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Abstract

Extracts with prebiotic activity or bioactive compounds from natural sources such as seaweeds or mushrooms, combining a broad spectrum of biological properties, may offer great potential for their use as functional ingredients enabling intestinal microbiota modulation. In this context, selected enzymatic extracts from Sargassum muticum, Osmundea pinnatifida and Pholiota nameko were evaluated in vitro. Faecal fermentations were conducted anaerobically under controlled temperature and pH over 24 h. Enzymatic extracts of Ph. nameko and of O. pinnatifida at 1% (w/v), lead to increases in Bifidobacterium spp. after 6 h of fermentation in comparison to negative control, suggesting a stimulatory effect. No significant changes over 24 h were observed of Lactobacillus spp. In particular, the Ph. nameko extract obtained with Flavourzyme not only stimulated growth and/or activity of Bifidobacterium spp. but also led to a decrease of Clostridium histolyticum group upon 24 h, thus potentially benefiting colonic health. Higher percentages of this extract (2 and 3%) impaired a C. histolyticum reduction confirming this selective action and prebiotic potential. Differences in short chain fatty acids (SCFA) and lactic acid production between the four extracts may indicate a potential relationship between their physico-chemical properties, which differ in composition and structures, and modulation of gut bacterial species.

Keywords: Seaweeds, mushrooms, enzymatic extracts, fluorescence *in situ* hybridization (FISH), prebiotic activity

1. Introduction

Edible seaweeds and mushrooms are an excellent source of bioactive compounds (Pádua, Rocha, Gargiulo, & Ramos, 2015; Ruthes, Smiderle, & Iacomini, 2016) and therefore research into the biological potential of enzymatic extracts of seaweeds *S. muticum* and *O. pinnatifida* and of mushroom *Ph. nameko* to be explored within the functional food perspective, were selected for *in vitro* fermentation study to consolidate their prebiotic potential (Rodrigues et al., 2015,2016).

Prebiotics are defined as substrates that improve the host health by selectively stimulating the growth and/or metabolic activity of one or a limited number of beneficial bacteria in the colon (Roberfroid et al., 2010). The potential prebiotic effect of the selected extracts has been evaluated by comparison with fructoligosaccharides (FOS), the gold standard in comparison studies, using pure cultures (*Lactobacillus acidophilus* La5; *Bifidobacterium animalis* BB12) (Rodrigues et al., 2015,2016). The human gastrointestinal tract represents a complex ecosystem where the available nutrients and the diverse microbiota will influence changes within the community (Roberfroid et al., 2010). Hence, to assess the possible importance that colonic catabolism of these extracts may have on human gut microbiota, *in vitro* batch culture fermentation experiments conducted with faecal inoculum from healthy volunteers, are in order to observe changes in the main bacterial groups present within (Eid et al., 2014; Sánchez-Patán et al., 2012). The human colon is considered the most metabolically active site in the human body with over 1000 species of microorganisms reaching up to 10^{12}-10^{13} bacteria per gram dry weight (Roberfroid et al., 2010). To study this diverse community pH controlled, anaerobic faecal batch cultures enable

assessment of the fermentability of substrates in the intestinal lumen, whilst simulating the conditions in the human distal colon (Bergillos-Meca, Costabile, Walton, Moreno-Montoro, Ruiz-Bravo, & Ruiz-López, 2015). A growing body of evidence suggests that the gut microbiota impacts on a wide range of host metabolic pathways, barrier function and immune modulatory function influencing the prevention and risk of a wide range of diseases, including inflammatory bowel disease, diarrhoea and colorectal cancer. Much of this impact is mediated through diet and the consumption of specific health-related foods, justifying the constant need to modulate diet or identify compounds that can positively modify the gut microbiota (Gibson, Scott, Rastall, & Tuohy, 2010).

Research has been focused on ‘prebiotics’, and in particular the ability of certain types of dietary fibre, especially indigestible oligosaccharides, to stimulate the growth of and/or activity of beneficial gut bacteria such as bifidobacteria and lactobacilli while retarding the development of *C. histolyticum*, leading to a concomitant positive effect on colonic health (Gibson et al., 2010; Aida, Shuhaimi, Yazid & Maaruf, 2009). Better understanding of the benefits of prebiotics has urged a need to search for and develop new and alternative sources of prebiotics. According to Zaporozhets et al. (2014), the prebiotic activity of extracts or of polysaccharides from marine seaweeds, combined with a broad spectrum of biological properties, evidences great potential for their use as functional nutrition ingredients enabling modulation of intestinal microbiota and of gastrointestinal tract (GIT) inflammation as well as normalization of the immune system. Therefore the main objective in this study was to evaluate the potential of the digested (closely simulating physiological conditions) seaweed and mushroom extracts containing different polysaccharide and oligosaccharide structures on gut

microbial ecology. To our best knowledge there are no studies regarding the impact of water-based enzyme-assisted extracts for seaweeds *S. muticum*, *O. pinnatifida* or mushroom *Ph. nameko* on gut microbial ecology confirm and consolidate the biological potential of these selected extracts, for their application as functional food and bioactive ingredient sources.

2. Material and methods

2.1. Selected seaweeds and mushrooms extracts

In this study water-based enzyme-assisted extracts from two seaweeds and one mushroom were selected following demonstration of potential prebiotic effect with pure cultures (Rodrigues et al., 2015,2016). Selected extracts included extracts of *S. muticum* obtained with Alcalase, *O. pinnatifida* obtained with Viscozyme and *Ph. nameko* obtained with Cellulase and with Flavourzyme. For each extract, 1g of dried mushroom or 2 g of dry seaweed was dispersed in 50 mL of deionised water and incubated in an agitated water bath for 10 min. After adjusting pH to specific enzyme optimum conditions (Alcalase: pH=8 – 50 ºC; Flavourzyme: pH=7.0 – 50 ºC; Cellulase: pH=4.5 – 50 ºC; Viscozyme® L: pH=4.5 – 50 ºC; All enzymes were obtained from Sigma-Aldrich), 100 mg of enzyme was added and incubated for enzymatic hydrolysis for 24h at 50 ºC. The enzymatic reaction was stopped by heating the sample at 90-100 ºC for 10 min followed by immediate cooling in an ice bath. The pH of enzymatic extracts was adjusted to pH 7.0 with 1M HCl and/or NaOH and then centrifuged, filtered and freeze-dried according to Rodrigues et al. (2015, 2016).

2.2. *In vitro* fermentation by human gut microbiota

126 Samples of the selected extracts were submitted to three consecutive steps: 1)
127 Simulated gastrointestinal digestion; 2) Faecal batch-culture fermentation and, 3)
128 Bacterial enumeration using FISH.

129 2.2.1. Simulated gastrointestinal digestion

130 To simulate the digestion of the selected seaweed and mushroom extracts
131 through the gastrointestinal tract and therefore evaluate the main effects of the
132 digested extracts on human microbiota, samples were treated according to Mills
133 et al. (2008), with slight alterations. Water (25 mL) was added to 10 g of
134 lyophilized extract, and the mixture was stomached (Seward, UK) for 5 min using
135 200 paddle-beats per min. The extract solution was then mixed with α-amylase
136 (A4551, Sigma; 3.33 mg) in $CaCl_2$ (0.001 M, pH 7.0; 1.04 mL) and incubated at 37
137 °C for 30 min and at 130 rpm in a shaker. Afterwards, the pH was decreased to
138 2.0 with 6 M HCl and pepsin (P 7000, Sigma; 0.45 g) dissolved in HCl (0.1 M;
139 4.16 ml) was added. The sample was incubated at 37 ºC for 2 h and at 130 rpm in
140 a shaker. After this period, the pH was increased to 7 with 6 M NaOH, and
141 pancreatin (P 8096, Sigma; 0.93 g) and bile (B 8631, Sigma; 0.58 g) in $NaHCO_3$
142 (0.5 M; 20.8 mL) were added. The extract solution was then incubated at 37 °C
143 and at 45 rpm in a shaker for 3 h and afterwards transferred to 1 kDa molecular
144 weight cut-off regenerated cellulose dialysis tubing (Spectra/Por® 6, Spectrum
145 Europe, Netherlands) and dialyzed against NaCl 0.01 M at 5 °C, to remove low
146 molecular mass digestion products. After 15 h the NaCl dialysis fluid was changed
147 and dialysis continued for an additional 2 h. Afterwards the digested samples
148 were frozen at -80 ºC and lyophilized in a freeze dryer (Armfield SB4 model,
149 Ringwood, UK). All chemicals were purchased from Sigma (St Louis, USA).

150 2.2.2. Faecal batch-culture fermentation

151 Three independent fermentation experiments were carried out. Faecal samples
152 were obtained fresh at the premises of the Department of Food and Nutritional
153 Sciences from 3 apparently healthy adult volunteers who ingested a normal diet,
154 had not ingested any antibiotics for at least 6 months and were not regular
155 consumers of pre or probiotics. Samples were collected into sterile vials and kept
156 in an anaerobic cabinet and used within 30 min of collection. A 1/10 (w/w) dilution
157 in phosphate buffer saline (PBS) was prepared and homogenized using a
158 stomacher (Serward, Worthing, UK) for 2 min at 460 paddle-beats per min.
159 Sterile stirred batch culture fermentation vessels (50 mL working volume) were set
160 up and aseptically filled with 45 mL sterile, pre-reduced, basal medium [peptone
161 water 2 g/L, yeast extract 2 g/L, NaCl 0.1 g/L, K_2HPO_4 0.04 g/L, KH_2PO_4 0.04 g/L,
162 $MgSO_4$.7H2O 0.01 g/L, $CaCl_2$.6H_2O 0.01 g/L, $NaHCO_3$ 2 g/L, Tween 80 2 mL/L
163 (BDH, Poole, UK), Hemin 0.05 g/L, vitamin K1 10 µL/L, L-cysteine-HCl 0.5 g/L,
164 bile salts 0.5 g/L, pH 7.0] and gassed overnight with O_2-free N_2 (15 mL/min) with
165 constant agitation. All media and chemicals were purchased from Oxoid
166 (Basingstoke, UK) and Sigma (St Louis, USA). The temperature was kept at 37 ºC
167 and pH was controlled between 6.7 and 6.9 using an automated pH controller
168 (Fermac 260, Electrolab, Tewkesbury, UK), which added acid or alkali (0.5 M HCl
169 and 0.5 M NaOH) in order to mimic conditions that resemble the distal region of
170 the human large intestine (Sánchez-Patán et al., 2012).
171 Six stirred pH-controlled batch fermenters were run in parallel. The different
172 digested extracts (1% w/v) were aseptically added to four vessels, the other two
173 vessels were used as controls. For the positive control vessel 1% (w/v) of FOS
174 (95% oligofructose, 0.5 kDa dextran with 25% α-1,2 branching, 1 kDa dextran with
175 32% α-1,2 branching; Orafti®*P95,* Oreye, Belgium) was used. To the negative

control vessel no source of carbon was added. The experiment was performed in triplicate, using one faecal sample given by a different donor for each run of six batch fermenters. Each vessel, with 45 mL sterile medium and digested extract, was inoculated with 5 mL of fresh faecal slurry (1/10 w/w). The batch cultures were run under anaerobic conditions for a period of 24 h, during which 5 mL samples were collected from each vessel at 0, 6, 12 and 24 h for FISH and analysis of lactic acid and short chain fatty acids (SCFA). For this latter analysis, samples were stored at -70 ºC until required.

In order to assess the effect of the addition of 2 and 3% of digested extracts of *O. pinnatifida* obtained with Viscozyme and of *Ph. nameko* obtained with Flavourzyme, faecal batch-culture fermentations were repeated, in triplicate, under similar conditions but non-pH controlled and at lower volumes (10 mL) and for a period of 12 h.

2.2.3. Bacterial enumeration using FISH.

To assess differences in bacterial composition, FISH was used with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA based on the method described by Daims, Stoecker, & Wagner (2005). A total of 6 different probes commercially synthesized and 5'-labelled with the fluorescent dye (Sigma Aldrich, St Louis, USA) were used in addition to an overall stain with 4,6-diamidino-2-phenylindole (DAPI), which measures all cells by staining DNA (Harmsen, Wildeboer-Veloo, Grijpstra, Knol, Degener, & Welling, 2000a; Harmsen et al., 2000b).

Samples (375 µL) obtained from each vessel and sampling time were fixed for a minimum of 4 h (4 ºC) in 1125 µL 4% (w/v) paraformaldehyde. Fixed cells were centrifuged at 13,000 g for 5 min and washed twice in 1 mL filtered sterilized PBS.

The washed cells were re-suspended in 150 µL filtered PBS and stored in 150 µl ethanol (99%) at -20 ºC until further processing. Samples were then diluted in a suitable volume of PBS in order to obtain countable fluorescent cells in each field of view and 20 µl of the above solution was added to each well of a 6 well PTFE/poly-L-lysine coated slide (Tekdon Inc., Myakka City, USA). The samples were dried for 15 min in a drying chamber (46 ºC).

To permeabilize cells for use with probes Bif164 and Lab 158 (Table 1), samples were treated with 20 µL of lysozyme at room temperature for 15 min before being washed briefly in water. Slides were dehydrated, using an alcohol series (50, 80 and 96% (v/v) ethanol) for 3 min in each solution. Slides were returned to the drying oven for 2 min to evaporate excess ethanol before adding the hybridization mixture to each well [(0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.01% sodium dodecyl sulphate and 4.55 ng/mL probe]. For probes EUB338 I-II-III, the hybridization mixture contained formamide [(0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0), 35% formamide, 0.01% sodium dodecyl sulphate and 4.55 ng/mL probe]. Hybridization occurred for 4 h in a microarray hybridization incubator (Grant-Boekel, Cambridge, UK). After hybridization, slides were washed in 40 mL washing buffer [0.9 M NaCl and 0.02 M Tris/HCl (pH 8.0)], and 0.005 M ethylenediaminetetraacetic acid for the EUB338 I-II-III probes with 20 µl nucleic acid stain 4', 6-diamidino-2- phenylindole (DAPI; 50 ng/µL) for 15 min. They were then dipped in cold water for a few seconds and dried with compressed air. Five microlitres of polyvinyl alcohol mounting medium with 1,4-diazabicyclo(2,2,2)octane (DABCO) was added onto each well and a cover slip was placed on each slide (20 mm, thickness No 1, VWR, Lutterworth, UK). Slides were examined by epifluorescence microscopy (Eclipse 400, Nikon, Surrey, UK)

using the Fluor 100 lens. For each well, 15 fields with a maximum of 300 positive cells were counted.

2.2.4. Lactic acid and SCFA analysis

Samples were collected from each batch culture at each sampling point (0, 6, 12 and 24 h) and frozen at -70 ºC until required. Samples were assessed for lactic acid and SCFA (acetic, propionic, butyric, isobutyric and isovaleric acids) using an HPLC apparatus from Merck LaChrom (Fullerton CA, USA), in a single run, based on calibration curves previously prepared with appropriate chromatographic standards; an Aminex HPX-87X cation exchange column from BioRad (Richmond CA, USA) was used for separation; the eluant was pumped at 0.8 mL/min and consisted of 13 mM H_2SO_4 (Merck); and detection was by UV absorbance at 220 nm. Prior to analysis, samples were defrosted, centrifuged (13,000 g for 10 min at 4 ºC) and filtered through a 0.22-µm membrane filter (Millipore, USA) to remove all particulate matter.

2.3. Statistical Analysis

A paired Student's *t* test was used to test for significant differences in the bacterial group populations between extracts and controls as well as for time *in vitro* fermentation experiments by human gut microbiota. All data of bacterial populations (Log_{10} cell/mL) are expressed as average of three replicates (donors) plus or minus standard deviation, justifying the high variability reported in several cases.

3. Results and discussion

3.1. Modulation of intestinal microbiota by seaweed and mushroom digested extracts.

Assessment of the prebiotic potential of bioactive compounds or extracts by *in vitro* fermentation with human faecal microbiota provides a cost-effective and rapid alternative to assess the fermentation and modulation capacity of different substrates on a laboratory scale comparative basis (Gullon, Gullon, Tavaria, Pintado, Gomes, Alonso, & Parajo, 2014). It is important to note that before performance of *in vitro* fermentations care was taken to submit each extract to simulated gastrointestinal digestion because resistance to gastric acidity and hydrolysis by mammalian enzymes are limiting factors that have to be assured in order to enable the substrate to reach the colon and be fermented by intestinal microbiota, meeting the pre-requisite for a prebiotic effect or gut modulation effect. According to Gibson et al. (2010) any dietary material that is non-digestible and enters the large intestine is a candidate prebiotic. The few studies that have evaluated the prebiotic potential of seaweed polysaccharides using *in vitro* fermentation (laminarin and low molecular weight polysaccharides from agar and alginate) did not undergo previous gastrointestinal digestion, hindering analysis of true effectiveness of intact compounds (Devillé, Gharbi, Dandrifosse, & Peulen, 2007; Ramnani et al., 2012). During the experimental time course (0, 6, 12 and 24 h) of the *in vitro* fermentation of the digested seaweed and mushroom extracts at 1% (w/v) changes in the different bacterial populations and accumulation of lactic acid and SCFA (acetic, propionic, butyric acids) were assessed. For comparative purposes, the same experimental strategy was used with the well-established prebiotic FOS (positive control) and with medium without carbon source present (negative control) (Fig. 1).

FISH was used to monitor the modifications among populations of selected bacterial species caused by the different digested *S. muticum*, *O. pinnatifida* and

276 *Ph. nameko* enzymatic extracts added at 1%, on a comparative basis. Depending
277 on the bacterial group different effects were observed. Both enzymatic extracts of
278 *Ph. nameko* and that of seaweed *O. pinnatifida* obtained with Viscozyme lead to
279 overall increases in *Bifidobacterium* spp. populations as compared to the negative
280 control between 6 and 24 h of fermentation, confirming a stimulatory effect (Fig.
281 1.a). Highest shift was observed for medium containing *Ph. nameko* extract
282 obtained with Flavourzyme raising bifidobacterial counts from 8.06±0.66 Log_{10}
283 cell/mL at 0h to 8.49±0.06 Log_{10} cell/mL at 24 h (p=0.391) in comparison to the
284 negative control at 24 h (p=0.021). Evidence for a potential prebiotic effect for
285 pure culture of *B. animalis* BB12 was observed for undigested *Ph. nameko*
286 enzymatic extracts (Rodrigues et al., 2016). The positive control FOS, did
287 however induce a higher increase in bifidobacterial numbers after 24 h of
288 fermentation; from 8.04±0.47 at 0 h to 8.98±0.13 at 24 h (p=0.017). Indeed a two-
289 fold higher increase was observed for FOS (0.94 log_{10}) when compared to *Ph.*
290 *nameko* extract obtained with Flavourzyme (0.43 log_{10}).
291 No significant lactobacilli populations changes were observed over the 24 h in
292 comparison to the negative control for any of the four extracts tested at 1% except
293 for FOS (Fig. 1.b). These results contrast with those reported in previous studies
294 obtained with pure cultures of *L. acidophilus* La-5; significant higher values
295 (p<0.05) of viable cells were observed for the majority of culture media enriched
296 with seaweed water-based extracts (Rodrigues et al., 2015) and with *Ph. nameko*
297 extracts (Rodrigues et al., 2016) after 24 h of incubation in comparison to growth
298 in media with glucose or FOS. Ramnani et al. (2012) also reported absence of
299 effect on gut lactobacilli populations by low molecular weight polysaccharides
300 from agar and alginate seaweeds.

The stimulation of growth and/or activity of beneficial gut bacteria such as *Bifidobacterium* by the digested extract of *Ph. nameko* obtained with Flavourzyme was associated with a decrease in numbers of the *C. histolyticum* after 24 h fermentation, in comparison to the negative control (Fig. 1.e). Such a change can be considered a concomitant positive effect on colonic health (Gibson et al., 2010; Aida et al., 2009). Furthermore, it can also be highlighted that although not statistically significant, the positive control FOS was associated with an increased number of cells of *C. histolyticum* after 12 and 24 h in comparison to the negative control (Fig. 1.e); in contrast, decreased numbers of *C. histolyticum* were observed in particular for the extracts of *O. pinnatifida*, *S. muticum* and *Ph. nameko* obtained with Flavourzyme. Although there is a clear difference in response between FOS and studied extracts, it must be mentioned that some authors have mentioned that an increase in *C. histolyticum* numbers may be a consequence of culture conditions rather than a specific effect mediated by the tested prebiotic compounds (Bergillos-Meca et al., 2015). Bergillos-Meca et al. (2015) reported such an increase in *C. histolyticum* numbers for both positive control (FOS) and for tested probiotic/prebiotic conditions which is not the case presented herein. The digested extract of *S. muticum* obtained with Alcalase seems to be the less promising of the tested extracts considering the absence of a positive shift for both the *Bifidobacterium* and lactobacilli groups and their positive influence on the *Clostridium* groups at 6 and 12 h (Fig 1.a,b and e).

The four digested extracts at 1% led to similar increases in numbers of total bacteria after 24 h fermentation in comparison to the negative control, which y reported a slight reduction in numbers by 24 h (Fig. 1.f). Furthermore, the four digested extracts at 1%, as well as the negative and positive controls, all led to a

decrease in numbers of the *Clostridium cocoides/E. rectale* group (Fig. 1.d), which is a major anaerobic population in the human gut. Statistically significant decreases ($p<0.05$) were observed for *S. muticum* extract alongside both negative and positive controls for *C. cocoides*/*E. rectal*e.

Bacteroides/Prevotella population (*Bacteroides* group) showed an increase over the 24 h of fermentation for all the four digested extracts, being statistically significant for the *S. muticum* extract and for both extracts of *Ph. nameko* in comparison to the negative control or to the positive FOS which revealed no significant shift in numbers over the 24 h fermentation period (Fig. 1c). It is known that these genera vary greatly with the nature of the diet and while studies have revealed increased proportions of *Bacteroides* in vegetarians (Matijasic, Obermajer, Lipoglavsek, Grabnar, Avgustin, & Rogelj, 2014), or upon ingestion of resistant starch type 4 (Martinez, Kim, Duffy, Schlegel, and Walter, 2010), further studies have detected no alterations in *Bacteroides* upon ingestion of formula diet containing FOS and pea fibre (Benus et al., 2010) or upon blueberry drink consumption (Vendrame, Guglielmetti, Riso, Arioli, Klimis-Zacas, & Porrini, 2011). In contrast, in a study by Vulevic, Juric, Tzortzis, & Gibson (2013), galactooligosaccharide consumption by overweight adults led to a reduction in the *Bacteroides* population. Although *Bacteroides*/*Prevotella* populations increased with the addition of the digested extracts to the medium it is important to correlate shift in population with the production of SCFA. *Bacteroides* and *Prevotella* genera are organisms capable of using a very wide range of substrates and are major producers of propionic acid. As may be seen from data listed in Table 2 and discussed further, propionic production is significant by 24 h fermentation, and of the same order of magnitude, for the positive control FOS and for both the *Ph.*

nameko extracts, yet branched-chain fatty acids are reduced, which is concurrent with a decrease in protein fermentation and of positive influence as far as *Bacteroides* modulation is concerned.

In order to observe if increasing concentrations of the digested enzymatic seaweeds and mushroom extracts would have a higher impact on gut microbiota modulation, similar *in vitro* fermentations were repeated with 2 and 3% (w/v) of extracts of *O. pinnatifida* obtained with Viscozyme and of *Ph. nameko* obtained with Flavourzyme. The selection of these two extracts was based on the best prebiotic potential selectivity effect demonstrated within each group of extracts, seaweeds and mushroom. The respective results are displayed in Figure 2. Interesting results were obtained for *Bifidobacterium* spp., *Lactobacillus* spp. and *C. histolyticum* group (Fig. 2.a, 2.b and 2.e), respectively; - an increase in the concentration of the digested extracts did not bring about a higher impact on *Bifidobacterium* spp. abundance and increases in population numbers were similar between digested extracts and the positive control FOS in comparison to the negative control which registered no alteration over 12 h fermentation; - abundance in the lactobacilli group was significantly increased with 2 and 3% digested extracts in comparison to the digested extracts at 1% fermentation (Fig. 1.b) where no significant increases had been observed for the extracts; - higher increases in lactobacilli populations, although not statistically significant ($p>0.05$), were observed between 0 and 6h for both concentrations of *Ph. nameko* extracts than with FOS at 2% and at 12 h of fermentation similar numbers of cells were observed for both *Ph. nameko* extracts and these were higher than those obtained with FOS 2% and with the negative control; - although the experiments with 2 and 3% *Ph. nameko* extract started with the lowest level of *C. histolyticum*

in the faecal inocula these extracts brought about the only statistical significant decreases of *C. histolyticum* by 12 h ($p=0.0003$ for 2% and $p=0.028$ for 3%, respectively) in comparison to 0 h and in comparison to FOS ($p>0.05$) and negative control ($p>0.05$). Numbers of *C. histolyticum* continued to diminish with higher percentages of *Ph. nameko* Flavourzyme extracts (in comparison to 1% extract) confirms their selectivity properties and prebiotic potential, although this result could be a consequence of the lower pH (non-pH controlled experiment) inhibitory effect on the microbial group, must not be overlooked. At higher percentages cross-feeding may become more predominant and selectivity could in fact be lost, yet this is not the case since *C. histolyticum* showed no proliferation and decreased in numbers.

3.2 Lactic acid and SCFA production

Lactic acid and SCFA, the main products arising from the microbial fermentation of carbohydrates, can provide energy to the colonic epithelium, modulate cholesterol and lipid metabolism, suppress pathogenic intestinal bacteria and modulate the immune system (Salazar, Prieto, Leal, Mayo, Bada-Gancedo, & Madiedo, 2009). Furthermore, they act as electron sinks of anaerobic respiration in the gut and decrease the intestinal pH, thus enhancing the bioavailability of minerals such as calcium and magnesium (Gullon et al., 2014). The concentration of acetic, propionic, butyric, isobutyric and isovaleric acids as well as lactic acid produced during 24 h fermentation of the different extracts added at 1% is shown in Table 2. Significant differences were found between donors with respect to the levels of the different SCFA, in particular with acetic and butyric acids. In some cases the SCFA were detected in only one donor, particularly for propionic,

butyric and branched-chain fatty acids. It is estimated that 90% of SCFA are absorbed and therefore low levels may be found in faeces

, SCFA production in the negative control was the lowest (14.3 mM by 24 h) in comparison with the media containing either the seaweed or the mushroom extracts. The lack of a carbohydrate may ascribe SCFA production in the negative control to protein degradation by putrefactive bacteria (Gullon et al., 2014) or to residual undigestive components within the faeces. The total SCFA concentrations achieved were highest for medium containing the positive control FOS (75.1 mM by 24 h), followed by medium added with the *Ph. nameko* extracts (50.9 and 50.5 mM by 24 h for *Ph. nameko* Flavourzyme extract and *Ph. nameko* Cellulase extract, respectively) and lastly by media added with *O. pinnatifida* Viscozyme extract (26.7 mM) and *S. muticum* Alcalase extract (20.0 mM by 24 h). These observations correlate well with the observed modulation by the associated extracts of SCFA producer bifidobacterial and lactobacilli bacterial populations discussed in the previous section. Importantly, most of the SCFA production occurred during the first 12 h of fermentation. Acetic and propionic acids were the main SCFA produced in all media containing any of the four extracts or FOS. Highest values for both acetic and propionic acids were observed in media containing FOS, yet importantly concentrations were also produced in media containing either of the *Ph. nameko* extracts. Propionic acid concentrations were highest for media containing FOS or the *Ph. nameko* extract obtained with Flavourzyme. These concentrations may be related to the high numbers of *Bacteroides* present or to the presence of specific compounds in the extracts. Broekaert, Courtin, Verbeke, Van de Wiele, Verstraete, & Delcour (2011), associated propionic acid production with the side chains found in

xilooligosaccharides. The acetic-to-propionic ratio increased along fermentation for all substrates except for the *Ph. nameko* Flavourzyme extract, which registered an effective decrease between 12 and 24 h (1.1 to 0.8). Low acetic-to-propionic ratios have been proposed as a positive marker for a hypolipidemic effect consequence of cholesterol biosynthesis inhibition (Salazar, Gueimonde, Hernández-Barranco, Ruas- Madiedo, & de los Reyes-Gavilán, 2008). All tested extracts led to the production of low levels of butyric acid by 24 h fermentation in comparison to the positive control FOS. In general, levels of butyric acid were either similar to those obtained by FOS, as is the case of *O. pinnatifida* extract (average values of 5.9 mM against 6.6 mM FOS) or ca. two-fold lower as for *Ph. nameko* extracts (average values of 3.0 and 2.3 mM); values reported for FOS were however quite moderate in comparison to values reported for other studies (for example 12-24 mM in Gullon et al., 2014) albeit a high variability between donors must be highlighted in this latter case. Related results were reported by Benus et al. (2010) who showed that butyric acid was reduced following the fibre-supplemented diet (FOS and pea fibre).

Concentrations of the branched chain fatty acids, isobutyric and isovaleric acids were either below detection limit or, in many cases, were detected in only one donor.

Lactic acid production was highest when FOS was used as a substrate correlating well with the predominant *Bifidobacterium*/*Lactobacillus* populations. Lactic acid was also produced in the media containing the tested substrates during the first 6 h of fermentation, in contrast to medium with FOS which achieved maximum lactic acid production by 12 h fermentation. Thereafter, lactic acid was consumed independently of the substrate in question. Consumption rate was highest in

media containing FOS. This observation may eventually suggest a cross-feeding mechanism (Gullon et al., 2014).

The differences observed in SCFA and lactic acid production for the four substrates tested tend to indicate that a relationship may exist between physico-chemical properties of extracts and modulation of individual bacterial species and SCFA production in the gut. The four extracts tested displayed different composition (for example, higher content of sugars was observed in extracts obtained with Viscozyme and Cellulase) and structures (less sulphated polysaccharides in *O. pinnatifida* extract or presence of α and β-glycosidic structures such as glucans and glucan-protein complexes in both *Ph. nameko* extracts) some of which may be more accessible for use (Rodrigues et al., 2015,2016). The different enzymatic treatments on the different seaweed or mushroom sources lead to the release of different oligomer residues from the structural and storage polysaccharides making these susceptible to degradation. Similarly, Ramnani et al. (2012) showed that low molecular weight extracts derived from agar and alginate seaweeds were fermentable by gut microbiota leading to important increases in acetate and propionate.

4. Conclusions

All tested extracts had an influence on the composition of human gut microbiota, albeit to different extents. The digested *Ph. nameko* extract obtained with Flavourzyme was found to hinder growth of *C. histolyticum* and growth of *members* of the *C. coccoides–E. rectale* group, while growth of *Bifidobacterium* spp. was enhanced and *Lactobacillus* spp. remained relatively unaffected. This

selective increase in bifidobacteria coupled to a consistent increase in total SCFA and lactic acid production suggest its potential prebiotic character.

Seaweed extracts, in particular that of *O. pinnatifida* obtained with Viscozyme, were fermentable by gut microbiota as indicated by an increase in SCFA. Increase in SCFA was not always correlated with an increase in bacterial populations for the seaweed extracts.

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623

624 **Figure captions**

625 **Figure 1.** Bacterial populations (a) *Bifidobacterium* spp.; b) *Lactobacillus*
626 spp./*Enterococcus* spp.; c) Bacteroides group; d) *C. cocoides*/*E. rectale* group; e)
627 *C. histolyticum* group; f) Total bacteria) analysed by FISH in batch cultures
628 containing 1% (w/v) of digested extracts of seaweeds *O. pinnatifida* obtained by
629 Viscozyme (O.pin_Visc) and *S. muticum* obtained by Alcalase (S.mut_Alc) and of
630 mushroom *Ph. nameko* obtained by Flavourzyme (Ph.nam_Flav) and by Cellulase
631 (Ph.nam_Cell) and the respective controls. Error bars indicate SD of the replicates
632 involving 3 adult donors. $^{a}p<0.05$; significantly different compared to 0h within the
633 same substrate. $^{*}p<0.05$; significantly different compared to negative control.
634 $^{\#}p<0.05$; significantly different compared to FOS, positive control.

635

636 **Figure 2.** Bacterial populations (a) *Bifidobacterium* spp.; b) *Lactobacillus*
637 spp./*Enterococcus* spp.; c) Bacteroides group; d) *C. cocoides*/*E. rectale* group; e)
638 *C. histolyticum* group; f) Total bacteria) analysed by FISH in batch cultures
639 containing 2 and 3% (w/v) of digested extracts of seaweed *O. pinnatifida* obtained

640 by Viscozyme (O.pin_Visc) and of mushroom *Ph. nameko* obtained by
641 Flavourzyme (Ph.nam_Flav) and the respective controls. Error bars indicate SD of
642 the replicates involving 3 adult donors. [a]$p<0.05$; significantly different compared to
643 0h within the same substrate. [*]$p<0.05$; significantly different compared to negative
644 control. [#]$p<0.05$; significantly different compared to 2% FOS, positive control.