

Prebiotic supplementation of In Vitro fecal fermentations inhibits proteolysis by gut bacteria, and host diet shapes gut bacterial metabolism and response to intervention

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1 **Prebiotic supplementation of *in vitro* faecal fermentations inhibits proteolysis by gut**
2 **bacteria and host diet shapes gut bacterial metabolism and response to intervention**

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14 **Abstract**

15

16 Metabolism of protein by gut bacteria is potentially detrimental due to production of toxic
17 metabolites, such as ammonia, amines, *p*-cresol, and indole. Consumption of prebiotic
18 carbohydrates produces specific changes in the composition and/or activity of the microbiota
19 that may confer benefits upon host wellbeing and health. Here, we have studied the impact of
20 prebiotics on proteolysis within the gut *in vitro*.

21 Anaerobic stirred batch cultures were inoculated with omnivore (n=3) and vegetarian (n=3)
22 faeces. Four protein sources (casein, meat, mycoprotein and soy protein) with and without
23 supplementation by a oligofructose enriched-inulin. Bacterial counts, and concentrations of
24 short chain fatty acids (SCFA), ammonia, phenol, indole, and *p*-cresol were monitored during

25 fermentation. Addition of the fructan prebiotic Synergy1 increased levels of bifidobacteria (p=
26 0.000019 and 0.000013 for omnivores and vegetarians respectively). Branched chain fatty
27 acids (BCFA) were significantly lower in fermenters with vegetarians' faeces (p=0.004),
28 reduced further by prebiotic treatment. Ammonia production was lower with Synergy1.
29 Bacterial adaptation to different dietary protein sources was observed through different patterns
30 of ammonia production between vegetarians and omnivores. In volunteer samples with high
31 baseline levels of phenol, indole, *p*-cresol and skatole, Synergy1 fermentation led to a reduction
32 of these compounds.

33

34 **Importance:** Dietary protein intake is high in Western populations which could result in
35 potentially harmful metabolites in the gut from proteolysis. In an *in vitro* fermentation model,
36 addition of prebiotics reduced the negative consequences of high protein levels.
37 Supplementation with a prebiotic resulted in a reduction of proteolytic metabolites in the model.
38 A difference was seen in protein fermentation between omnivore and vegetarian gut
39 microbiotas: bacteria from vegetarian donors grew more on soy and QuornTM, than on meat
40 and casein with reduced ammonia production. Bacteria from vegetarian donors produced less
41 BCFA.

42 **Introduction**

43

44 Dietary protein levels in western European populations can be as high as 105g/d according to
45 the Food and Agriculture Organization (1). However, the recommended dietary allowance
46 (RDA) is 56g/d for men and 46g/d for women (2). This may result in high residual colonic
47 nitrogen, with dietary protein having escaped digestion in the upper intestine entering the large
48 gut where it can become a substrate for the colonic microbiota. Approximately 16g of protein

49 will be present in the colon following ingestion of 105g protein/day of which 8g are
50 endogenous and 8g are exogenous (3, 4). Among the endogenous proteins, there are 69.2%
51 bacterial proteins, 16.9% mucin, 7.65% enzymes, and 6.2% mucosal cells (5, 6).

52

53 Anaerobic metabolism of carbohydrate by gut bacteria produces short chain fatty acids
54 (SCFA), and gases from different pathways. Production of SCFA, mainly acetate, propionate,
55 and butyrate, in the lumen is generally believed to mediate health benefits such as maintaining
56 colonic epithelial cell function, regulate energy intake and satiety, controlling inflammation,
57 and defend pathogen invasion (7). Microbial breakdown of protein not only generates SCFA
58 and gases, however, but also ammonia, amines, indolic and phenolic compounds, and branched
59 chain fatty acids (BCFA) through the deamination and decarboxylation of amino acids (8).
60 Though evidence on humans is scarce, in studies in rats and in *ex vivo* studies, ammonia at a
61 physiologically relevant dose can harm colon barrier function, shorten colonocyte lifespan, and
62 is co-carcinogenic in rats (9-11). Hydrogen sulphide can be produced from sulphur containing
63 amino acids and is toxic to colonocytes, damaging DNA and blocking utilisation of butyrate as
64 an energy source (12-15). Metabolism of tyrosine, phenylalanine and tryptophan produces
65 phenol, indole, *p*-cresol and skatole which are potential carcinogens; phenol and *p*-cresol can
66 reduce intestinal epithelial barrier function *in vitro* (10, 16, 17). BCFAs are generated from
67 branched chain amino acids such as valine, leucine, and iso-leucine which make them
68 biomarkers for bacterial proteolysis, however there are no human physiological roles for
69 BCFAs known (18).

70

71 Thus, foods entering the colon can have a health impact on the host, possibly by changing gut
72 microbiota composition and activity. The International Agency for Research on Cancer (19),
73 an agency under the World Health Organization (WHO) published a press release in October

74 2015: where it classified red meat as “probably carcinogenic to humans”, and processed meat
75 as “carcinogenic to humans”, with concerns over colorectal cancer (19). Some epidemiological
76 studies found reduced risk of colorectal cancer (CRC) with high consumption of dietary fibre,
77 while red meat and processed meat had a positive correlation with CRC (20-23). Animal
78 protein intake was associated with increased inflammatory bowel disease (IBD) risk in two
79 Japanese and French studies (24, 25).

80

81 Increased consumption of prebiotics, which can reach the colon resulting in specific changes
82 in the composition and/or activity in the gastrointestinal microflora, may counter the negative
83 effects of gut microbial proteolysis in persons ingesting high protein diets (26). Inulin-type
84 fructans can resist hydrolytic enzymes in the human GI tract and are resistant to small intestinal
85 absorption, subsequently they become a substrate source for the microbiota within the large
86 intestine. The impact of inulin on the gut microbiome has been studied using *in vitro* and *in*
87 *vivo* approaches (27-29). The aim of this study was to understand metabolism of gut bacteria
88 proteolysis in the distal colon and how prebiotics can affect the proteolysis, therefore, to
89 investigate the potential of consuming prebiotics to counteract the negative effect of having
90 high protein diet.

91 **Results**

92 **Bacterial Enumeration**

93

94 Total bacteria and most microbial groups that were monitored in this study reached the highest
95 number after 24 hours incubation. However lactobacilli, *Faecalibacterium prausnitzii* and
96 *Roseburia* numbers only increased in the first 10 hours with lactobacilli numbers in particular
97 declining after 10 hours. Bacterial populations from omnivores and vegetarians responded

98 differently to the proteins: faecal bacteria from omnivores had insignificant higher counts on
99 meat and casein than on soy protein and Quorn™ extract, while faecal bacteria from
100 vegetarians had higher counts on soy protein and Quorn™ extract ($8.75 \pm 0.40 \log_{10}$ CFU/ml)
101 than meat and casein ($8.38 \pm 0.47 \log_{10}$ CFU/ml) ($p=0.03$).

102 The vegetarian microbiota had higher bifidobacteria and lactobacilli counts at the beginning
103 compared to omnivore microbiota (Supplementary Tables 1 and 2).

104

105 In order to investigate proteolytic bacteria, independent *t* tests were performed to compare
106 samples with protein addition (casein, meat, mycoprotein and soy protein) and controls at 24
107 and 48 hours (Figure 1 and 2). Though there are studies confirming that many *Bacteroides*.
108 spp., are proteolytic (30), we found no significant changes in *Bacteroides*. spp. on protein
109 substrates. *Clostridium coccooides*, *Eubacterium rectale* and *Clostridium cluster XIVa* and
110 *XIVb* grew on protein substrates: bacteria from omnivore donors had higher counts comparing
111 to the control group ($p=0.055$) while those from vegetarian donors were significantly higher
112 ($p<0.01$). *Roseburia* number did not change with protein added. *Atopobium* cluster from both
113 omnivore and vegetarian donors grew on protein substrates with statistical significance.
114 Clostridial cluster IX populations in cultures inoculated with samples from vegetarian donors
115 increased on the protein substrates significantly, while cultures with omnivore samples were
116 not statistically different. Lower counts of clostridial cluster IX in vegetarian donors' controls
117 could explain the difference. *Desulfovibrio* counts were significantly higher with protein from
118 both omnivore and vegetarian donors. *Clostridium* clusters I and II also grew more on proteins
119 however, growth only reached statistical significance with inocula from vegetarians.

120

121 To investigate how prebiotics may modify the microbiota, independent *t* tests were used to
122 compare cultures with prebiotics and without, after 24 and 48 hours fermentation (Figure 1 and

123 2). Synergy1 addition significantly boosted the growth of total bacteria, bacteroides, clostridial
124 cluster IX, bifidobacteria, and lactobacilli with both omnivore and vegetarian inocula, with
125 bifidobacteria displaying the highest growth on Synergy1. In cultures with vegetarian donor'
126 samples, *Clostridium coccoides*, *Eubacterium rectale* and *Clostridium cluster XIVa* and *XIVb*,
127 *Roseburia*, *Faecalibacterium prausnitzii*, and *Atopobium* also had significant higher count with
128 prebiotics than without. There were no inhibitory effects of prebiotics found on any of bacterial
129 groups monitored in this study.

130 **Organic Acids**

131

132 Most organic acids accumulated during fermentation and reached their highest concentrations
133 at 24 or 48 hours fermentation, with the exception of lactate which transiently increased during
134 the first 10 hours then gradually decreased to below 1mM at 48 hours. Branched amino acids
135 such as leucine and isoleucine can be metabolised by faecal bacteria to produce BCFA
136 indicating proteolytic fermentation. Omnivores had higher BCFA production ($4.03\pm 5.25\text{mM}$)
137 while vegetarians had little production ($1.61\pm 1.60\text{mM}$) ($p=0.004$). For instance, while growing
138 on casein, bacteria from omnivores produced 10.19 ± 8.62 and 13.13 ± 10.93 mM of isobutyrate
139 and isovalerate respectively, while bacteria from vegetarians produced 2.03 ± 2.16 and
140 3.52 ± 3.29 mM of isobutyrate and isovalerate (Supplementary Table 1 and 2).

141 Comparing samples with protein and without at 24 and 48 hours, cultures inoculated with both
142 omnivore and vegetarian donors had significantly higher concentrations of acetate, propionate,
143 isobutyrate, butyrate, and isovalerate on protein (Figure 3 and 4). However, fermentation
144 samples with prebiotics had significantly elevated concentration of acetate and succinate at 24
145 and 48 hours, and significantly more lactate at 6 and 10 hours (Figure 3 and 5).

146

147 Butyric acid production was low in this study and no changes were found in cultures with

148 omnivores samples; this correlates with the lack of differences in populations of butyrate-
149 producing bacteria (*Roseburia* and *Faecalibacterium prausnitzii*). In samples with vegetarian
150 donors' inocula, butyrate producers (*Clostridium coccooides*, *Eubacterium rectale* and
151 *Clostridium cluster XIVa* and *XIVb*, *Roseburia*, *Faecalibacterium prausnitzii*) had
152 significantly higher counts, however, butyrate production was not significantly increased.
153 Concentrations of BCFA were lower on prebiotics although without statistical significance.
154 Variation in BCFA production between donors was seen in this study, therefore, two-way
155 ANOVA on isovalerate and isobutyrate was used to examine the effect of both treatment and
156 donor on production. A significant influence of donor on isobutyrate and isovalerate was found
157 with six donors ($p < 0.01$). Donor variation may indicate that a larger sample size is needed to
158 observe the inhibitory effect of prebiotics on BCFA production. (Supplementary Table 3)

159 **Volatile Organic Compounds**

160

161 This study quantified four potentially detrimental volatile organic compounds (VOCs) which
162 were indole, phenol, *p*-cresol and skatole. Production of these compounds varied with
163 individual donor and the effect of prebiotics on VOCs production also varied according to
164 donor diet. Production of VOCs, from highest to lowest, was indole, phenol, *p*-cresol and
165 skatole in most cases. However, with soy protein, phenol production was higher than indole
166 production. With all donors, comparing negative and positive controls, the production of
167 volatile compounds was reduced by Synergy1. However, comparing cultures on
168 protein+Synergy1 with cultures on the corresponding protein, production of indole, phenol, *p*-
169 cresol and skatole were inhibited by Synergy1 after 48 hours fermentation with inocula from
170 omnivore donor 1, omnivore donor 2 and vegetarian donor 1. These three donors produced
171 relatively high levels of phenol and indole on protein ($292.20 \pm 521.76 \mu\text{g/ml}$) compared with
172 others ($28.92 \pm 23.61 \mu\text{g/ml}$) ($p = 0.02$). Fermentation models inoculated with these high VOCs

173 producers, Synergy1+protein models produced significantly less phenol and indole
174 ($113.21 \pm 227.94 \mu\text{g/ml}$) ($p=0.046$).

175 Protein source affected production of VOCs. According to this study, casein resulted in the
176 highest concentration of VOCs in five donors, this was probably because casein is high in
177 aromatic amino acids which are the main substrates for bacteria to produce phenolic and indolic
178 compounds. Omnivore donor 3 had low phenolic production from casein correlating with this
179 donor's low total bacterial count (Supplementary Table 4).

180 **Ammonia**

181

182 Ammonia is a major metabolite of protein fermentation by faecal bacteria. Ammonia
183 concentrations increased gradually during fermentation on all substrates together with the
184 negative control when compared to the positive control. Ammonia concentrations on Synergy1,
185 however, remained at low levels ($17.55 \pm 4.53\text{mM}$ at 48 hours for omnivores and
186 $25.47 \pm 4.55\text{mM}$ for vegetarians) compared to all protein treatments in this study. The volunteer
187 diet also influenced the selective fermentation of faecal substrates. With faecal samples from
188 omnivores, fermentation resulted in higher ammonia levels on casein and meat extract,
189 however, with faecal samples from vegetarians, soy protein and Quorn extract produced more
190 ammonia (Figure 6).

191

192 Fermentation on protein for 24 hours resulted in significantly higher concentrations of
193 ammonia compared to fermentation without protein using both omnivore and vegetarian
194 samples ($p<0.001$). Fermentation on prebiotics resulted in significantly lower concentrations
195 of ammonia in cultures with omnivore donors' faecal bacteria (Table 1).

196 **Discussion**

197 Lactate production peaked at 10hours fermentation while other organic acids concentrations
198 kept increasing. This coincided with counts of lactobacilli and was to be expected as lactate
199 can be utilised by several bacteria to produce other SCFAs. Changes in propionic acid
200 producing *Bacteroides* and *Clostridium cluster IX* populations were seen and propionic acid
201 increased in vessels containing Synergy1 with the difference reaching significance with
202 omnivore donors' samples ($p=0.006$). Succinate is an intermediate product for propionate
203 production, the succinate pathway being widely present in bacteroides (31). The significantly
204 higher levels of succinate in samples with Synergy1 could be associated with propionate
205 production by bacteroides.

206

207 Faecal bacteria responded differently on various substrates in pH controlled stirred batch
208 cultures. Total bacteria number from vegetarians were significantly more on soy protein and
209 Quorn™ than meat and casein. Host dietary habits may explain a preference for different
210 protein sources. Growth of proteolytic bacteria from the human gut supported this: *Clostridium*
211 *coccoides* and *Eubacterium rectale* from omnivore microbiota and vegetarian microbiota grew
212 on meat/casein and soy/Quorn™ respectively (Supplementary Tables 1 and 2). Ammonia
213 concentrations also indicate that an omnivore microbiota and a vegetarian microbiota favour
214 different protein sources based on their host diet. A possible reason is differences in amino acid
215 composition among various proteins: bacteria that have adapted to the host diet can breakdown
216 peptides, metabolise amino acids or utilise coupled Stickland amino acid fermentation.

217 By observing fermentation characteristics of the negative controls: saccharolytic
218 bifidobacterial growth at 6 hours with omnivore faeces occurred, indicating that there was a
219 small amount of undigested saccharides within the omnivore faecal sample. However, this was
220 not seen from the vegetarian donors.

221 Even when total bacteria tend to be more saccharolytic, there were some proteolytic bacteria
222 present in the gut microbiota. The genus *Clostridium* contains more than 100 species and these
223 bacteria can be saccharolytic, proteolytic, or both. Within clostridial clusters I and II, there are
224 saccharolytic species such as *C. butyricum* and *C. beijerinckii*; *C. sporogenes* and *C.*
225 *acetobutylicum* are both saccharolytic and proteolytic; there are proteolytic species such as *C.*
226 *limosum* and *C. histolyticum* (32). This might explain why *Clostridium* spp. grew on prebiotics
227 with a vegetarian microbiota: saccharolytic types from this genus were likely to be stimulated
228 by prebiotics. This would also imply that these faecal bacteria from vegetarians are more
229 saccharolytic than clostridia from omnivore donors.

230

231 Vegetarian donor 1 had the highest production of phenolic and indolic compounds together
232 with the highest *E. coli* population which correlate with the ability of *E. coli* to produce
233 phenolic compounds (33). Indole and *p*-cresol are conjugated as indoxyl sulphate and *p*-cresol
234 sulphate in the human body; before they are excreted via urine, they are toxic to human
235 endothelial cells, can reflect the progression of chronic kidney diseases, and increase
236 cardiovascular disease risk for such patients (34-37). Therefore, reduced production of indole
237 and *p*-cresol can benefit human health in many ways.

238

239 Studies feeding rats with different protein sources did not find higher colonic toxicity of casein
240 comparing with soybean, which is contrary to the phenol and *p*-cresol results in this study (38,
241 39). Feeding red meat gave higher DNA damage than feeding casein in rats (40). Similar effects
242 were found in human epidemiological research: dairy products were inversely correlated with
243 colorectal cancer in Finnish men and New York University women; they speculated that this
244 protective effect may result from other nutrients in the dairy products but not from
245 macronutrients such as protein (41, 42). Mycoprotein is a relatively new protein source from

246 the filamentous fungus *Fusarium venenatum* source under the trade mark of Quorn™ (43).
247 Quorn™ products contain all the essential amino acids, are low in fat and high in dietary fibre.
248 However, in terms of protein fermentation by gut microbiota, Quorn™ was no different to other
249 proteins.

250 The use of pH controlled stirred batch culture systems allowed rapid analysis of different
251 protein fermentations by gut microbiota and the impact of prebiotics. This fermentation system
252 is limited however: SCFAs would be absorbed from the human colon and the digesta supply
253 would be continuous.

254

255 Some animal studies and human studies have revealed an inhibitory effect of proteolysis by
256 prebiotics such as resistant starch, FOS, and XOS (44-49). These were investigated by
257 analysing indolic/phenolic compounds, or nitrogen secretion in the urine and faeces. One of
258 these studies also compared DNA damage with and without resistant starch in rat colonic cells,
259 and found that the starch protected cells from DNA damage (46). One possible mechanism of
260 decreased proteolytic fermentation in the presence of prebiotics is through the enhanced growth
261 of saccharolytic bacteria requiring more amino acids for growth, reducing amino acid
262 availability for proteolytic bacteria.

263

264 Differences between the gut microbiotas from vegetarian and omnivore donors are not clear
265 with three donors, however, fermentation patterns on different substrates were seen in this study
266 such as the differences in BCFA, ammonia, and total bacteria. In terms of protein fermentation
267 by faecal bacteria, based on the different ammonia production and bacteria growth response to
268 different protein source: microbiota from vegetarian donors have adapted to vegetarian protein
269 sources and can utilise these proteins more efficiently. In addition, in this study, lower BCFA
270 production was found with vegetarians' gut bacteria; this could suggest that these donors had

271 lower branched chain amino acids in their diet. Prebiotic supplementation lowered proteolytic
272 metabolites more in cultures with omnivores' samples comparing to cultures with vegetarians'
273 bacteria: vegetarian donors are more likely to be on a high fibre diet and may need a higher
274 dose of Synergy1 to see a prebiotic effect (50).

275

276 Addition of Synergy1 at the beginning to 48 hour batch culture fermentation changed the
277 microbiota to a more saccharolytic nature by stimulation of bifidobacteria and lactobacilli
278 without a significant change of *Clostridium* and *E. coli*. Supplementation with Synergy1 also
279 reduced the concentration of protein metabolites (ammonia with significance and BCFA but
280 not reaching significance); in those donors with high production of VOCs, inhibition was also
281 found with Synergy1. An inulin rich diet could be beneficial in individuals with high protein
282 diet, however, this effective dose of inulin is relatively difficult to achieve, especially in people
283 consuming a Western diet (51, 52). Therefore, adding fructan prebiotics could potentially
284 reduce the negative consequences of ingesting high protein diets, although this would need to
285 be demonstrated *in vivo*. EFSA have approved the use of chicory inulin at a dosage of 12g per
286 day to maintain normal bowel function, however, the effective doses of prebiotics to regulate
287 bacterial proteolysis is unknown (53). In this study, 5g of inulin type fructans were effective *in*
288 *vitro*, but production of metabolites such as phenol and indole was only inhibited in some of
289 the donors. This needs to be validated *in vivo* and a higher dose might have a better inhibitory
290 effect and cover more of the population. This study also revealed the importance of host
291 habitual diet on the metabolic function of human gut microbiome. This infers that host diet
292 shapes the gut bacteria in a profound way. The individual difference is significant which again
293 could due to individual diet difference.

294 **Materials**

295 **Proteins**

296 Protein substrates used were casein hydrolysate (Sigma-Aldrich, Poole, UK, meat extract for
297 microbiology (Sigma-Aldrich, Poole, UK), soy protein acid hydrolysate powder (Sigma-
298 Aldrich, Poole, UK), and mycoprotein which was extracted from a commercial product
299 (Quorn™) purchased from a local supermarket.

300 **Prebiotic**

301 Inulin-type fructan was a mixture of oligofructose and inulin: 50%±10% DP (degree of
302 polymerisation) 3-9 and 50%±10% DP≥10 (Orafti®Synergy 1, BENEIO-Orafti, Tienen,
303 Belgium).

304 **Methods**

305 **Protein extraction**

306 Mycoproteins were extracted from Quorn™ based on the method described by R. J. H.
307 Williams et al. (54). Quorn™ mince (500g) was mixed with 1200ml water and then
308 homogenised in a blender. 60ml of formic acid was added after homogenisation and the pH
309 lowered to 1.6. Afterwards, 5g pepsin was added and the solution incubated at 37°C for 48
310 hours. Samples were centrifuged at 3000g for 15 minutes and the supernatants freeze-dried for
311 later use. After extraction, the nitrogen content of mycoproteins was quantified using the
312 Kjeldahl method (Campden BRI, UK) and was found to be 10.3%. The remaining mycoprotein
313 was stored at -20°C.

314 **Protein dose determination**

315 Based on previous validation work from *in vitro* batch culture experiments and in human trials,
316 the dose of 1% of substrate (w/v) equates to 5g inulin reaching the colon (27, 55). Synergy1

317 (1% w/v) was used in this study to investigate the prebiotic effect. The approach used in a
318 150ml batch culture experiments to simulate high protein ingestion is shown in Table 2. The
319 amount of casein, meat extract, mycoprotein and soy protein was adjusted based on their true
320 protein content which is shown in Table 3.

321

322 *In vitro* batch culture fermentation

323

324 **Faecal Sample Preparation**

325 Ethical approval of collecting faecal samples from healthy volunteers was obtained from
326 University of Reading University Research Ethics Committee in 2014. Faecal samples were
327 obtained from three healthy meat eating individuals and three healthy vegetarian volunteers
328 between the ages of 18 and 60 (vegetarians 34.44 ± 6.03 years old and omnivores 29.33 ± 3.06)
329 who had not taken antibiotics for at least six months prior to the experiment and had no history
330 of gastrointestinal disorders. None were taking prebiotic supplements. All volunteers were
331 following their diet for at least 5 years.

332 Faecal samples were diluted 1 in 10 (w/v) using 1M, pH7.4, anaerobically prepared phosphate
333 buffered saline (PBS, Oxoid, Hampshire, UK). This solution was homogenised in a stomacher
334 (Seward, stomacher 80, Biomaster) for 120 seconds at normal speed. 15mL of this was then
335 immediately used to inoculate batch culture vessels.

336 **Batch Culture Basal Nutrient Medium.**

337

338 Basal nutrient medium was prepared with chemicals obtained from Sigma-Aldrich, Poole, UK
339 unless otherwise stated. In one litre: 2g peptone water, 2g yeast extract (Oxoid, Hampshire,
340 UK), 0.1g NaCl, 0.04g K_2HPO_4 (BDH, Poole, UK), 0.04g KH_2PO_4 (BDH), 0.01g

341 MgSO₃.7H₂O (Fischer scientific, Loughborough, UK), 0.01g CaCl₂.6H₂O, 2g NaHCO₃
342 (Fischer), 0.5g L-cystine HCl, 2mL Tween 80, 10µL vitamin K1, 0.05g haemin, 0.05g bile
343 salts (Oxoid), 4ml resazurin (pH7).

344

345 **pH controlled, stirred batch culture fermentation**

346

347 Vessels with an operating volume of 300mL were set up. 135mL of basal nutrient medium was
348 autoclaved (121°C for 15 minutes) and aseptically poured into sterile vessels. This system was
349 left overnight with oxygen free nitrogen sparging into the medium at a rate of 15mL/min. After
350 4 hours of fermentation, nitrogen flow was stopped and gas outlets were clamped to trap gas.
351 pH meters (Electrolab pH controller, Tewksbury, UK) were connected to each vessel to regulate
352 pH 6.7 to 6.9 with the aid of 0.5M HCl or NaOH.

353

354 Each vessel was also temperature controlled at 37°C and stirred using a magnetic stirrer.
355 Prebiotic and relative protein treatment were added to the vessels prior to inoculation with
356 15mL of faecal inoculum. For each donor, 10 vessels were prepared for 10 treatments: casein,
357 meat extract, Quorn, soy protein, casein+Synergy1, meat extract+Synergy1, Quorn+Synergy1,
358 soy protein+Synergy1, Synergy1, and a negative control.

359

360 Samples were removed from the fermenters after 0, 6, 10, 24 and 48 hours incubation.

361 **Enumeration of faecal microbial populations by flow cytometry fluorescence *in situ*** 362 **hybridisation (FISH)**

363

364 A 750µl sample of batch culture fluid was centrifuged at 11337 × g for 5 minutes and the
365 supernatant discarded. The pellet was then suspended in 375µl filtered 0.1M PBS solution.

366 Filtered cold (4°C) 4% paraformaldehyde (PFA) (1125µl) was added and samples were stored
367 at 4°C for 4 hours. These were then washed thoroughly with PBS to remove PFA and re-
368 suspended in a mixture containing 300 µl PBS and 300 µl 99% ethanol. Samples were then
369 stored at -20°C prior to FISH analysis by flow cytometry. Filtered cold (4°C) 0.1M PBS (500
370 µl) was mixed with fixed samples (75µl), before centrifuged at $11337 \times g$ for 3 minutes. The
371 pellets were then resuspended in 100µl of TE-FISH (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8,
372 and filtered distilled water with the ratio of 1:1:8) containing lysozyme solution (1 mg/ml of
373 50,000 U/mg protein). Samples were then incubated in the dark at the room temperature for
374 10 minutes, and then centrifuged at $11337 \times g$ for 3 minutes. Pellets were washed with 500µl
375 filtered cold PBS, and then washed with 150µl hybridisation buffer (5 M NaCl, 1 M Tris/HCl
376 pH 8, formamide, ddH₂O, 10% SDS with the ratio of 180:20:300:499:1) and centrifuged at
377 $11337 \times g$ for 3 minutes. Pellets were then resuspended in 1ml of hybridisation buffer.
378 Aliquots (50µl) with 4µl of different probes (50 ng µl⁻¹) were incubated at 35°C for at least
379 10 hours. The probes used in this study are listed in Table 7. Non Eub, Eub338-I-II-III are
380 attached with fluorescence Alexa488 at the 5' end, and other specific probes are attached with
381 Alexa647. A set of Non Eub, Eub338-I-II-III are attached with fluorescence Alexa647 at the
382 5' end to be the controls. For samples to detect specific groups, 4µl of Eub338-I-II-III were
383 added together with 4µl specific probes. Hybridisation buffer (150µl) was added to each
384 aliquot after incubation, followed by 3 minutes centrifugation at $11337 \times g$. Supernatants
385 (150µl) were carefully removed before samples were centrifuged at $11337 \times g$ for 3 minutes.
386 Remaining supernatant was then removed, and pellets were resuspended in 200µl washing
387 buffer. Washing buffer was prepared as: 12.8µl of 5M Na Cl, 20µl of 1M Tris/HCl pH 8, 10µl
388 of 0.5 M EDTA pH 8, and 1µl of 10 % SDS in 956.2µl of filtered cold distilled water.
389 Samples were then incubated at 37°C for 20 minutes and centrifuged at $11337 \times g$ for 3
390 minutes. After supernatant removal, pellets were resuspended in different volume of filtered

391 cold PBS based on flow cytometry load. Bacteria counts were then calculated with the
392 consideration of flow cytometry reading and PBS dilution.

393 **Short chain fatty acid (SCFA) analysis by gas chromatography**

394 Samples were centrifuged at $11337 \times g$ for 10 minutes to remove all particulate matter.
395 Supernatants were then filtered through a $0.2 \mu\text{m}$ polycarbonate syringe filter (VWR,
396 Farlington, UK). Extraction was done with some modifications of a method from A. J.
397 Richardson et al. (69). Filtered sample ($500\mu\text{l}$) was transferred into a labelled $100 \text{ mm} \times 16 \text{ mm}$
398 glass tube (International Scientific Supplies Ltd, Bradford, England) with $25 \mu\text{l}$ of 2-
399 ethylbutyric acid (0.1 M , internal standard) (Sigma, Poole, UK). Concentrated HCl ($250\mu\text{l}$) and
400 1 ml diethyl ether were added to each glass tube and samples vortexed for 1 minute. Samples
401 were then centrifuged at $2000 \times g$ for 10 minutes. The diethyl ether (upper) layer of each sample
402 was transferred to a labelled clean glass tube. A second extraction was conducted by adding
403 another 0.5 ml diethyl ether, followed by vortexing and centrifugation. The diethyl ether layers
404 were pooled. Pooled ether extract ($400\mu\text{l}$) and $50 \mu\text{l}$ N-(tert-butyldimethylsilyl)-N-
405 methyltrifluoroacetamide (MTBSTFA) (Sigma-Aldrich, Poole, UK) were added into a GC
406 screw-cap vial. Samples were left at room temperature for 72 hours to allow lactic acid in the
407 samples to completely derivatise.

408
409 An Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS
410 $30\text{m} \times 0.25\text{mm}$ column with a $0.25\mu\text{m}$ coating (Crosslinked (5%-phenyl)-methylpolysiloxane,
411 Hewlett Packard, UK) was used for analysis of SCFA. Temperatures of injector and detector
412 were 275°C , with the column programmed from 63°C for 0 minutes to 190°C at $15^\circ\text{C min}^{-1}$
413 and held at 190°C for 3 minutes. Helium was the carrier gas (flow rate 1.7 ml min^{-1} ; head
414 pressure 133 KPa). A split ratio of 100:1 was used. Quantification of the samples was obtained
415 through calibration curves of lactic acid and acetic, propionic, butyric, valeric and branched

416 SCFA (iso-butyric and iso-valeric) in concentrations between 12.5 and 100 mM.

417

418 **Volatile organic compounds analysis by GC-MS**

419 **Entrapment of volatile compounds**

420

421 All fermentation samples were adjusted to a pH of 7.0 ± 0.3 using hydrochloric acid or sodium
422 chloride prior to volatile entrapment. Each sample (1 g) was placed in a 250mL conical flask
423 fitted with a Dreschel head. The flask was placed in a water bath maintained at a temperature
424 of 30°C for 1 hour. The flask was attached to oxygen-free nitrogen (40mL/min) which swept
425 volatile compounds from the headspace above the sample onto a glass trap (4 mm i.d., 6.35
426 mm o.d. x 90 mm long), containing 85mg of Tenax TA poly (a porous polymer absorbent based
427 on 2,6-diphenylene-oxide) (Supelco, Poole, UK). Following volatile extraction, 1 μ L of 1, 2
428 dichlorobenzene in methanol (653ng/ μ L) was added to each trap as an internal standard and
429 the trap was then flushed with oxygen free nitrogen to remove moisture (100mL/min) for 10
430 minutes.

431 **Gas Chromatography and Mass spectrometry (GC-MS)**

432

433 Volatile compounds collected on the Tenax adsorbent were analysed using a Perkin-Elmer
434 Claris 500 GC-MS, attached to an automated thermal desorber (Turbomatrix ATD, Perkin
435 Elmer, Beaconsfield, UK). Tenax traps were desorbed at 300°C for 10 min and the volatiles
436 cryofocused on the internal cold trap held at -30°C. After desorption, the cold trap was heated
437 to 300 °C at 40°C per second to release volatile material onto the GC column. GC separation
438 was carried out on a DB-5 non-polar column (60m x 0.32mm id, 1 μ m film thickness, J&W
439 Scientific from Agilent). Helium at 145 kPa was used as the carrier gas. The GC oven

440 temperature program was 2min at 40°C followed by an increase at 4°C/min up to 260°C, where
441 it was held for 10 min. All data were collected and stored using Turbomax software (version
442 3.5, Perkin Elmer). Compounds were identified from their mass spectra and identities
443 confirmed by comparison of retention time (linear retention index, LRI) and mass spectra with
444 those of authentic compounds analysed in online library database. Analyses were carried out
445 using Agilent 6890/5975 GC-MS system (Agilent Technologies, Palo Alto, CA, USA) fitted
446 with a Turbomatrix ATD.

447 Indole, *p*-cresol and phenol (Sigma-Aldrich, Poole, UK) were diluted using the same internal
448 standard which was 1, 2 dichlorobenzene in methanol (653ng/μL). Quantification of the
449 samples was obtained through calibration curves of phenol, *p*-cresol, indole and skatole in
450 concentrations between 25 and 100 μg/ml.

451

452 **Ammonia Analysis**

453 Samples at 0, 10 and 24 hours were diluted 1 in 50 v/v prior to analysis. Ammonia concentration
454 of diluted fermentation samples was analysed using a FluoroSELECT™ ammonia kit (Sigma-
455 Aldrich, Poole, UK). Reagent was prepared by combining 100 μL assay buffer, 4 μL reagent A
456 and 4 μL reagent B in the kit. 10 μL H₂O (blank) and 10 μL sample was added to each glass
457 vial. Afterwards, 100 μL reagent was added to each tube. Samples were kept in the dark for 15
458 minutes at room temperature before they were read in the fluorometer. Ammonia standards
459 were prepared by diluting 20 mmol/L NH₄Cl in distilled water and the concentration range was
460 0.25-1 mmol/L).

461 **Statistical analysis**

462 All statistical tests were performed with the use of IBM SPSS Statistics version 24 (IBM Corp,
463 US). Results are presented as means ± SD. Changes in specific bacterial groups, organic acids,
464 and ammonia were assessed among different treatments and time points using two-way

465 ANOVA. Significant differences were assessed by *post hoc* Tukey HSD test. In addition, to
466 monitor the influence of protein and prebiotics independent *t* tests were used for all variables.
467 For branched chain fatty acid and ammonia, two-way ANOVA was used to assess treatment
468 effect and donor difference.

469

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472

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475

476 We declare that there is no conflict of interest.

477 **Footnotes**

478

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480

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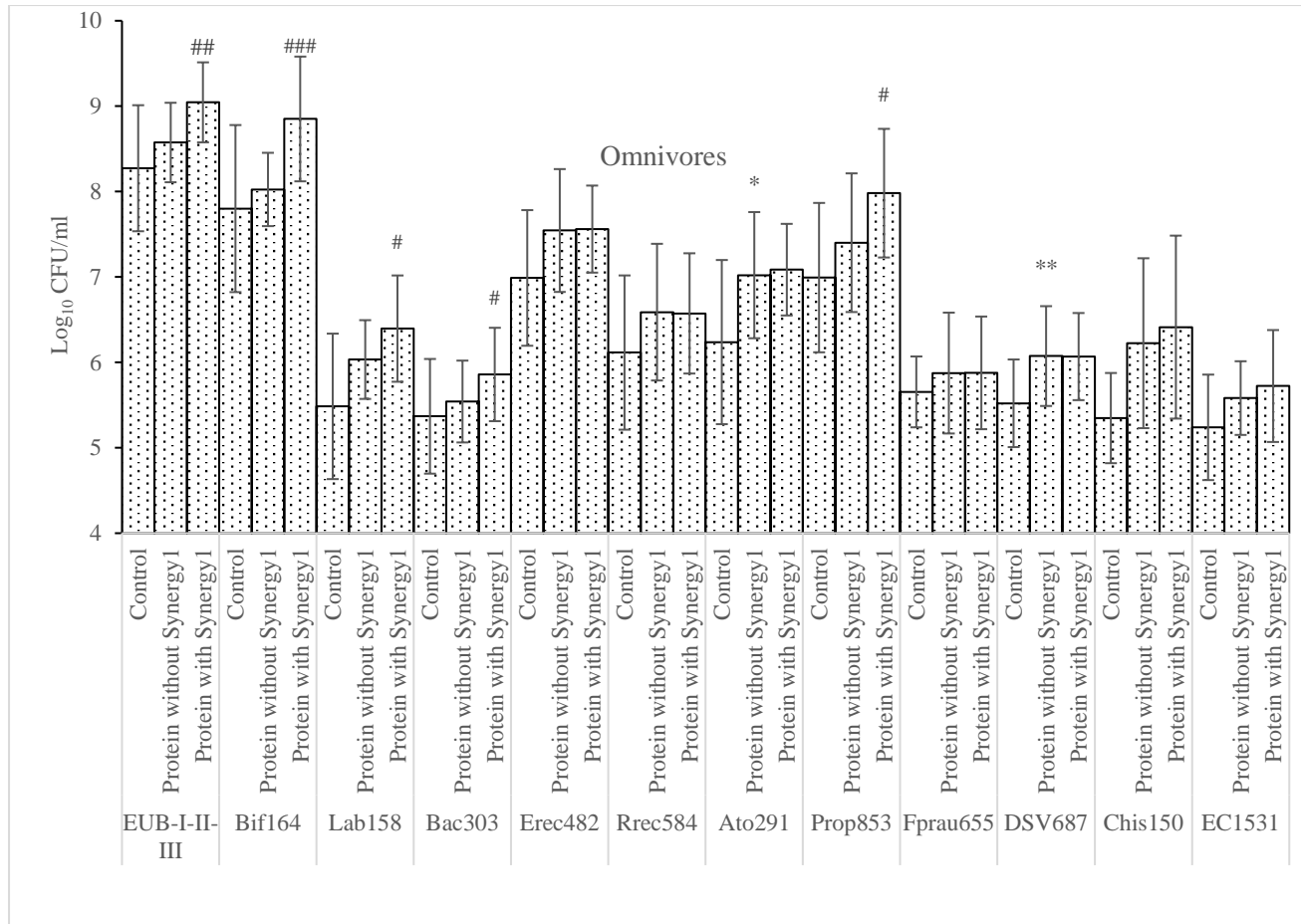
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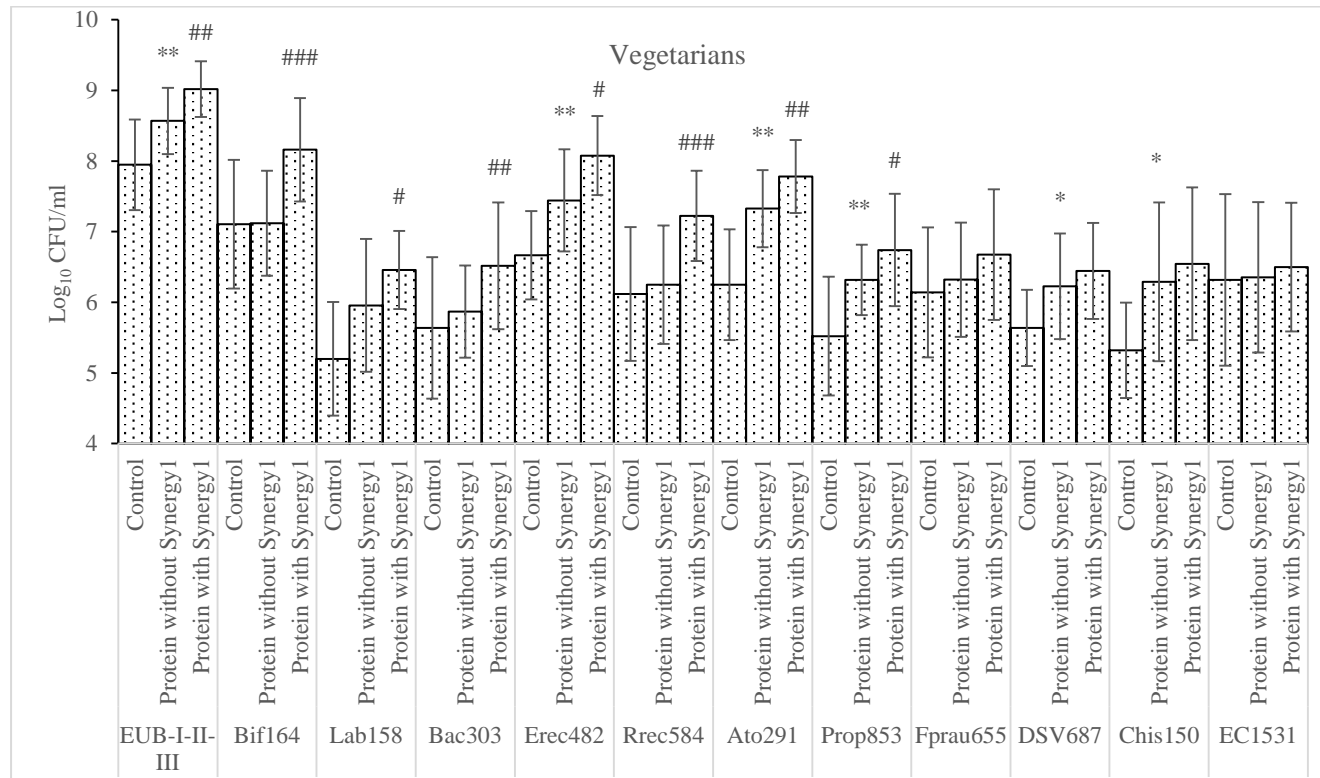
637

638 **Figure 1** Bacterial counts as log₁₀ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at 24 and 48 hours
 639 fermentation from 3 omnivores' microbiota ± standard deviation. a: * Mean values were significantly different between control and protein without
 640 Synergy1 (p<0.05). ** Mean values were significantly different between control and protein without Synergy1 (p<0.01). #: Mean values were

641 significantly different between protein with and without Synergy1 ($p < 0.05$). ## Mean values were significantly different between protein with and
 642 without Synergy1 ($p < 0.01$). ### Mean values were significantly different between protein with and without Synergy1 ($p < 0.001$).

643

644



645

646 **Figure 2** Bacterial counts as log₁₀ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at 24 and 48 hours

647 fermentation from 3 vegetarians' microbiota \pm standard deviation. * Mean values were significantly different between control and protein without
648 Synergy1 ($p < 0.05$). ** Mean values were significantly different between control and protein without Synergy1 ($p < 0.01$). # Mean values were
649 significantly different between protein with and without Synergy1 ($p < 0.05$). ## Mean values were significantly different between protein with and
650 without Synergy1 ($p < 0.01$). ### Mean values were significantly different between protein with and without Synergy1 ($p < 0.001$).

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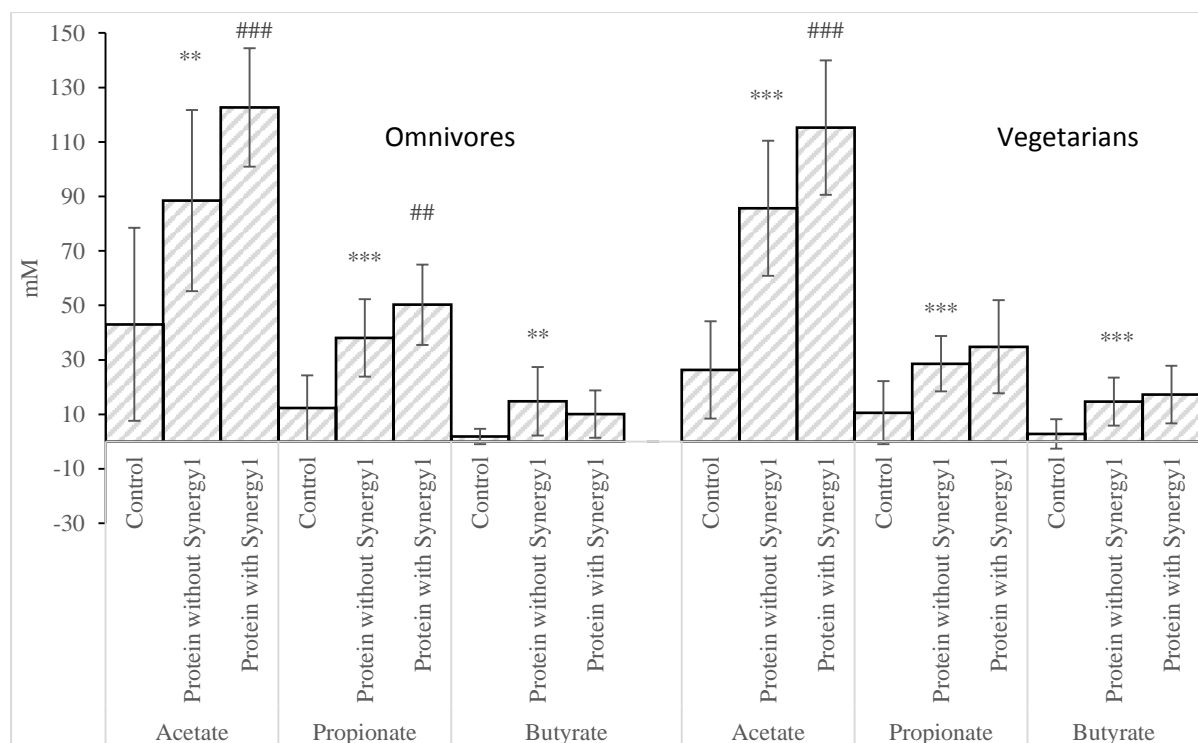
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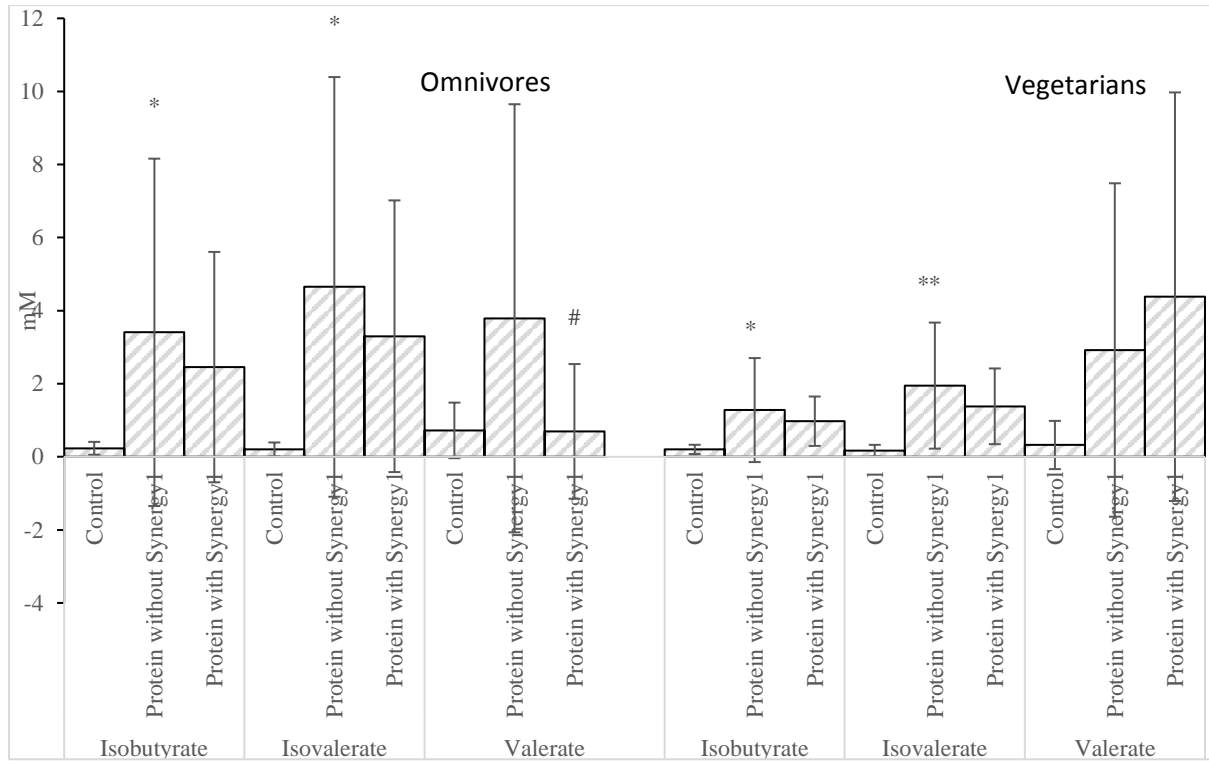
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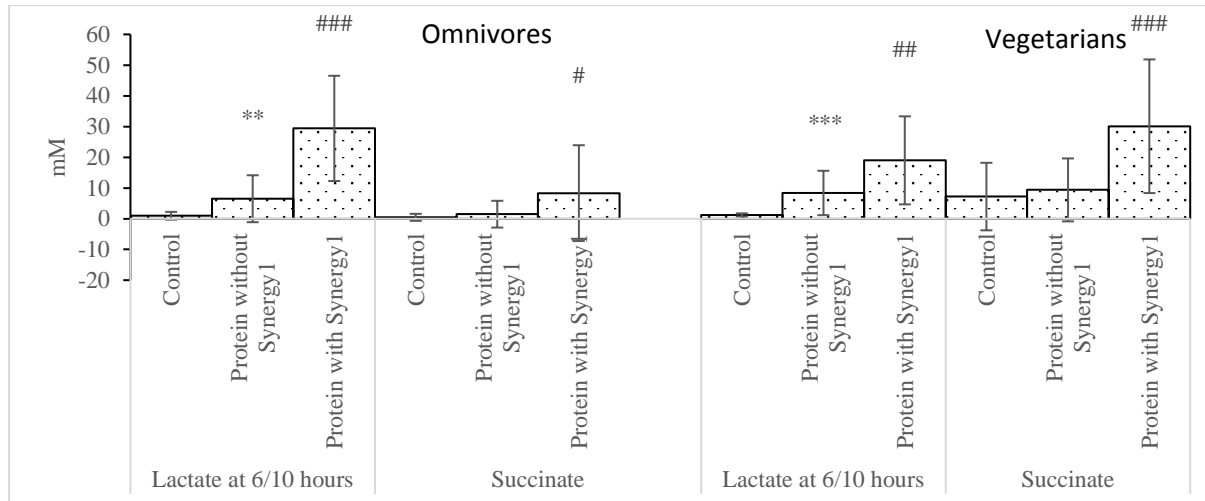
657

658 **Figure 3** SCFA differences between sample with and without protein as mM in the single stage batch culture. Values are mean values at 24 and 48
 659 hours fermentation from 3 omnivores' microbiota and 3 vegetarians' microbiota \pm standard deviation. * Mean values were significantly different
 660 between control and protein without Synergy1 ($p < 0.05$). ** Mean values were significantly different between control and protein without Synergy1
 661 ($p < 0.01$). *** Mean values were significantly different between control and protein without Synergy1 ($p < 0.001$). # Mean values were significantly
 662 different between protein with and without Synergy1 ($p < 0.05$). ## Mean values were significantly different between protein with and without
 663 Synergy1 ($p < 0.01$). ### Mean values were significantly different between protein with and without Synergy1 ($p < 0.001$).



664

665 **Figure 4** BCFA and valerate differences between sample with and without protein as mM in the single stage batch culture. Values are mean values
 666 at 24 and 48 hours fermentation from 3 omnivores' microbiota and 3 vegetarians' microbiota \pm standard deviation. * Mean values were significantly
 667 different between control and protein without Synergy1 ($p < 0.05$). ** Mean values were significantly different between control and protein without
 668 Synergy1 ($p < 0.01$). *** Mean values were significantly different between control and protein without Synergy1 ($p < 0.001$). # Mean values were
 669 significantly different between protein with and without Synergy1 ($p < 0.05$). ## Mean values were significantly different between protein with and
 670 without Synergy1 ($p < 0.01$). ### Mean values were significantly different between protein with and without Synergy1 ($p < 0.001$).



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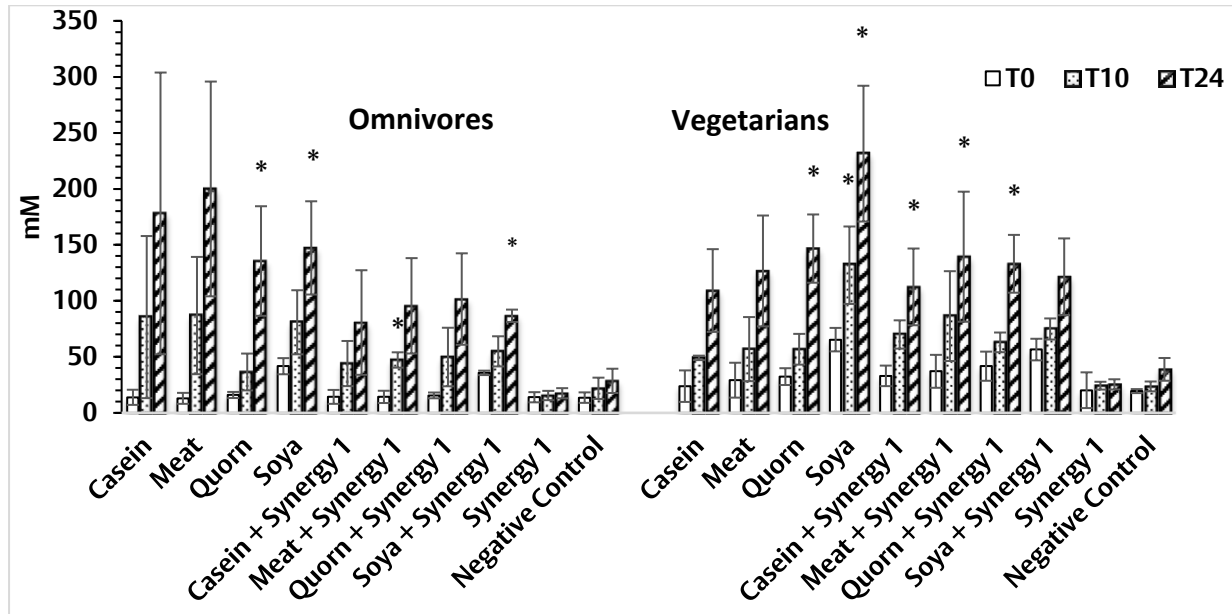
672 **Figure 5** Lactate and succinate differences between sample with and without protein as mM in the single stage batch culture. Values are mean
 673 values at 24 and 48 hours fermentation unless specified from 3 omnivores' microbiota and 3 vegetarians' microbiota \pm standard deviation. a: *
 674 Mean values were significantly different between control and protein without Synergy1 ($p < 0.05$). ** Mean values were significantly different
 675 between control and protein without Synergy1 ($p < 0.01$). *** Mean values were significantly different between control and protein without
 676 Synergy1 ($p < 0.001$). b: # Mean values were significantly different between protein with and without Synergy1 ($p < 0.05$). ## Mean values were
 677 significantly different between protein with and without Synergy1 ($p < 0.01$). ### Mean values were significantly different between protein with
 678 and without Synergy1 ($p < 0.001$).

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Figure 6 Changes in ammonia concentration

684 (mM) of batch culture sample over time. Values are mean values at three time points from 3 omnivore and 3 vegetarian faecal donor's \pm standard

685 deviation. * Mean values were significantly different from 0 hour fermentation samples ($p < 0.05$).

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	Omnivores			Vegetarians		
	Control	Protein without Synergy1 ^a	Protein with Synergy1 ^b	Control	Protein without Synergy1 ^a	Protein with Synergy1 ^b
	n=6	n=12	n=12	n=6	n=12	n=12
Ammonia in mM	23.07±9.58	165.24±77.44***	91.16±33.24**	32.02±8.97	153.53±62.69***	126.64±35.76

699 **Table 1** Ammonia concentration in samples as mM in the single stage batch culture. Values are mean values at 24 hours fermentation from 3
700 omnivores' microbiota and 3 vegetarians' microbiota \pm standard deviation. a: *** Mean values were significantly different between control and
701 protein without Synergy1 ($p < 0.001$). b: ** Mean values were significantly different between protein with and without Synergy1 ($p < 0.01$).

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In vitro fermentation dosage

Dietary protein	2.4g
Mucin	0.57g
Digestive enzymes	0.18g

Note: digestive enzyme is a mixture of 0.107g pepsin, 0.022g pancreatin, and 0.00079g α -amylase based on an *in vitro* upper gut digestion simulation paper (56)

714 **Table 2** Endogenous and exogenous protein dosage to simulate the *in vivo* effect of 105g dietary protein per day consumption for 150ml batch
715 culture experiment.

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Protein	Protein content	Protein dose
Casein	68.75%	3.5g
Soy protein	75%	3.2g
Meat extract	76%	3.2g
Mycoprotein	64.2%	3.7g

733 **Table 3** Protein dose that is equivalent to 2.4g dietary protein responding with protein content

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736 **Table 4** Name, sequence, and target group of oligonucleotide probes used in this study for FISH of bacterial enumeration

Probe name	Sequence (5' to 3')	Target groups	References
Non Eub	ACTCCTACGGGAGGCAG C	Control probe complementary to EUB338	(57)
Eub338	GCTGCCTCCCGTAGGAG T	Most Bacteria	(58)
Eub338II	GCAGCCACCCGTAGGTG T	Planctomycetales	(59)
Eub338II I	GCTGCCACCCGTAGGTG T	Verrucomicrobiales	(59)
Bif164	CATCCGGCATTACCACCC	<i>Bifidobacterium</i> spp.	(60)

Lab158	GGTATTAGCAYCTGTTTC CA	<i>Lactobacillus</i> and <i>Enterococcus</i>	(61)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(62)
Erec482	GCTTCTTAGTCARGTACC G	Most of the <i>Clostridium coccoides-Eubacterium rectale</i> group (<i>Clostridium</i> cluster XIVa and XIVb)	(63)
Rrec584	TCAGACTTGCCGYACCG C	<i>Roseburia</i> genus	(64)
Ato291	GGTCGGTCTCTCAACCC	<i>Atopobium</i> cluster	(65)
Prop853	ATTGCGTAACTCCGGC AC	Clostridial cluster IX	(64)
Fprau65 5	CGCCTACCTCTGCACTAC	<i>Feacalibacterium prausnitzii</i> and relatives	(66)

DSV687	TACGGATTTCACTCCT	<i>Desulfovibrio</i> genus	(67)
Chis150	TTATGCGGTATTAATCTY CCTTT	Most of the <i>Clostridium histolyticum</i> group (<i>Clostridium</i> cluster I and II)	(63)
EC 1531	CAC CGT AGT GCC TCG TCA TCA	<i>Escherichia coli</i> BJ4	(68)

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