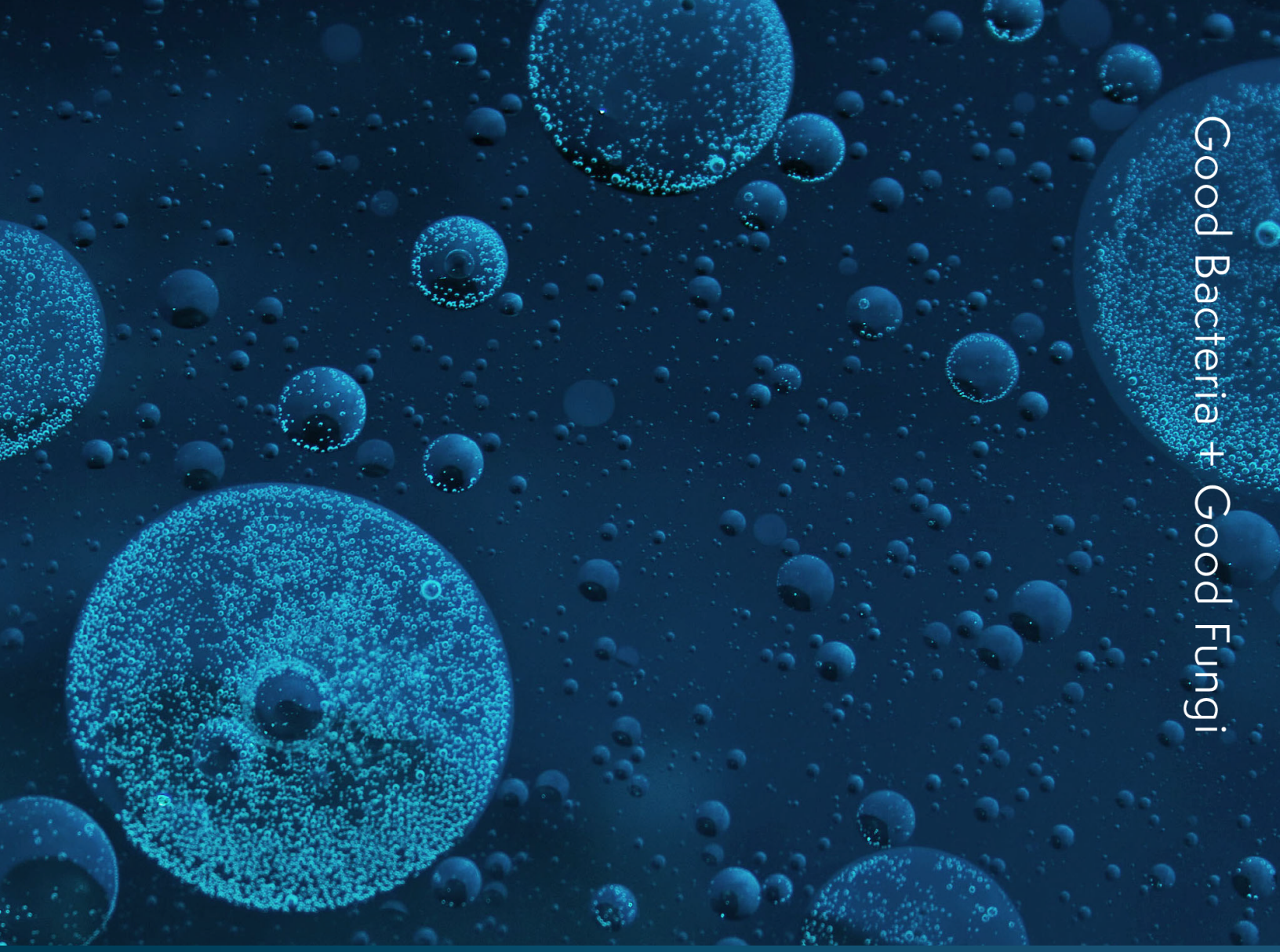

BIOHM®

Dossier of Published Clinical Trials

Updated March 1, 2024

B I O H M

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[1] In 2021, BIOHM completed a 49-person clinical trial over a 4-week period, which was entitled "Evaluation of Microbiome Alterations Following Consumption of BIOHM, a Novel Probiotic", and published in Current Issues in Molecular Biology.

[2] In 2022, BIOHM completed a 52-person, clinical trial over a 6-week period, which was entitled "A Probiotic Amylase Blend Reduces Gastrointestinal Symptoms in a Randomized Clinical Study", and published in Beneficial Microbes.

[3] In 2023, BIOHM completed a 40-person, clinical trial, which was entitled "Probiotic Supplementation of Pea-Derived Protein alters the gut microbiome balance in favor of increased protein degradation, reflected in increased levels of Essential Amino Acid in Human Plasma", and published in Gastroenterology & Hepatology.

current issues in molecular biology

MDPI

Article

Evaluation of Microbiome Alterations Following Consumption of BIOHM, a Novel Probiotic

Mahmoud A. Ghannoum [1,2,*], Thomas S. McCormick [1], Mauricio Retuerto [1], Gurkan Bebek [3], Susan Cousineau [4], Lynn Hartman [4], Charles Barth [4] and Kory Schrom [2]

1 Department of Dermatology, Case Western Reserve University, Cleveland, OH 44106, USA; tsm4@case.edu (T.S.M.); mauricio.retuerto@case.edu (M.R.)
2 University Hospitals Cleveland Medical Center, Cleveland, OH 44106, USA; Kory.Schrom@uhhospitals.or
3 Department of Nutrition, Case Western Reserve University, Cleveland, OH 44106, USA; gurkan@case.edu
4 Fermentation Festival, Santa Barbara, CA 93117, USA; connect@thefarmecologist.com (S.C.); vineescape@gmail.com (L.H.); charlesbartht@gmail.com (C.B.)
* Correspondence: mag3@case.edu; Tel.: +1-216-844-8580

Abstract: Gastrointestinal microbiome dysbiosis may result in harmful effects on the host, including those caused by inflammatory bowel diseases (IBD). The novel probiotic BIOHM, consisting of *Bifidobacterium breve*, *Saccharomyces boulardii*, *Lactobacillus acidophilus*, *L. rhamnosus*, and amylase, was developed to rebalance the bacterial–fungal gut microbiome, with the goal of reducing inflammation and maintaining a healthy gut population. To test the effect of BIOHM on human subjects, we enrolled a cohort of 49 volunteers in collaboration with the Fermentation Festival group (Santa Barbara, CA, USA). The profiles of gut bacterial and fungal communities were assessed via stool samples collected at baseline and following 4 weeks of once-a-day BIOHM consumption. Mycobiome analysis following probiotic consumption revealed an increase in Ascomycota levels in enrolled individuals and a reduction in Zygomycota levels (p value < 0.01). No statistically significant difference in Basidiomycota was detected between pre- and post-BIOHM samples and control abundance profiles ($p > 0.05$). BIOHM consumption led to a significant reduction in the abundance of *Candida* genus in tested subjects (p value < 0.013), while the abundance of *C. albicans* also trended lower than before BIOHM use, albeit not reaching statistical significance. A reduction in the abundance of Firmicutes at the phylum level was observed following BIOHM use, which approached levels reported for control individuals reported in the Human Microbiome Project data. The preliminary results from this clinical study suggest that BIOHM is capable of significantly rebalancing the bacteriome and mycobiome in the gut of healthy individuals, suggesting that further trials examining the utility of the BIOHM probiotic in individuals with gastrointestinal symptoms, where dysbiosis is considered a source driving pathogenesis, are warranted.

Keywords: probiotics; dysbiosis; microbiome; mycobiome; *Candida*

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1. Introduction

Human gastrointestinal (GI) microbiome research has primarily focused on resident bacteria and their associated bacterial–host interactions, both beneficial and detrimental. However, solely focusing on bacteria has neglected the potential influence of the host's fungal community (mycobiome) on health and disease. In a previous study, we characterized the gut bacterial microbiota (bacteriome) and the mycobiome in family members with Crohn's disease (CD) and their healthy relatives in an attempt to define the interactions leading to dysbiosis in CD. We identified a positive correlation between bacteria and fungi, wherein the bacteria, *Escherichia coli* and *Serratia marcescens*, and the fungus, *Candida tropicalis*, demonstrated increased abundance in the GI tract of CD patients when compared with their non-Crohn healthy relatives [1]. Subsequently, we showed that *C. tropicalis* and the two bacterial species cooperate in a strategic way to form in vitro pathogenic biofilms

capable of causing damage to the epithelial cell lining of the gut and initiating an inflammatory response [2]. Not only do these findings identify a possible new therapeutic targeting approach (i.e., bacterial–fungal interaction modulation) in patients with inflammatory bowel disease (IBD), they also highlight a possible avenue for improving human health and disease as a whole through microbiome modulation.

One approach to combat IBD symptoms by preventing and treating microbiome dysbiosis includes the use of probiotics, which the World Health Organization (WHO) has defined as live microorganisms that confer health benefits on the host when administered in adequate amounts [3]. The importance of research and development of probiotics for use in IBD is highlighted in a review by Sartor [4], who reported previously that minimal research has been carried out on probiotics in the setting of IBD, and studies that have been conducted are in relatively small trials with a low number of enrolled patients. Although the numbers of probiotic trials designed to address IBD have increased exponentially, modest cohort size and outcomes still hamper interpretation and limit the rigor of this research [5]. Clearly, there is a need for more clinical trials involving larger numbers of subjects powered sufficiently to statistically address the efficacy of probiotics in GI diseases.

Since the cooperative interaction of fungi and bacteria in the dysbiotic state has been shown to produce harmful effects on the host, it is logical to suggest that the introduction of different combinations of microbes in the form of probiotics to restore overall balance may help to counteract these detrimental effects. Probiotics have been shown to be effective in preventing and ameliorating various medical conditions, particularly those involving the GI tract in children. Recently, certain probiotic bacteria have been studied as a potential method to prevent opportunistic infectious diseases by stimulating the host immune system [6–8]. Previous studies have reported the positive effects of probiotics in a variety of diseases such as *Candida* vaginitis [9] and vulvovaginal candidiasis [10,11], oral candidiasis [12], GI infection [13], colon carcinoma [14], and recent probiotic studies on IBD [15–22].

Since it has been demonstrated that microbial dysbiosis is implicated in GI diseases such as IBD, ulcerative colitis, and CD, developing probiotics that can rebalance and maintain the gut microbiota is a reasonable approach to counteract the effect of dysbiosis. The development of the BIOHM probiotic was guided by microbiome analysis based on a large cohort of individuals who were analyzed through the BIOHM gut testing platform to design a probiotic that would affect organisms increased in individuals with intestinal dysbiosis. Our aim was to select appropriate microbes that target pathogenic bacterial and fungal strains while supporting beneficial ones. To achieve this, we conducted correlation analyses of bacterial–bacterial and bacterial–fungal interactions to identify appropriate probiotic strains. This work led to the development of a new probiotic, BIOHM, consisting of *Bifidobacterium breve 19bx, Saccharomyces boulardii 16mxg*, *Lactobacillus acidophilus 16axg, and L. rhamnosus 18fx*, combined with the enzyme amylase based on its anti-biofilm activity [23–25].

In order to determine the effect of BIOHM on the comprehensive intestinal microbiome (CIM, representing bacterial and fungal communities) of human subjects, in this study, we enrolled a cohort of 49 volunteers in collaboration with the Fermentation Festival group (Santa Barbara, California). The CIM profiles of bacterial and fungal communities were assessed at baseline and following 4 weeks of BIOHM use. We then compared the bacteriome of our subjects with those reported by the Human Microbiome Project (HMP) for healthy subjects as a control for bacterial abundance. For fungal controls, we used cumulative fungal abundance data generated through the BIOHM Gut Test data repository of healthy individuals.

2. Materials and Methods

2.1. Design of BIOHM Probiotic

Appropriate probiotic strain selection is critical to the probiotic design process. To select optimal probiotic strains that antagonize (inhibit the growth of) harmful microorgan-

isms while supporting beneficial ones, we conducted correlation analyses of bacterial–bacterial and bacterial–fungal interactions. Based on our results, we identified individual bacterial and yeast strains that antagonize *Candida* (*Lactobacillus rhamnosus 18fx* (2.38×10^{10} CFU/g), *Saccharomyces boulardii 16mxg* (5.6×10^{9} CFU/g), and *Lactobacillus acidophilus 16axg* (2.38×10^{10} CFU/g)), as well as a bacterium that antagonizes both *S. marcescens* and *E. coli* (*Bifidobacterium breve 19bx* (2.38×10^{9} CFU/g)) [26].

Based on our data, which showed that fungi and bacteria cooperate in strategic ways to form pathogenic, inflammation-inducing biofilms, we included the enzyme amylase in our formulation, which has been shown to inhibit biofilms and can be safely incorporated into a probiotic mixture [23].

Prior to reaching the small intestine, probiotics must first pass through the harsh acidic environment of the stomach. The pH of the stomach can increase to a range of 4.0–6.0 after ingestion of a meal but normally returns to the baseline acidic range of 1.5–3.5 within approximately 2 h [27]. It has been estimated that only 20–40% of probiotic cells survive this acidic exposure [28]. Previously, we evaluated the ability of selected BIOHM probiotic strains to survive at acidic conditions and showed that the *S. boulardii* and *L. rhamnosus* can survive at a pH of 1.5, while *L. acidophilus* and *B. breve* are able to survive the acidified stomach environment if ingested within 30 min of a meal [29].

2.2. Participants

To evaluate the effect of BIOHM on the microbiome structure of healthy individuals, we collaborated with the slow-food movement Fermentation Festival, Santa Barbara group (the slow-food movement was founded by Carlo Petrinin in 1986 as an alternative to "fast food"; proponents encourage traditional cooking of locally grown produce and livestock) to enroll in the present study [30]. Fecal samples were collected from volunteers (n = 49) who signed informed consent at baseline and following 4 weeks of once-a-day BIOHM consumption, these individuals are represented as "before" and "after" in all figures. In addition, a "normal" population was generated by comparing the bacteriome of our subjects to those reported by the Human Microbiome Project (HMP) for healthy subjects as a control for bacterial abundance (see below). For fungal "normal" controls, we used cumulative fungal abundance data generated through the BIOHM Gut Test data repository of healthy individuals.

2.3. HMP Patient Comparison Selection

To select the healthy normal subjects, we followed the inclusion and exclusion guidelines of the Human Microbiome Project [31]. Specifically, we excluded subjects that reported any chronic disease (e.g., diabetes, heart disease, overweight defined as having BMI > 35 kg/m^2, as well as subjects on medications (especially antibiotics, antifungals, acid reflux medications, etc.). This resulted in selecting 950 individuals considered healthy (age ranges included 18–34, 34–54, 55+), with a BMI of 18.6–34.9 kg/m^2, in our analysis.

2.4. DNA Extraction

Fecal samples were analyzed for their bacterial and fungal communities using Ion Torrent sequencing technology. Samples were transferred to tubes containing glass beads with the lysis solution included in the QiaAmpFast DNA Extraction Kit (QIAGEN, Germantown, MD, USA). Bacterial and fungal DNAs were isolated and purified following the manufacturer's instructions with minor modifications: In this regard, we incorporated an additional bead-beating step (Sigma-Aldrich beads, diameter = 500 µm), with the MP FastPrep-24 speed setting of 6 M/s and 2 × 40 s cycles. The quality and purity of the isolated genomic DNA were confirmed using a NanoDrop 2000 (Fisher Scientific, Waltham, MA, USA). DNA concentration was quantified using a Qubit 2.0 instrument applying the Qubit dsDNA HS Assay (Life Technologies, Carlsbad, CA USA) and adjusted to 100 ng per sample. Extracted DNA samples were stored at −20 °C.

2.5. Bacterial 16S rRNA Gene or Pan Fungal ITS Amplicon Library Preparation

For bacteria, the V3-V4 region of the 16S rRNA gene was amplified using 16S-515F: GTGCCAGCMGCCGCGGTAA and 16s-806R: GGACTACHVGGGTWTCTAAT primers, while the fungal ITS region was amplified using ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS 2 (GCTGCGTTCTTCATCGATGC) primers. The reactions were carried out on a 100 ng template DNA, in a 50 µL (final volume) reaction mixture consisting of Q5 PCR Master Mix (ThermoScientific, Waltham, MA, USA), for a final primer concentration of 400 nM. Initial denaturation at 94 °C for 3 min was followed by 30 cycles of denaturation for 30 s each at 94 °C, annealing at 57 °C (16 s) or 59 °C (ITS) for 30 s, and extension at 72 °C for 10 s. Following the 30-cycle amplification, there was a final extension time of 15 s at 72 °C. The size and quality of amplicons were screened on a 1.5% TAE agarose gel, separated using 100v, and electrophoresed for 45 min then stained with ethidium bromide. The PCR products were sheared for 20 min, using Ion Shear Plus Fragment Library Kit (Life Technologies, Carlsbad, CA, USA). The amplicon library was generated with sheared PCR products using Ion Plus Fragment Library Kit (<350 bp) according to the manufacturer's instructions. The library was barcoded with Ion Xpress™ Barcode Adapter and ligated with the A and P1 adaptors.

2.6. Next-Generation Sequencing, Classification, and Analysis

The adapted barcoded libraries were concentrated 4–6× in a speed-vac (ThermoScientific, Waltham, MA, USA) and the concentrated pooled libraries were then quantified using a TaqMan Quantitation Kit (ThermoScientific, Waltham, MA, USA). The libraries were adjusted to 100 pM and attached to the surface of Ion Sphere particles (ISPs) using an Ion PGM Template OT2 400 bp Hi-Q View Kit (LifeTechnologies, Carlsbad, CA, USA) according to the manufacturer's instructions, via emulsion PCR. The quality of ISP templates was checked using Ion Sphere™ Quality Control Kit (Part no. 4468656) with the Qubit 2.0 device. Sequencing of the pooled libraries was carried out on an Ion Torrent PGM System using the Ion Sequencing 400 bp Hi-Q View Kit (Life Technologies, Carlsbad, CA, USA) for 150 cycles (600 flows) with a 318 v2 chip, following the manufacturer's instructions. De-multiplexing and classification were performed using the Qiime Platform (ver. 1.8). The resulting sequence data were trimmed to remove adapters, barcodes, and primers during the de-multiplexing process. In addition, the sequence data were filtered for the removal of low-quality reads below the Q25 Phred score and de-noised to exclude sequences with a read length below 100 bp [32]. De novo OTU's were clustered using the Uclust algorithm and defined by 97% sequence similarity [33]. Classification at the species level was referenced using the Greengenes (v. 13.8) reference database [34] and taxa assigned using the nBlast method with a 90% confidence cut-off [35]. Abundance profiles for the microbiota were generated and imported into Partek Discover Suite v6.11 for principal components analysis (PCA). Diversity and correlation analyses and Kruskal–Wallis (non-parametric) analysis of variance were performed using abundance data and R statistical analysis software (CRAN, and Morgan) with packages (Psych and Vegan, Bioconductor). Diversity indices, including SDI, Richness (N), and PE, were calculated at all taxonomic levels.

2.7. Statistical Analyses

Pre- and post-BIOHM consumption data were analyzed for each sample. Statistical significance levels were calculated, comparing the changes across groups by *t*-test for a given genus, species, or phylum. A p value < 0.05 was considered significant.

3. Results

3.1. Effect of BIOHM on the Mycobiome Community

Figure 1 shows the phyla level profile of the mycobiome community before and after BIOHM consumption, compared with the level of fungal phyla observed in "normal" healthy individuals from the BIOHM gut testing platform cohort. Enrolled subjects had

significantly lower levels of the phylum Ascomycota at baseline, compared with controls, while the level of phylum Zygomycota of the participants was significantly higher at baseline. No significant difference in Basidiomycota was observed in enrolled individuals compared to the healthy profile.

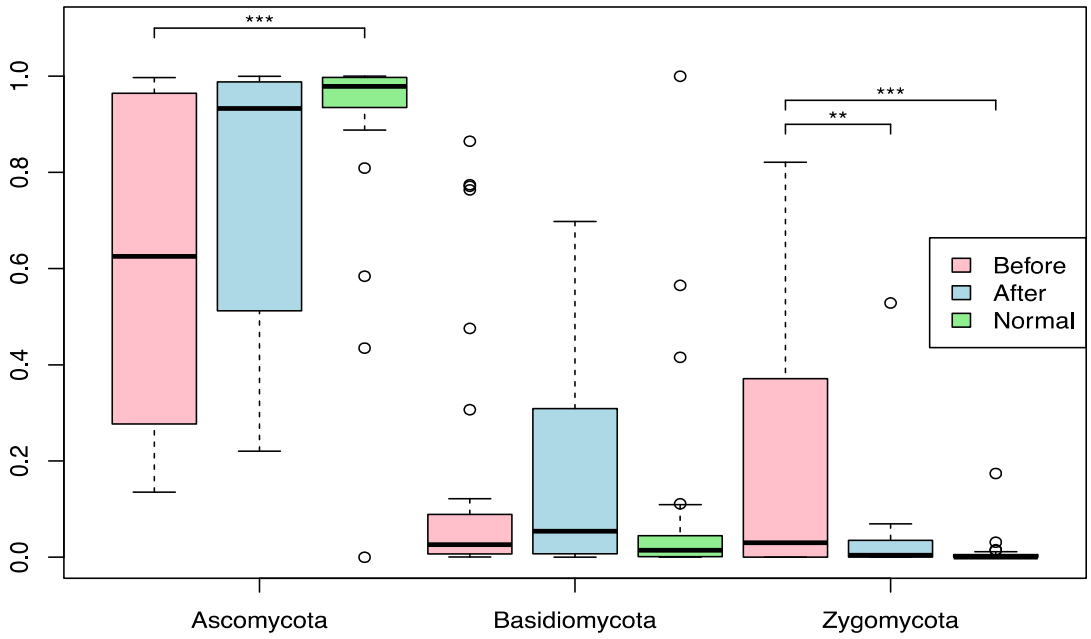

Figure 1. Phyla level abundance profile of the mycobiome community. Fecal samples were collected from subjects at baseline and following 4 weeks of once-a-day consumption of the probiotic BIOHM. The phyla level comparison of mycobiome abundance is shown for baseline (Before) and post-4 week consumption of BIOHM (After). Reference abundance levels (Normal) of the representative phyla are shown based upon the average abundance of a cohort of healthy individuals taken from the participants of the BIOHM gut survey (n = 950). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Mycobiome analysis following probiotic consumption ("after") showed an increase in Ascomycota levels in enrolled individuals, and the abundance of this phylum increased to levels observed in healthy control profiles, a reduction in Zygomycota levels (p value < 0.01) with a subsequent decrease in phylum abundance also matched healthy control profiles. No statistically significant difference in Basidiomycota was detected between pre- and post-BIOHM samples and control abundance profiles ($p > 0.05$).

3.2. Effect of BIOHM on Candida Genus and Species Level

Abundance levels of *Candida* genus and *C. albicans* before and after BIOHM are shown in Figures 2 and 3, respectively. Our data show that BIOHM consumption led to a significant reduction in the abundance of *Candida* genus in tested subjects (p value < 0.013), while the abundance of *C. albicans* also tended to be lower than before BIOHM use, albeit not reaching statistical significance, compared with healthy control profiles (Figure 2). The level of *C. albicans* at baseline also tended to be higher than the cumulative healthy subject average abundance (Figure 3).

3.3. Effect of BIOHM on the Bacteriome Community

Our data showed baseline enrolled subjects had significantly lower phylum levels of Bacteroidetes, compared with the HMP healthy control cohort, while the phylum level of Firmicutes of these subjects was higher at baseline (p value < 0.01). Subjects in the enrolled cohort had significantly higher phylum levels of Proteobacteria (known to be a red flag for inflammation) at baseline, compared with the HMP healthy control values (p value < 0.001). The phyla Actinobacteria, Tenericutes, and Verrucomicrobia were detected at low abundance in all subjects irrespective of the time of collection relative to BIOHM use (Figure 4).

A reduction in the abundance of Firmicutes at the phylum level was noted following BIOHM use, which approached levels reported for HMP controls. No significant changes before and after BIOHM use were noted in the other phyla.

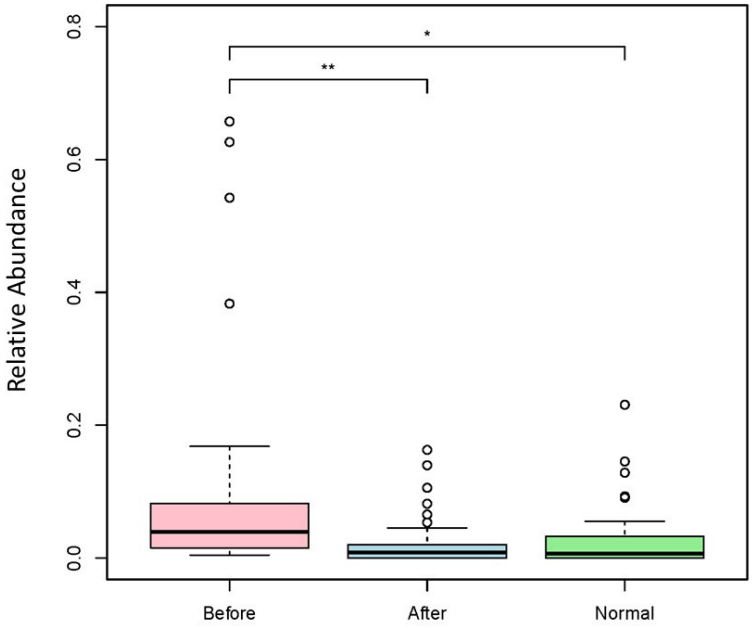

Figure 2. Genus level *Candida* spp. abundance levels. Fecal samples were collected from subjects at baseline and following 4 weeks of once-a-day consumption of the probiotic BIOHM. The genus-level comparison of *Candida* spp. abundance is shown for baseline (Before) and post-4 week consumption of BIOHM (After). Reference abundance levels (Normal) of *Candida* spp. were generated from the average abundance of *Candida* spp. in a cohort of healthy individuals who participated in providing samples to BIOHM for gut survey testing (n = 950). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

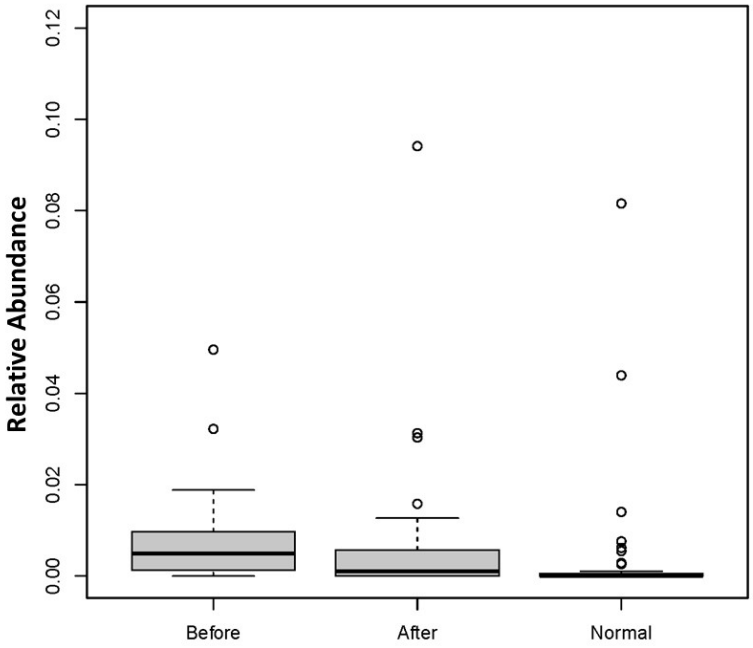

Figure 3. *Candida albicans* abundance levels before and after 4 weeks of BIOHM consumption. Fecal samples were collected from subjects at baseline and following 4 weeks of once-a-day consumption of the probiotic BIOHM. The *Candida albicans* abundance level is shown for baseline (Before) and post-4 week consumption of BIOHM (After). Reference abundance levels (Normal) of *Candida albicans* were generated from the average abundance of *Candida albicans* in a cohort of healthy individuals who participated in providing samples to BIOHM for gut survey testing (n = 950).

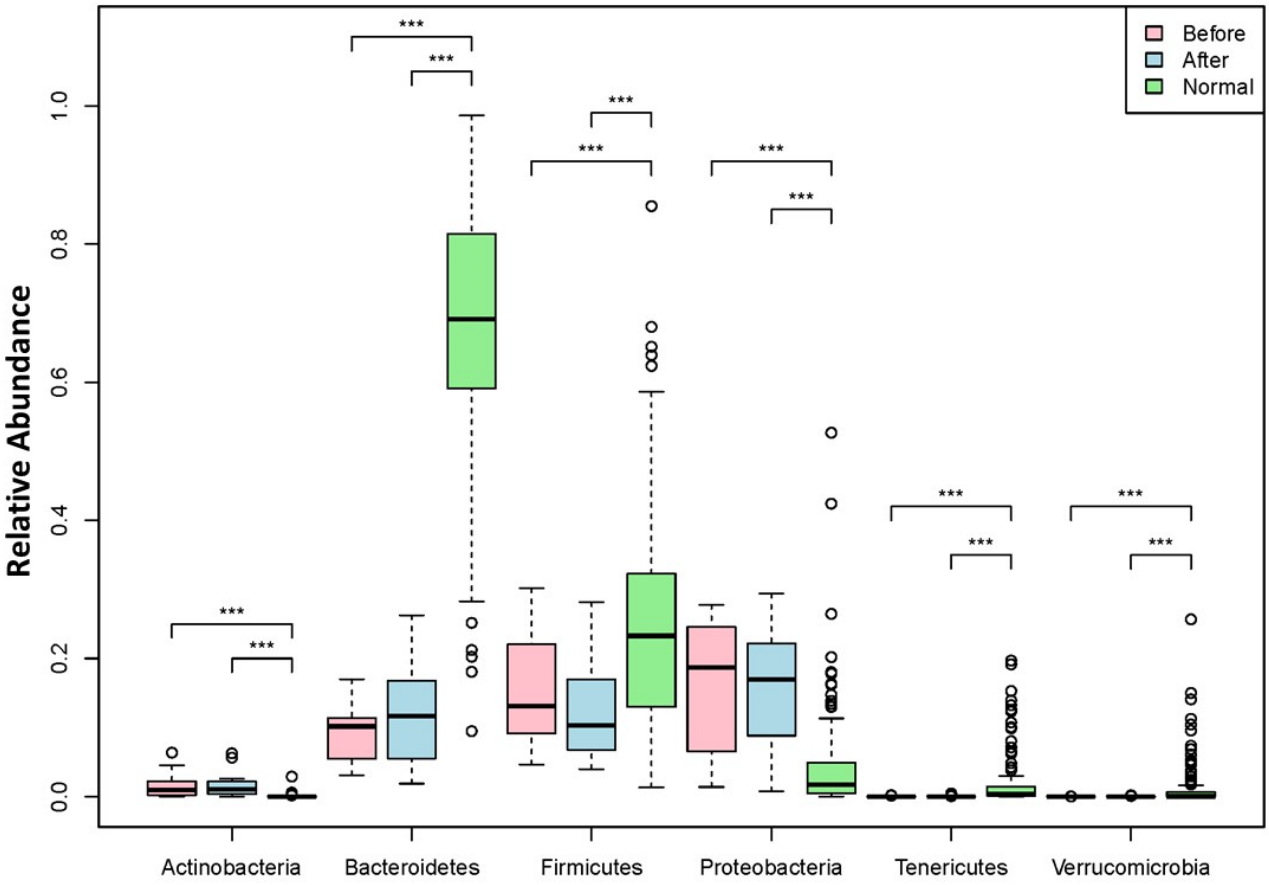

Figure 4. Phyla level abundance profile of the bacteriome community. Fecal samples were collected from subjects at baseline and following 4 weeks of once-a-day consumption of the probiotic BIOHM. The phyla level comparison of bacteriome abundance is shown for baseline (Before) and post-4 week consumption of BIOHM (After). Reference abundance levels (Normal) of the representative phyla are shown based upon the average abundance of these phyla in healthy control subjects who participated in the Human Microbiome Project (n = 250). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

4. Discussion

Several relevant changes occurred in the GI systems of subjects in the BIOHM cohort. A 4-week regimen of a once-a-day dosage of BIOHM reduced gut dysbiosis of *Candida* at the genus level, compared with the healthy control profile. Of particular significance to our study is the reduction in *Candida* numbers in the gut. Diarrhea is a common side effect of antibiotic use associated with the treatment of IBD, due to the eradication of beneficial along with harmful bacteria. As a result, *Candida* can overgrow in the GI tract, leading to further dysbiosis. For example, *C. tropicalis*, as well as *C. albicans*, have been shown to be elevated in CD [26,36].

Beneficial changes in the bacterial community following BIOHM consumption were also demonstrated. Noteworthy was the normalization of the abundance ratio between Bacteroidetes and Firmicutes bacterial phyla. In the healthy gut, Bacteroidetes will outnumber Firmicutes strains, and a disruption of this balance may lead to obesity or sleep disorders [37,38]. Thus, the increase in Bacteroidetes and decrease in Firmicutes following BIOHM use suggested an improved balance between these strains of organisms.

Our previous work demonstrated that *C. tropicalis*, *S. marcescens*, and *E. coli* are overabundant in CD patients, suggesting that these organisms may form a mixed-species biofilm in the gut. Data from our previously reported in vitro study demonstrated that the culture filtrate from the BIOHM probiotic strains inhibited fungal growth and germination, and possessed activity against both planktonic and biofilm forms of *Candida*, suggesting that this activity is mediated by secretory factors [2]. Given these observations, one potential

strategic approach to limiting the polymicrobial interactions observed in IBD would be through the judicious use of a probiotic nutritional supplement.

Traditional approaches to IBD treatment include the use of biologic therapies such as humanized monoclonal antibodies [39] that target and block specific immune pathways that drive mucosal inflammation. Although these types of therapies have proven to be successful in inducing and maintaining remission, patients often become recalcitrant to their effects over time [40].

In an effort to circumvent the associated risks of biologic therapy, antimicrobials have also been employed to control inflammatory symptoms resulting from pathogenic bacteria and fungi colonizing the gut. However, while some patients report relief of IBD symptoms during antibiotic therapy, concerns remain with respect to tolerability, long-term safety, and the emergence of resistant strains [41]. Equally relevant to gut health is the effect of antibiotic use on the bacteriome, or bacterial makeup, of the gut microbiome. Antibiotics may have several adverse effects, which may include the development of resistant antibacterial strains, reduction in beneficial bacteria that produce vitamins such as vitamin K, lower diversity of microbial species that may lead to increased susceptibility to pathogens, and changes to immune reactions in the gut [42]. Importantly, it is becoming clear that broad-spectrum antibiotic use leads to the eradication of pathogenic bacteria as well as beneficial ones, particularly in the gut [43]. As a consequence of the antibiotic effect, *Candida* living in the GI tract overgrow, leading to further dysbiosis.

In that regard, enteric colonization by *Candida* is the most important predictor of invasive fungal infections [44]. It is important to note, however, that *Candida* colonizes the GI tract in over half of healthy individuals as well [45], and the development of mucosal or systemic candidiasis can occur due to hormonal imbalance and immunosuppressive conditions in addition to antibiotic overuse [46]. Thus, designing new strategies that enhance beneficial microbes while inhibiting the expansion of detrimental organisms is desirable.

Recently, new over-the-counter probiotic products have been developed with the goal of preventing and ameliorating gut dysbiosis and IBD. In a previous in vitro study, we determined the effect of a novel formulation containing the probiotic strains *S. boulardii*, *B. breve, L. acidophilus*, and *L. rhamnosus* on pathogenic yeast and enteric bacteria, identified as possible contributors to the inflammatory process [2].

S. boulardii, a well-known probiotic species, is widely used for the prevention and/or treatment of intestinal disorders, including antimicrobial-associated diarrhea, recurrent *Clostridioides difficile* (previously *Clostridium difficile*) disease, acute diarrhea in adults and children induced by a variety of enteric pathogens, traveler's diarrhea, and relapses of CD or UC. Benefits of *S. boulardii* are believed to be related to direct enzymatic effects, modulation of the gut endogenous flora, and enhancement of the immune response. Samonis et al. evaluated the virulence of *S. boulardii* when used as a probiotic, and its role in preventing GI colonization by Candida in a murine model [47]. They showed that the gut colonization was proportional to the given dose but lasted only one week; no dissemination of the yeast was detected.

Lactobacillus spp., *Bifidobacterium* spp., and *S. boulardii* have shown efficacy against intestinal disorders, especially if treatment is introduced early. Orally administered *L. acidophilus* and *L. rhamnosus* (as cheese ingredients) have also been shown to reduce oral *Candida* colonization in denture wearers [48].

An in vitro study by Ribeiro et al. showed that both cells and supernatant of *L. rhamnosus* reduced *C. albicans* biofilm formation, filamentation, gene expression of adhesins (*ALS3* and *HWP1*), and transcriptional regulatory genes (*BCR1* and *CPH1*) [49]. Furthermore, probiotics have been described as a potential strategy to control opportunistic infections due to their ability to stimulate the immune system. In an in vivo study by Rossoni et al., strains of *L. paracasei*, *L. rhamnosus*, and *L. fermentum* were used in a *Galleria mellonella* larvae model to evaluate whether clinical isolates of *Lactobacillus* spp. are able to provide protection against *C. albicans* infection [50]. Their data demonstrated that *L. paracasei* strain

28.4 had the greatest ability to prolong the survival of larvae infected with a lethal dose of *C. albicans*, demonstrating that *Lactobacillus* can modulate the immune system.

Thus, a probiotic that will restore fungal and bacterial balance in the gut should be of enormous benefit to individuals suffering from IBD, as well as to the health of the general population. The ability of BIOHM to reduce polymicrobial biofilm formation may be an outcome of particular importance considering the pathogenesis associated with biofilms and the refractory nature of organisms incorporated in biofilms to traditional therapeutics [2]. The ability to limit biofilm formed by microbial pathogens may improve the overall ability to keep pathogenic organisms in check by decreasing the matrix of biofilms.

5. Conclusions

Our preliminary results show that BIOHM consumption results in the regulation of both bacterial and fungal abundance in the gut within 4 weeks of daily consumption. Importantly, the ability to significantly decrease the pathogenic genus *Candida* suggests that this probiotic should be further examined using expanded clinical trials including IBD patients, where we know imbalance in polymicrobial interactions is a key to dysbiosis and pathogenesis [1].

Limitations of the current study include the modest number of participants in the study as well as the lack of matched controls, although each participant did serve as their own control at baseline. Further limits include subject demographics and knowledge regarding potential dietary differences or the use of other potential probiotic regimens prior to participation in the current study. A more longitudinal sampling approach in future studies would provide more insight regarding the natural variability of the microbiome and how it reacts to external factors, such as changes in diet or the intake of probiotics.

Given our early success in demonstrating the ability of BIOHM to modulate the gut microbiome structure, more extensive placebo-controlled clinical trials are warranted to determine whether this novel probiotic could ameliorate or prevent symptoms in persons with IBD or gut dysbiosis. Further clinical implications regarding BIOHM consumption to consider are the face validity of being able to modulate both bacterial and fungal gut constituents. Modulation of the gut microbiome suggests that in addition to clinical approaches such as fecal microbiome transplant, it may be possible one day to tailor probiotics that would augment host microbial composition and may show efficacy as primary or adjuvant therapies for the treatment of diseases such as irritable bowel syndrome (IBS) or obesity. Indeed, the ability to modulate the microbiome through the rational design of probiotic would be useful in any number of clinical outcomes influenced by the gut microbiome, including potential immune modulation.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of University Hospitals Cleveland Medical Center (Studies 20180667 and 20191290, 9/2018.)

Informed Consent Statement: Participating subjects provided Informed consent prior to participation in the study.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available owing to the privacy concerns of research participants.

Conflicts of Interest: MAG is a co-founder of BIOHMHealthcare. He is a principal investigator and receives funding from Almirall, Scynexis, Partners Therapeutics, and BIOHMHealthcare (sponsored study). All other authors have no conflict of interest to declare.

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RESEARCH ARTICLE

A probiotic amylase blend reduces gastrointestinal symptoms in a randomised clinical study

M.B. La Monica, B. Raub, H.L. Lopez and T.N. Ziegenfuss*

The Center for Applied Health Sciences, 6570 Seville Dr., Canfield, OH 44406, USA;
*ml@appliedhealthsciences.org

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Abstract

A randomised, placebo-controlled, double-blind, parallel clinical study was performed to examine the effects of a probiotic- amylase (PRO) blend on gastrointestinal (GI) symptoms. Sixty men and women (44.4 ± 8.9 yr; 82.0 ± 18.4 kg; 170.3 ± 11.5 cm; 28.1 ± 4.6 kg/m^2) were randomised into PRO (n = 29) or placebo (PLA: n = 31) groups. Participants exhibited mild to moderate GI symptoms and severity [via Gastrointestinal Symptom Rating Scale (GSRS)] to be eligible for participation. Participants were tested before (Baseline) and after (POST) 6 weeks of supplementation on various gastrointestinal indices, the GSRS (to assess GI symptoms, frequency, and severity), an anxiety questionnaire (GAD-7), and an overall well-being questionnaire (SF-36). Two (PRO vs PLA) × 2 (Baseline vs POST) mixed factorial ANOVAs were completed to assess group, time, and (group × time) interaction effects. Fifty-two subjects who completed the entire study were analysed (PRO: n = 25, PLA: n = 27). There were statistically significant ($P \leq 0.05$) interactions for bloating, GSRS score, and abdominal discomfort but time effects for flatulence, constipation, stool regularity, and GAD-7 total score. PRO significantly reduced GSRS score (~60 vs 25%, d = 0.72), bloating (~49% vs 25%, d = –0.63) and abdominal discomfort (59% vs 32%, d = –0.66) to a greater degree than PLA. PRO significantly reduced subjective feelings of irritability, pain, and overall health interference. Oral supplementation of the probiotic-amylase blend was very well tolerated. Our study showed that the probiotic-amylase blend reduced the GSRS score and other GI symptoms to a greater degree than PLA.
Clinical trial registration: clinicaltrials.gov #NCT05614726

Keywords

gut health – bacteria – fungi – microorganisms – microbes

1 Introduction

Digestive health has emerged as an important topic for many consumers (Forssten *et al.*, 2011) and the use of microbes as medicine has been steadily gaining traction as increased understanding of the role of the microbiome in health and disease provided by improved genomics, metagenomics, and metabolomics platforms has emerged (Zmora *et al.*, 2019). In 2013, the International Scientific Association for Probiotics and Prebiotics (ISAPP) defined Probiotics as: 'Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.*, 2014). In the time since probiotics were officially defined, they have become a popular dietary supplement (Hill *et al.*, 2014). Probiotics have been used for over a century

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(Zmora *et al.*, 2019) and are found in supplement form as well as in specific foods (e.g. yoghurt, kefir, sauerkraut, tempeh, and kimchi).

The microbial specificity of each bodily site is dependent upon the habitat provided by the host and the commensal organisms that thrive in each habitat. The gastrointestinal tract (gut) represents the richest niche for microorganisms where inter-kingdom interactions between bacterial (bacteriome) and fungal (mycobiome) communities occur (Mukherjee *et al.*, 2015). The gut represents a very diverse area within the human host. However, the diversity represented within the gut can be altered by exogenous and endogenous factors including diet, lifestyle, generalised health, therapeutics, or other environmental factors.

Probiotics may beneficially modulate the gut microbiota in several ways including expansion of beneficial bacteria and yeast, increasing the mucus layer to improve the physiological barrier function within gut mucosal epithelial cells, keeping pathogens under control, and stimulating immune cells (Mukherjee *et al.*, 2015). Beyond the gut, probiotics may also have beneficial effects on anxiety, stress, and improving mood through the gut-brain axis (Butel, 2014). Since beneficial attributes of a given microbial species is strain specific, testing strain specific effects and formulating a mixture of strains to provide an optimal benefit for the host at an effective dose is necessary (Wallace and Milev, 2017).

Dysbiosis occurs when the gut microbial communities (e.g. bacterial and fungal) are imbalanced and may lead to gastrointestinal (GI) issues as well as numerous beyond the gut diseases (Bubnov *et al.*, 2018), although probiotics can help provide balance (symbiosis) within the gut (Gebrayel *et al.*, 2022) addressing the GI issues. Dysbiosis of the gut microbiota has been associated with adverse conditions, such as *Clostridioides difficile* infection (CDI) (Santiago *et al.*, 2019), metabolic syndrome (Scheithauer *et al.*, 2020), inflammatory bowel disease (IBD) (Zuo and Ng, 2018), and colorectal cancer (Wong and Yu, 2019). Previously, it had been shown that the abundance of pathogenic *Candida tropicalis* (a fungus), *Escherichia coli* and *Serratia marcescens* (bacteria) were elevated in Crohn's disease (CD) patients compared to their non-affected relatives (Hoarau *et al.*, 2016). Additionally, a previous investigation demonstrated that these pathogens formed thick polymicrobial biofilms (PMB) (Hoarau *et al.*, 2016). Recently, it was discovered that a novel formulation of probiotic strains (*Bifidobacterium breve* 19bx, *Lactobacillus acidophilus* 16axg, *Lacticaseibacillus rhamnosus* 18fx, and *Saccharomyces boulardii* 16 mxg) plus amylase was able to prevent and treat the formation of PMB (Hager *et al.*, 2019).

Recent evidence indicates that an overgrowth of fungus in the small intestine of non-immunocompromised subjects may contribute to unexplained GI symptoms. Furthermore, two recent studies showed that 26 and 25.3% of patients with unexplained GI symptoms had small intestinal fungal overgrowth (SIFO) which is characterised by the presence of an excessive number of fungal organisms in the small intestine, associated with GI symptoms (Erdoğan, 2014; Jacobs *et al.*, 2013). The most common symptoms observed in these patients were gas, bloating, intestinal cramps, altered bowel function, indigestion, nausea, diarrhea, belching, and rectal itching (Erdogan and Rao, 2015). While several different bacteria are known to occur in cases of small intestinal bacterial overgrowth (SIBO), *Candida* spp. are implicated in nearly all cases of SIFO (Martins *et al.*, 2014).

Recently, it was demonstrated that the probiotic formulation used in the current study was able to prevent and treat biofilm formation *in vitro* (Hager *et al.*, 2019). In the same *in vitro* study, it was shown that *Candida albicans* or *C. tropicalis*, compared to either *Trichosporon inkin* or *Saccharomyces fibuligera* formed significantly thicker polymicrobial biofilms (PMB) in combination with *E. coli* and *S. marcescens*, indicating that this interaction is *Candida* specific. Furthermore, it was shown that the probiotic could prevent or treat mature biofilms, and that *C. tropicalis* PMB exposed to this probiotic filtrate had reduced biofilm matrix, decreased thickness, and inhibited hyphal formation (Hager *et al.*, 2019).

In addition to *in vitro* work, an *in vivo* preclinical study evaluating the effect of the probiotic formulation using a spontaneous chronic CD like-ileitis animal model (SAMP1/YitFc) (Reuter *et al.*, 2011) was conducted. Three groups of 7-week-old SAMP mice were compared using (1) the current probiotic formulation (4 probiotic strains + amylase), (2) the probiotic supplement without amylase, and (3) control animals administered sterile phosphate-buffered saline alone. After treatment, mice were euthanised, and ilea were collected for histologic scoring of ileitis. Stool samples were evaluated by 16S ribosomal RNA and gas chromatography/mass spectrometry analyses. Histology scores showed that mice treated with the complete formulation (4 probiotic strains + amylase) had a significant decrease of ileitis severity compared to the other 2 groups. 16S ribosomal RNA and gas chromatography/mass spectrometry analyses showed that the abundance of species belonging to genus *Lachnoclostridium* and

the species *Mucispirillum schaedleri* were significantly increased compared to the other 2 groups, and this increase was associated with augmented production of short chain fatty acids (SCFAs) (Di Martino *et al.*, 2023). These findings suggest that administration of this novel probiotic formulation leads to functional changes that ameliorate the severity of CD-like ileitis. In addition, the hydrolytic activity of amylase appears to be essential for the anti-inflammatory effects of beneficial bacteria in the intestine (Di Martino *et al.*, 2023). Previous studies have reported that amylase causes a disruption in the biofilm matrix which supplies probiotic strains with the ability to inhibit pathogenic fungi and bacteria (Di Martino *et al.*, 2023; Gowen *et al.*, 2023; Hager *et al.*, 2019). This disruption in the biofilm matrix protects the intestinal barrier and causes marked alterations in faecal microbial population providing the murine model with greater resistance to colonization of microorganisms, which was dependent on the presence of amylase and the probiotic strains (Di Martino *et al.*, 2023). A follow-up pilot clinical trial showed that consumption of this novel formulation results in positively modifying both bacterial and fungal abundance in the gut within 4 weeks of daily consumption (Ghannoum *et al.*, 2021).

Given the demonstration that the probiotic plus amylase formulation was capable of altering biofilm formation *in vitro* and *in vivo*, combined with reports demonstrating the ability to modulate the gut microbiota structure, including significantly decreasing the pathogenic genus *Candida*, altering biofilm formation, and positively impacting the microbiota in an ileitis model, provided the rationale for performing further placebo-controlled clinical studies. We designed the current study to address the potential for the combined probiotic and amylase treatment to ameliorate or prevent common gastrointestinal symptoms often reported with IBD or gut dysbiosis. We initiated these studies in a cohort of individuals that reported mild to moderate GI symptoms, while excluding any individuals that reported chronic health issues. Thus, the cohort of interest was individuals that demonstrated only mild-moderate GI symptoms, and underlying chronic health issues would not confound the observations.

Therefore, this study examined the effects of oral supplementation with this novel formulation on gastrointestinal symptoms, stress response, and overall well-being, as assessed by quality-of-life metrics. We hypothesised that consumption of the designated probiotic + amylase formulation would decrease symptoms associated with GI discomfort and stress, and therefore enhance overall quality of life.

2 Materials and methods

2.1 *Experimental design*

The current study was a randomised, parallel, placebo-controlled, double-blind investigation consisting of three study visits. This study was conducted according to the Declaration of Helsinki guidelines and all procedures involving human subjects were approved by Pearl IRB on 6/18/21 (#21-CAHS-102). The study was registered on clinicaltrials.gov ('A Probiotic Amylase Blend Reduces Gastrointestinal Symptoms and Positively Impacts Gut Microbiota Modulation in a Randomized Study', #NCT05614726). Written informed consent was obtained from all subjects prior to enrolment. This study was conducted at a contract research organisation (CRO) in Northeast Ohio. Participants were recruited from the Northeast Ohio area and within the CRO's subject database via advertisements, phone calls, and word of mouth. During the study screening visit, each participant's medical history and routine blood work [Complete Blood Count (CBC), Comprehensive Metabolic Panel (CMP), and Lipid Panel] were collected, and a 24-h dietary recall was performed. At the next two visits (baseline and post- supplementation), body weight, baseline measurements of vital signs, visual analog scales (VAS) for flatulence, bloating, abdominal discomfort, stool consistency/regularity & constipation, and questionnaires that assess the participants' GI health [Gastrointestinal Symptom Rating Scale (GSRS)], overall well-being (e.g. SF-36), physical activity (e.g. Framingham), and general anxiety (i.e. GAD-7) were performed. On the third visit (post-supplementation) routine blood work (CBC, CMP, and Lipid Panel) was again collected. After completion of visit 2 (baseline testing), participants were randomised in a parallel, double-blind, placebo-controlled fashion to ingest either; a daily dose of a 575 mg [30 billion cfu probiotic + amylase blend (PRO (see Table 1))] consisting of *B. breve* 19bx, *L. acidophilus* 16axg, *L. rhamnosus* 18fx, *S. boulardii* 16 mxg, and alpha amylase 500 SKB (Alpha-amylase-Dextrinizing Units) (provided by BIOHM Health, LLC, Cleveland, OH, USA), or a placebo (PLA) consisting of rice oligodextrin for 6 weeks. Compliance was monitored and assessed with a daily supplement log in which subjects checked off each day they took the investigational product. Investigators reviewed the supplement log with the subjects at visit 3, and subjects returned their supplement bottles to the lab during their last visit to confirm that the correct dose was taken. Supplement bottles, from the manufacturer, were plain, labelled with a study code and instructions to take 1 capsule with their

TABLE 1 The composition of probiotic blend (PRO) BIOHM 30B, consisting of 575 mg [30 billion cfu] of *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Ligilactobacillus rhamnosus*, and *Saccharomyces boulardii* plus 500 SKB of amylase.[1]

Species	Strain
Lactobacillus rhamnosus ~12.8 billion cfu	18fx
Lactobacillus acidophilus ~12.8 billion cfu	16axg
Saccharomyces boulardii ~3 billion cfu	16 mxg
Bifidobacterium breve ~1.3 billion cfu	19bx
Other ingredients	
Amylase	Alpha-amylase-Dextrinizing Units (500 SKB)
Rice maltodextrin	
Vegetable cellulose (capsule)	
Magnesium stearate	
Amount per serving	
575 mg (30 billion cfu)	

1 Other inactive ingredients (components of the capsule) are listed.

TABLE 2 Anthropometrics and vitals on study participants total and in the active probiotic (PRO) and placebo (PLA) groups

Variable	Total (n = 52)		PRO (n = 25)		PLA (n = 27)		*P*-value
	Mean	SD	Mean	SD	Mean	SD	
Age (years)	44.4	8.9	44	10.0	44.7	7.9	0.78
Men	14 (26.9%)		8 (32%)		6 (22.2%)		
Women	38 (73.1%)		17 (68%)		21 (77.8%)		
Height (cm)	170.3	11.5	171.4	10.3	169.2		0.51
Weight (kg)	82	18.4	85.5	18.6	78.7	18.0	0.19
Body mass index (kg/m^2)	28.1	4.6	28.9	4.6	27.4	4.5	0.25
Systolic blood pressure (mm Hg)	123.4	11.3	124.6	10.1	122.3	12.3	0.46
Diastolic blood pressure (mm Hg)	79.3	7.0	79.6	7.5	79	6.7	0.79
Resting heart rate (bpm)	72.5	10.0	79	6.7	70.2	9.4	0.08

largest meal of the day, and marked as 'A' or 'B' for each respective group as they arrived to the research lab. The researchers were unblinded only after statistical analyses were finalised. Compliance to the supplementation regimen was >90% for all participants.

Prior to all study visits, participants were asked to replicate their initial dietary intake for the 24 h prior to their visit, refrain from caffeine and exercise for 24 h, and fast for 10 h. In addition to the clinical endpoints, a concurrent study (targeted for an independent publication on microbiome changes) was performed to assess the gut microbiota following supplementation, therefore individuals were requested to refrain from caffeine due to the potential impact of caffeine on the composition of the gut microbiota (i.e. association with greater alpha diversity) (Barandouzi *et al.*, 2021). The first participant was enrolled on 7/7/21, and data collection concluded on 2/15/22. Comprehensive side effect profile/adverse event monitoring took place throughout the study.

2.2 *Study participants*

Fifty-two (14 men and 38 women) participants were randomised (allocation ratio 1:1 via research randomizer, https://www.randomizer.org/) into the study. Twenty-seven individuals were assigned to PLA (44.7 ± 7.9 yr, 169.2 ± 12.7 cm, 78.7 ± 18.0 kg, 27.4 ± 4.5 kg/m^2), and 25 were assigned to PRO (44.0 ± 10.0 yr, 171.4 ± 10.3 cm, 85.5 ± 18.6 kg, 28.9 ± 4.6 kg/m^2) by the researchers. Table 2 presents the baseline demographics of the study cohort, showing that the two groups were similar at screening. Review of health/medical history documents and a physical exam showed that all study participants were free of chronic health issues. Inclusion criteria were established so that all participants were required to be between 30-60 years old, have a GSRS score ≥ 12 (corresponding to mild to moderate GI symptoms),

have a minimum body mass of 120 pounds (54.5 kg), and body mass index (BMI) between 20.0-34.99 kg/m^2. Inclusion also required participants to be normotensive (<140/<90 mm Hg) with a normal resting heart rate (<90 beats/min). Female participants were not eligible if they were determined to be pregnant, nursing, or trying to become pregnant. Exclusion criteria included any history of: unstable or new-onset cardiovascular or cardiorespiratory disease; stroke, diabetes, or other endocrine disorder; use of any nutritional supplement known to alter the gut microbiome/microflora, probiotic supplements, use of prebiotic supplements in the previous 4 weeks and for the duration of the study; use of any antibiotics, antifungals, antivirals, or antiparasitic within 8 weeks of the start of the study or throughout the study; any changes in diet within 4 weeks of study start date or throughout study duration; if the subject was unwilling to abstain from gut altering supplements for the duration of the study; malignancy in the previous five years except for non-melanoma skin cancer (basal cell cancer or squamous cell cancer of the skin); prior gastrointestinal bypass surgery (i.e. Lapband); any known gastrointestinal or metabolic diseases that might impact nutrient absorption or metabolism [e.g. short bowel syndrome, diarrheal illnesses, history of colon resection, gastroparesis, Inborn-Errors-of-Metabolism (such as PKU)]; any chronic inflammatory condition or disease (e.g. rheumatoid arthritis, Crohn's disease, ulcerative colitis, Lupus, HIV/AIDS, etc.); known sensitivity to any ingredient in the test formulations as listed in the certificates of analysis. Participants were excluded if they were currently participating in another research study with an investigational product or had participated in another research study in the past 30 days, or if they had any other diseases or conditions that, in the opinion of the medical staff, could confound the primary endpoints or place the subject at increased risk of harm if they were to participate. Figure 1 displays a Consort flow diagram.

2.3 *Procedures*

Standing height was determined using a stadiometer with participants in socks or bare feet with heels together. Body mass was measured using a Seca 767™ Medical Scale (Hamburg, Germany). Resting heart rate and blood pressure were measured using an automated blood pressure cuff (Omron HEM-780; Osaka, Japan) after participants had remained seated for a minimum of 5 min.

Health-associated questionnaires including Framingham Global Risk Assessment, Short Form Health Survey questionnaire (SF-36), a modified GSRS, a Generalised Anxiety Disorder Assessment (GAD-7), and VAS, to rate flatulence, bloating, abdominal discomfort, stool consistency/regularity and constipation were completed by each participant before and after 6 weeks of supplementation. VAS questions were constructed using a 10 cm line anchored by 'Lowest Possible' and 'Highest Possible' except for stool consistency which was anchored by 'Liquid' and 'Hard' and stool regularity which was anchored by 'Irregular' and 'Regular'. The validity and reliability of VAS to assess fatigue and energy have been previously established (Lee *et al.*, 1991) and reported (Lopez *et al.*, 2020; Ziegenfuss *et al.*, 2018). The Framingham physical activity questionnaire was used to assess physical activity habits throughout the study and to ensure participants complied with their instructions to maintain their physical activity habits. The SF-36 Health Survey was used to assess quality-of-life (McHorney, 1993; McHorney *et al.*, 1994; Orrell *et al.*, 2017; Wayne *et al.*, 2015). The modified GSRS was used to assess symptoms of gastrointestinal health (Dimenas, 1995). The GSRS is a self-administered questionnaire designed to subjectively evaluate the intensity, frequency, duration, and impact on daily living for commonly reported GI symptoms (Svedlund *et al.*, 1988). The questionnaire includes 15 symptoms and uses a graded Likert scale to assess the severity of symptoms and includes items that are frequently reported by patients with GI diseases (Dimenäs *et al.*, 1993). A zero on any one item within the questionnaire corresponds to the absence of a symptom whereas a higher overall total score on the GSRS indicates greater severity/frequency of symptoms and/or the existence of more GI symptoms. The total score on the GSRS can range from 0-45 and has previously demonstrated good construct validity and interrater reliability (Dimenäs *et al.*, 1993; Svedlund *et al.*, 1988). The GAD-7 was used to assess general anxiety (Spitzer, 2006). The GAD-7 assessed the frequency of 7 items over the previous two weeks ('Feeling nervous, anxious, or on edge', 'Not being able to stop or control worrying', 'Worrying too much about different things', 'Trouble relaxing', 'Being so restless that it's hard to sit still', 'Becoming easily annoyed or irritable', 'Feeling afraid as if something awful might happen'). A higher score on the GAD-7 indicates a greater degree of anxiety.

2.4 *Statistical analyses*

Primary outcome measures included subjective changes in flatulence, bloating, and abdominal discomfort, and GSRS score. Secondary outcome measures included subjective changes in stool consistency, stool regularity,

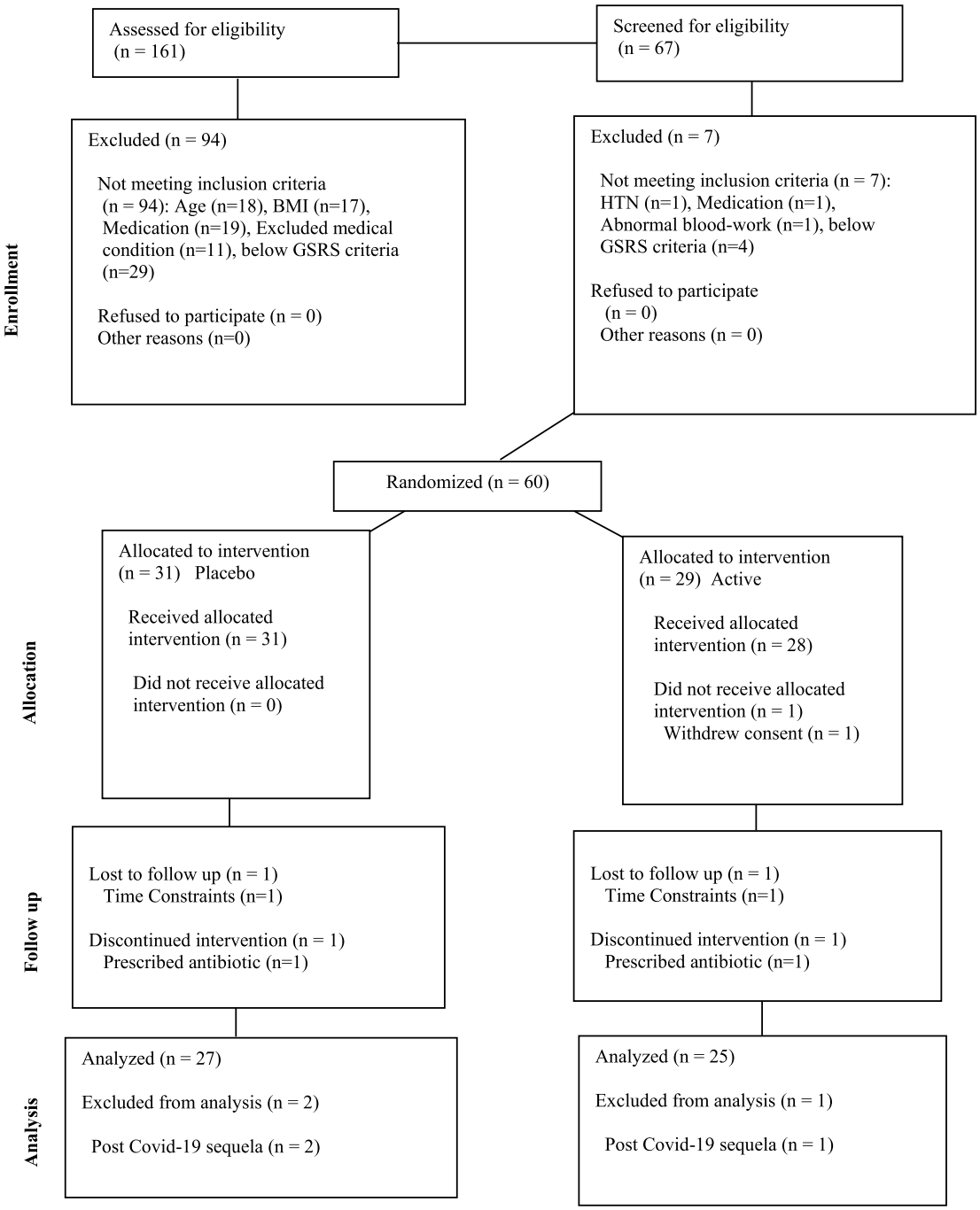

FIGURE 1 Consort diagram.

and constipation. Tertiary outcome measures included changes in anxiety/stress (GAD-7), changes in general health (SF-36), vital signs, bloodwork, side effect profile/adverse events monitoring and adverse events. Quaternary outcome measures included changes in physical activity (Framingham score) and body weight. *A priori* power analysis was conducted via G*Power (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower) for a mixed factorial ANOVA with repeated measures, a within-between interaction and a small effect size of 0.25 (as a conservative approach based on several of our primary outcome measures). With two groups and two time points a sample size of 34 was needed to achieve 80% power. Normality of each variable was assessed using the Shapiro-Wilk test. Two (PRO vs PLA) × 2 (week 0-Baseline, week 6-POST) mixed factorial ANOVAs were completed to assess group, time, and (group × time) interaction effects. When sphericity was violated, Greenhouse-Geisser corrected *P*-values were used for main effects/interactions. In the event of missing data, a mixed-effects model was utilised in

GraphPad Prism. Sidak post-hoc procedures were used to assess individual comparisons and adjust for multiple comparisons providing tighter bounds than Bonferroni between time points and/or groups. A significance level of ≤ 0.05 was accepted as statistical significance. Significant interactions were followed up with dependent t-tests to identify potential differences within groups or independent t-tests to identify potential differences between groups. For between-group changes over time, independent t tests (for data displaying normal distribution) and Mann-Whitney tests (for data not displaying normal distribution) were used to assess the change score (deltas) on all variables. Delta values were computed by the differences in time points relative to baseline (i.e. Post-Baseline). All data points less than –3SD or greater than +3SD were deemed outliers and removed before analyses. Effect sizes are expressed as Cohen's d with 95% confidence intervals and interpreted as ≥ 0.2 (small), ≥ 0.5 (moderate), and ≥ 0.8 (large). All analyses were completed with GraphPad Prism version 9.2.0 (GraphPad Software, San Diego, CA, USA).

3 Results

Fifty-two subjects who received the intervention, complied, and adhered to the intervention, and completed the clinical trial in-full were analysed (PRO: n = 25, PLA: n = 27).

3.1 *Visual analog scales and gastrointestinal symptom rating scale*

Table 3 presents VAS for (flatulence, bloating, abdominal discomfort, stool consistency & regularity, and constipation), and questionnaires that assess the participants' gastrointestinal health (GSRS) in comparison of baseline versus Post (Time) for treatment with PRO versus PLA. Significant time × group interactions and main effects of time were observed for bloating (P = 0.016 and P < 0.001, respectively), abdominal discomfort (P = 0.027 and P < 0.001, respectively), and GSRS score (P < 0.001 and P < 0.001, respectively). There were also significant main effects of time for flatulence (P = 0.002), stool regularity (P < 0.001), and constipation (P = 0.010).

Post-hoc testing for bloating indicated that Post was significantly lower than baseline in the PRO treatment group by an average of –2.8 cm (~49% reduction relative to baseline) (95% CI: –4.0 to –1.6) (P < 0.001, d = 1.14) and in PLA by an average of –1.3 cm (~25% reduction relative to baseline) (95% CI: –2.3 to –0.3) (P = 0.012, d = 0.58). In addition, the delta between groups for bloating indicated significant differences (P = 0.027, d = –0.63). On average PRO had a larger delta (i.e. reduction) in bloating than PLA (–2.8 ± 3.4 vs –1.3 ± 2.3 cm, respectively). Post-hoc testing for abdominal discomfort indicated that Post was significantly lower than baseline in the PRO group by an average of –3.2 cm (~59% reduction relative to baseline) (95% CI: –4.5 to –1.9) (P < 0.001, d = 1.14) and in the PLA group by an average of –1.5 cm (~32% reduction relative to baseline) (95% CI: –2.5 to –0.4) (P = 0.012, d = 0.65). The delta between groups for abdominal discomfort indicated significant differences (P = 0.021, d = –0.66). PRO had a larger average delta (i.e. reduction) in abdominal discomfort vs PLA (–3.2 ± 2.8 vs –1.5 ± 2.3 cm, respectively). Post-hoc testing for GSRS indicated that Post was significantly lower than baseline in PRO by an average of –9.1 au (~60% reduction relative to baseline) (95% CI: –11.0 to –7.2) (P < 0.001, d = 2.31) and in PLA by an average of –3.2 au (~25% reduction relative to baseline) (95% CI: –5.2 to –1.1) (P = 0.002, d = 0.72). The GSRS score for PLA was significantly greater than PRO at Post-treatment with a mean difference of 3.7 au (95% CI: 0.6 to 6.9) (P = 0.014, d = –0.89) indicating a more positive effect within the PRO-treated cohort. In addition, the delta between groups for GSRS indicated significant differences (P < 0.001, d = –1.46). PRO had a larger delta (i.e. reduction) in their GSRS score than PLA (–9.1 ± 3.9 au vs –3.0 ± 4.5 au, respectively). There were no significant differences noted for stool consistency (time: P = 0.45; group: P = 0.58; time × group: P = 0.48).

Individual scores (box and whisker plots) for flatulence, constipation, bloating, abdominal discomfort, GSRS total score, and changes in GSRS score before and after the intervention are shown in Figure 2.

3.2 *Quality of life measurements* (*GAD-7, SF-36, Framingham*)

Data from the generalised anxiety disorder (GAD-7) questionnaire is shown in Table 4. Within the GAD-7 questionnaire there was a significant time x group interaction (P = 0.048) for the question 'Becoming easily annoyed or irritable'. Post hoc testing indicated that post was significantly less than baseline for the PRO group (P = 0.025). There was a significant main effect of time (P = 0.024) on the GAD-7 total score. The delta between groups for total score on the GAD-7 indicated significant differences (P = 0.041). On average PRO had a larger delta (i.e. reduction) relative to baseline in their GAD-7 total score than PLA [–3.1 ± 4.8 au (~44%) vs –0.4 ± 2.3 au (~10%), respectively]. There was a significant main

TABLE 3 Visual analog scales (VAS) for (flatulence, bloating, abdominal discomfort, stool consistency/regularity & constipation), and questionnaires that assess the participants' gastrointestinal health [Gastrointestinal Symptom Rating Scale (GSRS)] are summarised before (Pre) and after (Post) active probiotic (PRO) and placebo (PLA) consumption.[1]

Variable	Time	n	PRO		n	PLA		Between group differences
			Mean	SD		Mean	SD	
Flatulence (*cm*)	Pre	25	4.8	2.1	27	4.2	2.2	
	Post	25	3.1	1.9	27	3.5	2.5	
Main effect for time			0.002					
Main effect for group			0.790					
Time × group			0.081					
Bloating (*cm*)	Pre	25	5.7	1.6	27	5.1	2.1	$P = 0.505$
	Post	25	2.9	2.0	27	3.8	2.0	$P = 0.078$
Within group differences			$P < 0.001$ (d = –1.14, 95%CI: –2.44 - –0.653)			$P = 0.012$ (d = –0.58, 95%CI: –1.407 - 0.139)		
Main effect for time			<0.001					
Main effect for group			0.710					
Time × group			0.016					
Abdominal Discomfort (*cm*)	Pre	25	5.4	2.1	27	4.7	1.7	$P = 0.372$
	Post	25	2.2	1.7	27	3.2	2.0	$P = 0.107$
Within group differences			$P < 0.001$ (d = –1.14, 95%CI: –2.59 - –0.76)			$P = 0.012$ (d = –0.65, 95%CI: –1.59 - –0.02)		
Main effect for time			<0.001					
Main effect for group			0.720					
Time × group			0.027					
GSRS score (*au*)	Pre	25	15.1	4.9	26	13	3.2	$P = 0.254$
	Post	25	6.0	3.5	27	9.8	4.8	$P = 0.014$ (d = 0.89, 95%CI: 0.33-1.47)
Within group differences			$P < 0.001$ (d = –2.31, 95%CI: –3.12 - –1.16)			$P = 0.002$ (d = –0.72, 95%CI: –1.34 - –0.22)		
Main effect for time			<0.001					
Main effect for group			0.421					
Time × group			<0.001					
Stool consistency (*cm*)	Pre	25	4.5	1.9	27	4.5	1.9	
	Post	25	4.9	1.2	27	4.5	1.5	
Main effect for time			0.451					
Main effect for group			0.583					
Time × group			0.479					
Stool regularity (*cm*)	Pre	25	4.9	2.8	27	4.8	2.1	
	Post	25	6.9	2.4	27	6.0	2.3	
Main effect for time			<0.001					
Main effect for group			0.352					
Time × group			0.319					
Constipation (*cm*)	Pre	25	3.6	2.8	27	3.3	2.4	
	Post	25	2.2	2.2	27	2.7	2.1	
Main effect for time			0.010					
Main effect for group			0.841					
Time × group			0.315					

1 SD = standard deviation. Between and within groups comparisons were only conducted when warranted.

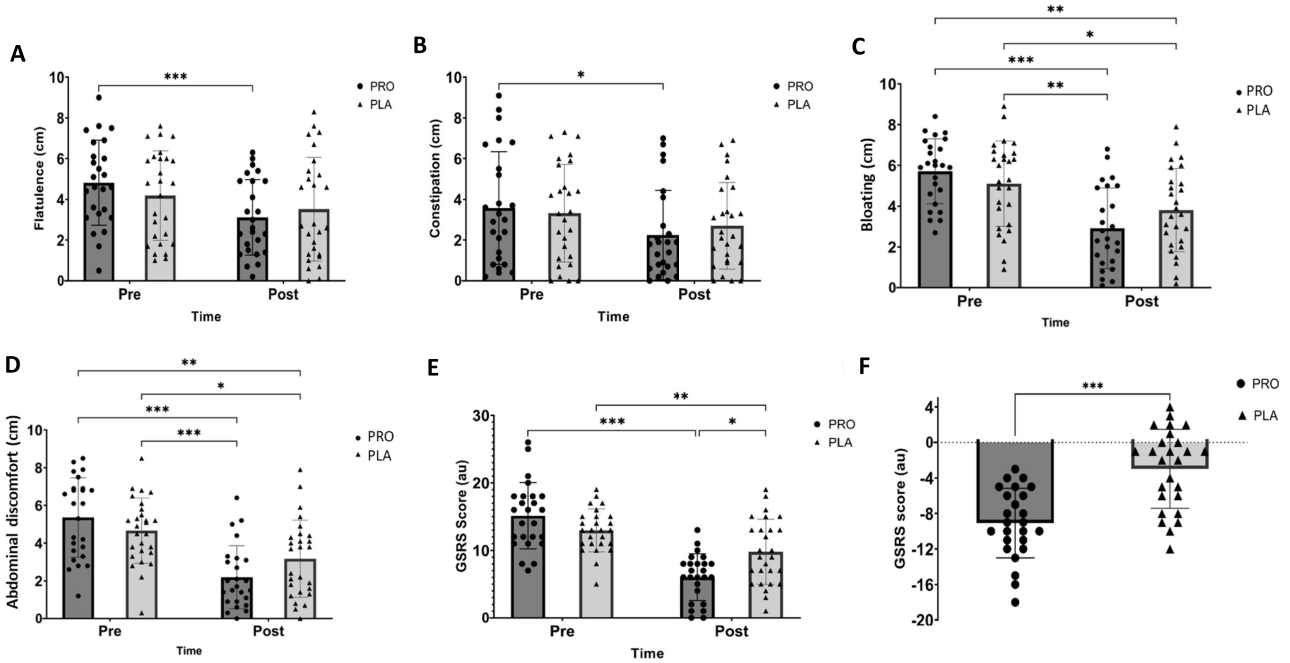

FIGURE 2 Box and whisker plots for flatulence (A), constipation (B), bloating (C), abdominal discomfort (D), GSRS scores (E), and GSRS change (delta) scores (F). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

effect of time ($P = 0.016$) and group ($P = 0.006$) on 'Feeling nervous, anxious, or on edge'. There was a significant main effect for group ($P = 0.034$) for 'Not being able to stop or control worrying'. There was a significant main effect of time ($P = 0.049$) for 'Trouble relaxing'. There were no significant differences ($P > 0.05$) in 'Worrying too much about different things', 'Being so restless that it's hard to sit still', or 'Feeling afraid as if something awful might happen'.

Data from the quality-of-life health survey questionnaire (SF-36) are summarised in Table 5. Specifically for the items within the SF-36, there was a significant interaction ($P = 0.016$) for the question 'Didn't do work/activities carefully due to emotional problems', however there were no post-hoc differences. The delta between groups for 'Didn't do work/activities carefully due to emotional problems' indicated a significant difference ($P = 0.019$) with PRO showing an improvement (by ~20%) while PLA had a poorer score (by ~4%) from baseline to post (16.7 ± 38.1 au vs –3.9 ± 19.6 au, respectively). There was a significant interaction ($P = 0.013$) for 'Health interfered with normal activities'. Post hoc testing indicated that post was greater than baseline within PRO ($P = 0.020$) showing an improvement after the intervention. In addition, the change score/delta between groups for 'Health interfered with normal social activities' indicated a significant difference ($P = 0.012$). On average PRO had an improvement while PLA had a worse score from baseline to post (13.5 ± 24.4 vs –1.9 ± 17.2 au, respectively). There was a significant interaction ($P = 0.048$) for Social Functioning, however there were no post hoc differences. There was a significant interaction ($P = 0.047$) for Pain. Post hoc testing indicated that post was significantly greater than baseline for PRO ($P = 0.019$) showing an improvement in Pain after the intervention. There was a significant main effect of time ($P = 0.032$) for the composite score of physical functioning. There were no significant main effect differences ($P > 0.05$) or interaction for several questions including; 'Cut down time on work/activities due to emotional problems', 'Accomplish less than would like due to emotional problems', 'Have you been nervous', 'Calm and peaceful', 'Role limitations due to physical health', 'Role limitations due to emotional health', 'Energy/Fatigue', 'Emotional well-being', 'General health', or 'Health change'.

No differences were noted in Framingham score at baseline or post intervention (PRO: 35.6 ± 5.2 au to 35.7 ± 5.2 au vs PLA: 35.6 ± 6.5 au to 36.6 ± 7.0 au, respectively).

3.3 *Adverse events*

Participants in both groups tolerated PRO and PLA well with ~10% of participants in both groups reporting possible grade 1 adverse events to either test article including: mild abdominal pain, vivid dreams, a maculopapular rash, vertigo, and headache (Table 6).

TABLE 4 General anxiety disorder (GAD-7) assessments before (Pre) and after (Post) active probiotic (PRO) and placebo (PLA) consumption.[1]

Variable	Time	n	PRO		n	PLA		Between group differences
			Mean	SD		Mean	SD	
Nervous/anxious (*au*)	Pre	15	1.4	1	15	0.6	0.6	
	Post	15	0.7	0.6	14	0.3	0.5	
Main effect for time			0.016					
Main effect for group			0.006					
Time × group			0.079					
Control worrying (*au*)	Pre	15	0.8	1	15	0.1	0.4	
	Post	15	0.4	0.5	15	0.3	0.6	
Main effect for time			0.340					
Main effect for group			0.034					
Time × group			0.068					
Worrying too much (*au*)	Pre	15	1.0	0.9	15	0.4	0.5	
	Post	15	0.7	0.7	15	0.6	0.7	
Main effect for time			0.795					
Main effect for group			0.143					
Time × group			0.085					
Trouble relaxing (*au*)	Pre	15	1.1	1.1	15	0.8	0.9	
	Post	15	0.7	0.6	15	0.6	0.7	
Main effect for time			0.049					
Main effect for group			0.405					
Time × group			0.404					
Restless (*au*)	Pre	15	0.8	1	15	0.7	1.2	
	Post	15	0.5	0.6	15	0.5	0.6	
Main effect for time			0.070					
Main effect for group			0.909					
Time × group			0.818					
Irritable (*au*)	Pre	15	1.4	1.1	15	1.1	1	$P = 0.582$
	Post	15	0.7	0.8	15	1	0.9	$P = 0.700$
Within group differences			$P = 0.025$ (d = –0.74, 95%CI: –1.773 - 0.317)			$P = 0.957$		
Main effect for time			0.077					
Main effect for group			0.912					
Time × group			0.048					
Feeling of doom (*au*)	Pre	15	0.5	0.8	14	0.1	0.3	
	Post	15	0.3	0.6	15	0.1	0.4	
Main effect for time			0.367					
Main effect for group			0.084					
Time × group			0.165					
Total score (*au*)	Pre	15	7.1	5.8	15	3.9	3.8	
	Post	15	4.0	3.4	15	3.5	3.7	
Main effect for time			0.024					
Main effect for group			0.157					
Time × group			0.073					

1 SD = standard deviation. Between and within groups comparisons were only conducted when warranted.

TABLE 5 36-Item Short Form Health Survey (SF-36) assessments before (Pre) and after (Post) active probiotic (PRO) and placebo (PLA) consumption to address health-related quality of life responses.[1]

Variable	Time	n	PRO		n	PLA		Between group differences
			Mean	SD		Mean	SD	
Cut down time on work/activities d/t emotional problems (*au*)	Pre	24	83.3	38.1	26	96.2	19.6	
	Post	25	96.0	20.0	27	92.6	26.7	
Main effect for time			0.354					
Main effect for group			0.412					
Time × group			0.103					
Accomplish less than would like d/t emotional problems (*au*)	Pre	24	70.8	46.4	26	84.6	36.8	
	Post	25	84.0	37.4	27	85.2	36.2	
Main effect for time			0.359					
Main effect for group			0.332					
Time × group			0.375					
Didn't do work/activities carefully d/t emotional problems (*au*)	Pre	24	83.3	38.1	26	100	0.0	$P = 0.084$
	Post	25	100	0.0	27	96.3	19.2	$P = 0.584$
Within group differences			$P = 0.080$			$P = 0.565$		
Main effect for time			0.122					
Main effect for group			0.122					
Time × group			0.016					
Health interfered w normal social activities (*au*)	Pre	24	79.2	28.2	26	93.3	13.3	$P = 0.092$
	Post	25	93.0	11.5	27	90.7	15.7	$P = 0.768$
Within group differences			$P = 0.020$ (d = 0.57, 95%CI: 2.0 - 25.6)			$P = 0.705$		
Main effect for time			0.063					
Main effect for group			0.174					
Time × group			0.013					
Have you been nervous (*au*)	Pre	24	75.0	30.8	26	80.0	18.8	
	Post	25	76.0	22.4	27	81.5	16.6	
Main effect for time			0.740					
Main effect for group			0.385					
Time × group			0.832					
Calm and peaceful (*au*)	Pre	24	63.3	27.5	26	66.9	26.5	
	Post	25	64.8	21.8	27	68.1	23.7	
Main effect for time			0.667					
Main effect for group			0.583					
Time × group			0.985					
Physical functioning (%)	Pre	24	89.0	19.5	26	87.1	19.6	
	Post	25	92.8	10.8	27	93.3	9.6	
Main effect for time			0.032					
Main effect for group			0.860					
Time × group			0.595					
Role limitations d/t physical health (%)	Pre		77.1	38.2		87.5	26.7	
	Post		89.0	24.0		84.3	26.1	
Main effect for time			0.377					
Main effect for group			0.670					
Time × group			0.150					

TABLE 5 (Continued)

Variable	Time	n	PRO		n	PLA		Between group differences
			Mean	SD		Mean	SD	
Role limitations d/t emotional	Pre	24	79.2	37.8	26	93.6	13.4	
health (%)	Post	25	93.3	16.7	27	91.4	23.7	
Main effect for time			0.176					
Main effect for group			0.207					
Time × group			0.064					
Energy/Fatigue (%)	Pre	24	55.6	23.1	26	63.3	20.7	
	Post	25	66.0	13.7	27	62.8	19.6	
Main effect for time			0.100					
Main effect for group			0.641					
Time × group			0.075					
Emotional well-being (%)	Pre	24	76.5	20.0	26	80	15.1	
	Post	25	81.1	14.1	27	83.1	12.6	
Main effect for time			0.700					
Main effect for group			0.500					
Time × group			0.755					
Social functioning (%)	Pre	24	81.8	25.0	26	91.8	15.8	$P = 0.286$
	Post	25	91.5	13.8	27	90.7	14.9	$P = 0.979$
Within group differences			$P = 0.052$			$P = 0.910$		
Main effect for time			0.085					
Main effect for group			0.318					
Time × group			0.048					
Pain (%)	Pre	24	74.7	26.6	26	84.0	21.6	$P = 0.385$
	Post	25	85.5	17.6	27	82.3	18.3	$P = 0.739$
Within group differences			$P = 0.019$ (d = 0.58, 95%CI: –0.09 - 1.05)			$P = 0.896$		
Main effect for time			0.104					
Main effect for group			0.591					
Time × group			0.047					
General health (%)	Pre	24	74.8	14.3	26	78.1	18.0	
	Post	25	79.8	16.6	27	81.3	19.1	
Main effect for time			0.053					
Main effect for group			0.630					
Time × group			0.765					
Health change (%)	Pre	24	50.0	25.5	26	58.7	21.1	$P = 0.423$
	Post	25	61.0	24.0	27	56.5	22.6	$P = 0.736$
Main effect for time			$P = 0.101$			$P = 0.887$		
Main effect for group			0.263					
Time × group			0.697					
Main effect for time			0.066					

1 SD = standard deviation. Between and within groups comparisons were only conducted when warranted.

TABLE 6 Adverse events (AE) summary

	Allocated subject (n = 60)	
	PRO (n = 29)	PLA (n = 31)
Severity		
Mild	4	5
Moderate		
Severe		
Relationship to study treatment		
Not related	4	5
Possible		
Definite		
Relationship to test article		
Not related		
Possible	4	5
Definite		
Body system and AEs		
Ear & labyrinth		
Vertigo		1
Gastrointestinal		
Abdominal pain	1	1
Nervous system		
Headache		1
Psychiatric disorder		
Sleep disturbance; vivid dreams	2	2
Skin & subcutaneous tissue		
Rash maculo-papular	1	
Total number of AE experienced during study	4	5
Total number of subjects experiencing AE events: n (%)	3/29 (10.3%)	3/30 (10%)

4 Discussion

This study examined the effects of a novel probiotic + amylase formulation on quantified digestive symptoms. Previous preliminary data testing the probiotic blend on *in vitro* biofilm formation, and preclinical studies indicated that the probiotic amylase formulation should reduce biofilm formation. In particular, we hypothesized that biofilms formed from bacterial interactions with fungal organisms (as observed in Crohn's Disease) would improve, based on our preliminary studies (Hager *et al.*, 2019). Thus, our rationale for the current study was to investigate the effect of the probiotic blend in a cohort of subjects that previously reported levels of mild-moderate GI symptoms. The current study showed that the probiotic and enzyme blend reduced the severity and frequency of overall GI symptoms and positively modulated specific clinical features (i.e. flatulence, bloating, stool regularity, constipation, and abdominal discomfort) to a greater degree than placebo. The changes in the GSRS, bloating and abdominal discomfort appear clinically relevant based on minimal important differences (Khanna *et al.*, 2017). In addition, PRO improved irritability, mitigated health interferences with normal social activities, reduced subjective feelings of pain, and improved the change in emotional problems that interfered with working or carrying out activities carefully. Importantly the probiotic + amylase formulation was very well tolerated in individuals in comparison to PLA.

Other studies investigating probiotics have demonstrated similar modulation of GI-associated endpoints. For example, Diop *et al.* (2008) reported reductions in abdominal pain and nausea/vomiting and decreased levels of flatulence and gas production during probiotic (*L. acidophilus* Rosell-52 and *Bifidobacterium longum* Rosell-175) supplementation compared to placebo. While the current investigation observed improvements in bloating, Verdenelli *et al.* (2011), reported that probiotic enhanced foods failed to alleviate constipation and flatulence, but enhanced stool regularity after 12 weeks. In support of the current results, Bonfrate *et*

al. (2020) reported that a double strain probiotic (*B. longum* BB536 and *L. rhamnosus* HN001) containing vitamin B6 was able to reduce abdominal pain (~49%), bloating (~36%), and GI-related interference with quality of life compared to placebo in IBS patients. Additionally, Kajander *et al.* (2005), showed a significantly greater reduction in GI symptoms (76% vs 43%) in the second half of a 6-month controlled trial with a 8-9 billion cfus blend of probiotics (*L. rhamnosus* LC705, *B. breve* Bb99, *Propionibacterium freudenreuchii* ssp. Shermanii JS) versus a placebo in IBS patients. The mixture of strains + amylase in the current probiotic accounted for greater changes in GI symptoms from baseline to Post-treatment time points compared to PLA, demonstrating that, similar to the work of Bonfrate and colleagues, combinations of *Bifidobacterium* and *Lactobacillus* can be beneficial to gut health (Bonfrate *et al.*, 2020).

The gut-microbiota and the brain communicate through a variety of different pathways that offer crosstalk to multiple organs and influences the behaviour of the host (Cryan *et al.*, 2019). Probiotic strains may modulate the microbiome structure including the hypothalamic-pituitary-adrenal (HPA) axis (leading to potential benefits supporting mental health) and the amygdala influencing social behaviour (Cryan *et al.*, 2019). Species such as the ones found in the tested probiotic (*L. acidophilus* and *L. rhamnosus*) have been shown to lower depression and anxiety (Cryan *et al.*, 2019). In the current investigation PRO was beneficial in mitigating irritability and overall health and emotional interference compared to PLA. The underlying mechanism behind probiotics enhancing mood and reducing anxiety is thought to be due to improving the integrity of the gastrointestinal lining, reducing leaky gut, and decreasing global inflammation which can improve neurotransmitter activity through the gut-brain axis and/or improve production of free tryptophan and subsequently improve serotonin secretion (Wallace and Milev, 2021). This may mirror preclinical studies that demonstrated a reduction in intestinal inflammation exhibited in the CD-like ileitis model SAMP1/YitFc mice following probiotic feeding (Di Martino *et al.*, 2023).

Although speculative, these neurological effects may be why the current investigation demonstrated improvements in items within the stress/anxiety questionnaire (GAD-7) and the health questionnaire (SF-36). Wallace and Milev (2017) reported a reduction in GAD-7 scores after a 4-week daily consumption of 2 probiotic strains (3 billion cfus of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in men and women with moderate depression (Wallace and Milev, 2021). On the other hand, a randomised controlled trial using *L. helveticus* R0052 and *B. longum* R0175 at 3 billion cfu for 8 weeks did not appear to improve depressive symptoms versus a placebo in mildly depressed subjects (Romijn *et al.*, 2017). Altogether, these results show that there may be strain specific effects for mental health that have not been fully elucidated and thus, more specific studies focused on anxiety and probiotic use should be undertaken.

In conjunction with depression and anxiety, probiotics may also have indirect implications on overall health and associated feelings of well-being. Given the link between the gut and the brain, particularly within the HPA axis (Cryan *et al.*, 2019), certain probiotic strains (i.e. *B. longum* NCC3001) may have an impact on brain activity which has been shown to coincide with less depression and improve quality-of-life in a 6-week intervention versus a placebo (Pinto-Sanchez *et al.*, 2017). In the current investigation PRO demonstrated potential improvements in a variety of health outcomes (i.e. mitigated irritability, pain, and health interference), whereas PLA did not. Several studies targeting a variety of clinical populations have also seen increased quality of life (e.g. vitality, social functioning, mental health, pain, physical functioning) following probiotic consumption (Jalali *et al.*, 2019; Mazzawi *et al.*, 2013; Ohigashi *et al.*, 2011; Pellino *et al.*, 2013; Preston *et al.*, 2018). Although probiotics alone did not achieve beneficial results, Kim *et al.* (2006), reported significant improvements in physical functioning and 'role-physical' domains within the SF-36 after 12 weeks with probiotics + fermented plant nutrients. This may suggest why the probiotic + amylase formulation is important, as a key interaction is the mechanism(s) of amylase, combined with the probiotic, as demonstrated in the preclinical model (Di Martino *et al.*, 2023), as well as *in vitro* work demonstrating an effect on reducing biofilm formation (Hager *et al.*, 2019), although more definitive research needs to be executed on the impact probiotics play on general health outcomes.

This study is not without limitations. The responses to the survey instruments are self-reported interpretation with no clinical verification regarding the response. The makeup of microbial constituents of the gut may be affected by diet (Singh *et al.*, 2017), which was not controlled in this study other than the 24 h diet repeat prior to each study visit. Although the study design was a randomised, placebo-controlled, double-blind parallel study, the potential for placebo effects in any clinical study is well documented (Gupta and Verma, 2013).

Lastly, future studies on this novel blend should consider including a comparator arm of amylase alone.

Our data demonstrate that consumption of the probiotic-amylase (PRO) formulation was well-tolerated with very mild side-effects. Ingestion reduced the severity and frequency of overall GI symptoms and positively modulated clinical features associated with gut discomfort (e.g. bloating, and abdominal discomfort) to a greater degree than PLA. In addition to the improved clinical features modulated by the probiotic + amylase formulation, there was also improvement in behavioural (irritability) symptoms and overall health (i.e. improved pain and lessened emotional and overall health interference) as measured by the validated SF-36 instrument.

Clinical implications regarding probiotic consumption and alteration of GI symptoms provide face validity that modulating microbiota gut constituents via probiotic supplementation can result in symptom improvement. Modulation of the gut microbiota suggests that it may be possible one day to tailor probiotics blends that would augment host microbial composition and may show efficacy as primary or adjuvant therapies for the treatment of diseases such as IBS, CD, or obesity (Fysekidis *et al.*, 2012; Pascal *et al.*, 2017). Indeed, the ability to modulate the microbiota through the rational design of probiotics would be useful in any number of clinical outcomes influenced by the gut microbiota.

Thus, we demonstrated herein that subjects consuming the complete probiotic + amylase formulation who completed the entire study were characterised by a significant ($P \leq 0.05$) reduction in GSRS score and other GI symptoms to a greater degree than individuals who consume the placebo formulation. In addition, oral supplementation of the probiotic + amylase blend was very well tolerated, suggesting incorporation of a probiotic formulation into the diet of individuals with gut-associated dysbiosis may improve overall outcomes.

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Author contributions

M.B.L. contributed to data collection, data analysis, interpretation of the findings, and manuscript preparation, B.R. contributed to data collection, H.L.L. contributed to the study design, interpretation of the findings, and helped review the manuscript, T.N.Z. contributed to the study design, interpretation of the findings, and helped prepare the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest

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Research Article
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Probiotic supplementation of pea-derived protein alters the gut microbiome balance in favor of increased protein degradation, reflected in increased levels of essential amino acid in human plasma

Abstract

Aim: The primary aim of this clinical study was to determine if dietary supplementation with the probiotic, BIOHM FX (BFX), altered the gut microbiome balance following ingestion of 15g pea protein (PP) and enhanced the absorption of non-animal proteins determined via quantification of essential amino acids (EAAs). Thus, we compared the effects of pea protein alone vs. pea protein + BFX on microbiome changes and plasma levels of EAAs.

Methods: A placebo-controlled crossover clinical study in active men (n=40) was performed during which quantification of abundance levels of gut bacterial and fungal (bacteriome and mycobiome) organisms were assessed. In addition, plasma EAAs were measured pre- and post- ingestion of the pea protein +/-BFX for 180 min. Stool samples were analyzed for changes in microbiome composition from baseline and compared for PP versus PP+BFX. Self-reported changes in gastrointestinal (e.g., bloating, flatulence) and quality of life (e.g., fatigue, mood, and energy) indices were also measured.

Results: Participants ingesting PP + BFX exhibited a distinct microbiome profile compared to baseline and ingestion of PP. Differences in plasma EAAs showed a trend for an interaction (P=0.097) and post hoc testing at 120 min showed a significant difference (P=0.047) between PP and PP+BFX. Microbiome analysis of stool samples showed that the pathogens *Escherichia coli, Prevotella copri, Shigella flexneri,* and *Brevundimonas diminuta* were lower in PP+BFX compared to PP. The abundance of *Candida albicans* was lower and the level of *Saccharomyces cerevisiae* was higher in PP+BFX compared to PP. Interestingly, the abundance of *Pseudomonas* species, cyanobacteria phyla and the fungal species *Galactomyces geotrichum* were elevated when the combination of PP+BFX were consumed by study subjects (P<0.05). Other than main effects of time there were no significant differences between treatments in self-reported gastrointestinal (GI) and well-being markers.

Conclusion: Our results indicate that the addition of BFX to PP alters the gut microbiome composition, aiding in the absorption of dietary non-animal proteins and increasing essential amino acid appearance in plasma.

Keywords: probiotic, gut health, amino acid absorption, microbiome

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Mauricio Retuerto,[1] Michael B La-Monica,[2] Tim N Ziegenfuss,[2] Betsy Raub,[2] Thomas S McCormick,[1] Mahmoud A Ghannoum[1,2]

[1]Department of Dermatology, Case Western Reserve University, USA

[2]Department of Dermatology, The Center for Applied Health Sciences, USA

Correspondence: Mahmoud Ghannoum, Integrated Microbiome, A Cancer Resource Core, Case Western Reserve University and University Hospitals Cleveland Medical Center 11100 Euclid Avenue, Wearn 311, Cleveland, Ohio 44106-5028, Tel 216-844-8580, Email mag3@case.edu

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Introduction

Increased interest in potential sources of non-animal proteins, whether due to environmental concerns, worldwide overpopulation, or increased awareness of food allergens, has resulted in greater attention being given to a variety of plant proteins. Plant proteins offer a sustainable, lower-cost alternative to animal proteins. Although soybeans are a major food source produced in North America, field peas (*Pisum sativum*) account for nearly 26% of the worldwide production of edible seeds.[1] Two commercial protein supplements (whey and pea protein powders) are popular with athletes, vegetarians, and vegans. Both whey and pea protein powders are considered complete proteins, as they contain all nine EAAs, though pea protein has very small amounts of methionine.[2] Importantly, while many types of whey products contain lactose and/or gluten allergens, pea proteins are free from the most common food allergens. Thus, these supplements could serve as an invaluable dietary source of easily renewable protein. Combining these protein supplements with beneficial probiotics is an upcoming area of interest.[3]

The role of the gut microbiome regarding mechanism(s) of nutrient absorption has been extensively studied in recent years. Several studies have shown that various probiotics are capable of increasing essential amino acid absorption. [4 5 6] Production of enzymes by beneficial gut microbes have been postulated to facilitate absorption of micronutrients such as vitamins, minerals, and amino acids through the mucosal layer of the small intestine, made up of enterocytes and mucin secreted by goblet cells.[7–9] However, disruption of this mucin layer, as occurs during dysbiosis of the gut microbiome, may enable invasion by pathogenic organisms, resulting in formation of thick biofilms which prevent nutrient absorption.[10]

Previously, our analysis of the gut microbiome of patients with Crohn's disease (CD) showed elevated levels of the pathogens *Candida tropicalis, Escherichia coli,* and *Serratia marcescens* compared to their healthy family members.[11] We subsequently demonstrated that the combination of these three organisms resulted in the production of robust biofilms *in vitro* and in a murine *in vivo* model.[10] Further studies using an *in vitro* biofilm model showed that the novel probiotic formulation BIOHM FX (BFX) (BIOHM Health, LLC, Cleveland,

OH), consisting of *Saccharomyces boulardii 16mxg*, *Lactobacillus acidophilus 16axg*, *L. rhamnosus 18fx*, and *Bifidobacterium breve 19bx* strains in combination with the enzyme amylase, significantly reduced the thickness of polymicrobial biofilms.[12]

Considering the *in vitro* activity of BFX on biofilm inhibition and thickness reduction, we hypothesized that BFX should enhance the absorption of micronutrients in the small intestine. Thus, the passage of vitamin C and casein, as representative of vitamins and proteins, respectively, through an epithelial monolayer was tested with and without the addition of BFX supernatant. Results showed that BFX significantly increased the permeability of both vitamin C and casein (*P* values <0.05 and 0.0001, respectively) through the Caco-2 cell monolayer overlaid with polymicrobial bacterial-fungal biofilms, elicited by *C. tropicalis, E. coli,* and *S. marcescens* exposure.[13]

Given the results demonstrating that BFX could reduce biofilm formation, leading to increased nutrient absorption *in vitro*, we wondered whether or not BFX would also enhance pea protein absorption in subjects consuming pea-derived protein supplements. A human clinical study was designed to compare the effects of a pea protein absorption in the presence or absence of BFX. Although several commercial supplements are considered complete proteins (e.g., whey and pea powders) that contain all nine essential amino acids, pea protein was chosen as the supplement because of its non-allergenic properties, as opposed to whey powder, which may contain lactose and/or gluten allergens.[2]

The aims of this clinical study were to: a) compare the effects of pea protein alone vs. pea protein + BFX on the microbiome composition of participating subjects using next generation targeted sequencing of bacteriome and mycobiome microbiota, b) compare the effects of pea protein alone vs. pea protein + BFX on plasma essential amino acid (EAA) levels, c) compare the effects of pea protein alone vs. pea protein + BFX on self-reported visual analog scales (VAS) for gastrointestinal bloating, flatulence, fatigue, mood, and energy, and d) assess safety and tolerability as determined by vital signs and adverse events.

Our hypothesis was that PP+BFX would alter the gut microbiome profile differentially from consumption of PP, and that the addition of BFX would lead to increased appearance of plasma EAA while also improving gastrointestinal (GI) tolerance.

Materials and methods

Clinical study design

The current study was registered on ClinicalTrials.gov (ID number NCT05657314). This double-blind, randomized, placebo-controlled, crossover clinical trial was designed to solely recruit recreationally active men (*n*=40). A consort diagram of the study design and enrollment features is shown in Figure 1. The subjects were recruited at a single investigational center in Ohio (The Center for Applied Health Sciences). Following informed consent, eligible subjects were randomly assigned in a 1:1 ratio to one of two study arms: 15g of Yantai Shuangta 85% pea protein with placebo (cellulose) dissolved in 12 fl. oz. of water (PP alone) or 15g of Yantai Shuangta 85% pea protein with 1 billion colony forming unit of BFX (PP+BFX) dissolved in 12 fl. oz. of water. Enrolled subjects ingested the assigned daily supplement for 4weeks, followed by a 1-week washout period (absent of any supplementation). Subjects were then given the alternate supplement for an additional 4 weeks. This crossover approach enabled each subject to serve as his own control, thus reducing inter-subject variability and enhancing statistical power.

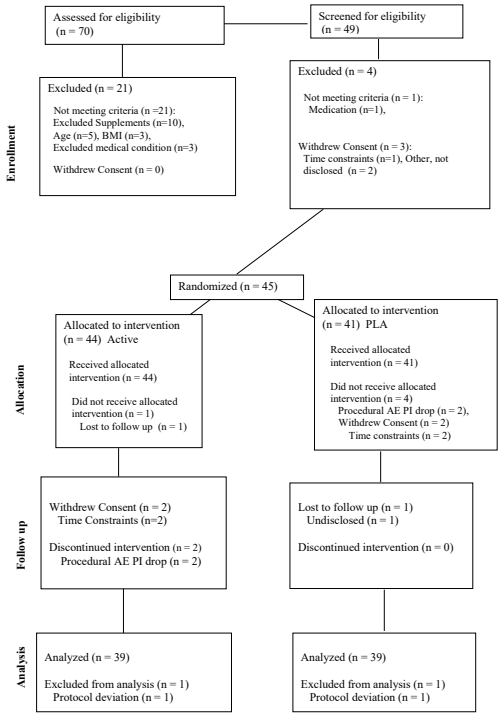

Figure 1 CONSORT Diagram.

Participants

40 healthy men completed all study visits, but 39 subjects were analyzed (See Table 1 for subject characteristics). All participants were in good health as determined by physical examination and medical history, recreationally active men (exercise ≥2-3d/wk for at least 1 year), between the ages of 18 and 55 years, and had a body mass index (BMI) of 18.5-29.9kg•m^{-2}. Prior to participation, all participants indicated their willingness to comply with all aspects of the experimental and supplement protocol. Participants were excluded if they: (a) had a history of diabetes or pre-diabetes; (b) had a history of malignancy in the previous 5 years except for non-melanoma skin cancer (basal cell cancer or squamous cell cancer of the skin); (c) had prior gastrointestinal bypass surgery; (d) known gastrointestinal or metabolic diseases that might impact nutrient absorption or metabolism (e.g. short bowel syndrome, diarrheal illnesses, history of colon resection, gastro paresis, Inborn-Errors-of-Metabolism); (e) had any chronic inflammatory condition or disease; (f) had a known allergy to any of the ingredients in the supplement or the placebo; (g) had currently been participating in another research study with an investigational product or have been in another research study in the past 30 days; (h) had excessive caffeine intake (>600 mg) per day; (i) used corticosteroids or testosterone replacement therapy (ingestion, injection, or transdermal); (j) had ever been diagnosed with liver, renal, cardiovascular, or other metabolic disease; (k) consumed more than 2 standard alcoholic drinks per day (or more than 10 drinks per week) or had a history of drug abuse/dependence; (l) use of any prescription medications (particularly antibiotics and/or anti-inflammatories), or probiotics within the past 2 months; (m) current smokers; (n) had any other diseases or conditions that, in the opinion of the medical staff, could confound the primary endpoint or place the participant at increased risk of harm if they were to participate; or (o) did not demonstrate a verbal understanding of the informed consent document.

Citation: Retuerto M, La-Monica MB, Ziegenfuss TN, et al. Probiotic supplementation of pea-derived protein alters the gut microbiome balance in favor of increased protein degradation, reflected in increased levels of essential amino acid in human plasma. *Gastroenterol Hepatol Open Access.* 2023;14(4):93–103. DOI: 10.15406/ghoa.2023.14.00553

Participants were instructed to follow their normal dietary habits throughout their participation in the study. Participants were required to complete a 24-hour diet record prior to arriving at the laboratory for their first initial screening visit. Participants were given a copy of this dietary record and instructed to duplicate all food and fluid intake 24 hours prior to their subsequent laboratory visits. Prior to each subsequent visit participants were asked to verbally confirm their 24-hour prior diet adherence and ensure they had a normal night's rest. In addition to replicating food and fluid intake for 24 hours prior, study participants were also asked to refrain from exercise for 72 hours prior, refrain from alcohol and caffeine 24 hours prior, and arrive 8 hours fasted to all testing sessions which were all verbally confirmed at the beginning of each study visit.

Clinical study visits

The study design and schedule of visits is shown in Figure 2. At Visit 1 (Screening), medical history and 24hr dietary recall were collected, along with routine safety blood work (CBC, CMP, and lipid panel). Following Visit 1, subjects underwent a baseline visit (Visit 2) consisting of body weight, blood pressure, heart rate, and a 3-day diet record. A baseline stool sample was provided at Visit 2 and collected at home prior to participant's initial supplementation and sent for microbiome analysis which included bacterial (bacteriome) and fungal (mycobiome) abundance quantification. Participants were provided with their first assigned treatment on Visit 2 and instructed to consume the investigational product after their stool sample was collected. After 4 weeks of daily consumption, participants came in for post testing (Visit 3) where they ingested their final dose of the product in the presence of the research staff. Three days prior to Visit 3, participants were instructed to collect another stool sample and send it out for analysis. Visit 3 consisted of several procedures; 1) blood was drawn before and 30, 60, 90, 120, 180 min post-ingestion of the protein supplement (either PP alone or PP+BFX) for EAAs; 2) perceived changes in GI flatulence, GI bloating, level of fatigue, overall mood, and level of energy were assessed using a 100 mm anchored VAS (visual analog scale) before supplement ingestion and 60, 120, 180 min post-ingestion, 3) vitals were recorded before and 60, 120, 180 min post-supplement ingestion; 4) a 3-day diet record was performed prior to each visit; and 5) stool samples were collected at home and sent for microbiome analysis. At the end of Visit 3, participants were provided with their second assigned treatment and told to begin consumption of the investigational product after a one-week washout period where none of the investigational products were consumed. A sufficient washout period (one week) was then performed to allow complete digestion of the consumed assigned treatment prior to switching to the second phase of treatment.[14] Again, 4 weeks later participants came in for post-testing (Visit 4) which included the same testing measures that were conducted at Visit 3. Participants were also instructed to collect another stool sample within three days prior to Visit 4 and send it out for analysis. The VAS provides a simple, reliable measure that is easy to follow and requires little time to complete. Participants can respond on continuous lines rather than Likert-type scales which allows them to rate their answer with little bias and any desirable amount of discrimination.[15] The validity and reliability of VAS to assess fatigue and energy have been previously established.[15]

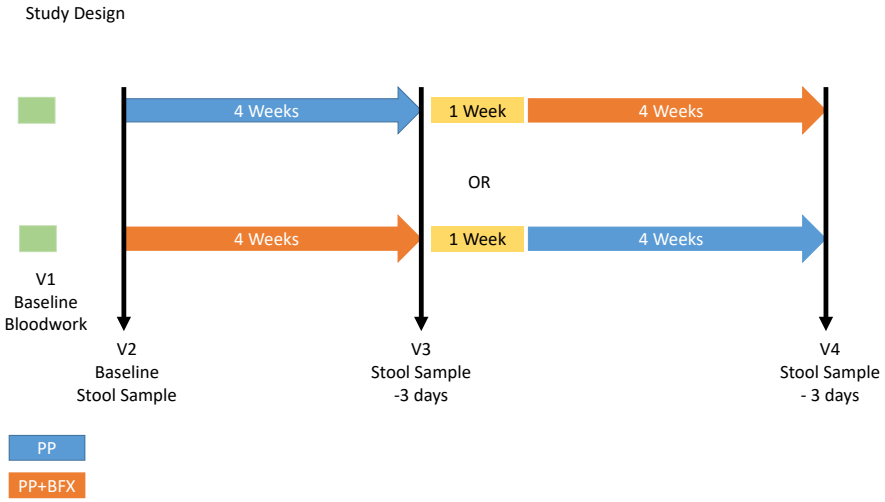

Figure 2 Shows the study design for this open label cross over study indicating the timing of the supplements, the samples obtained, the outcome measures, and the visit timing.

The study was conducted following ICH-GCP guidelines to ensure subject safety and scientific integrity of the data. Comprehensive side effect profile/adverse event monitoring took place throughout the study duration. There were no serious adverse events reported during the study. Mild adverse events were rare and included paresthesia and pre-syncope, associated with a muscle damage protocol (data not shown), rather than protein supplement ingestion. A table of adverse events is given in Supplemental Table 1.

Quantification of Essential Amino Acid (EAA) Concentration in plasma

Plasma amino acid concentrations were determined by liquid chromatography-mass spectrometry (LC-MS) using a QTrap 5500 Mass Spectrometer (AB Sciex, Foster City, CA, USA) and an internal standard method, as described previously. [16, 17] The analytes were derivatized with 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl; 23186, St. Louis, MA, USA). Ions of mass to charge ratio of 340/144 for threonine, 338/116 for valine, 370/47 for methionine, 352/130 for isoleucine and leucine, 425/203 for tryptophan, 386/164 for phenylalanine, 598/154 for histidine, and 589/145 for lysine were monitored with selected ion monitoring on quadrupole one and three, respectively. Quantification of each peak was determined using MultiQuant software (version 2.1: AB Sciex).

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Supplemental Table 1 Summary of Adverse Events

	Allocated Cross-Over (n=85)	
	A (n=44)	**B (n=41)**
Severity		
Mild	2	3
Moderate	3	1
Severe		
Relationship to Study Procedures		
Unlikely		1
Possible		
Probable	5	1
Relationship to Test Article		
Unlikely	4	1
Possible	1	3
Probable		
Body System and AEs		
GastoIntestinal		
Bloating		1
Flatulance		1
Gastrointestinal Pain		1
General Disorder		
Edema Face	1	
Nervous System		
Parathesia	1	
Pre-Syncope	3	1
Total Number of Adverse Events Experienced During Study	5	4
Total Number of Subjects Experiencing AEs: n (%)	5/44 (11%)	4/41 (10%)

Microbiome analysis of fecal samples

To determine the effect of supplementation with PP alone versus PP+ BFX on the microbiota profile, fecal samples were processed using our previously published methodology.[18]

DNA extraction

Fecal samples were analyzed for identification of their bacterial (bacteriome) and fungal (mycobiome) communities using Ion Torrent sequencing technology (Thermo Fisher Scientific, Waltham, MA, USA). Samples were transferred to tubes containing glass beads with the lysis solution included in the QiaAmpFast DNA Extraction Kit (QIAGEN, Germantown, MD, USA). Bacterial and fungal DNAs were isolated and purified following the manufacturer's instructions with minor modifications. In this regard, we incorporated an additional bead-beating step (Sigma-Aldrich beads, diameter =500µm), with the MP FastPrep-24 speed setting of 6M/s and 2×40 s cycles. The quality and purity of the isolated genomic DNA were confirmed using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). DNA concentration was quantified using a Qubit 2.0 instrument applying the Qubit dsDNA HS Assay (Life Technologies, Carlsbad, CA USA) and adjusted to 100ng per sample. Extracted DNA samples were stored at −20º C until needed.

Bacterial 16S rRNA gene or pan fungal ITS amplicon library preparation.

For bacteria, the V3-V4 region of the 16S rRNA gene was amplified using 16S-515F: GTGCCAGCMGCCGCGGTAA and 16s-806R: GGACTACHVGGGTWTCTAAT primers, while the fungal ITS region was amplified using ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS 2 (GCTGCGTTCTTCATCGATGC) primers. The reactions were carried out on a 100ng template DNA, in a 50µL (final volume) reaction mixture consisting of Q5 PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), for a final primer concentration of 400nM. Initial denaturation at 94º C for 3min was followed by 30 cycles of denaturation for 30 s each at 94º C, annealing at 57º C (16 s) or 59º C (ITS) for 30 s, and extension at 72º C for 10 s. Following the 30-cycle amplification, there was a final extension time of 15 s at 72º C. The size and quality of amplicons were screened on a 1.5% TAE agarose gels, separated using 100v, and electrophoresed for 45 min then stained with ethidium bromide. The PCR products were sheared for 20 min, using Ion Shear Plus Fragment Library Kit (Life Technologies, Carlsbad, CA, USA). The amplicon library was generated with sheared PCR products using Ion Plus Fragment Library Kit (<350 bp) according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). The library was barcoded with Ion Xpress™ Barcode Adapter and ligated with the A and P1 adaptors (Thermo Fisher Scientific, Waltham, MA, USA).

Next-generation sequencing, classification, and analysis

The adapted barcoded libraries were concentrated 4–6X in a speed-vac (Thermo Fisher Scientific, Waltham, MA, USA) and the concentrated pooled libraries were then quantified using a TaqMan Quantitation Kit (Thermo Fisher Scientific, Waltham, MA, USA). The libraries were adjusted to 100pM and attached to the surface of Ion Sphere particles (ISPs) using an Ion PGM Template OT2 400bp Hi-Q View Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, via emulsion PCR.

The quality of ISP templates was checked using Ion Sphere™ Quality Control Kit (Part no. 4468656- Thermo Fisher Scientific, Waltham, MA, USA) with the Qubit 2.0 device. Sequencing of the pooled libraries was carried out on an Ion Torrent PGM System using the Ion Sequencing 400bp Hi-Q View Kit (Life Technologies, Carlsbad, CA, USA) for 150 cycles (600 flows) with a 318 v2 chip, following the manufacturer's instructions. De-multiplexing and classification were performed using the Qiime Platform (ver. 1.8). The resulting sequence data were trimmed to remove adapters, barcodes, and primers during the de-multiplexing process. In addition, the sequence data were filtered for the removal of low-quality reads below the Q25 Phred score and de-noised to exclude sequences with a read

length below 100bp.[19] *De novo* OTU's were clustered using the Uclust algorithm and defined by 97% sequence similarity.[20] Classification at the species level was referenced using the Greengenes (v. 13.8) reference database [21] and taxa assigned using the nBlast method with a 90% confidence cut-off. [22] Abundance profiles for the microbiota were generated and imported into Partek Discover Suite v6.11 for principal components analysis (PCA). Diversity and correlation analyses and Kruskal–Wallis (non-parametric) analysis of variance were performed using abundance data and R statistical analysis software (CRAN, and Morgan) with packages (Psych and Vegan, Bioconductor). Diversity indices, including Shannon's Diversity Index (SDI), Richness (N), and Pielou's evenness (PE), were calculated at all taxonomic levels.

Statistical analysis

Based on previous data, as well as similar studies in the literature, a sample size calculation was performed with an effect size of 0.25, significance level of 0.05, power of 80%, and a dropout rate of ~20%. With these parameters, a total sample size of 40 subjects was chosen for this study. The primary outcome variable was plasma EAA response, and the secondary variables were changes in gut microbiome composition and GI tolerance/GI health (flatulence, bloating, fatigue, mood, energy). Descriptive statistics (mean and SD) were used to quantify subjects' physical characteristics. A table of demographics is shown for the study participants (Table 1). Two-way (group x time) linear mixed models were completed to assess group, time, and group x time interaction effects for EAAs and subjective rating for all VAS items. Paired samples t-tests or sidak post-hoc procedures were used to assess individual comparisons between time points and/or groups. SPSS and GraphPad Prism were used to perform these statistical analyses. For primary and secondary endpoints, post-hoc outcomes that indicated a significant difference (P value ≤ 0.05) or a trend (P value >0.51 to ≤ 0.1), Cohen's D effect sizes were calculated to evaluate the magnitude of the observed effect between treatment groups. Change scores between baseline and 30, 60, 90, 120, 180min for EAA were also calculated. For microbiome data, statistical significance levels were calculated comparing the changes across groups by Welch's t-test for a given genus, species, or phylum.

Table 1 Demographics summary

	Total (N=39)
Age (years)	26.3±7.9
Height (cm)	179.7±7.4
Weight (kg)	83.7±9.0
Body Mass Index (kg/m²)	26.0±3.0
Systolic Blood Pressure (mm Hg)	122.3±9.4
Diastolic Blood Pressure (mm Hg)	74.5±8.2
Resting Heart Rate (bpm)	66.6±9.8

Results

Quantification of Essential Amino Acid (EAA) concentration in plasma

All plasma amino acids displayed main effects of time (P≤0.001). A trend for a time by group interaction (P= 0.097) was noted for the plasma EAA temporal response. Post hoc testing indicated that PP+BFX had a significantly higher (P= 0.047; Cohen's D =0.30) plasma EAA concentration (1,333.9 ± 271.2 µmol/L) at 120 min as compared to PP alone (1,277.3 ± 258.3 µmol/L), a 4.4% increase. A trend for a time by group interaction (P = 0.066) was observed on the change from baseline for plasma EAA concentrations. Post-hoc testing indicated that PP+BFX had a trend (P= 0.081; Cohen's D=0.36) for a relative increase in plasma EAA concentrations from baseline to 120 minutes post-ingestion as compared to PP alone (1,040 ± 185.6 µmol/l vs. 996.7 ± 158.8 µmol/L, respectively) (Table 2). A trend for a time by group interaction (P=0.086) was observed on the change from baseline for plasma lysine concentrations. Post-hoc testing (data not shown) indicated that PP alone had a significantly greater (P= 0.029; Cohen's D=0.36) increase in plasma lysine concentrations from baseline to 90 minutes post-ingestion as compared to PP+BFX (229.6 ± 171.4 µmol/l vs. 182.8 ± 188.5 µmol/L, respectively).

Table 2 Plasma essential amino acids

Variable	Time*	PP+ BFX	PP alone
EAA (µmol/L)	0 min[a]	1074±164.2	1034.3±180.2
	30 min[b]	1750.6±450.6	1741.3±432.4
	60 min[b]	1686.5±465.2	1710.4±442.6
	90 min[c]	1451.9±331.6	1474.2±320.0
	120 min[d,#]	1333.9±271.2	1277.3±258.3
	180 min[e]	1176.9±257.2	1152.8±217.5

Subjective outcome variables and dietary records

There was a significant main effect of time (P≤0.001 – p=0.044) for flatulence, bloating, fatigue, mood, muscle tightness, and muscle soreness. There were no significant differences between groups or over time noted for energy (P>0.050). There were no differences over time or between groups in average calories, carbohydrates, fat, or protein (all $P>0.05$).

Microbiome analysis

Microbiome composition: stack plot

The supplementation of pea protein with BIOHM FX (PP +BFX) led to changes in the gut microbiota at the species composition level (Figure 3), particularly in bacterial and fungal species present in the gut at lower relative abundances, compared to both the baseline and PP alone. Similarly, subjects that received PP alone also exhibit patterns of microbiota change, largely in species of lower abundance, but to a lesser degree than PP + BFX (see Supplemental Figures 2A and 2B).

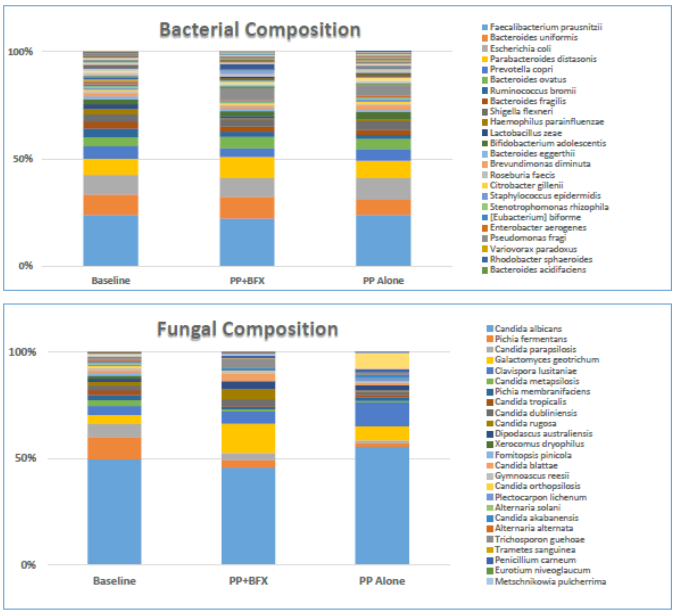

Figure 3 Microbial Composition: Stack Plot It shows the compositional differences in gut microbiota between treatment groups at the Species level. Relative Abundance of bacteria (16S-Bacteriome) and fungal (ITS-Mycobiome) composition is shown for the three treatment conditions (Baseline), Pea Protein + BFX (PP+BFX), and Pea Protein alone (PP alone).

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Alpha diversity analysis

Among baseline and the two treatments analyzed (baseline, PP+BFX, and PP alone) a similar level of richness, SDI, and PE was observed at the phylum level (Table 3) in the bacteriome and mycobiome profile. The bacteriome changes in diversity were mostly in the PP alone cohort primarily observed as an increase in SDI. In contrast, PP alone contained the lowest richness. In regard to the diversity of the mycobiome, the PP+BFX treatment cohort exhibited a modest increase in species SDI with the greatest effect at genus level.

The microbiota biodiversity

The Venn diagram shown in Figure 4A illustrates the bacterial species level biodiversity richness data among the three treatment conditions (baseline, PP, PP+BFX), demonstrating that 302 species are shared across all comparator groups. An interrelationship between PP+BFX and PP shows that they share 9 common species. Interestingly, 4 unique species, *Plantago atrata, Luteimonas mephitis, Pedobacter saltans,* and *Acidovorax caeni*, are exclusive to PP+BFX. Comparing Baseline and PP+BFX, there are 5 shared species. The Baseline and PP alone groups share 4 species, with no unique species on either side, suggesting PP alone may be a subset of Baseline. Baseline contains 4 unique species, *Prosthecobacter debontii, Weissella viridescens, Bdellovibrio bacteriovorus*, and *Clostridium difficile*, not found in the other two groups, suggesting changes to the bacterial biodiversity by the supplementation of PP+BFX.

Figure 4B demonstrates the impact of the fungal species biodiversity among the three treatment conditions (baseline, PP, PP+BFX), with a total of 412 species shared across all groups. However, the biodiversity is primarily influenced by the PP+BFX cohort, which shares 43 and 42 species with the Baseline and PP cohorts respectively. Most striking is the presence of 71 unique fungal species within the PP+BFX treatment, significantly contributing to the overall biodiversity. Notably, 20% of these unique species are from the *Penicillium* genera, underlining a key influence of this genus within the unique fungal biodiversity profile of the PP+BFX treatment.

Table 3A

Bacteriome				
Taxa	**Status**	**Richness**	**SDI**	**Pilou's Eveness**
Phylum	Baseline	17	0.92	0.34
	PP+BFX	16	0.96	0.35
	PP alone	15	0.97	0.37
Genus	Baseline	170	2.32	0.46
	PP+BFX	171	2.33	0.46
	PP alone	160	2.39	0.48
Species	Baseline	92	1.97	0.45
	PP+BFX	94	2.10	0.48
	PP alone	89	2.14	0.49

Table 3B

Mycobiome				
Taxa	**Status**	**Richness**	**SDI**	**Pilou's Eveness**
Phylum	Baseline	2	0.13	0.22
	PP+BFX	2	0.18	0.27
	PP alone	2	0.11	0.19
Genus	Baseline	57	0.80	0.38
	PP+BFX	54	0.95	0.45
	PP alone	47	0.84	0.43
Species	Baseline	41	1.09	0.50
	PP+BFX	38	1.10	0.54
	PP alone	36	0.93	0.50

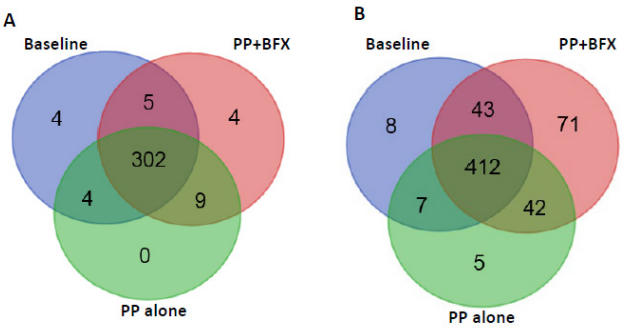

Figure 4 Venn diagram species level biodiversity richness for bacteriome (A) and mycobiome (B) among the three cohorts, it shows the overlap of bacteriome (A) and mycobiome (B) OTUs between.

Abundance microbiota analysis

A comparison of the microbiota composition of subjects at baseline versus PP+BFX shows that in PP+BFX treatment, *Pseudomonas nitroreducens* exhibited a significant increase in relative abundance, comparing baseline (0.0095%) with PP+BFX (0.0526%) (Table 4). This corresponds to a fold change of 5.5, (P = 0.02). Similarly, *Pseudomonas stutzeri* showed a significant (P = 0.037) 12-fold increase in relative abundance in PP+BFX (0.0704 OTUs) compared to baseline (0.0059). Another species belonging to the genera *Pseudomonas, Pseudomonas umsongensis*, increased in the PP+BFX cohort (P = 0.026), suggesting a substantial amplification of *Pseudomonas* following the intervention of PP+BFX.

In contrast, species such as *Alcanivorax dieselolei* and *Tindallia Anoxynatronum* show a decrease in relative abundance in the PP+BFX treatment group compared to the baseline. The reduction in these species may be related to either the pea protein or BFX exposure. However, because these species were not measured or found in PP alone, it cannot be distinguished whether it was the pea protein exposure or the BFX exposure that led to these changes. The presented data includes only those bacterial taxa that exhibited statistically significant changes as determined by a Welch's t-test (P < 0.05) and prevalence (>20%) for any single treatment, whereas fungal taxa shown exhibit significant changes as determined by Welch's t-test ($P<0.05$) and prevalence greater (>5%).

A comparison of the microbiota composition of subjects at baseline compared to PP alone highlights the effect of PP alone (Table 5). Notably, *Prevotella nanceiensis* was 0.00365%, at baseline whereas in PP alone, it decreased to 0.00014%. This corresponds to a fold change of 21.4, (P=0.02) suggesting a substantial reduction in the relative abundance of *P. nanceiensis* following pea protein supplementation alone.

A comparison of the bacteriome composition of subjects treated with PP+BFX compared to PP alone (**Table 6)** provides insights into the response to BFX supplementation when pea protein is included in both groups. The phyla *Bacteroidetes* exhibited a higher relative abundance in PP+BFX (35.1%) compared to PP alone (26.9%), with a fold change of 1.3 (P=0.034). Similarly, Spirochaetes and Cyanobacteria displayed increased relative abundances in PP + BFX, with fold changes of 1.6 (P=0.039) and 7.5 (P=0.049), respectively.

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Table 4 Changes in Bacteriome and Mycobiome Composition Comparing Baseline to PP+BFX

Taxa		Baseline RA(%)	PP+BFX RA (%)	Baseline Prevalence (%)	PP+BFX Prevalence (%)	PP+BFX: Baseline: (FC)	Elavated in	Baseline: PP+BFX (p value)
Bacterial Phyla	Crenarchaeota	0.0063	0.0139	71.1	73.7	2.2	PP+BFX	0.048
Bacterial Genus	Pseudomonas	0.74	6.25	100	100	8.4	PP+BFX	0.028
	Alcanivorax	0.0061	0.0018	55.3	31.6	-3.4	Baseline	0.041
	Brenneria	0.0091	0.0010	31.6	15.8	-8.8	Baseline	0.036
	Dethiosulfatibacter	0.00101	0.00018	21.1	7.9	-5.6	Baseline	0.041
Bacterial Species	Pseudomonas nitroreducens	0.0095	0.0526	23.7	52.6	5.5	PP+BFX	0.020
	Pseudomonas stutzeri	0.0059	0.0704	31.6	42.1	12.0	PP+BFX	0.037
	Acinetobacter rhizosphaerae	0.0032	0.0128	15.8	28.9	4.0	PP+BFX	0.046
	Pseudomonas umsongensis	0.0015	0.0068	13.2	26.3	4.5	PP+BFX	0.026
	Alcanivorax dieselolei	0.0101	0.0035	47.4	21.1	-2.9	Baseline	0.022
	Tindallia Anoxynatronum	0.00052	0.00002	21.1	2.6	-26.1	Baseline	0.015
Fungal Genus	Galactomyces	3.1	10.1	68.4	78.9	3.3	PP+BFX	0.048
	Plectocarpon	0.47	0.10	44.7	21.1	-4.8	Baseline	0.037
	Schwanniomyces	0.0019	0.0003	21.1	10.5	-5.5	Baseline	0.042
Fungal Species	Galactomyces geotrichum	4.1	12.0	68.4	78.9	2.9	PP+BFX	0.046
	Lasiodiplodia theobromae	0.00008	0.00222	7.9	13.2	28.6	PP+BFX	0.047
	Plectocarpon lichenum	0.70	0.15	44.7	21.1	-4.8	Baseline	0.037
	Stenocarpella maydis	0.0002	0.0024	7.9	15.8	11.9	PP+BFX	0.047
	Umbilicaria lyngei	0.0023	0.0002	15.8	5.3	-9.6	Baseline	0.045

Table 5 Changes in Bacteriome and Mycobiome Composition Comparing Baseline to PP alone

Taxa		Baseline RA(%)	PP aloneRA (%)	Baseline Prevalence (%)	PP alone Prevalence (%)	Baseline: PPalone (FC)	Elavated in	Baseline: PP alone (p value)
Bacterial Phyla	Gemmatimonadetes	0.0126	0.0053	68.4	65.6	2.0	Baseline	0.018
Bacterial Genus	Roseburia	1.03	0.72	97.4	96.9	2.1	Baseline	0.039
	Achromobacter	0.29	0.70	71.1	78.1	-15.0	PP alone	0.017
	Parvimonas	0.0031	0.0022	23.7	28.1	-4.1	PP alone	0.021
	Cellulosimicrobium	0.0276	0.0033	31.6	25.0	-9.3	PP alone	0.040
	Salinispora	0.0024	0.0010	23.7	18.8	-8.4	PP alone	0.024
	Prevotella nanceiensis	0.00365	0.00014	21.1	3.1	21.4	Baseline	0.020
Fungal Genus	Trametes	0.296	0.061	44.7	28.1	4.8	Baseline	0.033
Fungal Species	Candida flosculorum	0.00016	0.02640	7.9	15.6	-165.6	PP alone	0.033

Table 6 Changes in Bacteriome and Mycobiome Composition Comparing PP+BFX to PP alone

Taxa		PP+BFX RA(%)	PP aloneRA (%)	PP+BFX Prevalence(%)	PP Alone Prevalence(%)	PP+BFX: PP alone (FC)	Elavated in	PP+BFX:PP alone (p value)
Bacterial Phyla	Bacteroidetes	35.1	26.9	100	100	1.3	PP+BFX	0.034
	Spirochaetes	0.028	0.018	89.5	78.1	1.6	PP+BFX	0.039
	Cyanobacteria	0.074	0.010	92.1	75.0	7.5	PP+BFX	0.049
Bacterial Genus	Bilophila	0.382	0.197	89.5	87.5	1.9	PP+BFX	0.027
	Leadbetterella	0.001035	0.00021	26.3	9.4	4.9	PP+BFX	0.040
	Pyramidobacter	0.00105	0.00015	23.7	3.1	6.9	PP+BFX	0.045
Bacterial Species	Eubacterium dolichum	0.172	0.456	81.6	87.5	-2.6	PP alone	0.016
	Bulleidia moorei	0.0051	0.0155	31.6	34.4	-3.1	PP alone	0.041
	Corynebacterium simulans	0.032	0.197	36.8	53.1	-6.2	PP alone	0.050
Fungal Genus	Brodoa	0.000245	0.002055	10.5	18.8	-8.4	PP alone	0.031
Fungal Species	Candida khao-thaluensis	0.000412	0.005699	7.9	15.6	-13.8	PP alone	0.037
	Sclerotinia homoeocarpa	0.0105	0.001326	28.9	12.5	7.9	PP+BFX	0.042

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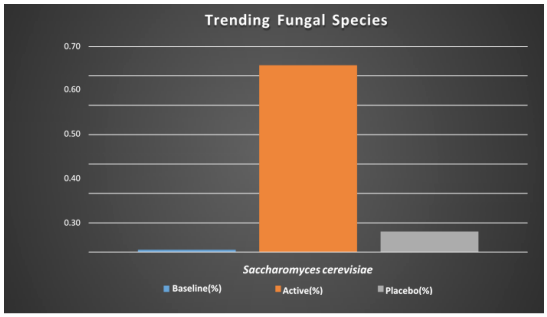
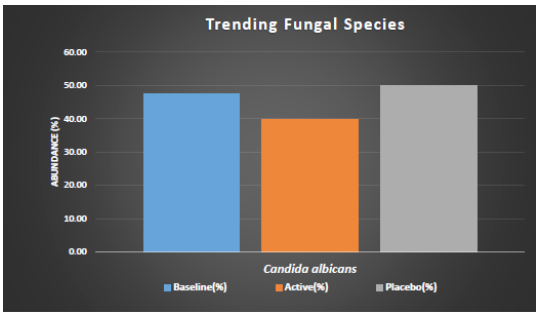
Bacteroides uniformis was highest in PP+BFX when compared to the baseline and PP alone, whereas the abundance of *Saccharomyces cerevisiae* was higher in PP+BFX compared with PP alone, as shown in Supplemental Figure 1. The mycobiome composition was more diverse in respects to the PP+BFX treatment with the key finding that *Galactomyces geotrichum* was significantly elevated when compared to baseline (P=0.046) (Table 4). *G. geotrichum* had a relative abundance of 4.1% at baseline and 12.1% in PP+BFX, demonstrating a 2.9-fold increase. Although not statistically significant, *Candida albicans* (39.7%) was decreased in the PP+BRF treatment compared to baseline (47.4%) or PP alone (49.8%) (Supplemental Figure 2).

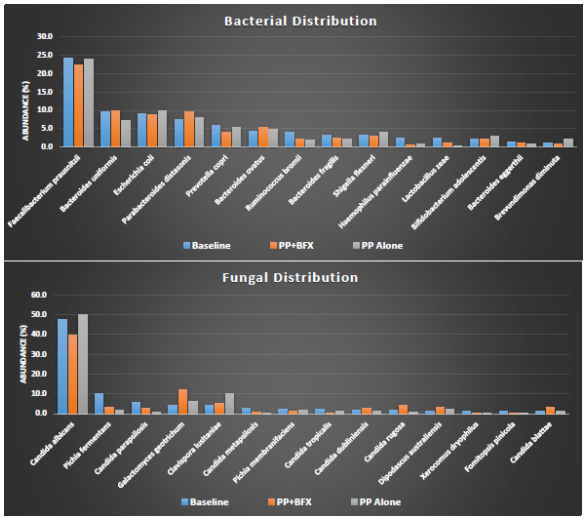


Supplemental Figure 1 Abundance of *Saccharomyces cerevisiae*.



Supplemental Figure 2 Abundance of *Candida albicans*.

The pathogens *Escherichia coli, Prevotella copri, Shigella flexneri,* and *Brevundimonas diminuta* were decreased in PP+BFX treatment when compared to PP alone, as shown in Supplemental Figures 3A & B.



Supplemental Figure 3 Bacterial and Fungal Species abundance Relative to Baseline.

Abundance OTUs of bacterial and fungal species corresponding to each treatment condition; Baseline, pea protein alone and pea protein + BFX.

Discussion

The primary aim of the current study was to determine if addition of the probiotic, BIOHM FX (BFX), to a pea protein supplement (15g pea protein-PP) altered the gut microbiome balance and enhanced the absorption of non-animal proteins determined via quantification of essential amino acids (EAAs) in plasma. A placebo-controlled crossover clinical study in active men (n=39 finishers) was performed and stool samples were analyzed for changes in microbiome composition from baseline and compared for PP versus PP+BFX. Plasma EAAs were measured pre- and post-ingestion of the pea protein +/-BFX for up to 180 min. Self-reported changes in gastrointestinal (e.g., bloating, flatulence) and quality of life (e.g., fatigue, mood, and energy) indices were also measured. We observed that subjects ingesting PP+BFX exhibited a distinct microbiome profile compared to subjects at baseline and following ingestion of PP alone. Differences were also observed in plasma EAA at 120 min post-ingestion, wherein PP+BFX values were greater than PP. Microbiome analysis of stool samples demonstrated that bacterial pathogens decreased following ingestion of PP+BFX compared to PP. Fungal species in the gut were also altered following consumption of PP+BFX. Interestingly, the abundance of several *Pseudomonas* species, cyanobacteria phyla and the fungal species *Galactomyces geotrichum* was elevated when the combination of PP+BFX were consumed by study subjects.

Previous investigators have shown that individuals consuming probiotic supplements containing lactic acid producing bacteria or *Bifidobacterium* have improved mucosal layer integrity and increased nutrient absorption.[23,24] Early studies of biofilm formation have shown that polymicrobial communities form within polysaccharide-rich extracellular matrices, with negative consequences that include alteration of gut permeability, decreased antimicrobial susceptibility, and reduction of host immune response.[25] In this regard, for proteins to be maximally absorbed, they must first be broken down into amino acids or oligopeptides by enzymes from enterocytes within the mucin membrane of the small intestine[8,26] with the expression of these enzymes in direct correlation to mucin thickness.[9] However, if partially digested food from the stomach does not come into direct contact with the mucosal layer, absorption cannot occur. In recent *in vitro* biofilm studies, the permeability of casein through an epithelial cell monolayer was significantly increased (P=0.0001) by the addition of BFX in the presence and/or absence of mixed-species biofilms.[13] Thus, these data provide evidence that BFX is capable of increasing the permeability of the epithelial lining of the gut.

In a similar study, investigators showed that plasma levels of EAAs tryptophan, and cysteine, and total amino acids were increased in a cohort of older women consuming plant-derived protein supplemented with the probiotic *Weizmannia coagulans* GBI-30, 6086 (BC30, formerly classified as *Bacillus coagulans*), at a level (4.4% increase) very similar to the observed change reported here for EAA.[27] Further, in a recent comparable clinical study, probiotic administration with pea-derived protein significantly increased methionine, histidine, valine, leucine, isoleucine, tyrosine, total BCAA, and total EAA maximum concentrations as compared to pea protein alone.[4] In this study, based on *in vitro* data, the investigators hypothesize that increased proteolysis and a synergistic effect with the combination of two probiotic strains (*L. paracasei* LP-DG® and *L. paracasei* LPC-S01) led to increased EAA absorption.

Interestingly, in this study, *Pseudomonas* species were elevated when the combination of PP+BFX were consumed by the study subjects. This observation is in agreement with a previous study

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showing *Pseudomonas* species following probiotic consumption.[28] *Pseudomonas* species are known to be involved in protein metabolism via their ability to degrade and utilize proteins as a source of nutrients. Although often associated with the pathogen *Pseudomonas aeruginosa*, many more *Pseudomonas* species are known, and the majority are not pathogenic, indeed beneficial *Pseudomonas* species are also known.[29] These bacteria possess a range of proteolytic enzymes, such as proteases, peptidases, and exopeptidases, which enable them to break down complex proteins into smaller peptides and amino acids. This protein degradation capacity allows *Pseudomonas* to access nitrogen and carbon sources necessary for their growth and survival.[30–32] Therefore, it is interesting to hypothesize that one of the potential underlying mechanisms by which BFX incorporation to PP increased amino acid absorption is by increasing the abundance of *Pseudomonas* spp. that enhanced pea protein degradation via endogenous increases in proteolytic enzymes, thus enhancing EAA detected in the plasma.

Table 6 provides evidence that the combination of PP+BFX supplementation has a significant impact on the gut microbiome. The PP+BFX treatment demonstrated an increase in the abundance of beneficial bacterial species, highlighting a potential synergistic effect between pea protein and BFX. In contrast, PP alone did not exhibit as many significant changes in the gut microbiome profile (**Table 5**).

An interesting key effect of BFX supplementation is an increase in the abundance of *Bacteroidetes* and *Cyanobacteria* when supplemented with PP+BFX compared to PP alone (**Table 6**). Bacterial species belonging to these phyla, contribute to the maintenance of gut homeostasis, nutrient metabolism, immune regulation, and overall host health.[33] Furthermore, *Cyanobacteria* has been shown to help in fiber (prebiotics) breakdown, and to produce α-amylase.[34,35] In addition, the breakdown of dietary fibers by anaerobic intestinal microbiota has been previously reported to produce short-chain fatty acids (SCFAs) which have been reported to exert multiple beneficial effects on mammalian energy metabolism.[36–38]

In the current study, BFX was shown to positively modulate the gut microbiome by influencing the abundance of several species. Importantly, the abundance of *S. cerevisiae* and *Galactomyces geotrichum* in stool samples following BFX ingestion increased dramatically from baseline and was much higher than the placebo group (PP alone). In a review of the gut mycobiota, Wu et al. noted that benefits of healthy levels of *S. cerevisiae* include the capacity to lessen the severity of gastroenteritis, prevent adherence of adherent-invasive *E. coli,* (which colonize the ileal mucosa of CD patients) in a mouse model, and relieve abdominal pain in irritable bowel diseases.[39] Additionally, *G. geotrichum*, a commensal fungus, is recognized for its ability to produce vitamin B2 and peptides that inhibit the angiotensin I converting enzyme,[40,41] thereby suggesting a potential role in maintaining gut health.

The abundance of the fungal pathogen *C. albicans* was lower in participants ingesting the PP+BFX-fortified supplement compared to baseline and the PP alone group (**Supplemental Figure 1**). *C. albicans* is a normal commensal fungal strain in the gut, but much attention has recently been given to multi-symptom conditions caused by *C. albicans* overgrowth. This overgrowth may be attributed to an imbalanced diet high in sugar and other refined carbohydrates that can cause a variety of conditions such as constipation, diarrhea, nausea, gas, cramps, and bloating.[42,43] Within the bacterial species isolated, abundance of the probable pathogens *E. coli, P. copri, S. flexneri,* and *B. diminuta* was lower under the PP+BFX treatment than the PP alone treatment.

While PP alone can implement change, the combination of PP+BFX results in distinct alterations in the microbiota, enhancing the relative abundance of certain beneficial microbes and reducing others. This highlights the potential for dietary interventions such as PP+BFX to modulate the gut microbiome, which may have important implications for health given the microbiome's role in many aspects of human physiology, including digestion, immune function, and even mood regulation.

One of the major limitations of this study was the crossover design, which although implemented to reduce bias in the protein absorption assay and increase statistical power by using each subject as their own control, had a potential impact on the microbiome analysis. This can be observed in **Supplemental Figure 1**, where the relative abundance of *S. cerevisiae* was low at baseline and increased substantially in PP+BFX but was lower in PP alone. Due to BFX containing *S. boulardii (*a sub-species of *S. cerevisiae*), this suggests increased abundance in the PP+BFX treatment was due to consumption of the BFX. However, in PP alone treatment, which had smaller amounts of this fungi, the lower abundance may be explained by the prior exposure of half of the subjects in the PP treatment had already been exposed to *S boulardii* in the first 4weeks of treatment prior to crossing over to the PP alone group. A second limitation was the lack of gathering information on baseline GI health, as only acute GI markers (gas and bloating on visits 3 and 4) were evaluated.

These findings underscore the importance of considering the combined effects of dietary interventions on the gut microbiome and suggest that the simultaneous supplementation of pea protein and BFX may have a more profound influence on gut microbial composition. Further research is warranted to elucidate the underlying mechanisms and explore the clinical implications of these findings.

Conclusion

In summary, our study indicated that the addition of BFX to the pea protein supplement resulted in a distinct change in the microbiome profile compared to baseline and ingestion of PP alone. Participants ingesting PP+ BFX had a significantly higher plasma EAA concentration (P= 0.047) at 120 min post-consumption as compared to PP alone. While several other commercially available probiotics have been shown to increase amino acid absorption, BFX appears to enhance the gut microbiome balance of organisms capable of producing increased levels of proteolytic enzymes, possibly leading to increased protein absorption (e.g., *Psuedomonas* spp.). Larger studies are warranted to confirm these findings and determine if there are additional benefits of BFX as an additive to plant protein supplements.

Author contributions

Conceptualization, Mahmoud Ghannoum; Data curation, Michael La Monica, Tim Ziegenfuss and Mauricio Retuerto; Formal analysis, Michael La Monica, Mauricio Retuerto and Thomas McCormick; Funding acquisition, Mahmoud Ghannoum; Investigation, Michael La Monica, Tim Ziegenfuss, Betsy Raub, and Mauricio Retuerto; Methodology, Mauricio Retuerto, Michael La Monica, and Thomas McCormick; Project administration, Tim Ziegenfuss, and Mahmoud Ghannoum; Writing – original draft, Michael La Monica and Tim Ziegenfuss; Writing – review & editing, Michael La Monica, Mauricio Retuerto and Thomas McCormick.

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Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

All data generated or analyzed during this study are included in this published article (and its supplementary information files). The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of interest

This research was sponsored in part by a grant from BIOHM Health, LLC. The presentation of results of this study does not constitute endorsement by any of the researchers or their affiliations. BIOHM Health, LLC had no role in the collection, analyses, or interpretation of the data. Mahmoud Ghannoum is the founding partner of BIOHM Health, LLC. Michael La Monica, Tim Ziegenfuss, and Betsy Raub are employees of The Center for Applied Health Sciences.

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