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Effects of phlorotannin and polysaccharide fractions of brown seaweed *Silvetia compressa* on human gut microbiota composition using an *in vitro* colonic model

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ABSTRACT

Few studies had demonstrated the interaction of phlorotannins and polysaccharides from algae on microbial diversity and growth of probiotic bacteria on the early stages of colonic fermentation. *Silvetia compresssa* hydroethanolic extract rich in phlorotannins and its isolated polysaccharides fraction enabled the proliferation of *Bifidobacterium* and *Lactobacillus* and increased total short-chain fatty acids synthesis compared to a negative control. Eckstolonol, dieckol and acacetin derivative in hydroethanolic extract were depleted after eight hours of *in vitro* colonic fermentation. After a 48 h fermentation, hydroethanolic extract showed high levels of acetic (104.82 μmol g^{-1} wet feces) and propionic acids (78.58 μmol g^{-1} wet feces), as well as an increase in *Bifidobacterium* (log_{10} 6.77 CFU g^{-1} wet feces) and *Lactobacillus* (log10 4.25 CFU g^{-1} wet feces) growth. These results showed no significant difference with inulin ($p > 0.05$), suggesting the potential of *Silvetia compresssa* hydroethanolic extract to improve human gut microbiota.

1. Introduction

The interaction between phenolic compounds and human gut microbiota (HGM) has gained scientific momentum due to its wide range of physiological functions involving metabolic and enzymatic activities that support digestive health (Espín, González-Sarrías, & Tomás-Barberán, 2017). The HGM protects the host against gastrointestinal pathogens (Vogt, Peña-Díaz, & Finlay, 2015), aids in nutrient processing (van Eunen et al., 2013) and modulates the intestine's immune response (Fernández et al., 2016). Disorders in HGM populations have also been linked to various serious chronic diseases, such as type 2 diabetes, obesity, and colon cancer (Fu et al., 2018).

Studies of the HGM have given rise to the concept of prebiotics. These are defined as non-digestible food components that are fermented preferentially by HGM and selectively stimulate the growth and/or activity of the intestinal microbes leading to health benefits of the host (Charoensiddhi, Conlon, Franco, & Zhang, 2017; Okolie, Mason, Mohan, Pitts, & Udenigwe, 2019). The development of *Bifidobacterium* and *Lactobacillus* is considered as a traditional sign of prebiosis (Charoensiddhi et al., 2017; Kleerebezem & Vaughan, 2009). The wide array of gastrointestinal diseases has paved the way for the development of a prebiotic market, which had an estimated 3.4 billion USD value by the year 2018 and it is expected to reach 8.34 billion by 2026 (Reports and Data, 2019). Thus, research focused on the development of effective prebiotics can have great scientific and economic impact.

Silvetia genus belongs to the Fucaceae family (Fucales, Phaeophyceae); it is used as a food ingredient in Asian countries (Pereira, 2016), as well as for bioremediation (Girardi et al., 2014). *Silvetia compressa* (J. Agardh; E. Serrão, T.O. Cho, S.M. Boo & Brawley), previously known as *Pelvetia fastigiata* (J. Agardh; De Toni), is a common member of the upper intertidal fucoid community in the Pacific coast of North America from Humboldt County, California, to Punta Baja, Baja California, Mexico,

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including coastal islands (Pedroche, Silva, Aguilar Rosas, Dreckmann, & Aguilar Rosas, 2008; Silva et al., 2004). *Silvetia compressa* has potential industrial applications as fertilizer, forage, and raw material for the extraction of alginate and other colloids used as thickeners in the food industry (Aguilar-Rosas, Aguilar-Rosas, Mateo-Cid, Mendoza-González, & Krauss-Cosio, 2002). Since phlorotannins and polysaccharides have potential health benefits, the conditions to recover both families of compounds from *S. compressa* was optimized using ultrasound assisted extraction (Vázquez-Rodríguez, Gutiérrez-Uribe, Antunes-Ricardo, Santos-Zea, & Cruz-Suárez, 2020). These phlorotannins (PT) and polysaccharides (PS) rich hydroethanolic extracts, obtained by our group, have been found to support a range of health-promoting activities, such as hypocholesterolemia in Wistar rats (Acevedo-Pacheco et al., 2020), antiviral (Morán-Santibañez et al., 2016), antioxidant, as well as α-glucosidase and α-amylase inhibitory activities (Múzquiz et al., 2019). This seaweed is rich in fucoidan (11–12.1%) and sulfated polysaccharides content (15–16%) (Hernández-Garibay, Zertuche-González, & Pacheco-Ruiz, 2019), soluble dietary fiber (9.1%), insoluble dietary fiber (50.1%), total phenolic (28%) and PT content (0.73%) (Múzquiz et al., 2019; Tapia-Salazar et al., 2019; Vázquez-Rodríguez et al., 2020). This dietary fiber and phenolic content are important characteristics to develop new functional foods incorporating *S. compressa* extracts.

Brown seaweeds are known to be a rich source of fiber with the presence of several non-digestible polysaccharides including laminarin, fucoidan, and alginate. These individual polysaccharides have previously been shown to favorably alter HGM composition and activity (Strain et al., 2020). Brown seaweeds are also a rich source of PT and PS, which selectively enhance the activity of beneficial bacteria and stimulate the production of functional metabolites such as short-chain fatty acids (SCFAs) that have a positive influence on host health (Charoensiddhi, Conlon, Vuaran, Franco, & Zhang, 2016; Zheng, Chen, & Cheong, 2020). Seaweed extracts containing PT or PS, separated or mixed, have hypoglycemic, hypolipidemic, anticancer, antiviral, antibacterial, and antioxidant activities (Charoensiddhi, Conlon, Vuaran, et al., 2017; Garcia-Vaquero, Rajauria, O'Doherty, & Sweeney, 2017; Praveen, Karthika Parvathy, Jayabalan, & Balasubramanian, 2019; Rodrigues et al., 2015). Prebiotic activity of certain red and brown seaweeds, along with their respective extracts, have been previously studied, particularly PS with low molecular weight derived from agar or alginate powders from *Gracilaria spp.*, *Gelidium sesquipidale* and *Ascophyllum nodosum* (Ramnani et al., 2012), crude PS-rich extract from *Laminaria japonica* (Kong, Dong, Gao, & Jiang, 2016), novel PS of *Sargassum thunbergii* (Fu et al., 2018), PS rich extract of *Laminaria digitata* (Strain et al., 2020), PS and PT enriched extracts of *Ecklonia radiata* (Charoensiddhi et al., 2016; Charoensiddhi, Conlon, Vuaran, et al., 2017) and PT rich extracts and fucoidan *of Ascophylum nodosum* (Corona et al., 2016; Okolie et al., 2019). Nevertheless, prebiotic effects of *S. compressa* extracts, particularly gut microbiota fermentation-induced variations in the fermenting bacterial populations, as well as the fermentation products including SCFAs, have not been deeply explored. More importantly, previous studies on gut microbiota have mainly focused on PS-rich extracts of brown algae but Charoensiddhi, Conlon, Vuaran, et al. (2017) demonstrated that PT from *Ecklonia radiata* affect microbial diversity and growth of probiotic bacteria on the early stages of colonic fermentation. Therefore, PT and PS contained in hydroethanolic extract must be evaluated before proposing a functional ingredient from brown algae.

The aim of this study was to evaluate the prebiotic effect of a hydroethanolic extract (EXT) and its PS fraction, both extracted from the brown seaweed *S. compressa*, using an *in vitro* methodology that simulates the passage of food through the digestive tract and its subsequent colonic fermentation by monitoring: (a) the changes in gut microbiota and the development of specific probiotic bacteria, (b) the synthesis of SCFA and (c) the changes in PT and carbohydrates present in the EXT and its PS fraction.

2. Materials and methods

2.1. Seaweed material and reagents

Silvetia compressa seaweed samples were collected from December 2014 to January 2015 from La Escalera Baja California Sur, Mexico. After collection, the seaweed thallus was rinsed with seawater to remove sand and epiphytes, drained on clean stone or clotheslines, and sun-dried inside a greenhouse shadow for several days until reaching a 10.9% moisture content. Finally, samples were ground (model 200, Pulvex), sieved with a 500-μm sieve, and vacuum packed until use.

2.2. Extract and polysaccharide fraction preparation

Extraction of PT and PS from dried ground seaweed samples was performed using ultrasound assisted extraction as detailed by Vázquez-Rodríguez et al. (2020). Experimental aliquots were suspended in 32.33% aqueous ethanol using a solvent/seaweed ratio of 30 mL g^{-1} of dried seaweed. Aliquots suspensions were placed in an ice bath and sonicated (SFX150 Cell Disruptor, Branson, New Carlisle, IN, USA) for 30 min with a power density of 3.8 W cL^{-1}, using a 1/16″ micro tip with an immersion depth of 10 cm and then mixed at 250 rpm for 90 min in an agitated hot plate at 50 °C. Afterwards, suspensions were centrifuged at 685 g for 15 min at 4 °C (SL16R Thermo Fisher Scientific Inc., Waltham, MA, USA) and the supernatant was transferred to an evaporator (Rocket Synergy evaporator, Genevac, Ipswich, UK) to completely remove ethanol and water. Finally, crude extracts were stored at −80 °C until further analysis. Polysaccharides' fraction (PS) was obtained by dissolving the concentrated extract (EXT) in pure methanol at a 1:10 (w/v) ratio and centrifuged for 10 min at 2739 × g. The methanol soluble PT were recovered, and the undissolved material (PS) was dried under a nitrogen flow, and then weighted and stored at −80 °C until analysis.

2.3. In vitro gastrointestinal digestion and colonic fermentation

In vitro gastrointestinal digestion was carried out according to Mosele, Macià, Romero, Motilva, and Rubió (2015) using the hydroethanolic extract (EXT), its polysaccharide fraction (PS), inulin (INU) as a positive control, and a negative control (CTRL) without any added substrate. The entire process was carried out at 37 °C with continuous agitation. Firstly, 1.5 g of each substrate were suspended in 100 mL of artificial saliva, at 200 rpm (phosphate buffer 5 mM, pH 6.9 with 0.04% NaCl and 0.044% $CaCl_2$), and incubated for 5 min. Following the α-amylase treatment, the pH was adjusted to 2.0 using HCl and it was incubated for 60 min at 200 rpm under the presence of pepsin (15 mg g^{-1} of sample) to simulate gastric digestion. After the gastric digestion step, the pH was adjusted to 6.5 with $NaHCO_3$ (0.5 N), and then a final digestion was performed adding pancreatin (20 mg g^{-1} of sample) and bile salts (133.33 mg g^{-1} of sample). The resulting mixture was dialyzed for 2 h in darkness in a membrane immersed in 100 mL of phosphate buffer (pH 7.5) at 200 rpm. During *in vitro* gastrointestinal digestion, temperature was maintained at 37 °C using a closed circuit of distilled water surrounding the system. After the dynamic duodenal digestion, two fractions were collected from the dialysis membrane: "IN" and "OUT". The "IN" fraction describes the digested material that reaches the colon, whereas the "OUT" fraction consisted of the phosphate buffer containing the compounds capable of crossing the membrane used in dialysis. This was considered the bioavailable fraction of the sample ("OUT").

The "IN" fraction was incubated *in vitro* in the presence of human gut microbiota. The HGM was obtained by making a pool of human feces that were collected from ten healthy volunteers. Criteria for selection required volunteers to be between 25 and 35 years of age and not to have taken antibiotics in the last 3 months. Feces samples were pooled together and stored for up to two hours at 4 °C in anaerobic conditions. A medium (10% feces) was prepared mixing feces with a pre-reduced

carbonate-phosphate buffer prepared according to Mosele et al. (2015). The pH of the carbonate medium was adjusted to 6.5 using HCl. Then, a 1:10 (w/v) dilution of the fecal samples with the anaerobic buffer was prepared and homogenized in a stomacher (1 min). One milliliter of fecal inoculum (10% feces) was aliquoted in 15 mL tubes and mixed with 9 mL of "IN" fraction and incubated for different time lengths (0, 2, 8, 24 and 48 h). These samples were prepared in parallel with two controls: one with fecal inoculum without the dialysis tube content, and another that was a carbonate-phosphate buffer with the dialysis tube content fraction without fecal inoculum. Two replicates of each incubation were carried out under anaerobic conditions at 37 °C in the absence of light without agitation. Samples obtained from each digestion time were stored at −80 °C until further analysis.

2.4. DNA extraction and Ilumina sequencing

Bacterial genomic DNA was extracted from each replicate of fermented samples. Genomic DNA extraction from fecal microbiota fermentations was carried out with the QIAamp DNA Kit Stool Mini Kit Handbook (Qiagen, Hilden, Germany) following the manufacturer's instructions. Consequently, bacterial DNA was quantified using a Nanodrop ND-1000 v3.5.2 spectrophotometer (Nanodrop Technology, Cambridge, UK); the samples were stored at −20 °C.

Microbiota diversity analysis based on the V3 and V4 region was carried out in only one replicate per fermentation experiment using the KAPA HiFI HotStart Ready (Kapa Biosystems, USA) for PCR amplification. Amplicons of the V3 and V4 regions were generated by employing one 16S amplicon forward primer = 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and one 16S Amplicon PCR Reverse Primer = 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. PCR tests were carried out on a G-storm PCR machine under the following conditions: 95 °C for 3 min followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s followed by 5 min at 72 °C after which the samples were held at 4 °C. The PCR solutions had a final volume of 25 μL and consisted of 12.5 μL of KAPA HiFi HotStart ready mix, 5 μL of Amplicon PCR forward primer (1 μM), 5 μL of Amplicon PCR reverse primer and 2.5 μL of Microbial DNA (5 ng μL^{-1}). The PCR product of each sample was cleaned using Agent- court AMPure XP kit (Beckman Coulter Genomics, UK) as per the manufacturer's protocol. The cleaned PCR products were eluted in 50 μL, and sample index PCR was carried out using a Nextera XT index kit (Ilumina, San Diego, CA, USA) following manufacturer instructions. Samples were denatured and pooled before adding PhiX according to the manufacturer's protocol. Sequencing was carried out using a MiSeq System (Ilumina, San Diego, CA, USA). Readings per sample, length of the sequences, and the quality of the readings were analyzed by QIIME software version 2. The minimum number of readings was 33,164 and the maximum 687215. The length of the sequences was 301 for forward (FW) and 300 for reverse (RV). Sequences were dereplicated, allowing at least ten errors per sequence. Chimeras were filtered and pooled into operational taxonomic units (OTUs). Subsequently, taxonomy was assigned using the Greengenes database (version 13_8), while alpha (Chao1, Simpson and Shannon indices) and beta diversities were calculated using PAST 4.0.

2.5. Microbial population enumeration by quantitative real-time PCR (Q-CPR)

Specific primers for total bacteria, *Bifidobacterium* and *Lactobacillus*, were used to quantify the fecal microbiota using real-time quantitative polymerase chain reaction (PCR) according to previous reports (Matsuki et al., 2002; Walter et al., 2001). Primer sequences were as follows: total bacteria forward: 5́TGGCTCAGGACGAACGCTGGCGGC-3́ and reverse: 5́-CCTACTGCTGCCTCCCGTAGGAGT-3́; *Bifidobacterium* forward: 5́-CTCCTGGAAACGGGTGG-3́ and reverse: 5́-GGTGTTCTTCCCGATATCTACA-3́; *Lactobacillus* genera forward: 5́-GGAAACAGRTGCTAATACCG-3́ and reverse: 5́-CACCGCTACACATGGAG-3́. Each PCR reaction contained 15 ng of fecal DNA and 2 μL of each primer at a concentration of 5 pmol μL^{-1} and amplification was performed using the iQ SYBR Green Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), in an iQ5 real-time PCR thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For total bacteria and *Lactobacillus* group quantification, the reaction was incubated at 95 °C for 10 min, followed by 35 cycles of 10 s at 95 °C, 30 s at 61 °C and 20 s at 72 °C. For *Bifidobacterium* group quantification, the reaction was incubated at 95 °C for 10 min, followed by 35 cycles of 10 s at 95 °C, 30 s at 55 °C and 20 s at 72 °C. Fluorescent products were detected at the last step of each cycle. The Ct values and melting curve analysis after amplification were used to calculate the bacterial population and distinguish target from non-target PCR products. Standard curves were constructed from eight 10-fold dilutions. Bacterial population was expressed as the logarithmic value of the colony forming unit ($\log_{10}$ CFU) per g^{-1} of wet feces using the equation (1) for total bacteria, (2) for *Bifidobacterium* and (3) for *Lactobacillus*. Where y is the Ct and × the bacterial concentration expressed in $\log_{10}$ CFU per g^{-1} of wet feces.

$$y = -3.103x + 28.927 \quad (1)$$

$$y = -3.346x + 33.413 \quad (2)$$

$$y = -3.428x + 32.056 \quad (3)$$

2.6. Short-chain fatty acid quantification

Fermentations were sampled at 0, 2, 8, 24 and 48 h for SCFA analysis and immediately stored at −80 °C prior to SCFA quantification. For GC-FID analysis, samples were centrifuged for 10 min at 10,000 g and 4 °C. Subsequently, the supernatant was collected and placed in a 2.5 mL crimp cap vial. Analysis was performed under standard laboratory conditions, these being 20–21 °C using a gas chromatograph (6850 Network GC System, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and an Agilent HP-FFAP column (25-m length × 0.32-mm internal diameter × 0.50-μm film thickness). The carrier gas was helium with a total flow rate of 8.7 mL min^{-1} and pressure of 6.1 psi. The temperature program began at 120 °C and elevated to 240 °C at a rate of 15 °C min^{-1}. Test samples were injected (1 μL) in a splitless mode. Based on the acquired GC data, including standard curves, the SCFA content was expressed as micromoles of SCFA per gram of wet fecal matter.

2.7. Identification and quantification of phlorotannins

Phlorotannin quantification in the EXT fermentation samples was carried out using high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD, Agilent Technologies 1200 series, USA). Separation was performed through a Luna C18 reverse column (250 mm, 4.6 mm, 5 μm particle size; Phenomenex, USA) with a flow rate of 1 mL min^{-1} at 30 °C and an injection volume of 10 μL. The mobile phase consisted of (A) acidified water (1% formic acid) and (B) 100% methanol. The elution was performed as follows: 0–5 min 10% B, 5–30 min 20–60% B, 30–35 min 60% B, 35–40 min 60–20% B, and 40–50 min 10% B (post time). Spectral data from all peaks were accumulated in the 230–550 nm range and chromatograms were recorded at 270 nm. Chromatographic data were collected using HP-Agilent Software for LC and phloroglucinol (Sigma-Aldrich, USA) was used as a quantification standard. Phlorotannin concentration was expressed as mg of phloroglucinol equivalents (PGE) per g wet feces (mg PGE g^{-1} wet feces) using equation $y = 1.7075x + 0.9081$ ($R^2 = 0.9994$) as a calibration curve, where y is the peak area and × is the concentration in mg PGE g^{-1} wet feces.

The same chromatographic conditions described above were used to

identify potentially bioactive compounds by mass spectrometry using a HPLC-MS-TOF model G1969A Agilent 1100 (Agilent Technologies, Santa Clara, CA, USA). Mass spectra were collected using electrospray source in positive mode (ESI +) under the following conditions: *m/z* range 150 to 1500, nitrogen gas, gas temperature 300 °C, drying gas flow rate 8 L min^{-1}, nebulizer pressure 20 psi, capillary voltage 4000 V and fragment voltage 70 V. Extracted ion chromatograms were obtained using the Analyst QS 1.1 software (Applied Biosystems, Carlsbad, CA, USA). Accurate mass and adducts with Na or K with an error range of 0.01 units were used to identify the different compounds based on previous reports.

2.8. Carbohydrate quantification

The carbohydrate quantification was performed with the modified method reported by Heredia-Olea et al. (2015). It was performed with a high-performance liquid chromatography (HPLC, Waters HPLC Breeze model, Milford, MA, USA) equipped with isocratic pumps (Waters 1515), a mobile phase degasser (AF Waters), an autosampler (Waters 717 plus), a refractive index detector (waters 2414), and the Empower 2 software to process data and command the equipment. A Shodex SP0810 column and a cation/anion deasher (Biorad) were used. Column temperature was set at 85 °C, whereas the detector and the autosampler at 50 and 4 °C, respectively. Standards of D-glucose, D-xylose, L-arabinose, D-mannose, D-galactose, D-fucose, glucuronic acid, and mannitol (Sigma Chemical Co. St. Louis, MO, USA) were used. Run time was 40 min. Carbohydrates were expressed as μmol of carbohydrate per gram of wet feces.

2.9. Statistical analysis

Beta diversity analysis was performed using principal coordinate analysis (PCoA) and Bray-Curtis distance. These analyses were used to quantify the compositional differences between microbial communities using PAST 4.0 and PRIMER 7 software.

A one-way ANOVA and Tukey-Krammer test were used to determine significance of substrates at each fermentation time on bacterial development, SCFA production, phlorotannin and carbohydrate concentration ($p < 0.05$). To assess correlation within the response variables, a Pearson test was performed ($p \leq 0.05$). All statistical analyses and tests were performed using JMP 14 software.

3. Results and discussion

3.1. Changes in total bacteria and beta diversity of human gut microbiota bacterial communities

Total bacteria count significantly increased after 8 h fermentation of the seaweed samples (EXT and PS) and INU when compared to negative control (Fig. 1-I). INU showed the highest total bacterial count after 24 h. Similar data on total bacteria development were reported by Charoensiddhi et al. (2016) for the *in vitro* fermentation of different *Ecklonia radiata* extracts and inulin. At 48 h, there was no significant difference between the INU and the EXT ($p > 0.05$) but PS and CTRL had lower total bacterial count compared to INU.

The beta-diversity and the effect of each substrate and fermentation time on the bacterial community composition was examined by PCoA and cluster analysis (Fig. 1-II and Fig. 1-III). INU and CTRL generated the most contrasting changes in the gut microbiota while EXT and PS were closer to the initial bacterial diversity from healthy human samples. Based on the Bray-Curtis distance, all initial fermentation times were clustered in two roots with an 80% similarity (Fig. 1-III). As fermentation time increased, bacterial communities in the CTRL group contrasted with the observed in INU, EXT and PS groups. Differentiation between populations occurred since the initial hours of fermentation, indicating the effects of initial nutrients availability that limited the growth of

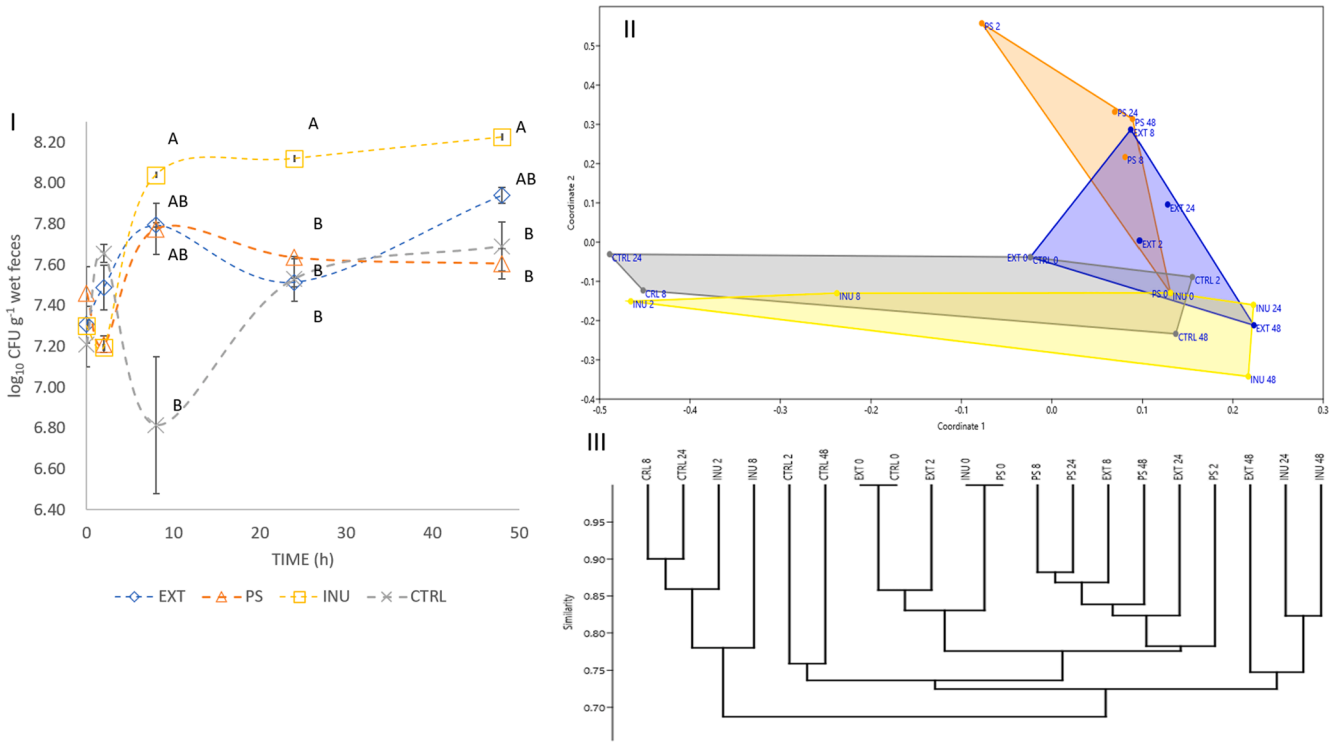

Fig. 1. Total bacteria count obtained by RT-PCR (I), Principal Coordinate Analysis (PCoA) of bacterial communities beta diversity from *in vitro* colonic fermentations of *Silvetia compresssa* hydroethanolic extract (EXT), its isolated polysaccharide fraction (PS), inulin (INU) and negative control (CTRL) (II) and (III) Cluster Analysis showing the contrasting results between CTRL and INU and the low changes observed for PS and EXT when compared to initial diversity of a healthy human gut microbiota. * Experiments not connected by letters denote significant median differences by Tukey-Krammer test ($p \leq 0.05$).

bacteria responsible for metabolizing the available substrates (primary degraders). Saccharides depletion promotes diversification by challenging bacteria to switch to other less efficient substrates and attenuates the growth of fast-growing species to favor more specialized competitors (Vieira-Sillva et al., 2016). This was observed for INU treatment after 24 h and then for EXT at 48 h (Fig. 1-III). Previous reports on other seaweed extracts, mainly polysaccharides, demonstrated differentiation in the diversity of gut microbiota (Duan et al., 2019; Xu, Aweya, Li, Deng, Chen, Tang, & Cheong, 2019; Zhang et al., 2020)

3.2. *Effect of the seaweed substrates on human gut microbiota composition and quantification of selected probiotic strains*

Firmicutes (55–69%) and Proteobacteria (16–18%) were the most predominant phylum followed by Bacteroidetes (7–20%) and Actinobacteria (3–4%) among the HGM total phyla identified at the baseline (0 h) (Fig. 2-I). Proteobacteria relative abundance increased in all treatments with the fermentation time reaching a maximum of 75, 60 and 54% at 48 h for CTRL, EXT and PS, respectively. In contrast, the maximum proteobacteria relative abundance in INU fermentation was observed at 8 h, reaching 41%. Proteobacteria is a vast and diverse phylum commonly found in fecal microbiota of healthy humans (Zhang et al., 2020). It is considered a subdominant phylum, representing no>10% of the HGM population (Gotteland et al., 2020; Binda et al., 2018) that responds to environmental factors, such as diet (Shin, Whon, & Bae, 2015). Under a healthy steady state, the relative abundance of Proteobacteria in the human gut might transiently increase up to 45% without clinical signs (Caporaso et al., 2011). The phylum Bacteroidetes presented a maximum relative abundance between 22 and 41% at 2 h followed by a continuous decrease reaching between 5 and 13% at the end of the fermentation. Bacteroidetes species are associated with polysaccharide degrading enzymes (Tandon et al., 2018) and typically produce a mixture of SCFA, such as acetic and propionic acids. Firmicutes relative abundance decreased with fermentation time from 55 to 69% to 16–30% at 48 h for all treatments except for INU. Firmicutes are associated with mono- and disaccharide fermentation (Vieira-Sillva et al., 2016) and butyric acid synthesis (Unno & Osakabe, 2018). Actinobacteria relative abundance was initially low (2–4%) for all treatments but reached a maximum of 9 and 13% at 8 h of fermentation with EXT and INU, respectively. Actinobacteria is another subdominant phylum that consists of gram-positive bacteria, mainly represented in HGM by the probiotic genus *Bifidobacterium*, whose presence is associated with the prevention of intestinal diseases and immune system modulation (Zhang et al., 2020).

At the lower taxonomic level and across all samples, 38 families and 50 genera were identified, with a relative abundance above 1% of the community set as a cut-off (Fig. 2-II and 2-III). After 48 h, the EXT treatment led to a lower abundance of the genera *Faecalibacetrium* (16–6%) and *Collinsella* (1·0%) and, at the same time, increased the proportion of the family *Enterobacteriaceae* (0.9 – 53.0%) and the genera *Catenibacterium* (2.2 – 14.4%) and *Phascolarctobacterium* (3.3 – 5.83%) compared with INU (Fig. 2-II and 2-III). Most notably, EXT increased the abundance of *Bifidobacterium* (18%) at 8 h and was similar to the abundance of INU (20%). After 48 h, the PS treatment also increased the proportion of *Enterobacteriaceae* (0.4 – 46.4%), *Catenibacterium* (3.4 – 13.6%), and *Clostridium* (0.0–6.5%) compared with INU (Fig. 2-II and 2-III). *Enterobacteriaceae* had a notable increase in all the evaluated substrates, even in INU experiments reaching up to 41%. Previous studies of *in vitro* colonic fermentations have reported pronounced development of this family using diverse polyphenols of oat bran (Kristek et al., 2019). This *Enterobacteriaceae* development was previously observed in other *in vitro* gut systems. Blatchford el al (2019) explained that this proliferation was attributed to altered growth conditions in *in vitro* assays such as (a) a richer growth medium, (b) suboptimal gaseous atmosphere (compromised anaerobiosis), and (c) the use of a static fermentation model that increases the accumulation of biomolecules and wastes that would normally be eliminated by absorption or excretion *in vivo*.

Correlation analysis between the concentration calculated by RT-

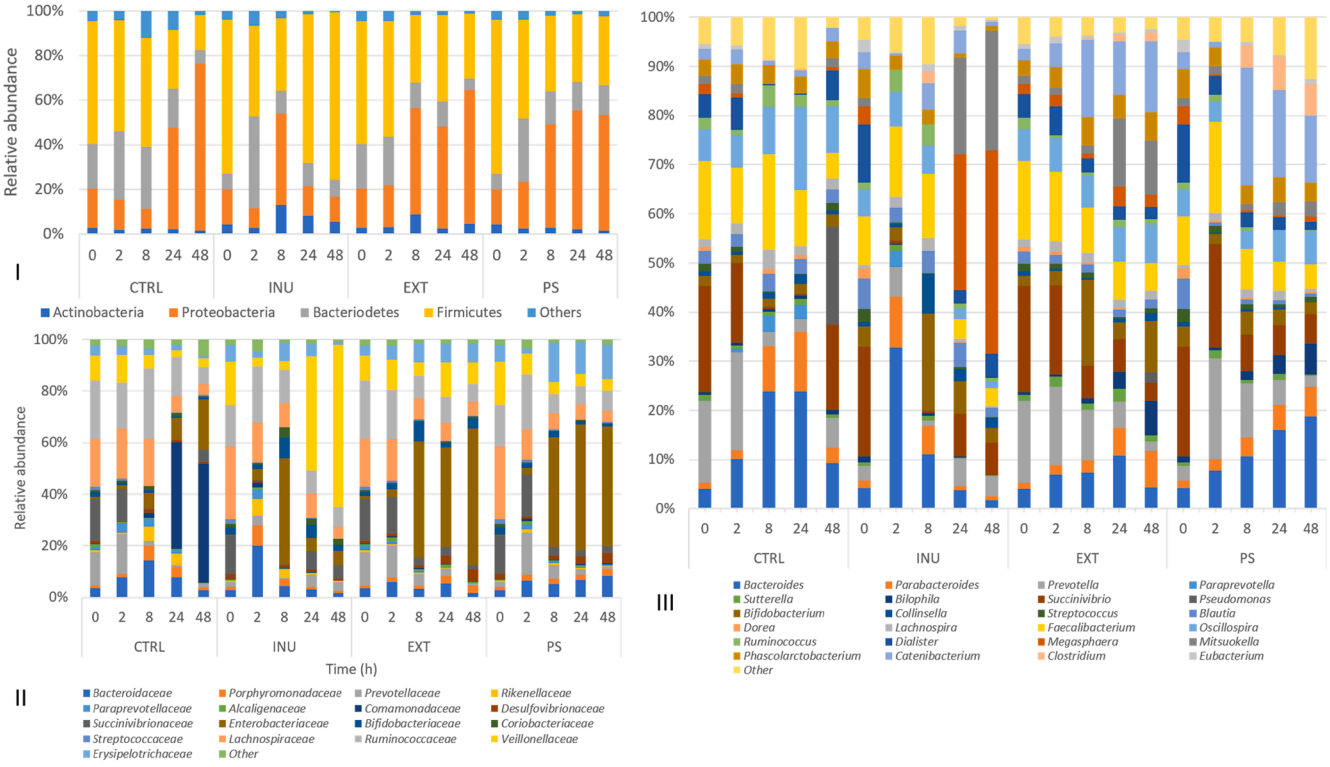

Fig. 2. Changes in human gut microbiota composition during *in vitro* colonic fermentations of *Silvetia compresssa* hydroethanolic extract (EXT), its isolated polysaccharide fraction (PS), inulin (INU), or a negative control (CTRL) at phylum (I), family (II) and genus level (III). *Others include organisms with a relative abundance below 1% or those that were not identified.

PCR (log_{10} CFU g^{-1} of wet feces) and the estimated log_{10} (X + 1) of the absolute abundances of *Bifidobacterium* and *Lactobacillus* genera obtained by 16S gene sequencing resulted in a significant correlation for *Bifidobacterium* ($p < 0.05$), but not for the *Lactobacillus* genus ($p > 0.05$) (Fig. 3). The correspondence between both techniques decreases at the very low end of the abundance range due to the relatively lower PCR amplification efficiency and increased stochasticity of the results for low abundance taxa in gene sequencing (Gonzalez, Portillo, Belda-ferre, & Mira, 2012; Jian, Luukkonen, Yki-Järvinen, Salonen, & Korpela, 2020). INU exerted the most pronounced development of *Bifidobacterium* and *Lactobacillus* at 24 h, with a result of log_{10} 7.25 and log_{10} 5.06 CFU g^{-1} of wet feces, followed by EXT (Fig. 3 I-B and II-B). PS fraction promoted bifidobacterial growth during the first 8 h but after that period there was no significant difference with CTRL ($p > 0.05$). In addition, *Lactobacillus* growth reached the maximum peak at 8 h of PS fermentation, being log_{10} 4.51 ± 0.09 CFU g^{-1} wet feces, and there was no significant difference with INU ($p < 0.05$). EXT fermentation reached its highest *Lactobacillus* count (log_{10} 4.42 ± 0.15 CFU g^{-1} wet feces) at 24 h, but it was only 43.04% of the count reached by INU. Charoensiddhi et al. (2016) reported similar findings between the extracts of *Ecklonia radiata*, with its polysaccharide fraction resulting in lower *Bifidobacterium* growth after 24 h fermentation in contrast to inulin or Celluclast extract.

3.3. Short-chain fatty acid synthesis

The substrate used in colonic fermentation and its duration had a significant impact ($p \leq 0.05$) on the concentrations of SCFA produced during the 48-h fermentation process (Fig. 4). At 48 h, total SCFA concentration (Fig. 4-I) in CTRL fermentation was the lowest (42.74 ± 6.09 μmol g^{-1} wet feces) in comparison with the media containing INU (410.44 ± 18.77 μmol g^{-1} wet feces), EXT (183.71 ± 0.86 μmol g^{-1} wet feces), or PS (110.02 ± 6.09 μmol g^{-1} wet feces). The INU and CTRL samples reached maximum concentrations of acetic and propionic acids at 24 h of fermentation, while the EXT and PS samples reached maximum concentrations after 48 h. After 48 h of fermentation, the EXT and PS produced 44.76 and 26.81% of the total SCFA obtained with INU after 24 h. Acetic and propionic acid production is considered a sign of prebiosis. Acetic acid has a key role as an energy source for peripheral tissue and a substrate for cholesterol synthesis (X. Wang et al., 2018). Propionic acid is a hypolipidemic factor, lowering serum cholesterol levels (Ramnani et al., 2012). EXT and PS reduced the acetate/propionate ratio after 24 h of fermentation (1.21 ± 0.06 and 1.41 ± 0.06, respectively), but EXT had a more pronounced decrease than INU (1.40 ± 0.25). This reduced ratio is considered a sign of hypolipidemic activity, inhibiting cholesterol biosynthesis by inhibiting HMG-CoA reductase activity in the host liver (Rodrigues et al., 2016; Strain et al., 2020; X. Wang et al., 2018; Weitkunat et al., 2015, 2016, 2017).

In agreement with other studies, acetic and propionic acids were the main SCFA detected in the fermentation of seaweeds extracts such as *S. muticum*, *O. pinnatifida* (Rodrigues et al., 2016), *Ecklonia radiata* (Charoensiddhi et al., 2016; Charoensiddhi, Conlon, Vuaran, et al., 2017), *Gracilaria spp*, *Gelidium sesquipidale* and *Ascophylum nodosum* (Ramnani et al., 2012). Significant differences ($p < 0.05$) were obtained by both seaweed substrates when compared to the CTRL and INU experiments (Fig. 4-II and 4-III). EXT produced 78% more acetic acid, 81% more propionic acid and 4.09% of the butyric acid produced by the CTRL. When compared to INU, the EXT produced 45%, 46%, and 7% of the previously mentioned SCFA. The PS produced 65.7% more acetic acid and 50.1% more propionic acid than the CTRL and only 5.28% of the butyric acid. When compared to INU, the PS produced 29.4% and 9.47% of the acetic acid and propionic acid, respectively. These results were similar to those reported for the *in vitro* and *in vivo* alginate and its low molecular weight derivatives (Li et al., 2016; Ramnani et al., 2012; Y. Wang, Han, Hu, Li, & Yu, 2006). Acetic and propionic acid concentrations and their improved ratio during EXT and PS fermentations have been previously associated to the growth of microorganisms belonging

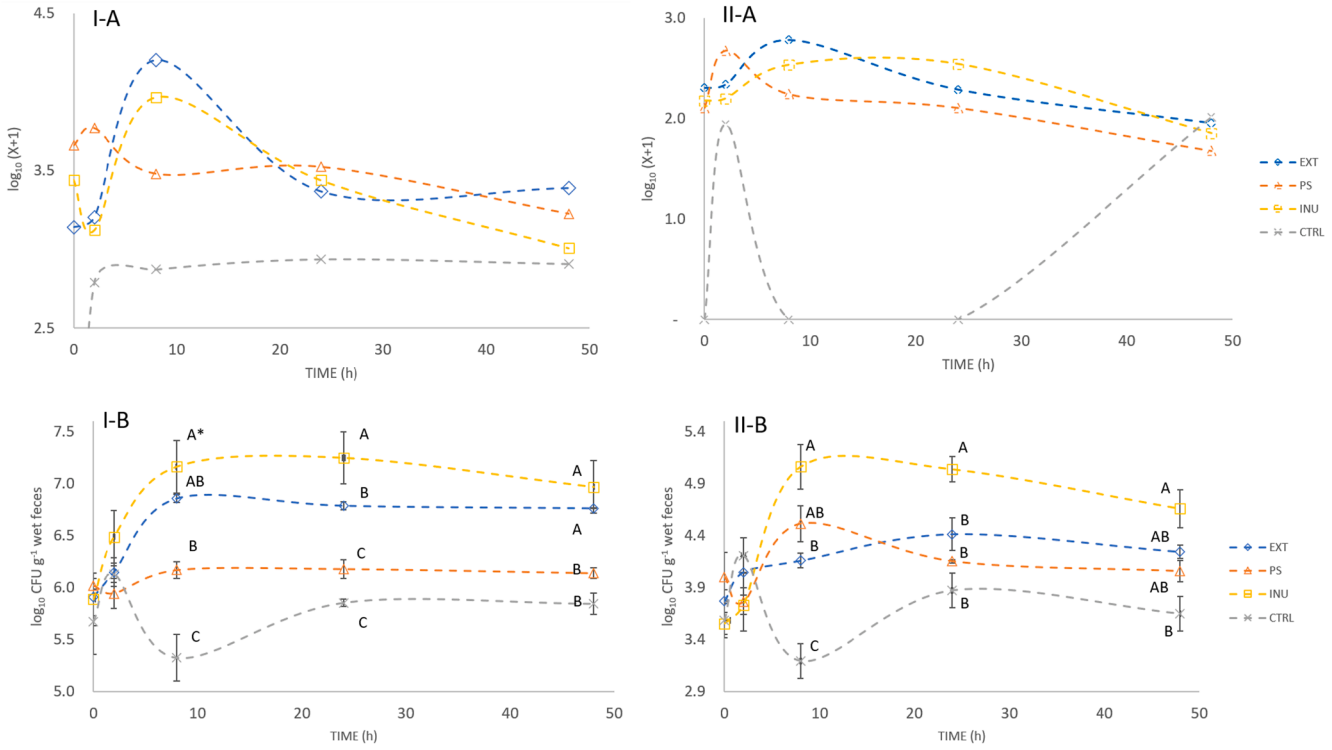

Fig. 3. Absolute abundance log_{10} (X + 1) of *Bifidobacterium* genera during the *in vitro* colonic fermentation of *Silvetia compresssa* hydroethanolic extract (EXT), its isolated polysaccharide fraction (PS), inulin (INU) or a negative control (CTRL) **(I-A)** and quantification using RT-PCR **(I-B)**, and Absolute abundance log_{10} (X + 1) of *Lactobacillus* genera **(II-A)** and quantification using RT-PCR **(II-B)**. * Different letters at each time point denote significant differences by Tukey-Krammer test ($p \leq 0.05$).

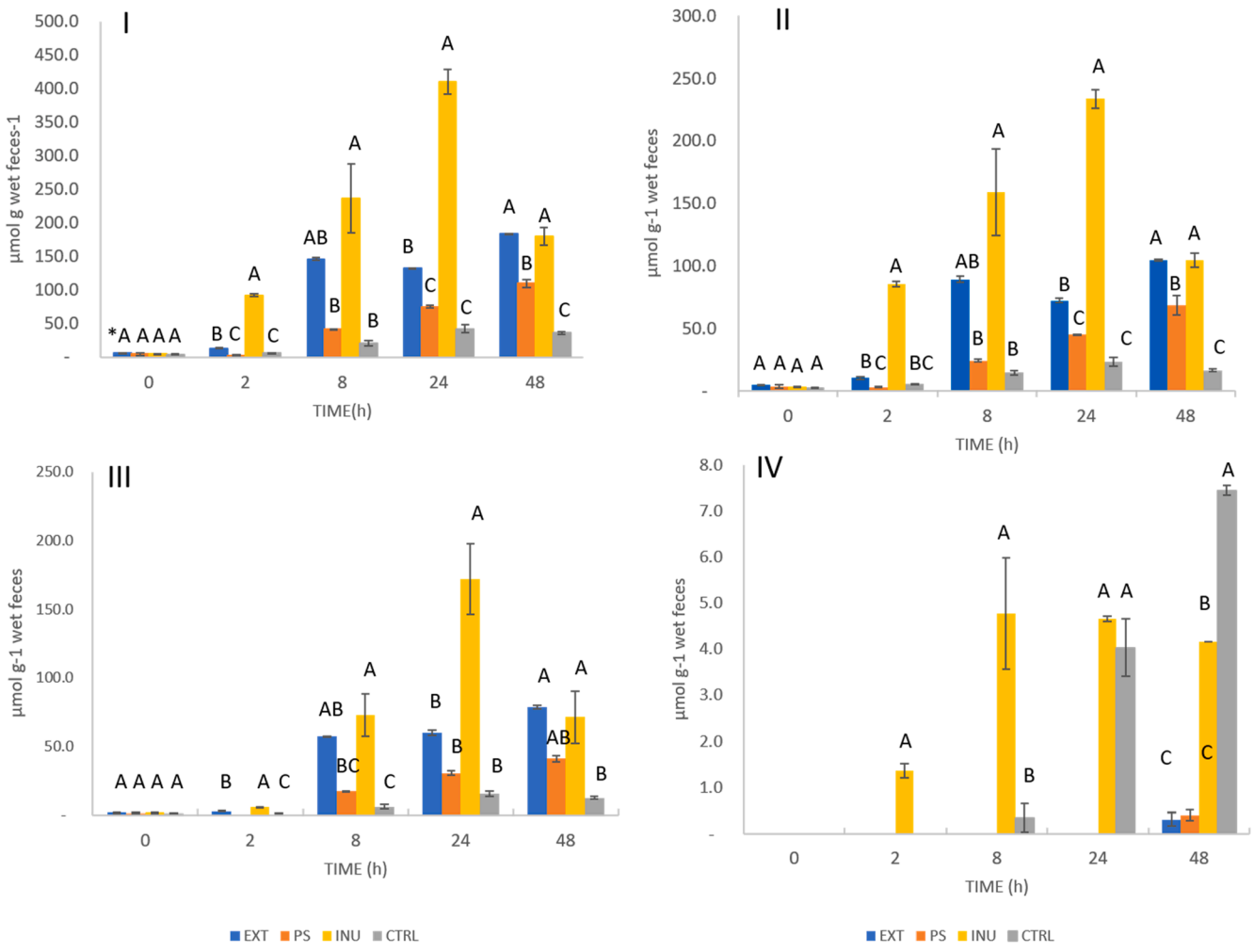

Fig. 4. Changes in concentrations of the most abundant short-chain fatty acids (SCFA) [**I.** Total SCFA, **II.** Acetic acid, and **III.** Propionic acid and **IV.** Butyric acid] during the *in vitro* colonic fermentation of *Silvetia compressa* hydroethanolic extract (EXT), its isolated polysaccharide fraction (PS), inulin (INU) or a negative control (CTRL). *Different letters denote significant differences by Tukey-Krammer test ($p \leq 0.05$).

to the genera *Bilophila, Catenibacterium, Phascolarctobacterium, Bifidobacterium,* and *Mitsuokella* (Fig. 2-III) (Gorham et al., 2017; Han et al., 2019; Ikeyama et al., 2020; Kageyama & Benno, 2000).

Butyric acid synthesis is considered an important prebiosis marker. This SCFA is an energy source for the colon epithelial cells and may play a role in the development of and gene expression in intestinal cells, possibly preventing colon cancer and colon inflammation (De Vuyst & Leroy, 2011). HGM is a competitive environment, and butyric acid synthesis has proven to be mediated by both primary degraders (partial hydrolysis of polysaccharides) and secondary degraders (acetic, lactic, and succinic acid dependent synthesis) (De Vuyst & Leroy, 2011). In INU and CTRL experiments, butyric acid synthesis was observed after two and eight hours of fermentation (Fig. 4-IV). Butyric acid production in CTRL fermentation may have been related to acetic acid consumption by secondary degraders, whereas in INU experiments primary degraders produced mono/oligosaccharides (FOS) available for consumption by other butyrogenic secondary degraders (Baxter et al., 2019). In CTRL fermentation, the Ilumina sequencing results showed that the relative abundance of primary degraders, such as *Lachonospira*, *Ruminococcus*, *Succinivibrio*, *Bifidobacterium,* and *Lactobacillus,* increased during the first 24 h which might have facilitated the growth of bacteria responsible for butyric acid production (Fig. 2-II). In contrast, FOS oligomers in INU fermentation have been previously associated with the growth of *Bifidobacterium* and *Lactobacillus* (De Vuyst & Leroy, 2011) and, in consequence, with the increase of secondary degraders (e.g. *Faecalibacterioum prausnitzii* and *Eubacterium rectale*) involved in butyric acid production (Fig. 2-II) (Flint, Scott, Duncan, Louis, & Forano, 2012).

3.4. Phlorotannin quantification in in vitro fermentation

Three main phenolic compounds were only detected in the EXT fermentations. These compounds include a flavonoid which is an acacetin derivative and eckstolonol and dieckol, which are two PT (Fig. 5). Eckstolonol was the most abundant with 3.28 ± 0.31 mg PE g^{-1} wet feces (Fig. 5 - IV). Previously, the only *in vitro* activities reported for the PT regarding the HGM were the (1) inhibition of pathogenic bacteria (Charoensiddhi, Conlon, Vuaran, et al., 2017), (2) possible transformation of high molecular weight PT in oligomers for its absorption in the large intestine (Corona et al., 2016, 2017), and (3) inhibition of HT-29 cell growth and reduction of H_2O_2-induced DNA damage in colon cells (Montero et al., 2016). Eckol administered *in vivo* increased the relative abundance of *Bifidobacterium* and *Lactobacillus* in mice and showed anti-inflammatory properties by recruiting $CD11c^+$ dendric cells into inflamed colon tissue (Zhu et al., 2020). In this study, an inverse correlation between SCFA production and total phlorotannin content (eckstolonol and acacetin derivative) was observed ($p \leq 0.05$). In general, PT content had an inverse correlation with *Bifidobacterium* ($p < 0001$), *Lactobacillus* ($p = 0.022$), and total bacteria ($p = 0.0148$), as well as acetic acid ($p = 0.0003$), propionic acid ($p < 0.0001$), and total SCFA ($p = 0.0001$) production. These effects were mainly attributed to the

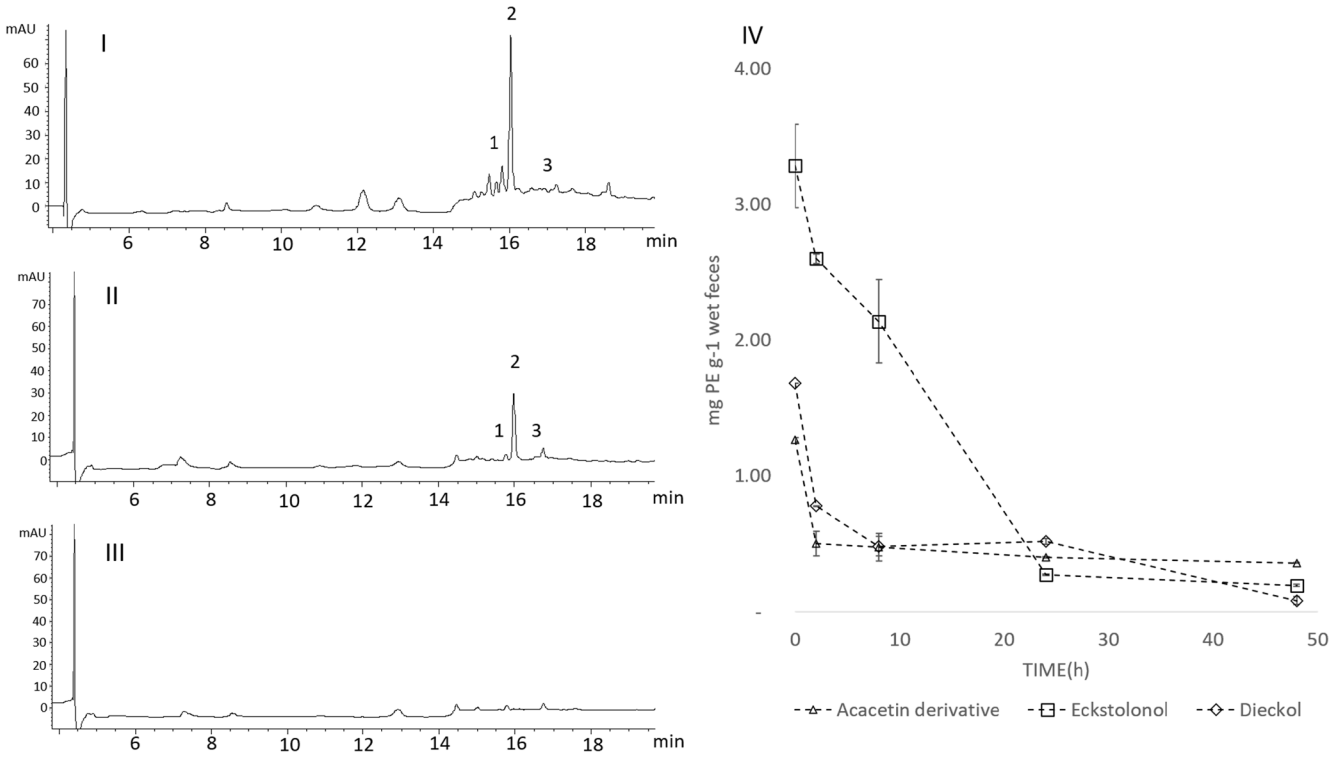

Fig. 5. Chromatograms obtained at 270 nm showing the changes in acacetin derivative (1), eckstolonol (2), and dieckol (3) concentrations in the supernatants obtained at 0 **(I)**, 8 **(II)** and 48 h **(III)** from *in vitro* colonic fermentation of *Silvetia compresssa* hydroethanolic extract (EXT) and changes expressed per gram of feces used as inoculum **(IV)**.

concentration of eckstolonol ($p < 0.05$). According to previous reports SCFA production is affected by phlorotannins, particularly when they are recovered from crude extracts (Charoensiddhi, Conlon, Vuaran et al., 2017). Acacetin flavonoids in HGM appear to be degraded and metabolized in less complex forms by members of the Enterobacteriaceae family (*Escherichia* and *Enterococcus* genera) due to being more easily absorbed (Gowd, Karim, Shishir, Xie, & Chen, 2019). In addition, acacetin could enhance the adhesion of *Lactobacillus acidophilus* in the gut by increasing SrtA activity, which is the enzyme responsible for the cell wall anchoring of sortase dependent proteins (Wu et al., 2020). Other phenolic compounds like flavonoids (e.g. quercetin and catechin) and resveratrol have shown to stimulate the growth of *Bifidobacterium* and *Lactobacillus* (Huang et al., 2016; Loo, Howell, Chan, Zhang, & Ng, 2020)

PT in conjunction with PS had an important role in prebiotic activity of the EXT. The range of prebiotic activities shown by phenolic compounds in *in vitro* colonic fermentations are related with mechanisms of cross feeding, probiotic development, and regulation of virulence factors from pathogen strains (Loo et al., 2020). Studies have also shown that flavonoids, phlorotannin extracts and several enriched polyphenolic extracts aid in the development of Proteobacteria, *Enterobacteriaceae*, *Escherichia,* and *Hafnia* (a novel probiotic anti-obesity lactic acid producing bacteria) (Charoensiddhi, Conlon, Vuaran, et al., 2017; Huang et al., 2016; Kristek et al., 2019). Proteobacteria development reveals an important role in HGM, and it is linked to the *Enterobacteriaceae* members' abilities to metabolize phenolic compounds due to their O-glucosidase, O-demethylase, and dehydroxylase activities (Cortés-Martín, Selma, Tomás-Barberán, González-Sarrías, & Espín, 2020). Phenolic acids may selectively attenuate the growth of pathogenic bacteria without compromising the commensal and probiotic bacteria in the gut, but further research is required to study the effect of phenolic compounds and phlorotannins on the commensal, probiotic, and pathogenic members of the *Enterobacteriaceae* family (Charoensiddhi, Conlon, Vuaran, et al., 2017; Loo et al., 2020).

3.5. Carbohydrate quantification in PS and EXT in vitro fermentation

The monosaccharides detected during the early stages of fermentation (Fig. 6) were related to the partial hydrolysis produced by the high energy of ultrasound-assisted extraction of polysaccharides. These compounds, which include laminarin, alginate and fucoidan, are mainly composed of galactose, fucose, mannose, glucuronic acid, guluronic acid, mannuronic acid and/or xylose (García-Ríos, Ríos-Leal, Robledo, & Freile-Pelegrin, 2012). The presence of mannitol is common in the Phaeophyceae family and is one of the main products of their photosynthesis (Lalegerie, Gager, Stiger-Pouvreau, & Connan, 2020). It is estimated that 25% of the ingested mannitol is absorbed in the small intestine and then excreted in the urine (Xiao, Li, Min, & Sakaguchi, 2013). The difference in the mannitol content between the EXT and the PS can be attributed to the PS precipitation step since it is slightly soluble in organic solvents such as methanol and ethanol (Lama-Muñoz et al., 2020; Su, Liu, Wang, Li, & Jia, 2020). Mannitol and glucose were the first carbon sources to be depleted, followed by galactose and the mannose + arabinose + fucose (MAF) fraction. Particularly, mannitol was not detected after 8 h of *in vitro* gastrointestinal digestion. Previous to this study, there is no report of mannitol being metabolized *in vitro* by fecal bacterial consortiums of HGM (Ruiz-Ojeda, Plaza-Díaz, Sáez-Lara, & Gil, 2019), but it has been reported to enhance the SCFA production and *Lactobacillus acidophilus* ATCC 4962 growth compared to maltodextrin or inulin (Liong & Shah, 2005). Mannitol supplementation promoted the development of isolated strains of *Lactobacillus sp* FTDC 2113 and *L. acidophilus* FTDC 8033 in soymilk and the synthesis of lactic acid (Yeo & Liong, 2010). This ability of lactic acid bacteria to metabolize mannitol is attributed to the alternative NADH-H + -oxidizing mechanism of NADH oxidase that affects the ability to grow on substrates more chemically reduced than glucose, such as mannitol.

Glucose and galactose were the second and third monosaccharides to be consumed. In EXT and PS fermentations, glucose was depleted after 24 h. Galactose decreased 42.8% and 39.62% during the first eight hours

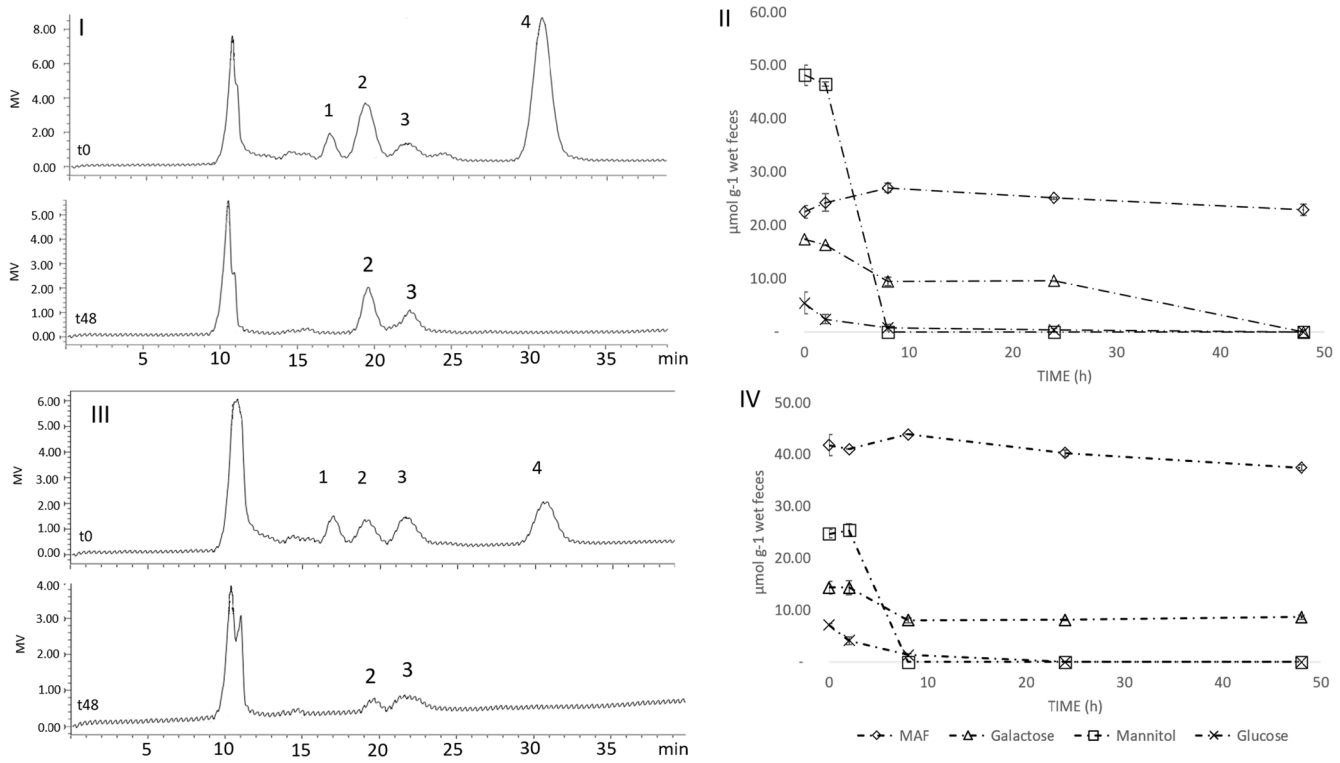

Fig. 6. Chromatograms obtained by HPLC-RI showing the differences in glucose (1), galactose (2), mannose + arabinose + fucose (MAF) (3) and mannitol (4) concentrations in the supernatants of a *Silvetia compresssa* hydroethanolic extract (EXT) *in vitro* colonic fermentation at 0 h (t0) and 48 h (t48) **(I)** and changes expressed per gram of feces used as inoculum for the 48 h EXT fermentation **(II)**, Chromatograms obtained for its isolated polysaccharide fraction (PS) **(III)** and corresponding concentration changes during the 48 h of fermentation **(IV)**.

of fermentation in EXT and PS respectively, maintaining its concentration practically stable onward. Glucose and galactose presented a strong significant correlation with the amounts of acetic acid, propionic acid, total SCFA as well as with *Bifidobacterium* growth, but the correlation with *Lactobacillus* bacteria was not significant ($p > 0.05$).

4. Conclusion

The hydroethanolic extract of brown seaweed *S. compressa* (EXT) and its PS fraction promoted changes in Proteobacteria, Bacteroidetes and Actinobacteria phyla in the HGM from ten healthy donors. EXT treatment significantly increased the production of SCFA compared to the PS treatment, but slightly less than INU. The differences in the SCFA synthesis and bacterial populations between EXT and PS were due to the interactions of phlorotannins and mannitol with HGM during the first 8 h of fermentation. Not only *S. compressa* polysaccharides were relevant for the maintenance and improvement of a healthy HGM since metabolic activity was positively influenced by phlorotannins and mannitol. However, further work is needed to confirm that this potential prebiotic effect and its associated health benefits occur *in vivo*.

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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