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# Colonic fermentation of enzymatically treated cocoa bean shells (CBSs) and short chain fatty acids (SCFAs) production.

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ABSTRACT

Cocoa bean shells (CBSs) are a by-product of the cocoa production chain characterized by a substantial dietary fiber (DF) content. The aim of this work was the evaluation of the prebiotic activity of DF from raw CBSs as well as defatted and dephenolized CBSs (polyphenols in their free form) treated with different enzymes mixtures to increase the fermentable fiber portion. Fermentability was tested through an in vitro model of colon fermentation using microbiota selectively adapted to more proximal and most distal compartments of the colon. Results showed a significant amount of short chain fatty acids (SCFAs) produced, particularly acetate, from fermentation of the lipid- and polyphenols-free CBS treated with the cellulase mixture. In both colonic regions, this sample boosted the production of SCFAs, suggesting the potential usefulness of this enzyme-driven processing to improve the prebiotic effect of CBS. Despite these findings, there was not a change in the DF content of the enzymatically treated samples, especially regarding the soluble dietary fiber (SDF) fraction. This outcome suggests that a structural change may have occurred in the fiber fraction increasing its fermentability. These outcomes open a new scenario in the bio-valorization of CBSs, in accordance with the concept of circular economy.

## 1. Introduction

Cocoa (*Theobroma cacao* L.) is the fruit of the cocoa tree and is massively consumed all over the world to produce chocolate and cocoa-associated food and beverage with fine flavour (da Cruz Ferraz Dutra et al., 2023; Erazo Solorzano et al., 2023). During the massive production of cocoa-related products, cocoa bean shells (CBSs) are produced in large amount as by-products of the roasting process, more precisely from the cocoa beans dehulling, and discarded (Younes et al., 2022). On average, ten times more waste is produced than final cocoa powder (da Cruz Ferraz Dutra et al., 2023). Despite being considered a by-product (or simply waste, in the past), CBSs is rich in dietary fiber and antioxidant compounds (Cinar et al., 2021). The richness of these bioactive compounds has led the attention over their functionalization and exploitation as ingredient for their prebiotic and antioxidant activity (Younes et al., 2022). CBSs are characterized by a very high fiber content, ranging from 18.3 to 59% dry matter (Lecumberri et al., 2007) and have a high functional value due to their phenolic composition (Delgado-Ospina et al., 2021).

Dietary Fibers (DF) are defined by the Codex Alimentarius as carbohydrate polymers with ten or more monomeric units, that are not hydrolyzed by the endogenous enzymes in the small intestine of humans (FAO/WHO, 2010). DF could be divided in two major groups: “soluble” (SDF) and “insoluble” (IDF), depending on their role in the intestine and the water solubility. However, the classification of fibers in terms of solubility depends on the analytical methods used for its quantification. More recently, the classification in “fermentable” and “non-fermentable” fiber is broadly accepted, depending on the capacity of the human intestinal microbial communities to process the matrix in the gut. Anyway, as traditionally classified, SDF such as pectins, gums, mucilages and oligosaccharides are commonly found in fruits (Arlorio et al., 2001), legumes, vegetables and plant extract (Mudgil, 2017). IDF such as cellulose, lignin, hemicellulose, resistant starch commonly found in plants, woody plants, cereal and also in processed foods can enhance osmolarity - hence water retention - increasing stool volume, speeding up stool transit and being a valuable substrate for distal colon microbial communities (Arayici et al., 2022). DF can be easily fermented by the colonic microorganisms, conferring health benefit to the host by triggering many physiological mechanisms. Whereas solubility could be roughly associated with fermentability both insoluble and soluble fiber fractions – principally depending on the polysaccharidic structure - could be metabolized by the microorganisms, showing prebiotic

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properties.

Many attempts are being made to enhance the fermentability of DF through different techniques such as thermomechanical treatments (M. Lamothe et al., 2021; Su et al., 2024; Tejada-Ortigoza et al., 2019), particle reduction treatments (Nsor-Atindana et al., 2020; Paepe et al., 2019) and enzymatic hydrolysis (M. Lamothe et al., 2021; Paepe et al., 2019), with promising results in terms of food ingredient with prebiotic effect and desirable textural characteristics. More specifically, despite CBSs richness in dietary fiber, most of the work that has been performed to valorize CBSs as functional ingredients, has focused on the antioxidant polyphenols, (Pavlović et al., 2020; Rojo-Poveda et al., 2019; Rossin et al., 2021) and little work has focused on CBSs as dietary fiber source (Nogueira Soares Souza et al., 2022). Up to date of writing, only one work has been performed to valorize CBSs dietary fiber through enzymatic treatment (Younes & Karboune, 2023).

The recent growing interest, in the last decades, on DF is justified for their beneficial effect on human health and the fact that regular fiber consumption has been associated with lower risk of many diseases such as cardiovascular diseases, gastrointestinal diseases, diabetes, hypertension, colon cancer risk and this effect is, in part, related to its fermentation by the gut microbiota and consequent short chain fatty acid (SCFAs) production (Arayici et al., 2022; Delgado-Ospina et al., 2021; Xu et al., 2022). DF selectively promotes the metabolism of SCFAs-producing bacteria and thus contributing to many positive physiological implications. DF, and more specifically SDF, it is reported that significantly increases the abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. and notably SCFA-producers like *Ruminococcus* spp. and *Butyricicoccus* spp. and negatively affect the relative abundances of *Proteobacteria* and *Actinobacteria* in animal model (Xu et al., 2022). Moreover, SCFA improve the gut health by enhancing the maintenance of intestinal barrier integrity, mucus production, protection against inflammation and reduction of colorectal cancer risk (Silva et al., 2020).

It is well known that gut microbiota varies in its functionality all along the intestinal tract with proximal colon characterized by microbial communities that are more prone to ferment carbohydrates (Huang et al., 2023; Marzorati et al., 2017). This event leads to a depletion of carbohydrates in favor of peptides and amino acids, which are utilized as energy source by bacteria present in more distal segments of the colon. Despite these differences in substrate availability, both colonic segments are characterized by the predominant abundances of *Bacteroidetes and Firmicutes* phyla (Huang et al., 2023) which in turn are impacted differently depending on the energy sources that are utilized through changes in their metabolic activity (Huang et al., 2023; Marzorati et al., 2017).

This study aimed to investigate the potentiality of cocoa bean shells as food ingredient with prebiotic property due to its high DF content. The novelty of the work lies on the assessment of different enzymatic treatments to enhance CBSs fermentability by colonic microorganisms. Moreover, being a matrix rich in polyphenols and dietary lipids, the study aims to investigate whether these compounds would interfere with the fermentability of the fibers by defatting and dephenolizing (polyphenols in their free form) CBSs. Samples underwent simulated digestion and simulated *in-batch* fecal fermentation in two different simulated colonic regions environment (proximal and distal colon) to understand if the DF would have different impacts on microbial communities inhabiting proximal or distal segments of the large intestine. The exploitation of this high-value food by product is still understudied, particularly the impact of CBSs on intestinal microbiota and the SCFA produced throughout the large intestine tract, by the intestinal microbial communities. The lack of precise information regarding the fermentation of cocoa shells fiber in different parts of the colon is the main gap that this work aims to fill.

## 2. Materials and methods

### 2.1. Materials

CBSs were kindly provided by an Italian leader manufacturer (Elah Dufour Novi SpA Group, Italy). Enzymes used for hydrolysis Prolyve 1000® and Extralyve LA2X® (cellulase ≥50 μkat/g; xylanase ≥50 μkat/g, polygalacturonase ≥20 μkat/g) were provided by an Italian supplier. All the chemicals used were purchased from Sigma Aldrich (St. Louis MO, USA), as well as other chemicals used in this study, unless stated otherwise.

### 2.2. Sample preparation

A fraction of CBSs was submitted to lipid extraction with a semi-automatic extraction system BÜCHI B-811 LSV (BÜCHI, Switzerland) using dichloromethane as solvent with a cycle of 6 h. Removal of free phenolics from the defatted CBS was performed by extracting 2.5 g of defatted CBS in 45 mL of an aqueous solution of acetone (80%, v/v) under constant stirring for 1 h at 4 °C. The mixture was centrifuged at 4000 rpm for 15 min at 5 °C, and the supernatant removed. Pellet was submitted to two additional extractions in aqueous solution of acetone (70%, v/v). The pellet was finally dried overnight under fume hood. The defatted and dephenolized sample is referred as "D" and the untreated fraction referred as "R".

### 2.3. Enzymatic hydrolysis

CBSs hydrolysates were produced in two ways: I) by a treatment with cellulase mixture Extralyve LA2X® for 4 h and II) by a pre-treatment with protease Prolyve 1000®. Briefly, raw and defatted/dephenolized CBSs were submitted to enzymatic treatment. The proteolytic step (referred as P) started by weighting 4 g of biomass in tubes of 50 mL and hydrated in phosphate buffer 0.5 mol/L (1:10 w/v) at room temperature for 1 h. Mixtures were than equilibrated at 50 °C and pH adjusted to 7.5 with NaOH 1 mol/L prior to the addition of protease (Prolyve 1000®) at an enzyme-substrate ratio (E:S) of 1:10 (v/w), for 2 h. The reaction was stopped by a heat-shock treatment (100 °C, 3 min) and then the samples were placed on ice and freeze-dried. The protease-treated sample and the non-treated samples were than submitted to cellulase treatment (referred as C). The biomasses were hydrated in citrate buffer 50 mmol/L (1:20 w/v) at room temperature for 1 h. The mixtures were then equilibrated at 30 °C and pH adjusted to 4.5 with HCl 1 mol/L prior to the addition of cellulase mixture of 1:1 (w/v) in citrate buffer, at an enzyme-substrate ratio (E:S) 1:10 (v/w). Incubation was performed for 4 h. Reaction was stopped through heat-shock (100 °C, 3 min) and then samples were placed on ice, then frozen at – 20 °C and finally freeze-dried. Samples were stored at room temperature for subsequent analysis. All the procedures were performed in duplicate.

### 2.4. Quantification of soluble and Insoluble Dietary Fiber

Soluble (SDF) and Insoluble Dietary Fiber (IDF) were quantified following the AOAC 991.43 method, using a MES-Tris buffer as reported for CBSs by Benítez et al. (2023). The samples were partially digested with alpha-amylase, and then further digested with protease and amyloglucosidase enzymes. The undigested IDF mass is removed by filtration, dried, and weighed. Ethanol is added to the filtrate to precipitate the soluble dietary fiber. The undigested SDF mass is isolated, dried, and weighed. Both IDF and SDF are calculated as the weight of the obtained residue minus the weight of the protein and ash, and finally reported as grams of dietary fibers in 100 g of the original dry weight.

### 2.5. Total phenolic content

Hydroalcoholic extracts of the hydrolyzed CBSs were produced as reported by Lessa et al. (2018). In brief, samples (1 g) were added with 7 mL of a solution of ethanol (80 %, v/v) in deionized water in 15 mL tubes and then shaked for 1 h at room temperature. Samples were then centrifuged at 5000 rpm, at 4 °C for 10 min, and the supernatant were collected for the analysis.

Total phenolic content was assessed in the extract described above using a modified version of the Folin–Ciocalteu's method (Papillo et al., 2019). 50 μL of Folin–Ciocalteu's reagent (Sigma-Aldrich) and 175 μL of aqueous $Na_2CO_3$ (5%, w/v) were added to the extracts. The solutions were then diluted with water to a final volume of 1.450 mL. The absorbance was read at 760 nm after 1 h, using an Evolution 60 S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Results were expressed as milligrams of catechin equivalents (CE) through a calibration curve.

### 2.6. *Antioxidant activity: DPPH assay*

Antioxidant activity was assessed on CBS extracts using the DPPH• (2,2-diphenyl-1-picryl-hydroxyl) assay according to the method described elsewhere (Papillo et al., 2019). Briefly, 700 μL of opportunely diluted sample or methanol (control) was added to the same volume of a 100 μmol/L DPPH• methanolic solution. This solution was shaken vigorously and left in the dark at room temperature for 20 min, after which the absorbance was read at 515 nm. The antioxidant activity was expressed as milligrams of Trolox equivalents (TE) through a calibration curve.

### 2.7. *In vitro digestion of cocoa beans shells*

The INFOGEST method (Brodkorb et al., 2019) was applied to the samples treated as described previously to produce digested CBSs. Simulated salivary fluid (SSF), simulated gastric fluid (SGF, pH 3.0 ± 0.05), and simulated intestinal fluid (SIF, pH 7.0 ± 0.05) were prepared according to the protocol. Briefly, 1.00 g of CBSs was mixed with ultra-pure water (1:1.5, w/w) and the mixture (2.5 g) was subjected to the oral phase by mixing with SSF for 2 min to obtain a final ratio of 50:50 (v/v). Following oral digestion, the resulting mixture (10 mL) was mixed with SGF to obtain a final ratio of 50:50 (v/v) to simulate the gastric phase of digestion. The pH was adjusted to 3.0 with 1 mol/L HCl and $CaCl_2$ was added to achieve 0.075 mmol/L in the mixture. Porcine pepsin was added to achieve an activity of 33.34 μkat/mL. The digest was incubated at 37 °C and shaken for 2 h. Finally, the pH of the gastric digest was raised to 7.0 with 1 mol/L NaOH and mixed with pre-warmed SIF. Fresh bile salts and $CaCl_2$ were added to achieve 10 mmol/L and 0.3 mmol/L, respectively, in the final digest. Pancreatin was added to achieve a lipase activity of 33.34 μkat/mL. The mixture was shaken at 37 °C for 2 h to simulate the intestinal phase. After the intestinal phase, the whole digesta (20 mL) underwent in vitro colonic fermentation.

### 2.8. *In vitro colonic fermentation*

*In vitro* colonic fermentation was applied by following established methods (Pérez-Burillo et al., 2021), with modifications. Briefly, fecal material collected from one healthy volunteer was inoculated in the SHIME® (Simulator of the Human Intestinal Microbial Ecosystem) designed to replicate the conditions of two colonic regions: proximal and distal. The fecal sample was prepared with anaerobic sterilized phosphate buffer (1:20, w/v), which consisted of 8.8 g/L of $K_2HPO_4$, 6.8 g/L of $KH_2PO_4$ and 0.1 g of sodium thioglycolate in demi-water as well as the addition of 15 mg/L sodium dithionite before use, using a Stomacher 400 circulator (Seward, UK). In this study, a stabilized fecal inoculum, previously prepared for another experiment, was employed. To establish a consistent baseline microbial composition, a basal period of 10 days preceded the application of experimental treatments. During this period, the microbial community was nurtured with the SHIME® basal medium (composed of 1.2 g/L arabinogalactan, 2.0 g/L of pectin, 0.5 g/L of xylan, 0.4 g/L of glucose, 3.0 g/L of yeast extract, 1.0 g/L of special peptone, 3.0 g/L of mucin, 0.5 g/L of L-cysteine-HCl, and 4.0 g/L of starch) as reported elsewhere (Rovalino-Córdova et al., 2020). This regimen facilitated the adaptation of the microbial community to the specific environmental conditions within the distinct colon vessels. After the equilibrium phase, it was freshly collected and directly inoculated in the fermentation vials. The batch fermentation was performed in sterilized penicillin bottles containing 43 mL of autoclaved basal medium and the entire digested material from the previous digestion. The basal medium consisted of 2.0 g/L of $NaHCO_3$, 2.0 g/L of yeast extract, 2.0 g/L of special peptone, 1.0 g/L of mucin, 0.5 g/L of L-cysteine HCl, and 2.0 % (v/v) of Tween 80. In addition, 2.11 g/L of $K_2HPO_4$ and 18.77 g/L of $KH_2PO_4$ were used in the fermentation with proximal colon bacteria to create a pH between 5.6 and 5.9 and 12.34 g/L of $K_2HPO_4$ and 10.88 g/L of $KH_2PO_4$ were used in the fermentation with distal colon bacteria for a pH between 6.6 and 6.9. Bottles were flushed with $N_2$ for 10 min to create anaerobic conditions before adding 7 mL of the SHIME® inoculum from each colon segment and from one donor, then incubated at 37 °C with gentle shaking. The batch fermentation was carried out in duplicate (a negative control with the same volume of sterilized water instead of the CBSs digesta was carried out). Samples were collected at three time-point: 0 h, 24 h and 48 h. After centrifugation at 14,000 rpm for 10 min, the supernatants and pellets were separated and stored at −20 °C for further analysis.

### 2.9. *SCFA extraction and determination*

SCFAs extraction and quantification was performed as reported elsewhere (Huang et al., 2021). Briefly, the supernatants of the fermentations were further centrifuged (14 000*g*, 5 min, 4 °C) and amounts of 2 mL were filtered (15 mm ⌀, 0.2 μm regenerated cellulose filter, Phenomenex, Torrance, USA). SCFA separation and quantification was performed after injection into a gas chromatography system equipped with a flame ionization detector (GC-FID, GC-2014, Shimadzu, Hertogenbosch, Netherlands) and a capillary fatty acid-free Stabil wax-DA column (1 μm × 0.32 mm × 30 m) (Restek, Bellefonte, PA, USA). The injection volume was 0.5 μL. Nitrogen was used as a carrier gas. The temperature of GC-FID started at 100 °C, then increased to 180 °C for 2 min at a rate of 10.8 °C/min. Then, it increased at 50 °C/min to 240 °C and was maintained at 240 °C for 2 min. Injector temperature was 100 °C and the temperature of the detector was 250 °C. Standard calibration curves of acetic (retention time, RT 2.50 min), propionic (RT 3.04) and butyric acid (RT 3.68) were prepared in the range of 0–1.5 mg/mL.

### 2.10. *Statistical analysis*

Differences in the concentrations of microbial metabolites were tested by one-way ANOVA followed by a Dunnet's multiple comparison test, with a $p < 0.05$ considered as statistical significance using GraphPad Prism Software. A multivariate analysis hierarchical clustering of principal component (HCPC) was performed using R software (4.2.2) and one-way ANOVA e post hoc Tukey test were performed using R for TPC, DPPH and TDF (R Core Team, 2022).

## 3. Results and discussion

The results of the analysis on the DF composition are shown in Fig. 1. The evidence suggests that the enzymatic treatment did not affect in a significant way the ratio of IDF and SDF in cocoa bean shells.

Fig. 1 also shows a slight but significant increase ($p < 0.05$) of DF in DU compared to RU. An explanation may be that DU lack lipids (the sample reported a loss 8.05 ± 0.13% of lipid) and free polyphenols. Moreover, a slight but significant ($p < 0.05$) decrease of both IDF and SDF content was observed in samples that underwent enzymatic hydrolysis, specifically RC and RCP. Similarly, data showed significantly

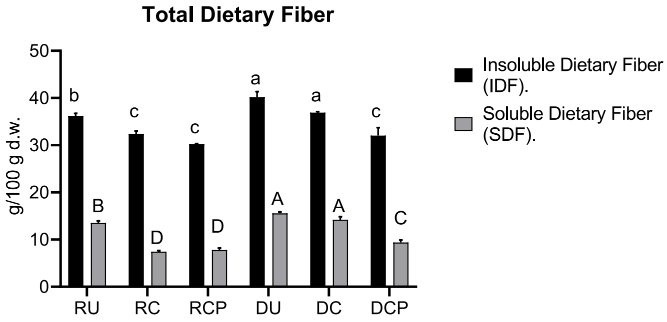

**Fig. 1.** Total Dietary Fiber content of cocoa bean shell samples expressed as g in 100 g of dry weight (different letters indicate sample significantly different; $p < 0,05$). Coding of the samples: RU, raw CBS untreated; RC, raw CBS treated with cellulase; RCP, raw CBS treated with protease and cellulase; DU, defatted and dephenolized CBS untreated; DC, defatted CBS treated with cellulase; DCP, defatted and dephenolized CBS treated with protease and cellulase.

lower amounts of SDF and IDF for DCP compared to DU. This could be attributed to the limitation of the assay used for DF quantification (reported in materials and methods), more specifically for the SDF quantification. Recent reports by McCleary and McLoughlin (2023) suggest that more advanced methods allow for the quantification of SDF that escapes the precipitation step. Similar results were obtained by Jagelaviciute et al. that treated apple pomace with a commercially available carbohydrase obtaining a decrease in IDF and SDF by yielding oligomers and monomers that do not precipitate in ethanol (Jagelaviciute et al., 2023). A recent work from (Younes & Karboune, 2023), reported an efficient enzymatic hydrolysis of CBSs to yield oligosaccharides with degree of polymerization (DP) 3–5 and 6–10 using the well-known Viscozyme® L.

Moreover, it has been reported that an enzymatic treatment may result in a higher SDF content when applied to a variety of food matrixes including corn bran (Haghighi-Manesh & Azizi, 2018), *Rhodomyrtus tomentosa* fruits (Deng et al., 2023), potato pulp (Meyer et al., 2009), cranberries (Jagelaviciute et al., 2022), dried ginger (Wang et al., 2020), citrus and barley bran (Karimi et al., 2018; Song et al., 2021).

Finally, it was also reported that the enzymatic treatment produced a 72.3% higher SDF content in tomato peels (with a total of 15.4% of SDF per gram of peels) over the non-treated peels (Gu et al., 2020).

The total phenolic content (TPC) of the CBSs samples is reported in Fig. 2a. It was observed a significant and substantial eightfold reduction in TPC in the defatted and dephenolized samples. However, the limitations of the Folin-Ciocalteu assay must be acknowledged, i.e., the lack of specificity since amino acids and other compounds containing a benzene ring are also detected (Amorati & Valgimigli, 2015).

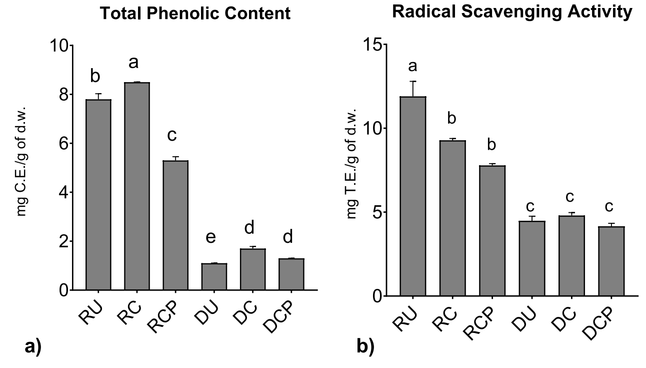

**Fig. 2.** a) Total Phenolic Content of cocoa bean shell samples expressed as mg of catechin equivalent (C.E.) in g of dry weight (different letters indicate sample significantly different; $p < 0,05$). b) Radical scavenging activity expressed as mg of Trolox equivalent (T.E.) in g of dry weight (different letters indicate sample significantly different; $p < 0,05$). Coding of the samples: RU, raw CBS untreated; RC, raw CBS treated with cellulase; RCP, raw CBS treated with protease and cellulase; DU, defatted and dephenolized CBS untreated; DC, defatted CBS treated with cellulase; DCP, defatted and dephenolized CBS treated with protease and cellulase.

Interestingly, it was observed a slight increase in total phenolic content on cellulase treated sample which could be attributed to the hydrolysis of fiber and the release of polyphenols bound on the polysaccharidic matrix. The fact that CBSs possess a large quantity of phenolic compounds bound to DF has been confirmed by the work of Rebollo-Hernanz and colleagues that reported that 51% of the total phenolics of CBSs are bound to the polysaccharidic matrix (Rebollo-Hernanz et al., 2020). The low TPC of the samples hydrolyzed with both cellulase and protease might be explained by a potential loss of polyphenols by the prolonged thermal treatment (Volf et al., 2014).

The radical scavenging activity of the CBSs samples is shown in Fig. 2b. A decrease in all the samples that were defatted and hydrolyzed was observed. This could be explained by a partial oxidation of the antioxidant compounds retained by CBSs after treatment (Volf et al., 2014). It was observed a 30% decrease of antioxidant activity in enzymatically hydrolyzed raw CBSs (RC and RCP) over non treated CBSs (RU), and a 70% reduction in defatted/dephenolized samples (DU, DC, DCP), due to the free polyphenols removal. As expected, the enzymatic hydrolysis on defatted and dephenolized samples did not affect the antioxidant capacity, contrary to that observed in enzymatic treatment of raw CBSs.

### *3.1. SCFAs quantification*

The level of SCFAs is reported in Figs. 3–5 for acetate, propionate, and butyrate respectively (see Fig. 6).

Acetate production (Fig. 3) was affected by the addition of CBSs. Looking at the proximal colon simulated environment for raw CBSs (Fig. 3a) it is possible to appreciate a statistically significant higher concentration of acetate for RC compared to RU and for DC compared to DU after 24 h of fermentation (Fig. 3c). Interestingly, after 48 h of fermentation, it is possible to observe an opposite trend. Samples hydrolyzed with cellulase, RC and DC have a lower concentration of acetate compared respectively to RU and DU, that could be explained by a depletion of the substrates.

Notably, for the distal colon simulated environment, at 24 h of fermentation the sample DC showed statistically significant higher concentration of acetate compared to DU (Fig. 3d), contrary to the RC sample that does not show statistically significance over the RU sample (Fig. 3b). At 48 h of fermentation, sample RC showed a statistically significant higher concentration of acetate compared to RU. The same trend is observed for DC and DU, confirming that the carbohydrase treatment was able to boost acetate production for both raw and defatted/dephenolized CBSs. A statistically significant higher amount of acetate is observed in samples that were hydrolyzed with protease, RCP compared to RU and DCP compared to DU respectively, but to a lower extent than sample hydrolyzed with cellulase only (RC and DC). The role of the treatment with protease resulted to be not significant for acetate production in both CBSs fractions, suggesting that the protein hydrolysis do not impact in a significant way the potential fermentability activity of CBSs.

The production of acetate by gut microbiota is positively correlated with health benefits such as protection against obesity and weight gain (García-Carrizo et al., 2020) and amelioration of metabolic disorders (Aoki et al., 2017), immune modulation (Bui et al., 2021; Erny et al., 2021) and protection against cognitive impairment in murine model (Zheng et al., 2021).

Acetate-producing bacteria use carbohydrates as a substrate to produce acetate. The monomer galacturonic acid (Chen et al., 2016) that, together with the monomer rhamnose, are constituent of the polysaccharide pectin (Picot-Allain et al., 2021; Zdunek et al., 2021). The

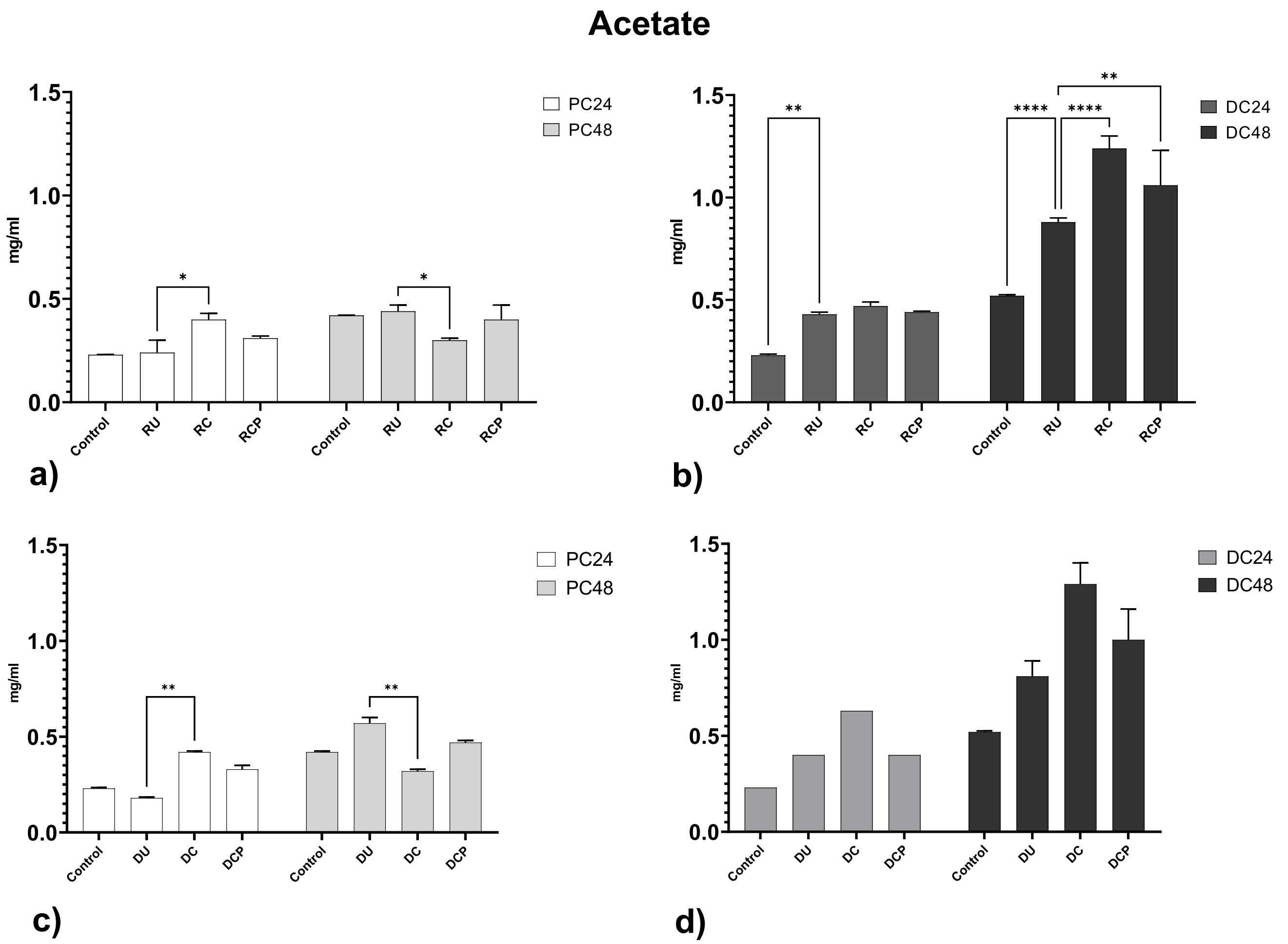

**Fig. 3.** Concentration of acetate after 24 and 48 h of in vitro fermentation of CBS samples and using microbiota inoculums adapted to different colon simulated environments: a) raw CBS in proximal colon measured at 24 and 48 h; b) raw CBS in distal colon simulated environment measured at 24 and 48 h. c) defatted and dephenolized CBS in proximal colon measured at 24 and 48 h; d) defatted and dephenolized CBS in distal colon measured at 24 and 48 h * concentration of acetate significantly different $p < 0.05$; ***p* < 0.01; ***; p < 0.001; ****p < 0.0001. Coding: control, fermentation without the addition of sample; RU, raw CBS untreated; RC, raw CBS treated with cellulase; RCP, raw CBS treated with protease and cellulase; DU, defatted and dephenolized CBS untreated; DC, defatted CBS treated with cellulase; DCP, defatted and dephenolized CBS treated with protease and cellulase; PC24, Proximal Colon at 24 h; PC48, proximal colon at 48 h; DC24, distal colon at 24 h; DC48, distal colon at 48 h.

commercial enzymatic mixture used contained the enzyme endopolygalacturonase (EC 3.2.1.15) which acts as a hydrolytic enzyme capable of hydrolyzing the α-1,4 glycosidic bonds of pectic acid resulting in random degradation of the pectic chain (Deng et al., 2023). DF of CBSs are mainly composed of cellulose, hemicellulose, lignin and a high proportion of pectin up to 6.1% (Arlorio et al., 2001; Barišić et al., 2023; Belwal et al., 2022). This could explain the efficiency of the enzyme to release oligosaccharides of galacturonic acid, easily degraded by the gut microorganisms. The study of Younes and colleagues further investigate the role of CBSs fibers and they found that the CBSs cell walls where particularly effective in boosting the growth of some *Lactobacillus* spp., well known probiotic microorganisms correlated with many positive health effects (Younes et al., 2023).

Acetate production is a typical fermentation end-product for various genera of bacteria. For example, *Ruminococcus* spp., despite being known to be butyrate-producing bacteria are also acetate-producers in the same way that *Prevotella* spp., being known as propionate-producers, are able to produce acetate (Duncan et al., 2002, 2004; Fukuda et al., 2011, 2012; Nogal et al., 2021). Acetate is also a fundamental substrate for butyrate producing bacteria thanks to their metabolic pathway of Coenzyme-A transferase that convert acetate into butyrate. Bacteria possessing this ability are represented by some *Firmicutes* such as *Eubacterium rectale, Roseburia* spp.*, Coprococcus catus, Faecalibacterium prausnatzii* (Duncan et al., 2004; Flint et al., 2015). The observation that the enzymatic hydrolysis resulted in higher acetate production could be explained by a possible higher accessibility to substrates utilized by acetate-producing bacteria, in line with data reported in literature (Lei et al., 2022).

Fig. 4 reports the concentration of propionate in the different colonic simulated regions at 24 and 48 h. It is possible to evaluate a peculiar inhibitory effect of CBSs in proximal colon at 24 and 48 h, compared to the control (Fig. 4a and c).

The situation is different for the distal colon simulated environment, where at 24 h of fermentation, no statistically significant difference with the control is observed, with a spike in propionate production after 48 h. The contribution of enzymatically treated samples above their non-treated counterparts is particularly noteworthy. In particular, the RC sample depicts a significant difference versus the RU (Fig. 4b). Different metabolic pathways were theorized for propionate production of which the succinate, the acrylate and the propanediol pathways with the latter implicated in some bacteria to ferment rhamnose (Hosseini et al., 2011; Reichardt et al., 2014). This could explain the effect of the enzymatic

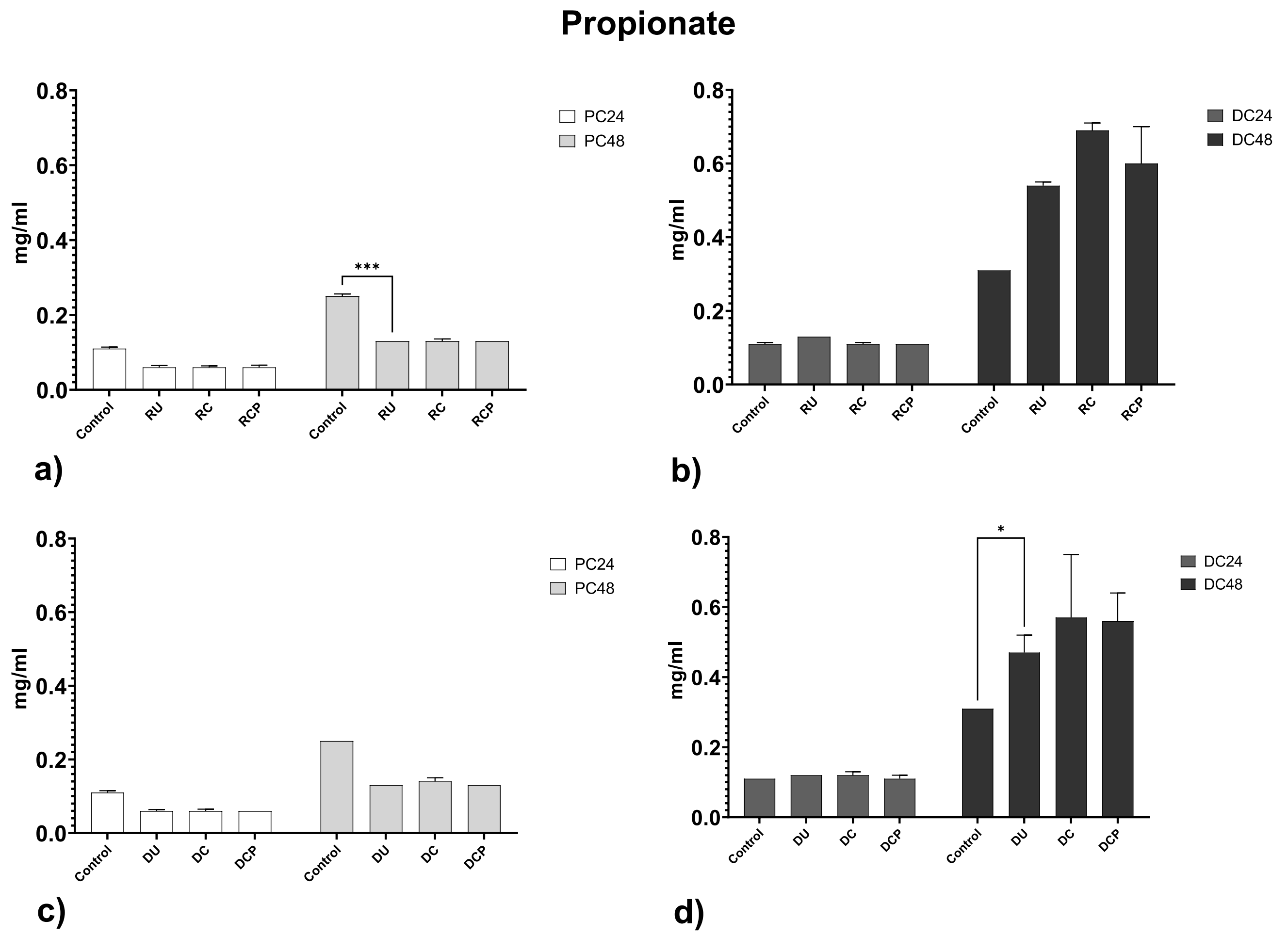

**Fig. 4.** Concentration of propionate after 24 and 48 h of in vitro fermentation of CBS samples and using microbiota inoculums adapted to different colon simulated environments: a) raw CBS in proximal colon measured at 24 and 48 h; b) raw CBS in distal colon simulated environment measured at 24 and 48 h. c) defatted and dephenolized CBS in proximal colon measured at 24 and 48 h; d) defatted and dephenolized CBS in distal colon measured at 24 and 48 h * concentration of acetate significantly different $p < 0.05$; **$p < 0.01$; ***; $p < 0.001$; ****$p < 0.0001$. Coding: control, fermentation without the addition of sample; RU, raw CBS untreated; RC, raw CBS treated with cellulase; RCP, raw CBS treated with protease and cellulase; DU, defatted and dephenolized CBS untreated; DC, defatted CBS treated with cellulase; DCP, defatted and dephenolized CBS treated with protease and cellulase; PC24, Proximal Colon at 24 h; PC48, proximal colon at 48 h; DC24, distal colon at 24 h; DC48, distal colon at 48 h.

hydrolysis on propionate production due to the breaking of pectin that contains rhamnose together with galacturonic acid as previously supposed. Different types of bacteria are implicated in propionate production of which *Ruminococcus* spp. and *Roseburia* spp. are probably involved (Flint et al., 2015; Hosseini et al., 2011; Reichardt et al., 2014). Propionate is associated with many positive health implications, such as appetite control, apoptosis of colorectal cancer cells, positive effect on hepatic lipid synthesis (Hosseini et al., 2011).

Butyrate production is represented in Fig. 5 where the samples show a strong influence over the proximal colon segment in comparison to distal colon that did not show a significant impact of CBSs. Proximal colon butyrate production was strongly impacted by the CBSs degradation and consumption by the fecal microbiota. The raw CBSs hydrolyzed with the cellulase mixture (RC) showed a higher production of butyrate compared to the untreated counterpart (RU samples) after 24 h in the proximal colon simulated environment (Fig. 5a). Similar situation is observed for the defatted/dephenolized CBSs hydrolyzed with the cellulase mixture (DC sample) that showed a marked influence over the non-enzymatically treated counterpart DU (Fig. 5c). The situation drastically changes at 48 h of fermentation where the sample RCP resulted in a significant butyrate production when compared to the RU (Fig. 5a) and for DCP compared to DU (Fig. 5c). Butyrate-producing bacteria are a fundamental subset of microorganisms in the gut of humans, they are mainly represented by gram positive *Firmicutes* (Flint et al., 2015; Louis & Flint, 2009). Different metabolic pathways lead to the production of butyrate by these bacteria as said, via acetate CoA-transferase pathway that convert acetate into butyrate (Flint et al., 2015). Moreover, the butyrate kinase pathway is utilized by some *Coprococcus* spp. to convert different carbohydrates into butyrate (Flint et al., 2015). Butyrate is associated with the integrity of the intestinal epithelium being the substrate preferred by colonocyte as energy source (Fu et al., 2019). Moreover, it is correlated with other many positive physiological implications, such as cardiovascular health, immune regulation and appetite control (Nogal et al., 2021).

Bacteria of the proximal colon segment tend to be characterized by a higher affinity to an acidic environment and that is the case for some butyrate producing bacteria, such as some *Clostridium* spp., more specifically from the cluster IV and XIVa that are particularly active in degrading xylo-oligosaccharides (XOS), as well as many *Bifidobacterium* spp. (Fu et al., 2019). This particular ability could explain the effect of the enzymatic treatment in augmenting butyrate production, specifically at 48 h, in the proximal colon environment. CBSs are a good source

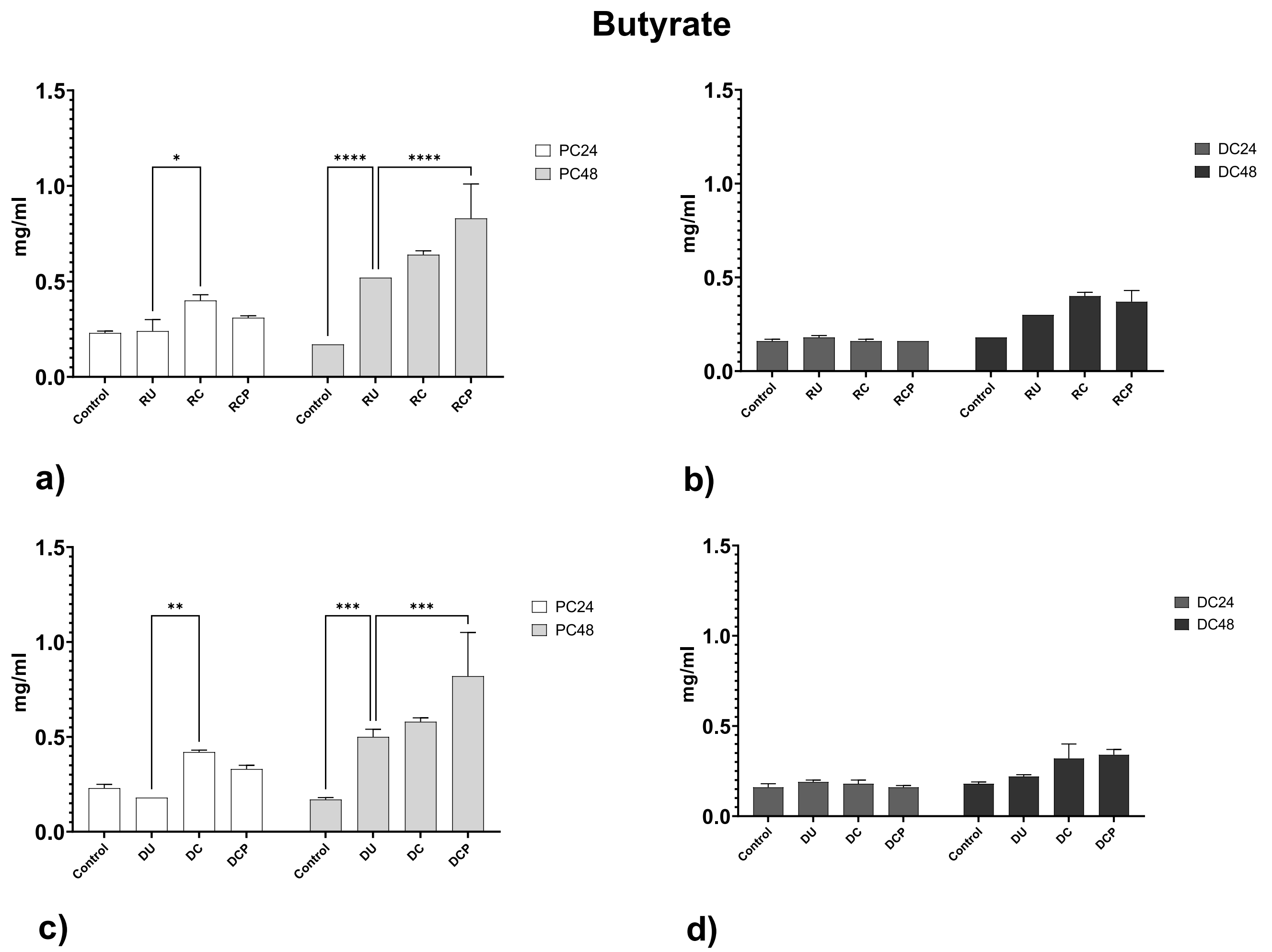

**Fig. 5.** Concentration of butyrate after 24 and 48 h of in vitro fermentation of CBS samples and using microbiota inoculums adapted to different colon simulated environments: a) raw CBS in proximal colon measured at 24 and 48 h; b) raw CBS in distal colon simulated environment measured at 24 and 48 h. c) defatted and dephenolized CBS in proximal colon measured at 24 and 48 h; d) defatted and dephenolized CBS in distal colon measured at 24 and 48 h * concentration of acetate significantly different $p < 0.05$; **$p < 0.01$; ***; $p < 0.001$; ****$p < 0.0001$. Coding: control, fermentation without the addition of sample; RU, raw CBS untreated; RC, raw CBS treated with cellulase; RCP, raw CBS treated with protease and cellulase; DU, defatted and dephenolized CBS untreated; DC, defatted CBS treated with cellulase; DCP, defatted and dephenolized CBS treated with protease and cellulase; PC24, Proximal Colon at 24 h; PC48, proximal colon at 48 h; DC24, distal colon at 24 h; DC48, distal colon at 48 h.

of xylan-derived polysaccharides (Barišić et al., 2020; Belwal et al., 2022) and the use of xylanase as part of the enzymatic mixture may explain the effect. These results are in line with data reported in literature by Liu and colleagues that showed a higher butyrate concentration and a higher relative abundance of *Bifidobacteriaceae* and butyrate-producing bacteria in proximal colon simulated environment compared to distal colon for processed starch (Liu et al., 2021). It is also known that *in-vivo* concentration of SCFAs is higher in the proximal segment of the colon, decreasing in the distal (Cummings et al., 1987) but this may depend on the progressive depletion of the fiber substrate as the digesta progress towards more distal segments of the colon and/or the removal of SCFA from the lumen of the large intestine because of their absorption. In fact, Huang et al. report that the distal colon microbial communities produce more SCFAs than the proximal colon bacteria, given the same amount of dietary fiber (Huang et al., 2023).

The multivariate analysis (Fig. 7) shows the clustering of the different treatments over the various metabolites produced in both colonic regions simulated environment and fermentation time-point. It is possible to appreciate three main clusters: i) control, ii) RU and DU, iii) DC, RC, DCP and RCP. This would suggest the efficiency of the enzymatic treatment on SCFAs production (and so potentially for a prebiotic effect on intestinal microorganisms), confirming results from previous studies and different food matrixes such as *Morus alba* L. (Chen et al., 2016) and black mulberry (Ai et al., 2022).

The accessibility of dietary fiber is a key factor for SCFAs production by bacteria of the human intestinal microbiota (Fu et al., 2019). Lei et al. reported that, by optimizing the extraction of soluble fiber from *Morchella importuna,* they were able to enhance the bioaccessibility of the SDF and as a result they observed an enhancement in SCFAs production in an in vitro model (Lei et al., 2022). This observation is in line with the obtained results that could theoretically be explained by the fact that the enzymatic hydrolysis augmented the bioaccessibility of the substrates for the microbial communities.

The influence of SDF on gut microbiota as substrate for their metabolism is positively correlated with health benefits (Xu et al., 2022) but it is also desirable food component for its rheological properties (Mrabet et al., 2017; Wen et al., 2017) and antioxidant capacity through bound residual phenolics (Mrabet et al., 2017). The removal of free polyphenols, and particularly the fat fraction, showed to not interfere particularly with SCFAs production. Results and information reported in

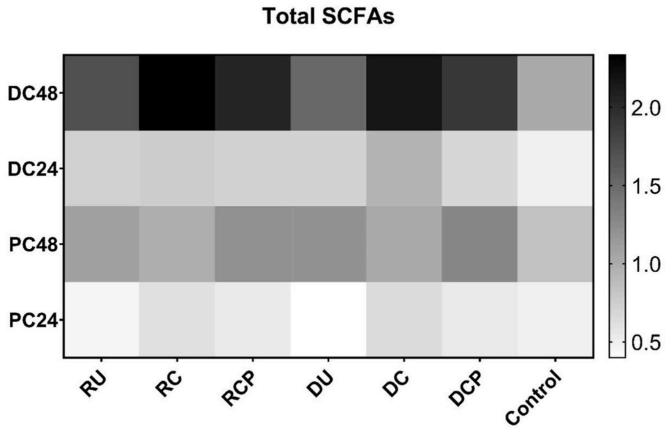

**Fig. 6.** Heatmap showing the total short chain fatty acids at the different simulated colonic region. Coding: control, fermentation without the addition of sample; RU, raw CBS untreated; RC, raw CBS treated with cellulase; RCP, raw CBS treated with protease and cellulase; DU, defatted and dephenolized CBS untreated; DC, defatted CBS treated with cellulase; DCP, defatted and dephenolized CBS treated with protease and cellulase; PC24, Proximal Colon at 24 h; PC48, proximal colon at 48 h; DC24, distal colon at 24 h; DC48, distal colon at 48 h.

literature about polyphenols are controversial because of the duplicity nature of this large family of compounds: on one hand, they can inhibit enzymatic activity, some with some antibiotic properties; on the other hand they can modulate microbial communities favoring species able to produce SCFA (Garcia-Alonso et al., 2022; Guerra-Valle et al., 2022). The obtained results do not support any of these statements (Magistrelli et al., 2016; Álvarez-Cilleros et al., 2020). The unbalanced and diversified action of polyphenols can be probably correlated to the modulation of the microbiota, even if this effect is difficult to be controlled and clearly evaluated. Unfortunately, it is not possible to discriminate if the contribution of lipid or polyphenols alone would have impacted differently the microorganisms of both proximal and environments being the sample DU both defatted and dephenolized.

## 4. Conclusions

The enzymatic hydrolysis of CBSs was intended to increase the proportion of SDF. Although no substantial change was seen in the level of SDF, the enzymatic treatment enhanced the amount of SCFA generated in an in vitro model of colon fermentation. This could be justified by I) the potential hydrolysis of the DF into fermentable oligosaccharides in the SDF fraction, that were not quantified through the assay used in this work, and II) a higher accessibility of the microbial communities to the fermentable DF. More investigations are needed to further elucidate the chemical structure of the DF before and after the enzymatic hydrolysis. Moreover, only the contribution of free polyphenols was investigated in this work, and it could be potentially interesting to assess the contribution of total or bound polyphenols in future studies. Up to date of writing, this study was the first attempt to uncover the potentiality of enzymatic treatment to modulate the prebiotic effect of CBSs on human intestinal microbiota. These outcomes open new scenarios for CBSs exploitation as source of functional ingredients. More studies are needed to further optimize the enzymatic treatment of CBSs and to characterize the DF composition and the potentiality to have a positive impact on intestinal microbiota and human health. A specific factorial experimental design will be useful to identify the best conditions of use for single- and mixed-enzymes preparations, allowing to optimize the valorization of CBSs in terms of production of SCFAs.

## CRediT authorship contribution statement

**Vincenzo Disca:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Edoardo Capuano:** Writing – review & editing, Conceptualization. **Marco Arlorio:** Writing – review & editing, 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.

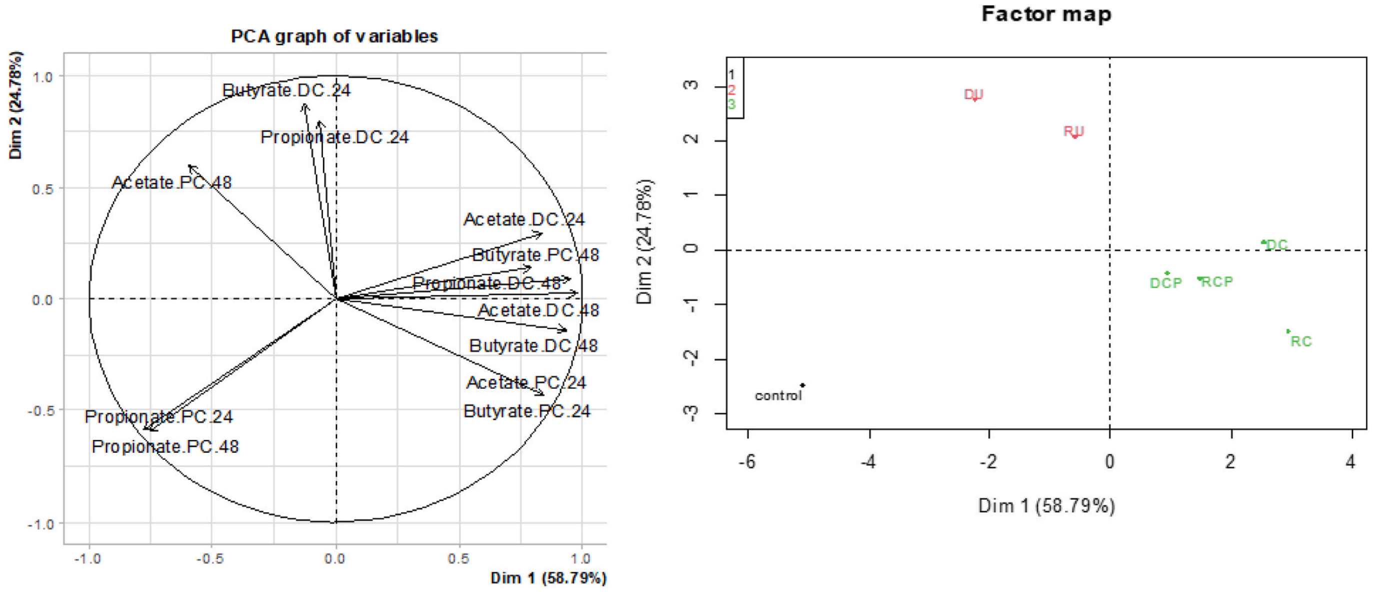

**Fig. 7.** Hierarchical clustering on the factor map (HCPC) based on Principal Component Analysis (PCA). Coding of the samples: control, fermentation without the addition of sample; RU, raw CBS untreated; RC, raw CBS treated with cellulase; RCP, raw CBS treated with protease and cellulase; DU, defatted and dephenolized CBS untreated; DC, defatted CBS treated with cellulase; DCP, defatted and dephenolized CBS treated with protease and cellulase; PC24, Proximal Colon at 24 h; PC48, proximal colon at 48 h; DC24, distal colon at 24 h; DC48, distal colon at 48 h.

## Data availability

No data was used for the research described in the article.

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