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Safety, tolerability, and preliminary effects of cricket chitin for adults with IBS: a double-blind randomized crossover pilot trial

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Irritable Bowel Syndrome (IBS) is a gastrointestinal disorder affecting around 11% of the global population. Increased production of inflammatory mediators and altered gut microbiota are common with IBS, and evidence suggests these factors are integral to IBS pathophysiology. Prebiotic dietary fiber interventions are being investigated to improve functional gastrointestinal symptoms by remediating gut microbiota and reducing intestinal inflammation. *Objective*: To investigate the safety and tolerability of a unique and sustainable fiber source, cricket-derived chitin, in a sample of adults (n = 18) diagnosed with IBS, and to gather preliminary data regarding IBS symptoms, inflammatory biomarkers, and gut microbiota to power future randomized, controlled trials. *Methods*: In this randomized, double-blind, 2 × 2 crossover pilot intervention, participants consumed cacao patties containing either 4 grams of cricket-derived chitin (n = 10) or a maltodextrin comparator (n = 8) daily for 30 days, followed by a 14 day washout period and additional 30 day intervention period where participants engaged in in the opposite intervention group. The primary outcome of safety and tolerability (*e.g.*, adverse events monitoring, physiologic and metabolic biomarkers, quality of life) and secondary outcomes (*e.g.*, inflammatory biomarkers, gastrointestinal symptoms, stool consistency, and gut microbiota) were assessed before and after each intervention. *Results*: All safety and tolerability criteria were met. Additionally, while participants reported improved GI symptoms following both treatments ($p < 0.05$), the relationship was more pronounced following the chitin intervention ($p < 0.01$); on average, the chitin treatment resulted in a significantly greater reduction in serum TNF-alpha than the comparator group by 47.41% (95% CI: −90.37, −4.44; p-value = 0.0350). Both interventions resulted in differentially abundant microbial taxa. *Conclusions*: Results suggest that while both chitin and cacao may be safe and tolerable in individuals with IBS and support symptom management, the chitin additive may provide an additional benefit for lowering inflammatory cytokines. This trial is registered on Clinicaltrials.gov (NCT06397924) and funded by a Colorado Agricultural Experiment Station grant.

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1 Introduction

Irritable Bowel Syndrome (IBS) is a chronic gastrointestinal disorder characterized by functional gastrointestinal (*e.g.*, abdominal pain, bloating, and altered bowel habits) and extra-intestinal symptoms (*e.g.*, fatigue, migraines) that can greatly impact quality of life. There are four classified IBS subtypes, including diarrhea-predominant IBS (IBS-D), constipation-predominant IBS (IBS-C), mixed IBS (IBS-M) alternating between constipation and diarrhea, and unspecified IBS (IBS-U) where symptoms vary.[1,2] Although each subtype is characterized by differing predominant symptoms, irregular bowel habits, bloating, and abdominal pain are common among all.[3] While underlying causes are still incompletely understood, IBS pathology is thought to involve a complex interplay of immune responses to various environmental triggers, coupled with gut-brain signaling disruptions and visceral hypersensitivity.[4,5] More recently, a role for gut microbiota and intestinal barrier function have emerged, with altered microbial populations and increased intestinal permeability noted in individuals with IBS.[6,7]

Around two-thirds of the IBS population attribute symptom exacerbation to the consumption of specific foods, with those

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rich in fat, biogenic amines, and certain carbohydrates commonly reported.[8,9] Consequently, a growing focus of IBS-related clinical research is to improve symptom management and reduce flare-ups through dietary modification.[10] Although the exact mechanisms remain unclear, the relationship between food consumption and IBS-related GI symptoms is likely multi-factorial and personalized.[11] Not only can specific foods trigger immune responses in susceptible individuals (*e.g.*, IgE- or IgG-mediated sensitivities;[12,13]), but diet also shapes microbial populations and their metabolic activities, impacting food component degradation, production of bioactive metabolites, and nutrient bioavailability.[14–16] Accordingly, evidence from dietary intervention studies suggests benefits from both targeted food restrictions (*e.g.*, low-FODMAP, gluten-free diets;[17]) and the inclusion of prebiotics (*e.g.*, fiber) to positively influence gut microbial dynamics and anti-inflammatory activities.[18]

Prebiotic dietary fibers are key interventions studied for their potential to alleviate global gastrointestinal symptoms in IBS patients.[19,20] As an energy source for gut microbiota, dietary fiber metabolism promotes the growth of beneficial commensal organisms and supports microbial-mediated anti-inflammatory activities.[21,22] However, evidence indicates that the impact of fiber on IBS varies by IBS subtype (*e.g.*, IBS-C, IBS-D), fiber type, and fiber solubility.[23,24] For instance, the different solubilities of specific fiber types will determine their capacity for supporting either softer or harder stools,[24,25] subsequently having different implications for IBS-C and IBS-D subtypes. Additionally, the physiochemical properties of certain fibers may exacerbate symptoms, offsetting their prebiotic benefits.[24] This highlights the need to explore novel dietary strategies that provide prebiotic support and mitigate IBS pathology without triggering symptoms.

Chitin ($(C_8H_{13}O_5N)n$) is a naturally occurring long-chain polymer analogous to cellulose. It is a unique source of fiber in the diet primarily derived from insect, crustacean, and arthropod exoskeletons, as well as fungal cell walls. Structurally, chitin from crustaceans and insects are high molecular weight polymers conjugated to melanin or other proteins while fungal chitin is a lower molecular weight polymer conjugated to glucans[26] In contrast, to fungal chitin, which has been studied for its gastrointestinal benefits,[27,28] there is limited information on the prebiotic properties of animal chitins. Chitin itself is considered an insoluble fiber, but its biotransformation in the human gut may result in more soluble derivatives (*e.g.*, chitosan, chitooligosaccharides) with enhanced prebiotic potential.[26] While limited research on the fate of chitin in digestion exists, there is some evidence that human gastric juice may contain low levels of chitinases that can degrade chitin.[29]

Although uncommon in the United States, people across the globe frequently consume dietary chitin from edible insects, including crickets,[26] and a recent *in vitro* study demonstrated that edible insects contain fermentable substrates that cause a shift in microbiome diversity.[30] In a prior clinical trial, the daily consumption of 25 grams of whole cricket powder, containing ~2 g chitin, was determined to be safe and tolerable in healthy adults, and lead to a decrease in the proinflammatory cytokine TNF-alpha and a 5.7-fold increase in the probiotic bacterium *Bifidobacterium animalis*.[31] Additionally, the use of chitin-glucan, a chitin derivative from fungi, in a murine model of IBS led to an up-regulation of genes related to barrier function, reduced visceral pain perception, and decreases in pro-inflammatory cytokine production; in summary, chitin-glucan mitigated IBS-like symptomology and pathology in the murine model.[32]

Understanding how dietary insect chitin affects health is a timely endeavor. Insects have been consumed by humans throughout history,[33] with more than 2100 edible species documented in the literature.[34] Despite Western bias against the practice, around 2 billion people currently live in contexts where insects are eaten,[35] and increasing access to edible insects has recently been proposed as one way to ameliorate global malnutrition and food insecurity.[36] Insects are both nutrient-dense and environmentally sustainable—requiring less feed, land, and water per unit to produce than conventional livestock while emitting significantly fewer greenhouse gases.[37,38] Moreover, insects generally contain relevant quantities of digestible animal-sourced protein[39,40] and micronutrients including iron and zinc.[41] Many species provide all essential amino acids for human nutrition,[42,43] and depending on the species, some are also good sources of B vitamins[44,45] and polyunsaturated fatty acids.[46] The added benefit of fiber is understudied but could offer important health benefits beyond nutrition, especially for food insecure and other vulnerable populations, such as those with IBS. More research is warranted given the dearth of studies evaluating direct health impacts of insect consumption and inclusion of insect-derived chitin in the diet.[47]

Thus, the primary aim of this study was to evaluate the safety and tolerability of 4 g day^{-1} cricket-derived chitin consumption in individuals with IBS, assessed *via* adverse events, metabolic profiles, and gastrointestinal symptom and IBS-related quality of life questionnaires. The secondary aims were to gather preliminary data on (a) if consuming cricket-derived chitin reduces the duration and severity of symptoms related to IBS and normalizes bowel habits; (b) if consumption of cricket-derived chitin is associated with reduced biomarkers of inflammation; and (c) if consuming cricket-derived chitin modulates the gut microbiota.

This study provides novel insights into the safety and tolerability of cricket-derived chitin for individuals with IBS and explores its potential to improve IBS-related outcomes. Additionally, our findings may be relevant for other functional gastrointestinal disorders, such as environmental enteropathy, which is often prevalent in regions where entomophagy—the practice of consuming insects—is culturally acceptable. Examining insect-derived chitin in this context presents a valuable opportunity to support global health efforts by exploring a nutrient-dense, sustainable food additive with potential benefits for gut health.

2 Materials and methods

2.1 Study design

This randomized, double-blind, 2 × 2 crossover pilot trial was approved by the Institutional Review Board (IRB) for Human Subjects Research at Colorado State University [IRB #2973] and conducted in accordance with the Declaration of Helsinki. The trial is also registered on Clinicaltrials.gov under NCT06397924 and was conducted following CONSORT guidelines (Table S1). As IBS pathology and gut microbiome measures can vary greatly between individuals, a crossover design was chosen to limit inter-individual variability in between-group comparisons while using a limited sample size. All participants provided written informed consent prior to enrollment in the study. There were two 30 day intervention periods separated by a 14 day washout period to allow sufficient time for clearance of intervention products and for the gut microbial ecosystem to return to baseline; the total study duration was 74 days (Fig. 1). After enrollment, each study participant was randomly assigned to start the first intervention period with either the chitin or comparator treatment, using a random number generator. Participants were then provided with 30 cacao patties (22 grams each) containing either 4 grams of cricket-derived chitin or 4 grams of a maltodextrin comparator and were asked to consume one per day for 30 days. Maltodextrin is a common comparator in studies examining gut microbiota and gastrointestinal health and has not resulted in gut microbiota modulation or modification of physiologic markers when used as a placebo in previous intervention studies.[48–50] Following the washout period, participants engaged the opposite treatment group for the second intervention period (*e.g.*, participants receiving the chitin treatment in the first intervention period were assigned to comparator treatment in the second intervention period; Fig. 1).

Participants visited the Food and Nutrition Clinical Research Laboratory (FNCRL) in the Department of Food Science and Human Nutrition at Colorado State University four times during the trial to provide fasting blood samples, stool samples, daily stool logs, three-day food records, and to complete questionnaires such as the Gastrointestinal Symptom Severity questionnaire, Quality of Life questionnaire, and a seven-day physical activity recall. The first study visit served as the first baseline data collection point and occurred prior to starting the first intervention period; the second visit occurred after the first intervention period (30 days); the third visit occurred after the washout period, which also served as a baseline for the second intervention period (~day 44); the fourth and final visit occurred after the second intervention period (~day 74). Study participants were provided with 30 chocolate patties at the start of each intervention period in accordance with their treatment group and were asked to return any uneaten chocolates post-intervention to assess compliance. The complete study design is outlined in Fig. 1. The facility producing the cacao patties created the blinding code and provided this information in a sealed envelope to a CSU employee that was not involved in the design or conduct of the study. All investigators remained blinded to treatment assignment until after data were collected and statistically analyzed.

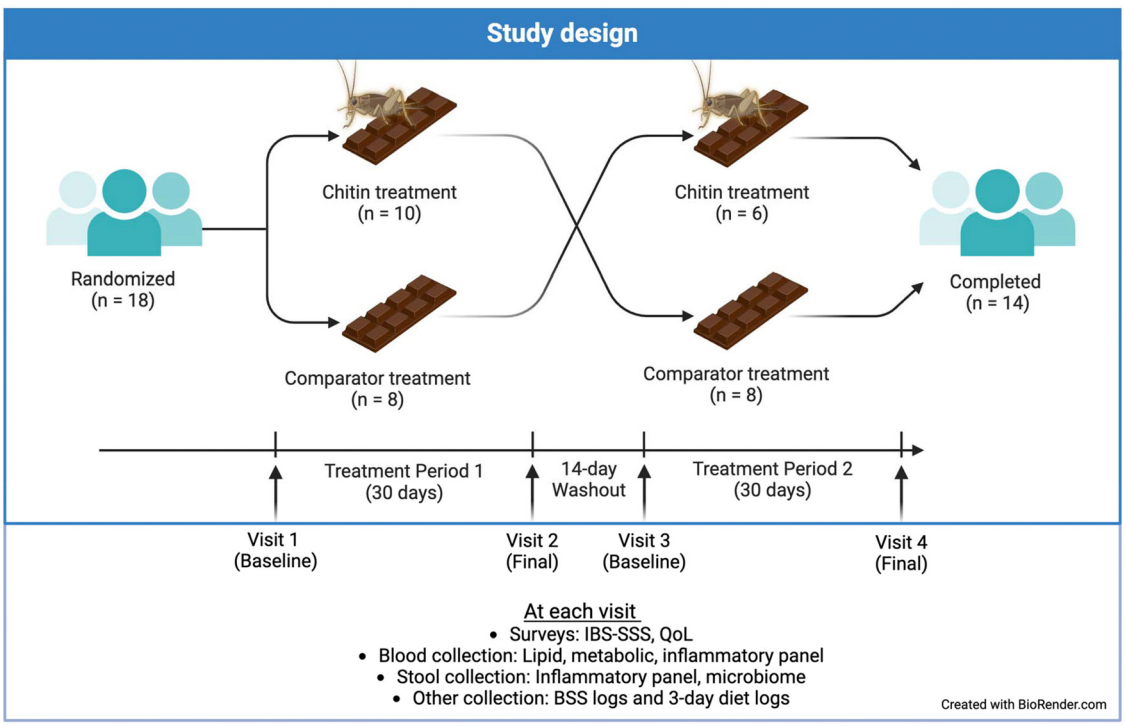

Fig. 1 Shown is an overview of the study design, including timeline of study visits and treatment periods, and outcomes assessed at each visit.

2.2 Participant selection and randomization

Adults aged 18–65 were recruited *via* email announcement and flyers at Colorado State University (Fort Collins, Colorado, USA) from June 2023 through August 2024. A G*Power[51,52] calculation indicated that repeated measures analyzing within and between group differences across 2 experimental groups and 4 time points, with a moderate effects size (0.50) and 80% power, would require a sample size of 12. Target enrollment was n = 18 to allow for 20% attrition. Participants were included if they were at least 18 years of age and had either a medical diagnosis of IBS or met 2 of the 3 criteria for IBS diagnosis according to the Rome IV assessment at screening. Participants were excluded if (a) they were younger than 18 years old or older than 65, (b) did not meet at least 2 out of 3 of the Rome IV diagnostic criteria for IBS, (c) were pregnant or breastfeeding, (d) had a diagnosis for any intestinal or metabolic disease, such as inflammatory bowel disease, cancer, liver or kidney disease, (e) self-reported presence of food allergies to shellfish, milk or soy, (f) used antibiotics in the last 2 months, or (g) were currently medicated or on dietary supplementation designed to impact gut microbiota. Eligibility was determined by a phone call interview using a screening questionnaire and later confirmed at the initial clinic visit prior to enrollment. After enrollment, each study participant was randomly assigned by a study coordinator to start the first intervention period with either the chitin or comparator treatment, using a random number generator.

2.3 Study intervention products

Chitin was extracted from 18 kg of dried *Gryllodes sigillatus* crickets enriched in exoskeleton-containing parts from a commercial insect supplier (Entomo Farms Norwood, Ontario, Canada). Briefly, crickets were ground to a fine powder in a Ninja Bullet blender (SharkNinja, Needham, MA, USA) and extracted in a BrewMagic Sabco brewing system (Sabco Craft Equipment, Toledo, OH, USA) following previously published methods.[53–56] Briefly, incubation in 1.0 M HCl was used to demineralize the cricket powder and 1M NaOH incubation was used for deprotonation prior to drying and yield assessment. Chitin from the extracts was confirmed by comparison of FTIR spectra (Q laboratories, Cincinnati, OH, USA) of the cricket extract with commercially purchased chitin from shrimp (Sigma, St. Louis, MO). Ratios for absorbance at 1620/1305 suggest that the degree of acetylation was ~99–100%. Chocolate patties used in the study were prepared by Nuance Chocolate (Fort Collins, CO, USA) and infused with either 4 grams of cricket-derived chitin (chitin patties) or 4 grams of tapioca maltodextrin (comparator patties; Pike Global Foods, Hawley, PA, USA).

Composition of the chitin and comparator patties was confirmed *via* third party analysis (IEH-Warren Analytical Laboratories, Greeley, CO, USA; Report No. WAL-211120), and both the chitin and comparator patties were similarly matched in their macronutrient content (Table S2). The chitin patties contained 8.26 g per 100 g protein, 39.12 g per 100 g fat – acid hydrolysis, 51 g per 100 g carbohydrates, 11.0 g per 100 g dietary fiber, and a total of 625 kcal per 100 g. The comparator patties contained 11.55 g per 100 g protein, 39.12 g per 100 g fat – acid hydrolysis, 51 g per 100 g carbohydrates, 6.5 g per 100 g dietary fiber, and a total of 588 kcal per 100 g. Micronutrient composition of the cricket chitin was assessed by inductively coupled plasma mass spectrometry (ICP-MS; Table S3) at CSU's Analytical Research Core (ARC), to ensure products did not contain high levels of heavy metals, according to previously published methods.[57] Finally, absence of microbiologic contaminants was confirmed by third party testing (Q Laboratories, Cincinnati, OH).

2.4 Outcomes

The primary outcome is safety and tolerability. Assessments of adverse events and metabolic panels served as measures of safety throughout the study, whereas assessments of treatment compliance and quality of life served as measures of tolerability. This treatment is considered safe if no serious adverse events are reported and all metabolic measures remain within clinically normal ranges for the duration of the study, and this treatment is considered tolerable with >80% compliance and no negative impact on quality of life. GI symptoms in individuals with IBS are often exacerbated by specific foods.[8] Studies demonstrate that anywhere from around one half to two-thirds of IBS patients experience exacerbation of IBS symptoms within 60 minutes after eating a triggering food, with a smaller subset of individuals experiencing symptom onset within hours, and even less beyond 3 hours.[9,58,59] Thus, regarding IBS-specific safety and tolerability, we made an evidence-informed estimation that 30 days of consuming a new food daily was sufficient to reveal whether that food exacerbates IBS pathology in our sample, resulting in any adverse events or reduced quality of life.

The secondary outcomes include concentration of fecal and serum inflammatory biomarkers, IBS symptom severity, stool consistency, fecal short chain fatty acids, and gut microbiome measures (diversity and taxa abundance). All measures and instruments are further defined in detail below.

2.5 Participant-reported measures

The IBS Symptoms and Severity Questionnaire (IBS-SSS)[60] and the IBS Quality of Life (QoL) questionnaire[61] are validated scales for measurement of severity of symptoms in an IBS population and were administered to the participants at each clinic visit. The IBS-SSS is composed of 5 subcategories related to abdominal pain severity, abdominal pain frequency, abdominal distention severity, satisfaction with bowel habits, and interference with quality of life. Each subcategory is scored on a scale of 0 to 100, with total possible IBS-SSS scores ranging from 0 to 500. A higher score indicates more severe symptoms and can be categorized as mild (scores 75–174), moderate (scores 175–299), or severe (300 or greater). For the purposes of this study, symptom severity score was analyzed as a continuous variable.

The IBS QoL questionnaire contains 34 statements related to the degree to which IBS interferes with different aspects of life and are divided across the follow 8 subcategories: (1) dysphoria, (2) interference with activities, (3) body image, (4) health worry, (5) food avoidance, (6) social reaction, (7) sexual, and (8) relationships. Participants are asked to rate their experience with each statement on a scale of 1 to 5, with 1 being Not at All and 5 being either Extremely or a Great Deal. These scores are then summed, averaged, and transformed to a scale of 0–100, with higher scores indicating a better IBS-specific quality of life. For the purposes of this study, we only used the overall QoL scores and did not perform any subcategory analyses.

Participants were asked to track the consistencies of their bowel movements daily using the Bristol Stool Scale.[62] The Bristol Stool Scale uses a visual aid to help participants categorize their stools in ranges of hard (Types 1–2), normal (Types 3–5), and loose (Types 6–7) consistencies. Bristol Stool Scale logs were given to participants at visits 1 and 3 for tracking of stools during treatments. For analysis, the number of stools in each consistency category (hard, normal, or loose) was first tallied, and then hard and loose categories were added together and classified as "abnormal". The number of abnormal stools were compared against the number of normal stools during specific timepoints to create a ratio of abnormal to normal stools. This ratio was then used in statistical analysis.

Finally, participants were also asked to report their habitual diet in 3 day food logs, with 2 days recorded Monday–Thursday and 1 day recorded Friday–Sun. Food logs were completed the week prior to each of the 4 study visits and returned at each study visit. All meals, drinks, candies, snacks, and the study intervention product should be included in the food log. Aside from the food/beverage item, participants were also asked to record time of consumption, amount consumed, food preparation (if applicable), and calories (if known). Total calories in addition to macronutrient and micronutrient intake were then calculated using Nutritionist Pro Version 8.1.0 (Axxya Systems LLC, Stafford, TX, USA). Reported in Table S2 is the average intake of calories (kcal), protein (g), fat (g), carbohydrates (g), protein percent of total kcal, fat percent of total kcal, carbohydrate percent of total kcal, total dietary fiber (g) total cholesterol (mg), saturated fat (g), and alcohol percent of total kcal.

2.6 Biological specimen collection

Both blood and stool samples were collected from participants at each study visit to the FNCRL in the Department of Food Science and Human Nutrition at Colorado State University. During each visit, venous blood samples were collected in lithium heparin and ethylenediaminetetraacetic acid (EDTA) tubes. Plasma was collected and stored immediately after centrifugation of the EDTA tubes and stored at −80 °C until analysis of circulating inflammatory markers. Two-hundred microliters of lithium heparin whole blood was analyzed immediately for lipid and metabolic biomarkers. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) was also processed immediately after each visit and stored in liquid nitrogen. At each clinical visit, participants provided a stool sample which was collected at home either the day prior to or the morning of the visit. Participants were instructed to keep stool samples frozen until delivery. Once delivered, samples were stored at −80 °C until analyzed for microbiome-related outcomes.

2.7 Blood chemistry for lipid and metabolic panel outcomes

Two-hundred microliters of lithium heparin whole blood per participant was analyzed using the Piccolo MetLyte Plus CRP and Lipid Panels on a Piccolo Xpress blood chemistry analyzer (Abaxis Inc., Union City, CA, USA). All analyses were carried out according to the manufacturer's instructions.

2.8 Inflammatory biomarker outcome detection *via* ELISA

The inflammatory biomarkers tumor necrosis factor alpha (TNF-α), C-reactive protein (CRP), interleukin-10 (IL-10), interleukin-6 (IL-6), and serum lipocalin were measured from participant plasma samples *via* enzyme linked immunosorbent assay (ELISA). The following kits were used: Human TNF-alpha ELISA KIT (Eagle Biosciences Catalog Number TNA31-KO1); Human high sensitivity C-Reactive Protein ELISA Kit (Biomatik Catalog Number EKC34032); Human IL-10 PicoKine™ ELISA Kit (Boster Catalog Number EK0416), Human IL-6 PicoKine™ ELISA Kit (Boster Catalog Number EK0410), Human Lipocalin-2/NGAL ELISA Kit (Biomeda Catalog Number RD191102200R). Each analysis was done according to the manufacturer's instructions. An ELISA microplate reader recorded the optic densities (OD) as directed. Standard curves were used to quantify the concentration of each serum inflammatory marker in either pg mL^{-1} (IL-10, TNF-alpha, IL-6, and hs-CRP) or ng mL^{-1} (serum lipocalin).

The inflammatory and intestinal health biomarkers secretory immunoglobulin A (sIgA), fecal lipocalin, and fecal calprotectin were measured from participant fecal samples *via* ELISA. The following kits were used: Human sIgA ELISA Kit (ImmuChrom GmbH Catalog Number IC6100), IDK® Calprotectin ELISA Kit (ImmunDiagnostik Catalog Number KR6927), Human Lipocalin-2/NGAL ELISA Kit (Biomeda Catalog Number RD191102200R). Each analysis was done according to the manufacturer's instructions. An ELISA microplate reader recorded the optic densities (OD) as directed. Standard curves were used to quantify the concentration of each fecal inflammatory marker and normalized to mg g^{-1} fecal weight.

2.9 Fecal microbial DNA extraction, analysis, and processing

Fecal samples were collected and stored at −80 °C until sequencing. The FastDNA™ SPIN Kit for feces (MP Biomedicals, USA) was used to extract microbial DNA from participant fecal samples according to the manufacturer's instructions, and used for downstream qPCR and 16s rRNA gene sequencing.

DNA was diluted to 10 ng μL^{-1} in nuclease-free water, and 1 μL (≈10 ng) was used per reaction, run in duplicate. Absolute quantification of total Bifidobacteria was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using Sso Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). Each reaction comprised 5 μL of 2× SYBR Green Supermix, 0.5 μM each of forward primer Bif164f (5′-GGGTGGTAATGCCGGATG-3′) and reverse primer Bif662r (5′-CCACCGTTACACCGGGAA-3′), 1 μL of template DNA (10 ng), and nuclease-free water to a final volume of 10 μL. Thermocycling conditions were: 95 °C for 3 min; 30 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min. Standard curves were generated from ten-fold serial dilutions of purified genomic DNA from *Bifidobacterium breve* ATCC 15700 (catalog no. 15700D-5; ATCC), yielding efficiencies of ~95%. Ct values were converted to absolute 16S rRNA gene copy numbers per ng of fecal DNA.

Amplicon libraries were generated for the 16s rRNA hypervariable V4 region using 515F-806R primer set according to protocols published for the Earth Microbiome Project (16S Illumina Amplicon Protocol : earthmicrobiome). Amplicon sequencing was performed on an Illumina MiSeq (Illumina INc., San Diego, CA, USA) using 2 × 250 bp paired end reads by the Next Generation Sequencing Core Facility at Colorado State University (Fort Collins, CO, USA). The resulting 16S rRNA amplicon dataset was processed using QIIME2 (v2023.5).[63] The DADA2 pipeline[64] within QIIME2 was used to quality check and trim reads with a quality score of greater than 35, followed by denoising and clustering amplicon sequence variants (ASVs). Clustered ASVs were then taxonomically classified using the SILVA database (v138).[65] The trimmed and denoised data was exported from QIIME2 and converted into a comma-delimited file. The data were then filtered and normalized in the MicrobiomeAnalyst online platform.[66] To remove features that may be a result of sequencing error or low-level contamination, a low count filter removed reads with less than 2 counts and reads that were present in less than 10% of the samples; no low variance filter was used. Data were then normalized *via* total sum scaling. The resulting filtered and normalized data were used in downstream analyses.

2.10 Short chain fatty acid quantification

Approximately 20 mg of fecal sample was spiked with 0.34 mL cold 3 M HCl and 0.06 mL internal standard (1 mg mL^{-1} $^{13}C_2$-acetate, 0.1 mg mL^{-1} $^{13}C_4$-butyrate), then shaken at 4 °C for 15 min, sonicated 10 min in a cold bath, shaken another 15 min at 4 °C, and centrifuged (15 000*g*, 15 min, 4 °C). Two hundred microliters of supernatant were extracted with 350 μL cold MTBE (10 × 3 s vortex), centrifuged (3000*g*, 5 min, 4 °C), and the top layer (~80 μL) stored at −20 °C. A 1 μL aliquot was injected (5 : 1 split) onto a Trace 1310 GC–Thermo ISQ-LT MS fitted with a 30 m DB-WAX UI column (0.25 mm ID, 0.25 μm film); the oven ran 100 °C (0.5 min) → 175 °C @ 10 °C min^{-1} →240 °C @ 40 °C min^{-1} (3 min), with helium at 1.2 mL min^{-1} and transfer line/ion source at 250 °C. SCFAs were monitored in SIM mode (*m*/*z* 45, 60, 62, 73, 74, 88 at 10 scans per s), QC samples were interleaved every six injections, and calibration curves from serial dilutions of acetate, propionate, and butyrate were generated post-run in Chromeleon 7.2.10 software (Thermo Scientific) for peak integration and normalization to the internal standards.

2.11 Microbiome analyses

Alpha diversity measures (Shannon Index, Observed Richness, and Chao1) for each time point and treatment were generated using the MicrobiomeAnalyst web-based platform.[66] Then, differences in alpha diversity measures between groups and time points were assessed using mixed effects models in GraphPad Prism 10 Version 10.2.2, including treatment, time, sequence, and sequence*treatment as fixed effects and subject as a random effect (alpha = 0.05). A Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics was used to observe differences in bacterial communities between the chitin and comparator groups baseline and the final visits. Differences in Bray–Curtis distances were statistically analysed using a permutational analysis of variance (PERMANOVA). Then, baseline to final differences in Bray Curtis dissimilarity metrics was calculated for each individual in the chitin and comparator groups, and mean differences were compared between groups using a two-tailed T test (alpha = 0.05). To assess differences in relative microbial taxa abundance between the chitin and comparator groups baseline and the final visits, we used Microbiome Multivariable Association with Linear Models (MaAsLin2;[67]) in MicrobiomeAnalyst to employ a zero-inflated negative binomial model with treatment and sequence as fixed effects and subjects as a random effect, while controlling for false discovery rate (FDR) *via* Benjamini–Hochberg correction. To assess whether the chitin intervention altered total abundance of *Bifidobacterium* specifically, as previously reported,[31] we used a mixed effects models in GraphPad Prism 10 Version 10.2.2, adjusting for treatment, time, sequence, and sequence × treatment as fixed effects and subject as a random effect (alpha = 0.05).

2.12 Statistical analyses

All statistical analyses related to inflammatory biomarkers (fecal and serum) and participant-reported outcomes were performed using GraphPad Prism 10 Version 10.2.2 (GraphPad Software, San Diego, CA, USA). All data were assessed for normality using QQ plots and Shapiro-Wilks statistical tests for normality. All inflammatory biomarker outcomes and participant-reported outcomes were analyzed using linear mixed effects models in two stages. First, two *a priori* mixed effects models were performed to aid in model selection and determine which biomarkers to pull forward into additional and final analyses. The first *a priori* mixed effects model (Model 1) included treatment, sequence, and sequence*treatment as fixed effects and subject as a random effect. The second *a priori* mixed effects model (Model 2) included treatment, time, and treatment*time as fixed effects and subject as a random effect. Both Model 1 and Model 2 were performed for each bio-

marker and compared using goodness of fit tests. Any biomarkers and participant-reported outcomes showing significant relationships with fixed covariates (alpha = 0.05) were brought forward into additional analyses (*e.g.*, multiple comparisons test and percent change), with results of the mixed effects analysis reported using the model of best fit. Multiple comparisons were conducted using the *post-hoc* Sidak test adjusted for multiple comparisons; accordingly, all reported *p*-values for the multiple comparisons are adjusted p-values. All mixed effects models were run as intent-to-treat (ITT; n = 18) and as a modified intent-to-treat (mITT); for each outcome, the associated mITT analysis included the subset of participants who successfully completed at least one intervention period and had matching data for that particular outcome within the intervention period. Paired two-tailed T-tests or non-parametric Wilcoxon tests were used to determine if there were any differences in the percent change of baseline-to-final values between the chitin and comparator treatments. For all statistical tests, p-values < 0.05 were considered statistically significant, with p-values < 0.1 being reported as non-significant trends.

Effect sizes for outcomes significantly different by treatment group or time were then calculated by dividing the mean difference by the standard deviation of the difference. Finally, relationships between significant outcomes and IBS-SSS were explore using a repeated measures correlation.

Finally, to further evaluate relationships between significant outcomes (identified *via* LMM), taxa (identified *via* Maaslin2), and IBS-SSS, we conducted a repeated measures Spearman's correlation in R (v4.4.0). Using the rmcorr package (v 0.4.1), which fits a common regression line per variable pair to account for non-independence, we tested the null hypothesis that the slope equals zero. Data were analyzed in three subsets —Chitin, Comparator, and all subjects combined—and within each, Spearman's ρ was calculated for each pair of outcome, taxon, and IBS-SSS. *P*-Values were annotated on heatmaps created with ggplot2 (v 3.4.4) and the tidyverse (v 1.3.2), with significance denoted as t (p < 0.10), * (p < 0.05), ** (p < 0.01), and *** (p < 0.001).

3 Results

3.1 Study participants

Eighteen eligible adults were enrolled in this study. Participants were a majority biological females (94.4%) identifying as Non-Hispanic White (88.8%) and were between the ages of 20 and 66 (M = 33.8, SD = 11.06). Additional details regarding participant demographics and health history can be found in Table 1. Of the 18 participants enrolled, only 14 completed the study. Of the four participants who dropped out, two reported increased vomiting, one reported increased GI symptoms, and one reported decreased digestive health. Two dropouts occurred during the washout period and two additional dropouts occurred after the third study visit; thus, only 14 participants were analysed in the second treatment allocation (Fig. 2). Baseline and final total kilocalorie intake and macronutrient intake of participants within each treatment group are reported in Table S3; change in participant macronutrient intake throughout the study, reported as percentage of total kilocalories ingested, was under 1% for protein, fat, carbohydrates, and fiber (Table S3). Furthermore, a detailed overview of participant physiological measures throughout the study, such as average biomarker concentrations pre- and post-interventions, are reported in Table S4.

Table 1 Study Participants. Shown are study participant demographics and health history. M = mean; SD = standard deviation; n = count; % = percentage

	Total	Sequence AB (n = 8)	Sequence BA (n = 10)
Age M (SD)	33.8 (11.1)	33.6 (9.9)	34.0 (12.5)
Sex (female/male)	17/1	7/1	10/0
BMI M (SD)	24.9 (5.6)	23.5 (4.4)	26.0 (6.4)
Race/ethnicity n(%)			
White	16 (88.9)	8 (100)	8 (80)
Native American	1 (5.5)	0 (0)	1 (10)
Asian	1 (5.5)	0 (0)	1 (10)
Non-hispanic	18 (100)	8 (100)	10 (100)
Smoking n(%)			
Ever smoker	5 (27.7)	2 (25)	3 (30)
Current smoker	1 (5.5)	1 (12.5)	0 (0)
Participants using Rx medication n(%)			
None	3 (16.6)	2 (25)	1 (10)
Contraceptives only	7 (38.8)	2 (25)	5 (50)
Contraceptives total	9 (50)	3 (37.5)	6 (60)
Respiratory/allergy	4 (22.2)	1 (12.5)	3 (30)
Neuropsychiatric	3 (16.6)	0 (0)	3 (30)
Antiviral	1 (5.5)	1 (12.5)	0 (0)
Dermatologic	2 (11.1)	1 (12.5)	1 (10)
Migraine therapy	1 (5.5)	0 (0)	1 (10)
GI Agents for IBS	4 (22.2)	3 (37.5)	1 (10)
Other	2 (11.1)	1 (12.5)	1 (10)
Participants with food allergies or sensitivities n(%)			
Fiber or high fiber foods	3 (16.6)	1 (12.5)	2 (20)
Gluten or wheat	2 (11.1)	1 (12.5)	1 (10)
Lactose or dairy	4 (22.2)	0 (0)	4 (40)
Nuts	1 (5.5)	1 (12.5)	0 (0)
Other foods	4 (22.2)	0 (0)	4 (40)

3.2 Safety and tolerability of intervention in IBS participants

Assessments of adverse events, metabolic panels, and lipid panels served as measures of safety throughout the study. Consuming four grams of chitin daily for 30 days did not result in any serious adverse events in our population with IBS. Some adverse events were reported, however, most overlapped with existing IBS symptoms and were not specifically related to the chitin intervention period. Over the course of the entire study, mild to moderate bloating was reported two times with the comparator and once with the chitin treatment; mild constipation was reported once each for the placebo and the treatment; mild to moderate flatulence was reported three times with the placebo and once with the treatment; mild to moderate fatigue was reported twice with the placebo and three times for the treatment; abdominal pain was reported twice with the treatment; mild to moderate anxiety or depression was reported twice with the placebo and once with

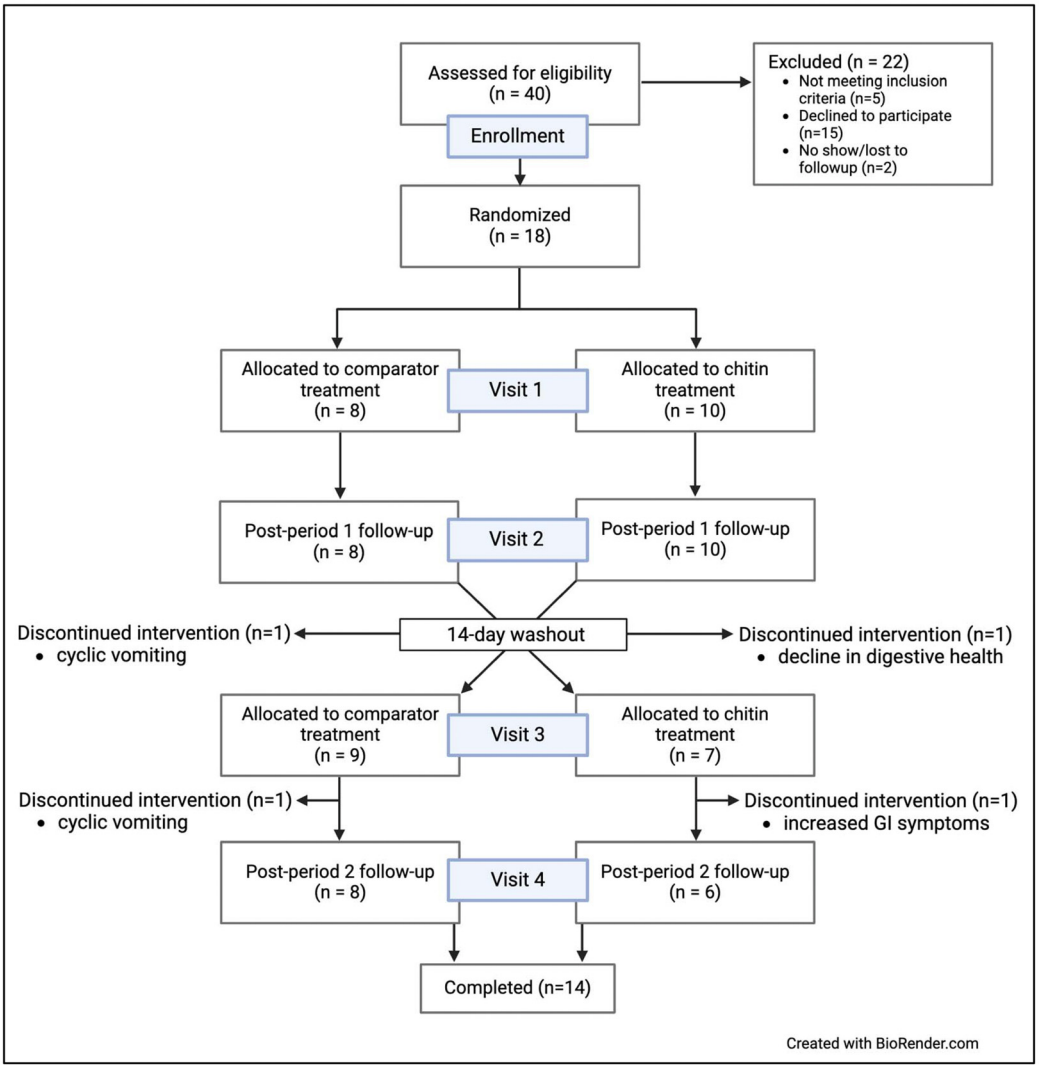

Fig. 2 Shown is the CONSORT flow diagram outlining participant enrollment, randomization, treatment allocation, participant drop out, cross-over, and completion.

the chitin treatment; and difficulty concentrating was reported once during the treatment. While there were some statistically significant shifts in biomarkers on the metabolic panel (*e.g.*, glucose and creatinine) and lipid panel (*e.g.*, high density lipoprotein, triglycerides, and ratio of total cholesterol-to-high density lipoprotein), all values remained well within clinically normal ranges throughout the study (Table S4). Accordingly, we considered metabolic and lipid panels to meet our safety criteria.

Treatment compliance and the participant-reported QoL questionnaire was used to assess tolerability of the intervention. Participants had an average of 95% compliance consuming the chitin patties and 97.5% compliance for the placebo patties, determined by percent of patties consumed. In addition, given that individuals with IBS frequently experience IBS-related disruptions in QoL and heightened sensitivity to various foods, it was essential to ensure that the daily inclusion of cacao and/or chitin in participants' diets did not impose an additional burden on their QoL. We observed that neither the chitin nor the comparator intervention had negative impacts on the QoL score. QoL score increased after the comparator intervention by an average of 5.25 points (95% CI: −9.686, −0.8189) and 5.29 points (95% CI: −9.709, −0.8613) for the ITT and mITT analyses, respectively. Although these increases in score were statistically significant (ITT p-value = 0.0181; mITT p-value = 0.0171), they may not be clinically relevant based on suggested MCID score changes values of 10 to 14 points.[68]

3.3 IBS symptom severity

There was a significant relationship between IBS-SSS scores and time (*i.e.*, baseline values *vs.* final values), but not treatment (*i.e.*, chitin *vs.* comparator) in both the ITT analysis (p-value = 0.0002, F = 17.74) and the mITT analysis (p-value =

0.0002, F = 18.60), after adjusting for all other covariates. Significant within-subject variability was observed across all analyses (p < 0.0001). Multiple comparisons tests indicated that IBS-SSS scores were significantly lower after both the comparator treatment (ITT p-value = 0.0157; mITT p-value 0.0138) and the chitin treatment (ITT p-value = 0.0082, mITT p-value = 0.0065), when compared to baseline values (Fig. 3A); However, the significance of the relationship was greater in the chitin group. The mean difference and 95% CI for each are reported in Table 2. The effect size of the mean difference following chitin treatment was Cohen's d = 0.73 and 0.75 for the ITT and mITT analyses, respectively, indicating medium-to-large effects. Similarly, the comparator treatment yielded Cohen's d = 0.67 and 0.68 for the ITT and mITT analyses, respectively, also reflecting medium-to-large effects.

Although IBS-SSS scores remained, on average, within the "moderate" clinical category of symptom severity after both the chitin and comparator treatments (*i.e.*, scores between 175–299), average scores shifted from the higher end to the lower end of the category post-treatment. There was no significant difference between the chitin and comparator group when comparing percent change in scores from baseline (Fig. 3B; p-value = 0.6092).

3.4 Stool consistency evaluation using the BSS

There was no significant difference in the ratio of normal to abnormal stools for either the chitin or placebo treatments. The 30 day stool log was divided into four sections: days 1–3 was baseline, days 4–10, days 11–20, and days 21–30. For each of these four sections, no statistical significance was found when comparing change in ratios of normal to abnormal stools from baseline within and between treatment groups (Fig. 4; p-values > 0.05). There was, however, significant within subjects variation for all analyses (p-value < 0.0001).

3.5 Serum biomarkers of inflammation

For serum biomarkers of inflammation, no significant relationships between fixed covariates and hsCRP, IL-10 or serum lipocalin were observed (p-values > 0.05). However, in the ITT analysis there was a significant relationship between serum TNF-alpha and treatment effect (*i.e.*, comparator *vs.* chitin; p-value = 0.0473, F = 4.277), but *not* time effect (*i.e.*, baseline *vs.* final), after adjusting for all other covariates. In the mITT analysis, the relationship between serum TNF-alpha and treatment effect was a non-significant trend (p-value =

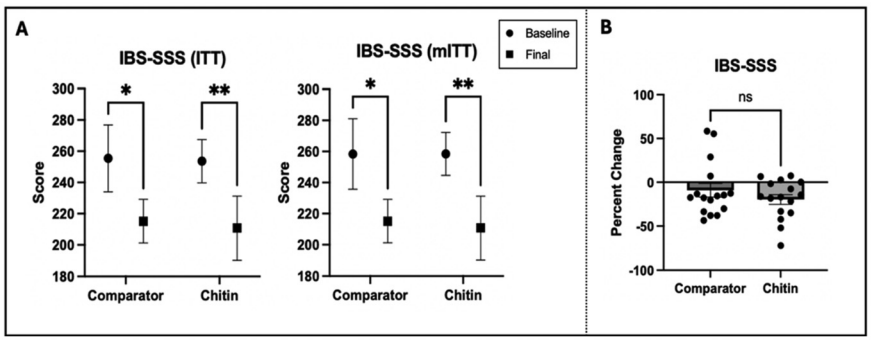

Fig. 3 Shown are (A) average baseline and final IBS-SSS scores for the intent-to-treat (ITT) and modified intent-to-treat (mITT) analyses; and (B) percent change in IBS-SSS scores from baseline to final time points for the comparator and chitin treatment group. Significance of mean differences from baseline to final were evaluated *via* multiple comparisons test *post hoc* to a mixed effects model. Percent change differences were evaluated by paired t test. ns = not significant; p-value* = <0.05; p-value** = <0.01.

Table 2 IBS-SSS multiple comparisons test for differences by time and treatment

IBS-SSS	Multiple comparisons of mean difference by time (baseline – final)					
	Comparator			Chitin		
	$Mean_{diff}$ (SE_{diff})	Adj. p-value [95% CI_{diff}]	t	$Mean_{diff}$ (SE_{diff})	Adj. p-value [95% CI_{diff}]	t
ITT	−42.18 (14.81)	0.0157* [−77.04 to −7.325]	2.849	−46.01 (14.81)	0.0082** [−80.86 to 11.15]	3.107
mITT	−43.19 14.90	0.0138* [-78.25 to −8.127]	2.900	−47.65 (14.90)	0.0065** [−82.71 to −12.59]	3.199
	Multiple comparisons of mean difference by treatment (comparator – chitin)					
	Baseline			Final		
ITT	1.78 (24.79)	0.997 [−55.04 to 58.60]	0.072	5.61 (25.23)	0.9693 [−52.21 to 63.42]	0.222
mITT	−0.08 (25.65)	0.999 [−58.92 to 58.77]	0.003	4.38 (25.65)	0.982 [−54.47 to 63.23]	0.171

$Mean_{diff}$ = mean difference of baseline – final values; SE_{diff} = standard error of the mean difference; CI_{diff} = confidence interval of the mean difference; Adj. = adjusted; t = t score; ITT = intent-to-treat; mITT = modified intent-to-treat; IBS-SSS = IBS symptom severity score; QoL = quality of life; p-value* = <0.05; p-value** = <0.01.

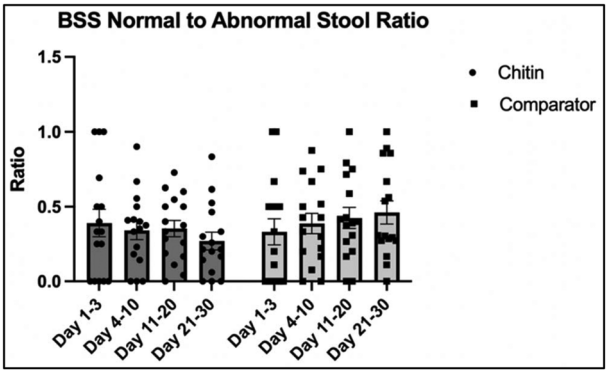

Fig. 4 Shown are the average ratios of normal to abnormal stools at four multi-day intervals throughout each treatment period; individual participant ratios are a lot plotted. Normal stools were defined as BSS scores 3–5, whereas abnormal were defined as BSS scores 1–2 (hard stools) or scores 6–7 (loose stools). No significant differences were observed.

0.0675, F = 3.666). For all analyses, we observed significant within subjects variation (p-value < 0.0001).

Interestingly, although results of the *post hoc* adjusted multiple comparisons test demonstrated no difference in baseline to final values within each treatment group (Fig. 5A and Table 3), a comparison of the percent change in TNF-alpha concentrations from the baseline to final visits between each treatment group (Fig. 5B) corroborates results of the mixed effects analysis and suggests the chitin group saw a significantly greater percent change from baseline than the comparator (p-value = 0.0350). On average, the chitin treatment resulted in a 47.41% greater reduction in serum TNF-alpha than the comparator group (95% CI: −90.37, −4.44), when compared to baseline. This corresponded to a Cohen's d of 0.72, indicating a medium-to-large effect. It is worth noting that within the chitin group, three participants saw a percent change of 100%, with their final serum TNF-alpha concentrations being undetectable.

3.6 Fecal Biomarkers of Inflammation

Regarding fecal inflammatory biomarkers, there were no significant relationships between any fixed covariates and fecal calprotectin or fecal lipocalin; (p-values > 0.1), although there was significant within subjects variation for all analyses (p-values < 0.0001). However, we observed a significant relationship between sIgA and sequence (*i.e.*, Comparator/Chitin *vs.* Chitin/Comparator sequence) in the ITT analysis (p-value = 0.0487, F = 4.553) and a non-significant trend between sIgA and sequence in the mITT analysis (p-value = 0.0501, F = 4.487); there was also a non-significant trend between sIgA and sequence*treatment in the ITT analysis (p-value = 0.067, F = 2.577). For all analyses, we observed significant within subjects variation (p-value < 0.0001).

The adjusted multiple comparisons test indicated that both the baseline and final sIgA concentrations for the comparator intervention were, on average, trending higher in the comparator/chitin sequence group when compared to the Chitin/Comparator sequence group (Fig. 6 and Table 4). It is worth noting that the baseline and final comparator values for the Comparator/Chitin sequence group (the group with the higher values) were measured in the first treatment period, during visits 1 and 2; the baseline and final comparator values for the Chitin/Comparator sequence group (the group with the lower values) were measured in the second treatment period, during visits 3 and 4. However, when comparing the pre-post percent change in IgA concentrations between treatments by sequence, there was no significant difference (Fig. 6B; p-value = 0.1655).

3.7 Microbial diversity analyses

Three alpha diversity measures were compared between treatment groups and time points (Fig. 7A), while adjusting for sequence. There were no changes in Shannon Index, Chao1, or Observed richness following chitin and comparator treatment and no significant relationships between any of the fixed covariates and alpha diversity measures (p-values > 0.05). There was, however, significant within subjects variation for each diversity measure (p-value < 0.0001).

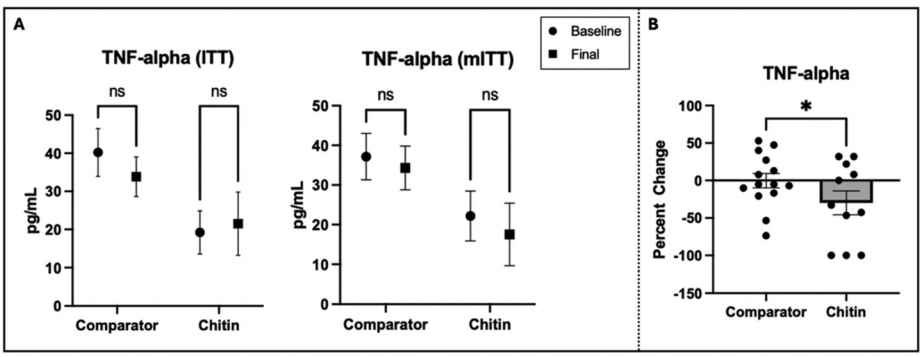

Fig. 5 Shown are (A) average baseline and final concentrations of TNF-alpha for the intent-to-treat (ITT) and modified intent-to-treat (mITT) analyses; and (B) percent change in TNF-alpha from baseline to final time points for the comparator and chitin treatment group. Significance of mean differences from baseline to final were evaluated *via* multiple comparisons test *post hoc* to a mixed effects model. Differences in percent change were evaluated by paired t test. ns = not significant; p-value* = <0.05.

Table 3 Multiple comparisons test for mean differences in TNF-alpha by time and treatment

TNF-alpha	Multiple comparisons of mean difference by time (baseline – final)					
	Comparator			Chitin		
	$Mean_{diff}$ (SE_{diff})	Adj. *p*-value [95% CI_{diff}]	*t*	$Mean_{diff}$ (SE_{diff})	Adj. *p*-value [95% CI_{diff}]	*t*
ITT	−3.326 (3.173)	0.5175 [−10.920 to 4.264]	1.048	−2.337 (3.564)	0.7681 [−10.860 to 6.188]	0.655
mITT	−2.872 (3.173)	0.7494 [−10.48 to 4.736]	0.905	0.7367 (3.569)	−3.275 [−11.83 to 5.283]	0.917
	Multiple comparisons of mean difference by treatment (comparator – chitin)					
	Baseline			Final		
ITT	18.19 (8.897)	0.0897^{t} [−2.290 to 38.66]	2.044	17.20 (8.865)	0.1121 [−3.206 to 37.60]	1.940
mITT	16.35 (8.999)	0.1510 [−4.481 to 37.19]	1.814	16.76 (8.943)	0.1344–3.950 to 37.47	1.874

$Mean_{diff}$ = mean difference of baseline – final values; SE_{diff} = standard error of the mean difference; CI_{diff} = confidence interval of the mean difference; adj. = adjusted; *t* = t score; ITT = intent-to-treat; mITT = modified intent-to-treat; *p*-value^{t} = non-significant trend.

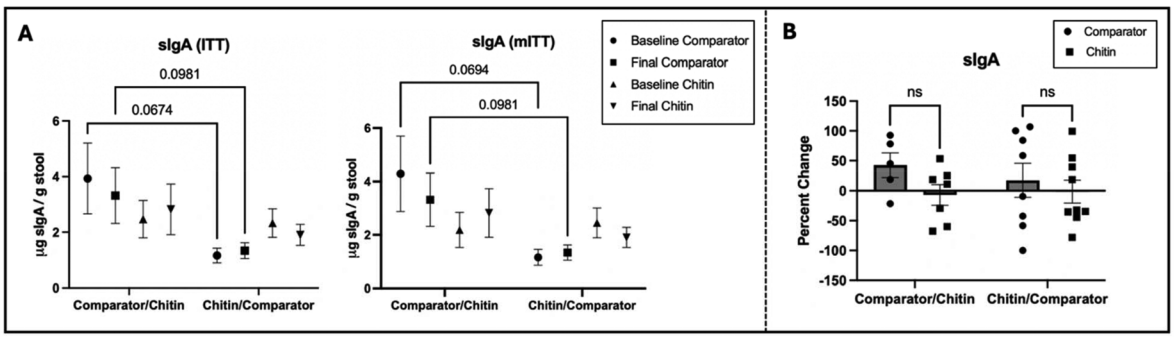

Fig. 6 Shown are (A) average baseline and final concentrations of serum IgA (sIgA) for the intent-to-treat (ITT) and modified intent-to-treat (mITT) analyses. These average values are separated by treatment sequence (*e.g.,* comparator/chitin sequence and chitin/comparator sequence); and (B) percent change in sIgA from baseline to final time points for the comparator and chitin treatment groups, also separated by treatment sequence. Significance of mean differences from baseline to final were evaluated *via* multiple comparisons test *post hoc* to a mixed effects model. Differences in percent change were evaluated by paired *t* test. ns = not significant.

Table 4 Multiple comparisons test for mean differences in sIgA between sequences

sIgA	Multiple comparisons of mean difference by sequence (comparator/chitin – chitin/comparator sequence)					
	Comparator baseline			Comparator final		
	$Mean_{diff}$ (SE_{diff})	Adj. p-value [95% CI_{diff}]	*t*	$Mean_{diff}$ (SE_{diff})	Adj. *p*-value [95% CI_{diff}]	*t*
ITT	2.770 (1.300)	0.0674^{t} [−0.2534, 5.794]	2.131	1.979 (1.037)	0.0981^{t} [−0.4744, 4.432]	1.908
mITT	3.128 (1.442)	0.0694^{t} [−0.3306, 6.587]	2.170	1.979 (1.037)	0.0981^{t} [−0.4744, 4.432]	1.908

$Mean_{diff}$ = mean difference of baseline – final values; SE_{diff} = standard error of the mean difference; CI_{diff} = confidence interval of the mean difference; adj. = adjusted; *t* = t score; ITT = intent-to-treat; mITT = modified intent-to-treat; *p*-value^{t} = non-significant trend.

Although there was no significant difference between baseline and final beta diversity values (Bray Curtis dissimilarity) within each treatment group (Fig. 7B and C), the average pre-post change values between each treatment group were significantly different (*p*-value <0.0001). On average, an individual's beta diversity decreased after the comparator treatment when compared to the chitin treatment (Fig. 7D).

3.8 Identifying taxon biomarkers of each treatment group

Using a zero inflated negative binomial model (MaAsLin2), we identified taxa at the feature level that were significantly different from the baseline to final time points within each treatment group, while controlling for false discovery rate and adjusting for sequence (Table S5). Several taxa were identified as biomarkers of both treatment groups. An uncultured bacterium from the family Ruminococcaceae and *Barnesiella* were decreased after both treatments, whereas *Coprococcus*, *Lachnospiraceae_NK4A136_group*, and an uncultured genus *UCG_010* from the order Oscillospirales were increased after both treatments. Conversely, a specific amplicon sequence variant of *Streptococcus* was higher after the comparator treatment but lower after the chitin treatment. Additionally, many

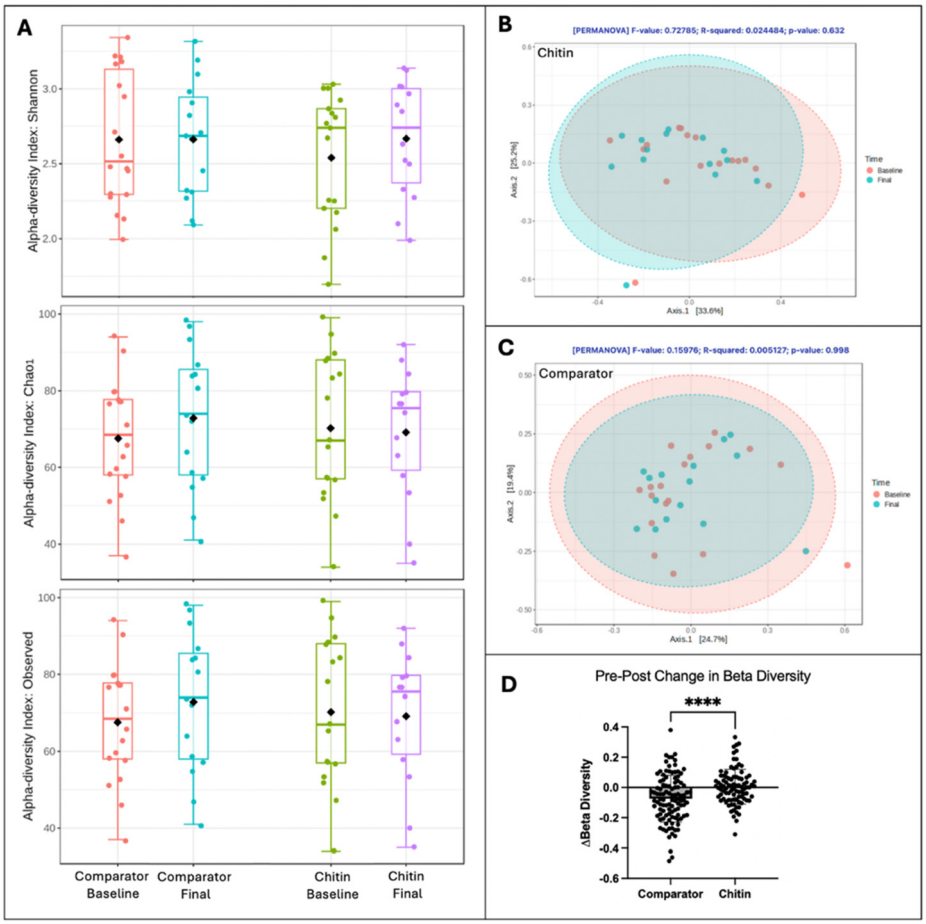

Fig. 7 Shown are (A) average baseline and final alpha diversity measures for the comparator and chitin group. No significant differences were identified; (B) PCoA of baseline and final Bray Curtis dissimilarity metrics for the chitin treatment group and non-significant PERMANOVA results; (C) PCoA of baseline and final Bray Curtis dissimilarity metrics for the comparator treatment group and non-significant PERMANOVA results; and (D) comparison of individual pre-post change in Bray Curtis distances between the comparator and chitin group. *p*-value**** = <0.0001.

taxa were identified as being biomarkers of either the chitin treatment or the comparator treatment. Although all taxa identified as significant biomarkers of each treatment group are outlined in Table S5, here we describe significant, differentially abundant taxa that also meet an effect size threshold of 2 (*i.e.*, –Log2FC absolute value of 2; Fig. 8). Out of the identified taxon biomarkers of the chitin treatment that also met our effect size threshold, those that were increased in abundance following the chitin treatment included *Coprococcus*, two amplicon sequence variants of *Colstridia_UCG-014*, *Lachnospiraceae_NK4A136_group*, and *Colidextribacter*; Those that were decreased in abundance include *Streptococcus* and a third, different amplicon sequence variant of *Colstridia_UCG-014*.

Out of the identified taxon biomarkers of the comparator treatment that also met our effect size threshold, those that were increased in abundance following the comparator treatment included *Eubacterium ventriosum group*, *Campylobacter hominis*, and Oscillospirales_UCG-010. Those that were decreased in abundance include *Eubacterium siraeum group*, Ruminococcaceae uncultured bacterium, *Eubacterium oprostanoligenes group*, *Eubacterium nodatum group*, Ruminococcaceae, *Lachnospiraceae_UC5-1-2E3*, and a Rhodospirillales uncultured bacterium.

3.9 Analysis of changes in Bifidobacterium abundance

Next, based on a previous study observing increased *Bifidobacterium* in response to a cricket powder intervention in healthy adults, we examined changes in *Bifidobacterium* total (targeted qPCR; Fig. 9A) abundance and relative abundance (16s rRNA sequence data; Fig. 9B). There were, however, no significant changes in *Bifidobacterium* in response to either intervention, after adjusting for sequence (*p*-values > 0.05; Fig. 9).

3.10. Correlations between IBS symptom severity, microbiome and inflammatory outcomes

Although SCFAs did not significantly differ by treatment or time-point (data not shown), we included SCFAs in a repeated measures correlative analysis with microbial taxa abundance, serum TNF-alpha, and IBS-SSS (Fig. 10).

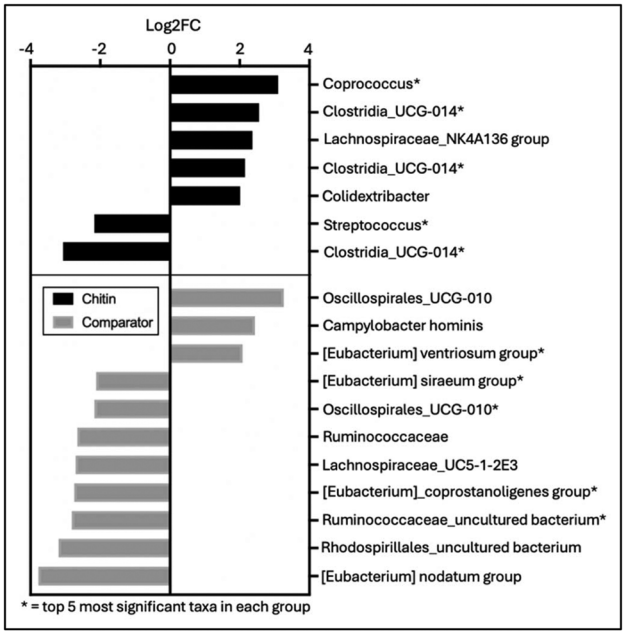

Fig. 8 Shown are the taxa that significantly increased or decreased following either the chitin or comparator intervention after controlling for FDR, which also met an effect size threshold of 2 (*i.e.,* −Log2FC absolute value of 2). Any taxa that appear more than once in each group are different amplicon sequence variants of that particular species or genus. * = top 5 smallest *p*- and *q*-values and thus considered top 5 most significant taxa.

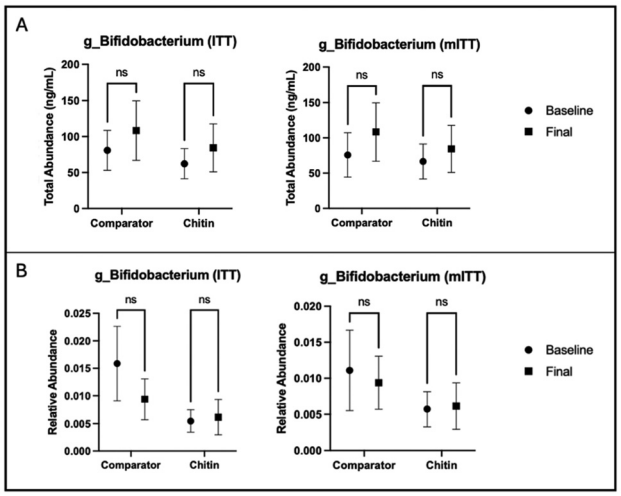

Fig. 9 Shown are average changes in relative abundance and total abundance (ng mL^{-1}) of *Bifidobacterium* from baseline after each treatment for both the (A) intent-to-treat (ITT) and (B) modified intent-to-treat (mITT) analyses. No significant changes were observed. ns = non-significant.

No taxa were correlated with IBS-SSS in the Chitin group, although *Eubacterium siraeum group* was significantly and positively correlated with IBS-SSS in the Comparator group (rho = 0.577; *p*-value = 0.019), and when considering all individuals regardless of group (rho = 0.381; *p*-value = 0.008). Conversely, *Eubacterium ventriosum group* was significantly and inversely correlated with IBS-SSS in the Comparator group (rho = −0.501; *p*-value = 0.048), and trending significance when considering all individuals (rho = −0.259 *p*-value = 0.082). Within the Comparator group, several other taxa were also inversely correlated with IBS-SSS in relationships that were trending toward significance (Fig. 10; *p*-values < 0.1).

Campylobacter hominis inversely correlated with acetate, propionate, and butyrate; this relationship was most pronounced when considering all individuals, but still present when subset by Comparator and, to a lesser degree, Chitin (Fig. 10). *Clostridia*_UCG-014 ASV2 was also inversely associated with SCFAs and, similarly, this relationship was most pronounced when considering all individuals (Fig. 10). Out of the SCFAs, propionate had the greatest number and strength of correlations with taxa, followed by butyrate and then acetate.

Finally, neither TNF-alpha nor any SCFAs were significantly correlated (*p*-values > 0.05) nor trending significance (*p*-values > 0.1) with IBS-SSS (Fig. 11).

4 Discussion

This pilot study aimed to assess whether the daily consumption of a chitin-infused cacao patty was safe and tolerable in adults with IBS, as well as explore the preliminary effects of the intervention on gastrointestinal symptoms, inflammation, and gut microbiota. Overall, results provide support for the safety and tolerability of cricket chitin-infused cacao in our sample of adults with IBS. Results also suggest that while both chitin and cacao may contribute to a reduction of gastrointestinal symptoms and shifts in microbial populations, chitin appears to provide enhanced symptom relief and a concurrent reduction in an inflammatory biomarker (*i.e.*, TNF-alpha).

4.1 Safety and tolerability of the intervention

We observed no serious adverse events throughout the study, and an assessment of metabolic and lipid biomarkers provided additional support for the safety and tolerability of the intervention in adults with IBS. Despite some statistically significant shifts in values for glucose, creatinine, high density lipoprotein, triglycerides, and ratio of total cholesterol-to-high density lipoprotein, all pre- and post-intervention values in both treatment groups remained well within normal healthy ranges and were not clinically significant shifts. Of note, while there was an increase in blood glucose in the comparator group, possibly related to the added maltodextrin, values still fell within normal healthy ranges for an adult. The slight increase in triglyceride levels following chitin intervention is possibly due to higher fat content of the chitin intervention although, again, values still fell within normal healthy ranges for an adult. Although unconventional, the inclusion of a lipid panel as a safety and tolerability measure was particularly important for this intervention and this population, as evidence suggests chitin and its derivative chitosan can bind

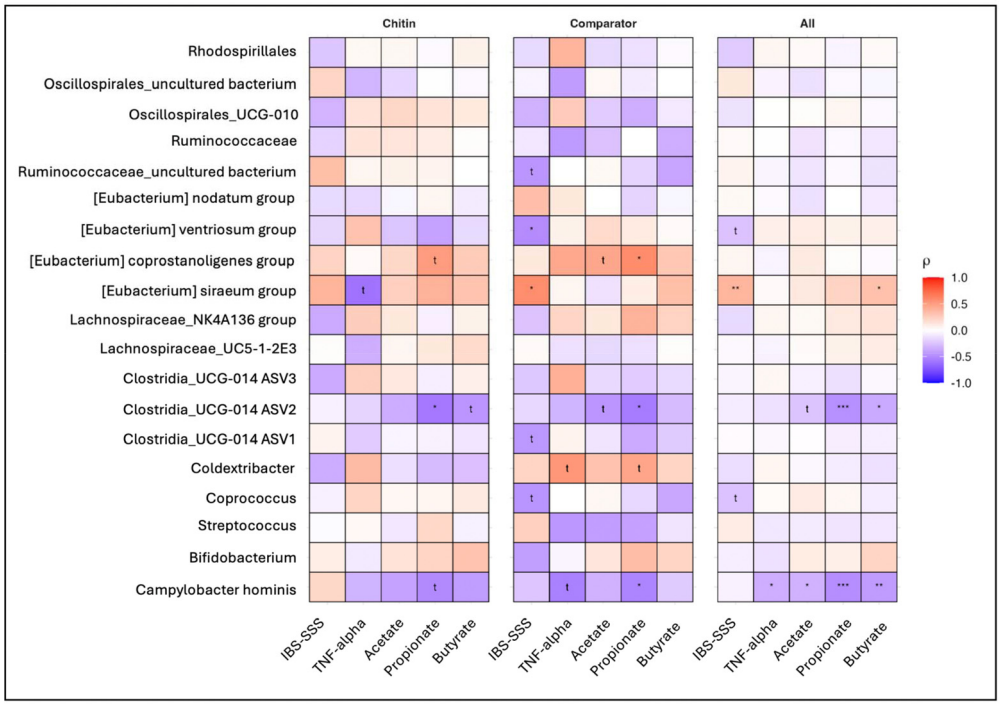

Fig. 10 Shown is a heatmap of correlation coefficients generated by a repeated measures Spearman's correlation between IBS-SSS and taxa, TNF-alpha, and SCFAs (*e.g.*, acetate, butyrate, and propionate), subset by group (*e.g.*, comparator, chitin, or all). Any p-values < 0.1 = *t*, p-values < 0.05 = *, p-values < 0.01 = **, and p-values < 0.001 = ***.

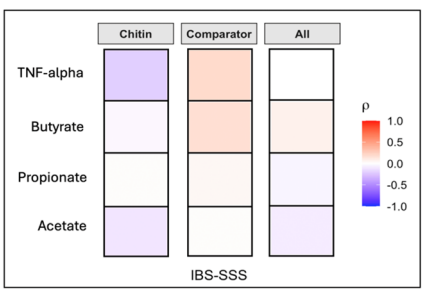

Fig. 11 Shown is a heatmap of correlation coefficients generated by a repeated measures Spearman's correlation between IBS-SSS and inflammatory (TNF-alpha) and SCFA (acetate, butyrate, and propionate) outcomes. No significant correlations were observed.

lipids in the intestinal lumen and prevent fat absorption.[69,70] The consequence of this lipid binding is enhanced fat excretion[69,71] which, in a population already experiencing irregular bowel habits, could potentially further promote loose and abnormal stools (*i.e.*, further exacerbate symptoms).

The results of this study also suggest that the daily inclusion of cacao and/or chitin in participants' diets did not negatively impact IBS-related quality of life, which was reported by participants before and after each intervention. As IBS-related disruptions in quality of life are often triggered by specific foods, it was important to determine whether the daily inclusion of cacao and/or chitin in participants' diets negatively impacted these individuals. In fact, a survey of patients with IBS-C revealed that chocolate was one of the several food items those participants perceived as worsening their constipation.[72] In contrast to these perceptions, we observed statistically significant increases in QoL score after the comparator intervention, although the magnitude of these shifts may not translate to clinical relevance based on suggested MCID values.[68] Additionally, due to the small sample size we were also unable to discern whether effects on QoL scores were dependent on IBS subtype, and future investigations should be designed to establish sub-type specific effects.

4.2 Effects of chitin on gastrointestinal, immune, and microbiome outcomes

While we did not observe changes in participants' bowel habits throughout the study (*i.e.*, ratio of normal to abnormal stools), participants reported improvements in their IBS-related GI symptoms following both interventions, as evidenced by lower IBS-SSS scores. Both the chitin and comparator intervention contained a cacao base, which is rich in polyphenols. Research suggests dark chocolate polyphenols possess diverse bioactivities including anti-inflammatory,[73] barrier permeability regulating,[74] gut microbiota-mediating effects,[75] which have positively supported gastrointestinal health in contexts other than IBS. For example, animal models of ulcerative colitis utilizing cocoa-based interventions observed a downregulation in inflammatory cytokine production,[76] reduced colon damage, and decreased immune cell infiltration into the intestinal mucosa.[77] Cacao polyphenol

extracts also improved intestinal barrier damage in a Caco-2 *in vitro* model.[74] Thus, although cacao-based interventions and cacao polyphenols have not been directly studied in IBS, evidence of cacao's gut health-promoting bioactivities in other relevant contexts leads us to hypothesize that the presence of cacao polyphenols in both treatments may have contributed to the observed reductions in IBS-SSS scores.

Interestingly, while both treatments demonstrated improvements in gastrointestinal symptoms, the reduction in IBS-SSS scores was significantly greater following the chitin intervention when compared to the cacao alone. This stronger association may indicate a synergistic effect, where the bioactive cacao polyphenols combined with the effects of chitin enhance the intervention's impact on IBS-SSS outcomes. Although existing evidence is limited to chitin-glucan, which is derived from fungi and is more soluble than chitin, two *in vivo* IBS mouse models that administered chitin-glucan alone[32] or with a probiotic[78] and one clinical study using chitin-glucan in combination with an IBS-medication[79] suggest improvements in GI symptoms. Specifically, the recent prospective, open-label multicenter study by Talbodec *et al.* (2024),[79] in which 120 individuals with IBS consumed a combination of chitin-glucan and simethicone daily for 4 weeks, observed improvements in abdominal pain, bloating, abdominal distension, stool consistency, and the impact of global GI symptoms on daily life. Similar to our own observations, Talbodec *et al.* (2024)[79] also observed no serious adverse events and good-to-very good tolerability. Moreover, in the two *in vivo* mouse models, which did not include co-administration of IBS medication with chitin-glucan, both observed reductions in visceral pain sensitivity and inflammatory cytokines.[32,78] To our knowledge, this is the first report of insect-based chitin, which is mainly a highly insoluble form of chitin-melanin, on improving gastrointestinal symptoms in a population with IBS.

Our study demonstrates that chitin consumption may also reduce certain inflammatory outcomes in adults with IBS. The chitin treatment resulted in, on average, a greater decrease in serum TNF-alpha than seen after comparator treatment; of note, three individuals no longer had detectable levels of plasma TNF-alpha following the chitin treatment. This corroborates results from our previous study in healthy participants that observed a decrease in TNF-alpha in response to cricket-derived chitin consumption *via* whole crickets in muffins and smoothies.[31] Whether chitin or its derivatives (*e.g.*, chitosan) elicit pro- or anti-inflammatory responses appears to be partially dependent on molecule size.[80] It is suggested that whereas large chitin molecules are generally immunologically inert and intermediate sized chitin molecules promote inflammatory responses and the production of pro-inflammatory cytokines, small chitin molecules may promote a more anti-inflammatory immunological tone (*e.g.*, enhanced IL-10 and lower TNF-alpha secretion when compared to intermediate sized chitin).[81] As a small chitin molecule, the cricket-derived chitin used in this study appears to demonstrate immunomodulatory effects in accordance with these size-dependent reports. Combined with our findings, this supports the use of crickets as a specific chitin source for anti-inflammatory purposes, although future studies comparing effects of various chitin sources in human populations is warranted. Moreover, as previous research demonstrated a positive correlation between TNF-alpha levels and IBS-SSS scores in adults with IBS,[82] the enhanced reduction of TNF-alpha observed in our study following the chitin treatment may partially explain the parallel, more significant decrease in IBS-SSS scores we observed in response to chitin as well.

Regarding gut microbial outcomes, the chitin treatment also appeared to result in greater changes from baseline between individuals pre- and post-intervention (*e.g.*, increase in beta diversity). This could signify the development of a more diverse or distinct microbial community in response to chitin, potentially due to metabolism by and modulation of specific microbial taxa. In contrast, the average reduction in beta diversity seen in the control group pre- to post-intervention could indicate a homogenization of gut microbiota over time. This may suggest that the absence of chitin in the comparator cacao patties limits dynamic changes in microbial composition. Chitin's effects on beta diversity could stem from its role as a substrate for specific gut bacteria;[83–86] however, our results suggest that smaller responses by a greater number of microbial taxa may be responsible for promoting shifts in community composition that differentiate treated individuals from their baseline. In relation to health, a higher beta diversity might reflect beneficial functional changes, as a more diverse microbiome is often associated with improved gut health and resilience.[87] However, we did not see any treatment related differences in alpha-diversity parameters, limiting our ability to interpret the beta-diversity differences.

Unexpectedly, we did not see changes in *Bifidobacterium* relative or absolute abundance. This contrasts with our previous study in healthy adults where we observed increased *Bifidobacterium adolescence* abundance[31] and mechanistic observations regarding the ability of several *Bifidobacterium* species to utilize chitin as a carbon source.[83] While the study was likely underpowered to detect changes in *Bifidobacterium*, evidence also suggests that relative abundance of *Bifidobacterium* may be lower in individuals with IBS compared to healthy controls.[88–90] Thus, it is possible we did not observe a bifidogenic effect of chitin in this study due to a pre-existing lower relative abundance of *Bifidobacterium* in the IBS population compared to the healthy population tested in our initial study. Future studies in this population could explore whether co-administration of chitin with *Bifidobacterium* improves persistence or engraftment of the probiotic.

The relative abundance of several other microbial taxa did shift in response to the chitin treatment and the comparator. While some of these taxa were previously identified as being increased or decreased (or both) in adults with IBS (*e.g.*, *Coprococcus*, *Streptococcus*, *Campylobacter*, several members of Ruminococcaceae, Clostridales and *Clostridium*, and others), systematic reviews highlight conflicting results regarding several of these taxa across studies and/or highlight potential

differences in microbial taxa by IBS-subtype.[89–91] It should be noted that the low taxonomic resolution provided by 16s rRNA data limits interpretation of findings across studies as different ASVs mapping to the same taxa can have different effects. For example, two ASVs of Clostridium UCG_014 had opposing relationships with the chitin intervention.

The largest increase in relative taxa abundance after the chitin treatment in our study was seen in *Coprococcus*. Although existing evidence is conflicting and limited,[92–94] some studies suggest *Coprococcus* may be associated with a healthier microbiome in IBS. Relative abundance of *Coprococcus* was increased in the microbiomes of healthy controls when compared to IBS participants[94] and was also increased in remediated microbiomes of IBS participants following a dietary and probiotic intervention;[93] in the latter study, this increase in *Coprococcus* was correlated with improved IBS symptoms. Interestingly, our own results may corroborate this. We observed inverse relationships that were trending towards significance (p-values < 0.1) between *Coprococcus* and IBS-SSS scores in the comparator group and when considering all individuals, regardless of treatment group.

We also observed a decrease in relative abundance of *Streptococcus* after the chitin treatment in our study. Although, as with *Coprococcus*, evidence is conflicting regarding *Streptococcus* in IBS and may be influenced by sub-type, elevated *Streptococcus* abundance in IBS participants was noted in several studies.[89,90] One study in particular determined that *Streptococcus* sp. abundance was positively associated with IBS symptom severity[95] and decreased after treatment. We did not, however, observe any relationships between *Streptococcus* and IBS-SSS that were either significant or trending towards significance.

Finally, distinct amplicon sequence variants (ASVs) of *Clostridium_UCG-010* were observed to either increase (two ASVs) or decrease (one ASV) following chitin treatment, although none were correlated with IBS-SSS scores. While *Clostridium_UCG-010* has not yet been identified in IBS studies, the abundance of other *Clostridium* species or clusters was reported as either elevated (*e.g.*, *Clostridium perfungins* and *Clostridium difficile*) or reduced in IBS (*e.g.*, *Clostridium sesnu strico*, *Clostridium cluster IV*, and *Clostridium cluster XI*).[89,90,96] This variability highlights the complex and context-dependent roles of *Clostridium* species in gut health, leaving the precise health implications of *Clostridium_UCG-010* yet to be fully elucidated.

Campylobacter hominis relative abundance increased following the comparator intervention—and this change correlated inversely with levels of short-chain fatty acids both in the comparator group and across all participants. Prior work has linked elevated *Campylobacter* spp. to post-infectious IBS and heightened inflammatory signaling,[97] so its inverse relationship with protective SCFAs is biologically plausible. In contrast to this, we also unexpectedly observed inverse relationships between *Campylobacter hominis* and TNF-alpha. Notably, this rise in *Campylobacter* was absent in the chitin arm; we speculate whether the chitin addition to the cacao patty mitigated an increase in *Campylobacter*. We did not identify any existing literature specifically involving cacao or dark chocolate and *Campylobacter* abundance, and only limited literature involving chitin derivatives and *Campylobacter*. However, both *in vitro* and *in vivo* animal studies suggest that chitosan may interfere with *Campylobacter* virulence factors, such as quorum sensing and host cell adhesion.[98,99]

4.3 Strengths and limitations

A notable strength of this study is the utilization of cricket-sourced chitin as a novel and sustainable dietary fiber source. Moreover, the application of unmodified chitin as a prebiotic is relatively unique, as many studies investigating chitin focus on derivatives or conjugates that enhance solubility. Employing cricket-derived chitin in its unaltered form enhances the relevance of the findings to populations where insect chitin is part of the habitual diet. However, this study is not without limitations. The small sample size restricted our ability to examine potential differences in chitin's impact across IBS subtypes, which is significant given the distinct characteristics of each subtype and their potential for varying dietary responses. Additionally, the lack of diversity in sex and race among participants further limits the generalizability of the findings. Future studies with larger sample sizes and defined IBS subtypes are needed to enhance generalizability of findings to the broader IBS population. Finally, one aspect of this study that could be considered both a strength and a limitation is the minimal control over participants' diets. Although consistency was assessed *via* a 3 day food log, the variability in dietary intake could have influenced the outcomes. However, this pragmatic approach better reflects real-world conditions, allowing for insights into how this nutritional intervention may interact with regular dietary practices in everyday settings.

5 Conclusion

Our results suggest that while both chitin and dark chocolate are safe and tolerable and improve symptoms in our sample of adults with IBS, chitin may provide an additional benefit of lowering inflammatory cytokines and enhancing the reduction of symptom severity. Investigating the use of cricket chitin for remediating gastrointestinal symptoms and associated inflammation provides insight into the utility of a unique prebiotic source for a population that frequently experiences food sensitivities. Notably, these results may have implications beyond populations with IBS. Crickets, and other edible insects, are considered a highly sustainable food source with farming practices that have low environmental impacts (especially when compared to conventional animal foods). This sustainability, coupled with their integration into traditional diets in regions affected by other functional gastrointestinal disorders (*e.g.*, environmental enteropathy), underscores the potential to translate these findings into broader global health initiatives. Future research could explore the role of cricket-derived chitin

in promoting GI health and addressing nutritional challenges in diverse populations, particularly in resource-limited settings.

Author contributions

Conceptualization – V. S. and T. W.; data curation – A. A. V., S. T., C. M., R. A. H., T. A., I. D., T. W.; formal analysis – A.A.V.; funding acquisition – V. S. and T. W.; investigation – S. T., C. M., A. M. K., R. A. H., P. M., J. W. W., J. C., Y. W.; methodology – V. S., T. W., P. M., J. C., Y. W., A. A. V.; project administration – A. M. K., V. S., T. W.; resources – V. S. and T. W.; software – A. A. V.; supervision – V. S., T. W., A. M. K.; validation – T. W., A. M. K., A. A. V.; visualization – A. A. V., S. T., C. M.; writing – A. A. V., S. T., C. M.; writing – review & editing – A. A. V., T. W., V. S.

Conflicts of interest

There are no conflicts to declare.

Data availability

Additional data supporting this article are included as part of the SI. These data include the CONSORT checklist and supplemental tables with nutrient composition of the intervention and comparator, micronutrient composition of the chitin extraction, measured physiologic parameters of participants, and a comprehensive list of altered microbial taxa. See DOI: **https://doi.org/10.1039/d5fo01540e**.

Sequence data are available through the QIITA repository at UCSD under study number 15938. **https://qiita.ucsd.edu**.

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