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Baseline intestinal microbiota composition influences response to a real-world dietary fiber intervention

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The intestinal microbiota is shaped by fiber-rich ingredients, such as unripe banana flour (UBF), high in resistant starch (RS). We investigated the effects of RS-rich UBF and inulin on gut microbiota and intestinal function in a double-blind, randomized, placebo-controlled pilot trial. Forty-eight healthy adults consumed maltodextrin (control), inulin, or UBF three times weekly for six weeks. Microbiota composition and function were analyzed using 16S rRNA gene sequencing and PICRUSt, alongside fecal short-chain fatty acids, blood biochemistry, and gastrointestinal parameters. We observed two microbiota clusters at baseline, one *Prevotella*-rich (P) and one *Bacteroides*-rich (B), with distinct responses to the interventions. Only cluster P subjects consuming UBF showed significant global microbiota shifts (weighted Unifrac Beta diversity, PERMANOVA $p = 0.007$) and major functional changes (533 KEGG orthologs, FDR < 0.05). Inulin produced modest modulation (19 KOs) on cluster P, and no effects were observed on cluster B. RS-rich UBF modulated gut microbiota in a composition-dependent manner, supporting the potential of microbiota-based stratification to improve dietary fiber interventions.

The human microbiome has been the subject of intense research and so far, the results have shown a high degree of diversity among subjects[1,2] and within the same subjects throughout life[3]. In healthy individuals, we observe symbiosis, where microorganisms with a potentially beneficial effect on health predominate numerically over those with potentially damaging effects. As such, substances that positively stimulate this relationship have a key role in human well-being and health[4–6].

Diet is a significant modulator of the gut microbiome composition, with long-term dietary patterns being able to influence not just a person ´s gut microbiota profile but also shape their enterotype[2,3,7,8]. Furthermore, short but intense alterations in diet can trigger a rapid response on the gut microbiome[9], and comparable foods can have distinctive effects on different people's microbiomes[7,10–12]. Diet and food-derived molecules are promising targets to stimulate symbiosis, inducing the growth of microorganisms with a potential beneficial effect on health over those potentially damaging[5,13].

One of the most studied substances shown to influence the gut microbiome is highly fermentable, non-digestible carbohydrates, many of which are classified as prebiotics[5,6,14,15]. The fermentation of these molecules by the gut microbiome has a wide array of effects on health, such as controlling appetite through the release of gastrointestinal hormones[16]; controlling the host's intestinal barrier function and immunity[17]; improving glucose metabolism[11], reducing insulin resistance[18], and improving molecular markers related to stress[19]. A murine model study demonstrated that a soluble fiber-containing diet was the primary influencer of the gut microbiota's structure, diversity,

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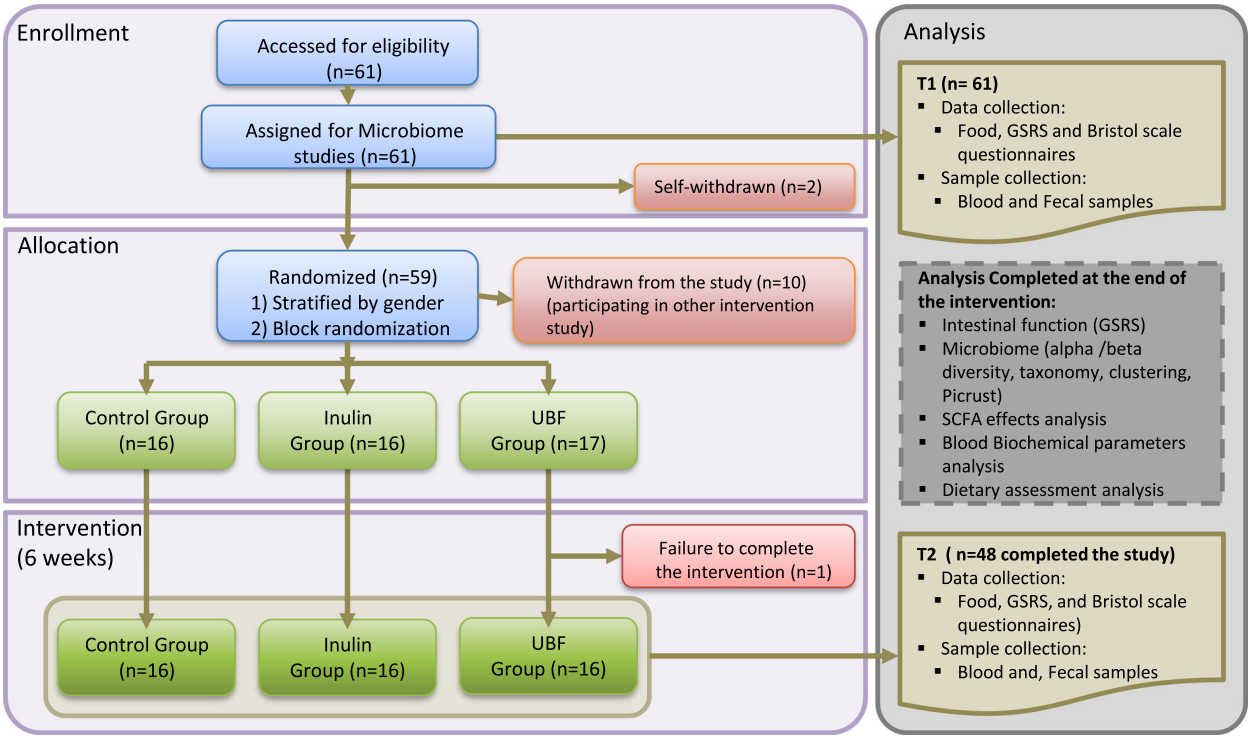

Fig. 1 | Experimental design of the study. GSRS gastrointestinal symptom rating scale, SCFA short chain fatty acids, UBF unripe banana flour. CONSORT flow diagram.

and composition, even when using diets with distinct fat contents[20]. Resistant Starch (RS) and other carbohydrates classified as prebiotics, such as Inulin, are undergoing substantial investigation for their physiological effects on the host[4,6,21–23]. Supplementation with inulin has been shown in some in vitro[22], animal[17], and human studies[12,23–25] to induce microbiome changes associated with health effects. The effects of RS consumption on the intestinal microbiome are less studied than inulin, but increased butyrate and propionate intestinal concentrations induced by RS consumption have been observed in animal studies[4,26,27], while in human studies, a significant variation in fecal butyrate concentration has been observed[10,28]. Unripe banana flour (UBF) is a good source of RS[29] and has been shown in vitro and in animal studies to be highly fermentable, with increased production of short-chain fatty acids (SCFA)[30].

Studies in humans using different types of fiber often present mixed outcomes[12,28,31]. However, in vitro studies using carbohydrates with different chemical structures have shown that inocula dominated by *Prevotella* versus *Bacteroides* (identified as key genera in the gut) impart distinct SCFA production profiles[32]. Furthermore, a study in elderly healthy individuals using wheat bran arabinoxylan oligosaccharides induced a bifidogenic effect only in individuals with *Prevotella-enriched* gut microbiota[33]. Therefore, these distinct effects depend on both the carbohydrate structure and whether gut microorganisms contain the machinery to act as their primary degraders, or the competition among them[34–37]. Carbohydrate degradation by these bacteria can also support the growth of other potentially beneficial bacteria, thereby affecting the production of various metabolites, such as SCFA[34,35]. In humans, identical meals can elicit a variable postprandial glucose response due to a combination of host and microbiome metabolic features[11,38].

As such, non-digestible carbohydrates appear to be a natural choice for targeted interventions aimed at treating and preventing diseases through modulation of the gut microbiome. Nevertheless, the significant interpersonal variability present in the gut microbiome worldwide demands a deeper understanding of the host-microbiome-diet relationship. Here, we investigate the effects of a dietary supplementation containing RS or Inulin on the intestinal microbiota, evaluate their effects on the intestinal function of healthy subjects, and assess the influence of gut microbiota variability.

Results

Intervention description

This was a double-blind, parallel, randomized, placebo-controlled study performed over 6 weeks, registered at clinicaltrial.org under trial number NCT02467972. We recruited 61 healthy subjects to participate in this according to the study criteria (Fig. 1). Of those, 49 subjects were randomly distributed into the Control, Inulin, and UBF treatment groups, and 48 finished the study per protocol. Our pilot trial was designed to test an intervention with (near) real-life conditions that would be easy to incorporate into future routine habits and that would still bring about the desired health benefits. As such, the intervention used a small sachet containing the functional ingredient or the placebo to be added to a meal-replacement standardized soup used as a vehicle, which was consumed 3 times a week. The anthropometric characteristics of enrolled subjects before the interventions are presented in Supplementary Table 1 and did not differ among groups. The intervention´s primary outcomes were published previously[16,39]. Here, we present the results of the intervention on the gut microbiota and intestinal function.

Gut microbiota of healthy subjects before the dietary intervention

We have detected a total of 129 known bacterial genera across all 61 subjects. Within-subject genera count ranged from 33 to 72 distinct genera per person. Operational taxonomic units (OTUs) detected ranged from 697 to 2925 per sample/subject, averaging 1557 OTUs per sample and 82,883 sequences per sample.

Clustering analysis of subjects using Jensen-Shannon Distance (JSD) [1], based on their baseline gut bacterial composition, detected two clusters in the intestinal microbiota of the subjects, one enriched in the *Prevotella* genus (cluster P), and one by the *Bacteroides* genus (cluster B) (Fig. 2). We used three clustering algorithms to separate the gut clusters, with good agreement on the results obtained in the different clustering techniques (Prediction strength of 0.93, 0.94, and 0.67 for the K-Means, Partition

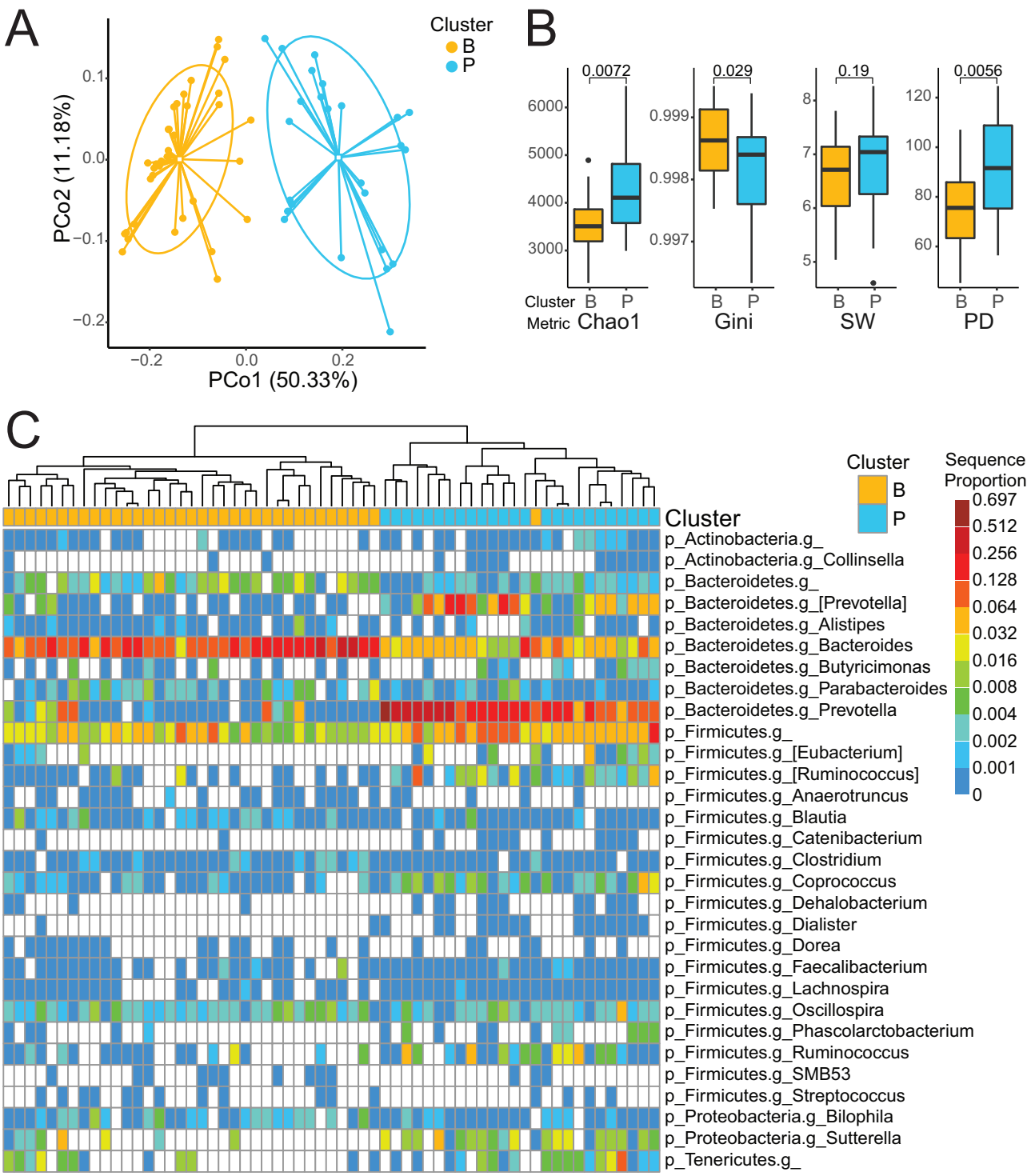

Fig. 2 | Brazilian subjects are separated into two microbiome clusters. Microbiome samples were sequenced before any intervention (T01) and analyzed using JSD, followed by clustering analysis. **A** PCoA analysis of samples at T01 showing their cluster assignment (axes show the % variance explained by each coordinate). **B** Alpha diversity estimates for each cluster at T01 (Chao1 Chao1 richness estimator, PD Faith's phylogenetic diversity, Gini Gini index, SW Shannon-Weaver diversity index. **C** Heatmap of the OTUs that were detected with different abundances between the two clusters, grouped at genus level classification. Rows and columns are clustered based only on the genera presented in the figure. The sample cluster assignment was performed using the Jensen-Shannon distance, calculated using the sequencing data obtained, as described in the Methods. The clustering presented in this figure was performed using only data from the genera shown in the figure. B cluster B assigned samples. P cluster P assigned samples.

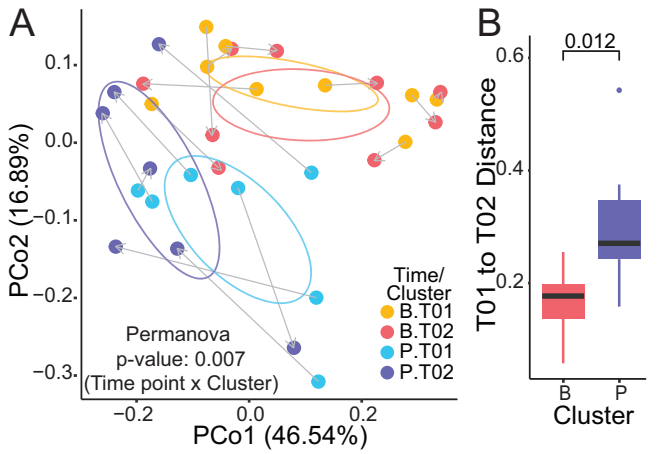

Fig. 3 | Global microbiome changes are observed with UBF treatment in cluster P subjects only. A Principal coordinate analysis of weighted Unifrac distances for subject samples before and after UBF treatment (Permanova analysis), and **B** intra-individual weighted Unifrac distances between T01 and T02 for each UBF cluster (Mann–Whitney analysis).

Around Medoids, and Hierarchical Clustering methods, respectively)[40]. The separation between clusters follows the ratio of *Prevotella* to *Bacteroides* genus proportions, which can be clearly visualized in a principal coordinate analysis (Supplementary Fig. 1A–F). Gut microbiota cluster classification results for the samples obtained after the intervention indicated a cluster switching for 13 out of 49 volunteers for whom we had multiple sampling points. The switching was observed in volunteers for initial samples near the interface between the two clusters (Supplementary Figs. 2A and 1E). No significant differences were observed in total JSD travelled between the start of the study and the last sample studied regarding either cluster assignment or treatment. However, a slightly larger, although not statistically significant, distance was travelled by cluster P volunteers under the UBF treatment (Supplementary Fig. 2B).

Many alpha diversity metrics are used to evaluate how many taxa are present in a given ecosystem (i.e., Chao1 richness index), how evenly distributed these taxa are within the community (Gini evenness index), and if these microorganisms are more closely related to each other or not (i.e., Faith's Diversity Index)[41]. Our analysis revealed differences in alpha diversity between clusters B and P (Fig. 2B and Supplementary Fig. 1G–R). Richness and evenness indexes were higher in cluster P individuals, as measured by the Chao1 and Gini indexes, respectively ($p = 0.0072$, $p = 0.029$). Additionally, bacteria within cluster P were more phylogenetically diverse than in cluster B, according to Faith's phylogenetic diversity index ($p = 0.0056$).

Random forest classification, followed by a non-parametric Wilcoxon rank sum test, detected 287 OTUs in different abundances in each cluster (Supplementary Data 1). Cluster B individuals had higher amounts of OTUs classified at the species level as *Bacteroides ovatus*, *Bacteroides plebeius, Bacteroides uniformis, Alistipes indistinctus* and *Clostridium citroniae*, as well as several OTUs belonging to genera *Dorea, Blautia, Bilophila, Anaerotruncus* and *Clostridium*, while cluster P subjects had higher amounts of OTUs classified at species level as *Prevotella copri*, *Prevotella stercorea*, *Bacteroides caccae, Ruminococcus gnavus, Eubacterium biforme* and at genus level as *Sutterella*, *Ruminococcus*, *Coprococcus, Collinsella, Catenibacterium, Dialister*, *Phascolarctobacterium*, and *Prevotella* (Fig. 2C and Supplementary Data 1). Four OTUs were detected and shared among all individuals, including one classified as *Bacteroides uniformis*, with 2.6% and 0.6% mean relative abundances on cluster B and P individuals, respectively. Cluster B individuals shared only 5 OTUs (1 *Bacteroides uniformis*, 1 *Bacteroides* sp., 2 *Lachnospiraceae*, and 1 *Ruminococcaceae*), while 29 OTUs were common among all cluster P subjects, including 2 *Prevotella copri*, 3 *Prevotella stercorea*, 2 *Faecalibacterium prausnitzii*, 1 *Dorea formicigenerans*, 1 *Blautia*, and 1 *Roseburia*. When considering 90% presence, 50 OTUs were shared among all subjects, 61 OTUs were shared by cluster B subjects, and 99 OTUs were shared among cluster P individuals. (Supplementary Data 1).

The pre-existing microbiota influenced the outcome of a RS-rich dietary intervention

Our initially established goals were to measure the effects of the dietary intervention on intestinal function, and secondary outcomes on microbiota composition and SCFAs production. We observed noticeable gastrointestinal function effects, measured by the GSRS questionnaire and Bristol scale, for the UBF and Inulin interventions (Supplementary Fig. 5). However, there were no evident changes induced by the interventions on the gut microbiota using a global analysis (Weighted and Unweighted Unifrac distances, Permanova test; Supplementary Table 7). Therefore, we postulated that the two clusters of individuals we detected at baseline could respond differently to each intervention. As such, we proceeded to perform an analysis considering the microbiota cluster membership to evaluate both physiological and microbiota responses.

A global change in the gut microbiota was observed after 6 weeks of UBF consumption only for cluster P individuals (weighted Unifrac Permanova, Cluster x Time interaction, $p = 0.007$) (Fig. 3A, Supplementary Table 7). The average intra-individual microbiota distance between before and after intervention timepoints was different between the two clusters of subjects (Fig. 3B) (Mann–Whitney test, $p = 0.012$). The distance between the start and end points was markedly larger for subjects from cluster P for UBF treatment (Fig. 3B). The Control group did not show differences between the start and the end of the intervention for either cluster (Supplementary Fig. 3B, Supplementary Table 7A). Due to random subject assignments to intervention groups, only three subjects belonging to cluster P were assigned to the Inulin treatment. As such, no statistically significant changes were observed between the start and end of the intervention period for the Inulin treatment (Supplementary Fig. 3A, Supplementary Table 7A). A Beta diversity Permanova pairwise analysis (function Adonis 2), Time 1 vs Time 2, was also analyzed for each cluster in each treatment individually (UBF cluster B, $p = 0.789$; UBF cluster P, $p = 0.062$) (Supplementary Table 7B).

No Beta diversity changes were observed for cluster B subjects in Control, Inulin, or UBF treatments (Supplementary Table 7A). Beta diversity analysis based on unweighted Unifrac distances showed no difference with treatment on any cluster/diet combination (Supplementary Table 7C).

We carried out a LEfSe analysis to determine which taxa were involved in the changes observed in the Beta diversity (Permanova analysis). Several OTUs, such as those classified as *Phocaeicola vulgatus, Bacteroides thetaiotaomicron, Paraprevotella clara, Prevotella copri, Phocaeicola dorei, Waltera intestinalis, Lachnospira eligens, Blautia wexlerae, Massiliimalia massiliensis, Faecalibacterium prausnitzii, Vescimonas fastidiosa* and *Oscillibacter valericigenes*, were modulated by the UBF treatment in cluster *Prevotella* individuals only, and the inulin treatment induced changes in *Faecalibacterium prausnitzii*, *Oscilospira sp*. and Clostridiaceae for cluster *Bacteroides* individuals (Supplementary Table 9).

A functional profile was estimated using PICRUSt2 to determine if the treatments modulated metabolic pathways. A distinct response pattern was observed with UBF treatment, whereas no effects were observed for cluster B individuals, with 533 KEGG orthologs modulated in cluster P (FDR ≤ 0.05). KEGG pathways were tested using Fisher's exact test (Supplementary Data 2 and Supplementary Fig. 4) to determine which pathways were enriched or depleted. Lipopolysaccharide (LPS) biosynthesis (ko00540) and transporters (ko02000) pathways were enriched, while Ribosome (ko03010), Aminoacyl-tRNA biosynthesis (ko00970) and Transcription machinery (ko03021) had fewer orthologs than expected (Fig. 4, Supplementary Data 2, and Supplementary Fig. 4). There was a decrease in the sequence quantity of all LPS orthologs predicted after UBF treatment. Although only 3 cluster P subjects were

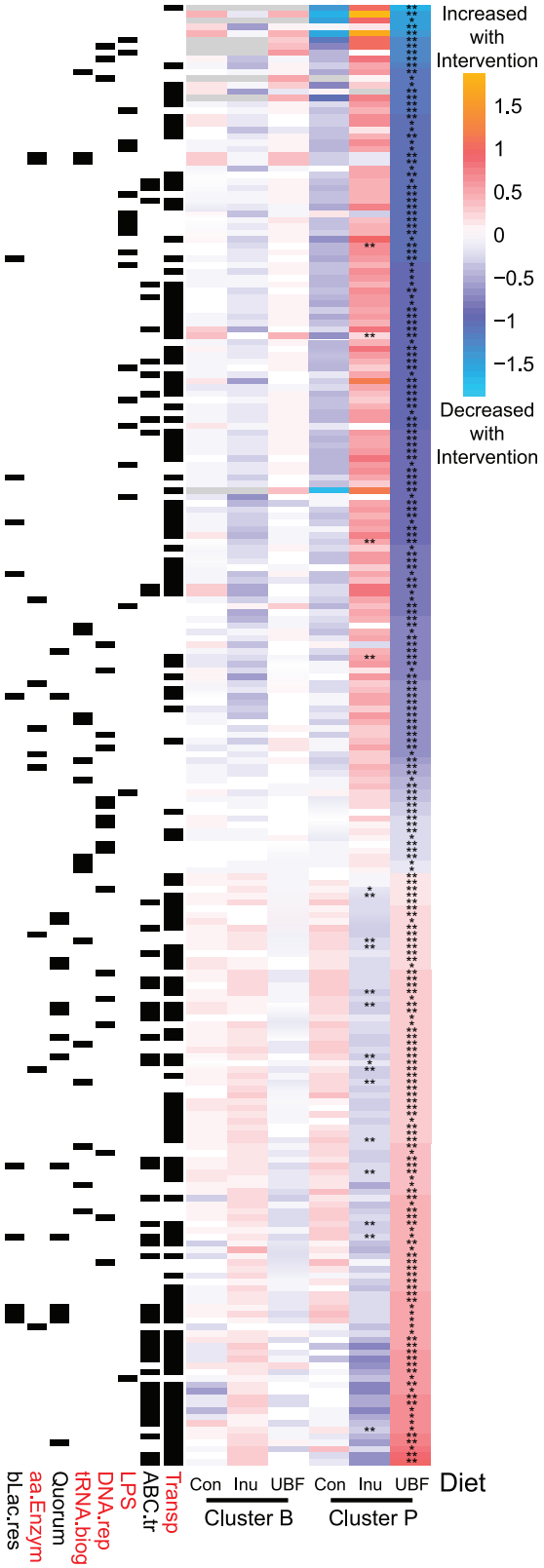

Fig. 4 | Heatmap of significantly KEGG orthologs inferred to respond to intervention with UBF and Inulin on cluster P subjects. KEGG orthologs belonging to enriched KEGG pathways are shown in the figure with representative pathway membership annotations on the left (full annotation available in Supplementary Data 2; red denotes a pathway considered enriched or depleted). Color scale represents the log2(fold change) for each ortholog after the intervention (*indicates a significant fold-change difference at FDR ≤ 0.05, and ** at FDR ≤ 0.01). Transp: Transporters (ko02000); ABC.tr ABC transporters (ko02010), LPS Lipopolysaccharide biosynthesis proteins / Lipopolysaccharide biosynthesis (ko01005/ko00540), DNA.rep DNA repair and recombination proteins (ko03400), tRNA.biog Transfer RNA biogenesis (ko03016), Quorum Quorum sensing (ko02024), aa.Enzyme Amino acid related enzymes (ko01007), bLac.res beta-Lactam resistance (ko01501). No significant changes were observed on cluster B subjects or for the cluster P Control group.

assigned to consume inulin, the effect was consistent enough to yield 17 KEGG orthologs modulated by this treatment (FDR ≤ 0.05), with the overall modulation pattern being opposite to that observed with UBF treatment. However, due to the very small sample size in this group resulting from randomization, care should be taken when interpreting these results. No significant changes were observed for cluster B subjects with either treatment or the control group.

SCFA profile is influenced by the dietary fiber ingested

We determined the metabolic effects of the different dietary interventions on the gut microbiome by measuring the concentration of SCFA in the stool samples (acetate, propionate, butyrate, valerate, isobutyrate, isovalerate). There were no significant changes detected in the absolute SCFA for UBF treatment, although the same general trend was observed in the data, congruent with a large amount of inter-individual variation at baseline values (overall coefficient of variation of 59% at baseline) (Supplementary Table 4A). The SCFA absolute values detected for each treatment showed that Inulin treatment decreased acetate and increased propionate levels; nevertheless, great variability was observed (Supplementary Table 4B). When SCFA were standardized to proportions the variability was minimized and statistical differences were evident, as shown below. A principal component analysis (PCA) of relative SCFA amounts measured in the stool samples indicated that the Inulin intervention influenced the proportions of SCFA measured, in a cluster-independent manner (Fig. 5A, B). The RS intervention was not able to induce a shift in the measured proportions of SCFA (Fig. 5A). The consumption of inulin induced a significant increase in propionate proportion measured in the gut microbiome, with a simultaneous decrease in the proportion of acetate for individuals of cluster B. The same trend was observed for individuals of cluster P (Fig. 5B). Correlations between SCFA and individual OTUs were modest and did not survive FDR correction.

The intestinal function of the subjects is improved in a cluster-dependent manner

Stool consistency, assessed by the Bristol Stool Scale (BSS), was changed only with UBF consumption by subjects belonging to Cluster P; all other intervention groups or clusters did not present changes. The intestinal function was evaluated using the Gastrointestinal Symptom Rating Scale (GSRS) questionnaire, which covers 15 intestinal function parameters (Fig. 6). Subjects belonging to the Cluster P showed a decrease in 4 parameters after UBF consumption: stomachache (Q01), acid reflux occurrence (Q03), hunger pains (Q04), and hard stools (Q13), while also observing an increase in Bristol scale measurements (i.e., increase in stool softening). Cluster P subjects who received inulin had a change in 7 parameters: decrease in stomachache (Q01), heartburn (Q02), acid-reflux occurrence (Q03) and hunger pains (Q04), and increase in flatulence (Q09), loose stools (Q12) and hard stools (Q13). Cluster B subjects showed changes in 2 parameters for UBF only: a decrease in constipation (Q10), and a decrease in hard stool (Q13). Inulin increased the occurrence of flatulence (Q09) in individuals from cluster B (Fig. 6). Constipation symptoms were relieved by UBF consumption regardless of cluster type, while inulin consumption improved constipation only for cluster P individuals. However, there was an increase in flatulence (Q09) reported by subjects consuming inulin, for both clusters, while UBF consumption did not change this parameter (Supplementary Fig. 5). An improvement was observed in symptoms related to the upper

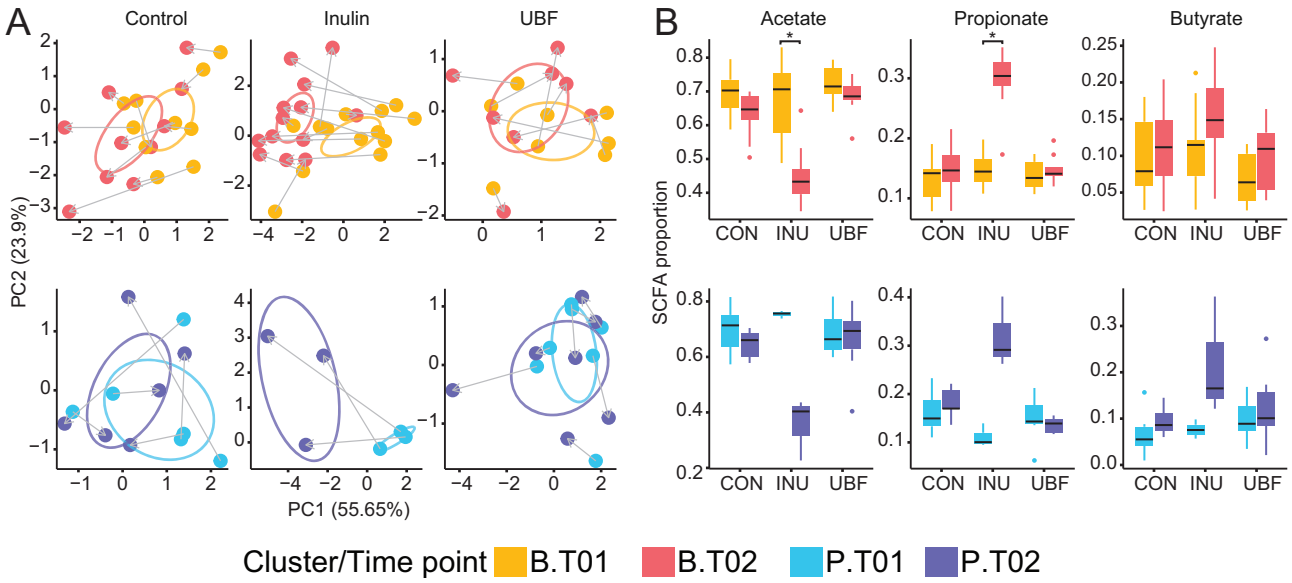

Fig. 5 | Principal component analysis of short chain fatty acids (SCFA) measured in the gut microbiome for each treatment group. A Proportions of SCFA within each sample were used to conduct a PCA analysis of all samples (the PCA analysis was carried out using all samples, but microbiome cluster/intervention groups were plotted separately for clarity). Arrows connect samples obtained from the same subjects, from T01 (pre-intervention) to T02 (post-intervention). Ellipses represent 95% confidence intervals. **B**. Proportions of acetate, propionate and butyrate for each cluster, before and after each intervention. Con (Control), UBF (unripe banana flour) and Inu (Inulin). (*indicates a significant change at FDR ≤ 0.05).

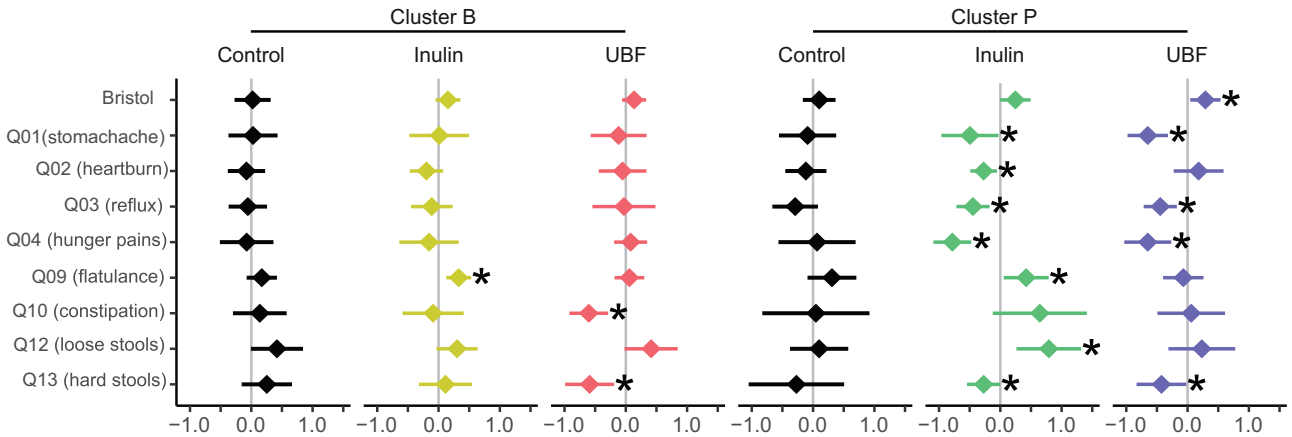

Fig. 6 | Changes in gastrointestinal function induced by the UBF and Inulin treatments for the two microbiome clusters were detected. A larger number of symptoms, as assessed by the GSRS questionnaire and BSS, were changed in cluster P subjects. GSRS questions: stomachache (Q01), heartburn (Q02), acid-reflux occurrence (Q03), hunger pains (Q04), flatulence (Q09), constipation (Q10), loose stools (Q12), hard stool (Q13). No statistically significant changes were observed for the Control group, which is shown for comparison purposes only. (Poisson regression estimates and confidence intervals are shown; *indicates a significant change from baseline at $p \leq 0.05$).

intestinal tract both with inulin and UBF consumption, particularly an improvement in acid-reflux occurrence (Q02) and in hunger pains (Q04) (Fig. 6).

Diet evaluation showed that cluster B volunteers had a lower consumption of dietary fiber ($p = 0.02$), total carbohydrates ($p = 0.007$), available carbohydrates ($p = 0.009$), a higher ratio Lipids/ Fiber than cluster P volunteers ($p = 0.03$) (Supplementary Table 5).

Biochemical parameters measured (total, HDL and LDL cholesterol, triglycerides, and AST and ALT hepatic enzymes) were all inside normal range for healthy adults, before and after the intervention (Supplementary Table 6), and a statistically significant decrease was observed for total cholesterol values both clusters B and cluster P individuals consuming UBF, and for cluster B individuals consuming Inulin (Supplementary Table 8).

Discussion

Over the past few years, several studies have linked diet intake and health through the modulation of the human gut microbiome[3,42–45]. Current dietary guidelines would benefit from incorporating such diet-host-microbe interactions[46]. Dietary interventions aimed at modulating the gut microbiome have clearly shown two distinct groups of individuals, responders and non-responders, raising awareness of the importance of a personalized approach[11,42,46–48]. Here, we show that a person's gut microbiota composition can determine their response to distinct dietary fibers, such as RS and inulin. This further demonstrates that the gut microbiota composition should be considered a priori in randomized controlled trials (RCT) aimed at dietary interventions, to either adjust the statistical analysis model or as a minimization factor for

randomization[49,50], instead of considering the microbiota a posteriori, as it is currently done[24,33,51].

Previously published data using a similarly sized sample set, but composed only by women, has shown that the *Prevotella* cluster is associated with loose stool when assessed by the BSS[51]. We did not observe this relationship in our study, perhaps due to intrinsic population differences present in the gut microbiome of the Brazilian population. Unlike a previous description of the gut microbiota present in the Brazilian population[52] we detect only two clusters of individuals, marked by the presence of *Prevotella* or *Bacteroides* genera. We observe a much higher proportion of people belonging to the *Prevotella* cluster: 40% in our Brazilian sample set versus 13–24% in other western populations[1,2,34,51]. On the other hand, traditional agriculturalist populations present gut clusters almost exclusively belonging to the *Prevotella* group. This gut microbiota pattern shift seems to be driven by changes in diet, particularly an increase in the consumption of meats and processed foods, accompanied by a decrease in the consumption of starch-rich foods[53].

People belonging to the *Prevotella* cluster had higher daily consumption of dietary fiber (DF), total carbohydrates, and protein, while cluster B showed a higher ratio of consumption lipids/DF (Supplementary Table 6), a relationship similar to that observed in other populations around the globe[2,11].

Brazil is currently undergoing a nutritional transition[54], and the Brazilian urban population could be placed between traditional agriculturalist populations, almost exclusively belonging to the *Prevotella* cluster, and Western countries, where most individuals are members of the *Bacteroides* cluster. It is worth noting that dietary habits in Brazil vary significantly from region to region, and the study was conducted in the cosmopolitan city of São Paulo, which may limit the generalizability of this pattern to the entire Brazilian population.

The interventions with UBF and inulin in this study had distinct effects on the gut microbiota depending on the starting gut cluster. Inulin consumption elicited a significant change in the proportion of acetate and propionate measured in the fecal samples from *Bacteroides* cluster individuals. In contrast, no changes were observed in SCFA levels with the consumption of UBF. On the other hand, we observed a global change in the microbiota only with the consumption of UBF specifically for *Prevotella* cluster individuals. Only a few OTU-level changes were observed for the inulin treatment, while UBF elicited changes in several OTU belonging to Bacteroidetes and Firmicutes, indicating a wider microbiota reconfiguration, which was also detected using Unifrac and Permanova tests. A recent study examining a large population set detected *Ruminococcus torques* as a signature of omnivore diets and pointed out a negative correlation with host metabolic health[55]. Notably, although we detected several OTUs classified as *Ruminococcus* in the volunteers' gut microbiota (Supplementary Fig. 6), there were no significant changes in *Ruminococcus bromii*, which has been previously implicated in the degradation of resistant starch[56,57]. Nevertheless, we detected changes in several other Firmicutes taxa, perhaps indicating differences in the gut microbiota of this population. Similarly, although inulin is widely considered bifidogenic, and although we detected many OTUs classified as *Bifidobacterium*, their relative abundances remained low in our cohort and were not significantly modulated by inulin consumption (Supplementary Fig. 6). This may be due to the amount of inulin used in the intervention, the low baseline levels of *Bifidobacterium*, or other intrinsic microbiome characteristics of this cohort.

There is ample evidence of inulin's potential to improve bowel function[21,51,58], although reports on SCFA measured concentration upon inulin consumption vary greatly, ranging from a lack of detectable effect to changes in specific SCFA, all the way to changes in the total pool of SCFA[28,42,59,60]. Microbial carbohydrate metabolism within the gut microbiome is well known to involve syntrophic relationships. Many different microorganisms are necessary to ferment polysaccharides into SCFA in a context-dependent relationship, and that provides distinct degradation pathways depending on the consortium members present in the gut[20,32,34–36]. It has recently been observed that minor differences in DF chemical structure affect the gut microbiota, resulting in distinct SCFA profile outputs[28,61]. The functional predicted data we present here could help future research investigate metabolites and pathways that are not involved in SCFA production.

We have also observed a decrease in genes related to LPS production, perhaps indicating a reorganization of the microbiota towards a state containing more gram-positive members in the gut microbial ecosystem. Increased levels of circulating LPS have been linked to various metabolic disorders, inducing low-grade inflammation and potentially helping drive the pathogenesis of these diseases[62]. Reducing LPS biosynthesis pathways upon the consumption of RS could provide a potential avenue to help treat and prevent such conditions.

UBF is a good source of RS, providing as much as 64% of RS per dry weight and only 19% of available starch. In contrast, high-amylose maize offers 56% RS and 40% digestible starch. RS consumption has been linked to numerous effects on bowel function[63] and metabolic responses, including lipid and glucose metabolism[4,39]. Inulin consumption has also been linked to improved bowel function and decreased inflammatory markers[23,24,64], with noticeable exceptions[12,20]. However, many inconsistent reports of its effects are available in the relevant literature[6,63,65]. We observed a slight variation in some biochemical parameters, all within typical values for a healthy population, unlike a recent report that demonstrated an increase in hepatic enzyme function after an intervention with a higher dose of inulin consumption[12]. This may be due to the different doses used in each study, as well as the distinct microbiota compositions present in the two populations.

We designed our intervention to represent a real-world scenario in which people consumed the UBF or the Inulin added to a standardized soup, replacing three of their regular meals per week. The consumption of the UBF-added soup (5 g equivalent of RS, 17 g total fiber) significantly improved stool consistency, as measured by BSS, in people belonging to the *Prevotella* cluster only.

Both UBF and Inulin had several differential effects on intestinal functioning, as assessed by the GSRS questionnaire, including a significant improvement in the frequency and consistency of the stool, a decrease in abdominal pain, and a feeling of incomplete evacuation in the subjects who consumed UBF. Inulin consumption is often reported to elicit increased flatulence[66]. The entire inulin group in our study reported the same, as did the individuals in the *Prevotella* and *Bacteroides* cluster, unlike UBF, which did not elicit these adverse events.

An in vitro study demonstrated that a specific fructan was more effective in stimulating gas production from a basal inoculum of normal-weight subjects than from a morbidly obese basal inoculum, highlighting the need for developing population-specific products[67]. Gastrointestinal tolerance is critical when prescribing probiotics[68] and DF intake, mainly when used as a supplement in food products. Studies providing less than 10 g/day of RS often show no increase in laxative effects, which are typically observed with consumption of 20 to 25 g/day. Doses above 30 g/day affect other gastrointestinal symptoms, such as increased bloating, abdominal pain, and distension[31,67]. Such variation in effect reported in the literature could be attributed to the fact that these studies used volunteer populations with heterogeneous gut microbiota compositions. Indeed, a recent intervention observed different gut microbiota responses elicited by the same RS intervention[69], and the authors also indicated that non-responders may require different dietary fiber sources. Previous studies[70,71], inferred the *Prevotella/Bacteroides* genera ratio in participants in dietary intervention, after randomization. This parameter remained stable for 6 months and did not affect the intervention results in one study[70]. However, when participants were also characterized by their metabolic profile (larger waist circumference), their response to the intervention was microbiota-dependent[71]. At that time, the methodology used was qPCR, which brought limitations in detecting broader ecological variations. As such, microbiota characterization, or perhaps measuring the gut *Bacteroides/Prevotella* ratio, could be used to select the appropriate DF for an intervention with a specific aim, thus increasing the chances of a successful outcome with the least likelihood of inducing side effects. Finally, as

clinicians and end-users start to use microbiota characterization sequencing services, a more comprehensive understanding of diet-microbiome interactions may lead to more tailored dietary recommendations and interventions.

In summary, consumption of RS-rich UBF in a regular and intermittent way for 6 weeks by healthy subjects induced an improvement in intestinal function without causing gastrointestinal discomfort side effects, broadening its potential use as a functional ingredient. Additionally, markedly distinct effects were observed on the gut microbiota in a cluster-dependent manner after the consumption of two different dietary fibers. Inulin consumption induced SCFA changes in the gut microbiome, while RS improved gastrointestinal motility in *Prevotella* cluster individuals without the onset of gastrointestinal discomfort. Although UBF treatment modulated a broad range of KEGG orthologs uniquely in cluster P individuals, these results should be interpreted with caution, as PICRUSt-based methods are limited by the quality of the underlying annotation database. First, EdgeR identified differentially abundant KEGG orthologs in each treatment group, as predicted by PICRUSt, in a microbiota cluster dependent manner. Then, a Fisher's exact test enrichment analysis highlighted pathways where these modulated orthologs were disproportionately represented. Despite these limitations, the results demonstrate the potential for the use of UBF to promote modulation of the intestinal microbiota in a real-world scenario intervention, which people can easily follow. Moreover, the functionally predicted data we present here could help future research investigate metabolites and pathways that are not involved in SCFA production. Finally, this is the first sequencing-based intervention study in healthy subjects to demonstrate that an individual's gut microbiota cluster can influence the response to a dietary intervention, with potential benefits for the health of the host.

Methods

Study design

This was a double-blind, parallel, randomized, placebo-controlled study performed over 6 weeks (Fig. 1, Experimental design). We sampled the gut microbiome of 61 healthy subjects before any intervention (T01), and 49 of these subjects were randomly assigned to the control, inulin, and UBF treatment groups. Forty-eight subjects completed the study according to protocol. Gastrointestinal assessment, stool samples, and other evaluations were performed before (T01) and after (T02) the intervention.

The inclusion criteria to participate in the study were: subjects who were in good overall health, defined as the absence of hyperthyroidism and renal and gastrointestinal diseases; with no previous diagnosis or family history of diabetes mellitus, and were not using any medication, particularly antibiotics, during the study period. Exclusion criteria were: subjects classified as obese (BMI ≥ 30 kg/m^2) or underweight (BMI ≤ 18.6 kg/m^2) according to the criteria of the World Health Organization[72] and those reporting any disease, pregnancy, breastfeeding, or treatment of any kind (including for possible eating disorders).

The anthropometric characteristics of all subjects before the interventions are presented in Supplementary Table 1. The study was approved by the Research Ethics Committee of the School of Pharmaceutical Science at the University of Sao Paulo (approval number: CEP/FCF/194), following national and international guidelines following the declaration of Helsinki. All subjects signed an informed consent form before the start of the intervention. This trial is registered at clinicaltrials.gov as NCT02467972, 2015-06-03, (https://clinicaltrials.gov/study/NCT02467972).

The primary outcomes of the trial were changes in satiety (hormones PYY and Ghrelin, satiety scale), changes in bowel function (GSRS, Bristol Scale), and changes in the colonic bacterial population (through gut microbiota's 16S rRNA gene sequencing). Secondary outcomes were changes in biochemical profile (total, HDL, LDL cholesterol; triglycerides; ALT, AST, creatinine) (Supplementary Tables 6 and 8). However, not all volunteers consented to every assessment: only 11 volunteers in each group agreed to participate in the hormone measurements. The hormone and satiety results were previously published[16,39]. Due to the smaller sample set size for these measurements, no analysis was conducted comparing the hormone data with the microbiota data. The main outcome study design evaluated only time points T01 (before intervention) and T02 (after the intervention). However, the study participants were given a choice to bring up to two more stool samples after the T02 sample was collected in the hope of allowing us to investigate the washout period.

Dietary intervention

The premise of the study was to test an intervention with (near) real-life conditions that would be easy to incorporate into future routine habits and that would still bring about the desired health benefits. As such, the intervention used a small sachet containing the functional ingredient or the placebo to be added to a standardized soup and used as a vehicle, which would be consumed 3 times a week. The sample size was estimated using previous UBF and Inulin studies conducted by our group and others[23,73,74]. Preliminary studies in our group have indicated that changes in intestinal function can be achieved in as little as 14 days with daily consumption of 8 g of UBF in a group of 14 participants[73]. An acute UBF intervention with a group size of 9 participants was able to elicit changes in glycemic response[74]. Additionally, Inulin was able to induce effects on intestinal function in groups of 15 individuals, as reported in a parallel study design[23].

We enrolled 61 healthy subjects after applying inclusion and exclusion criteria; 2 self-withdrew before any intervention started, and 10 volunteers joined another study and were withdrawn from this study (Nisin arm). The Nisin arm was a parallel study designed to use the same control group but with no intention of being part of this research question related to fiber effects on health and gut microbiome (it was designed to value participants' time and willingness, not repeating placebo groups).

The randomization of the 49 participants was achieved by (1) stratifying by gender and (2) block randomization ($n = 3$). The randomized, allocated patients received one of 3 treatments: UBF, inulin (used as a reference/positive control due to extensive literature indicating this fiber as a probiotic), or a control (placebo). Trial participants, individuals enrolling them, and individuals collecting and analyzing biological samples were kept blind to the intervention groups throughout the trial.

All 49 randomized participants received 18 individual portions of frozen ready-to-eat soup in two different flavors (9 units of each flavor) with an approximate energy intake of 1100 kJ (260 kcal) and an average weight of 450 g, which were used as the functional ingredient vehicle, and 18 sachets containing functional ingredient supplements (or control). They were instructed to add the supplements after thawing and heating the frozen soup (8 min in a microwave), and to consume three servings per week for 6 weeks. The Control group received sachets containing 2 g of maltodextrin, and the UBF Group received sachets with 8 g of UBF (equivalent to 5 g resistant starch). The inulin group received soups already containing 8 g of inulin added during soup manufacturing and sachets with 2 g of maltodextrin (to keep treatment blindness and caloric energy equivalency) (Supplementary Table 3). The study was completed per protocol by 48 participants.

Stool and blood sample collection

Gastrointestinal and dietary assessments, gut microbiota fecal samples, and blood samples were collected before (T01, from all 61 subjects) and after the intervention (T02, from 48 subjects who completed the study according to protocol). The T02 stool and blood samples (post-intervention time point) were collected at least one day after the last soup portion was consumed. Both the functional ingredient sachets and the soups were provided to the volunteers in bi-weekly batches, with instructions to return any sachet that was not used on subsequent visits. Each volunteer had to complete a diary-style spreadsheet reporting side effects, day of soup/sachet consumption, and any other observation they deemed relevant. Participants were instructed to maintain their usual dietary intake, apart from the meal substitution made here, and to report any over-the-counter medicine intake and /or medical issues. All volunteers who completed the intervention consumed all functional ingredient doses given (compliance), as self-reported, with no medical occurrences reported. One volunteer failed to

complete the intervention, as reported in the diary spreadsheet, and was removed from post-intervention analyses. The average time between baseline and T02 sample collections was 45 days for the control group, 46 days for the Inulin group, and 45 days for the UBF group. The first time point of washout (T03) was collected on average 30 days after the T02 stool sample for the control and Inulin groups ($n = 13$ and 12 respectively), and 25 days for the UBF group ($n = 13$), however, there was significant variation in this time interval, with as little as 5 days and as much as 85 days. Only 12 participants handed in a T04 sample, with an average of 16 days after the T03 stool sample for the control groups ($n = 3$), 20 days for the inulin group ($n = 5$), and 19 days for the UBF group ($n = 4$); again, there was significant variation in this interval, with as little as 6 days and as much as 29 days between samples. All stool samples were sequenced, but there was a large variation between time points T02 and T03, and between T03 and T04, no analyses were carried out regarding the washout period other than a cluster classification procedure described below.

Soup preparation

Soups used a vehicle for the functional ingredients were prepared by a commercial manufacturer in a semi-industrial scale (BFR S.A.) following Brazilian legislation[75] for the category of Frozen Ready-to-eat Foods. The same soup was used in a previously published work by ref. 39. Soups were prepared in two different flavors, while maintaining the total calorie intake isocaloric for each soup portion. One flavor was "Meat, pasta, and vegetable soup" (Ingredients: Ringlet pasta, frozen carrots, potatoes, broccoli, cauliflower, onion, green beans, leeks, oregano, celery, ground beef, tomatoes, beef broth), and the other was "Bean and vegetable soup" (Ingredients: Beans, potatoes, carrot, sausage, garlic, onion, olive oil, bay leaf, parsley, chives). Soups were prepared, portioned, and frozen for the entire duration of the study. Frozen soup portions were given in bi-weekly batches to the volunteers with instructions to keep them frozen until ready to be consumed in the intervention. Soup's characteristics, ingredients and chemical composition are available in Supplementary Tables 2 and 3.

Chemical composition analysis

Chemical composition analysis was carried out for the soups, as well as the UBF, using standard procedures described below. Soups were analyzed following the same instructions given to the participants: soups were thawed and heated up in a microwave for 8 min, to reach a temperature people would be able to consume it, and immediately prior to consumption the supplement sachets were added and mixed in. These prepared soups were freeze-dried (Lyophilizer – Freeze- Dryer, model Super Modulo 220 TC60 Tray Cell; Thermo Fisher Scientific, Waltham, MA, USA), grinded (60 mesh) and stored at −20 °C until chemical analysis.

The proximate composition (Protein, Lipid, Moisture and Ash) was assessed in triplicate according to the AOAC method[76]. Briefly, protein content was determined by the total nitrogen present using the micro-Kjeldahl technique (AOAC 960.52) and considering a conversion factor 6.25; lipid content was determined using the Soxhlet method (AOAC 920.39); and ash content by calcination in a muffle furnace at 550 °C to a constant weight (AOAC 923.03)[76]. Moisture content was determined using a vacuum oven at 70 °C (AOAC 920.151) using both the freeze-dried, ground soup samples, as well as the original soup.

Total starch (TS) was determined using AOAC method 2002.02[77] for the quantification of the total starch, the resistant starch (RS) quantification was performed as described by Cordenunsi and Lajolo[78], and the free glucose content was determined by the enzymatic method (glucose oxidase/peroxidase/2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)[79]. Corn and potato RS (Megazyme K-RSTCL; Megazyme International Ireland Ltd., Wicklow, Ireland) and cooked 'carioca' beans (*in house*) were used as reference materials for determination of RS; potato starch (Sigma S-2004) was used as the standard for determining the TS. The concentration of available starch (AS) was obtained by the difference between the total and resistant starch (AS = TS - RS). The dietary fiber (DF) content was determined in the lyophilized, crushed (60 mesh), and degreased samples by the enzymatic-gravimetric method AOAC 991.43, after pre-treatment with dimethyl sulfoxide in a bath at 100 °C for 30 min to solubilize all RS present, in order to avoid overlapping RS values, as proposed by McCleary and Rossiter[80]. Total soluble sugars (TSS) were extracted and quantified as previously described[78]. Glucose, fructose and sucrose (Sigma, Chemical CO, Saint Louis, MO, USA) were used as reference materials. Fructans (FR) were measured by AOAC 999.03 (Megazyme K-FRUC). Energy was calculated (Atwater factor; FAO (2003)) as: [protein (g) × 17 kJ] + [lipids (g) × 37 kJ] + [total available carbohydrates × 17 kJ] + [non-available carbohydrates × 8 kJ] (1 kJ = 0.239 kcal). All chemical analyses were performed in triplicate, and the results expressed in grams, percent, or g/100 g of wet weight (d.w.), as necessary.

Test ingredients

The UBF was produced as described[29]. We used *Musa acuminata* (AAA group), sub-group Cavendish, at stage I of maturity (unripe). Chemical composition was published elsewhere[16]. Volunteers in the UBF arm consumed 8 g of UBF each time, as described above. We used 2 g of Maltodextrin (code: MOR-REX 1920 - Ingredion) as a placebo for blinding and keeping the isoenergetic values of the meals. We chose this amount as derived from the UBF chemical composition analysis, which showed that the intervention portion contained 2 g of available carbohydrates. Each 2.0 g of maltodextrin contains 1.9 g of available carbohydrates. The portion has the equivalent of 32 kJ. The inulin used was Beneo GR (granulated Inulin powder, average DP ≥ 10) from Beneo-OraftiTM- Tienen, Belgium, and it was added directly to the soups used for this intervention arm during their production.

16S rRNA gene sequencing and bioinformatic analysis

Freshly voided stool samples were collected by each study participant in sterile polypropylene containers and kept refrigerated for up to 4 h. Stool samples were received and processed immediately into ~200 mg aliquots, which were kept frozen at −80 °C until further processing. Frozen stool sample aliquots were used for total DNA extraction using the PSP Spin Stool DNA Plus Kit (Stratec Molecular), with prior bead beating using the Environmental Lysing Matrix E (MPbiosciences)[2,53]. The 16S rRNA gene v1v2 region was amplified by PCR and sequenced using an Illumina MiSeq v2 Reagent 500 cycle kit according to the manufacturer's instructions[81]. Sequences were bioinformatically processed using Qiime 1.9 with default parameters[82]. Alpha diversity calculations were performed with a rarefied dataset containing 50,000 sequences per sample. For all other analyses, singletons and doubletons OTUs were removed from the OTU table. Shared taxa across samples were identified using the unrarefied OTU table. Beta diversity was calculated using Weighted and Unweighted Unifrac distances[83] and visualized using Principal Coordinate Analysis (PCoA). Taxonomy was assigned in Qiime using the Greengenes 13_8 database. OTUs were chosen as an ecological representation for our study[84]. Sequence data is deposited online and will be made openly available upon manuscript publication.

Clustering analysis and taxa differences between clusters

Clustering was performed using Jensen-Shannon distance and the Partition Around Medoids (PAM) as previously described[1]. Additionally, we used k-means and hierarchical clustering using Ward's criterion. Clustering quality was assessed using prediction strength, with a significant cutoff of 0.7 or higher[85]. A majority rule based on all three clustering algorithms was used to assign clusters. Differences in OTUs between the clusters were detected using a random forest classifier with OTUs present in at least 20% of the samples. OTUs with a mean decrease in accuracy >0.0001 were further tested with a Wilcoxon Rank Sum test, and OTUs were deemed significant at a $p <= 0.05$ after FDR correction (multiple tests throughout the study were controlled with False Discovery Rate (FDR) correction calculated by the Benjamini-Hochberg method. The same cluster classification procedure used for the samples obtained prior to the dietary intervention was used to classify samples collected at timepoints 2, 3, and 4. Each post-intervention

sample was classified separately using all the T01 samples, and removing the corresponding T01 for the same individual, to avoid confounding effects due to having two samples from the same person. Results from this classification procedure are shown in Supplementary Fig. 2A.

SCFA determination

Stool sample SCFA extracts and SCFA standard curves were analyzed by GC-FID, based on methods previously described[86], and adapted[30,74]. Briefly, frozen stool sample aliquots were re-suspended in sterile water, vortexed, and centrifuged to separate the fecal matter residue. The supernatant was filtered using a 0.2 µm syringe filter (Corning), and the filtered suspension was used for the SCFA determination. Formic acid was used as an acidifying agent (12%) and 2-metil-valeric acid (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard (0.25%). Standard curves were done using volatile acids standards (acetate, butyrate, propionate, valerate, isobutyric, isovaleric) (0.1 mM–10 mM) (Volatile Acid Standard Mix, Sigma-Aldrich, St. Louis, MO, USA). The prepared supernatants and standard curves were transferred into a 250 µl glass insert and placed in a 2 mL amber GC vial and sealed with a *PTFE* cap (Agilent), and measurements were performed in triplicate.

Intestinal function, Bristol scale and 24 h dietary recall questionnaires

The intestinal function was evaluated with the GSRS, translated and validated to Brazilian Portuguese[87] and the BSS[88]. Three 24-h recalls[89] were collected by trained dietitians, before (T1) and after (T2) the intervention, on non-consecutive days, to assess the macronutrient energy distribution of the diet in relation to WHO/FAO recommendations. Food nutrients were calculated using the Brazilian Food Composition Table (TBCA)[90]. Energy was calculated according to FAO[91].

Intervention outcome and other statistical analyses

Intervention outcome analysis was conducted only for subjects who successfully completed the study (per-protocol analysis). Changes in intestinal function GSRS and BSS scores were assessed using a generalized linear model, with a Poisson distribution where each question was the outcome and the dietary intervention was the predictor (before intervention: *adlib* diet, after intervention: control group, UBF group, INU group). The test was initially performed for all subjects combined. The global microbiota effects of dietary intervention were assessed using Beta diversity, measured using weighted and unweighted Unifrac distances, with a Permutational analysis of variance (PERMANOVA) test using a block design[83]. As no significant effects were observed on the microbiota for any of the interventions, the full analysis was carried out for each microbiota cluster separately. Taxa modulated by the interventions were tested using LEfSe[92]. Species-level taxonomy was assigned for OTU's detected by LEfSe using BLAST[93] and the nr/nt database with all default parameters, except that the taxa parameter was restricted to 'Bacteria', and 'uncultured/environmental sample sequences' were excluded from the search. A linear mixed model was used to test the intervention effects on blood biochemical parameters and lipid profile, with subjects considered a random effect. Differences in SCFA were determined using a Wilcoxon Rank Sum test on the proportions of each SCFA from the total measured, as well as the absolute amounts of SCFA per gram of stool. Proportions of SCFA within each sample were used to conduct a PCA of all samples. Multiple comparisons were controlled using FDR. Samples from one individual were not assessed for SCFA and were used for these analysis. All statistical tests were carried out within the R statistical environment for scientific computing[94].

Functional inference and pathway enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs were inferred using PICRUSt 2.0[95,96]. The R package EdgeR[97] was used to determine differences in the inferred orthologs' relative abundances. The analysis was carried out in a paired fashion, with each subject serving as their own baseline (before vs after the intervention), and performed separately for each intervention group (control, UBF and Inulin) and for each microbiota cluster detected. Orthologs were considered differentially modulated by the intervention at a $p \le 0.05$ after FDR correction. Enrichment in KEGG pathways was tested using Fisher's exact test with all orthologs detected in that pathway included in the analysis. To allow for a more comprehensive pathway-level assessment, we included all orthologs with FDR-corrected $p \le 0.1$ in the EdgeR analysis. The EdgeR analysis detects statistical differences in individual KEGG orthologs predicted by PICRUSt, reflecting genes/functions that are relatively enriched or depleted after each treatment. The Fisher's exact test then asks whether the number of modulated orthologs assigned to a given pathway is significantly higher or lower than expected by chance, given the total number of orthologs detected for that pathway. This result should be interpreted as a deviation in the proportion of modulated orthologs within a pathway, relative to all PICRUSt-inferred orthologs.

R packages used

All analyses were performed using R version 4.4.2. Key packages used included ggplot2 (v3.5.1), ggpubr (v0.6.0), ggsignif (v0.6.4), edgeR (v4.4.2), limma (v3.62.2), lme4 (v1.1-36), lmerTest (v3.1-3), Matrix (v1.7-1), msm (v1.8.2), pheatmap (v1.0.12), plyr (v1.8.9), reshape2 (v1.4.4), RColorBrewer (v1.1-3), viridis (v0.6.5), viridisLite (v0.4.2), tidyr (v1.3.1), vegan (v2.6-10), permute (v0.9-7), fpc (v2.2-13), cluster (v2.1.6), sandwich (v3.1-1), and lattice (v0.22-6).

Biochemical parameter measurements

Fasting blood was collected for biochemical analysis parameters (glucose, insulin, cholesterol, triglyceride, liver enzymes, and creatinine). The glucose levels were analyzed using the enzymatic method (hexokinase) with automated equipment (ADVIA 1800 Chemistry Analyzer; Siemens, Tokyo, Japan). The insulin levels were analyzed using the immunoassay method with chemiluminescence and automated equipment (Advia Centaur XP, Dublin, Ireland, Siemens). Cholesterol fractions, triacylglycerol, liver enzymes, and creatinine were analyzed using specific kits (Labtest Diagnostica S.A., São Paulo, SP, Brazil).

Data availability

The sequence dataset generated in this study has been deposited in the European Nucleotide Archive under project accession number PRJEB66278.

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Author contributions
F.A.H.S., E.B.G., G.S.S.M., A.O., and S.B.R.P. performed the experiments. F.A.H.S., E.B.G., C.R.T., C.C.T., K.B., F.D.B., E.W.M., and C.H. provided protocols, reagents and/or designed experiments. E.W.M., E.B.G., F.A.H.S., and C.H. designed the study. F.A.H.S. and C.H. analyzed and interpreted the data. F.A.H.S. prepared all the tables. F.A.H.S. and C.H. prepared all the figures. F.A.H.S. and C.H. drafted the manuscript. All authors critically revised the manuscript for important intellectual content.

Competing interests
The authors declare that there are no competing interests. BRF S.A. supplied the soup used in the study but was not involved in the design, implementation, analysis, or interpretation of data. E.W.M. and C.C.T. have an Unripe Banana Flour production patent issued. All other authors have no relationships or activities that could appear to have influenced the submitted work.

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