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Modulation of microbiota composition and markers of gut health after *in vitro* dynamic colonic fermentation of plant sterol-enriched wholemeal rye bread

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ABSTRACT

A human oral phase followed by a dynamic gastrointestinal digestion and colonic fermentation (simgi®) has been applied to wholemeal rye bread (WRB) and PS-enriched WRB (PS-WRB). The aim of this study was to evaluate the impact of these solid and high-fiber food matrices on the metabolism of PS, modulation of the microbiota and production of short-chain fatty acids (SCFA) and ammonium ion after a simulated chronic intake (5 days). In both breads, campesterol, campestanol, stigmasterol, β-sitosterol, sitostanol, Δ5-avenasterol, Δ5,24-stigmastadienol, Δ7-stigmastenol, and Δ7-avenasterol were identified, of which only β-sitosterol was metabolized to sitostenone after PS-WRB treatment. The presence of fiber in both breads exerted a prebiotic effect after fermentation by the increase in Firmicutes (*Lactobacillus* genus, maximum abundance of 89–99 %) and Actinobacteria (*Bifidobacterium* genus, maximum abundance of 30–31 %), reflected in an increase of SCFA content. The reduction of proteolytic activity confirmed by the decrease in ammonium ion contents is related to a reduction in the Proteobacteria phylum. Thus, PS-WRB could be considered as a healthy staple food choice since, besides the known hypocholesterolemic effect of PS, rye bread fiber preserves the beneficial microbiota and exerts a positive impact on markers of gut health.

1. Introduction

The absorption rate of dietary plant sterols (PS) in the small intestine (SI) is between 2 and 3 %, the non-absorbed PS reach the colon, interacting with the microbiota. The influence of PS on the microbiota and their metabolism has been scarcely reviewed (Cuevas-Tena, Alegría, & Lagarda, 2018), indicating that, up to now, the only genus involved in the biotransformation pathways of β-sitosterol, campesterol, and stigmasterol is *Eubacterium* (Eyssen et al., 1973). Microbiota involved in cholesterol metabolism are *Bifidobacterium*, *Eubacterium*, and *Bacteroides*, producing hydrophobic metabolites of cholesterol (cholestenone and coprostanone), which have been involved in colorectal carcinogenesis through their cytotoxic activity on non-tumor colon cells (Makran, Garcia-Llatas, et al., 2023). However, an increased intake of PS may reduce cholesterol conversion (Cuevas-Tena, Alegría, & Lagarda, 2018), as well as production of metabolites such as ethylcoprostanol, which have demonstrated antiproliferative effect and alleviated the cytotoxicity induced by cholestenone (Makran, Garcia-Llatas, et al., 2023).

Bread is a highly consumed food worldwide, and it is intriguing to incorporate a wholemeal alternative into the diet, such as wholemeal rye bread. Therefore, considering the main beneficial hypocholesterolemic effect of PS (reduction of plasma cholesterol by 12 % consuming 1.5–3 g of PS (EFSA, 2012), the European Union has authorized the

Abbreviations: AC, ascending colon; BHT, butylhydroxytoluene; BSTFA, N,O-bis(trimethylsilyl)-trifluoroacetamide; CIAL, Institute of Food Science Research; CSIC, Spanish National Research Council; DC, descending colon; F/B, Firmicutes/Bacteroidota; FL, fermentation liquids; GOS, galactooligosaccharides; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; PCoA, principal coordinate analysis; PERMANOVA, Permutational multivariate analysis of variance; PS-WRB, plant sterol-enriched wholemeal rye bread; PS, plant sterol; SCFA, short-chain fatty acids; SI, small intestine; TC, transverse colon; TMCS, trimethylchlorosilane; WRB, wholemeal rye bread.

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enrichment with PS and commercialization of rye bread in order to reach the effective intakes (Decision 2006/58/EC, 2006/59/EC). The fiber present in rye cereal is mainly insoluble (arabinoxylan, fructan, resistant starch, and β-glucan) (Åman et al., 2010), having a bifidogenic effect and positively modulating abundance of health-beneficial microbes in the gut. In particular, *Bifidobacterium* sp., *Lactobacillus* sp, *Akkermansia* sp, *Fecalibacterium* sp., *Roseburia* sp., *Bacteroides* sp., *Prevotella*, *Roseburia*, *Clostridium lepum*, and *Ruminococcus intestinalis* have been demonstrated to be modulated by these fiber components (Yang et al., 2020).

In vitro methods for simulated gastrointestinal and colonic fermentation have proven to be useful tools to study the effects and responses of food components and food matrices on the composition of the microbiota (Tsitko et al., 2019). In relation to PS, different formulations (PS from a source ingredient and a PS-enriched milk-based fruit beverage) have evidenced the modulation of the microbiota in batch fermentation studies (Cuevas-Tena, del Pulgar, et al., 2018) and dynamic models (Cuevas-Tena et al., 2019; Blanco-Morales et al., 2020; Blanco-Morales et al., 2021). In wholegrain rye breads, grain, and flour, the microbiota composition has been modified or not using *in vitro* (batch or dynamic models) (Maccaferri et al., 2012; Ibrügger et al., 2014; Tsitko et al., 2019; Pirkola et al., 2023; Xu et al., 2023) and *in vivo* (human studies) (Lappi et al., 2013; Vuholm et al., 2017; Laatikainen et al., 2019; Eriksen et al., 2020; Iversen et al., 2022) methods. To the best of our knowledge, the effect of a PS-enriched solid food matrix, such as rye bread, on the modulation of the microbiota and the production of metabolites (PS metabolites, short-chain fatty acids (SCFA), or ammonium ion) has not been evaluated. The use of a dynamic model (simgi®) (Tamargo et al., 2022; Faubel et al., 2024) with a previous human oral phase (Faubel et al., 2022) show a better approximation to physiological situation.

Therefore, the aim of this study is to assess, for the first time, if the enrichment with PS of a high-fiber solid food matrix (wholemeal rye bread (WRB)) affects the modulation of gut microbiota and metabolite production after a dynamic gastrointestinal digestion and colonic fermentation during a 5-day simulated chronic intake of WRB or PS-enriched wholemeal rye bread (PS-WRB).

2. Material and methods

2.1. Reagents

The supplier of commercial wholemeal rye flour was HARINERA LA META S.A. (part of La Meta Group, the Vall Companys Group's flour division, Barcelona, Spain). The PS ingredient used was microencapsulated free PS (purity of 74.7 %, w/w) from tall oil (Lypophytol ME dispersible, palm-free) supplied by Lipofoods (Barcelona, Spain), which also provided a blank ingredient with the same composition but without PS. According to Miedes et al. (2023), the PS profile of the ingredient is as follows: β-sitosterol (78.8 %), sitostanol (10.2 %), campesterol (7.4 %), campestanol (1.4 %), Δ5-avenasterol (0.7 %), Δ7-stigmastenol (0.6 %), stigmasterol (0.5 %), Δ7-avenasterol (0.4 %), and Δ5,24-stigmastadienol (0.2 %). L-ascorbic acid was purchased from Merck LifeScience S.L.U. (St. Louis, MO, USA) with a purity of ≥99.0 %, w/w. The digestive enzymes used were pepsin from porcine gastric mucosa (Ref: P6887) and pancreatin from porcine pancreas (Ref: P1625) purchased from Merck LifeScience S.L.U. (Madrid, Spain) and difcoTM Oxgall Dehydrated Fresh Bile (Ref: 212820) from Thermo Fisher Scientific (Madrid, Spain). The PS standards used were 5α-cholestane as internal standard (IS) (99.9 %), stigmasterol (97.4 %), and stigmastanol (67.5 %), which were purchased from Merck LifeScience S.L.U. (Madrid, Spain). −Sitosterol (98.8 %) and campesterol (98.6 %) were purchased from Chengdu Biopurify Phytochemicals Ltd. (Sichuan, China). Coprostanol (96.0 %) and sitostenona (98.0 %) were purchased from Cymit Química (Barcelona, Spain). Anhydrous pyridine from Acros Organics (Geel, Belgium) and N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) [1 % trimethylchorosilane (TMCS)] were purchased from Merck LifeScience S.L.U. (Madrid, Spain). Butylhydroxytoluene (BHT) and potassium hydroxide were purchased from Merck LifeScience S.L.U. (Madrid, Spain). The ethanol was purchased from VWR (Briare, France). Hexane was purchased from Scharlau (Barcelona, Spain). Water purification was done with a Milli-Q system (Milford, MA, United States).

2.2. Samples

The WRB (55.3 mg PS/100 g) and PS-WRB (1.6 g PS/100 g) were made as stated in Makran, Cilla, et al. (2023). The dough of PS-WRB was made with 300 g of wholemeal rye flour, 2.5 % compressed yeast (based on flour weight), 1.6 % sodium salt (based on flour weight), water (adjusted for optimal absorption, 500 BU, 67 % based on flour weight), 0.01 % ascorbic acid (based on flour weight), and 4.3 % of the flour weight represented by the PS-containing ingredient. The WRB was made by replacing the ingredient that contained PS with a blank ingredient (1.1 % based on flour weight). The elaboration of the breads and the chemical composition are described in Makran, Cilla, et al. (2023).

2.3. Oro-gastrointestinal digestion and colonic fermentation

2.3.1. Description of the model

For the human oral phase, based on a previous study performed with 6 volunteers (Faubel et al., 2022), the one who fulfilled the requirements of a food/saliva ratio of 1:1 (w/w) or a 100 % increase in bolus and providing a bolus consistency not thicker than tomato or mustard paste (Brodkorb et al., 2019) was selected. In the present study, boluses of WRB (n = 5) and PS-WRB (n = 5) were obtained after human mastication (144.28 ± 3.94 g/bolus) corresponding to 81.45 g ± 1.14 g of bread.

For the dynamic gastrointestinal digestion and colonic fermentation, the simgi® system was used, developed by the Institute of Food Science Research (CIAL) of the Spanish National Research Council (CSIC-UAM) (Madrid, Spain) and described in Faubel et al. (2024). This system comprised five compartments with different pH at each phase (stomach 1.8, SI 7, ascending colon (AC) 5.6, transverse colon (TC) 6.3, and descending colon (DC) 6.8). A constant temperature of 37 °C and a flow of enzyme solutions was used. The stomach compartment was replaced with a double-jacketed glass reactor vessel to conduct the gastric phase of the model because of the inability to produce peristaltic movements and problems with stomach emptying that resulted in tube blockage in the system. The gastric content was manually incorporated to the SI, and magnetic stirring (at 800 rpm at the gastric and 1000 rpm at intestinal reactors) was used to homogenize and attempt to mimic peristaltic movements. The gradual addition of enzymes and solutions to maintain pH was carried out as usual in the simgi® system (Tamargo et al., 2022). In the SI, AC, TC, and DC the pipes connect those compartments and the peristaltic valve pumps were automatically controlled. This model is show in Fig. 1.

For the colonic fermentation, the fecal sample was obtained from a healthy donor with the following exclusion criteria: not received any anabolic, hormonal, antibiotic or hypocholesterolemic treatment, not consumed herbal products, enriched foods, vitamin, carotenoid, pro/prebiotics, PS, or phytoestrogen supplements, who had not suffered any acute inflammation or gastrointestinal diseases, did not take any chronic medication, and that was not vegan nor vegetarian. Feces were collected at the CIAL as stated in Rodriquez-Saavedra et al. (2023). The sample was placed in a sterile plastic bottle and transferred it immediately into a gas-tight bag that contained an anaerobic generator system (BD, USA). The fecal sample was transported to the laboratory in a period no longer than 3 h. The experimental procedure of feces collection was approved by the Ethics Committee of the CSIC under the internal registration code AGL2015-64522 and was compliant with the Declaration of Helsinki. The fecal sample was prepared as indicated by Tamargo et al. (2018). An aliquot of 4 g of freshly fecal sample was diluted (20 %, w/v) with 20 mL

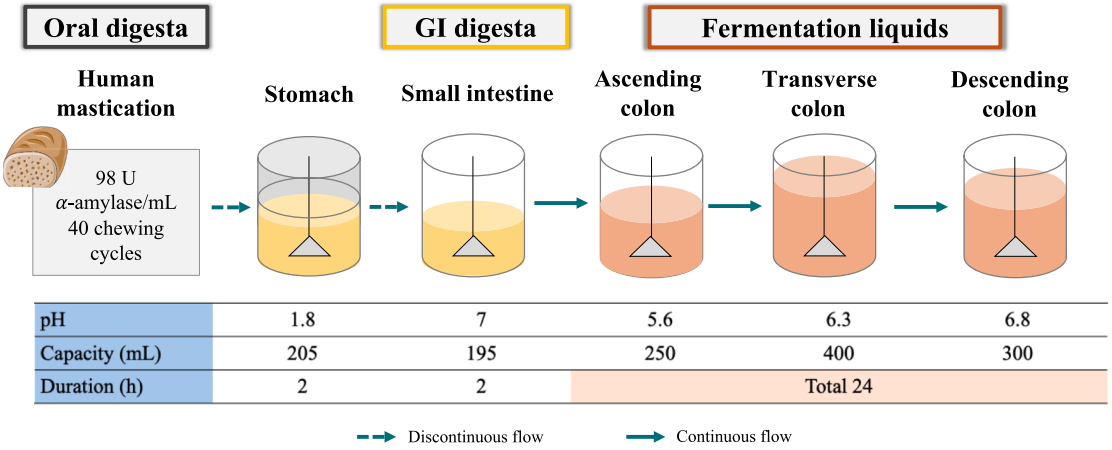

Fig. 1. Oro-gastrointestinal digestion and colonic fermentation.

of sterilized phosphate buffer (0.1 mol/L, pH 7.0), containing 1 g/L sodium thioglycolate as reducing agent. A stomacher (Stomacher 400 Circulator, SEWARD, U.K.) was used to homogenize the fecal mixture during 3 min at 230 rpm.

The AC, TC, and DC compartments were inoculated with the diluted fecal sample and filled with culture medium (AC: 250 mL; TC: 400 mL; DC: 300 mL). The culture medium contains arabinogalactan (1 g/L), pectin from apple (2 g/L), xylan (1 g/L), potato starch (3 g/L), glucose (0.4 g/L), yeast extract (3 g/L), peptone (1 g/L), mucin (4 g/L), and L-cysteine (0.5 g/L). All compounds were dissolved in distilled water and sterilized at 121 °C for 15 min. The transfer volume between the colonic compartments were 145 mL at a rate of 5 mL/min every 8 h.

2.3.2. *Experimental design of gastrointestinal digestion and colonic fermentation*

The gastric digestion was carried out introducing an oral bolus of WRB or PS-WRB into the modified stomach compartment (Fig. 1). To initiate the gastric phase lasting 2 h, gastric juice containing 2000 U/mL of pepsin, dissolved in a total of 15 mL, and delivered at a rate of 3.9 mL/min in 150 mM NaCl, was automatically added. The intestinal digestion and colonic fermentation were performed in the simgi® system feeding the SI during 5-days to simulate a chronic intake (24, 48, 72, 96, 120 h) incorporating the gastric digesta manually into the SI compartment. The intestinal phase was conducted by 2 h introducing intestinal/pancreatic juice, totaling 40 mL and administered at a rate of 5 mL/min, include pancreatin at a concentration of 0.9 g/L and Oxgall dehydrated fresh bile at 6 g/L.

The colonic fermentation latest 32 days and is shown in Fig. 2. A 13-day stabilization period was carried out after inoculation with fecal microbiota and before the WRB treatment, adding 80 mL of culture medium with 40 mL of intestinal/pancreatic juice to the SI three times per day. Once the microbiota was stabilized, the system was fed (5 mL/min) from the SI by adding 145 mL of gastrointestinal digesta which corresponds to 40 g of WRB (of a total of 80 g per day) and, after 8 h, another 145 mL of gastrointestinal digesta corresponding to other 40 g of daily consumption of WRB was added.

A 9-day washout period was performed after the last collection of the WRB treatment in the DC by adding 80 mL of culture medium with 40 mL of intestinal/pancreatic juice in the SI. After the washout period, the PS-WRB treatment was carried out by feeding the equipment with gastrointestinal digesta of PS-WRB in the same way as aforementioned for WRB.

The FL (5 mL) of AC, TC, and DC were taken at S0 h (end of stabilization period), W0 h (end of washout period), 24, 48, 72, 96 and 120 h from both bread treatments.

The FL immediately collected (1 mL) during the stabilization and washout periods, as well as S0, W0h and 72 h were used for the microbial plate count to confirm the maintenance of the microbial population. The FL were decimally diluted in a physiological solution (0.9 % NaCl) and plated (10 μL) on Wilkins Chalgren agar (Difco™ BD) for determination of total anaerobes. The plates were incubated at 37 °C for 48 h in an anaerobic cabinet (BACTRON Anaerobic/Environmental Chamber, SHEL LAB, Cornelius, OR, USA). Colonies were counted using SC6PLUS colony counter (Stuart, UK) (Tamargo et al., 2022). The microbial population levels (log UFC/mL) remained constant without differences during the stabilization and washout period, as well as at S0, W0, and 72 h of fermentation for both bread treatments (AC: 7.8–9.3; TC: 8.4–9.4; DC: 8.2–9.2).

For sterol determination, 2 mL of all the FL collected were stored at −20 °C for further analysis. Another 2 mL of the FL were centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant obtained was filtered through a 0.22 μm filter and stored at −20 °C for SCFA and ammonium

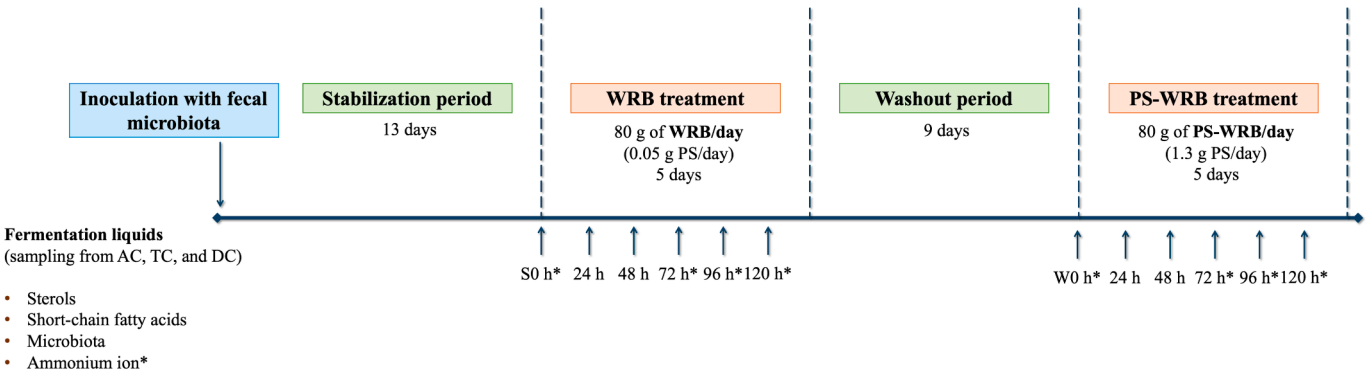

Fig. 2. Experimental design of the colonic fermentation.

ion determination. The pellet obtained after centrifugation was stored at −80 °C for microbial population analysis by PCR.

2.4. Sterol and metabolite determination

The content of sterols and metabolites in FL samples was determined (by triplicate) by applying the methodology used by Blanco-Morales et al. (2020), with slight modifications. To ensure a homogeneous sampling, due to the presence of undigested bread fibers in the FL, a phase separation was performed sampling under continuous stirring. For samples obtained from the WRB treatment, 400 μL of FL were taken. In the case of samples from the PS-WRB, different volumes were employed (50 μL for the analysis of campesterol, β-sitosterol, and sitostanol and 250 μL for coprostanol, cholesterol, campestanol, stigmasterol, Δ5-avenasterol, Δ5,24-stigmastadienol, Δ7-stigmastenol, Δ7-avenasterol and sitostenone). As IS, 10 μg of 5α-cholestane was added. All samples were subjected to hot saponification and extraction of the insaponificable fraction. Subsequently, the derivatization was carried out and the trimethylsilylether derivatives were filtered and dissolved in 100 μL of hexane. For the determination of PS, 0.7 μL were used for the analysis by FAST GC-FID (Shimadzu GC-2025, Kyoto, Japan) equipped with a capillary column Restek Rxi-5Sil MS (10 m x 0.10 mm x 0.10 mm film thickness, Bellafonte, Pennsylvania, USA). The conditions used for the chromatographic analysis are described in Faubel et al. (2024), being the temperature of the oven set initially at 220 °C, increased to 300 °C at a rate of 15.5 °C/min, and then maintained at 325 °C for 0.65 min. Hydrogen was the carrier gas (28.7 mL/min), the temperature of the injector and detector was 325 °C for both, and a split ratio of 1:40 was used. For sterol determination, calibration curves using PS standards were employed (Table S1). The β-sitosterol calibration curve ranged between 0.48 and 14.52 μg in assay was developed to quantify Δ5-avenasterol, Δ5,24-stigmastadienol, Δ7-stigmastenol, and Δ7-avenasterol, since no commercial standards are available. The sitostanol curve was also used for the quantification of campestanol.

Additionally, the background response of S0 and W0 h samples in each colon compartment was used to calculate the limit of detection (LOD) and limit of quantitation (LOQ) of sterols (Table S2). The limits were determined in accordance with recommendations from the U.S. Food and Drug Administration (FDA, 2019). LOD = 3SD/S and LOQ = 10SD/S (where SD is the standard deviation of the method blanks, and S is the slope of the calibration curve).

2.5. DNA extraction and 16S rRNA gene amplicon sequencing

Total DNA from the pellet obtained after the aforementioned centrifugation of the FL samples were isolated (by triplicate) using the QIAamp Fast DNA Stool Mini Kit (SO) (Qiagen, Hilden, Germany), according to the instructions by the manufacturer. Variable V3 and V4 regions of the 16S rDNA gene were amplified following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15,044,223 Rev. A). Gene-specific primers PCR1_f/PCR1_r containing Illumina adapter overhang nucleotide sequences were selected according to Klindworth et al. (2013). After 16S rDNA gene amplification, the multiplexing step was performed using Nextera XT Index Kit. Then, 1 μL of the PCR product was run on a Bioanalyzer DNA 1000 chip to verify the amplicons size (~550 bp). After size verification, the libraries were sequenced using a 2 × 301 pb paired-end run (MiSeq Reagent Kit v3) on a MiSeq Sequencer according to manufacturer's instructions (Illumina, Inc., San Diego, U.S.A). Sequencing data were demultiplexed using Illumina bcl2fastq© program. Demultiplexed paired FASTQ sequence were imported into the QIIME2 v2023.2 for analysis. Quality control was carried out using the DADA2 pipeline incorporated into QIIME2 (Caporaso et al., 2010). The DADA2 program filtered out phiX reads removed chimeric sequences and assigned reads into Amplicon Sequence Variants (ASVs) (Callahan et al., 2016). Taxonomic annotation was obtained using the SILVA v138 database.

2.6. Short-chain fatty acid determination

The SCFA determination (by duplicate) in the FL of the AC, TC, and DC during stabilization, washout and bread treatments was carried out by GC-FID (Agilent 6890A chromatograph equipped with an automatic injector G2613A). An DB-WAXetr column (100 % polyethylene glycol, 60 m x 0.32 mm x 0.25 μm) was employed, with helium serving as the carrier gas at a flow rate of 1.5 mL/min. The temperature gradient was programmed as follows: initial temperature of 50 °C for 2 min, then a ramp of 15 °C/min to 150 °C, 5 °C/min to 200 °C, and finally 15 °C/min to 240 °C, maintaining this temperature for 20 min. Calibration curves (IS: methylvaleric acid) of each compound (acetic, butyric, propionic, valeric, *iso*-butyric, *iso*-valeric, caproic, and heptanoic acids) were obtained by diluting the original stock standard solution (from 5 g/L to 0.005 g/L of all volatile fatty acids) in PBS 0.1 M (Tamargo et al., 2022).

2.7. Ammonium ion analysis

The proteolytic activity of the microbiota was assessed (by duplicate) by quantifying the generation of ammonium ions (expressed as mg NH_4^+/L) as described in Tamargo et al. (2022) with modifications. It was employed the Kit Photometric Spectroquant® Ammonium Test (Merck, Kenilworth, USA) at 690 nm and 25 °C. The data for the ammonium determination in the FL was obtained from a linear regression analysis using an ammonium standard solution within the range of 2 to 75 mg $NH4^+$/L. The samples were diluted with deionized water to adjust their concentration to within the measurement range.

2.8. Statistical analysis

Significant differences ($p < 0.05$) in sterol, metabolites, SCFA or ammonium ion contents in the FL at different fermentation times were evaluated using a one-way ANOVA, followed by Tukey's post hoc test for each fermentation treatment (WRB and PS-WRB) in each colon compartments. The statistical analysis was conducted using the GraphPad Prism (version 9.5.1) software package (GraphPad Software, LLC., Boston, MA, U.S.A.).

MicrobiomeAnalyst version 2.0 was used to calculate α and β-diversities, Pearson r correlation analysis, core microbiome, phyla and genera percentage abundance in the microbiota composition, and to visualize the results. It was used the total sum scaling normalized data and at all statistical tests it was performed a multi-testing adjustment based on Benjamini-Hochberg procedure (FDR, hereinafter referred to as *p*).

The α-diversity differences between bread treatments for the same colon compartment was assessed using the Shannon and Simpson indexes, and statistical comparisons were performed using the Welch/T-test at the genus level ($p < 0.05$). Rarefaction analysis indicate that the number of OTUs per sample reached a maximum, indicating that the sequencing effort captured the diversity of all samples (Fig. S1).

For β-diversity differences across fermentation times for the same colon compartment, the ordination method used was principal coordinate analysis (PCoA) plots, applying a Bray-Curtis index for distance method. Permutational multivariate analysis of variance (PERMANOVA) test was applied to determine the strength and statistical significance of samples clustering based on the distance matrix at the genus level ($p < 0.05$).

Pearson r correlation analysis was performed to test statistically significant correlation ($p < 0.05$) between relative abundance and fermentation times at phylum and genus level. Core microbiome was used to visualize the detected high fraction of the population above a giving abundance threshold (shared taxa present in at least 70 % of samples with relative abundance ≥1 %). Single-factor statistical comparison was performed for a better understanding of the Actinobacteria phylum and *Bifidobacterium* genus modulation during fermentation times of both bread treatments in the AC, TC, and DC (differential

abundance analysis for statistical method using DESeq2 of the MicrobiomeAnalyst version 2.0 program, $p < 0.05$). For the abundance of the microbiota composition across fermentation times of both bread treatments (WRB and PS-WRB) in the AC, TC, and DC it was included the 5 phyla and 10 genera top taxa, expressed as percentage abundance.

3. Results and discussion

3.1. Evolution of sterol contents and metabolite production

The PS content in the FL in the AC, TC, and DC compartments during the fermentation treatments with WRB and PS-WRB is showed in Fig. 3. The mean of values of sterol contents ± standard deviation (Tables S3 and S4) and chromatograms of FL from WRB and PS-WRB (Fig. S2) are included in the Supplementary Material.

The PS identified in the FL of WRB and PS-WRB were campesterol, campestanol, stigmasterol, β-sitosterol, sitostanol, Δ5-avenasterol, Δ5,24-stigmastadienol, Δ7-stigmastenol, and Δ7-avenasterol. These sterols were the same identified in the bread samples (WRB and PS-WRB) (Faubel et al., 2024), except Δ5-avenasterol which was not identified in the FL of WRB.

Regardless of the colon compartment, the lowest values for total and individual PS were shown at 24 h of fermentation of the WRB, except for Δ5,24-stigmastadienol in the AC, for which the contents were constant throughout fermentation. In the AC, at 48 h it was observed a statistically significant increase ($p < 0.05$) in total and individual PS (except Δ5,24-stigmastadienol as aforementioned). In the TC, the same trend was observed, this time after 72 h, with a statistically significant increase ($p < 0.05$) for total and individual PS, except for Δ5,24-stigmastadienol, Δ7-stigmastenol, and Δ7-avenasterol which increased at 48 h. After that, the content of all the PS remained constant until 120 h with no statistically significant differences ($p > 0.05$). In the DC, at 96 h it was stated a statistically significant increment ($p < 0.05$) for total PS, campesterol, campestanol, β-sitosterol, sitostanol, and Δ7-avenasterol, except stigmasterol and Δ7-stigmastenol, for which the statistically significant increase ($p < 0.05$) was at 72 h. Unlike the WRB, the total and individual PS content after PS-WRB fermentation in all colon compartments did not follow a trend, showing variations depending on the fermentation time (Fig. 3 and Table S4).

After fermentation of WRB, no PS were metabolized, contrary to PS-WRB treatment, in which sitostenone was identified (Table S4) as the only metabolite of PS from the fermentation of β-sitosterol, showing a ratio β-sitosterol/sitostenone between 0.0006–0.001. Its content in the AC held stable during fermentation, with a statistically significant decrease ($p < 0.05$) at 120 h, while in the TC increased (statistically significant, $p < 0.05$) at 72 h. The sitostenone could only be quantifiable at 72 and 96 h in the DC. The metabolism of PS from PS-WRB may be attributed to the higher amount of PS (1600 mg/100 g PS-WRB) compared to WRB (55.3 mg/100 g WRB), which could enhance the metabolism of PS.

The microbiota present in the colon compartments during treatments could also be involved in the PS metabolism. It has been shown that only *Eubacterium* genus has been able to biotransform sterols (Cuevas-Tena, Alegría, & Lagarda, 2018). Exactly, *Eubacterium* 21,408 isolated from rat

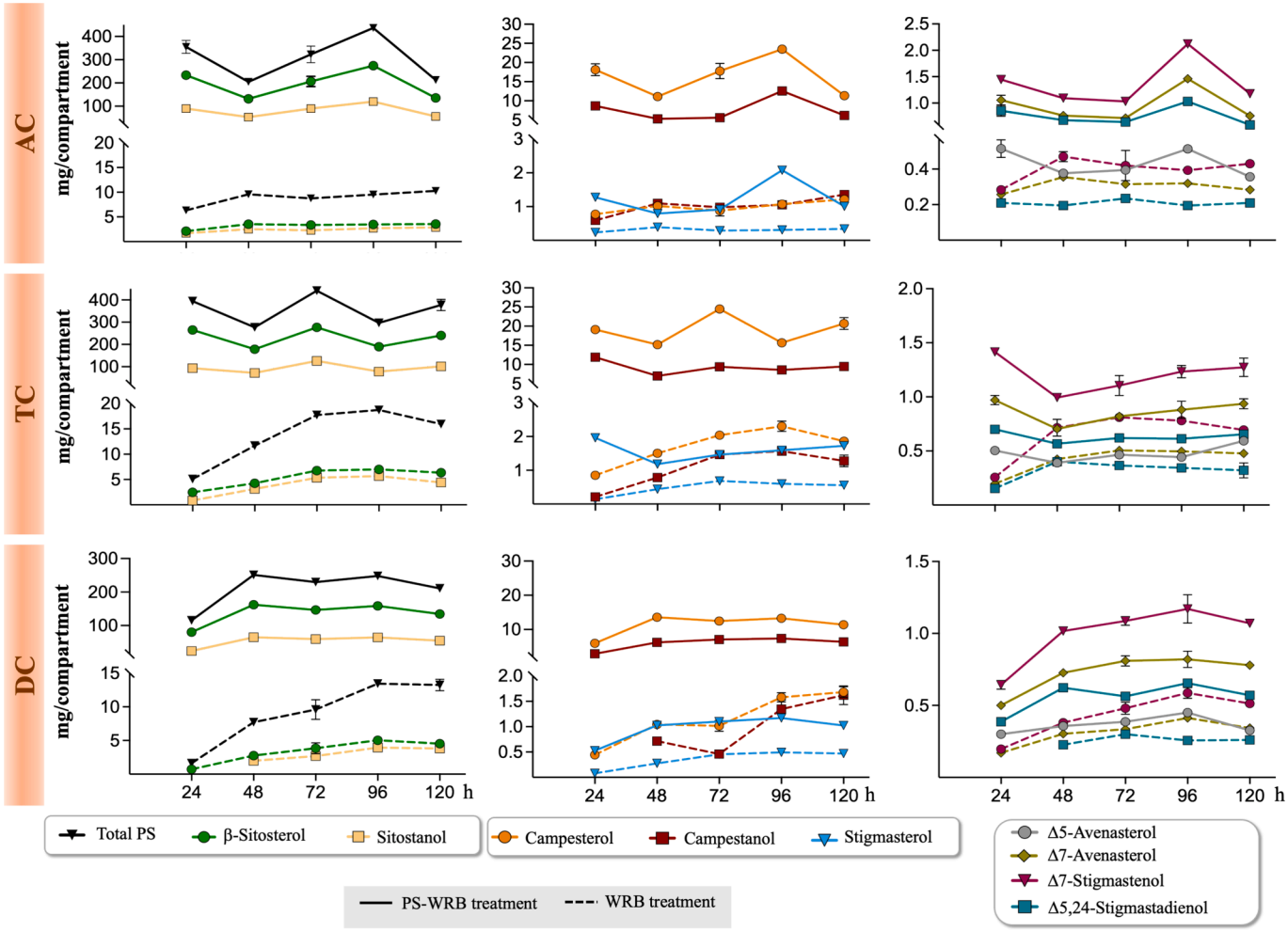

Fig. 3. Plant sterol content in the AC, TC, and DC compartments during fermentation times (24–120 h) of WRB and PS-WRB.

cecal content was able to transform more than 90 % of β-sitosterol, campesterol, and stigmasterol. This microorganism reduces the 5,6-double bond of these sterols yielding 5β-stanols (Eyssen et al., 1973). The metabolism of PS has been evaluated using an *in vitro* 48 h-batch colonic fermentation with the residue obtained after simulated gastrointestinal digestion of a PS-enriched milk-based fruit beverage (Cuevas-Tena, del Pulgar, et al., 2018). A decrease in PS content was observed on the score of an increase in certain metabolites (ethylcoprostanol, ethylcoprostanone, methylcoprostanone, and stigmastenol), which could be explained by the presence of *Eubacterium hallii* after fermentation of the PS residue beverage (Cuevas-Tena et al., 2018). However, two *in vitro* dynamic models (TIM-2 at 24, 48, and 72 h and D-CGD at 24, 48, 72, 96, 120, 144, and 168 h) were used in order to evaluate PS metabolism (Cuevas-Tena et al., 2019; Blanco-Morales et al., 2020; Blanco-Morales et al., 2021). When a PS-source ingredient (Cuevas-Tena et al., 2019) and a PS-enriched milk-based fruit beverage with GOS (Blanco-Morales et al., 2020) were used, PS metabolites (ethylcoprostanol, ethylcoprostanol, ethylcoprostanone, methylcoprostanol, methylcoprostanone, and sitostenone) increases, although none of *Eubacterium*. Hence, Blanco-Morales et al. (2020) suggested that other genera might also play a role in PS metabolism, although specific genera were not identified. However, the use of a PS-enriched milk-based fruit beverage (Blanco-Morales et al., 2021) did not lead to an increase in PS metabolites, possibly because of the absence of GOS, a dietary fiber which could enhance sterol metabolism. Thus, the lack of PS metabolism in our study could be explained by the food matrix (liquid *vs.* solid), the fiber types (soluble fiber such as GOS *vs.* insoluble fiber mainly arabinoxylans in rye bread), and the absence of *Eubacterium* genus and other genera involved in the PS metabolism.

As shown in Fig. S2, cholesterol and coprostanol were identified in the FL. The cholesterol (0.75–1.13 mg in all colon compartment at 0 h) comes from digestion reagents such as bile salts and pancreatin, as reported by Makran et al. (2022) and was identified below LOQ at the rest of the fermentation times. This origin can be corroborated by the fact that Faubel et al. (2024) have analyzed the sterol contents in the same breads, and no cholesterol was identified as expected. In addition, the coprostanol comes from the metabolism by the inoculated microbiota of cholesterol included in the digestion reagents. The formation of coprostanol from cholesterol has been associated with the presence of *Bifidobacterium* (chemical conversion) and *Bacteroides* (reduction) (Cuevas-Tena, Alegría, & Lagarda, 2018). In our study, coprostanol was detected (contents below LOQ) at 24 and 48 h in TC and DC after both bread treatments, agreeing with the fermentation times with the highest abundance of the involved genera (Fig. 9).

3.2. Composition of gut microbiota and metabolites in the colonic fermentation

The α-diversity, using the Shannon and Simpson indexes, identifies local microbial richness an evenness at genus level in the AC, TC, and DC between bread treatments. The Shannon and Simpson indexes revealed a statistically significant decrease ($p < 0.05$, Welch/T-test) in species richness and evenness only in the AC during the PS-WRB treatment. Fig. 4 shows a representation of the Shannon index. The lack of statistically significant differences ($p > 0.05$) in the TC and DC for both bread treatments implied a similar microbial richness and evenness. The Bray-Curtis index dissimilarity β-diversity, using the PCoA, visualizes the genera biodiversity across fermentation times in the AC, TC, and DC, regardless of the bread treatments (Fig. 5). The microbial composition in the AC did not show statistically significant differences ($p > 0.05$, PERMANOVA) across 48–120 h, while a more biodiverse microbial composition was observed in TC (non-statistically significant differences ($p > 0.05$) between 96 and 120 h) and DC (all fermentation times were statistically significant different ($p < 0.05$)).

After observing that there are generally no differences in microbial composition between the two bread treatments (α-diversity), and that the differences are mainly showed across the fermentation times for the same colon compartment and bread (β-diversity), we proceeded to evaluate the differences in phyla and genera abundance at each compartment level and across fermentation times. For this, Pearson r correlation analysis (Fig. 6) was used, indicating that the main phylum that was statistically enriched ($p < 0.05$) across fermentation times was Firmicutes for both bread treatments and all colon compartments. The genus corresponding to this enriched phylum was *Lactobacillus* (Fig. 6) ($p < 0.05$). Actinobacteria phylum did not show a statistically enrichment ($p > 0.05$) in the Pearson r correlation analysis (Fig. 6), however, from 48 to 120 h of colonic fermentation times compared to 0 h it was observed a statistically significant increment ($p < 0.05$, DESeq2) when applying a single-factor statistical comparison for both bread treatments (Table S5), being *Bifidobacterium* the only genus detected (Table S6). Therefore, the core microbiome analysis (Fig. 7) showed that Firmicutes and Actinobacteria phyla were detected in at least 70 % of samples (FL of both bread treatments in the AC, TC, and DC) with relative abundance ≥1 %, specifically *Lactobacillus* and *Bifidobacterium*.

Before bread treatments (0 h), the microbiota composition in the three colon compartments (AC, TC, and DC) showed that the predominant phyla were Firmicutes and Bacteroidota (representing the 70–79 % of total abundance), except in the AC before PS-WRB treatment (highest abundance for Proteobacteria) (Fig. 8). The principal phyla of human gut microbiota are Firmicutes and Bacteroidota, as well as Actinobacteria, Fusobacteria, Proteobacteria, and Verrucomicrobia (Eckburg et al., 2005). However, Firmicutes and Bacteroidota abundance in our

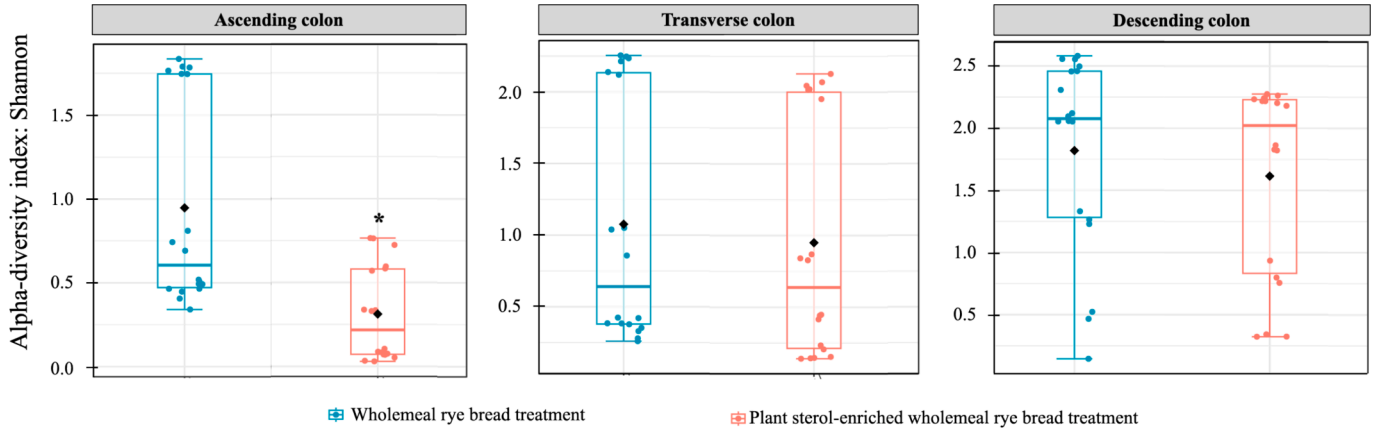

Fig. 4. Shannon index for α-diversity analysis of microbiota genera composition in the AC, TC, and DC between WRB and PS-WRB treatments.

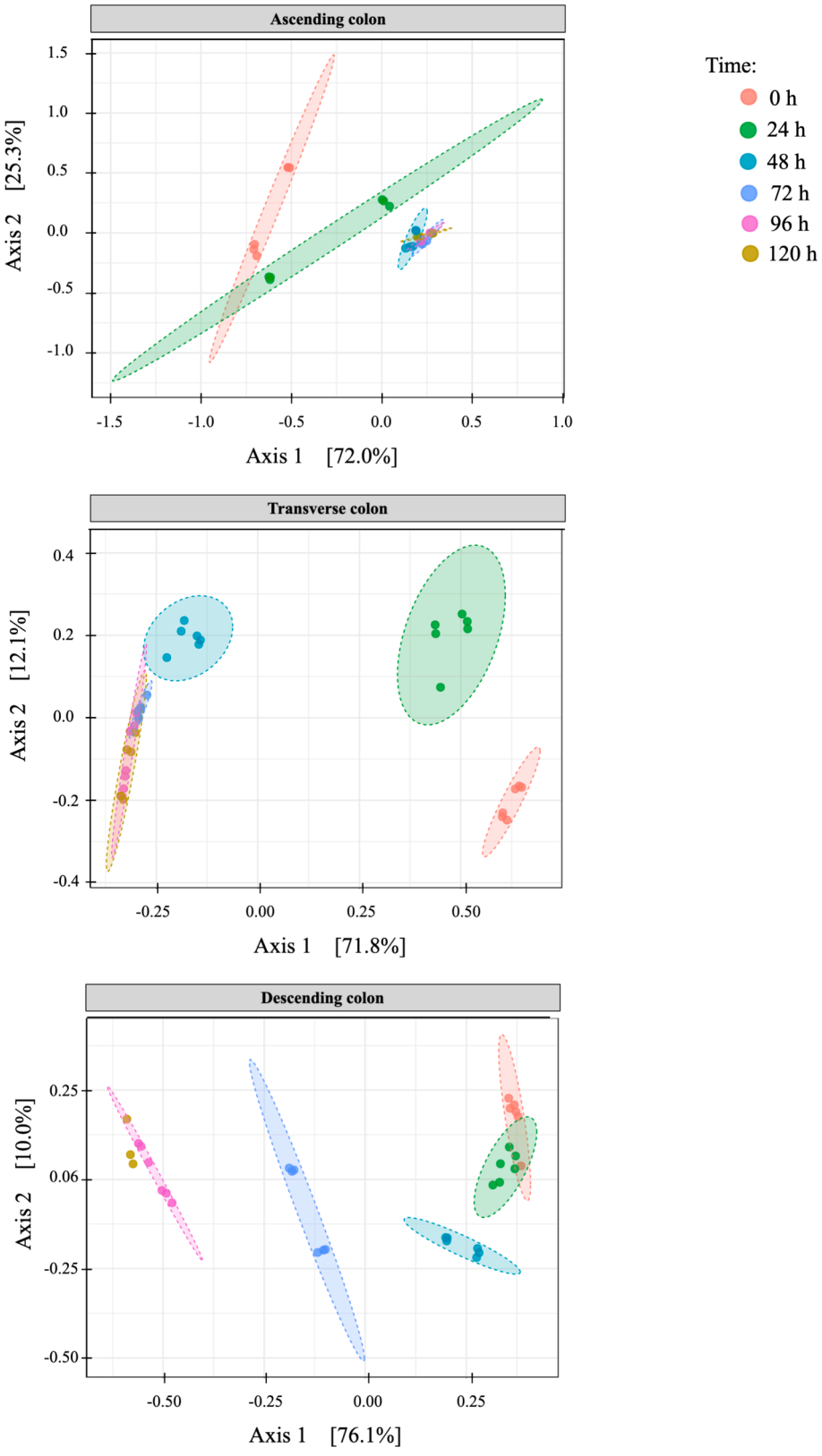

Fig. 5. Principle Coordinate Analysis 2D plot of β-diversity analysis of microbiota genera composition in the AC, TC, and DC across fermentation times of WRB and PS-WRB treatments.

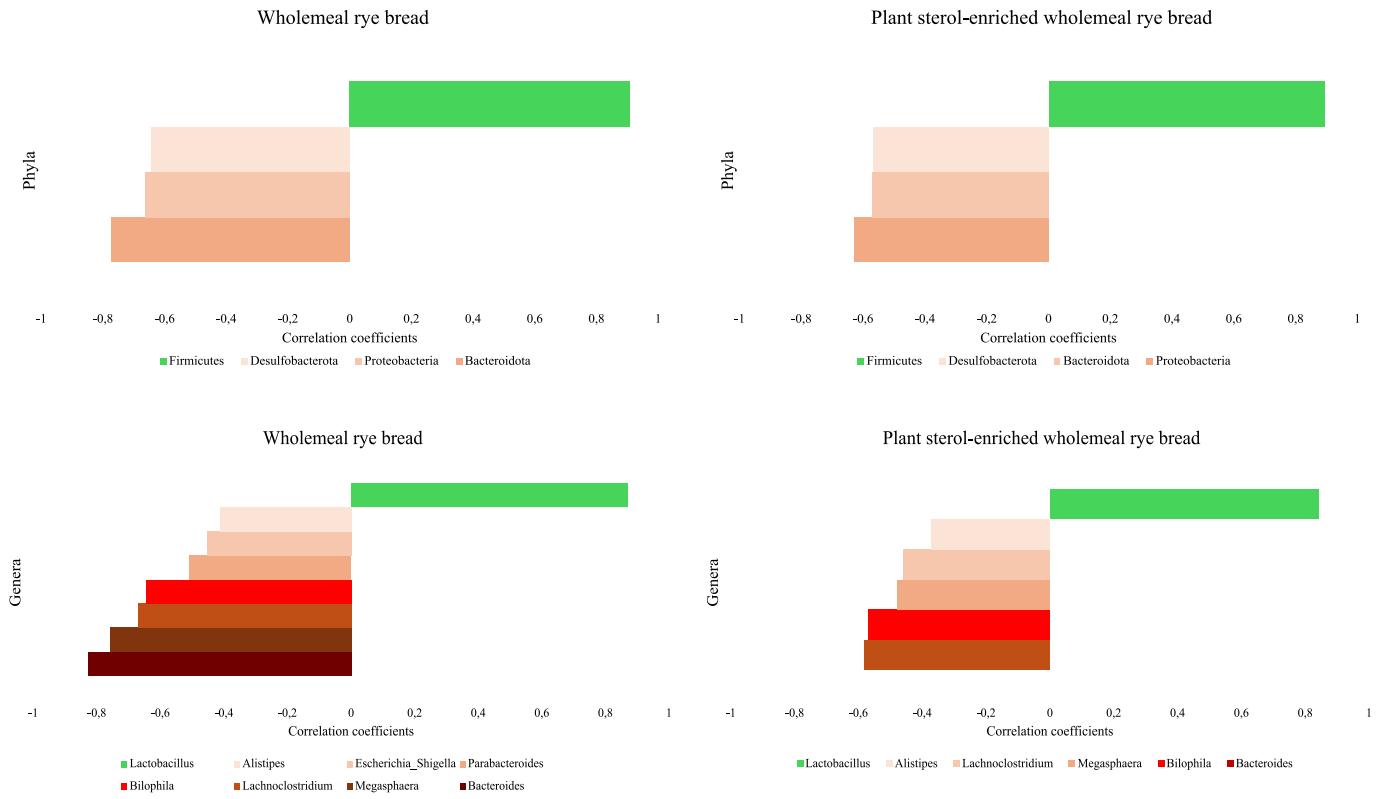

Fig. 6. Pearson r correlation analysis of microbiota phyla and genera abundance across fermentation times of WRB and PS-WRB treatments.

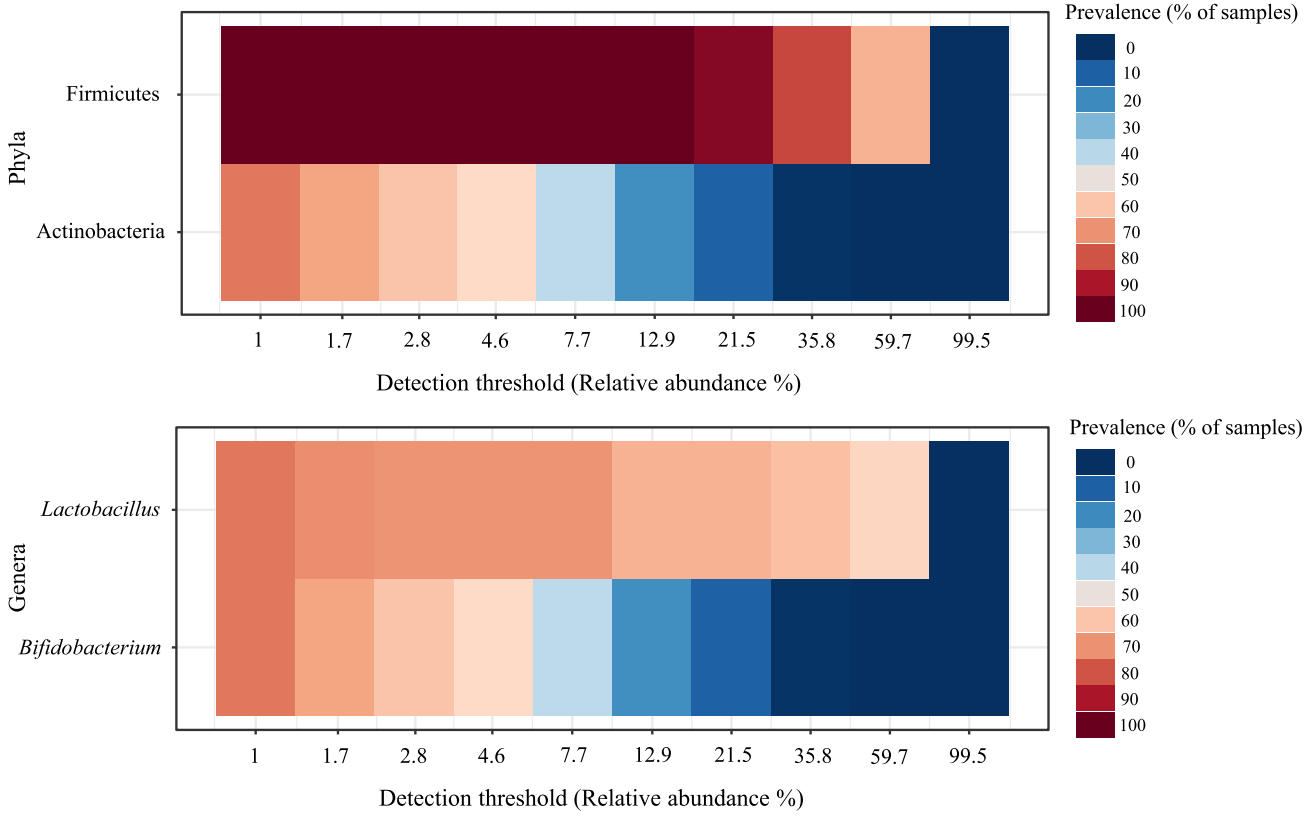

Fig. 7. Core microbiome representation of the most prevalent genera and phyla present in the AC, TC, and DC in all fermentation times of WRB and PS-WRB treatments (plotted as a heatmap including at least 70 % of samples with relative abundance ≥1 %).

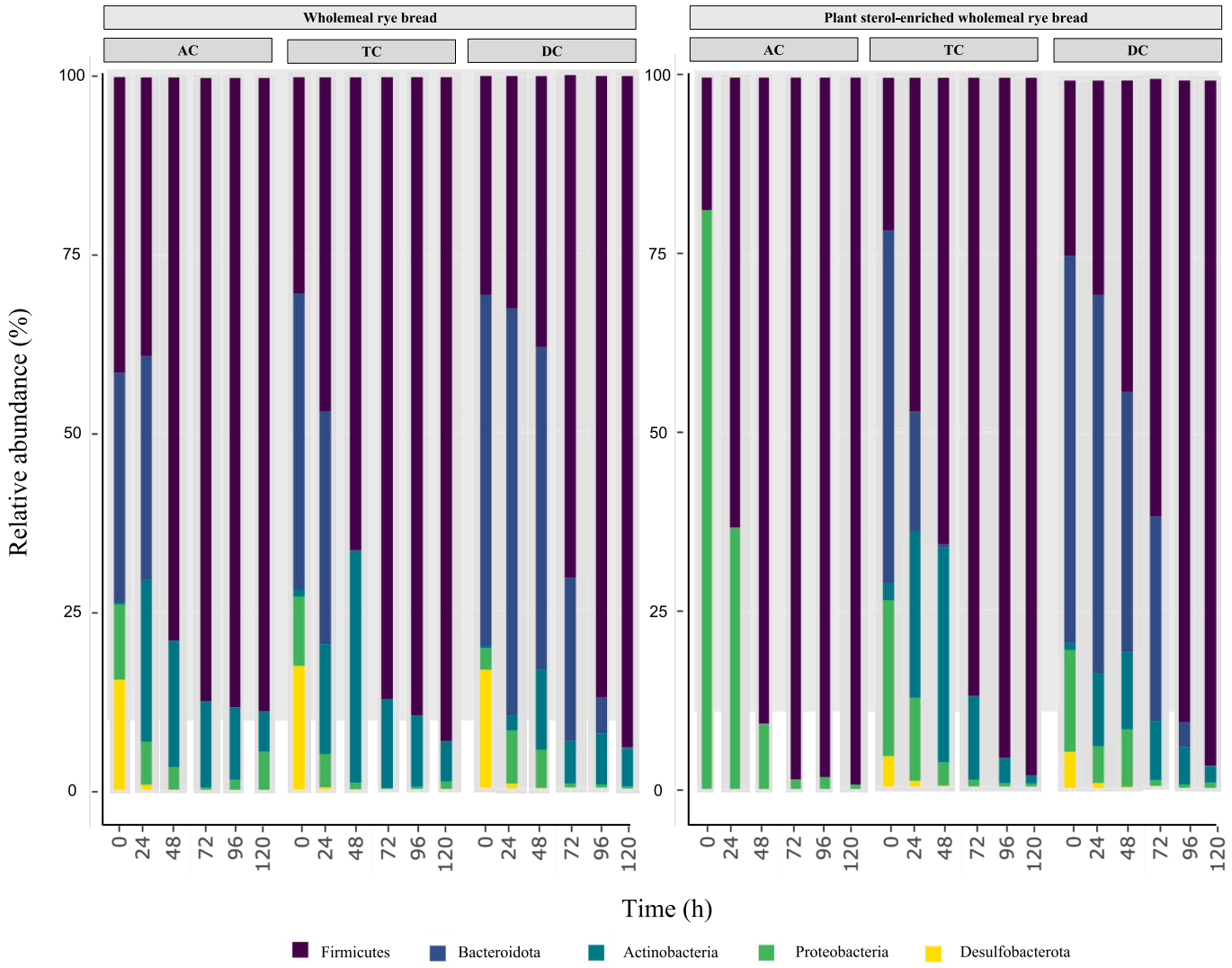

Fig. 8. Evolution of microbiota phyla composition in the AC, TC and DC across fermentation times of WRB and PS-WRB treatments.

study is below the percentage indicated in the human gut microbiota for these phyla (over 90 %) (Eckburg et al., 2005; Ley et al., 2006), possibly due to the abundance of Proteobacteria (3–22 %) and Desulfobacterota (4–17 %) at 0 h, which explains a higher diversity of the microbiota. These differences are reasonable as there is a high inter-individual variability (12 and 2,187-fold) even in the most common 57 species present in 90 % of individuals (Qin et al., 2010).

Thereafter, both bread treatments modulated the microbiota (confirmed by the Pearson r correlation analysis) showing a statistically significant increase ($p < 0.05$) in the phylum Firmicutes (reaching an abundance of 89–99 %) and decrease ($p < 0.05$) in Bacteroidota to negligible values (Fig. 8). Consumption of non-digestible but fermentable carbohydrates, such as resistant starch present in the breads (22.4 g/100 g according to Makran, Cilla, et al. (2023)), enhances fermentative activity resulting in increased acid production (Flint et al., 2012). At acidic pH (5.5), in *in vitro* studies it has been reported a reduction of *Bacteroides* (belonging to Bacteroidota phylum), while butyrate-producing Firmicutes populations are likely to be favored (Walker et al., 2005; Duncan et al., 2009). In addition, the lower intestinal pH provides an unfavorable environment for microbial pathogens and pathogenic bacteria to thrive (Montroy et al., 2020). The increase in the ratio Firmicutes/Bacteroidota (F/B) has been frequently considered as a possible marker for obesity (Magne et al., 2020). However, opposite results (decrease in F/B ratio) or even no association between obesity and F/B ratio has been reported in overweight and obese individuals (Brahe et al., 2013). The increase in Actinobacteria phylum after bread treatments, indicated in the single-factor statistical comparison (Table S5), was a desirable outcome, since this phylum includes *Bifidobacterium* genus with health-promoting effects (Picard et al., 2005). The statistically significant and highest increase ($p < 0.05$) in Actinobacteria abundance was observed at 24 h in the AC only after WRB treatment (abundance of 23 %) and 48 h in TC and DC (30–33 and 11 %, respectively). Another positive outcome was the progressively and statistically significant decreased ($p < 0.05$) of the phylum Proteobacteria (Fig. 8) after both bread treatments (lowest values of 0.3–5 % abundance at 120 h), since this phylum is related to the incidence of inflammatory diseases (Azimirad et al., 2022).

Regarding genera abundance (Fig. 9), the predominant genera at 0 h in all colon compartments were generally *Lachnoclostridium* (11–23 % abundance) and *Megasphaera* (4–12 % abundance) (belonging to Firmicutes phylum), *Bacteroides* (23–29 % abundance) and *Parabacteroides* (0.4–25 % abundance) (Bacteroidota phylum), *Escherichia_Shigella* (2–17 % abundance, Proteobacteria phylum), and *Bilophila* (4–16 % abundance, Desulfobacterota phylum). Both bread treatments shift microbiota genera composition during fermentation process. Despite the aforementioned increase in the phylum Firmicutes, there was a statistically significant decrease ($p < 0.05$) in the genera *Lachnoclostridium* and *Megasphaera* up to 120 h (Fig. 6), yielding *Lactobacillus* as the only

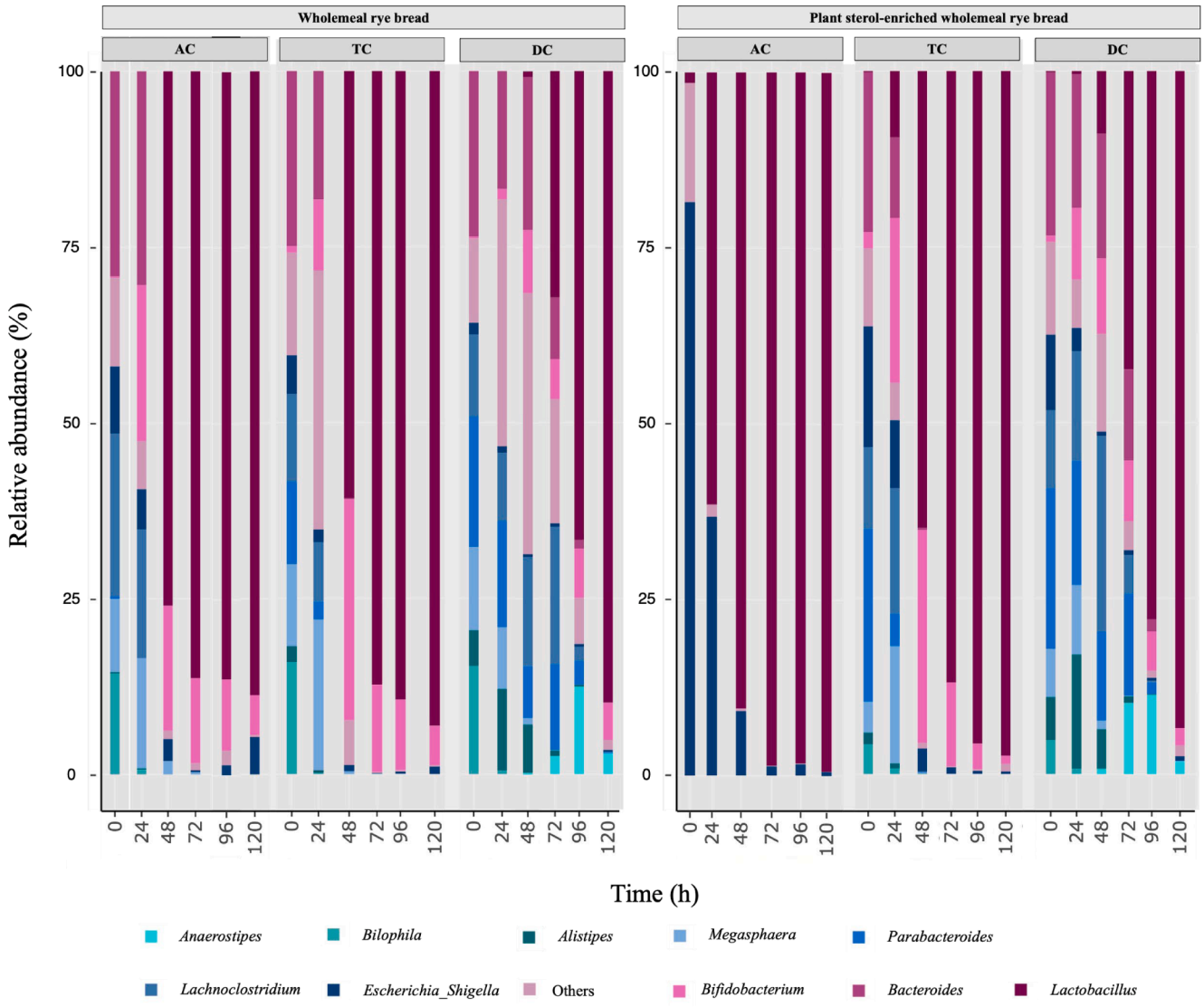

Fig. 9. Evolution of microbiota genera composition in the AC, TC and DC across fermentation times of WRB and PS-WRB treatments.

predominant genus in this phylum (89–99 % of abundance after bread treatments). An elevated abundance of Lactobacillus is considered a beneficial output in the gut health, and it has widely been used as a probiotic microorganism (Abdelazez et al., 2018). Regardless of the mentioned higher ratio F/B after bread treatments possibly related to obesity, some genera (from Firmicutes phylum) that are strongly associated with obesity (*Blautia, Coprococcus, Eubacterium,* and *Ruminococcus*) were not present after the treatments with both breads. *Lactobacillus* genus has been proved to reduce the F/B ratio in murine models and obese adolescents (Stojanov et al., 2020). Regarding *Bifidobacterium* (the only genus present belonging to Actinobacteria), a statistically significant increase ($p < 0.05$) in its abundance (highest values of 30–31 % abundance) was observed after 48 h of bread treatments (Table S6), except in the AC after treatment with PS-WRB, possibly due to the total absence of the genus at 0 h (Fig. 9). It has been noted that dietary fiber, particularly arabinoxylan, fructan, resistant starch, and β-glucan of the rye grain (Åman et al., 2010), enhances fecal abundance of *Bifidobacterium* and *Lactobacillus* due to its prebiotic effect (So et al., 2018; Leeming et al., 2019). Since *Bifidobacterium* genus has been demonstrated as capable to degrade resistant starch in 72 h-batch culture incubations, it utilizes the breakdown products (pullulan, isomaltose, and panose) for growth (Ze et al., 2012).

Regarding wholegrain rye breads, a study using an *in vitro* 24 h-batch colonic fermentation (Ibrügger et al., 2014) observed a similar trend as in our study, with a major density of *Bifidobacterium* and a lower relative quantity of *Bacteroides* compared to control. However, Pirkola et al. (2023), using the same fermentation model with two different microbiota donors, indicates differences in the modulation of the microbiota associated to the initial donor rather than the sample. The whole grain rye bread may act as a substrate to enhance the growth of the predominant species initially present (principal increase of *Bacteroides* and *Escherichia/Shigella* in donor I and *Subdoligranulum* and *Bifidobacterium* in donor II). These differences points to the importance of the initial microbiota in its modulation, reflected by the aforementioned high variability of microbiota between subjects (Qin et al., 2010). The increase of *Lactobacillus* in our study, not reported by the aforementioned studies performed with rye bread, could be due to different colonic fermentation (dynamic *vs.* batch). Additionally, the bread composition may be a factor behind the differences, since our bread contains only rye as grain ingredient, compared with a 58 % of rye and addition of other cereals (wheat and barley) by Pirkola et al. (2023). Ibrügger et al. (2014) do not indicate the ingredients of the bread.

In contrast, the only two human studies on rye bread (Lappi et al., 2013; Laatikainen et al., 2019) and one study incorporating cereal products elaborated with rye grain (Vuholm et al., 2017) showed non-significant changes of the relative abundance of any bacterial taxa,

regardless the health status of human (metabolic syndrome, irritable bowel disease, or healthy adults, respectively) and the intervention period (12, 4 or 6 weeks, respectively). These results could be due to the habitual consumption of high-whole grain food by the subjects previous to the study (67 previously *vs.* 75 g whole grain/day in the intervention period) (Lappi et al., 2013). The addition or increment of high-fiber foods does not alter the microbial diversity as long as the individual continues with his/her usual diet (Vuholm et al., 2017; Laatikainen et al., 2019). Whereas an increase in *Bifidobacterium* genus has been observed in subsequent studies incorporating similar cereal products elaborated with rye grain (reaching 30 g dietary fiber/day) during 8 weeks in men with metabolic syndrome (Eriksen et al., 2020) and during 12 weeks in healthy humans (Iversen et al., 2022).

An *in vitro* 48 h-batch colonic fermentation of an enzymatically treated rye bran (using a mixture of commercial hydrolytic enzyme preparation containing α-amylase, as in our study in the oral phase)

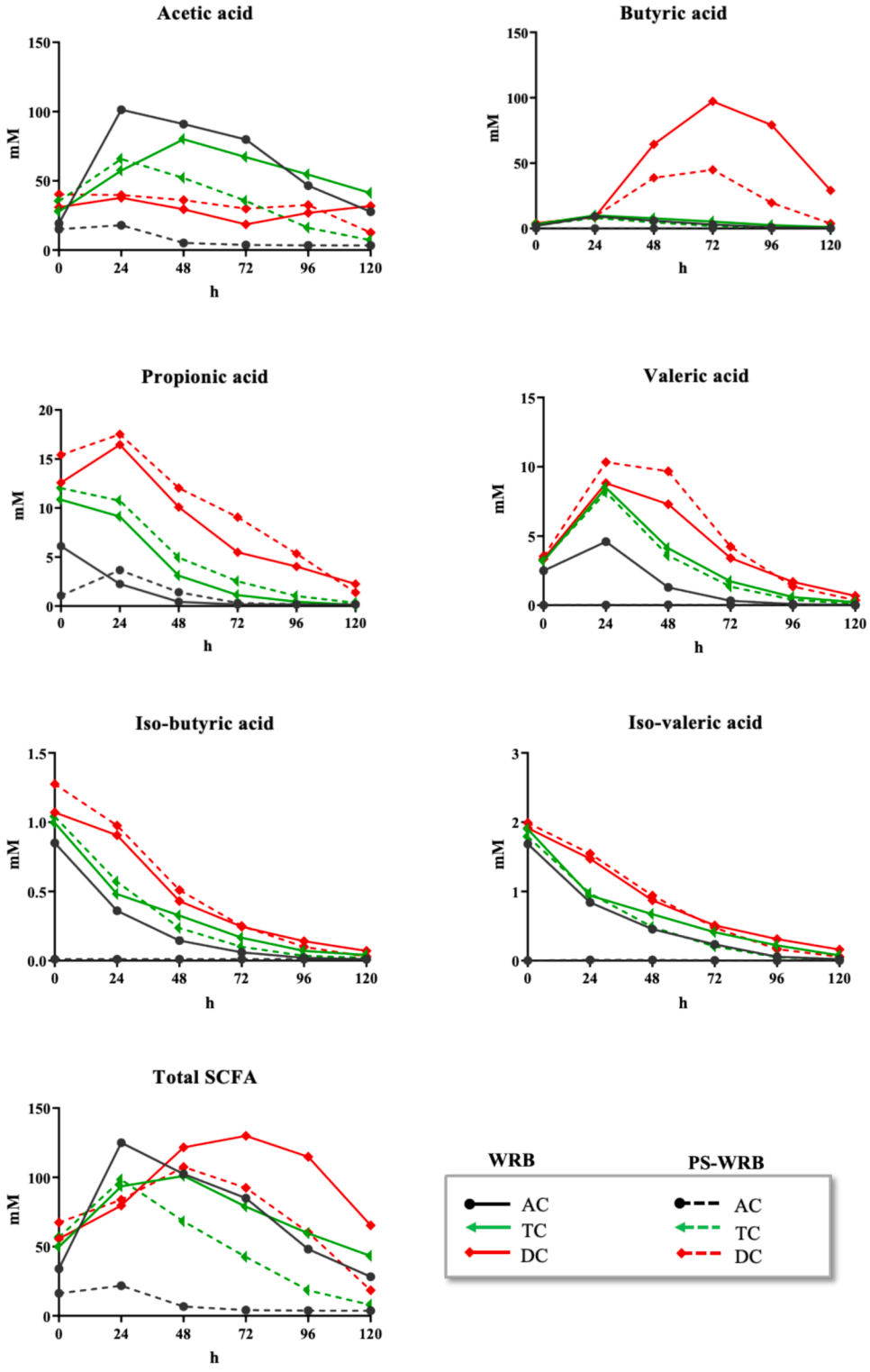

Fig. 10. Total and individual SCFA contents in the AC, TC, and DC during fermentation of WRB and PS-WRB.

(Tsitko et al., 2019) or a three-stage continuous culture gut model of wholegrain rye flour (Maccaferri et al., 2012) has been used. As in our work, both studies indicate an increase of Bifidobacteriaceae and Lactobacillaceae families. The importance of dietary fiber composition has been assessed by Tsitko et al. (2019), reporting a higher abundance of the Bifidobacteriaceae family in enzymatically treated rye bran with high insoluble fiber content (37.1 g/100 g sample) *vs.* higher abundance of Lactobacillaceae family with only 6.6 g soluble fiber/100 g sample. As in our study, an improvement of the abundance of *Bifidobacterium* and *Lactobacillus* has been observed in milled grains during an *in vitro* 24 h-batch colonic fermentation of a 2:3 mixture of rye and wheat (Xu et al., 2023).

SCFA are metabolites produced by intestinal microbiota showing various bioactivities in *in vivo* (animals and humans) and *in vitro* (cells) studies, such as anti-inflammatory and immunoregulatory effects, as well as preventive and therapeutic effects on several diseases, including obesity, cardiovascular disease, liver disease, diabetes mellitus, irritable bowel disease, diarrhea, constipation, neurodegenerative diseases, neuropsychiatric diseases, cancers, arthritis, and periodontal disease (Xiong et al., 2022). SCFA contents in the FL from AC, TC, and DC after fermentation of WRB and PS-WRB are shown in Fig. 10. The values of mean SCFA contents ± standard deviation (Tables S7 and S8) are included in the Supplementary Material.

In both bread treatments, the same SCFA (acetic, butyric, propionic, valeric, *iso*-butyric, *iso*-valeric, caproic, and heptanoic acids) were identified. Higher total SCFA contents were observed after WRB (101–130 mM) *vs.* PS-WRB treatment (22–107 mM). The highest total SCFA contents ($p < 0.05$) were observed at 24 h in the AC only after WRB treatment (3.7-fold), and at 24–48 h in the TC (1.7–2.0-fold), and 48–72 h in the DC (1.6–2.3-fold) for both bread treatments (Fig. 10). The statistically significant increment ($p < 0.05$) in acetic acid in the AC (24 h after WRB treatment) and TC (24–48 h in both bread treatments) could explain the aforementioned high content of total SCFA. This increase could be related to the highest abundance of *Bifidobacterium* and *Bacteroides* at 24 h, known as acetic acid-producing bacteria (Nogal et al., 2021). During the fermentation of PS-WRB, the absence of the aforementioned genera could be the reason for an 82 % lower content of acetic acid in the AC. At 72 h in the DC, a statistically significant increment ($p < 0.05$) was observed in the butyric acid content after both bread treatments (24- and 12-fold for WRB and PS-WRB), which contributes to a high SCFA content in the DC compartment. Theses increase of butyric acid could be attributed to the appearance of *Anaerostipes* at 72 h in the DC, which uses acetic acid (Liang et al., 2023) and resistant starch (22.4 g/100 g of both breads) (Topping & Clifton, 2001) to produce butyric acid. Propionic acid showed a statistically significant increase ($p < 0.05$) only at 24 h in the DC, which could be explained by the highest abundance of *Alistipes* (propionic acid-producing bacteria) at this fermentation time (Parker et al., 2020). In contrast, valeric, *iso*-butyric, *iso*-valeric, caproic, and heptanoic acids generally decrease ($p < 0.05$) throughout both bread fermentation assays.

As in our study, an increase in total SCFA content has been observed using whole grain rye bread (Ibrügger et al., 2014; Pirkola et al., 2023) and mixed grains (wheat + rye) (Xu et al., 2023) after an *in vitro* 24 h-batch colonic fermentation, as well as enzymatically treated rye bran submitted to an *in vitro* 48 h-batch colonic fermentation (Tsitko et al., 2019). These studies reported a generalized total SCFA increment (between 1.7- and 17-fold), especially acetic (or acetate) and butyric (or butyrate) acids due to the growth of *Bifidobacterium*. Pirkola et al. (2023) indicates a clear difference in the total SCFA production after colonic fermentation of whole grain rye bread with different fecal microbiota composition since higher initial contents in donor II (85 *vs.* 40 mM) led to higher final contents (125 *vs.* 100 mM). In our work, the total SCFA contents after *in vitro* 24 h-dynamic colonic fermentation of both breads are between 22 and 125 mM for all colon compartments, range included among the values reported by Ibrügger et al. (2014) (33 mM) and Pirkola et al. (2023) (100–125 mM).

There are only two human studies assessing fecal SCFA contents incorporating cereal products elaborated with rye grain (Vuholm et al., 2017; Eriksen et al., 2020). In subjects with metabolic syndrome (Eriksen et al., 2020), a significant increment was only observed for butyrate after 8 weeks of rye products consumption. However, controversial results have been observed in healthy humans (Vuholm et al., 2017) since the content of total SCFA and acetate decreased after 6 weeks of rye products consumption, which could be explained by the lack of changes in the microbiota composition, as well as a high baseline fecal SCFA concentration.

PS content could also affect the production of SCFA during colonic fermentation. In this regard, in our study a lower production (18–78 %) of total SCFA after PS-WRB compared to WRB treatment has been observed. However, studies of dynamic colonic fermentation using a PS-source ingredient (Cuevas-Tena et al., 2019) or PS-enriched beverages (Blanco-Morales et al., 2020; Blanco-Morales et al., 2021) reported an increment of total SCFA contents, being acetate the most predominant as in our study. Cuevas-Tena et al. (2019) indicates the different pattern on SCFA depending on the initial microbiota, since obese donor inoculum did not produce an increment in the SCFA after PS fermentation. The production of SCFA after colonic fermentation of a PS-enriched milk-based fruit beverage with GOS has been evaluated, reporting an increase up to 80–130 mM at 120 h (Blanco-Morales et al., 2020). However, the same beverage without GOS (Blanco-Morales et al., 2021) results in a lower increase of SCFA (52–85 mM) at the same fermentation time, which could indicate the contribution of fiber to SCFA production. However, in our study, lower SCFA content (3.7–18.5 mM) was observed at 120 h, which could be attributed to several factors: different food matrix (liquid *vs.* solid, respectively); fiber contents (1.8 g/100 g *vs.* 15.3–15.7 g/100 g) and fiber type (GOS soluble fiber *vs.* mainly insoluble fiber); different microbiota at 0 and 120 h after fermentation (*Clostridium*, *Bacteroides*, and *Klebsiella vs. Lactobacillus*); as well as the dynamic colonic fermentation model (D-CGD *vs.* simgi®).

The ammonium ion produced by intestinal microbiota was used to assess the proteolytic activity after WRB or PS-WRB in the three colon compartments (Table 1). Highest content of ammonium ion was observed at 0 h in TC and DC compared to AC. A statistically significant decrease ($p < 0.05$) was observed in the ammonium ion contents after the feeding periods with both bread treatments. A general recovery of the ammonium ion content was observed after the washout period of the WRB treatment. The desirable decrease in ammonium ion levels could be attributed to the protein content of WRB and PS-WRB, which is 6.3 g and 5.9 g/100 g bread, respectively. This relatively lower protein content, compared to the fiber content (15.3–15.7 g/100 g), could inhibit

Table 1
Ammonium ion content (mg/L) in the fermentation liquids in the ascending (AC), transverse (TC), and descending (DC) colon during the wholemeal rye bread (WRB) and plant sterol-enriched wholemeal rye bread (PS-WRB) treatments.

		0 h	72 h	96 h	120 h
WRB	AC	279.26 ± 20.84^{a}	79.85 ± 2.63^{b}	46.57 ± 0.12^{b}	27.65 ± 0.67^{b}
	TC	368.79 ± 16.48^{a}	67.08 ± 1.57^{b}	54.50 ± 0.65^{bc}	41.11 ± 1.34^{c}
	DC	417.96 ± 10.45^{a}	18.59 ± 0.25^{b}	26.87 ± 0.16^{b}	31.84 ± 1.84^{c}
PS-WRB	AC	179.82 ± 4.53^{a}	31.36 ± 0.85^{b}	39.76 ± 0.06^{bc}	44.46 ± 0.01^{c}
	TC	375.52 ± 5.30^{a}	39.76 ± 2.00^{b}	41.59 ± 0.81^{b}	42.17 ± 0.21^{b}
	DC	465.90 ± 1.47^{a}	54.82 ± 1.05^{b}	57.01 ± 0.13^{b}	47.58 ± 0.60^{c}

Data expressed as mean ± standard deviation (n = 2). Statistically significant differences ($p < 0.05$) between fermentation times for each colon compartment and bread treatment indicated with superscript letters (a-c).

proteolytic fermentation. In addition, the statistically significant decrease ($p < 0.05$) of Proteobacteria phylum, especially *Escherichia_-Shigella* genus could promote the reduction in proteolytic activity since this genus is related with protein fermentation. The suppression of protein fermentation by dietary fiber is thought to be due to the decreasing demand for amino acids as an energy source, and lower pH from SCFA production inhibiting proteolytic enzymes. Furthermore, increased carbohydrate fermentation and bacterial growth can decrease ammonia concentrations in the gut due to higher incorporation of nitrogen into microbial cells (Diether & Willing, 2019). These results are considered desirable since negative effects of ammonium production and presence in the colon have been observed in *in vitro* and animal models (histological damage of the colonic mucus layer and reduction of colonocyte lifespan, decline in the absorptive capacity of colonocytes, greater expression of marker genes reflecting mucosal cell turnover and synthesis of pro-inflammatory cytokines, inhibition of butyrate oxidation and acetate and propionate oxidation, and increment in epithelial permeability) (Yao et al., 2016).

4. Conclusions

The scarcity of PS metabolites (only sitostenone present after colonic fermentation of PS-WRB) could be related to the absence of microbiota involved in their biotransformation (principally *Eubacterium*). The digestion and fermentation of the WRB and PS-WRB exerted a growth of *Lactobacillus* and *Bifidobacterium* genera, possibly explained by the bifidogenic effect of the rye bread fiber, leading to beneficial outcomes in markers of gut health (increment of total SCFA and reduction of ammonium ion). Therefore, the novelty of this study is the confirmation that the presence of PS, does not modify the beneficial modulation of the microbiota composition exerted by the WRB fiber. Thus, the PS-WRB can be considered a healthy staple food alternative to other more consumed bread, preserving the beneficial microbiota enhanced by rye bread fiber and its positive impact on markers of gut health.

CRediT authorship contribution statement

Nerea Faubel: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Virginia Blanco-Morales:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Data curation. **Vicente Sentandreu:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis, Data curation. **Reyes Barberá:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Guadalupe Garcia-Llatas:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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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.2024.115570.

Data availability

Data will be made available on request.

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