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In vitro assessment of the impact of astragalus, pineapple stem and bergamot extracts on human fecal bacteria

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Abstract

Background Botanical extracts are increasingly incorporated into functional foods and dietary supplements due to their purported health benefits; however, their impact on the healthy adult gut microbiota remains insufficiently characterized. This study aimed to assess the microbiota-modulating potential of three commercially relevant botanicals (*Astragalus membranaceus* root extract, pineapple stem extract, and bergamot extract) in both native and digested forms, simulating gastrointestinal passage via the standardized INFOGEST protocol.

Methods Antimicrobial activity was first evaluated in monocultures of nine gut-associated bacterial strains representative of the healthy adult intestinal microbiota, including *Lactobacillus acidophilus* LA14, *Lacticaseibacillus paracasei* DG, *Hafnia alvei* HA4597, *Bifidobacterium longum* BB536, and *B. animalis* subsp. *lactis* BL-04. Botanicals were tested up to a supraphysiological concentration (100 mg/mL, worst-case exposure). Subsequently, each extract (native and digested) was incubated for 48 h under anaerobic conditions with a complex bacterial consortium derived from multiple fecal aliquots obtained from a single healthy adult donor. Microbial composition was assessed using 16 S rRNA gene profiling. α-diversity (Shannon index) and β-diversity (Bray-Curtis distances) were computed, and differential abundance was analyzed.

Results In monoculture, botanical extracts exhibited minimal and strain-specific antimicrobial activity at 100 mg/mL, with inhibition observed primarily for *Akkermansia muciniphila*, *Bacteroides fragilis*, and *Collinsella aerofaciens*. In the fecal consortium model, none of the botanical treatments, regardless of digestion status, produced significant alterations in α- or β-diversity. Modest taxonomic changes were detected, including a reproducible decrease in *Collinsella* spp. following treatment with digested *Astragalus* extract. Notably, core beneficial taxa such as *Faecalibacterium prausnitzii* and *A. muciniphila* remained unaffected.

Conclusions At physiologically relevant concentrations, *Astragalus membranaceus*, pineapple stem, and bergamot extracts, in both native and digested forms, do not induce disruptive effects on the structure of the human gut microbial community, supporting their microbiome compatibility under the conditions tested.

Keywords Botanical extracts, Gut microbiota, Shannon index, *In vitro* digestion

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Background

Botanical extracts have emerged as major components of the functional food and nutraceutical markets. Their widespread use extends beyond foods and food supplements to encompass cosmetic and pharmaceutical applications, often as highly processed and standardized ingredients [1]. These products are typically marketed based on their bioactive content and associated health benefits, which may include antioxidant, anti-inflammatory, immunomodulatory, and metabolic regulatory effects [2, 3]. However, accumulating evidence indicates that botanical ingredients can also influence the composition and functionality of the intestinal microbiota, a key endogenous ecosystem contributing to host homeostasis [4, 5].

Historically, research on plant–microbiota interactions focused predominantly on the prebiotic properties of dietary fibers and indigestible carbohydrates found in plant material [6]. These substrates are known to selectively stimulate the growth of beneficial gut bacteria, such as *Bifidobacterium* spp. and *Lactobacillus* spp [7, 8]. However, botanical extracts are chemically complex mixtures that go well beyond carbohydrate content, delivering diverse phytochemicals (such as polyphenols, catechins, flavonoids, saponins, tannins, and terpenes) that can modulate the intestinal microbial ecosystem through distinct and sometimes antimicrobial mechanisms [9, 10]. These compounds are typically poorly absorbed in the upper gastrointestinal tract, resulting in low systemic bioavailability but prolonged exposure in the colon. This pharmacokinetic profile enables extended interaction with resident microbial communities and supports the hypothesis that these compounds can substantially shape the gut microbiota community structure.

In this context, multiple plant-derived compounds have demonstrated the capacity to inhibit enteric pathogens, including *Salmonella* spp. and *Escherichia coli*, by disrupting bacterial membrane integrity, altering cell wall synthesis, and interfering with critical metabolic and signaling pathways [11–14]. However, such antimicrobial activities are not inherently selective. Indeed, the same antimicrobial pressure may unintentionally decrease the abundance of commensal keystone taxa such as *Bifidobacterium* spp. and *Faecalibacterium prausnitzii*, and reduce overall microbial diversity [15–17], potentially triggering dysbiosis, an ecologically disrupted microbial configuration mechanistically linked to a variety of chronic pathologies such as obesity, type 2 diabetes, inflammatory bowel disease, and metabolic dysfunction-associated steatotic liver disease (MASLD) [18, 19]. Despite these potential consequences, the impact of botanical extracts on gut microbial ecosystems is rarely considered in their safety and efficacy evaluations. This knowledge gap highlights the need for studies that systematically investigate how plant-derived bioactives influence the gut microbiome, especially when these ingredients are used in functional foods and supplements.

With the primary aim of assessing microbiome compatibility and excluding adverse effects on gut microbial diversity and keystone taxa of the healthy adult gut microbiota, rather than demonstrating prebiotic or microbiota-enhancing efficacy, the present study evaluates the effects on gut microbiota composition of three commercially available botanical ingredients: *Astragalus membranaceus* root extract, pineapple stem extract, and bergamot extract. In particular, the *A. membranaceus* extract is rich in typical saponins and polysaccharides, and has been reported to attenuate inflammation and mitigate joints disorders [20]. The pineapple extract supplies bromelain, a cysteine protease with documented anti-inflammatory and immunomodulatory activities able to down regulate the activity of COX-2 [21]. The bergamot extract delivers flavonoids intended to attenuate inflammatory responses and improve plasma lipid profiles, offering promise for the dietary management of hyperlipidaemia and other metabolic disorders [22]. Each botanical extract, both in its native form and after simulated in vitro digestion, was evaluated using two complementary systems: (i) mono-culture growth assays with selected commensal strains and (ii) a complex consortium of microorganisms derived from human feces. This dual strategy allowed us to assess microbial responses at both targeted and community levels, with the primary aim of determining whether these extracts can modulate microbiota composition without impairing overall diversity or depleting essential keystone taxa.

Methods

Botanicals under investigation

The three botanical preparations examined in this study, named Axtragyl (AX), Bromeyal (BR), and Kalita (KA), are commercially available branded ingredients used in food supplements. AX is a standardized hydroalcoholic extract from the root of Astragalus membranaceus Bunge, sourced from plants cultivated in the Sichuan and Gansu provinces of China. BR is a proteolytic bromelain enzyme-rich preparation derived from the stem of Ananas comosus (pineapple). KA is obtained through spray-drying of secondary juices extracted from Citrus bergamia Risso (bergamot) fruits, following the procedure described by Della Vedova et al. [23]. All three preparations were kindly provided by Giellepi S.p.A. (Milan, Italy).

Cultivation of human intestinal bacterial strains in the presence of botanical ingredients

The following bacterial strains, selected for their human intestinal origin and relevance to human health, were used to assess the potential modulatory effects of the three botanical preparations: *Lactobacillus acidophilus* LA14, *Lacticaseibacillus paracasei* DG, *Hafnia alvei* HA4597, *Bifidobacterium longum* subsp. *longum* BB536, *Bifidobacterium animalis* subsp. *lactis* BL-04, *Odoribacter splanchnicus* S57, *Bacteroides fragilis* NCTC 9343, *Akkermansia muciniphila* DSM 22959, *Collinsella aerofaciens* DSM 3979.

L. acidophilus, L. paracasei, and H. alvei were isolated from commercial food supplements. The two Bifidobacterium strains were obtained from Giellepi S.p.A. (Milan, Italy), while O. splanchnicus S57 [24] was sourced from the microbial collection of the Department of Biotechnology and Biosciences (BtBs), University of Milano-Bicocca. A. muciniphila DSM 22959 and C. aerofaciens DSM 3979 were acquired from the Leibniz Institute DSMZ (Braunschweig, Germany). Finally, the B. fragilis strain originated from the culture collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan.

All bacterial strains were cultivated under anaerobic conditions using a Baker Ruskinn Bugbox Plus anaerobic chamber (Carli Biotec S.r.l., Frascati, Italy) maintained with a gas mixture of 90% N_2, 5% CO_2, and 5% H_2. Strict anaerobes were manipulated exclusively with pre-reduced media and buffers inside the anaerobic environment. Cultivation conditions were as follows: *L. acidophilus* and *L. paracasei* were grown in de Man, Rogosa, and Sharpe (MRS) medium (Difco, Franklin Lakes, NJ, USA). *Hafnia alvei* was cultured in tryptic soy broth (TSB), whereas both *Bifidobacterium* strains were cultured in MRS supplemented with 0.5 g/L cysteine hydrochloride (Sigma, St. Louis, MO, USA). *O. splanchnicus* and *B. fragilis* were grown in anaerobe basal broth (ABB) (Oxoid, Basingstoke, UK), while *A. muciniphila* was cultured in brain heart infusion (BHI) medium supplemented with 2 g/L bovine mucin (Sigma). *C. aerofaciens* was cultivated in Modified Reinforced Clostridial Medium (MRCM), composed of: tryptose (10 g/L), beef extract (10 g/L), yeast extract (3 g/L), dextrose (5 g/L), NaCl (5 g/L), soluble starch (1 g/L), L-cysteine hydrochloride (0.5 g/L), sodium acetate (3 g/L), and resazurin (4 mL of a 0.025% stock solution per liter).

To evaluate the impact of the botanical preparations on bacterial growth, the basal, strain-specific broth was first prepared at 1.25 × concentration. Each botanical product was dissolved in sterile Milli-Q water to yield a 500 mg mL^{-1} stock solution (corresponding to 5-fold the highest target test level).

Working media containing the botanicals at 1, 10, and 100 mg mL^{-1} were obtained by mixing appropriate volumes of the 1.25 × broth stock, the 500 mg mL^{-1} botanical stock, and sterile Milli-Q water. Before mixing, the pH of both the concentrated broth and each botanical stock was individually adjusted to the standard medium pH (7.0 ± 0.1). After dilution to the final 1 × strength, the pH of every preparation was re-checked; in > 90% of the cases the value remained within ± 0.05 pH units, confirming that the additional Milli-Q water did not measurably alter medium acidity.

Aliquots of 5 mL were distributed into sterile culture tubes sealed with cotton plugs and pre-reduced overnight under anaerobic conditions prior to inoculation. Pre-inocula were prepared 24 h in advance by transferring 50 µL of frozen glycerol stock into the appropriate medium. Prior to inoculation, the bacterial density of the pre-inoculum was determined using a counting chamber (Marienfeld, Lauda-Königshofen, Germany) and microscopy, and each tube was inoculated to achieve a final concentration of approximately 10^6 cells/mL. The following controls were included: (i) inoculated media without botanical ingredients, (ii) uninoculated media, and (iii) uninoculated media containing the highest concentration of each botanical product. Samples were collected at 48 h, serially diluted, and plated on strain-specific agar to determine viable counts. All experiments were conducted in triplicate.

In vitro *digestion of botanical ingredients*

Gastrointestinal digestion was simulated in vitro using the standardized INFOGEST protocol [25], a widely accepted method for mimicking human digestive processes. In brief, each of the three botanical powders underwent sequential digestion through oral, gastric, and intestinal phases. Initially, 1 g of each product was homogenized with simulated salivary fluid using a Stomacher 3500 peristaltic homogenizer (Seward, Worthing, UK) to replicate the oral phase. The homogenate was adjusted to a volume of 2 mL and agitated for 2 min at 37 °C. Subsequently, simulated gastric fluid was added along with rabbit gastric extract containing pepsin (RGE15, Lipolytech, Marseille, France), and the mixture was incubated at 37 °C for 2 h to simulate gastric digestion. For the intestinal phase, simulated intestinal fluid was introduced with appropriate pH adjustments, followed by the addition of porcine pancreatin (8× USP, Sigma-Aldrich, St. Louis, USA) and bovine bile salts. The final volume of each digested sample was adjusted to 8 mL. The samples were then incubated under agitation at 37 °C for another 2 h. Enzymatic activity was terminated by heating the digesta at 90 °C for 10 min in a water bath. All digestion experiments were performed in triplicate, and the resulting samples were stored at − 80 °C until further analysis.

Preparation of the microbial consortium

Three independent fecal samples were collected from a single healthy adult volunteer, corresponding to three distinct bowel movements obtained within a short time frame. Each sample was processed, suspended in pre-reduced PBS supplemented with glycerol, and cryopreserved separately under anaerobic conditions. The use of multiple samples from a single donor was intended to reduce intra-individual temporal variability while maintaining a controlled, donor-specific microbial background. The donor had not consumed antibiotics or probiotics for at least one month prior to sample collection. The collection was performed on-site using sterile collection containers, and the samples were processed (start to finish) within two hours of collection. Processing involved the weighing of the samples, followed by the insufflation of nitrogen gas into a Whirl-Pak bag (Nasco, Pleasant Prairie, Wisconsin, USA). Subsequently, an equal volume of pre-reduced phosphate-buffered saline (PBS) at pH 7 and 15% glycerol, equivalent to the weight of the fecal matter, was added under anaerobic conditions within the Baker Ruskinn Bugbox Plus anaerobic chamber. The fecal sample was then meticulously mixed by hand manipulation through the bag under these controlled conditions, aliquoted into 10 mL samples under the anaerobic chamber, and promptly stored at -80 °C. To assess the overall cell viability and integrity over time, multiple sample tubes were periodically thawed, diluted, and plated onto Brain Heart Infusion (BHI) agar with 2% (w/v) glucose and 0.3% (w/v) yeast extract (gyBHI). Culture-based enumeration and flow cytometry were used exclusively as functional quality controls to verify overall cell viability and membrane integrity after cryopreservation, and were not intended to assess taxonomic composition or relative abundance of individual microbial groups. Additionally, for flow cytometry analysis, samples were diluted and stained with SYTO24 and propidium iodide according to ISO 19344:2015 guidelines. The flow cytometry was performed using a BD Accuri™ C6 Plus Flow Cytometer (BD, Milan, Italy) after incubation at 37 °C for 15 min in the dark with the staining agents.

Incubations of the microbial consortium with botanical ingredients

The design of this experiment is reported in Fig. 1. In detail, on the day of incubation, aliquots of the microbial consortium (MC) were thawed inside the anaerobic cabinet. The three botanical preparations (either in their native form or after INFOGEST in vitro digestion) were added to the respective MC aliquots. Tested concentrations were selected based on physiological relevance, considering an estimated average bowel movement weight of 100 g and a recommended daily intake of approximately 500 mg per product. Accordingly, three concentrations were tested: 50 mg, 500 mg, and 5000 mg per 100 g of starting fecal material. The highest concentration was intentionally included as a worst-case exposure scenario to maximize the likelihood of detecting any adverse antimicrobial effects on gut microbial members. To achieve these dosages, botanical powders were resuspended and diluted in sterile, pre-reduced PBS, and 1 mL of each preparation was added to the corresponding fecal aliquots. Then, the inoculated suspensions were incubated for 48 h at 37 °C under anaerobic conditions. Samples (200 µL) were collected at 0, 12, 24, and 48 h, under strict anaerobic handling, and immediately stored at – 80 °C until subsequent DNA extraction.

DNA extraction from fecal suspensions and 16 S rRNA gene profiling

Fecal samples were stored at – 80 °C until DNA extraction. Upon thawing at 4 °C, the suspensions were homogenized by vortexing for 60 s at maximum speed. Then, 150 mg of each suspension was weighed and processed using the QIAsymphony PowerFecal Pro Kit (Qiagen, Hilden, Germany) on a QIAsymphony SP automated extraction platform, according to the manufacturer's instructions. DNA concentrations were quantified with the Qubit Broad Range Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and the resulting extracts were subjected to metataxonomic analysis via 16 S rRNA gene profiling. Amplicon sequencing targeted the V3–V4 hypervariable regions of the 16 S rRNA gene and was performed on the Illumina NovaSeq 6000 platform with paired-end 2 × 250 bp reads (NovaSeq 6000 SP Reagent Kit, 500 cycles). PCR amplification employed primers 341 F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (LC Sciences, Houston, TX, USA). Raw sequencing reads were processed in the QIIME 2 (v2022.2) environment, using the DADA2 algorithm [26] for quality filtering, denoising, and chimera removal. Taxonomic assignment of Amplicon Sequence Variants (ASVs) was carried out against the Greengenes (gg_13_8) reference database. To minimize batch effects and technical variability, all samples were sequenced in a single NovaSeq run. After ASV inference, singleton variants (i.e., ASVs represented by only one read across the entire dataset) were filtered out. The resulting ASV table was then rarefied to the minimum library size across samples (9598 reads per sample) to standardize sequencing depth. Read abundances underwent centered log-ratio transformation (CLR); α-diversity was estimated by calculating the Shannon index (H′) from the rarefied ASV Table (9598 reads per sample). Shannon index calculations were performed using the "diversity" plugin in QIIME 2 (v2022.2) on the rarefied ASV feature table. Weighted UniFrac distance matrices were generated with the "diversity" plugin of

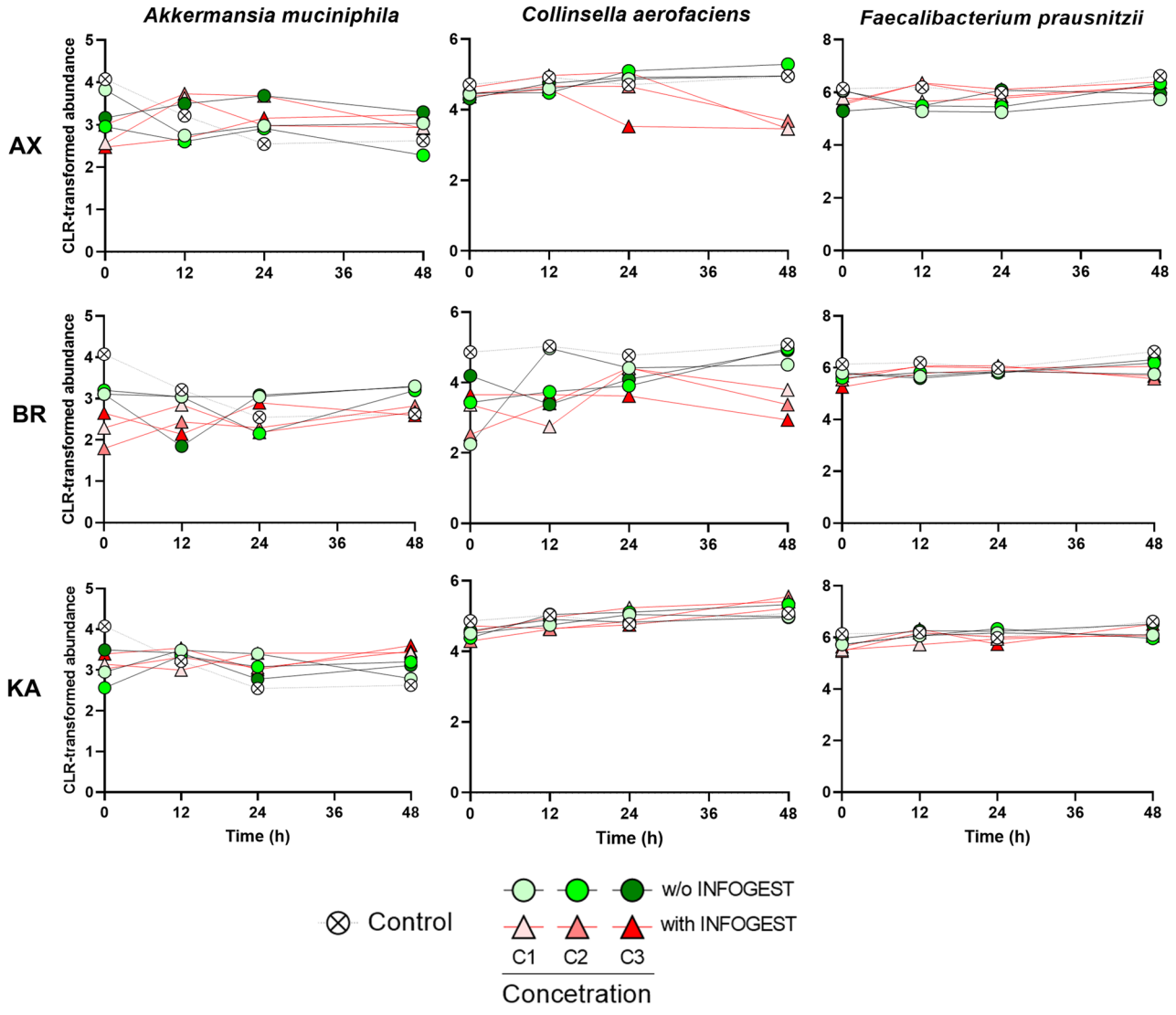

Fig. 1 Experimental design of microbial consortium (MC) incubations with botanical ingredients. MC samples were incubated with three different botanical preparations, either in their native form or after simulated gastrointestinal digestion (INFOGEST protocol). Each preparation was tested at three concentrations (C1, C2, and C3 = 50, 500, and 5000 mg per 100 g of MC suspension) under anaerobic conditions at 37 °C for 48 h. Samples were collected at 0, 12, 24, and 48 h for metataxonomic analysis

QIIME 2 (v2022.2) using the rarefied ASV table. Principal Coordinates Analysis (PCoA) was then applied to the distance matrices to visualize sample clustering patterns and assess community dissimilarities.

Statistical analysis

Colony-forming units were expressed as decimal logarithms ($\log_{10}$ CFU/mL) prior to analysis to stabilize variances and approximate normality. For each bacterial strain, the effect of treatment (four levels: control medium, AX, BR, or KA) and dose (three concentrations: 1, 10 and 100 mg/mL) on bacterial growth was evaluated by a one-way analysis of variance (ANOVA) performed independently for each strain. Normality of the residuals and homoscedasticity were verified with the Shapiro–Wilk and Levene's tests, respectively; all datasets met parametric assumptions. When the global ANOVA was significant ($P < 0.05$), pair-wise differences among group means were resolved with Tukey's honestly significant difference (HSD) post hoc test, which controls the family-wise Type I error rate for multiple comparisons. Data are presented as mean ± standard deviation (SD) obtained from three independent biological replicates. All statistical computations were executed with GraphPad Prism v8.4.3 (GraphPad Software, LCC, San Diego, CA, USA), and significance was defined at a two-tailed α level of 0.05.

16 S rRNA profiling data lack the independent observations required to estimate residual variance, because every extract–dose combination was represented by a

single biological replicate; consequently, either α-diversity and single taxa relative abundances are reported descriptively and have been interpreted qualitatively.

The analysis of β-diversity was performed through Pairwise Analysis of Similarities (ANOSIM) to assess differences in microbial community structure between two experimental conditions (between incubation times, and with vs. without INFOGEST), using sample scores on the first two axes (PC1 and PC2) of a weighted UniFrac PCoA. Euclidean distance matrices were computed from the two-dimensional coordinate matrix using the pdist function in SciPy (v1.7.1). The ANOSIM R statistic was calculated according to Clarke [27]. Statistical significance was assessed by 999 random permutations of group labels, with the permutation-based p-value defined as the proportion of permuted R values that were greater than or equal to the observed R. All computations were implemented in Python (v3.8) with NumPy (v1.20.3) and pandas (v1.3.4).

Results

Impact of botanical extracts on the growth of individual gut microbial strains

The potential inhibitory effects of the botanical extracts under investigation were assessed on the growth of nine human gut bacterial strains. Each extract was evaluated at three concentrations (1, 10, and 100 mg/mL) to determine any dose-dependent responses. Following 48 h of incubation in strain-specific optimal culture media, the results indicated that AX exerted negligible influence on the growth of bacterial strains. A notable exception was Akkermansia muciniphila, whose proliferation was markedly suppressed at the highest AX concentration (100 mg/mL) (Fig. 2). Additionally, a significant reduction of approximately 1 log CFU was observed for Bacteroides fragilis at 10 and 100 mg/mL, as well as for Odoribacter splanchnicus. A milder yet statistically significant decrease (less than 0.5 log CFU) was detected for Collinsella aerofaciens and Bifidobacterium longum.

BR demonstrated a modest inhibitory effect, characterized by an approximate 1 log CFU reduction, exclusively against *C. aerofaciens* across all tested concentrations. A slight decrease (under 0.5 log CFU) was also noted for *B. longum* at 1 and 10 mg/mL (Fig. 2). Similarly, KA induced a slight but statistically significant reduction in *B. longum* counts at 10 and 100 mg/mL. Notably, KA exhibited a more pronounced dose-dependent inhibitory activity against *B. fragilis* and significantly reduced *A. muciniphila* growth only at the highest concentration tested (Fig. 2).

Collectively, these findings suggest that the botanical extracts possess limited inhibitory activity, predominantly at the highest concentration (100 mg/mL), with primary effects observed on the Gram-negative bacteria *B. fragilis* and *A. muciniphila*. Importantly, BR did not elicit substantial inhibition of any of the tested human gut bacterial strains.

Stability and taxonomic composition of the MC preparations

To evaluate the effects of botanical extracts on a complex microbial ecosystem, we prepared a MC based on the microorganisms of human fecal samples. Prior to conducting the main experiments, preliminary assessments were performed to determine whether the addition of prereduced PBS and glycerol could preserve bacterial viability during storage at − 80 °C.

Cultivation assays on gyBHI agar demonstrated that, in the absence of cryoprotectants, viable bacterial counts decreased by approximately 1.5 log CFU over a 7-day storage period. Conversely, samples supplemented with PBS and glycerol exhibited no significant reduction in viable counts under identical storage conditions (Fig. 3(a)). Flow cytometry analyses further corroborated these findings, revealing that the proportion of bacterial cells with intact membranes remained stable in samples treated with PBS and glycerol, whereas a decline was observed in untreated controls (Fig. 3(b)). Subsequently, three aliquots of the MC preparations stored at − 80 °C were subjected to 16 S rRNA gene profiling to assess the taxonomic composition. The analysis revealed a microbial community dominated by families commonly associated with the human colonic microbiota, including Ruminococcaceae, Lachnospiraceae, Coriobacteriaceae, Bacteroidaceae, and Bifidobacteriaceae (Fig. 3(c)).

Collectively, these results indicate that the implemented cryopreservation strategy effectively maintains both the viability and taxonomic integrity of bacterial populations during storage at − 80 °C, thereby facilitating subsequent in vitro experimentation with preserved MC aliquots.

Impact of botanical extracts on the bacterial community structure of stabilized MC suspensions

The influence of botanical preparations on the microbial community within stabilized MC suspensions was evaluated at three concentrations (C1, C2, and C3) over a 48-h incubation period, with sampling at 0, 12, 24, and 48 h. The resulting metataxonomic data were analyzed descriptively and interpreted qualitatively, as detailed in Sect. 2.7 (Statistical Analysis).

Analysis of α-diversity (assessed using the Shannon index, which accounts for both richness and evenness of bacterial taxa) showed no significant differences between treatment conditions, with Shannon values remaining consistently near 8 throughout the incubation (Fig. 4). Shannon index values are presented to illustrate temporal trends and overall stability of α-diversity across

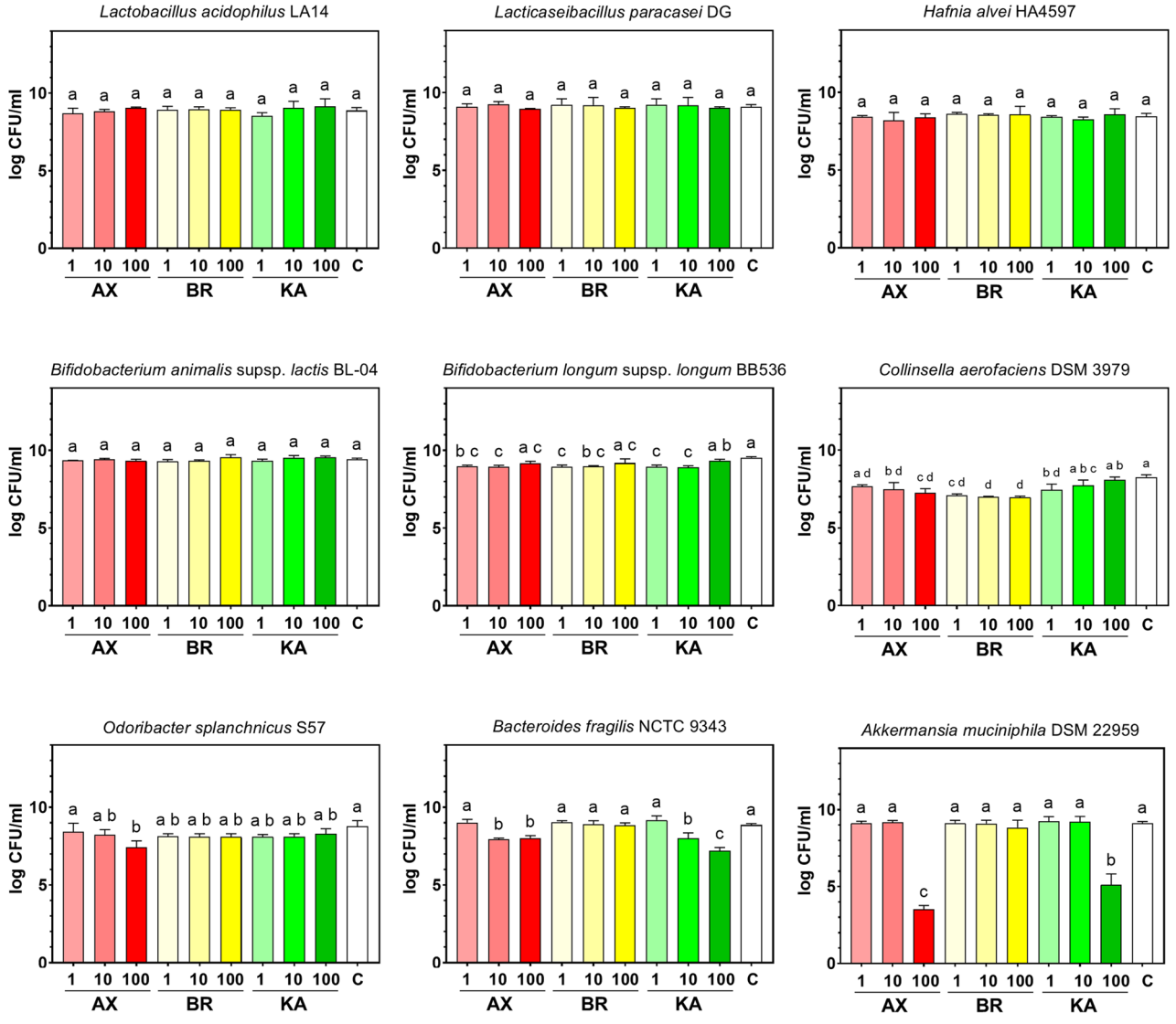

Fig. 2 Impact of botanical extracts on the growth of selected human gut bacterial strains at varying concentrations (1, 10, and 100 mg/mL). The botanical extracts evaluated include *Astragalus membranaceus* root extract (AX), pineapple stem extract (BR), and bergamot extract (KA). Bacterial growth was quantified by enumerating colony-forming units (CFU) on agar plates, expressed as the decimal logarithm (log_{10} CFU). The control (C) represents cultures incubated in media devoid of botanical additives. Each bar denotes the mean ± standard deviation derived from three independent experiments. Distinct letters above the bars signify statistically significant differences ($P < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test

treatments, rather than to emphasize small quantitative differences between conditions.

The analysis of β-diversity, based on the weighted UniFrac metric, indicated that temporal progression was the primary driver of changes in overall community structure, rather than the specific botanical treatments or their concentrations. In fact, ANOSIM showed significant differences between samples at T0 and subsequent time points: T0 vs. T12 ($n = 19$ per group; $R = 0.280$; $P = 0.004$), T0 vs. T24 ($R = 0.439$; $P = 0.001$), T0 vs. T48 ($R = 1.147$; $P = 0.001$), and T24 vs. T48 ($R = 0.472$; $P = 0.01$). Notably, ANOSIM did not reveal significant differences between samples subjected to INFOGEST pre-treatment and those that were not ($n = 36$ vs. $n = 40$; $R = 0.008$; $P = 0.319$) (Fig. 5(b)).

Effect of botanical extracts on the abundance of specific bacterial taxa in the MC suspension

Given the overall stability of community structure, taxon-specific analyses were restricted to bacterial groups showing consistent and biologically meaningful trends across treatments. The impact of botanical preparations on the relative abundance of specific bacterial taxa was further assessed by monitoring the top 10 most abundant families and genera in the initial MC sample over the 48-h incubation period. Overall, none of these taxa exhibited drastic reductions in relative abundance (Supplementary Figure S1). The most notable decreases were observed in the genus Oscillospira with AX treatment without INFOGEST pre-treatment at lower concentrations, and

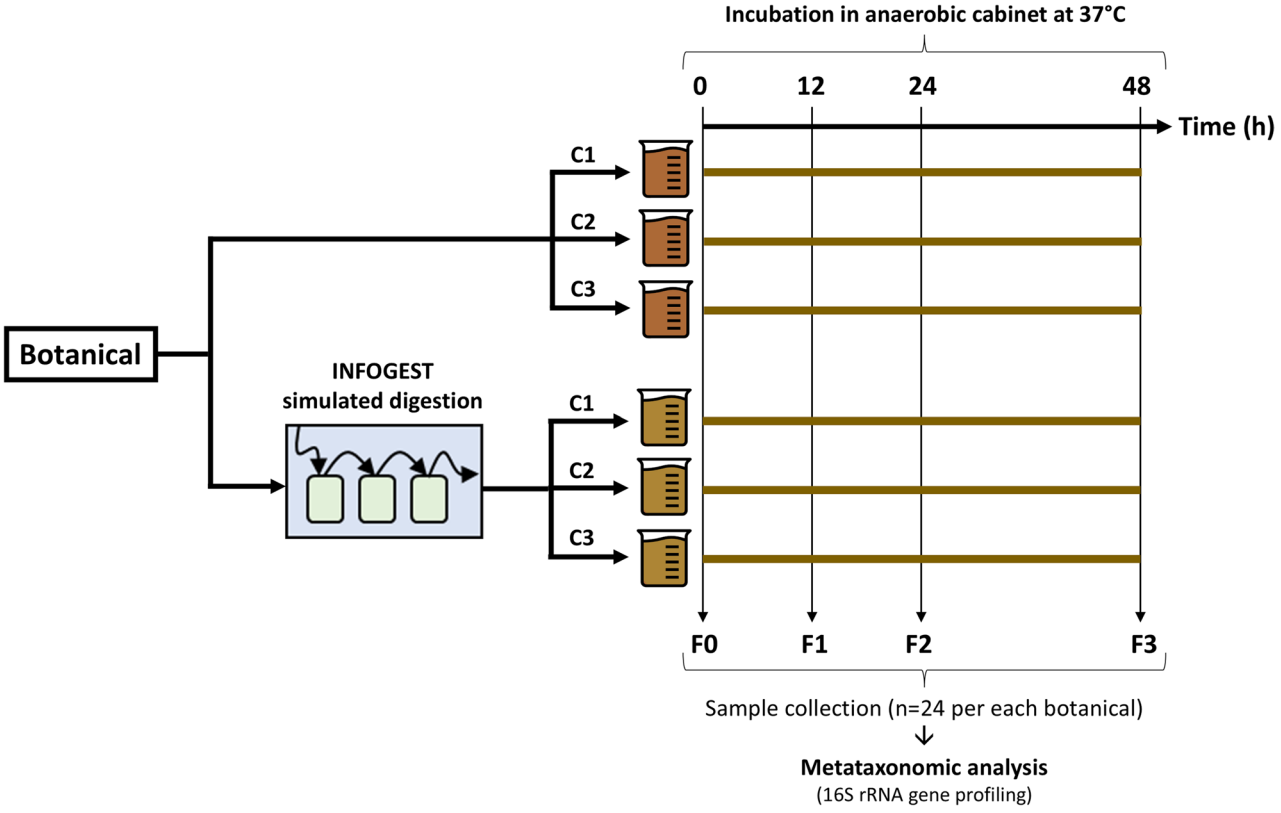

Fig. 3 Microbiological characterization of the stabilized microbial consortium suspension utilized in incubation experiments with botanical extracts. The sample was analyzed with (treated) and without (control) the addition of phosphate-buffered saline (PBS) and glycerol. Viable bacterial counts were determined by plating on brain heart infusion (BHI) agar medium before and after 3 and 7 days of storage at −80 °C (panel a). The same samples were subjected to flow cytometry following dual staining with SYTO and propidium iodide to assess membrane integrity; cells with intact (green), damaged (grey), and disrupted (red) membranes were differentially quantified (panel b). Three aliquots of the stabilized MC suspension (treated) were analyzed through 16 S rRNA gene profiling to determine taxonomic composition (panel c). Different letters in panels (a) and (b) indicate statistically significant differences at $P < 0.05$, as determined by one-way ANOVA followed by Tukey's post hoc test

in the genus Collinsella following treatment with BR (in a dose-dependent manner) and AX, both after simulated INFOGEST digestion. Manual taxonomic analysis of amplicon sequence variants (ASVs) via BLASTn confirmed that sequences assigned to the genus Collinsella corresponded to Collinsella aerofaciens (Fig. 6). Similarly, ASVs corresponding to Akkermansia muciniphila and Faecalibacterium prausnitzii, both recognized for their relevance to human health, were evaluated. The relative abundances of these species remained mostly unaffected across all incubation conditions, with the only exception of an increase in the relative abundance of A. muciniphila with KA compared with the control (Fig. 6).

Discussion

Plant-derived preparations are promoted for antioxidant, anti-inflammatory and metabolic benefits, yet many of their signature molecules (flavanones, triterpenoid saponins, cysteine proteases, catechins, thiosulfinates or essential-oil terpenes) have been frequently reported to display antibacterial properties [28–30]. Since 90–95% of dietary polyphenols and related phytochemicals reportedly escape absorption in the small intestine and reach the colon [31], it can be hypothesized that these compounds may also act upon gut-resident microbes, potentially altering community structure and reducing global diversity.

To explore this hypothesis, we conducted in vitro testing of three commercial botanical preparations, originally developed for health benefits not primarily related to intestinal function, for their capacity to impact human gut microbiota. We selected three formulations containing distinct categories of phytochemicals with known broad-spectrum antimicrobial properties. AX contains *Astragalus*-derived saponins (e.g., astragaloside IV) and flavonoids that permeabilize bacterial membranes; ethanolic extracts inhibit a wide array of pathogens and commensals in vitro [32, 33]. BR supplies bromelain, a cysteine protease that degrades surface adhesins and peptidoglycan in *E. coli* and other Gram-negative bacteria [34–36]. Finally, Bergamot juice powders such as KA are enriched in glycosylated flavanones (naringin,

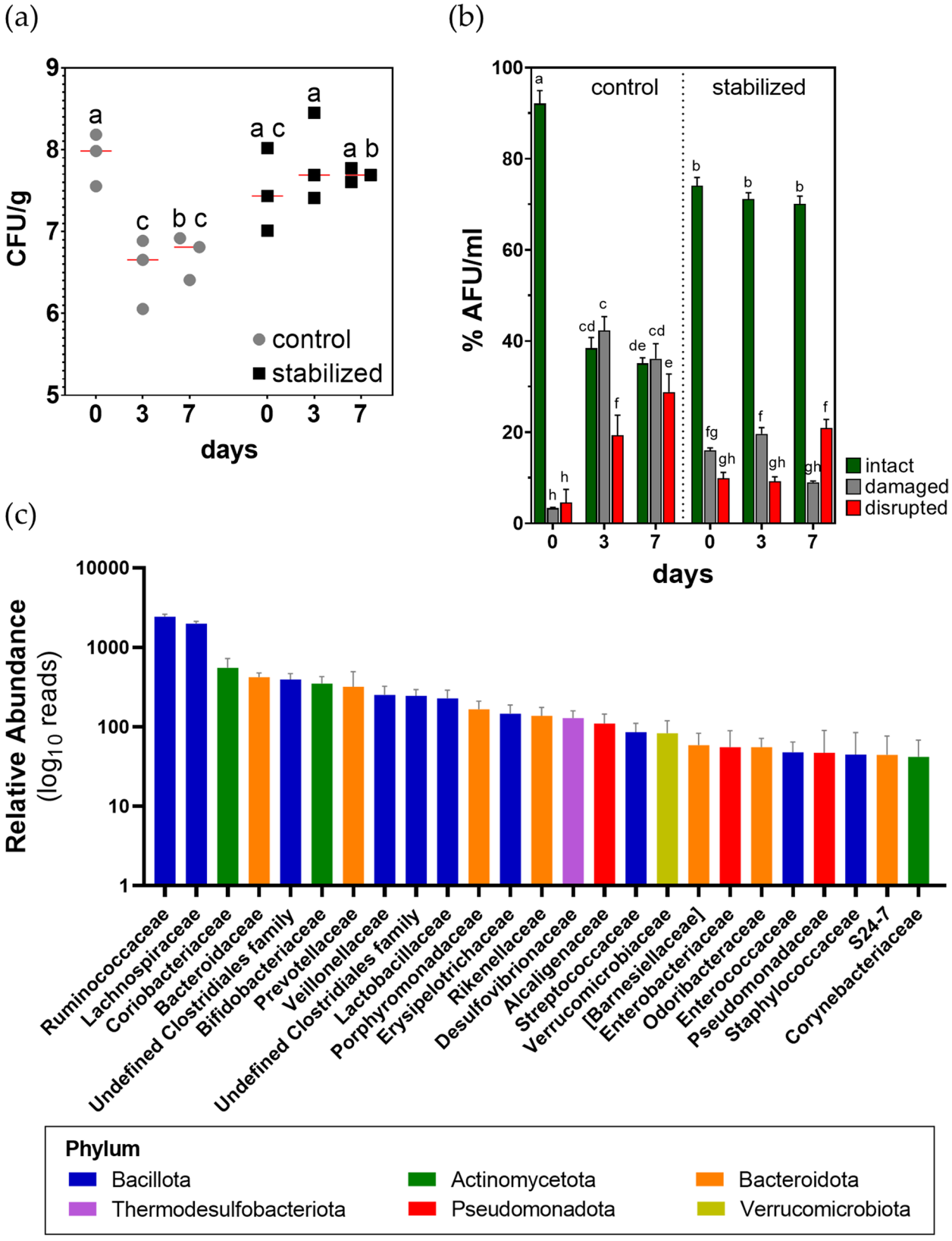

Fig. 4 α-diversity analysis using the Shannon index of MC samples incubated with three botanical extracts (AX, *Astragalus membranaceus* root extract; BR, pineapple stem extract; KA, bergamot extract), with or without INFOGEST pre-treatment. Extracts were added at concentrations of 50 (C1), 500 (C2), and 5000 (C3) mg per 100 g of MC material

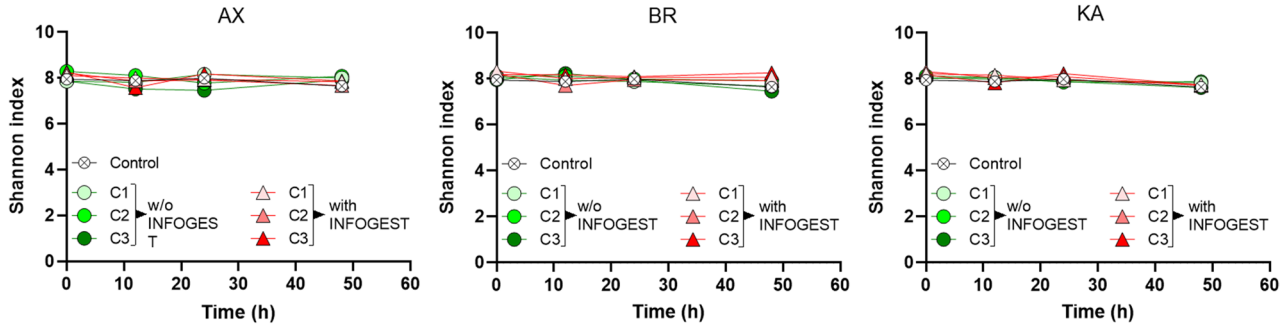

Fig. 5 β-diversity analysis of microbial consortium (MC) samples based on weighted UniFrac distances. MC samples were incubated with three botanical extracts, with or without INFOGEST pre-treatment, at concentrations of (a) 50 mg/100 g, (b) 500 mg/100 g, and (c) 5000 mg/100 g of MC material. Principal coordinate analysis plots show sample clustering according to incubation time points (T0 = baseline, T12, T24, and T48 h; panel A) or according to INFOGEST pre-treatment (panel B). C, control (MC incubated without botanical extracts); AX, *Astragalus membranaceus* root extract; BR, pineapple stem extract; KA, bergamot extract

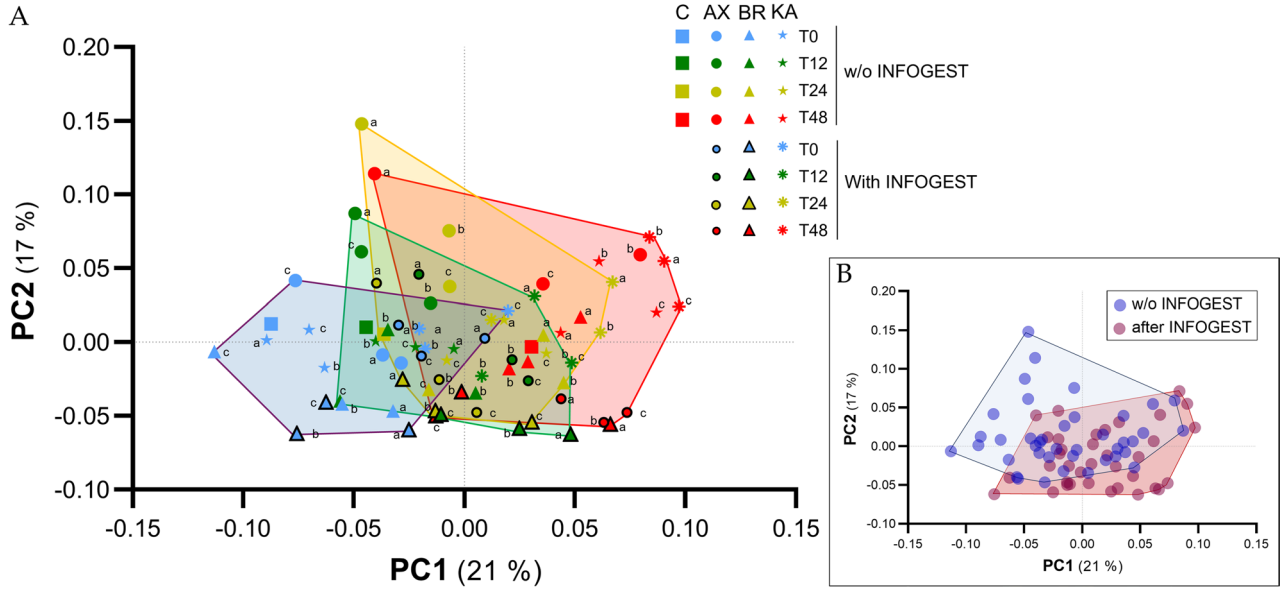

Fig. 6 Impact of botanical preparations on the relative abundance of selected bacterial species in the microbial consortium (MC) suspension. Extracts were applied at 50 (C1), 500 (C2), and 5000 (C3) mg per 100 g of MC material. AX, *Astragalus membranaceus* root extract; BR, pineapple stem extract; KA, bergamot extract Abundances are shown as centered log-ratio (CLR) transformed read counts. Each botanical was tested both in its native form (w/o INFOGEST) and after simulated in vitro digestion using the INFOGEST protocol (w/ INFOGEST)

neohesperidin, neoeriocitrin) that, once deglycosylated, disrupt bacterial membranes and inhibit both Gram-negative and Gram-positive species, including lactic acid bacteria [37]. Notably, pure naringenin suppresses *Escherichia coli*, *Streptococcus* spp. and *Staphylococcus aureus* at micromolar levels [38, 39].

These compositional features suggest that botanical preparations may pose a risk of unintended antimicrobial activity against beneficial gut microbes. Nevertheless, current regulatory frameworks, such as those of EFSA and FDA, primarily focus on toxicological endpoints (e.g., acute or sub-chronic toxicity, contaminant screening) and do not require evaluation of gut microbiota structure or function [40]. This regulatory gap remains, despite growing evidence linking dysbiosis to multiple chronic diseases [41].

To address this issue, we first evaluated the antimicrobial activity of the three botanical preparations against specific bacterial strains cultivated in monoculture in their respective optimal media. Five of the selected strains have intestinal origin and exhibit documented probiotic potential: *Lactobacillus acidophilus* LA14 (shown to increase lymphocyte counts and protect against liver injury in mice; [42, 43]), *Lacticaseibacillus paracasei* DG (enhances antiviral responses, and modulates immune responses and gut microbiome; [44–46]), *Hafnia alvei* HA4597 (produces the ClpB protein with satiety-mimetic activity; [47–49]), *Bifidobacterium longum* subsp. *longum* BB536 (a well-characterized probiotic with gastrointestinal and immune benefits; [50–52]), and *Bifidobacterium animalis* subsp. *lactis* BL-04 (supports antiviral immunity

and reduces upper respiratory infections; [53, 54]). None of these five strains exhibited impaired growth in monoculture upon incubation with the botanical preparations, even at the highest tested concentration (100 mg/mL), suggesting compatibility of AX, BR, and KA with probiotic supplementation under the tested conditions.

In contrast, mild inhibitory effects were observed against four additional gut-associated bacterial strains. Specifically, AX exhibited slight inhibition of *Odoribacter splanchnicus* S57, a butyrate-producing anaerobe that releases anti-inflammatory outer membrane vesicles [24, 55]. However, this effect was only significant at 100 mg/mL, far exceeding the expected luminal concentration in the colon, given the recommended daily intake of 480 mg. AX and, especially, KA moderately inhibited *Bacteroides fragilis* NCTC 9343, a non-toxigenic strain producing capsular polysaccharide A (PSA), known to induce regulatory T cells and restore Th1/Th2 balance in germ-free mice [56]. In contrast, enterotoxigenic *B. fragilis* (ETBF) strains carrying the *bft* gene are associated with diarrheal disease and colitis [57]. All three botanicals, particularly KA, also showed moderate inhibition of *Collinsella aerofaciens* DSM 3979, an anaerobic bacterium involved in bile acid metabolism. Increased *Collinsella* levels have been associated with obesity, NAFLD, IBS, and rheumatoid arthritis, possibly via promotion of low-grade inflammation [58–60]. The only marked inhibition was observed for *Akkermansia muciniphila* DSM 22,959, a mucin-degrading strain considered a next-generation probiotic with proven metabolic benefits (e.g., increased acetate/propionate, improved insulin sensitivity; [61–63]). However, this inhibition occurred only at the unrealistic dose of 100 mg/mL, making in vivo suppression unlikely under physiological conditions.

Phytochemicals often exert antimicrobial activity in their aglycone form, whereas they occur predominantly as glycosides in plants [64–66]. Human colonic microbiota can enzymatically release these aglycones [67], thereby uncovering bioactivities that would otherwise remain undetectable in axenic culture. Therefore, we next evaluated the impact of the three preparations on a complex microbial community prepared on the basis of the microorganisms present in human feces. We also assessed the effect of a simulated gastrointestinal digestion step on their bioactivity, as the matrix in which phytochemicals are embedded can influence their accessibility and, consequently, their biological activity.

For this experiment, we adopted specific measures for the preparation of the MC suspension to preserve microbial viability, including the use of pre-reduced PBS supplemented with glycerol and strict anaerobic handling in a workstation flushed with a reducing gas mixture. This protocol proved highly effective in maintaining microbial viability, even after storage at – 80 °C. Consequently, we were able to perform the experiment using aliquots that were compositionally stable and microbiologically consistent throughout the study. The microbial consortium was prepared with the fecal microorganisms from a single donor, resulting in a taxonomic profile encompassed key constituents of the adult gut microbiota, including families of the phylum Bacillota such as Ruminococcaceae and Lachnospiraceae (major butyrate producers and metabolically active fermenters [68, 69], families of the phylum Bacteroidota such as Bacteroidaceae and Prevotellaceae (dominant in two of the three canonical human enterotypes; [70]), and Gram-positive families Bifidobacteriaceae and Lactobacillaceae, whose strains confer a number of documented health benefits. Nonetheless, it must be emphasized that the design of this study limits the interpretation to a predominantly qualitative assessment of the results.

Unexpectedly, we found no evidence that INFOGEST-pretreated botanicals induced more substantial changes in the MC microbiota than their native (non-digested) forms, despite the simulated digestion reagents having known antimicrobial activity. This result can be explained primarily by the fact that, in accordance with the INFOGEST protocol, samples were subjected to heat treatment to inactivate enzymes prior to their addition to the MC suspensions. Furthermore, the intestinal bacteria present in the MC suspension are inherently adapted to withstand antimicrobial molecules, such as bile salts, which are naturally abundant in their ecological niche.

The primary finding of this study is that, despite the botanical preparations used containing substantial quantities of antimicrobial molecules, none of them triggered major compositional disruptions or a reduction in overall microbial diversity. Overall, we observed only few taxon-specific changes, the most relevant being the reduction of *Collinsella* spp., and specifically *C. aerofaciens*, following simulated digestion of AX. From a translational standpoint, the selective attenuation of *Collinsella* could be metabolically advantageous, as these taxa have been associated with pathological conditions such as rheumatoid arthritis and non-alcoholic fatty liver disease [60, 71]. In the context of food supplements, the absence of microbiota disruption should be interpreted as a safety-relevant outcome, rather than as a lack of microbiota-modulating efficacy.

It should be acknowledged that 16 S rRNA gene profiling is a DNA-based approach that does not discriminate between viable cells and DNA derived from damaged or recently lysed microorganisms. As such, this method is not designed to provide direct information on microbial viability following exposure to potentially antimicrobial compounds. However, the primary objective of the present study was to detect treatment-associated alterations in community structure and relative abundance, rather than to quantify taxon-specific viability. In this

framework, the absence of major shifts in α-diversity, β-diversity, and dominant taxa suggests that the tested botanical preparations did not exert disruptive effects on the gut microbial ecosystem. Dedicated approaches, such as viability PCR, RNA-based profiling, or cultivation-based analyses, would be required to specifically address microbial viability at the taxon level. The chemical composition of the botanical extracts following INFOGEST digestion was not analytically characterized; therefore, digestion-induced molecular changes cannot be directly linked to specific microbiota effects observed in this study. In addition, functional analyses and metabolite profiling were not performed; therefore, the present study does not address treatment-associated changes in microbial metabolic activity or the production of bioactive end products relevant to host physiology. pH was not monitored during the in vitro fecal incubations, as the study was not designed to investigate fermentation-associated metabolic outputs; therefore, potential treatment-related pH variations cannot be formally excluded.

In summary, neither α-diversity nor β-diversity exhibited significant treatment-dependent alterations, with time being the predominant driver of the observed community shifts. Several factors may account for this microbial resilience. First, within the gastrointestinal context, 90–95% of dietary polyphenols and related phytochemicals escape absorption in the small intestine and reach the colon embedded in a protein- and fiber-rich matrix. This matrix sequesters amphipathic aglycones via hydrogen bonding and hydrophobic interactions [72], reducing their freely diffusible concentration by one to two orders of magnitude, well below most reported minimum inhibitory concentrations. Second, unabsorbed compounds undergo extensive microbial biotransformation. For example, Bacteroides-derived β-glucosidases cleave naringin into naringenin, which is subsequently reduced, ring-cleaved, and dehydroxylated into phenyl-γ-valerolactones, which are metabolites with attenuated antimicrobial activity and potentially prebiotic effects [72]. Saponins follow a similar pathway, in which terminal glucose residues are sequentially removed, generating sapogenins that integrate into micelles rather than bacterial membranes [73]. Findings from studies on garlic, tea, cranberry, oregano, and crucifer-derived isothiocyanates support these observations: although bactericidal in broth-based assays, they elicit only modest shifts in microbial composition in vivo [28, 30, 74]. Our results extend this paradigm to bergamot flavanones, *Astragalus*-derived saponins, and bromelain, reinforcing the notion that predictions based solely on reductionist in vitro assays must be tempered by ecological considerations.

Conclusions

This study integrated mono-culture assays with a stabilized microbial consortium, providing a multilayered perspective on microbial responses to botanical exposures. The results indicate that the three tested commercial botanical preparations rich in potentially antimicrobial phytochemicals, did not induce significant reductions in overall microbial diversity or inhibit key members of the human gut microbiota when tested at physiologically relevant concentrations. These findings support the classification of such botanical preparations as microbiome-compatible ("***microbiome-friendly***") functional ingredients, while highlighting the importance of conducting community-scale monitoring using the whole botanical matrix, rather than relying solely on taxon-specific antimicrobial evaluations with isolated phytochemicals, to accurately predict the intestinal footprint of plant-derived bioactives.

Abbreviations

AX	Astragalus membranaceus root extract (Axtragyl®)
BR	pineapple stem extract (Bromeyal®)
KA	bergamot extract (Kalita®)
MC	microbial consortium
CFU	colony-forming units
ASV	amplicon sequence variant
PCoA	principal coordinates analysis
ANOSIM	analysis of similarities
CLR	centered log-ratio

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

R.D. and S.G. drafted the original manuscript. R.D., R.R., and S.G. conceived the study. R.D., G.M., and G.G. developed the methodology and performed the experiments. G.G. and S.G. conducted the formal data analysis. R.D., G.G., and S.G. curated the data. R.R. provided materials and resources and, together with S.G., secured funding. S.G. supervised the project. All authors reviewed and approved the final manuscript.

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Data availability

The 16 S rRNA gene amplicon sequencing data generated in this study have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB91377. All other data supporting the findings of this study are included in this published article and its supplementary information files or are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The study involved exclusively ex vivo incubation of fecal material donated by a single healthy adult volunteer, with no additional interventions on human participants. Ethical approval was obtained from the Research Ethics Committee of the University of Milan (opinion no. 89/22). Written informed consent to participate in the study was obtained from the donor prior to sample collection. All procedures complied with the principles of the Declaration of Helsinki and with applicable institutional and national guidelines.

Consent for publication
Written informed consent for the publication of anonymized data was obtained from the donor prior to sample collection.

Competing interests
R.R. is employed by Giellepi S.p.A. and was not involved in the data analysis or interpretation of results. R.D.'s doctoral fellowship is co-funded by Giellepi S.p.A.

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