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Phenolic compounds' impact on gut microbiota: Insights from *in vitro* batch fecal fermentation for composition modulation

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ABSTRACT

The relationship between phenolic compounds and gut microbiota (has been widely studied to explore the health benefits of these bioactive dietary compounds. Phenolic compounds are metabolized by gut microbiota, while also modulating its composition. However, the individual effects of these compounds on human gut microbiota remain underexplored. To address this, three phenolic compouds—ellagic acid, naringenin, and phloroglucinol—underwent *in vitro* batch fermentation with fecal samples from healthy donors. Samples were analyzed through 16S metagenomics sequencing, and short-chain fatty acids (SCFAs) were measured using gas chromatography.

Results showed that ellagic acid and phloroglucinol had prebiotic properties, producing SCFAs like acetic, propanoic, and butyric acids and promoting the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*. In contrast, naringenin was linked to the growth of pathogenic genera like *Escherichia* and *Salmonella*. This study provides valuable insights into how specific phenolic compounds influence gut microbiota composition, contributing to potential pharmaceutical or nutraceutical developments.

1. Introduction

Human gut microbiota (GM) is currently regarded as an isolate metabolizing "forgotten organ," unique as a fingerprint (Gowd et al., 2019). It consists in a complex microbial community (mainly bacteria) that colonize the human intestinal tract (Espín et al., 2017), with about 10^{14} bacterial cells and more than 1000 microbial species (Cardona et al., 2013; Duda-Chodak et al., 2015). Humans and these gut microorganisms have developed a symbiotic relationship (Espín et al., 2017). However, GM greatly influences human health (Ozdal et al., 2016) and is essential to the immunological system and metabolic functions (Espín et al., 2017). So, when an alteration of GM composition and/or function occurs, it could result in some human diseases (namely, obesity, inflammatory bowel disease (IBD), cardiovascular and even mental diseases, among others), defined as dysbiosis (Espín et al., 2017; Gentile & Weir, 2018; Veiga et al., 2020). GM bacteria, which correspond in majority to the most common phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia, could be determinant to intestine health, being their ratio, especially the one of F/B an important marker (Duda-Chodak et al., 2015; Rinninella et al., 2019). A wide array of factors influences GM composition, including internal factors related to the host—such as age, immune system function, and genetics—as well as external factors, notably dietary habits, antibiotic use, and environmental conditions (Duda-Chodak et al., 2015; Espín et al., 2017; Rinninella et al., 2019).

Diet has been seen as a composition and function modulator of GM (Espín et al., 2017), with some compounds, such as phenolic compounds (PCs), being poorly absorbed in the small intestine (only about 10–15 %) and reaching the colon, where they could be metabolized by GM (Gowd et al., 2019; Ozdal et al., 2016). PCs, which are secondary metabolites well-known by their wide structural diversity and commonly present in a wide variety of plants and food products, have been broadly studied due to their vast range of biological activities (antioxidant, anti-inflammatory, anti-proliferative, and antimicrobial activities, among others), essential to prevent/ treat some health disorders (Ozdal et al., 2016). However, these beneficial effects on human health are strictly

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related to their structure; thus, phenolic metabolites, resulting from GM activity, could be more or less bioactive-promising depending on their structural features (Dueñas et al., 2015; Gowd et al., 2019).

Given the identification of thousands of PCs with diverse structures and their structure-activity relationship, the human health beneficial effects of PCs' metabolites produced by GM activity may vary significantly (Dueñas et al., 2015; Gowd et al., 2019). The scientific community has established a two-way relationship between GM and dietary PCs. In addition to the metabolism of PCs by GM mentioned above, these compounds and their metabolites also simultaneously affect the GM composition (Braune & Blaut, 2016; Espín et al., 2017; Ozdal et al., 2016).

Dietary PCs and their metabolites can be considered prebiotics because of their selective ability to promote the growth of beneficial gut bacteria, such as lactobacilli and bifidobacteria, or to act as antimicrobial agents against pathogenic bacteria, thereby offering health benefits to the human host (Gowd et al., 2019; Ozdal et al., 2016). In fact, the modulatory effect on GM composition of some PCs has been widely evaluated, as for example in the cases of gallic acid, caffeic acid, resveratrol, and quercetin (QUE) (Bialonska et al., 2009; Duda-Chodak, 2012; Gwiazdowska et al., 2015; Hervert-Hernández et al., 2009; Hidalgo et al., 2012; Kawabata et al., 2013; Larrosa et al., 2009; Lee et al., 2006; Parkar et al., 2008, 2013; Qiao et al., 2014; Raimondi et al., 2015; Selma et al., 2012; Volstatova et al., 2017; Yang et al., 2019).

However, some of published studies only evaluate the effect of PCs in isolated intestinal bacteria, hindering the understanding of their effect in the presence of a complex bacterial community (Bialonska et al., 2009; Duda-Chodak, 2012; Parkar et al., 2008).

Moreover, the modulatory effects of some PCs have been associated to the effects of complex food matrices or enriched extracts, namely tannins or proanthocyanidins from grape, cranberry, blueberry and respective byproducts, among others (Anhê et al., 2015; Choy et al., 2014; Rodríguez-Daza et al., 2020; Roopchand et al., 2015; Yamakoshi et al., 2001). Furthermore, some of these studies usually employ complex communities of gut bacteria, associated with a disease condition (Anhê et al., 2015; Etxeberria et al., 2015; Larrosa et al., 2009; Qiao et al., 2014; Rodríguez-Daza et al., 2020; Roopchand et al., 2015; Yang et al., 2019), which contribute to the knowledge of PCs' prebiotic capacity or even their ability in disease control. However, their effect in a "healthy"-regarded GM community was not accessed, which could be important in the development of nutraceuticals or pharmaceuticals formulations to determine if these compounds could lead to undesirable effects.

Hence, this study aims to evaluate the influence of some specific PCs on human GM composition, with the goal of understanding their potential beneficial and/or adverse effects on GM modulation and their consequent effects on human health. For that, five PCs were selected for the study, namely ellagic acid (EA), QUE, naringenin (NAR), naringin (NARN) and phloroglucinol (PG), taking into consideration the results obtained in a previous study of their bioaccessibility and intestinal absorption (Pais et al., 2024).

To achieve that goal, the antimicrobial activity or prebiotic effects of the selected PCs was first evaluated. Based on the obtained results and the lack of information regarding the impact of isolated compounds on GM composition in literature, EA, NAR and PG were subjected to an *in vitro* batch fermentation with human fecal inoculum and the collected samples from different fermentation times were subsequently analyzed through 16S amplicon metagenomics sequencing. To the best of our knowledge, the modulatory effects of EA and NAR have been commonly studied using isolated intestinal bacteria, whereas the modulatory effect of PG has not yet been reported.

2. Material and methods

2.1. Materials

QUE (95 % purity), EA (96 % purity), PG (99 % purity), D-(+)-Maltose monohydrate (63419), D-(+)-glucose (G8270), resazurin sodium salt (199303), bile salts (48305), vitamin K1 (V3501), tris-ethylenediamine tetraacetic acid (EDTA) buffer (TE, 10× concentrate, PPB010), lysozyme (P00698), diethyl ether (HPLC grade, 99 % stabilized with ethanol, 1009212500), butyric acid (B10350-0), propionic acid (P1386), isobutyric acid (58360), isovaleric acid (59850), and valeric acid (94530) were purchased from Sigma-Aldrich (Madrid, Spain). Dimethyl sulfoxide (DMSO, ≥ 99.9 %) and fructooligosaccharides (FOS) were obtained from Sigma-Aldrich (St. Louis, MA, USA). NAR (98 % purity) and NARN (95 % purity) were supplied from Biosynth ® CarboSynth (Bratislava, Slovakia). Acetic acid (20104.323), soya peptone (84616.0500) and tween 80 (28830.291) were purchased from VWR Chemicals (Pennsylvania, USA). Mueller–Hinton (MH) and Man-Rogosa-Sharpe (MRS) broths were purchased from Biokar Diagnostics (Beauvais, France) Yeast extract (A1202) and tryptone (A1401 HA) were from Biokar Diagnostics (Cedex, France). Trypticase soya broth (TSB) without dextrose was purchased from BBL (Lockeysville, USA), whereas bactopeptone was obtained from Amersham (Buckinghamshire, UK). Sodium chloride (31434) was supplied from Honeywell (North Carolina, USA), and potassium dihydrogen phosphate (1.04871.1000), sodium bicarbonate (1.06329.1000), L-cysteine hydrochloride (1.02839.0100) and calcium chloride (102378), tetracosane (99 % purity), pyridine (≥ 99.5 % purity), (99 % purity) *N,O*-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99 % purity) were from Merck (New Jersey, USA). Heme chloride (A11165) was from Alfa Aesar (Massachusetts, USA). Magnesium sulfate hexahydrate (459337) was supplied from Carlo Erba (Emmendingen, Deutschland). The NZY Tissue gDNA kit for DNA extraction (MB13502) was purchased from NZYTech (Lisbon, Portugal).

2.2. Preliminary screening of antimicrobial and prebiotic effects

Initially, five PCs (EA, QUE, NAR, NARN, and PG) were solubilized in pure DMSO, and their antimicrobial and/or prebiotic abilities were assessed using 4 % (v/v) DMSO: growth medium solutions (Alves et al., 2012; Araújo-Rodrigues et al., 2022; Bordiga et al., 2019). Pathogenic (*Escherichia coli* and *Salmonella enterica*) and probiotic strains (*Bifidobacterium animalis* subsp. *Lactis* BB-12 and *Lactobacillus casei* LC1), were incubated with MHB and MRS media, respectively. So, using a 96-well microplate, for each 4 % (v/v) DMSO PC solution, previously filtered through a 0.22 μm Ø filter, a range of final concentrations of selected PCs between 25 and 1600 μg mL^{-1} (except for EA, which was 12.5–200 μg mL^{-1}, due to its low solubility) was tested. Subsequently, each bacteria strain's inoculum was added (10 μL). Negative controls (one containing only the respective medium and another with the PCs solution (4 % (v/v) DMSO), at the highest concentration) and a positive control (comprising the respective inoculum and medium) were included in this growth assay. The incubation time for pathogenic bacterial strains was 24 h at 37 °C, while probiotic strains were incubated for 48 h at 37 °C (under anaerobic conditions using paraffin only to BB-12). Cell growth was determined by measuring the cultures' OD at 600 nm. All experiments were conducted in triplicate.

2.3. In vitro fermentation assays

2.3.1. Pool of human fecal inoculum

Following the methodology outlined by de Carvalho et al. (2021), fresh fecal samples were obtained from five healthy human donors and promptly transferred into sterile plastic containers to maintain anaerobic conditions. Before collection, it was assured that donors were healthy, between the ages of 18 and 65, without any food intolerances,

and that they had not taken any prebiotic, probiotic, or antibiotic supplements within the past six months. Prior to their participation in the study, all human donors provided informed consent.

Collection and processing of feces were completed within a maximum of 2 h. Similar quantities of each donor fecal sample were pooled together to have a uniform and representative inoculum. Then, the fecal inoculum pool was diluted at 10 % (w/w) in phosphate-buffer saline (PBS) solution (0.1 M, pH 7.4) and subjected to homogenization in a stomacher for 2 min at 460 paddle-beats per min (de Carvalho et al., 2021).

Ethical review and approval were waived for this study, as it was conducted according to internal rules legally established, based on research ethics recommendations and with the informed consent of all subjects involved in the study.

2.3.2. Fermentation media

Following the methodology elucidated by Campos et al. (Campos et al., 2020), the nutrient base medium (NBS) used comprises constituents including TSB without dextrose (5.0 g L^{-1}), bactopeptone (5.0 g L^{-1}), yeast nitrogen base (5.0 g L^{-1},), cysteine-HCl (0.5 g L^{-1},), 1.0 % (v/v) of salt solution A (100.0 g L^{-1} NH_4Cl, 10.0 g L^{-1} $MgCl_2{\cdot}6H_2O$, 10.0 g L^{-1} $CaCl_2{\cdot}2H_2O$), 0.2 % (v/v) of salt solution B (200.0 g L^{-1} $K_2HPO_4{\cdot}3H_2O$), 0.2 % (v/v) resazurin solution (0.5 g L^{-1},) and 1 % (v/v) trace minerals (ATCC, 10 mL L^{-1}). These constituents were dissolved in distilled water, and the pH was adjusted to 6.8 before sterilization by autoclave.

2.3.3. In vitro fermentation

Fecal fermentations were carried out, using a previously reported methodology (Campos et al., 2020; de Carvalho et al., 2021) in an anaerobic cabinet (5 % H_2, 10 % CO_2, and 85 % N_2) after sterilization of all components. Prior to inoculation, DMSO solutions of PCs were introduced into tubes containing NBS (not exceeding 4 % of DMSO). The final concentration targeted, namely 100 and 200 μg mL^{-1} of EA, 200 and 400 μg mL^{-1} of NAR, and 200 and 800 μg mL^{-1} of PG. Then, each tube was inoculated with fecal slurry (final concentration of 2 % (v/v)) and incubated at 37 °C for 48 h without agitation (Campos et al., 2020; de Carvalho et al., 2021). Negative (only fecal inoculum) and positive (with a well-known prebiotic, FOS) controls were also carried out, as well as a respective control of each PC at 200 μg mL^{-1} with inoculum. Samples were collected at 0, 6, 12, 24, and 48 h of fermentation and immediately centrifuged at 16,000*g* for 5 min. Both resulting pellet and supernatants were stored at −80 °C, the pellet conserved for genomic DNA extraction, whereas the supernatants were stored for further identification and quantification of PCs and resultants metabolites and SCFAs analysis. Before freezing, the pH values were measured (Micro pH 2002, Crison, Spain).

2.4. Analysis of SCFAs

SCFAs were analyzed by gas chromatography-flame ionization detector (GC-FID) as described by Scortichini et al (Scortichini et al., 2020), using a gas chromatograph Agilent Technologies 6850 GC (Agilent, USA), equipped with a split/splitless injector and FID. A nitroterephthalic acid-modified polyethylene glycol (PEG) column (DB-FFAP, 25 m, 0.25 mm i.d., 0.25 μm film thickness, purchased from Agilent Technologies, (Agilent, USA) was used. The GC injector and FID temperature were 280 and 250 °C, respectively, and the injection was carried out in splitless mode (splitless time 3 min). Initially, the oven temperature was maintained for 3 min at 40 °C, then increased to 20 °C/min until 160 °C. After that, the temperature rose to 245 °C, with a 40 °C/min rate, and maintained for 1.87 min. The flow rate of hydrogen was 3.70 mL min^{-1}. Firstly, 200 μL of sulfuric acid (50 % *w/v*) was added to each sample supernatant (250 mg) and mixed by vortex for 1 min. Additionally, 10 μL of an internal standard solution (*n*-valeric acid, final concentration of 450 μM) in ethyl ether was added to the acidified sample, followed by the addition of 800 μL of ethyl ether and centrifuged (5 min, at 2800×*g*). This extraction was performed three times after removing the organic phase to a vial. SCFAs were identified and quantified, comparing the relative Rts of sample peaks with those of standard components (acetic (C2), propionic (C3), *n*-butyric (C4), i-butyric (*i*C4), *i*-valeric (*i*C5) and *n*-caproic (C6) acids) and using the respective calibration curves, represented in Table S1 (Supplementary material).

2.5. Gut microbiota evaluation

For metagenomics analysis of the fermented samples, 4 mL of each fecal fermentation sample was initially centrifuged at 4000×*g* for 10 min at 4 °C. The resultant pellet was resuspended in 1 mL of tris-EDTA buffer and centrifuged again under the same conditions. The pellet was then treated with 180 μL of lysozyme solution (10 mg/mL in tris-EDTA buffer) and incubated at 37 °C for 2 h before a final centrifugation at 4000×*g* for 10 min at 4 °C. DNA was subsequently extracted from the pellet using the NZYTissue gDNA Isolation Kit, following the manufacturer's instructions.

The 16S amplicon metagenomics sequencing analysis was performed by Novogene (Cambridge, UK). The 16S rRNA genes from the V3-V4 regions were amplified using specific primers attached to barcodes (5′-CCTAYGGRBGCASCAG-3′ and 5′-GGACTACNNGGGTATCTAAT-3′). The PCR was performed with 15 μL of Phusion® High-Fidelity PCR Master Mix, 0.2 μM of each primer, and 10 ng of template DNA. The amplification protocol included an initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The amplicons were verified by electrophoresis on a 2 % agarose gel and purified using the Universal DNA Purification Kit.

Libraries for sequencing were prepared using the NEB Next® Ultra™ II FS DNA PCR-free Library Prep Kit, and index codes were assigned. Library quality was assessed using the Qubit system, real-time PCR, and a bioanalyzer to verify size distribution. The libraries were then pooled and sequenced on an Illumina platform.

Bioinformatic analysis commenced with the splicing and filtering of raw data to produce clean data. Noise reduction was achieved using DADA2, and amplicon sequence variants (ASVs) were annotated to determine species information and abundance distribution. ASV abundance and α-diversity were calculated to assess species richness and uniformity. Phylogenetic trees were constructed from multiple sequence alignments of ASVs, facilitating community structure analysis through β-diversity by Principal Coordinates Analysis (PCoA) and dendrogram visualization. *β*-diversity was also calculated, employing the generalized UniFrac distance to compare community composition complexity between sample groups. Differences in community structure across groups were evaluated using statistical methods such as analysis of Similarities (ANOSIM) and Linear discriminant effect size (LefSe) analysis. All bioinformatic processes, including ASV identification, species annotation, phylogenetic analysis, and diversity assessments, were conducted using QIIME2 software (version QIIME2-2023.9).

2.6. Statistical analysis

The statistical analysis for the SCFAs quantification results was carried out using OriginPRO 2024 v10.1.0.170. We first checked whether the data followed a normal distribution. In cases where we confirmed a normal distribution, we assessed the homogeneity of variances using Levene's test (p-value >0.05).

For samples with normal distribution and homogeneous variance, we compared the concentration of each SCFA along fermentation time through a one-way ANOVA, and at each time of fermentation, the concentration of the identified SCFAs was compared. To determine multiple separations of the means, we applied Tukey's post-hoc test. However, we opted for the nonparametric Kruskal-Wallis test for cases where the data did not follow a normal distribution or when the homogeneity of

variances was not confirmed.

Nonparametric MANOVA (ADONIS) analysis, a multivariate statistical method based on permutational multivariate analysis of variance (PERMANOVA), was employed to assess the differences in microbial community structures between groups according to the distance matrix used for PCoA. This method can analyze the explanation of grouping factors on the difference of samples and estimate the significance of grouping by permutation test. This analysis was performed using the software R-Studio (R version 4.3.1) with the library "vegan".

ANOSIM was used to evaluate the statistical differences in the microbial community. This indicated that there was variation among groups significantly larger than within groups, which helped to evaluate the reasonability of the division of groups. It provided an R statistic that quantifies the degree of separation between groups; an R-value close to 1 suggests dissimilarity between groups, while a value close to 0 indicates no significant difference. This analysis was performed using the software R-Studio (R version 4.3.1) with the libraries "vegan" and "permute".

LefSe analysis was used to analyze and detect biomarkers (Segata et al., 2011). It emphasizes statistical significance, biological consistency, and effect correlation, allowing researchers to identify abundance characteristics and related classes. The result comprised a histogram of LDA scores, the cladogram, and the histogram of statistically different biomarkers' relative abundance among groups. This analysis was performed using the software lefse (version 1.1.01), with a high threshold of 4.

3. Results and discussion

3.1. Phenolic compounds selection

Before exploring the impact of individual PCs in the presence of a complex gut bacteria community, we examined how these PCs can influence the bacterial growth of isolated species, including probiotics and pathogenic strains. The strains *B. animalis* subsp. *Lactis* BB12 and *L. casei* LC1 were incubated with the selected PCs for 48 h, while the pathogenic strains *E. coli* and *S. enterica* were incubated for 24 h (data not shown).

Regarding their impact on the growth of probiotic strains, EA (12.5–200 μg mL^{-1}), NAR (50–400 μg mL^{-1}), and PG (50–400 μg mL^{-1}) were found to prolong the beginning of *L. casei* death stage and to enhance its growth. In contrast, QUE and NARN triggered an earlier onset of the death stage. All five PCs had an inhibitory effect on *B. animalis* subsp. *Lactis* BB12 growth at all tested concentrations, being EA the one with the least pronounced impact.

After 24 h of incubation, all the PCs inhibited *S. enterica* growth. Although the inhibitory effect on *E. coli* was less marked, the tested concentrations of EA and NAR inhibited *E. coli* growth from 18 h onward. Higher concentrations (800 and 1600 μg mL^{-1}) were tested for all PCs except EA due to its solubility limitations. These concentrations had a stronger inhibitory effect on all bacterial strains. While this may be advantageous for limiting the growth of pathogenic strains, it is less desirable for *L. casei*.

Therefore, the selection of PCs and their respective concentrations prioritized those with minimal negative effects on probiotic strains' growth while inhibiting the growth of pathogenic strains. In order to ensure the representativeness of all the PC classes, the following compounds were selected: EA, NAR, and PG. The results obtained at 100, 400, and 800 μg mL^{-1} concentrations were found to be the most promising. For comparative purposes, a concentration of 200 μg mL^{-1} was also chosen for all three compounds.

3.2. pH and SCFAs profile during in vitro fecal fermentation

Fig. 1 and Table S2 (Supplementary material) present the pH variation observed along fecal fermentation for each concentration of the studied PCs – EA (100 and 200 μg mL^{-1}), NAR (200 and 400 μg mL^{-1}) and PG (200 and 800 μg mL^{-1}). After 6 h of fermentation, the pH values of all samples decreased significantly, followed by a slight increase at 12 h. Thus, in most cases, after 12 h of fermentation, the pH values did not change significantly. Throughout 48 h, the fermentation with FOS (positive control) exhibited the most significant decrease in pH values. The pH trends were consistent across all tested concentrations of PCs and control groups, except for NAR at 400 μg mL^{-1}, which showed significant deviations from the positive control throughout the fermentation period. Therefore, the pH variation obtained during fecal fermentation with these PCs may indicate that the fermentation environment did not undergo a significant alteration of pH values. This could suggest that a healthy gut environment was maintained, without compromising the favorable conditions for beneficial bacteria important for human health.

The SCFAs profile and the contents obtained for each concentration of the studied PCs – EA (100 and 200 μg mL^{-1}), NAR (200 and 400 μg mL^{-1}) and PG (200 and 800 μg mL^{-1}) are represented in Fig. 2 and Fig. 3

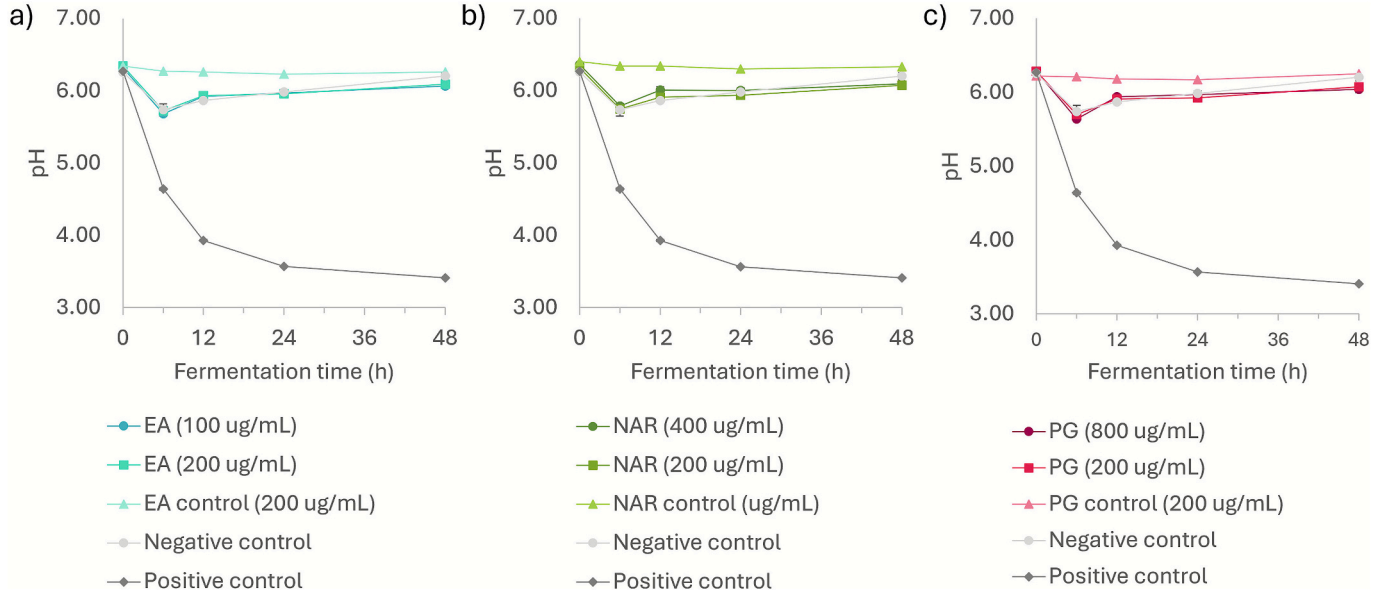


Fig. 1. pH variation throughout fecal fermentation of a) ellagic acid (EA), b) naringenin (NAR) and c) phloroglucinol (PG).

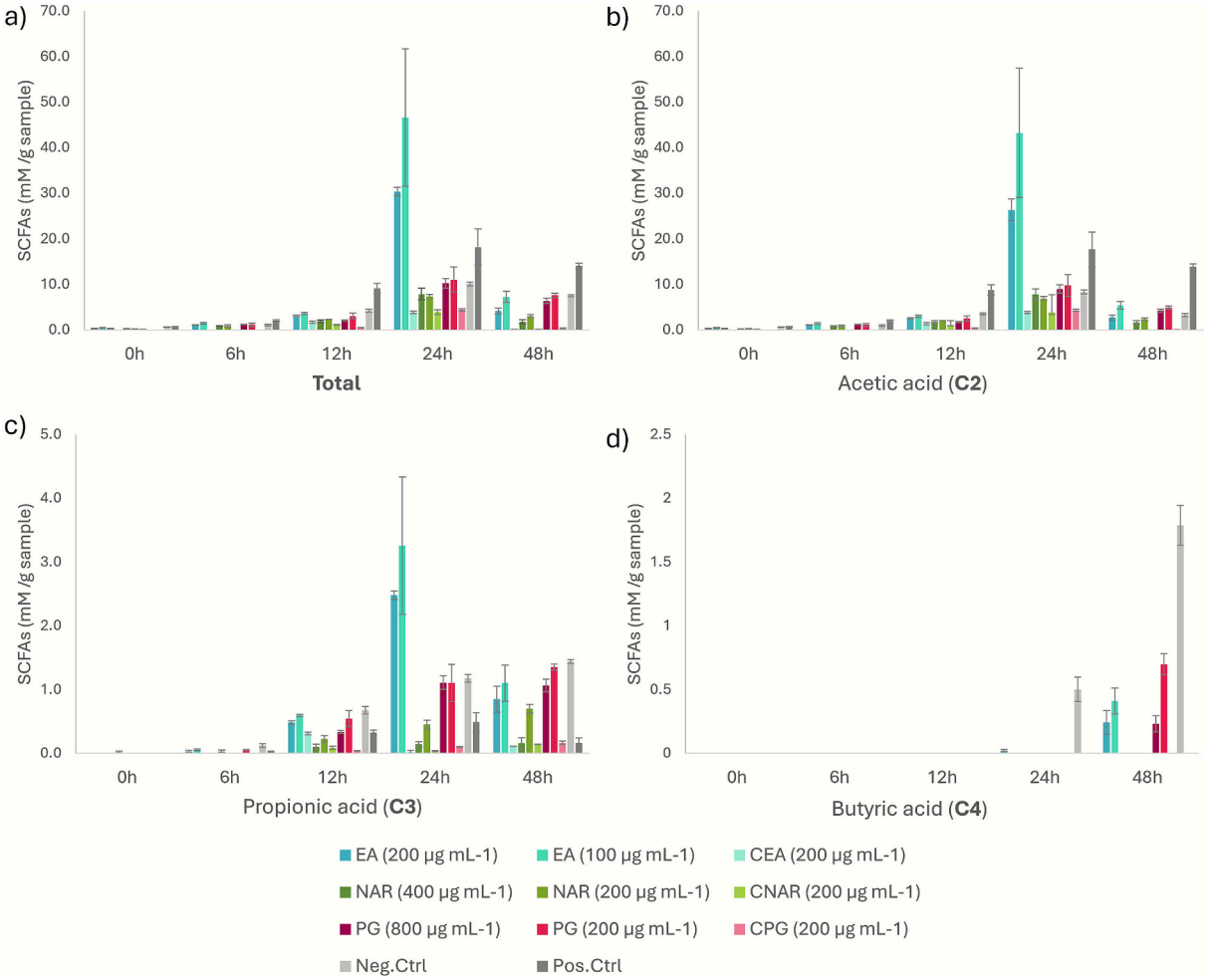


Fig. 2. SCFAs concentrations (µM/mg sample) a) total, b) acetic (C2), c) propionic (C3) and d) butyric (C4) acids throughout fecal fermentation of ellagic acid (EA), naringenin (NAR) and phloroglucinol (PG).

and Table S2 (Supplementary material). SCFAs are well-known fermentation products of unabsorbed or undigested food components generated by the activity of the GM (Ribeiro et al., 2021). Previous literature identifies specific SCFA-producing bacteria (Ribeiro et al., 2021; Ríos-Covián et al., 2016); for instance, members of the Bacteroidetes phylum produce acetic and propionic acids, while Firmicutes are known to produce butyric acid (Venegas et al., 2019). The study investigated the modulatory effects of PCs on GM composition and their influence on SCFA production during fermentation. SCFAs are known to confer numerous health benefits, including the prevention and treatment of conditions such as metabolic syndrome, IBD, and cancer (Gullon et al., 2015). Notably, during fermentation, the concentration of each SCFA significantly increased by 24 h, reflecting an overall rise in the total SCFA levels.

In the colon, about 90–95 % of the SCFAs correspond to acetic (C2), propionic (C3), and butyric (C4) acids (Ríos-Covián et al., 2016). The results of this study were consistent with the hypothesis that C2 is the major organic acid found in fecal fermentation with PCs, as it was present in all samples, including negative and positive controls. C3 was the second most abundant SCFA found in these fecal fermentation samples, with an increase observed from 12 h onward.

C2 was identified in samples collected at the beginning of fermentation (0–6 h), which may result from gut bacteria metabolism or derive from endogenous compounds (Ribeiro et al., 2021). C2 appeared to increase between 12 and 24 h in all fermentation samples, followed by a decrease at 48 h. These findings suggest potential beneficial effects for host health, as C2 is crucial in energy homeostasis, inflammatory status, and anxiety (Chambers et al., 2018). However, there is some controversy regarding the effects of C2 on host health, as it may reduce proinflammatory cytokines or, conversely, promote cancer cell survival (Rahman et al., 2023). At both concentrations (100 and 200 µg mL^{-1}), EA led to the highest amount of C2, whereas its amount in NAR samples (200 and 400 µg mL^{-1}) was significantly lower. Indeed, compared to the positive control (FOS fermentation), NAR (400 µg mL^{-1}) exhibited a significantly lower amount of C2 at 6 h and 48 h.

C3 is responsible for appetite regulation and colon cancer prevention (Chambers et al., 2018), and its primary producers in the colon belong mainly to the Bacteroidetes group, abundantly found in healthy humans' guts (Ribeiro et al., 2021). At 6 h, the amounts of C3 in the samples of EA (200 µg mL^{-1}), NAR (200 µg mL^{-1}), and the positive control were significantly lower than in the negative control. These differences between the negative control and the other samples has also been reported in other studies (Yao et al., 2022; Zhao et al., 2023). In this study, the production of SCFAs in fermentation with FOS (positive control) could be attributed to the acidic environment resulting from the accumulation of lactic acid, which serves as a substrate for SCFAs bacterial production (Ribeiro et al., 2021). However, no significant differences were found throughout fermentation time between EA (100 and 200 µg mL^{-1} and the, positive and negative controls. Furthermore, at 24 h, EA (200 µg mL^{-1}) exhibited the highest C3 concentration (3.26 ± 1.08 µM/mg sample). On the other hand, NAR showed the lowest C3 content at both concentrations. Once more, EA and NAR demonstrated the most

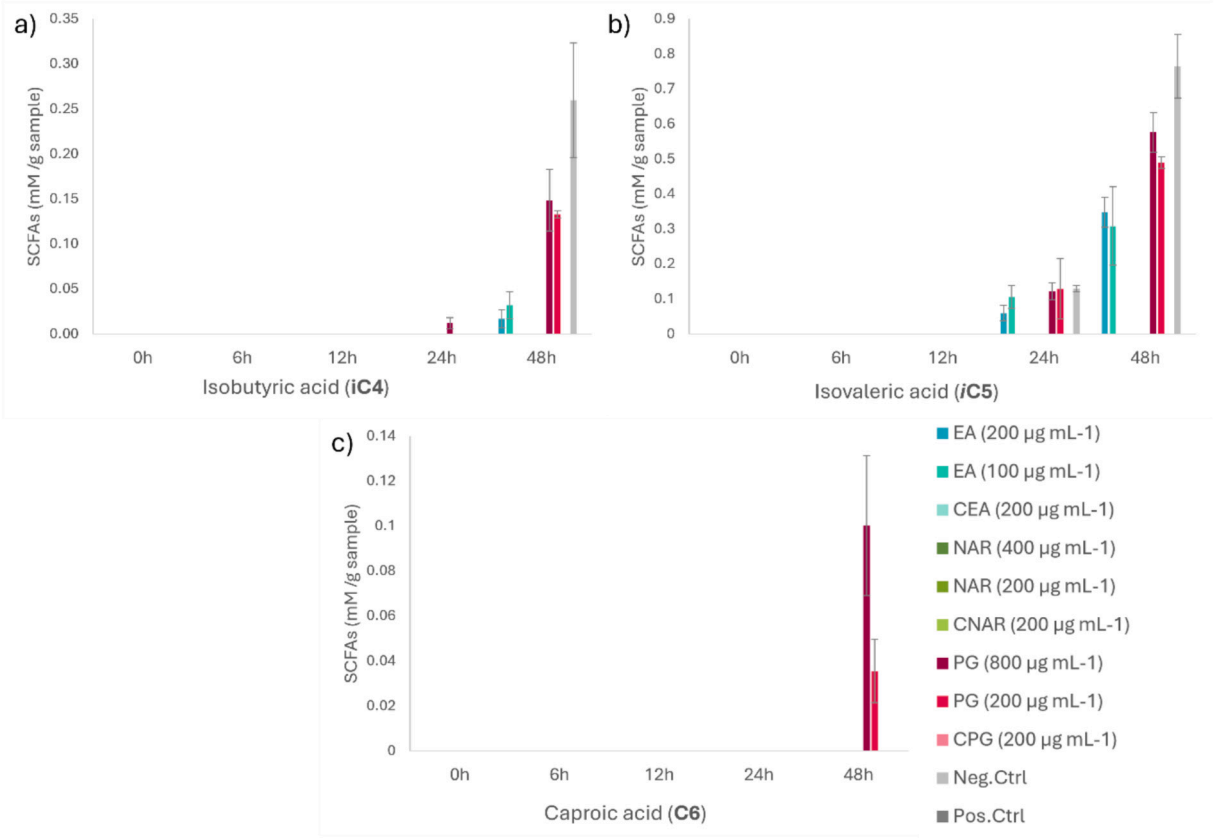


Fig. 3. SCFAs concentrations (µM/mg sample) – a) isobutyric (iC4), b) isovaleric (iC5) and c) caproic (C6) acids throughout fecal fermentation of ellagic acid (EA), naringenin (NAR) and phloroglucinol (PG).

significant positive and negative influences on C3 production, respectively, at both concentrations.

C4 plays an important immunomodulatory role, regulating intestinal homeostasis through anti-inflammatory activity, restoring intestinal barrier function in an inflammatory context and, in the presence of pathogens, stimulating the production of antimicrobial peptides that act as a first line defense (Venegas et al., 2019). It was only detected after 24 h of fermentation, possibly due to its production from carbohydrates or other organic acids metabolism, which occurs through acetate conversion (Chambers et al., 2018; Venegas et al., 2019). As previously stated, the primary producers of C4 belong to the Firmicutes phylum (Chambers et al., 2018; Ribeiro et al., 2021). The detection of C4 in samples of EA (100 and 200 µg mL^{-1}) and PG (200 and 800 µg mL^{-1}) may be indicative of a modulatory effect of these PCs on the composition of the GM. At 48 h, the presence of C4 was also observed in the negative control.

In addition to the most abundant SCFAs (C2, C3, and C4), the remaining 5–10 % comprises isobutyric (iC4) and isovaleric (iC5) acids – branched SCFAs (BCFAs) (Fig. 3 and Table S2, supplementary material) (Ríos-Covián et al., 2016). As observed for C4, iC4 was only found in the 48 h samples of EA (100 and 200 µg mL^{-1}), PG (200 and 800 µg mL^{-1}), and negative control samples. At 24 h, iC4 was only found in PG at 800 µg mL^{-1}. Moreover, iC5 was only found in the 24 h and 48 h samples of EA (100 and 200 µg mL^{-1}) and PG (200 and 800 µg mL^{-1}), as well as in those of negative control. At 48 h, the samples of both concentrations of EA exhibited a significantly lower amount of iC5 than those of PG (200 and 800 µg mL^{-1}), and both were lower than the negative control. The BCFAs are usually produced from protein metabolism (Ríos-Covián et al., 2016). Their human health effects are still not fully understood (Rios-Covian et al., 2020), representing a potential topic of interest for further scientific investigation.

The origin of caproic acid (C6) (Fig. 3c) seemed to be related to GM activity, although some species have been identified as capable of producing C6 through a lactate cross-feeding mechanism (Rios-Covian et al., 2020). In this study, C6 was only produced in PG fermentation at both concentrations at 48 h, with a dose-dependent effect.

Furthermore, the beneficial effects of SCFAs on the human body have been extensively documented. These compounds can reach other organs, where they play essential roles as signaling molecules and/or energy substrates (Ribeiro et al., 2021). Given that SCFAs are well known for their human beneficial effects, it seems that EA and PG may possess prebiotic properties, as they promote increased production of these beneficial metabolites. Moreover, since the GM bacteria responsible for the production of these metabolites have already been identified, as previously mentioned, the fermentation of these PCs leading to SCFAs production suggests that EA and PG may positively influence GM composition. Alternatively, they may not harm SCFAs producing-bacteria, thereby supporting GM diversity. The significative difference in SCFAs production, observed between FOS and the negative control could be related with the accumulation of lactic acid in FOS fermentation (Ribeiro et al., 2021), as mentioned above. Since lactic acid is a substrate for bacterial production of SCFAs (such as acetic, propionic or butyric acids) (Ribeiro et al., 2021), the observed lower pH value and reduced amount of SCFAs in the *in vitro* fecal fermentation of FOS may indicate that FOS fermentation leads to an acidic intestinal environment.

3.3. *Gut microbiota profile during in vitro fecal fermentation*

3.3.1. *Relative abundance*

The microbial landscape of the human gut is a dynamic and complex ecosystem that plays a crucial role in the well-being of its host (Clemente et al., 2012). In addition to the pH and SCFAs profile during *in vitro* batch

fecal fermentation, this study investigated the modulatory effects of the three distinct PCs (EA, NAR, and PG) on human fecal microbiota. A meticulous taxonomic analysis was conducted at multiple hierarchical levels, as represented in Fig. 4, including a) phylum, b) class, c) order, d)

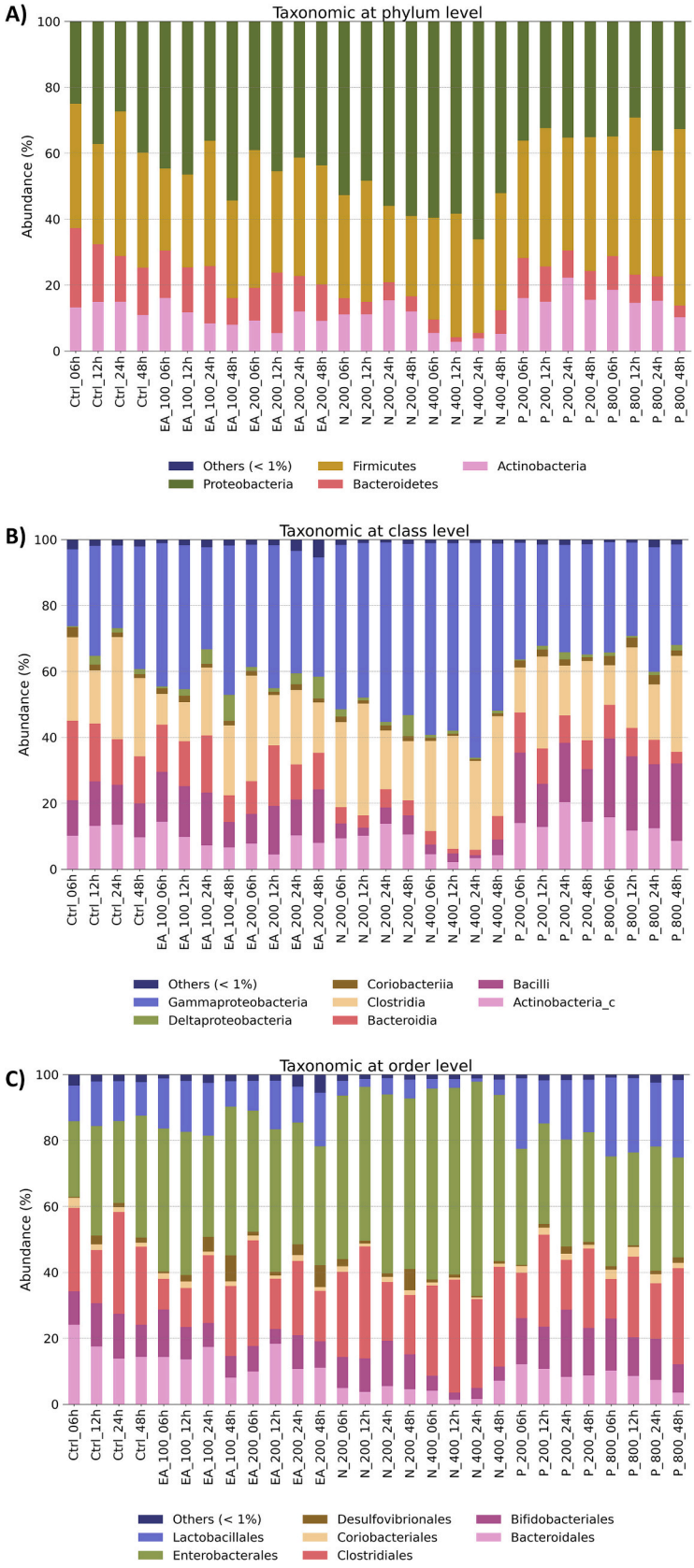


Fig. 4. Microbial relative abundances at a) phylum, b) class, c) order, d) family, e) genus, and f) species levels during the 48 h of *in vitro* batch fecal fermentation with ellagic acid (EA, at 100 and 200 μg mL^{-1}), naringenin (N, at 200 and 400 μg mL^{-1}) and PG (P, at 200 and 800 μg mL^{-1}).

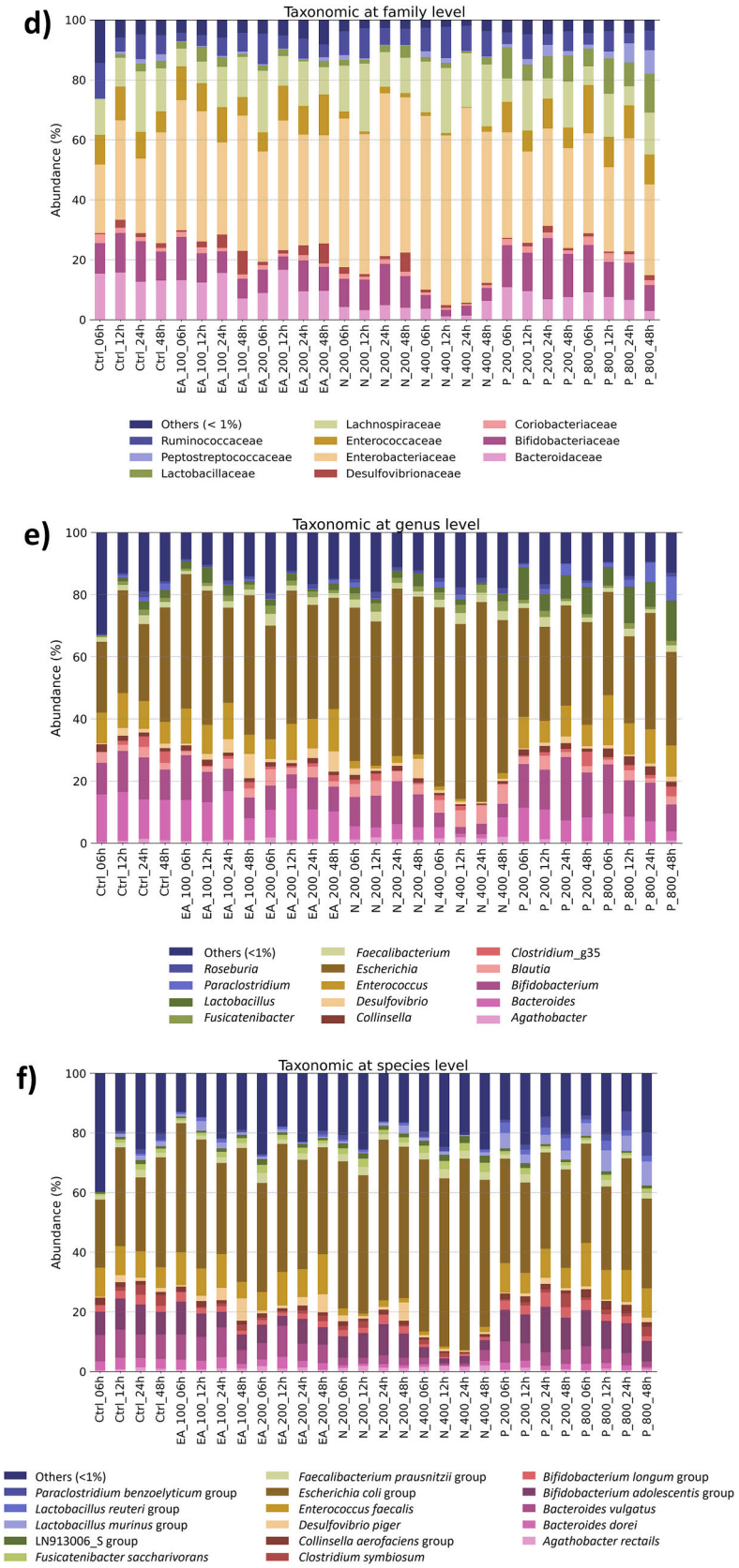


Fig. 4. (*continued*).

family, e) genus, and f) species levels. The effect of studied PCs at each level revealed a dose-dependent microbial community composition over the 48 h.

Considering that about 98 % of all GM bacterial species belong to four phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Duda-Chodak et al., 2015), they were found in all samples, and

notable shifts were induced following exposure to PCs. The higher tested concentration of EA (200 μg mL^{-1}) at the phylum level (Fig. 4a) appeared to influence the proportional balance of these dominant phyla. These changes indicate that this phenolic acid may alter the gut environment and microbial competition. Specifically, the presence of EA affected the classes Bacilli and Clostridia; at a concentration of 100 μg mL^{-1}, it reduced the growth of Clostridia over 48 h (Fig. 4b). This effect of EA aligns with the findings of Bialonska et al. (2009), who reported that this compound inhibited the growth of two gram-positive intestinal pathogenic species, namely *Clostridium perfringens* and *C. clostridioforme*.

Furthermore, EA seemed to promote Bacilli, which is a class within the phylum Firmicutes that includes many probiotic species, particularly *Lactobacillus* species (Huang et al., 2016). When the effect of EA was evaluated on the growth of isolated bacterial strains, it was found that the growth of *L. acidophilus*, *L. pentosus*, and *L. ramnosus* was inhibited in the presence of EA (Bialonska et al., 2009), in contrast to the results obtained in this study, which could be due to differences in the tested concentrations or may reflect a specific effect on isolated strains. These *Lactobacillus* species have been well-known for their health-promoting abilities and role in PC metabolism (dos Santos et al., 2019). At the genus level (Fig. 4e), EA was found to promote the growth of *Bifidobacterium*, a genus well-known for its symbiotic relationship with the human host (Alves-Santos et al., 2020; Wu & Wang, 2019). This prebiotic effect was evident at the species level (Fig. 4f) since all studied PCs showed a positive impact on *Faecalibacterium prausnitzii* group, known for its anti-inflammatory properties (He et al., 2021), and the *L. reuteri* group, recognized for its health-promoting capabilities (Mu et al., 2018).

NAR had varying effects on the Gammaproteobacteria and Deltaproteobacteria classes, which include genera such as *Escherichia*, *Salmonella*, and *Desulfuvibrio*, indicating changes that could affect gut redox states and sulfate reduction pathways (Ouwehand & Vaughan, 2006; Shin et al., 2015). After 24 h, this flavanone at 400 μg mL^{-1} showed the highest percentage of the Gammaproteobacteria class (Fig. 4b At the order level, the presence of NAR at both tested concentrations induced shifts in Enterobacterales and Desulfovibrionales (Fig. 4c), which may have complex implications for gut health, mainly associated with intestinal inflammation or other enterohepatic conditions due to their diverse functional roles (Baldelli et al., 2021; Hu et al., 2022). In fact, the highest percentage of Desulfovibrionales was observed at 200 μg mL^{-1}, after 48 h. In the presence of 400 μg mL^{-1} of NAR, the highest percentage of Enterobacterales was observed after 24 h, followed by a slight decrease at 48 h of fermentation. Moreover, NAR's impact on the *E. coli* group and *Desulfovibrio* genus was consistent with the patterns described at the class and order levels, which suggests the complex effects of this flavanone on microbial communities, potentially affecting pathogenicity and sulfur metabolism (Baldelli et al., 2021; Hu et al., 2022; Ouwehand & Vaughan, 2006; Shin et al., 2015).

However, previous studies have shown that NAR is one of the most antibacterially active PCs studied (particularly when compared to caffeic, chlorogenic, and coumaric acids, catechin and epicatechin, DAID and genistein) against a commensal (*E. coli*) and two pathogenic bacteria (*Staphylococcus aureus* and *S. typhimurium*) (Parkar et al., 2008). NAR has been considered a promising active component in the treatment of some diseases, namely polycystic ovary syndrome and central nervous system diseases, due to their ability to modulate the GM composition since NAR regulates the abundance of various beneficial bacteria (such as *Parabacteroides* and *Lactobacillus*) and simultaneously decrease those associated to the disease condition (such as Deltaproteobacteria and Desulfovibrionaceae) when compared with the GM of diseased animals (Liu et al., 2023; Wu et al., 2022). This NAR effect contrasts with those obtained in this study, which could potentially be a side effect of the tested doses. Moreover, Liu et al. (2023) also stated that NAR has a role in gut-brain axis regulation by leading to the production of specific metabolites in feces and brain, such as lipid and flavonoids metabolites, among others.

PG relative abundances analysis showed that at the phylum level (Fig. 4a), PG at 800 μg mL^{-1} seemed to increase F/B ratio through the increase of Firmicutes and decrease of Bacteroidetes along the 48 h of fermentation. This ratio between these phyla has been associated to various human health conditions. Higher levels of Firmicutes relative to Bacteroidetes have been associated with body weight and are commonly observed in obese individuals, as these phyla can effectively harvest metabolic energy from the metabolism of undigested remaining food (Parkar et al., 2013; Xue et al., 2016). However, this association remains controversial, because this ratio could be influenced by other lifestyle-associated factors, as well as by the selection/characterization of the subjects included in studies and by the interpretation of the results acquired through diverse methods of sample preparation or DNA sequence analysis (Magne et al., 2020). The results on the order level, shown in Fig. 4c, provide additional resolution by revealing changes in orders such as Lactobacillales and Bifidobacteriales. PG presence at both concentrations seemed to promote the growth of these orders during the fermentation time. Lactobacillales and Bifidobacteriales (where the well-known *Lactobacillus spp.* and *Bifidobacterium spp.*, respectively) are often associated with health-promoting attributes, such as the production of beneficial metabolites (Alves-Santos et al., 2020). Additionally, the PG (at 200 and 800 μg mL^{-1}) results on the family level (Fig. 4d) highlight the PG's prebiotic potential and suggest that it may create a conducive environment to beneficial microbes. This PC also had a pronounced effect on Coriobacteriaceae, a family associated with bile acid metabolism and, thus, cholesterol homeostasis and host metabolism (Wegner et al., 2017).

In accordance with the previous level, the results at the species level (Fig. 4f) demonstrate that PG has a positive impact on beneficial bacteria, namely *F. prausnitzii* group and *L. reuteri* group (He et al., 2021; Mu et al., 2018).

To the best of our knowledge, this is the first time that the effect of PG, the monomeric unit of phlorotannins, on GM composition has been studied. The fate of two brown seaweed (*Fucus vesiculosus* and *Silvetia compressa*) phlorotannin extracts through the gastrointestinal tract as well as their modulatory effect on GM composition has previously been investigated (Catarino et al., 2021; Vázquez-Rodríguez et al., 2021), however, these are phlorotannin-enriched extracts that contain other components (Catarino et al., 2021; Vázquez-Rodríguez et al., 2021), meaning that the effects of isolated phlorotannins had not yet been evaluated, so the simplest structure of this subfamily of compounds was considered in this study.

Broadly, the data suggest that these PCs selectively modulate the fecal microbiota, with implications that extend beyond mere alterations in microbial composition. Modifications at different taxonomic levels indicate potential shifts in metabolic functions expected to influence broader aspects of gut physiology and health. The dynamic interplay between dietary PCs and the gut microbiome is highlighted by the dose-dependent and time-dependent nature of these changes.

3.3.2. α-Diversity analysis

Regarding the α-diversity analysis at genus level of ASVs (Fig. 5), results were displayed in three boxplots representing different α-diversity indices: a) phylogenetic diversity (which represents the total branch length of the phylogenetic tree that spans a set of taxa within a community), b) Shannon index (which allows the analysis of the abundance and evenness of the present species), and c) Chao1 index (which provides information on species richness of the samples).

Fig. 5a shows the boxplot that represents the distribution of phylogenetic diversity values across different treatment groups. The control group exhibits a wide interquartile range, indicating a significant variation in phylogenetic diversity within the control samples. The presence of PCs appears to result in a narrower interquartile range. The exposure of NAR and PG shows a downward shift in median phylogenetic diversity compared to the control. All treatment groups overlap substantially with the control, implying that the PCs may have some influence. However, according to the Kruskal-Wallis test, there are no significant

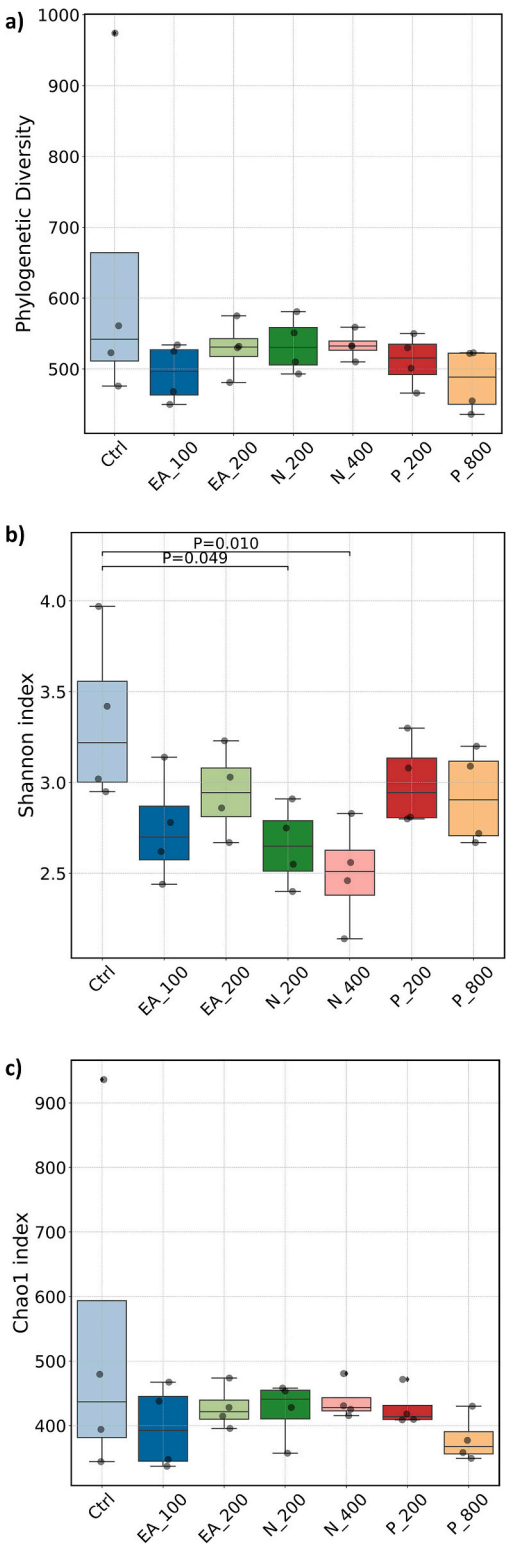


Fig. 5. α-Diversity indices – a) phylogenetic diversity, b) Shannon index, and c) Chao1 index, of the samples of different PCs – ellagic acid (EA, at 100 and 200 μg mL^{-1}), naringenin (N, at 200 and 400 μg mL^{-1}) and phloroglucinol (P, at 200 and 800 μg mL^{-1}). *p*-Values indicate the statistical significance in comparison with the control sample.

differences between the samples of each PC, so the overall phylogenetic diversity of the microbial communities is relatively conserved.

Shannon index (Fig. 5b) shows a significant decrease in microbial diversity with NAR (at both concentrations, 200 and 400 μg mL^{-1}) compared to the control. This is indicated by the lower median values and the statistical significance (p-value = 0.049 and p-value = 0.010, respectively). This statement suggests that NAR's presence may selectively affect the microbial community by reducing their diversity.

Moreover, by interpreting the boxplot Chao1 index (Fig. 5c), and according to the Kruskal-Wallis test, no significant differences in species richness were observed across the samples of different PCs.

Therefore, the potential trend towards reduced species richness with these compounds was not as pronounced as the reduction observed in the Shannon index.

Together, these indices provide a comprehensive understanding of the impact of PCs on the GM's diversity. The significant in the presence of NAR. However, the phylogenetic diversity and Chao1 indices suggest that these PCs have minimal effects on overall phylogenetic breadth and species richness. These findings suggest that PCs may have a selective effect on specific microbial taxa, leading to changes in community composition without significantly altering overall species richness or phylogenetic diversity. Thus, these PCs did not seem to significantly disturb the microbiota ecosystem's equilibrium significantly, which is a crucial influence on the host's health, particularly in processes like inflammation, immunity, gut-brain axis regulation, and metabolism (Espín et al., 2017).

3.3.3. *β-Diversity analysis*

The comparison of the similarities and differences in microbial community composition among samples was performed by the unweighted pair-group method with an arithmetic mean (UPGMA) cluster tree (Fig. 6) based on the generalized UniFrac distance. This distance-based statistical test was preferred since it is more accurate for detection of abundance change either in rare or highly abundant lineages, when compared with unweighted and weighted UniFrac distances (Chen et al., 2012). The dendrogram in Fig. 6a shows that the control samples cluster closely at the beginning, indicating high similarity among control samples across time points since shorter branch lengths indicate a greater degree of similarity in microbial communities.

EA samples show varying degrees of similarity to the control; however, they tend to cluster more closely with the control than the other treatments, potentially indicating a less pronounced effect on community composition at the genus level. The samples of NAR form a distinct cluster, with those from higher concentrations (at 400 μg mL^{-1}) and later time points diverging more from the control. PG samples also form a distinct cluster away from the control, especially at the earlier time points, suggesting a significant impact of this treatment on the microbiota structure. Therefore, these findings reinforce that different PCs and concentrations can distinctly influence the gut microbiome.

In addition to UPGMA clustering, PCoA and ADONIS analysis offer complementary insights into community structure. PCoA provides a graphical representation of the beta diversity among microbial communities. Whereas ADONIS analysis partitions the sum of squares of a multivariate dataset into components associated with one or more factors or variables. It then assesses the significance of the partitioning using permutations, providing a robust approach to analyzing the variability in community composition, attributing it to explanatory variables while controlling for the effects of other variables in the model. The results of the ADONIS analysis (Tables S3–S4, supplementary material) allow for a more profound comprehension of the influence of PCs on microbial diversity and structure. This is achieved by statistically testing the hypothesis that the microbial community composition differs significantly disparate across experimental groups defined by disparate treatments or conditions.

The PCoA plot, shown in Fig. 6b and based on generalized UniFrac distances at the genus level, illustrates distinct clusters for each PC and

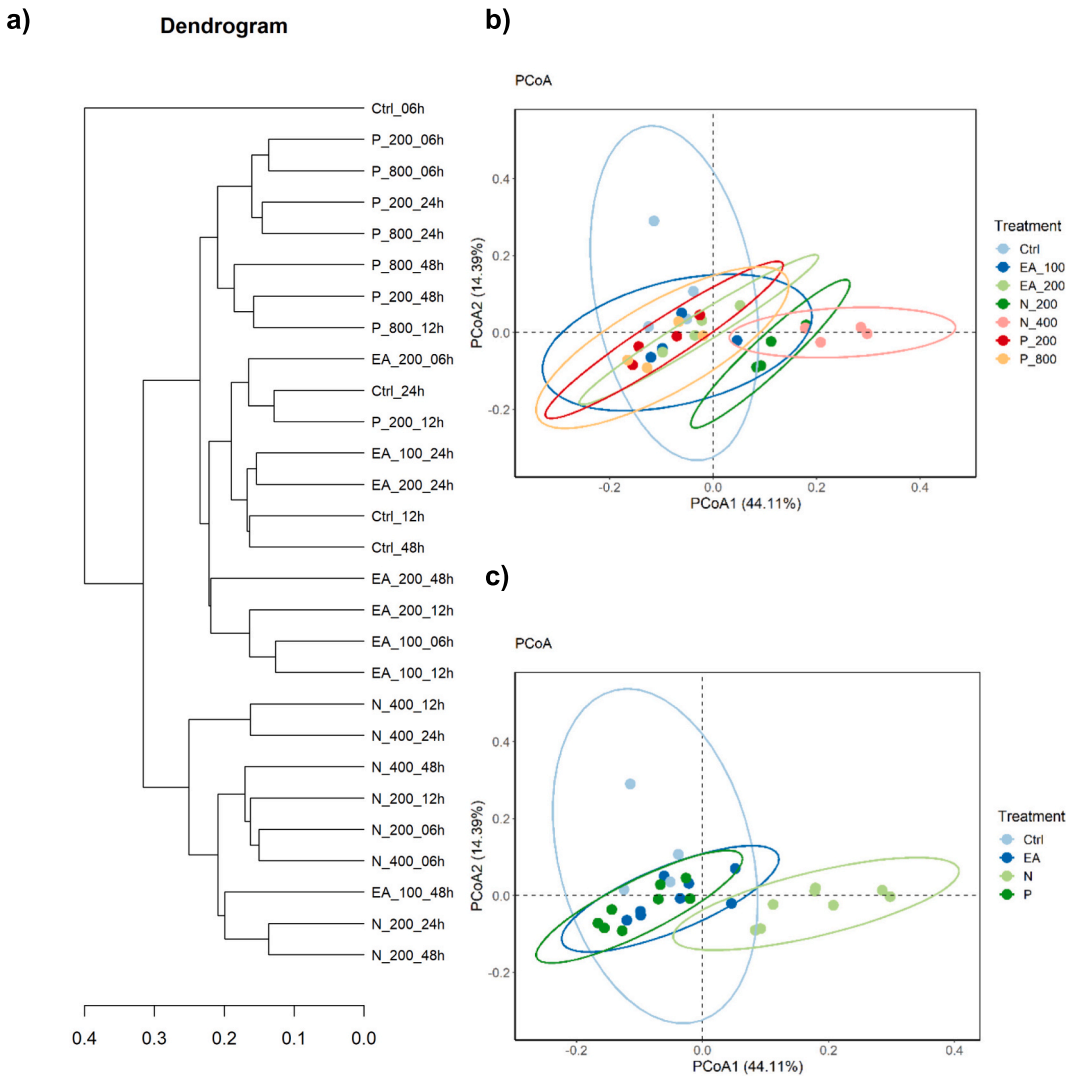


Fig. 6. Beta diversity analysis - a) dendrogram resultant from UPGMA clustering, b) and c) principal coordinates analysis (PCoA) of the samples of different PCs – ellagic acid (EA, at 100 and 200 μg mL^{-1}), naringenin (N, at 200 and 400 μg mL^{-1}) and phloroglucinol (P, at 200 and 800 μg mL^{-1}) along 48 h of fermentation.

their respective concentrations, with some overlap. The first PCoA (PCoA1) explains 44.11 %, and the second (PCoA2) is 14.30 % of the total variation, indicating that these axes together capture a substantial portion of the data's variability.

Fig. 6b also shows that the control samples appear as an individual cluster, indicating a unique microbial community structure in the absence of PCs.

The ANOSIM is a non-parametric test that ranks the dissimilarities between and within groups based on a chosen distance or dissimilarity measure. It then tests whether the ranks of between-group dissimilarities are significantly greater than within-group dissimilarities. The test (Table S5, supplementary material) is particularly useful for determining whether variations in community structure are statistically significant across different experimental conditions or in the presence of PCs.

EA at concentrations of 100 and 200 μg mL^{-1} showed some overlap, consistent with the ANOSIM results indicating no significant difference (Table S5, supplementary material). NAR at 200 and 400 μg mL^{-1} concentrations formed distinct clusters, aligning with the ANOSIM and ADONIS results (Table S3 and S5, supplementary material), which showed significant differences, justifying treating them as separate groups. In contrast, PG at 200 and 800 μg mL^{-1} exhibited significant overlap (Table S3 and S5, supplementary material), indicating similar microbial community structures at different concentrations.

The second PCoA plot in Fig. 6c shows that the samples cluster by PC, indicating that the PCs have a distinct impact on the microbial community composition. The corresponding ADONIS analysis (Table S4, supplementary material) supports these visual observations with statistical significance. The pairwise comparison results indicate that all PC samples differ significantly from the control, with *p*-values well below the 0.05 threshold after FDR adjustment. Furthermore, the study found significant differences in the effects of EA, NAR, and PG on the gut microbiome, indicating that each PC uniquely impacts the microbial community composition.

In the second PCoA plot (Fig. 6c), the presence of PC is grouped homogeneously without differentiation by concentration. Clear separation between the PCs groups and the control is observed, confirming that PCs significantly affect the microbial community structure, particularly in the case of NAR. The ADONIS analysis reveals significant differences between all PCs and the control and among the PCs themselves. These findings suggest that PCs, even when considered as homogeneous groups, have a significant and distinct impact on the β-diversity of the microbiota, influencing the microbial community structure in unique ways.

3.3.4. Community differences analysis

According to the ANOSIM (Table S5, Supplementary material), the results of EA (100 and 200 μg mL^{-1}) and PG (200 and 800 μg mL^{-1}) indicate that their different concentrations can be grouped for further analysis as no significant differences were detected, with an R statistic of −0.1667 and −0.05208 and a significance level of 0.882 and 0.555, respectively. However, the different concentrations for NAR should be considered separately due to significant differences between 200 and 400 μg mL^{-1}, with an R statistic of 0.5729 and a significance level of 0.036.

In order to obtain a detailed overview of microbial modulation in response to PCs, allowing an understanding of the relationship between diet, the GM, and host well-being, the LEfSe analysis was performed extensively, identifying the taxa most likely to present differences between the PCs effects, including all taxonomic levels from phylum to species, and with a high threshold of 4 to ensure that only the most robustly differentiated taxa between the groups are highlighted. This conservative threshold enhances the reliability of the biomarkers identified, although it may also exclude taxa with more subtle but potentially relevant differences.

Initially, LEfSe analysis was performed to include the effect of each PC concentration, depicted in Fig. 7. Subsequently, a more generalized analysis was conducted without differentiating between concentrations, as shown in Fig. 8. Each LEfSe analysis produces a histogram of LDA scores and a cladogram. The histogram identifies taxa that shows significant differences between the control and each PC or concentration based on the magnitude of their effects. The cladogram illustrates where these significant changes occur within the broader taxonomy, highlighting specific areas of the phylogenetic tree most affected by each PC. This approach also demonstrates how these variations integrate into the overall microbial diversity.

Hence, the analysis of the control samples, which serve as the reference point for comparison, revealed no significant biomarkers, indicating a stable and balanced microbial ecosystem under normal conditions.

EA was not included in the graphical representation of LEfSe analysis (Fig. 7 and Fig. 8) since it did not yield specific biomarkers at the stringent threshold set in the LEfse analysis. This could be because its impact on the microbiota may not be as pronounced or distinctive as other PCs or due to a more uniform distribution of EA effects across the microbial community, affecting many taxa to a lesser extent that falls below the threshold for detection. Moreover, it might indicate that EA has a minimal or neutral impact on the microbial taxa examined.

Concerning the first LEfSe analysis, which provides insights into how each NAR and PG, at two concentrations, influenced the GM, it revealed that the different tested concentrations of NAR (200 and 400 μg mL^{-1}) had different effects on GM composition, as mentioned before.

In the presence of NAR (200 μg mL^{-1}), the abundance of *Paraprevotella* strain seemed to increase compared to the control. *Paraprevotella* strains are intestinal commensal bacteria in human GM that can degrade trypsin. The presence of these bacteria can significantly contribute to maintaining intestinal homeostasis and protecting against pathogenic infections (Li et al., 2022).

Whereas NAR at 400 μg mL^{-1} increased the Escherichia/Shigella genera, which contain both harmless and pathogenic strains, and the bacteria belonging to these genera are considered as pathobionts – commensal bacteria that become pathogens due to environmental conditions and host genetics (Baldelli et al., 2021; Pakbin et al., 2021). Although some strains of *E. coli* are harmless, others can cause gut disturbances or infections (Pakbin et al., 2021). This suggests a potentially negative impact on gut health, highlighting the importance of developing a nuanced understanding of the effects of NAR.

Moreover, the NAR modulatory effect seemed to be dose-dependent, which could be important information for supplement formulation to take advantage of the most favorable effects of this flavanone, already reported in the literature (Liu et al., 2023; Wu et al., 2022).

On the other hand, PG seemed to lead to an increase in beneficial taxa, particularly Lactobacillaceae and Bifidobacteriaceae, at 800 and 200 μg mL^{-1}, respectively. These taxa are associated with various health benefits, including supporting gut barrier integrity and modulating the immune response (Alves-Santos et al., 2020). Therefore, this treatment may positively affect gut health, potentially enhancing the gut microbiome's resilience and function.

Regarding the results of the second LEfSe analysis (Fig. 8), which was performed to identify several key taxa associated with the control, EA, NAR, and PG homogenized groups, the analysis once again showed that the EA samples did not reveal a significative association. Similarly to what was observed in the previous LEfSe analysis, NAR samples showed increased taxa such as Enterobacteriales, including commensal and pathogenic bacteria, indicating a complex influence on gut health (Baldelli et al., 2021; Pakbin et al., 2021). Whereas the PG samples exhibited associations with beneficial taxa, such as *Lactobacillus* and *Bacteroides*, which play a role in fiber degradation and the production of critical metabolic by-products (Alves-Santos et al., 2020; L. Li et al., 2023).

Overall, this analysis highlights the selective modulation of the GM by PCs. This has potential implications for host health that require further exploration, particularly regarding the metabolic functions and ecological interactions of these significant taxa within the gut ecosystem.

4. Conclusions

The modulatory potential of three PCs, selected based on their antimicrobial and prebiotic properties, was investigated through *in vitro* fecal fermentation, revealing that EA and PG exhibit a promising effect on GM composition. The fermentation with these PCs led to the production of SCFAs, predominantly acetic acid, propanoic acid, and butyric acid, which are well-known for their beneficial effects on human health. Moreover, the production of these SCFAs has been associated with specific bacteria belonging to the Bacteroidetes and Firmicutes phyla.

Furthermore, according to the results concerning the GM profile of each PC fermentation, the findings indicate that both EA and PG could contribute to the growth of beneficial bacteria, namely *Lactobacillus* and *Bifidobacterium* species. These may be responsible for specific functions within the gut ecosystem, such as fermenting dietary fibers, producing vitamins, or protecting against pathogens. Although EA did not yield LEfSe biomarkers, its consistent ability to preserve diversity while modestly promoting key beneficial taxa positions it as a mild but potentially useful microbiota-modulating compound. In contrast, NAR influenced GM composition by promoting changes in other genera, which could have a pathogenic effect on human health, namely *Escherichia* and *Salmonella*.

Therefore, this study offers novel insights that extend current knowledge on phenolic compound-microbiome interactions, demonstrating that PCs can individually shape the gut's metabolic landscape, with potential implications for host health. These findings are key for the development of new dietary supplements or nutraceuticals, given the PC's ability to modulate GM composition and promote a healthy, balanced gut ecosystem. Future research should focus on combining various PCs to enhance their regulatory effects and maximize the production of beneficial metabolites and fermentation products, thereby optimizing health benefits.

CRediT authorship contribution statement

Adriana C.S. Pais: Writing – original draft, Investigation, Formal analysis. **Tânia B. Ribeiro:** Writing – review & editing, Validation, Investigation. **Ezequiel R. Coscueta:** Writing – review & editing, Validation, Investigation. **Ana Sofia Salsinha:** Writing – review & editing, Investigation. **Maria Manuela Pintado:** Writing – review & editing,

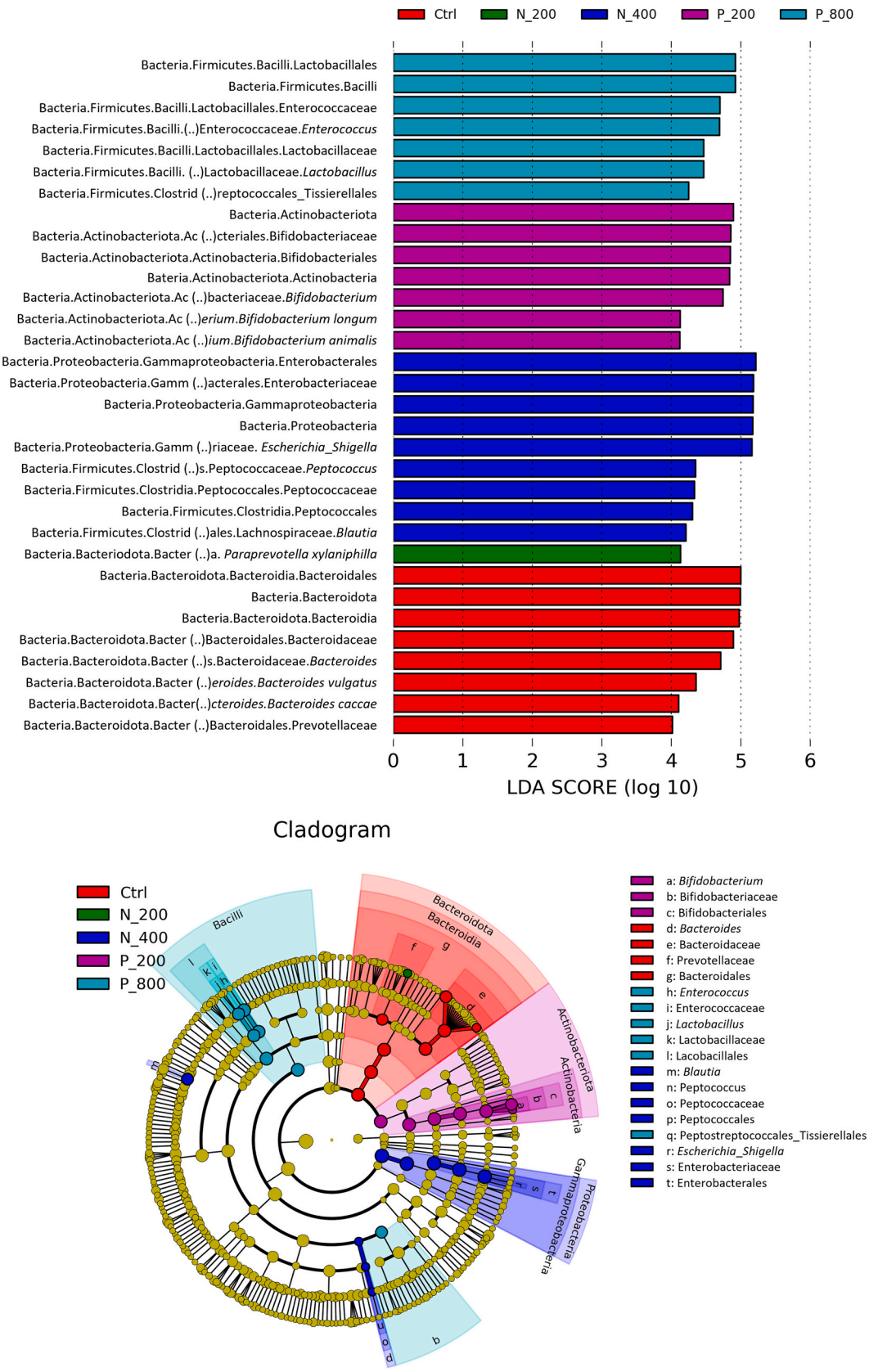
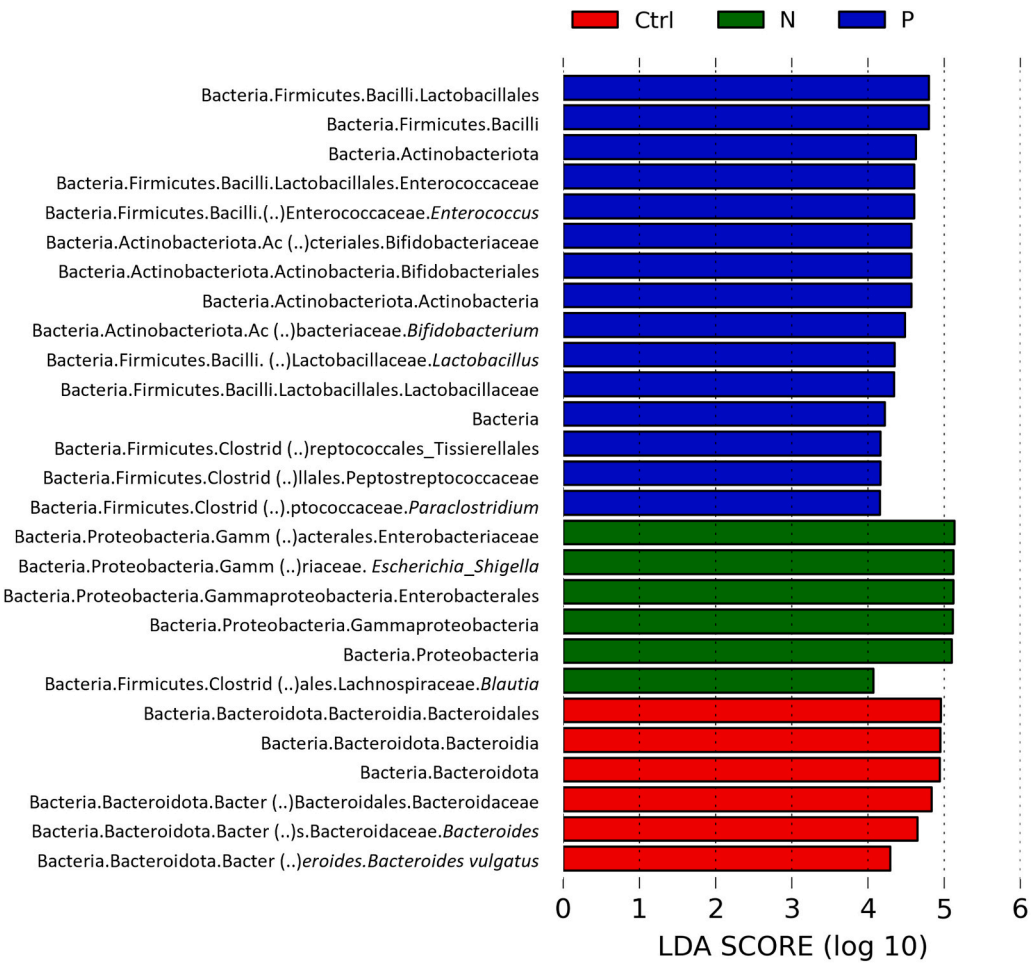


Fig. 7. LefSe histogram and cladogram of the GM composition after fermentation with naringenin (N, at concentrations 200 and 400 μg mL^{-1}) and phloroglucinol (P, at concentrations 200 and 800 μg mL^{-1}).



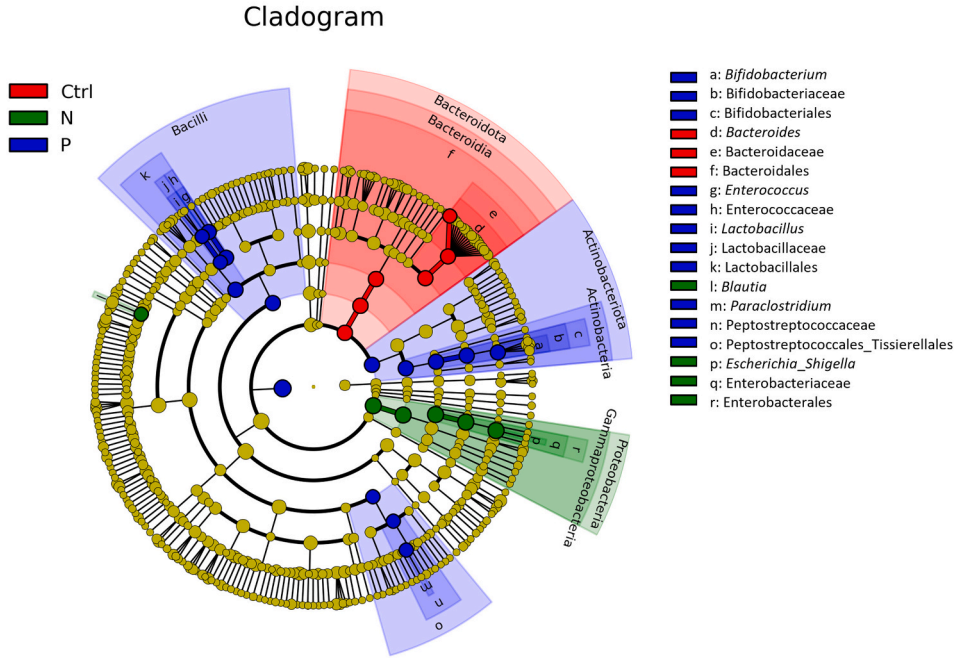


Fig. 8. LefSe histogram and cladogram of the GM composition after fermentation with naringenin (N) and phloroglucinol (P).

Supervision, Funding acquisition. **Armando J.D. Silvestre:** Writing – review & editing, Supervision, Funding acquisition. **Sónia A.O. Santos:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.118167.

Data availability

Data will be made available on request.

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