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1 ***In vitro* fermentation and prebiotic potential of selected extracts from**

2 **seaweeds and mushrooms**

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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

76 assessment of the fermentability of substrates in the intestinal lumen, whilst

77 simulating the conditions in the human distal colon (Bergillos-Meca, Costabile,

78 Walton, Moreno-Montoro, Ruiz-Bravo, & Ruiz-López, 2015). A growing body of

79 evidence suggests that the gut microbiota impacts on a wide range of host

80 metabolic pathways, barrier function and immune modulatory function influencing

81 the prevention and risk of a wide range of diseases, including inflammatory bowel

82 disease, diarrhoea and colorectal cancer. Much of this impact is mediated through

83 diet and the consumption of specific health-related foods, justifying the constant

84 need to modulate diet or identify compounds that can positively modify the gut

85 microbiota (Gibson, Scott, Rastall, & Tuohy, 2010).

86 Research has been focused on 'prebiotics', and in particular the ability of certain

87 types of dietary fibre, especially indigestible oligosaccharides, to stimulate the

88 growth of and/or activity of beneficial gut bacteria such as bifidobacteria and

89 lactobacilli while retarding the development of *C. histolyticum*, leading to a

90 concomitant positive effect on colonic health (Gibson et al., 2010; Aida, Shuhaimi,

91 Yazid & Maaruf, 2009). Better understanding of the benefits of prebiotics has

92 urged a need to search for and develop new and alternative sources of prebiotics.

93 According to Zaporozhets et al. (2014), the prebiotic activity of extracts or of

94 polysaccharides from marine seaweeds, combined with a broad spectrum of

95 biological properties, evidences great potential for their use as functional nutrition

96 ingredients enabling modulation of intestinal microbiota and of gastrointestinal

97 tract (GIT) inflammation as well as normalization of the immune system. Therefore

98 the main objective in this study was to evaluate the potential of the digested

99 (closely simulating physiological conditions) seaweed and mushroom extracts

100 containing different polysaccharide and oligosaccharide structures on gut

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

106

107 **2. Material and methods**

108 2.1. Selected seaweeds and mushrooms extracts

109 In this study water-based enzyme-assisted extracts from two seaweeds and one
110 mushroom were selected following demonstration of potential prebiotic effect with
111 pure cultures (Rodrigues et al., 2015,2016). Selected extracts included extracts of
112 *S. muticum* obtained with Alcalase, *O. pinnatifida* obtained with Viscozyme and
113 *Ph. nameko* obtained with Cellulase and with Flavourzyme. For each extract, 1g
114 of dried mushroom or 2 g of dry seaweed was dispersed in 50 mL of deionised
115 water and incubated in an agitated water bath for 10 min. After adjusting pH to
116 specific enzyme optimum conditions (Alcalase: pH=8 – 50 ⁰C; Flavourzyme:
117 pH=7.0 – 50 ⁰C; Cellulase: pH=4.5 – 50 ⁰C; Viscozyme® L: pH=4.5 – 50 ⁰C; All
118 enzymes were obtained from Sigma-Aldrich), 100 mg of enzyme was added and
119 incubated for enzymatic hydrolysis for 24h at 50 ⁰C. The enzymatic reaction was
120 stopped by heating the sample at 90-100 ⁰C for 10 min followed by immediate
121 cooling in an ice bath. The pH of enzymatic extracts was adjusted to pH 7.0 with
122 1M HCl and/or NaOH and then centrifuged, filtered and freeze-dried according to
123 Rodrigues et al. (2015, 2016).

124

125 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 $^{\circ}$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 $^{\circ}$C 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 $^{\circ}$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 $^{\circ}$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 $^{\circ}$C 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

Three independent fermentation experiments were carried out. Faecal samples were obtained fresh at the premises of the Department of Food and Nutritional Sciences from 3 apparently healthy adult volunteers who ingested a normal diet, had not ingested any antibiotics for at least 6 months and were not regular consumers of pre or probiotics. Samples were collected into sterile vials and kept in an anaerobic cabinet and used within 30 min of collection. A 1/10 (w/w) dilution in phosphate buffer saline (PBS) was prepared and homogenized using a stomacher (Serward, Worthing, UK) for 2 min at 460 paddle-beats per min.

Sterile stirred batch culture fermentation vessels (50 mL working volume) were set up and aseptically filled with 45 mL sterile, pre-reduced, basal medium [peptone 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, $MgSO_4.7H2O$ 0.01 g/L, $CaCl_2.6H_2O$ 0.01 g/L, $NaHCO_3$ 2 g/L, Tween 80 2 mL/L (BDH, Poole, UK), Hemin 0.05 g/L, vitamin K1 10 µL/L, L-cysteine-HCl 0.5 g/L, bile salts 0.5 g/L, pH 7.0] and gassed overnight with O_2-free N_2 (15 mL/min) with constant agitation. All media and chemicals were purchased from Oxoid (Basingstoke, UK) and Sigma (St Louis, USA). The temperature was kept at 37 °C and pH was controlled between 6.7 and 6.9 using an automated pH controller (Fermac 260, Electrolab, Tewkesbury, UK), which added acid or alkali (0.5 M HCl and 0.5 M NaOH) in order to mimic conditions that resemble the distal region of the human large intestine (Sánchez-Patán et al., 2012).

Six stirred pH-controlled batch fermenters were run in parallel. The different digested extracts (1% w/v) were aseptically added to four vessels, the other two vessels were used as controls. For the positive control vessel 1% (w/v) of FOS (95% oligofructose, 0.5 kDa dextran with 25% α-1,2 branching, 1 kDa dextran with 32% α-1,2 branching; Orafti®*P95,* Oreye, Belgium) was used. To the negative

176 control vessel no source of carbon was added. The experiment was performed in

177 triplicate, using one faecal sample given by a different donor for each run of six

178 batch fermenters. Each vessel, with 45 mL sterile medium and digested extract,

179 was inoculated with 5 mL of fresh faecal slurry (1/10 w/w). The batch cultures

180 were run under anaerobic conditions for a period of 24 h, during which 5 mL

181 samples were collected from each vessel at 0, 6, 12 and 24 h for FISH and

182 analysis of lactic acid and short chain fatty acids (SCFA). For this latter analysis,

183 samples were stored at -70 ºC until required.

184 In order to assess the effect of the addition of 2 and 3% of digested extracts of *O.*

185 *pinnatifida* obtained with Viscozyme and of *Ph. nameko* obtained with

186 Flavourzyme, faecal batch-culture fermentations were repeated, in triplicate,

187 under similar conditions but non-pH controlled and at lower volumes (10 mL) and

188 for a period of 12 h.

189 2.2.3. Bacterial enumeration using FISH.

190 To assess differences in bacterial composition, FISH was used with

191 oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA

192 based on the method described by Daims, Stoecker, & Wagner (2005). A total of

193 6 different probes commercially synthesized and 5'-labelled with the fluorescent

194 dye (Sigma Aldrich, St Louis, USA) were used in addition to an overall stain with

195 4,6-diamidino-2-phenylindole (DAPI), which measures all cells by staining DNA

196 (Harmsen, Wildeboer-Veloo, Grijpstra, Knol, Degener, & Welling, 2000a; Harmsen

197 et al., 2000b).

198 Samples (375 µL) obtained from each vessel and sampling time were fixed for a

199 minimum of 4 h (4 ºC) in 1125 µL 4% (w/v) paraformaldehyde. Fixed cells were

200 centrifuged at 13,000 g for 5 min and washed twice in 1 mL filtered sterilized PBS.

201 The washed cells were re-suspended in 150 µL filtered PBS and stored in 150 µl

202 ethanol (99%) at -20 ºC until further processing. Samples were then diluted in a

203 suitable volume of PBS in order to obtain countable fluorescent cells in each field

204 of view and 20 µl of the above solution was added to each well of a 6 well

205 PTFE/poly-L-lysine coated slide (Tekdon Inc., Myakka City, USA). The samples

206 were dried for 15 min in a drying chamber (46 ºC).

207 To permeabilize cells for use with probes Bif164 and Lab 158 (Table 1), samples

208 were treated with 20 µL of lysozyme at room temperature for 15 min before being

209 washed briefly in water. Slides were dehydrated, using an alcohol series (50, 80

210 and 96% (v/v) ethanol) for 3 min in each solution. Slides were returned to the

211 drying oven for 2 min to evaporate excess ethanol before adding the hybridization

212 mixture to each well [(0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.01% sodium

213 dodecyl sulphate and 4.55 ng/mL probe]. For probes EUB338 I-II-III, the

214 hybridization mixture contained formamide [(0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0),

215 35% formamide, 0.01% sodium dodecyl sulphate and 4.55 ng/mL probe].

216 Hybridization occurred for 4 h in a microarray hybridization incubator (Grant-

217 Boekel, Cambridge, UK). After hybridization, slides were washed in 40 mL

218 washing buffer [0.9 M NaCl and 0.02 M Tris/HCl (pH 8.0)], and 0.005 M

219 ethylenediaminetetraacetic acid for the EUB338 I-II-III probes with 20 µl nucleic

220 acid stain 4', 6-diamidino-2- phenylindole (DAPI; 50 ng/µL) for 15 min. They were

221 then dipped in cold water for a few seconds and dried with compressed air. Five

222 microlitres of polyvinyl alcohol mounting medium with 1,4-

223 diazabicyclo(2,2,2)octane (DABCO) was added onto each well and a cover slip

224 was placed on each slide (20 mm, thickness No 1, VWR, Lutterworth, UK). Slides

225 were examined by epifluorescence microscopy (Eclipse 400, Nikon, Surrey, UK)

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

227 cells were counted.

228 2.2.4. Lactic acid and SCFA analysis

229 Samples were collected from each batch culture at each sampling point (0, 6, 12

230 and 24 h) and frozen at -70 ⁰C until required. Samples were assessed for lactic

231 acid and SCFA (acetic, propionic, butyric, isobutyric and isovaleric acids) using an

232 HPLC apparatus from Merck LaChrom (Fullerton CA, USA), in a single run, based

233 on calibration curves previously prepared with appropriate chromatographic

234 standards; an Aminex HPX-87X cation exchange column from BioRad (Richmond

235 CA, USA) was used for separation; the eluant was pumped at 0.8 mL/min and

236 consisted of 13 mM H_2SO_4 (Merck); and detection was by UV absorbance at 220

237 nm. Prior to analysis, samples were defrosted, centrifuged (13,000 g for 10 min at

238 4 ⁰C) and filtered through a 0.22-µm membrane filter (Millipore, USA) to remove

239 all particulate matter.

240 2.3. Statistical Analysis

241 A paired Student's *t* test was used to test for significant differences in the bacterial

242 group populations between extracts and controls as well as for time *in vitro*

243 fermentation experiments by human gut microbiota. All data of bacterial

244 populations (Log_{10} cell/mL) are expressed as average of three replicates (donors)

245 plus or minus standard deviation, justifying the high variability reported in several

246 cases.

247

248 **3. Results and discussion**

249 3.1. Modulation of intestinal microbiota by seaweed and mushroom digested

250 extracts.

251   Assessment of the prebiotic potential of bioactive compounds or extracts by *in*

252   *vitro* fermentation with human faecal microbiota provides a cost-effective and

253   rapid alternative to assess the fermentation and modulation capacity of different

254   substrates on a laboratory scale comparative basis (Gullon, Gullon, Tavaria,

255   Pintado, Gomes, Alonso, & Parajo, 2014). It is important to note that before

256   performance of *in vitro* fermentations care was taken to submit each extract to

257   simulated gastrointestinal digestion because resistance to gastric acidity and

258   hydrolysis by mammalian enzymes are limiting factors that have to be assured in

259   order to enable the substrate to reach the colon and be fermented by intestinal

260   microbiota, meeting the pre-requisite for a prebiotic effect or gut modulation effect.

261   According to Gibson et al. (2010) any dietary material that is non-digestible and

262   enters the large intestine is a candidate prebiotic. The few studies that have

263   evaluated the prebiotic potential of seaweed polysaccharides using *in vitro*

264   fermentation (laminarin and low molecular weight polysaccharides from agar and

265   alginate) did not undergo previous gastrointestinal digestion, hindering analysis of

266   true effectiveness of intact compounds (Devillé, Gharbi, Dandrifosse, & Peulen,

267   2007; Ramnani et al., 2012). During the experimental time course (0, 6, 12 and 24

268   h) of the *in vitro* fermentation of the digested seaweed and mushroom extracts at

269   1% (w/v) changes in the different bacterial populations and accumulation of lactic

270   acid and SCFA (acetic, propionic, butyric acids) were assessed. For comparative

271   purposes, the same experimental strategy was used with the well-established

272   prebiotic FOS (positive control) and with medium without carbon source present

273   (negative control) (Fig. 1).

274   FISH was used to monitor the modifications among populations of selected

275   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

326 decrease in numbers of the *Clostridium cocoides/E. rectale* group (Fig. 1.d), which

327 is a major anaerobic population in the human gut. Statistically significant

328 decreases ($p < 0.05$) were observed for *S. muticum* extract alongside both negative

329 and positive controls for *C. cocoides/E. rectal*e.

330 *Bacteroides/Prevotella* population (*Bacteroides* group) showed an increase over

331 the 24 h of fermentation for all the four digested extracts, being statistically

332 significant for the *S. muticum* extract and for both extracts of *Ph. nameko* in

333 comparison to the negative control or to the positive FOS which revealed no

334 significant shift in numbers over the 24 h fermentation period (Fig. 1c). It is known

335 that these genera vary greatly with the nature of the diet and while studies have

336 revealed increased proportions of *Bacteroides* in vegetarians (Matijasic,

337 Obermajer, Lipoglavsek, Grabnar, Avgustin, & Rogelj, 2014), or upon ingestion of

338 resistant starch type 4 (Martinez, Kim, Duffy, Schlegel, and Walter, 2010), further

339 studies have detected no alterations in *Bacteroides* upon ingestion of formula diet

340 containing FOS and pea fibre (Benus et al., 2010) or upon blueberry drink

341 consumption (Vendrame, Guglielmetti, Riso, Arioli, Klimis-Zacas, & Porrini, 2011).

342 In contrast, in a study by Vulevic, Juric, Tzortzis, & Gibson (2013),

343 galactooligosaccharide consumption by overweight adults led to a reduction in the

344 *Bacteroides* population. Although *Bacteroides/Prevotella* populations increased

345 with the addition of the digested extracts to the medium it is important to correlate

346 shift in population with the production of SCFA. *Bacteroides* and *Prevotella*

347 genera are organisms capable of using a very wide range of substrates and are

348 major producers of propionic acid. As may be seen from data listed in Table 2 and

349 discussed further, propionic production is significant by 24 h fermentation, and of

350 the same order of magnitude, for the positive control FOS and for both the *Ph.*

351 *nameko* extracts, yet branched-chain fatty acids are reduced, which is concurrent

352 with a decrease in protein fermentation and of positive influence as far as

353 *Bacteroides* modulation is concerned.

354 In order to observe if increasing concentrations of the digested enzymatic

355 seaweeds and mushroom extracts would have a higher impact on gut microbiota

356 modulation, similar *in vitro* fermentations were repeated with 2 and 3% (w/v) of

357 extracts of *O. pinnatifida* obtained with Viscozyme and of *Ph. nameko* obtained

358 with Flavourzyme. The selection of these two extracts was based on the best

359 prebiotic potential selectivity effect demonstrated within each group of extracts,

360 seaweeds and mushroom. The respective results are displayed in Figure 2.

361 Interesting results were obtained for *Bifidobacterium* spp*., Lactobacillus* spp. and

362 *C. histolyticum* group (Fig. 2.a, 2.b and 2.e), respectively; - an increase in the

363 concentration of the digested extracts did not bring about a higher impact on

364 *Bifidobacterium* spp. abundance and increases in population numbers were

365 similar between digested extracts and the positive control FOS in comparison to

366 the negative control which registered no alteration over 12 h fermentation; -

367 abundance in the lactobacilli group was significantly increased with 2 and 3%

368 digested extracts in comparison to the digested extracts at 1% fermentation (Fig.

369 1.b) where no significant increases had been observed for the extracts; - higher

370 increases in lactobacilli populations, although not statistically significant ($p>0.05$),

371 were observed between 0 and 6h for both concentrations of *Ph. nameko* extracts

372 than with FOS at 2% and at 12 h of fermentation similar numbers of cells were

373 observed for both *Ph. nameko* extracts and these were higher than those

374 obtained with FOS 2% and with the negative control; - although the experiments

375 with 2 and 3% *Ph. nameko* extract started with the lowest level of *C. histolyticum*

376 in the faecal inocula these extracts brought about the only statistical significant

377 decreases of *C. histolyticum* by 12 h (p=0.0003 for 2% and p=0.028 for 3%,

378 respectively) in comparison to 0 h and in comparison to FOS (p>0.05) and

379 negative control (p>0.05). Numbers of *C. histolyticum* continued to diminish with

380 higher percentages of *Ph. nameko* Flavourzyme extracts (in comparison to 1%

381 extract) confirms their selectivity properties and prebiotic potential, although this

382 result could be a consequence of the lower pH (non-pH controlled experiment)

383 inhibitory effect on the microbial group, must not be overlooked. At higher

384 percentages cross-feeding may become more predominant and selectivity could

385 in fact be lost, yet this is not the case since *C. histolyticum* showed no proliferation

386 and decreased in numbers.

387 3.2 Lactic acid and SCFA production

388 Lactic acid and SCFA, the main products arising from the microbial fermentation

389 of carbohydrates, can provide energy to the colonic epithelium, modulate

390 cholesterol and lipid metabolism, suppress pathogenic intestinal bacteria and

391 modulate the immune system (Salazar, Prieto, Leal, Mayo, Bada-Gancedo, &

392 Madiedo, 2009). Furthermore, they act as electron sinks of anaerobic respiration

393 in the gut and decrease the intestinal pH, thus enhancing the bioavailability of

394 minerals such as calcium and magnesium (Gullon et al., 2014). The concentration

395 of acetic, propionic, butyric, isobutyric and isovaleric acids as well as lactic acid

396 produced during 24 h fermentation of the different extracts added at 1% is shown

397 in Table 2. Significant differences were found between donors with respect to the

398 levels of the different SCFA, in particular with acetic and butyric acids. In some

399 cases the SCFA were detected in only one donor, particularly for propionic,

400 butyric and branched-chain fatty acids. It is estimated that 90% of SCFA are

401 absorbed and therefore low levels may be found in faeces

402 , SCFA production in the negative control was the lowest (14.3 mM by 24 h) in

403 comparison with the media containing either the seaweed or the mushroom

404 extracts. The lack of a carbohydrate may ascribe SCFA production in the negative

405 control to protein degradation by putrefactive bacteria (Gullon et al., 2014) or to

406 residual undigestive components within the faeces. The total SCFA

407 concentrations achieved were highest for medium containing the positive control

408 FOS (75.1 mM by 24 h), followed by medium added with the *Ph. nameko* extracts

409 (50.9 and 50.5 mM by 24 h for *Ph. nameko* Flavourzyme extract and *Ph. nameko*

410 Cellulase extract, respectively) and lastly by media added with *O. pinnatifida*

411 Viscozyme extract (26.7 mM) and *S. muticum* Alcalase extract (20.0 mM by 24 h).

412 These observations correlate well with the observed modulation by the associated

413 extracts of SCFA producer bifidobacterial and lactobacilli bacterial populations

414 discussed in the previous section. Importantly, most of the SCFA production

415 occurred during the first 12 h of fermentation. Acetic and propionic acids were the

416 main SCFA produced in all media containing any of the four extracts or FOS.

417 Highest values for both acetic and propionic acids were observed in media

418 containing FOS, yet importantly concentrations were also produced in media

419 containing either of the *Ph. nameko* extracts. Propionic acid concentrations were

420 highest for media containing FOS or the *Ph. nameko* extract obtained with

421 Flavourzyme. These concentrations may be related to the high numbers of

422 *Bacteroides* present or to the presence of specific compounds in the extracts.

423 Broekaert, Courtin, Verbeke, Van de Wiele, Verstraete, & Delcour (2011),

424 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

474 selective increase in bifidobacteria coupled to a consistent increase in total SCFA

475 and lactic acid production suggest its potential prebiotic character.

476   Seaweed extracts, in particular that of *O. pinnatifida* obtained with Viscozyme,

477 were fermentable by gut microbiota as indicated by an increase in SCFA.

478 Increase in SCFA was not always correlated with an increase in bacterial

479 populations for the seaweed extracts.

480

492

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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.