplementation communities for placebo- or calcium-treated participants (Fig. 4A). In contrast to these results in colon biopsy material, stool sample microbial communities were not different between or within groups either at baseline or at endpoint (Fig. 4C and D).

Alpha diversity was assessed in both colon and stool samples using the Shannon diversity index. In Aquamin-treated participants, there was a decrease in Shannon diversity (Fig. 4E and F). For colon biopsy samples, Shannon diversity was significantly decreased ($P = 0.0037/q = 0.0026$) as compared with the placebo.

Alterations in the relative abundance of major gut phyla

First, we determined the total number of phyla and genera identified in each sample type. In colon biopsies, there were 15 different phyla and 224 genera identified. In stool samples, there were 10 phyla and 213 genera found. Overall, there were 7,267 OTUs detected; out of those, 2,573 were present in colon biopsies, 6,642 were present in stool samples, and 1,948 were common in both. A list containing all the phyla and genera identified are presented in Supplementary Tables S3 and S4.

Next, we assessed alterations in the relative abundance of individual OTUs representing the major gut phyla to compare intergroup differences in each of these phyla at endpoint. Bacteroidetes, Firmicutes, and Verrucomicrobia were decreased with Aquamin, whereas Actinobacteria and Proteobacteria were increased (with $P \leq 0.0001/q \leq 0.0001$). Trends were similar in both colon and stool specimens (Fig. 4G and H). Firmicutes OTUs also showed a drop with calcium intervention (colon biopsies only; $P < 0.0001/q < 0.0001$), but there was little change in the other phyla. Little change in OTU relative abundance in any of the major phyla was seen with placebo.

### Identification of OTUs that differed with Aquamin supplementation

Differentially abundant OTUs were identified by LEfSe to explain the pre- and postsupplementation differences with Aquamin treatment. In the Aquamin-supplemented group, 82 OTUs were identified in colon samples (Tables 3A and 3B), and only 9 OTUs were identified in stool samples (Tables 3C and 3D) with an LDA score >2 and significance $P < 0.05$ ($q < 0.05$). Overall, more OTUs decreased in relative abundance than increased after supplementation. Differences between pre- and post-Aquamin supplementation were driven principally by an increases in OTUs within the normally less abundant phyla Proteobacteria, and Actinobacteria, along with three OTUs of phylum Firmicutes (Table 3A) and a decrease in OTUs within the normally higher abundance phyla Firmicutes and Bacteroidetes (Table 3B). Similar trends were also observed in stool samples (Tables 3C and 3D).

Effects on bile acid concentrations

Alterations in bile acid levels were assessed in stool samples from Aquamin- and calcium-treated participants in comparison with placebo as shown in Fig. 5. Aquamin treatment resulted in a net decline in total bile acids ($P = 0.0375/q = 0.0262$) with the major portion of this change being in unconjugated forms (the major components of the fecal bile acid pool; Fig. 5A). The major unconjugated primary bile acids—i.e., cholic acid (CA) and, particularly, chenodeoxycholic acid [CDCA; measured concurrently with deoxycholic acid (DCA)]—were significantly lower in posttreatment Aquamin stool samples ($P = 0.0074/q = 0.0052$ and $P = 0.0310/q = 0.0217$ respectively; Fig. 5B). This was not observed with calcium.

Of the measurable secondary bile acids, taurine-conjugated ursodeoxycholic acid (TUDCA), measured concurrently with taurohyodeoxycholic acid (THDCA), was decreased ($P = 0.0287/q = 0.0161$), as were the α and ω muricholic acids (minor secondary bile acid components; $P = 0.0012/q = 0.0009$ and $P = 0.0003/q < 0.0001$ respectively; Fig. 5C, inset). Two other unconjugated secondary bile acids [hyocholic acid (HCA) and hyodeoxycholic acid (HDCA)] were significantly decreased with Aquamin as compared with placebo ($P = 0.015/q = 0.010$ and $P < 0.0001/q < 0.0001$ respectively; Fig. 5C). HDCA is a byproduct of gut microbial metabolism (39), utilizing HCA and muricholic acids after microbial deconjugation and enzymatic modification. Overall, these Aquamin-associated bile acid changes in stool are consistent with both a decreased bile acid pool and decreased bacterial conversion of primary to secondary bile acids. Of interest, these effects were unique to Aquamin. Calcium supplementation did not show a measurable effect on total fecal bile acids, and only HDCA was significantly decreased (Fig. 5C).

Bile acid levels were also assessed in colon biopsy samples from Aquamin- and calcium-treated groups in comparison
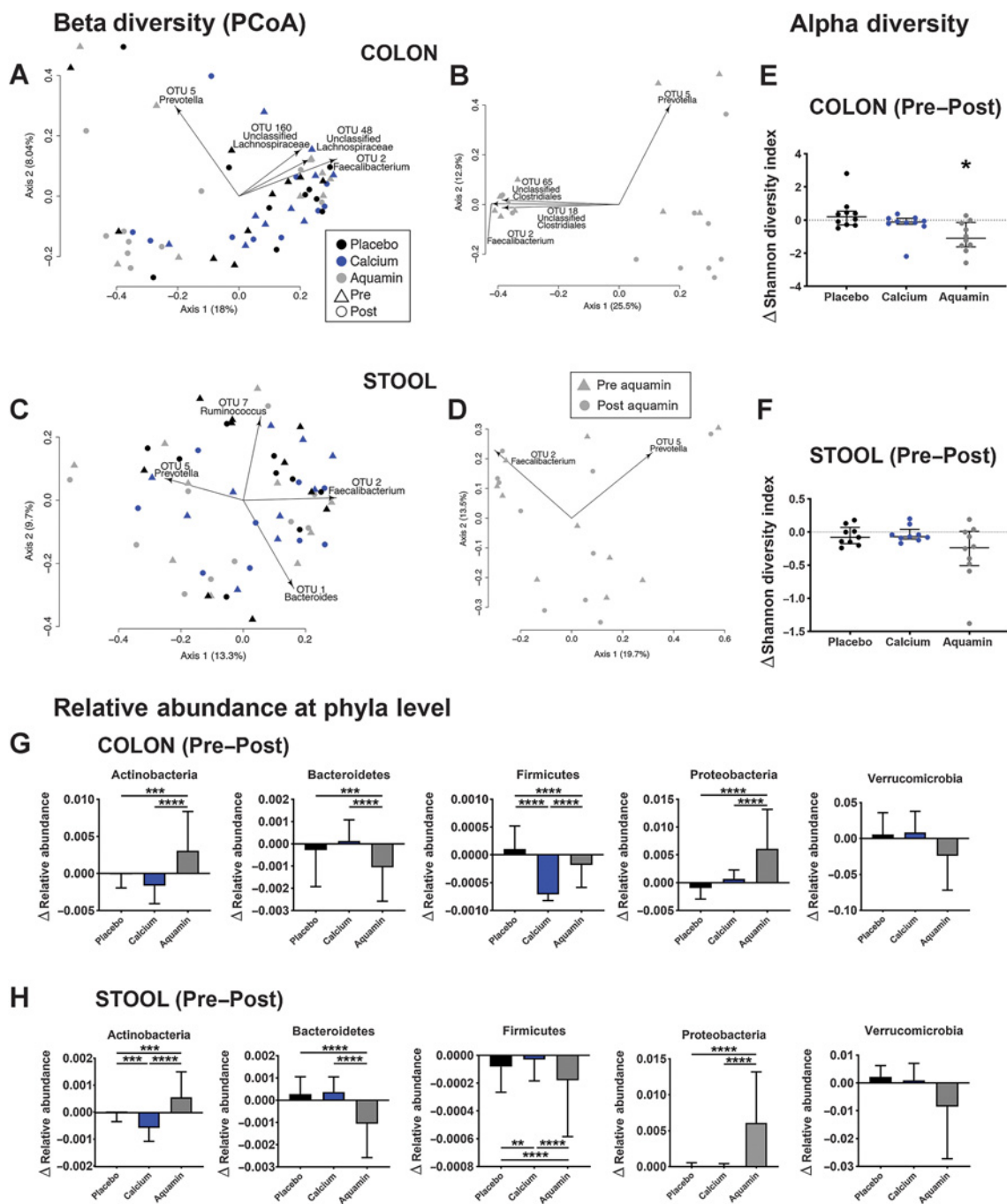
Figure 4.
Segregation of gut microbial communities and gut microbial diversity. Biplot figures depicting PCoAs of θ_{YC} distances between colon biopsy and stool samples based on Illumina sequencing of the V4 region of 16S rRNA. θ_{YC} distance is a measure of difference in pre- and posttreatment microbial populations from individual participants with each of the three interventions. Data from colon specimens are shown in **A** and **B**, whereas stool specimen data are presented in **C** and **D**. Some of the OTUs driving the observed segregation between the groups are shown in the biplots. **A,** Differences in colon microbiota between postintervention Aquamin compared with postintervention placebo and calcium were seen: Aquamin samples with AMOVA P values of 0.005 (compared with placebo) and 0.009 (compared with calcium). **B,** Postintervention colon samples from Aquamin group were also significantly different relative to preintervention Aquamin samples with an AMOVA P value of 0.022. **C** and **D,** There were no significant differences found in stool samples. **E** and **F,** Shannon diversity index. Aquamin intervention reduced gut microbial (alpha) diversity as compared with the placebo in colon biopsy samples (*, $P < 0.05$). No significant change was observed in the gut microbial diversity in stool samples. **G** and **H,** Alterations in the relative abundance of major gut phyla with Aquamin supplementation. The change in the relative abundances of the top 1,000 OTUs (pooled by phyla) and assessed by pre- and postintervention analysis among three interventions in colon and stool specimens. For this analysis, 43 to 48 OTUs across the three treatment groups were pooled for Actinobacteria, 101 to 110 OTUs for Bacteroidetes, 543 to 591 OTUs for Firmicutes, 31 to 39 OTUs for Proteobacteria, and 4 to 6 OTUs for Verrucomicrobia phyla. **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

Table 3A. LefSe data for OTU with elevated relative abundance in colon post-Aquamin.[a]

OTU	Taxonomic ID	Order (family)	Phylum (class)	LDA score[b]	P value	q value
Otu0034	*Ralstonia*	Burkholderiales (*Burkholderiaceae*)	Proteobacteria (Betaproteobacteria)	4.68	0.001	0.024
Otu0052	*Comamonadaceae (uc)*	Burkholderiales (*Comamonadaceae*)	Proteobacteria (Betaproteobacteria)	4.48	0.009	0.027
Otu0092	*Leifsonia*	Actinomycetales (*Microbacteriaceae*)	Actinobacteria (Actinobacteria)	4.07	0.002	0.024
Otu0082	*Stenotrophomonas*	Xanthomonadales (*Xanthomonadaceae*)	Proteobacteria (Gammaproteobacteria)	4.03	0.002	0.024
Otu0095	*Rhodococcus*	Actinomycetales (*Nocardiaceae*)	Actinobacteria (Actinobacteria)	3.98	0.013	0.027
Otu0028	*Dorea*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.49	0.027	0.040
Otu0067	*Lachnospiraceae (uc)*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.36	0.035	0.044
Otu0157	*Flavonifractor*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	3.01	0.006	0.027

[a]Criteria for inclusion: LDA values greater than 2 with $p/q < 0.05$.
[b]LDA values reported as absolute value. OTU: Operational taxonomic unit. uc: unclassified.

Table 3B. LefSe data for OTU with decreased relative abundance in colon post-Aquamin[a].

OTU	Taxonomic ID	Order (family)	Phylum (class)	LDA score[b]	P value	q value
Otu0001	*Bacteroides*	Bacteroidales (*Bacteroidaceae*)	Bacteroidetes (Bacteroidia)	4.40	0.016	0.030
Otu0004	*Blautia*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	4.15	0.023	0.039
Otu0008	*Fusicatenibacter*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	4.00	0.005	0.027
Otu0009	*Anaerostipes*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.92	0.032	0.043
Otu0031	*Akkermansia*	Verrucomicrobiales (*Verrucomicrobiaceae*)	Verrucomicrobia (Verruccomicrobiae)	3.88	0.021	0.036
Otu0026	*Ruminococcus*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.83	0.008	0.027
Otu0016	*Parabacteroides*	Bacteroidales (*Porphyromonadaceae*)	Bacteroidetes (Bacteroidia)	3.81	0.013	0.027
Otu0043	*Lachnospiraceae (uc)*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.80	0.026	0.040
Otu0029	*Bacteroides*	Bacteroidales (*Bacteroidaceae*)	Bacteroidetes (Bacteroidia)	3.68	0.006	0.027
Otu0020	*Bacteroides*	Bacteroidales (*Bacteroidaceae*)	Bacteroidetes (Bacteroidia)	3.63	0.029	0.041
Otu0018	*Clostridiales (uc)*	Clostridiales (*Clostridiales_uc*)	Firmicutes (Clostridia)	3.60	0.024	0.039
Otu0076	*Lachnospiraceae (uc)*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.52	0.003	0.026
Otu0019	*Blautia*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.51	0.004	0.027
Otu0010	*Collinsella*	Coriobacteriales (*Coriobacteriaceae*)	Actinobacteria (Actinobacteria)	3.33	0.048	0.048
Otu0070	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.29	0.001	0.024
Otu0045	*Coprococcus*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.27	0.009	0.027
Otu0025	*Streptococcus*	Lactobacillales (*Streptococcaceae*)	Firmicutes (Bacilli)	3.18	0.018	0.035
Otu0110	*Parasutterella*	Burkholderiales (*Sutterellaceae*)	Proteobacteria (Betaproteobacteria)	3.16	0.021	0.036
Otu0054	*Ruminococcus2*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.15	0.031	0.043
Otu0107	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.14	0.002	0.026
Otu0087	*Bacteroides*	Bacteroidales (*Bacteroidaceae*)	Bacteroidetes (Bacteroidia)	3.08	0.019	0.035
Otu0037	*Dialister*	Selenomonadales (*Veillonellaceae*)	Firmicutes (Negativicutes)	3.08	0.019	0.035
Otu0069	*Coprococcus*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.07	0.045	0.048
Otu0074	*Bacteroides*	Bacteroidales (*Bacteroidaceae*)	Bacteroidetes (Bacteroidia)	3.06	0.007	0.027
Otu0068	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.03	0.009	0.027
Otu0091	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	3.03	0.027	0.040
Otu0128	*Clostridiales_uc*	Clostridiales (*Clostridiales*)	Firmicutes (Clostridia)	3.01	0.004	0.027
Otu0066	*Alistipes*	Bacteroidales (*Rikenellaceae*)	Bacteroidetes (Bacteroidia)	3.00	0.048	0.048
Otu0084	*Dorea*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.98	0.048	0.048
Otu0145	*Clostridium_IV*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.93	0.012	0.027
Otu0089	*Oscillibacter*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.90	0.007	0.027
Otu0148	*Clostridiales_uc*	Clostridiales (*Clostridiales_uc*)	Firmicutes (Clostridia)	2.90	0.036	0.044
Otu0113	*Clostridium_XlVa(98)*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.85	0.013	0.027
Otu0080	*Ruminococcaceae_uc*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.85	0.021	0.036
Otu0132	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.81	0.006	0.027
Otu0160	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.80	0.026	0.040
Otu0134	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.80	0.041	0.047
Otu0228	*Proteobacteria_uc*	Proteobacteria_uc (*Proteobacteria_uc*)	Proteobacteria_uc (Proteobacteria_uc)	2.78	0.013	0.027
Otu0136	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.76	0.002	0.024
Otu0265	*Clostridium_XlVa*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.71	0.029	0.041
Otu0090	*Alistipes*	Bacteroidales (*Rikenellaceae*)	Bacteroidetes (Bacteroidia)	2.70	0.026	0.040
Otu0198	*Ruminococcaceae_uc*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.65	0.015	0.030

(Continued on the following page)

Table 3B. LefSe data for OTU with decreased relative abundance in colon post-Aquamin[a]. (Cont'd)

OTU	Taxonomic ID	Order (family)	Phylum (class)	LDA score[b]	P value	q value
Otu0215	*Faecalicoccus*	Erysipelotrichales (*Erysipelotrichaceae*)	Firmicutes (Erysipelotrichia)	2.64	0.010	0.027
Otu0227	*Clostridium_XIVa*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.64	0.025	0.040
Otu0101	*Ruminococcaceae_uc*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.63	0.036	0.044
Otu0192	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.61	0.009	0.027
Otu0276	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.61	0.006	0.027
Otu0138	*Ruminococcaceae_uc*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.56	0.001	0.024
Otu0149	*Clostridium_IV*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.55	0.001	0.024
Otu0088	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.55	0.021	0.036
Otu0156	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.53	0.039	0.045
Otu0120	*Lactococcus*	Lactobacillales (*Streptococcaceae*)	Firmicutes (Bacilli)	2.52	0.010	0.027
Otu0190	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.50	0.013	0.027
Otu0179	*Blautia*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.49	0.039	0.045
Otu0187	*Clostridium_XIVb*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.49	0.036	0.044
Otu0141	*Blautia*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.42	0.045	0.048
Otu0102	*Streptococcus*	Lactobacillales (*Streptococcaceae*)	Firmicutes (Bacilli)	2.41	0.039	0.045
Otu0278	*Peptoniphilus*	Clostridiales (*Peptoniphilaceae*)	Firmicutes (Clostridia)	2.36	0.045	0.048
Otu0194	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.31	0.031	0.043
Otu0164	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.29	0.015	0.030
Otu0201	*Clostridiales_uc*	Clostridiales (*Clostridiales_uc*)	Firmicutes (Clostridia)	2.28	0.013	0.027
Otu0218	*Ruminococcaceae_uc*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.25	0.039	0.045
Otu0165	*Eggerthella*	Coriobacteriales (*Coriobacteriaceae*)	Actinobacteria (Actinobacteria)	2.24	0.009	0.027
Otu0298	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.21	0.041	0.046
Otu0195	*Ruminococcaceae_uc*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.19	0.013	0.027
Otu0207	*Ruminococcaceae_uc*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.19	0.033	0.044
Otu0307	*Clostridiales_uc*	Clostridiales (*Clostridiales_uc*)	Firmicutes (Clostridia)	2.19	0.007	0.027
Otu0301	*Coriobacteriaceae_uc*	Coriobacteriales (*Coriobacteriaceae*)	Actinobacteria (Actinobacteria)	2.13	0.013	0.027
Otu0185	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.08	0.036	0.044
Otu0224	*Clostridium_XVIII*	Erysipelotrichales (*Erysipelotrichaceae*)	Firmicutes (Erysipelotrichia)	2.04	0.013	0.027
Otu0452	*Oscillibacter*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.02	0.013	0.027
Otu0341	*Holdemania*	Erysipelotrichales (*Erysipelotrichaceae*)	Firmicutes (Erysipelotrichia)	2.02	0.009	0.027
Otu0396	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.02	0.009	0.027
Otu0772	*Ruminococcaceae_uc*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.02	0.013	0.027

[a]Criteria for inclusion: LDA values greater than 2 with $p/q < 0.05$.
[b]LDA values reported as absolute value. OTU: Operational taxonomic unit. uc: unclassified.

Table 3C. LefSe data for OTU with elevated relative abundance in stool post-Aquamin[a].

OTU	Taxonomic ID	Order (family)	Phylum (class)	LDA score[b]	P value	q value
Otu0034	*Ralstonia*	Burkholderiales (*Burkholderiaceae*)	Proteobacteria (Betaproteobacteria)	3.15	0.002	0.010
Otu0194	*Lachnospiraceae_uc*	Clostridiales (*Lachnospiraceae*)	Firmicutes (Clostridia)	2.75	0.043	0.050
Otu0052	*Comamonadaceae (uc)*	Burkholderiales (*Comamonadaceae*)	Proteobacteria (Betaproteobacteria)	2.48	0.002	0.010

[a]Criteria for inclusion: LDA values greater than 2 with $p/q < 0.05$.
[b]LDA values reported as absolute value. OTU: Operational taxonomic unit. uc: unclassified.

Table 3D. LefSe data for OTU with decreased relative abundance in stool post-Aquamin[a].

OTU	Taxonomic ID	Order (family)	Phylum (class)	LDA score[b]	P value	q value
Otu0149	*Clostridium_IV*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	3.02	0.015	0.022
Otu0120	*Lactococcus*	Lactobacillales (*Streptococcaceae*)	Firmicutes (Bacilli)	2.73	0.014	0.022
Otu0272	*Bacteroides*	Bacteroidales (*Bacteroidaceae*)	Bacteroidetes (Bacteroidia)	2.46	0.014	0.022
Otu0234	*Actinomyces*	Actinomycetales (*Actinomycetaceae*)	Actinobacteria (Actinobacteria)	2.43	0.049	0.050
Otu0301	*Coriobacteriaceae_uc*	Coriobacteriales (*Coriobacteriaceae*)	Actinobacteria (Actinobacteria)	2.36	0.050	0.050
Otu0354	*Faecalibacterium*	Clostridiales (*Ruminococcaceae*)	Firmicutes (Clostridia)	2.08	0.010	0.022

[a]Criteria for inclusion: LDA values greater than 2 with $p/q < 0.05$.
[b]LDA values reported as absolute value. OTU: Operational taxonomic unit. uc: unclassified.
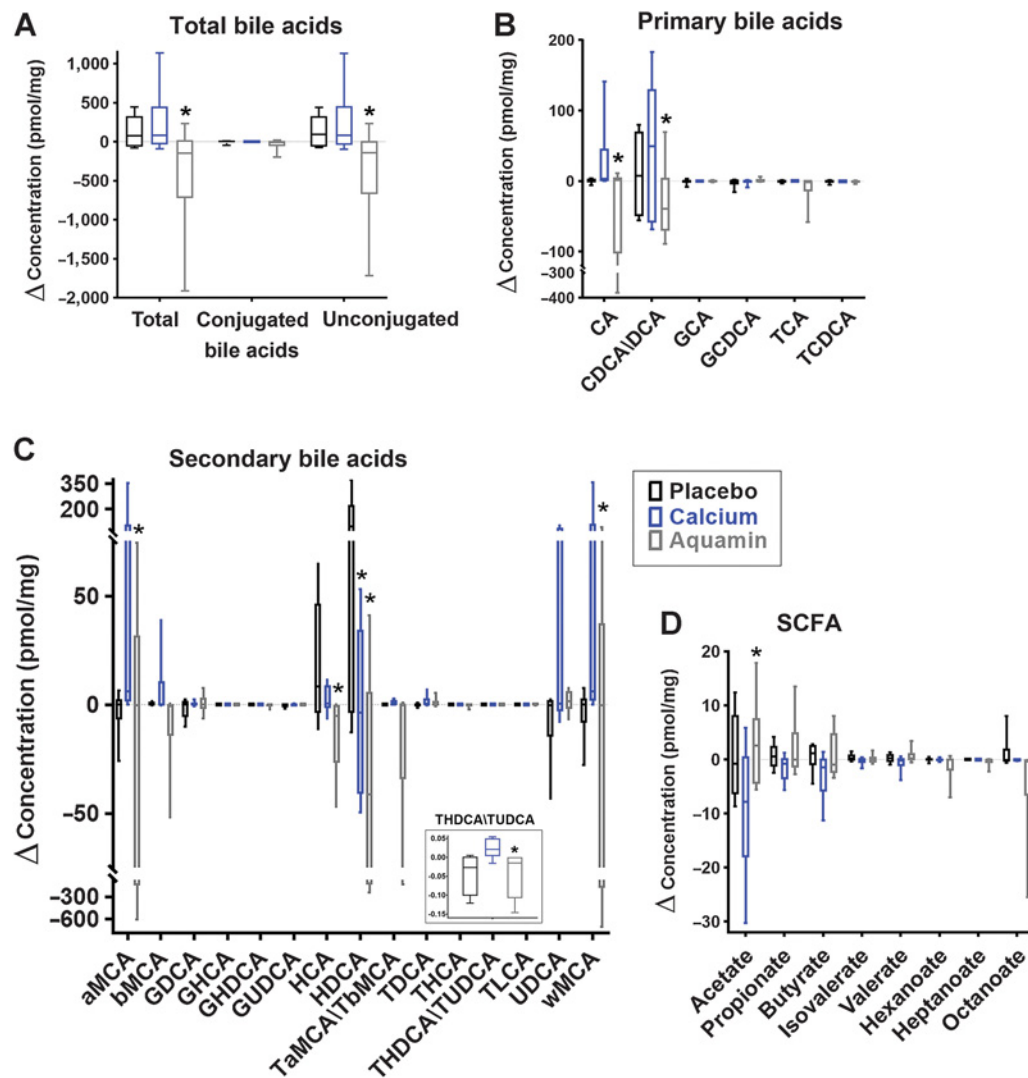
Figure 5.
Decrease in bile acids and increase in SCFAs in stool specimens. Bile acids and SCFA were assessed as described in the Materials and Methods Section. Values shown represent concentration differences between preintervention and postintervention samples. Asterisks represent statistical significance. **A,** Total bile acids (sum of the total conjugated and total unconjugated bile acids) are shown along with conjugated and unconjugated forms. * reflects decrease with Aquamin at $P = 0.0375$ (total) and at $P = 0.0527$ (unconjugated) versus calcium. **B,** Primary bile acids. CA and CDCA measured concurrently with DCA were significantly decreased with Aquamin at $P = 0.0074$ (CA) and $P = 0.0310$ (DCA/CDCA) versus calcium. **C,** Secondary bile acids. HDCA was significantly decreased with Aquamin and calcium versus placebo with P value < 0.0001 and $= 0.0149$ respectively. HCA and TUDCA, measured concurrently with THDCA were decreased with Aquamin relative to calcium, whereas α and ω muricholic acids were also reduced relative to calcium. For HCA, $P = 0.015$; for TUDCA/THDCA, $P = 0.0287$; for αMCA, $P = 0.0012$; and for ωMCA, $P = 0.0003$. With calcium supplementation, HDCA was also decreased relative to placebo ($P = 0.0149$). Inset: TUDCA/THDCA. **D,** SCFA. Acetate was significantly increased with Aquamin relative to calcium alone ($P < 0.0001$).

with placebo (Supplementary Fig. S2). Similar trends to those seen in stool specimens were observed, but none of the changes with Aquamin or calcium intervention reached statistical significance in comparison with placebo except HDCA which was significantly reduced with Aquamin ($P = 0.0456/q = 0.0319$) as compared with placebo.

Effects on SCFA concentrations

SCFA levels were assessed in both stool and colon samples. Apart from a modest (but statistically significant)

increase in acetate in stool samples from Aquamin-treated individuals relative to calcium ($P < 0.0001/q < 0.0001$), no other statistically significant alterations were observed in either stool (**Fig. 5D**) or colon (Supplementary Fig. S2) specimens.

As part of the analyses, we attempted to correlate bile acid changes and changes in SCFAs with alterations in microbial parameters. A weak correlation between total bile acid levels and θYC was observed, but this was not statistically significant (due, in large part, to small sample size).

Effects on colonic eicosanoid concentrations

There was no significant change from baseline detected for any eicosanoid apart from an increase in 13S-hydroxy-octadecadienoic acid (13S-HODE) in calcium-supplemented colon samples (Supplementary Fig. S3).

Discussion

This study demonstrated that 90-day dietary intervention with Aquamin was well-tolerated by healthy individuals. There were no changes in liver injury/function and kidney function markers; there were no serious adverse events; and minor adverse events were no higher with Aquamin than with calcium alone or placebo. These findings are consistent with the previous reports (25–27). Although a much larger database will be required to establish safety, all of the data to date suggest that safety and tolerability will not be issues with Aquamin. At the same time, intervention with Aquamin resulted in measurable changes in the colonic microbial community and the attendant bile acid profile. We are currently carrying out a phase I/II therapeutic trial with Aquamin in ulcerative colitis in remission. The same microbial and metabolomic endpoints assessed here are being measured in the ongoing trial. It is our hope that these microbial and metabolomic findings will prove useful as biomarkers going forward in the interventional trial.

The microbial population present in the colon of an individual is sensitive to environmental influences, including diet (40, 41). Past studies have demonstrated that calcium (18, 19) and Aquamin itself (20) can influence the colonic microbial community in mice. In the present study, we found that Aquamin supplementation in human participants resulted in a decrease in total colonic microbial DNA and an overall decrease in OTUs within the major gut phyla Firmicutes and Bacteroidetes, with a decrease in gut microbial diversity. Thus, the major impact may simply be a decrease in total gut bacteria. The impact of the decrease in bacteria on colonic health is unknown at this time but poses interesting questions for future exploration. Germ-free mice have shown increased resistance to chemical or dietary-induced colon cancers (42). Because our own previous studies have shown that Aquamin-treated mice have decreased incidence of colon polyps (10, 11) as well as fewer liver tumors (43), the possibility that Aquamin-associated microbial decreases may play a mechanistic role in these observations warrants further exploration. Of interest, many of the observations made with samples from Aquamin-treated participants were not seen in samples from individuals ingesting calcium alone. This argues that although calcium is the major mineral component in Aquamin, the effects of Aquamin on the gut microbial community cannot be attributed to calcium alone.

The changes we observed in the post-Aquamin treatment specimens are similar to the effects of broad-spectrum antibiotic treatment, which have been shown to cause a significant reduction in the proportions of Firmicutes and Bacteroidetes and an increase in Proteobacteria (44). As a follow-up to the observations reported here, it would be informative to assess the potential antimicrobial activity of Aquamin supplementation directly in a minimum inhibitory concentration assay. Metals, particularly cations, have previously been utilized as antimicrobial agents (45). For example, colloidal or nanoparticulate silver and mineral-rich clays are known to have broad-spectrum antimicrobial effects (46, 47). Notably, in our study, the administration of Aquamin did not result in diarrhea and caused only a small drop in bacterial diversity in colon samples; both common adverse effects of broad-spectrum antibiotic use. Thus, the antimicrobial effects of Aquamin appear to be mild in comparison with antibiotics and may allow some of the benefit of reduced gut microbial populations (e.g., reduced potentially carcinogenic secondary bile acids) without inducing symptoms that would render the intervention intolerable.

In parallel with microbial changes, Aquamin administration decreased the levels of total bile acids and selected primary and secondary bile acids. This finding is consistent with the decrease in total bacteria and may be potentially beneficial to colon health. At high concentrations, bile acids are membrane-damaging and cytotoxic (21). In addition, several secondary bile acids, most notably lithocholic acid (LCA) and DCA (22, 23), are known to be carcinogenic. Although these species could not be individually measured in this study for methodological reasons, the combination of DCA and CDCA (the primary bile acid precursor of LCA) was significantly lower in the Aquamin-supplemented group (**Fig. 5**). This suggests that one beneficial effect of Aquamin supplementation might be through effects on bile acid metabolism. A caveat in the interpretation of bile acid data is that it is not currently known whether the reduction in gut bile acid pools is due to altered bacterial composition or, potentially, to a direct effect on the bile acids, themselves. Previous studies have shown that calcium, the major component of Aquamin, has the capacity to precipitate bile acids (48). Although mineral binding and precipitation might interfere with detection, this could be beneficial if it prevented potentially harmful secondary bile acids from binding to colonic epithelium or entering the circulation. Arguing against direct binding and precipitation is the finding that calcium alone did not mimic the effects of Aquamin on the total bile acid pool.

A decrease in gut bacteria could, potentially, be harmful to colon health if it resulted in decreased concentration of protective bacterial metabolites, such as the colon-protective SCFAs (24). However, in this study there was no decrease in the colonic concentration of butyrate in the Aquamin-treated group and an actual increase in acetate. Thus, even with the apparent decline in total bacteria, the size and/or composition of the microbial pool was sufficient to maintain presupplementation levels of certain SFCAs.

Although the data strongly suggest an Aquamin-associated decrease in gut microbial bacteria, the specific shifts in gut microbial populations are harder to interpret. One complication of low microbial biomass samples during microbial sequencing is greater likelihood of detecting reagent or

laboratory contaminant microbes in the amplification step. This has been documented in low-biomass samples such as glacier ice, air, rocks, etc. (49), but is not likely to be a major issue in the normal high biomass of the colon.

It should be noted that the antimicrobial effect of Aquamin might not be a direct consequence of the mineral components making up the supplement. Our recent studies employing colonoid culture technology demonstrated upregulation of proteins having antimicrobial activity upon treatment with Aquamin (50). These included lactotransferrin (51), natural resistance–associated macrophage protein-2 (52), and Ly6/PLAUR domain–containing protein 8 (53). Thus, it is possible that the antimicrobial effects seen with Aquamin *in vivo* are mediated, in part, through its effects on the colonic mucosa, itself. Finally, decreased bacteria, especially as it relates to colon biopsy specimens, might reflect altered bacterial adhesion. In our colonoid culture study (50), several mucins and CEA-CAMS were altered by Aquamin. Studies by others have noted that alterations in mucosal surface proteins affect microbial interactions with the mucosal wall (15, 16, 53).

In addition to the microbial and metabolomic changes, there are other, direct, beneficial effects of Aquamin on the epithelium of the colonic mucosa. Specifically, Aquamin suppressed colon epithelial proliferation and induced differentiation in colonoid culture. Effects on proliferation were seen, primarily, in colonoids derived from large adenomas (14), whereas improved differentiation—including upregulation of multiple cell–cell and cell–matrix adhesion molecules and barrier proteins—was observed in normal tissue-derived colonoids (50) as well as in adenoma colonoids (14). Direct effects on the colonic mucosa and indirect effects resulting from changes in microbial/metabolomic profiles are, of course, not mutually exclusive. Further, as noted above, the indirect effects (i.e., antimicrobial activity) may reflect changes induced in the colonic mucosa.

The primary limitations of this study were the small sample size and short duration, which were by design, as this was an initial tolerability study rather than an efficacy study. Although the study was too short and too small to provide definitive results with several endpoints, it was sufficiently powered to detect significant differences in several microbial and metabolomic (bile acid) endpoints in Aquamin-treated participants relative to the other two interventions. To better define effects on efficacy-related endpoints, we are currently beginning a 180-day interventional trial with Aquamin in participants with ulcerative colitis in remission. Another limitation was the potential for intrasubject (as well as intersubject) variability that needs to be acknowledged. Pre and postinterventional differences in the microbial community were observed in all subjects, but intrasubject variability was less than variability between individuals (Supplementary Fig. S1).

In summary, this pilot study demonstrated that 90-day dietary intervention with a calcium- and magnesium-rich multimineral supplement was well-tolerated in healthy human volunteers. Adverse events were mild and largely consisted of gastrointestinal symptoms (constipation, diarrhea, etc.) that did not preclude completion of the study. Reports of adverse events were actually less frequent with Aquamin than with calcium alone. Consistent with this, liver function/liver injury and renal function markers and other serum biochemical markers did not vary significantly with intervention. With respect to microbial and metabolomic findings, Aquamin supplementation resulted in an overall decrease in gut microbial numbers and a decrease in bile acids, including potentially carcinogenic secondary bile acids or their precursors. Despite the antimicrobial-like effects, concentrations of the colon-protective SCFAs were maintained. These findings, along with previous *in vitro* and animal data (10–14, 50) showing a beneficial effect of Aquamin on colonic health, support future longer-term interventional studies in human participants. Finally, the observation that Aquamin had a more pronounced effect on gut microbial populations and bile acid levels than calcium alone supports the view that the beneficial activity of Aquamin (calcium in conjunction with additional trace elements) cannot be attributed to calcium alone. This conclusion supports findings from a recent epidemiologic study suggesting that calcium in combination of additional minerals may be linked with the lower risk of colorectal cancer (54).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M.N. Aslam, C.M. Bassis, S.M. Zick, D.K. Turgeon, J. Varani
Development of methodology: M.N. Aslam, C.M. Bassis, S.M. Zick, D.K. Turgeon, J. Varani
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.N. Aslam, C.M. Bassis, I.L. Bergin, K. Knuver, D.K. Turgeon, J. Varani
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.N. Aslam, C.M. Bassis, I.L. Bergin, K. Knuver, A. Sen, J. Varani
Writing, review, and/or revision of the manuscript: M.N. Aslam, C.M. Bassis, I.L. Bergin, K. Knuver, S.M. Zick, A. Sen, D.K. Turgeon, J. Varani
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.N. Aslam, C.M. Bassis, J. Varani
Study supervision: M.N. Aslam, D.K. Turgeon, J. Varani

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Cancer Prevention Research

A Calcium-Rich Multimineral Intervention to Modulate Colonic Microbial Communities and Metabolomic Profiles in Humans: Results from a 90-Day Trial

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Supplementary material

Supplement Table 1: A complete list of elements detected in Aquamin® (Mineral composition)

Supplement Table 2: Demographic characteristics of the participant population

Supplement Table 3: The distribution of Phyla based on sample type (all samples)

Supplement Table 4: The distribution of Genera based on sample type (all samples)

Supplement Figure 1: Microbial variability – intra- and inter-subject differences.

Supplement Figure 2: Metabolomic (Bile acids and SCFA) profile of colon biopsies

Supplement Figure 3: The evaluation of eicosanoid metabolites in colon tissue

Supplemental Figure Legends

Supplement Figure 1. Intra-subject versus inter-subject variability. θ_{YC} distances as a comparison of intra-subject vs inter-subject differences in bacterial community composition for colon (left) and stool (right) samples. For both data sets, intra-subject variation was measured as θ_{YC} distances between pre and post-intervention samples for each individual ('within' column). Inter-subject variation was measured as θ_{YC} distances between each pre-intervention sample and all other samples ("between" column). Higher values reflected greater differences. Inter-subject variation for both sample types was greater than intra-subject variation. *** reflects significance was at $p=0.0001$ and **** reflects significance was at $p<0.0001$.

Supplement Figure 2. Alteration in the metabolomic profile of colonic specimens. **A**. Total bile acids. **B**. Primary bile acids. **C**. Secondary bile acids. **D**. SCFA. No statistically-significant differences were detected in these data sets but trends were to those observed in stool samples except HDCA which was significantly reduced with Aquamin® ($p=0.0456$ / $q=0.0319$) as compared to placebo.

Supplement Figure 3. Eicosanoid metabolites in colon biopsy specimens. Eicosanoid metabolites were assessed as described in the Materials and Methods Section. The left panel represents lipoxygenase metabolites and the right panel reflects cyclooxygenase metabolites. Values shown represent differences in concentration between pre-intervention and post-intervention samples. No statistically-significant differences were detected in these data sets. Eicosanoid acronyms:

5-OxoETE	5-oxo-eicosatetraenoic acid
12-OxoETE	12-oxo-eicosatetraenoic acid
12(S)-HETE	12(S)-Hydroxy-eicosatetraenoic acid
15(S)-HETE	15(S)-Hydroxy-eicosatetraenoic acid
20(S)-HETE	20(S)-Hydroxy-eicosatetraenoic acid
5(S)-HETE	5(S)-Hydroxy-eicosatetraenoic acid
11-HETE	11-Hydroxy-eicosatetraenoic acid
8-HETE	8-Hydroxy-eicosatetraenoic acid
9-HETE	9-Hydroxy-eicosatetraenoic acid
13-S-HODE	13(S)-hydroxy-octadecadienoic acid
LKT B4	Leukotriene B4
6k PSG F1a	6-keto Prostaglandin F1-alpha
PSG A2	Prostaglandin A2
PSG B2	Prostaglandin B2
PSG D2	Prostaglandin D2
PSG E2	Prostaglandin E2
PSG F2a	Prostaglandin F2a (PGF2alpha)
RSV D1	Trihydroxy-docosahexaenoic acid D1 (Resolvin D1)
RSV D2	Trihydroxy-docosahexaenoic acid D2 (Resolvin D2)
TB B2	Thromboxane B2

Supplement Figure 1
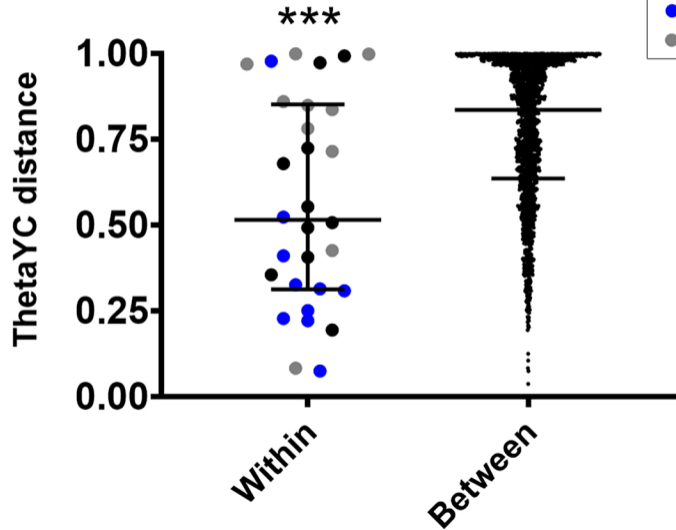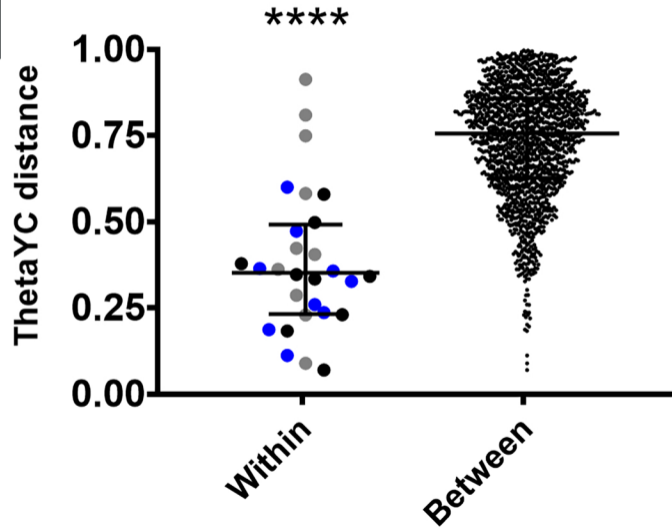
Supplement Figure 2   (Colon)

A. Total Bile Acids

B. Primary Bile Acids
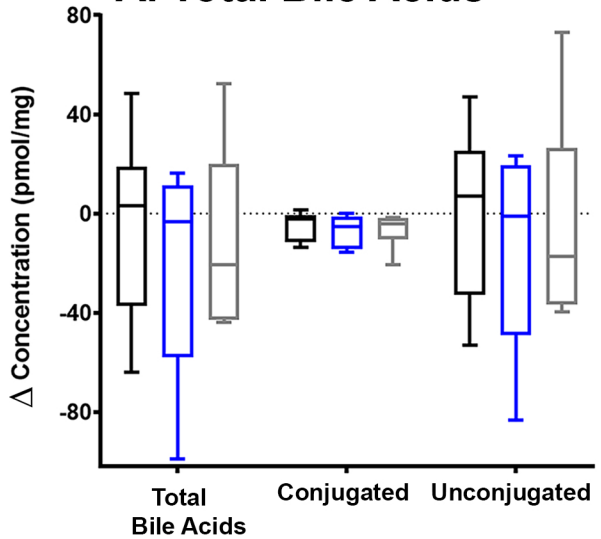
C. Secondary Bile Acids
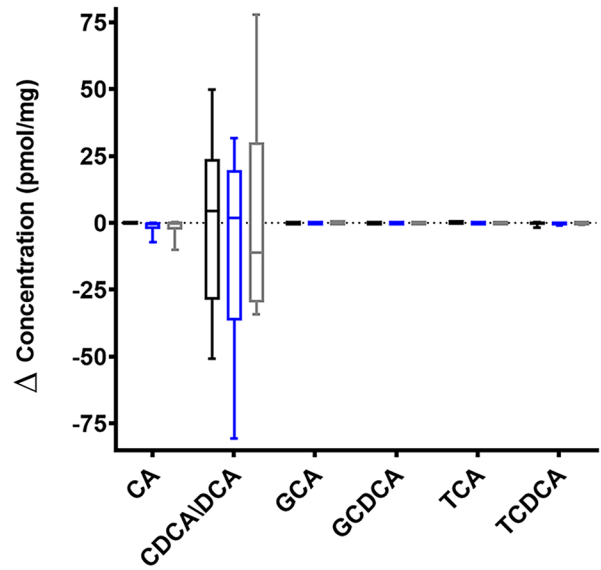
D. SCFA
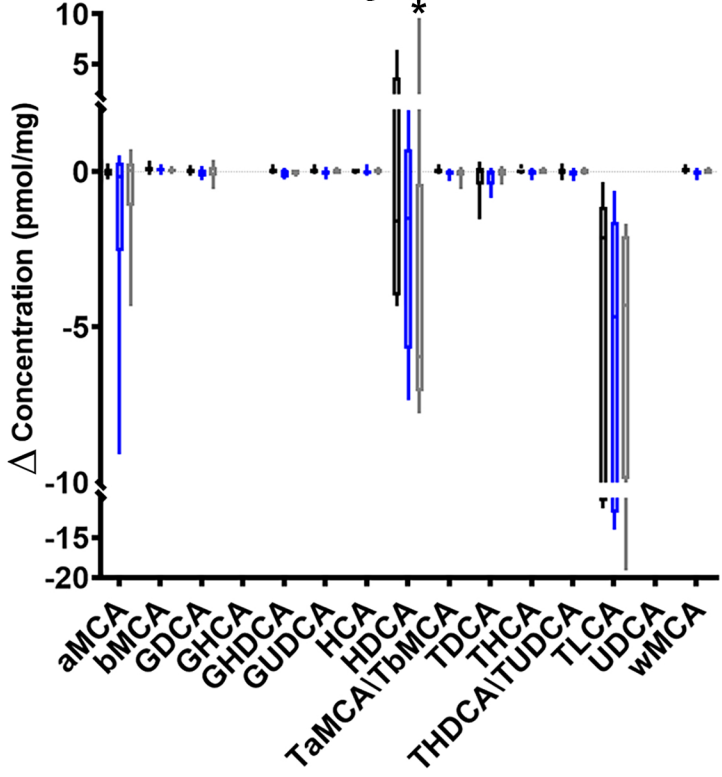
Supplement Figure 3
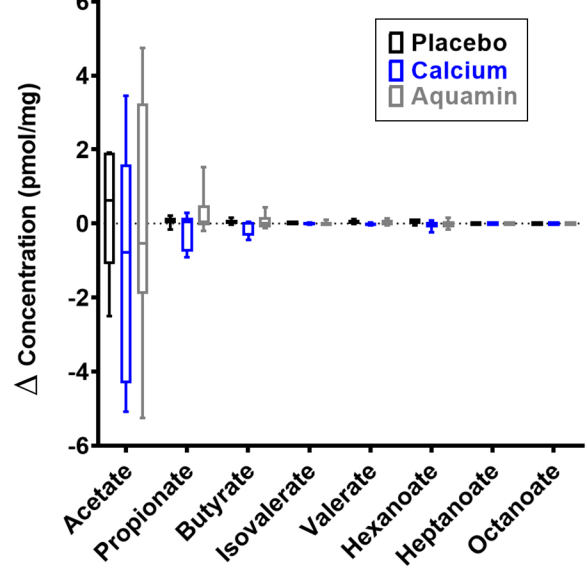
Colon eicosanoids (Pre-Post)
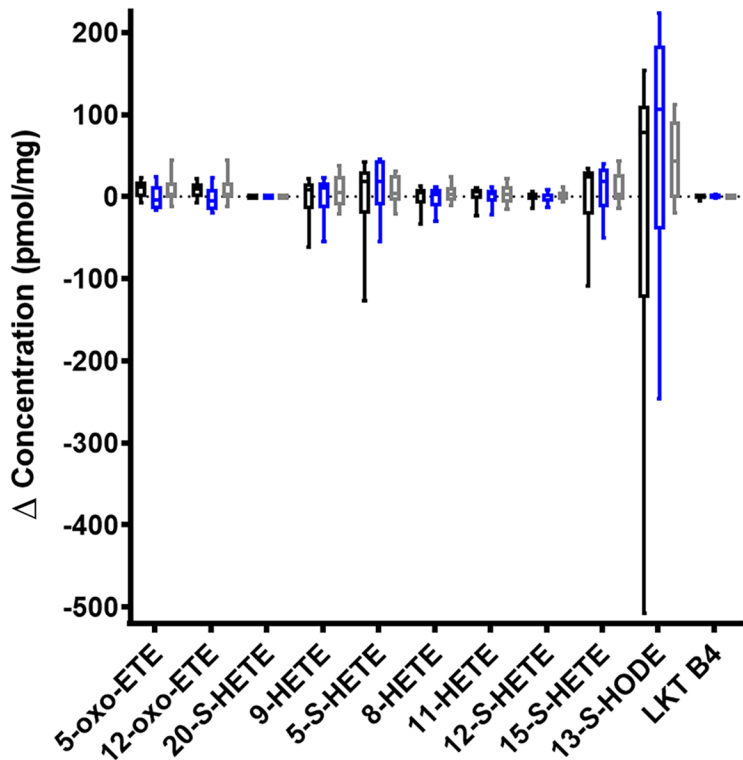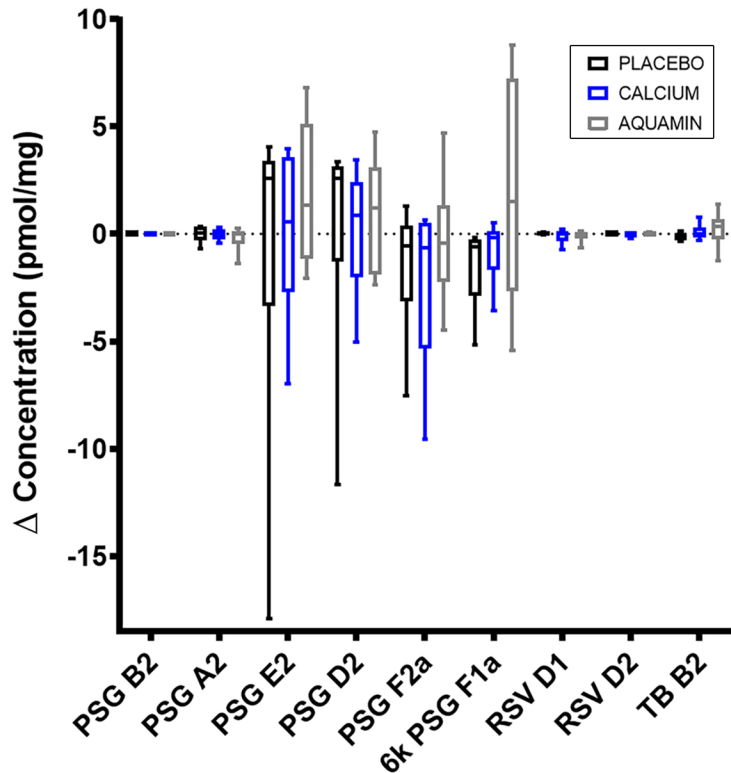
Supplement Table 1. Mineral composition of the Aquamin® TG.

Element	µg/g	Element	µg/g	Element	µg/g
Aluminum	208	Hafnium	0.057	Rubidium	0.019
Antimony	<0.5	Holmium	0.013	Ruthenium	2.271
Arsenic	0.840	Indium	<0.001	Samarium	0.042
Barium	4.67	Iodine	19.12	Scandium	1.028
Beryllium	<0.5	Iridium	<0.001	Selenium	<0.5
Bismuth	<0.5	Iron	689	Silicon	109
Boron	34.5	Lanthanum	<0.5	Silver	3.06
Cadmium	0.526	Lead	0.084	Sodium	4,971
Calcium	340,900	Lithium	<0.5	Strontium	2,265
Carbon	128,000	Lutetium	0.017	Sulfur	3,554
Cerium	0.307	Magnesium	14,190	Tantalum	0.019
Cesium	0.001	Manganese	36.9	Tellurium	<0.5
Chloride	869	Mercury	<0.001	Terbium	0.009
Chromium	<0.5	Molybdenum	<0.5	Thallium	<0.5
Cobalt	0.61	Neodymium	0.179	Thorium	6.91
Copper	1.99	Nickel	<0.5	Thulium	0.005
Dysprosium	0.057	Niobium	<0.5	Tin	0.106
Erbium	0.041	Osmium	<0.001	Titanium	31.7
Europium	0.014	Palladium	0.247	Tungsten	<0.5
Fluoride	1.01	Phosphorous	559	Vanadium	2.73
Gadolinium	0.056	Platinum	0.003	Ytterbium	0.036
Gallium	0.138	Potassium	338	Yttrium	<0.5
Germanium	<0.001	Praseodymium	0.041	Zinc	11.4
Gold	4.37	Rhenium	0.002	Zirconium	5.21
		Rhodium	0.189		

Source: 2015 Test Certificate of Aquamin® TG (Food Grade) used in the trial, Mineral composition was tested by Advanced Laboratories, Inc. (Salt Lake City), for client Marigot Limited (Ireland). Most of the levels of individual trace elements were determined by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) except Carbon (determined by LECO), Chloride, Iodine (determined by Titration), and Fluoride (determined by AOAC 939.11).

Supplement Table 2. Demographic data of participants who completed the 90 days trial.

A. Study population

	Female		Male		Both Genders	
Ethnic Category	N	%	N	%	Total	%
Hispanic or Latino	0	0.00%	0	0.00%	0	0.00%
Not Hispanic or Latino	22	73.33%	8	26.67%	30	100.00%
Total	**22**	**73.33%**	**8**	**26.67%**	**30**	**100.00%**
Racial Category	N	%	N	%	Total	%
American Indian/Alaska Native	0	0.00%	0	0.00%	0	0.00%
Asian	0	0.00%	0	0.00%	0	0.00%
Native Hawaiian or Other Pacific Islander	0	0.00%	0	0.00%	0	0.00%
Black or African American	5	16.67%	0	0.00%	5	16.67%
White	17	56.66%	8	26.67%	25	83.33%
Total	**22**	**73.33%**	**8**	**26.67%**	**30**	**100.00%**
Age at Enrollment	N	%	N	%	Total	%
18 - 21 years	0	0.00%	1	3.33%	1	3.33%
22 - 29 years	0	0.00%	0	0.00%	0	0.00%
30 - 39 years	1	3.33%	0	0.00%	1	3.33%
40 - 49 years	1	3.33%	0	0.00%	1	3.33%
50 - 59 years	14	46.67%	2	6.67%	16	53.33%
60 - 69 years	6	20.00%	5	16.67%	11	36.67%
70 - 79 years	0	0.00%	0	0.00%	0	0.00%
> 80 years	0	0.00%	0	0.00%	0	0.00%
Total	**22**	**73.33%**	**8**	**26.67%**	**30**	**100.00%**

B. Demographics by group.

Group	Gender	Age (Years) Mean±SD	Capsule intake %
Placebo	Male:4 / Female:6	57.4±6.0	96.1±12.8
Calcium	Male:2 / Female:8	50.3±15.2	96.7±11.2
Aquamin®	Male:2 / Female:8	58.2±4.8	97.5±9.6

Age presented in years at the start of the study.

Compliance is reflected by daily capsule intake log and capsule count at study end.

Supplement Table 3. The distribution of Phyla based on sample type (all samples).

	Colon	Stool	Common OTUs
Total OTUs: 7267	2573	6642	1948
Range (Sequence count)	1 - 205,463	1 - 250,238	

# of Phyla:	**Colon: 15**		**Stool: 10**

Colon:	**OTUs present**	**Stool:**	**OTUs present**
Firmicutes	1770	Firmicutes	5244
Bacteroidetes	364	Bacteroidetes	681
Bacteria_unclassified	135	Bacteria_unclassified	244
Proteobacteria	126	Actinobacteria	238
Actinobacteria	119	Proteobacteria	159
Verrucomicrobia	32	Verrucomicrobia	54
Tenericutes	6	Synergistetes	8
Fusobacteria	5	Fusobacteria	7
Lentisphaerae	4	Lentisphaerae	5
Spirochaetes	4	Candidatus_Saccharibacteria	2
Synergistetes	4		
Acidobacteria	1		
Candidatus_Saccharibacteria	1		
Deinococcus-Thermus	1		
Planctomycetes	1		

Supplement Table 4. The distribution of Genera based on sample type (all samples).

# of Genera: Colon: 224 / Stool: 213

Colon:

Phyla	Genus	# of OTUs
Firmicutes	Lachnospiraceae_unclassified	420
Firmicutes	Ruminococcaceae_unclassified	271
Firmicutes	Clostridiales_unclassified	223
Bacteroidetes	Bacteroides	164
Bacteria_unclassified	Bacteria_unclassified	135
Firmicutes	Blautia	100
Firmicutes	Firmicutes_unclassified	89
Firmicutes	Faecalibacterium	58
Bacteroidetes	Prevotella	48
Firmicutes	Ruminococcus2	47
Firmicutes	Ruminococcus	41
Firmicutes	Roseburia	37
Verrucomicrobia	Akkermansia	31
Firmicutes	Streptococcus	30
Firmicutes	Clostridium_IV	29
Firmicutes	Clostridium_XIVa	25
Firmicutes	Clostridia_unclassified	24
Firmicutes	Fusicatenibacter	24
Firmicutes	Anaerostipes	22
Bacteroidetes	Butyricimonas	22
Firmicutes	Oscillibacter	22
Firmicutes	Erysipelotrichaceae_unclassified	21
Bacteroidetes	Parabacteroides	21
Bacteroidetes	Alistipes	19
Actinobacteria	Coriobacteriaceae_unclassified	19
Bacteroidetes	Paraprevotella	19
Actinobacteria	Bifidobacterium	16
Proteobacteria	Enterobacteriaceae_unclassified	16
Firmicutes	Lactobacillus	14
Bacteroidetes	Porphyromonadaceae_unclassified	14
Firmicutes	Coprococcus	13
Firmicutes	Dorea	13
Bacteroidetes	Barnesiella	12
Firmicutes	Clostridium_sensu_stricto	12
Firmicutes	Clostridium_XIVb	12
Actinobacteria	Corynebacterium	12
Firmicutes	Megasphaera	12
Firmicutes	Dialister	11
Actinobacteria	Collinsella	10
Firmicutes	Anaerococcus	9
Bacteroidetes	Bacteroidetes_unclassified	9
Firmicutes	Butyricicoccus	9
Firmicutes	Holdemanella	9
Firmicutes	Peptoniphilus	9

Actinobacteria	Actinomyces	8
Bacteroidetes	Bacteroidales_unclassified	8
Proteobacteria	Ralstonia	8
Proteobacteria	Alphaproteobacteria_unclassified	7
Proteobacteria	Betaproteobacteria_unclassified	7
Firmicutes	Intestinimonas	7
Firmicutes	Phascolarctobacterium	7
Bacteroidetes	Porphyromonas	7
Proteobacteria	Acinetobacter	6
Firmicutes	Anaerotruncus	6
Proteobacteria	Burkholderiales_unclassified	6
Firmicutes	Clostridium_XVIII	6
Bacteroidetes	Odoribacter	6
Actinobacteria	Olsenella	6
Proteobacteria	Proteobacteria_unclassified	6
Tenericutes	Anaeroplasma	5
Firmicutes	Catenibacterium	5
Proteobacteria	Escherichia/Shigella	5
Firmicutes	Peptostreptococcaceae_unclassified	5
Firmicutes	Romboutsia	5
Firmicutes	Veillonellaceae_unclassified	5
Actinobacteria	Actinomycetales_unclassified	4
Spirochaetes	Brachyspira	4
Proteobacteria	Campylobacter	4
Proteobacteria	Comamonadaceae_unclassified	4
Bacteroidetes	Coprobacter	4
Proteobacteria	Cupriavidus	4
Proteobacteria	Desulfovibrio	4
Firmicutes	Ezakiella	4
Firmicutes	Faecalicoccus	4
Firmicutes	Flavonifractor	4
Firmicutes	Lactobacillales_unclassified	4
Firmicutes	Megamonas	4
Proteobacteria	Pasteurellaceae_unclassified	4
Actinobacteria	Slackia	4
Firmicutes	Sporobacter	4
Firmicutes	Turicibacter	4
Lentisphaerae	Victivallis	4
Firmicutes	Acidaminococcus	3
Actinobacteria	Actinobacteria_unclassified	3
Bacteroidetes	Alloprevotella	3
Actinobacteria	Atopobium	3
Firmicutes	Christensenella	3
Synergistetes	Cloacibacillus	3
Firmicutes	Clostridiaceae_1_unclassified	3
Firmicutes	Clostridiales_Incertae_Sedis_XI_unclassified	3
Firmicutes	Coprobacillus	3
Firmicutes	Lactococcus	3
Firmicutes	Mogibacterium	3
Firmicutes	Murdochiella	3

Bacteroidetes	Prevotellaceae_unclassified	3
Actinobacteria	Rothia	3
Firmicutes	Subdoligranulum	3
Proteobacteria	Sutterella	3
Firmicutes	Veillonella	3
Firmicutes	Allobaculum	2
Bacteroidetes	Anaerorhabdus	2
Actinobacteria	Arcanobacterium	2
Proteobacteria	Bilophila	2
Firmicutes	Butyrivibrio	2
Firmicutes	Catabacter	2
Firmicutes	Eubacterium	2
Fusobacteria	Fusobacterium	2
Actinobacteria	Gordonibacter	2
Firmicutes	Holdemania	2
Firmicutes	Intestinibacter	2
Proteobacteria	Janthinobacterium	2
Firmicutes	Lactobacillaceae_unclassified	2
Firmicutes	Leuconostoc	2
Actinobacteria	Microbacteriaceae_unclassified	2
Actinobacteria	Mobiluncus	2
Proteobacteria	Neisseria	2
Proteobacteria	Parasutterella	2
Firmicutes	Peptococcus	2
Proteobacteria	Pseudomonas	2
Proteobacteria	Rhodobacteraceae_unclassified	2
Firmicutes	Solobacterium	2
Firmicutes	Staphylococcus	2
Proteobacteria	Stenotrophomonas	2
Proteobacteria	Succinivibrio	2
Actinobacteria	Varibaculum	2
Firmicutes	Abiotrophia	1
Firmicutes	Acetanaerobacterium	1
Proteobacteria	Achromobacter	1
Actinobacteria	Actinoplanes	1
Proteobacteria	Aeromonas	1
Firmicutes	Allisonella	1
Actinobacteria	Alloscardovia	1
Firmicutes	Anaerofustis	1
Firmicutes	Anaeroglobus	1
Firmicutes	Anaerosporobacter	1
Actinobacteria	Aquihabitans	1
Actinobacteria	Asaccharobacter	1
Actinobacteria	Blastococcus	1
Proteobacteria	Bradyrhizobium	1
Actinobacteria	Brevibacterium	1
Proteobacteria	Brevundimonas	1
Firmicutes	Bulleidia	1
Proteobacteria	Burkholderia	1
Candidatus_Saccharibacteria	Candidatus_Saccharibacteria_unclassified	1

Bacteroidetes	Capnocytophaga	1
Proteobacteria	Cardiobacterium	1
Verrucomicrobia	Cerasicoccus	1
Bacteroidetes	Cloacibacterium	1
Firmicutes	Clostridiales_Incertae_Sedis_XIII_unclassified	1
Firmicutes	Clostridium_XI	1
Proteobacteria	Cronobacter	1
Deinococcus-Thermus	Deinococcus	1
Proteobacteria	Desulfovibrionaceae_unclassified	1
Firmicutes	Dolosigranulum	1
Actinobacteria	Eggerthella	1
Firmicutes	Eisenbergiella	1
Proteobacteria	Enhydrobacter	1
Firmicutes	Enterococcus	1
Firmicutes	Facklamia	1
Firmicutes	Faecalitalea	1
Firmicutes	Finegoldia	1
Bacteroidetes	Flavobacterium	1
Actinobacteria	Gardnerella	1
Firmicutes	Gemella	1
Actinobacteria	Georgenia	1
Acidobacteria	Gp6_unclassified	1
Firmicutes	Granulicatella	1
Proteobacteria	Haemophilus	1
Firmicutes	Howardella	1
Firmicutes	Hungatella	1
Proteobacteria	Hyphomicrobium	1
Actinobacteria	Janibacte	1
Firmicutes	Lachnoanaerobaculum	1
Firmicutes	Lactonifactor	1
Actinobacteria	Leifsonia	1
Fusobacteria	Leptotrichia	1
Fusobacteria	Leptotrichiaceae_unclassified	1
Actinobacteria	Micrococcaceae_unclassified	1
Actinobacteria	Micrococcus	1
Firmicutes	Mitsuokella	1
Firmicutes	Moryella	1
Firmicutes	Murimonas	1
Actinobacteria	Mycobacterium	1
Tenericutes	Mycoplasma	1
Proteobacteria	Myxococcales_unclassified	1
Firmicutes	Negativicoccus	1
Actinobacteria	Nocardioidaceae_unclassified	1
Actinobacteria	Nocardioides	1
Firmicutes	Oribacterium	1
Proteobacteria	Oxalobacteraceae_unclassified	1
Firmicutes	Parvimonas	1
Firmicutes	Pediococcus	1
Firmicutes	Pelospora	1
Firmicutes	Peptoniphilaceae_unclassified	1

Firmicutes	Peptostreptococcus	1
Proteobacteria	Phenylobacterium	1
Proteobacteria	Phreatobacter	1
Proteobacteria	Phyllobacterium	1
Planctomycetes	Planctomycetaceae_unclassified	1
Firmicutes	Planococcaceae_unclassified	1
Proteobacteria	Polynucleobacter	1
Actinobacteria	Propionibacterium	1
Firmicutes	Pseudoflavonifractor	1
Synergistetes	Pyramidobacter	1
Proteobacteria	Rhizobiaceae_unclassified	1
Actinobacteria	Rhodococcus	1
Proteobacteria	Rhodopseudomonas	1
Proteobacteria	Rhodospirillaceae_unclassified	1
Proteobacteria	Salinisphaera	1
Actinobacteria	Scardovia	1
Actinobacteria	Senegalimassilia	1
Fusobacteria	Sneathia	1
Proteobacteria	Sphingomonas	1
Firmicutes	Staphylococcaceae_unclassified	1
Firmicutes	Stomatobaculum	1
Firmicutes	Succiniclasticum	1
Proteobacteria	Tepidimonas	1
Firmicutes	Terrisporobacter	1
Proteobacteria	Tolumonas	1
Actinobacteria	Trueperella	1
Firmicutes	Weissella	1
Proteobacteria	Xanthomonas	1
Proteobacteria	Yersinia	1

Stool:

Phyla	Genus	# of OTUs
Firmicutes	Lachnospiraceae_unclassified	1624
Firmicutes	Ruminococcaceae_unclassified	757
Firmicutes	Clostridiales_unclassified	722
Bacteroidetes	Bacteroides	368
Firmicutes	Firmicutes_unclassified	245
Bacteria_unclassified	Bacteria_unclassified	244
Firmicutes	Blautia	235
Firmicutes	Faecalibacterium	185
Firmicutes	Ruminococcus2	150
Firmicutes	Roseburia	130
Firmicutes	Ruminococcus	115
Firmicutes	Coprococcus	88
Firmicutes	Fusicatenibacter	86
Bacteroidetes	Prevotella	63
Firmicutes	Streptococcus	60
Actinobacteria	Collinsella	59
Firmicutes	Dorea	53
Verrucomicrobia	Akkermansia	52

Firmicutes	Anaerostipes	50
Firmicutes	Clostridium_IV	50
Bacteroidetes	Parabacteroides	45
Actinobacteria	Coriobacteriaceae_unclassified	44
Firmicutes	Erysipelotrichaceae_unclassified	43
Firmicutes	Clostridium_XIVa	40
Bacteroidetes	Alistipes	39
Bacteroidetes	Butyricimonas	37
Firmicutes	Clostridia_unclassified	36
Firmicutes	Dialister	36
Actinobacteria	Bifidobacterium	34
Firmicutes	Oscillibacter	33
Firmicutes	Phascolarctobacterium	29
Proteobacteria	Enterobacteriaceae_unclassified	27
Bacteroidetes	Paraprevotella	27
Firmicutes	Clostridium_XIVb	26
Firmicutes	Clostridium_sensu_stricto	23
Firmicutes	Holdemanella	22
Bacteroidetes	Porphyromonadaceae_unclassified	20
Firmicutes	Lactobacillus	19
Bacteroidetes	Barnesiella	18
Firmicutes	Flavonifractor	18
Bacteroidetes	Odoribacter	17
Firmicutes	Subdoligranulum	17
Firmicutes	Megamonas	16
Firmicutes	Romboutsia	16
Bacteroidetes	Bacteroidales_unclassified	15
Firmicutes	Anaerotruncus	14
Firmicutes	Catenibacterium	14
Firmicutes	Clostridiaceae_1_unclassified	14
Proteobacteria	Sutterella	14
Actinobacteria	Actinomyces	13
Firmicutes	Acidaminococcus	12
Proteobacteria	Bilophila	12
Firmicutes	Intestinimonas	12
Firmicutes	Butyricicoccus	11
Firmicutes	Megasphaera	11
Actinobacteria	Slackia	11
Firmicutes	Veillonellaceae_unclassified	11
Firmicutes	Lactobacillales_unclassified	10
Firmicutes	Lactococcus	10
Firmicutes	Peptostreptococcaceae_unclassified	10
Actinobacteria	Actinomycetales_unclassified	9
Proteobacteria	Alphaproteobacteria_unclassified	9
Bacteroidetes	Bacteroidetes_unclassified	9
Proteobacteria	Burkholderiales_unclassified	9
Firmicutes	Clostridium_XVIII	9
Firmicutes	Intestinibacter	9
Synergistetes	Cloacibacillus	8
Proteobacteria	Desulfovibrio	8

Firmicutes	Eisenbergiella	8
Proteobacteria	Succinivibrio	8
Firmicutes	Turicibacter	8
Actinobacteria	Asaccharobacter	7
Proteobacteria	Betaproteobacteria_unclassified	7
Actinobacteria	Corynebacterium	7
Actinobacteria	Eggerthella	7
Firmicutes	Mogibacterium	7
Firmicutes	Veillonella	7
Bacteroidetes	Alloprevotella	6
Actinobacteria	Bifidobacteriaceae_unclassified	6
Firmicutes	Faecalicoccus	6
Actinobacteria	Gordonibacter	6
Firmicutes	Holdemania	6
Actinobacteria	Olsenella	6
Proteobacteria	Parasutterella	6
Firmicutes	Peptoniphilus	6
Firmicutes	Sporobacter	6
Firmicutes	Acidaminococcaceae_unclassified	5
Firmicutes	Anaerococcus	5
Firmicutes	Butyrivibrio	5
Firmicutes	Eubacterium	5
Proteobacteria	Pasteurellaceae_unclassified	5
Bacteroidetes	Porphyromonas	5
Proteobacteria	Proteobacteria_unclassified	5
Firmicutes	Pseudoflavonifractor	5
Actinobacteria	Senegalimassilia	5
Lentisphaerae	Victivallis	5
Actinobacteria	Actinobacteria_unclassified	4
Firmicutes	Bacilli_unclassified	4
Proteobacteria	Campylobacter	4
Firmicutes	Christensenella	4
Firmicutes	Coprobacillus	4
Proteobacteria	Escherichia/Shigella	4
Firmicutes	Leuconostoc	4
Firmicutes	Murimonas	4
Firmicutes	Anaerofustis	3
Firmicutes	Catabacter	3
Bacteroidetes	Coprobacter	3
Proteobacteria	Desulfovibrionaceae_unclassified	3
Firmicutes	Hungatella	3
Fusobacteria	Leptotrichia	3
Firmicutes	Parvimonas	3
Firmicutes	Pediococcus	3
Bacteroidetes	Prevotellaceae_unclassified	3
Proteobacteria	Pseudomonas	3
Actinobacteria	Rothia	3
Firmicutes	Terrisporobacter	3
Firmicutes	Abiotrophia	2
Proteobacteria	Acinetobacter	2

Firmicutes	Allobaculum	2
Bacteroidetes	Anaerorhabdus	2
Firmicutes	Anaerosporobacter	2
Actinobacteria	Atopobium	2
Firmicutes	Bacillales_unclassified	2
Firmicutes	Bulleidia	2
Candidatus_Saccharibacteria	Candidatus_Saccharibacteria_unclassified	2
Verrucomicrobia	Cerasicoccus	2
Firmicutes	Clostridiales_Incertae_Sedis_XIII_unclassified	2
Proteobacteria	Cupriavidus	2
Firmicutes	Ezakiella	2
Firmicutes	Finegoldia	2
Fusobacteria	Fusobacterium	2
Firmicutes	Gemella	2
Firmicutes	Granulicatella	2
Firmicutes	Lactobacillaceae_unclassified	2
Firmicutes	Lactonifactor	2
Actinobacteria	Mobiluncus	2
Firmicutes	Murdochiella	2
Proteobacteria	Neisseria	2
Firmicutes	Peptococcus	2
Proteobacteria	Tolumonas	2
Firmicutes	Weissella	2
Actinobacteria	Actinomycetaceae_unclassified	1
Proteobacteria	Aeromonas	1
Firmicutes	Allisonella	1
Actinobacteria	Alloscardovia	1
Firmicutes	Anaeroglobus	1
Actinobacteria	Arcanobacterium	1
Firmicutes	Bacillus	1
Proteobacteria	Bosea	1
Actinobacteria	Brachybacterium	1
Proteobacteria	Bradyrhizobium	1
Proteobacteria	Burkholderia	1
Firmicutes	Calditerricola	1
Proteobacteria	Cardiobacterium	1
Firmicutes	Carnobacteriaceae_unclassified	1
Actinobacteria	Clavibacter	1
Bacteroidetes	Cloacibacterium	1
Firmicutes	Clostridiales_Incertae_Sedis_XI_unclassified	1
Firmicutes	Clostridium_III	1
Firmicutes	Clostridium_XI	1
Proteobacteria	Comamonadaceae_unclassified	1
Proteobacteria	Cronobacter	1
Actinobacteria	Cryptobacterium	1
Proteobacteria	Deltaproteobacteria_unclassified	1
Proteobacteria	Diplorickettsia	1
Firmicutes	Dolosigranulum	1
Bacteroidetes	Dysgonomonas	1
Firmicutes	Enterococcus	1

Firmicutes	Eubacteriaceae_unclassified	1
Firmicutes	Facklamia	1
Firmicutes	Faecalitalea	1
Proteobacteria	Gammaproteobacteria_unclassified	1
Actinobacteria	Gardnerella	1
Proteobacteria	Haemophilus	1
Proteobacteria	Halomonas	1
Firmicutes	Howardella	1
Bacteroidetes	Hymenobacter	1
Proteobacteria	Kingella	1
Proteobacteria	Labilithrix	1
Firmicutes	Lachnoanaerobaculum	1
Actinobacteria	Leifsonia	1
Fusobacteria	Leptotrichiaceae_unclassified	1
Firmicutes	Listeria	1
Bacteroidetes	Macellibacteroides	1
Actinobacteria	Micrococcaceae_unclassified	1
Firmicutes	Mitsuokella	1
Proteobacteria	Moraxella	1
Actinobacteria	Mycobacterium	1
Proteobacteria	Oligoflexus	1
Firmicutes	Oribacterium	1
Proteobacteria	Oxalobacteraceae_unclassified	1
Firmicutes	Pelospora	1
Firmicutes	Peptoniphilaceae_unclassified	1
Firmicutes	Peptostreptococcus	1
Proteobacteria	Phyllobacterium	1
Actinobacteria	Pseudonocardiaceae_unclassified	1
Proteobacteria	Ralstonia	1
Proteobacteria	Rhizobiaceae_unclassified	1
Proteobacteria	Rhodopseudomonas	1
Proteobacteria	Rhodospirillaceae_unclassified	1
Proteobacteria	Salinisphaera	1
Actinobacteria	Scardovia	1
Proteobacteria	Schlegelella	1
Firmicutes	Selenomonas	1
Fusobacteria	Sneathia	1
Firmicutes	Solobacterium	1
Firmicutes	Staphylococcus	1
Proteobacteria	Stenotrophomonas	1
Firmicutes	Stomatobaculum	1
Proteobacteria	Sulfurospirillum	1
Firmicutes	Syntrophomonadaceae_unclassified	1
Actinobacteria	Varibaculum	1
Proteobacteria	Yersinia	1