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Article

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

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Abstract

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

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

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

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

Introduction

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

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

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

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

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

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

Results

Bacterial Enumeration

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

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

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

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

To investigate how prebiotics may modify the microbiota, independent *t* tests were used to 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±5.25mM)
137 while vegetarians had little production (1.61±1.60mM) ($p=0.004$). 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

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

Volatile Organic Compounds

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

producers, Synergy1+protein models produced significantly less phenol and indole (113.21±227.94 µg/ml) ($p=0.046$).

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

Ammonia

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

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

Discussion

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

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

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

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

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

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

246 the filamentous fungus *Fusarium venenatumsource* under the trade mark of Quorn^{TM} (43).
247 Quorn^{TM} 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^{TM} 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

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

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

Materials

Proteins

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

Prebiotic

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

Methods

Protein extraction

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

Protein dose determination

Based on previous validation work from *in vitro* batch culture experiments and in human trials, 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 $MgSO_3.7H_2O$ (Fischer scientific, Loughborough, UK), 0.01g $CaCl_2.6H_2O$, 2g $NaHCO_3$
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.

Filtered cold (4°C) 4% paraformaldehyde (PFA) (1125µl) was added and samples were stored at 4°C for 4 hours. These were then washed thoroughly with PBS to remove PFA and re-suspended in a mixture containing 300 µl PBS and 300 µl 99% ethanol. Samples were then stored at -20°C prior to FISH analysis by flow cytometry. Filtered cold (4°C) 0.1M PBS (500 µl) was mixed with fixed samples (75µl), before centrifuged at 11337 × g for 3 minutes. The pellets were then resuspended in 100µl of TE-FISH (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8, and filtered distilled water with the ratio of 1:1:8) containing lysozyme solution (1 mg/ml of 50,000 U/mg protein). Samples were then incubated in the dark at the room temperature for 10 minutes, and then centrifuged at 11337 × g for 3 minutes. Pellets were washed with 500µl filtered cold PBS, and then washed with 150µl hybridisation buffer (5 M NaCl, 1 M Tris/HCl pH 8, formamide, ddH2O, 10% SDS with the ratio of 180:20:300:499:1) and centrifuged at 11337 × g for 3 minutes. Pellets were then resuspended in 1ml of hybridisation buffer. Aliquots (50µl) with 4µl of different probes (50 ng μl^{-1}) were incubated at 35°C for at least 10 hours. The probes used in this study are listed in Table 7. Non Eub, Eub338-I-II-III are attached with fluorescence Alexa488 at the 5′ end, and other specific probes are attached with Alexa647. A set of Non Eub, Eub338-I-II-III are attached with fluorescence Alexa647 at the 5′ end to be the controls. For samples to detect specific groups, 4µl of Eub338-I-II-III were added together with 4µl specific probes. Hybridisation buffer (150µl) was added to each aliquot after incubation, followed by 3 minutes centrifugation at 11337 × g. Supernatants (150µl) were carefully removed before samples were centrifuged at 11337 × g for 3 minutes. Remaining supernatant was then removed, and pellets were resuspended in 200µl washing buffer. Washing buffer was prepared as: 12.8µl of 5M Na Cl, 20µl of 1M Tris/HCl pH 8, 10µl of 0.5 M EDTA pH 8, and 1µl of 10 % SDS in 956.2µl of filtered cold distilled water. Samples were then incubated at 37°C for 20 minutes and centrifuged at 11337 × g for 3 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 × g for 10 minutes to remove all particulate matter.
395 Supernatants were then filtered through a 0.2 µm 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 (500µl) was transferred into a labelled 100 mm×16 mm
398 glass tube (International Scientific Supplies Ltd, Bradford, England) with 25 µl of 2-
399 ethylbutyric acid (0.1 M, internal standard) (Sigma, Poole, UK). Concentrated HCl (250µl) 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 × 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 (400µl) and 50 µl 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 30m×0.25mm column with a 0.25µm 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°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

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

Volatile organic compounds analysis by GC-MS

Entrapment of volatile compounds

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

Gas Chromatography and Mass spectrometry (GC-MS)

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

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

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

Ammonia Analysis

Samples at 0, 10 and 24 hours were diluted 1 in 50 v/v prior to analysis. Ammonia concentration of diluted fermentation samples was analysed using a FluoroSELECT™ ammonia kit (Sigma-Aldrich, Poole, UK). Reagent was prepared by combining 100 µL assay buffer, 4 µL reagent A and 4 µL reagent B in the kit. 10 µL H_2O (blank) and 10 µL sample was added to each glass vial. Afterwards, 100 µL reagent was added to each tube. Samples were kept in the dark for 15 minutes at room temperature before they were read in the fluorometer. Ammonia standards were prepared by diluting 20 mmol/L NH_4Cl in distilled water and the concentration range was 0.25-1 mmol/L).

Statistical analysis

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

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

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We declare that there is no conflict of interest.

Footnotes

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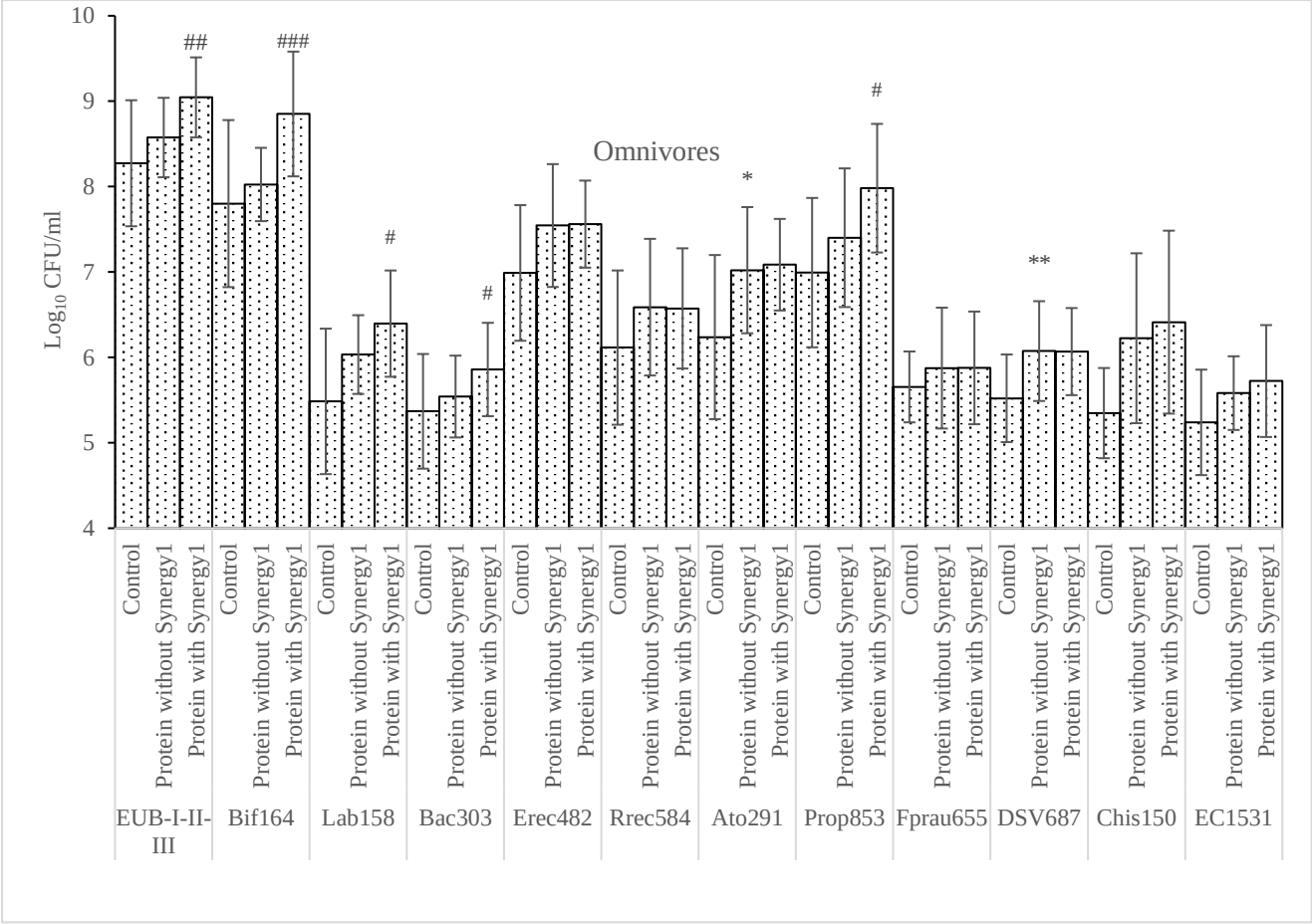
Figures



637

638 **Figure 1** Bacterial counts as log_{10} 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 ($p<0.01$). b: # 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 ($p<0.001$).

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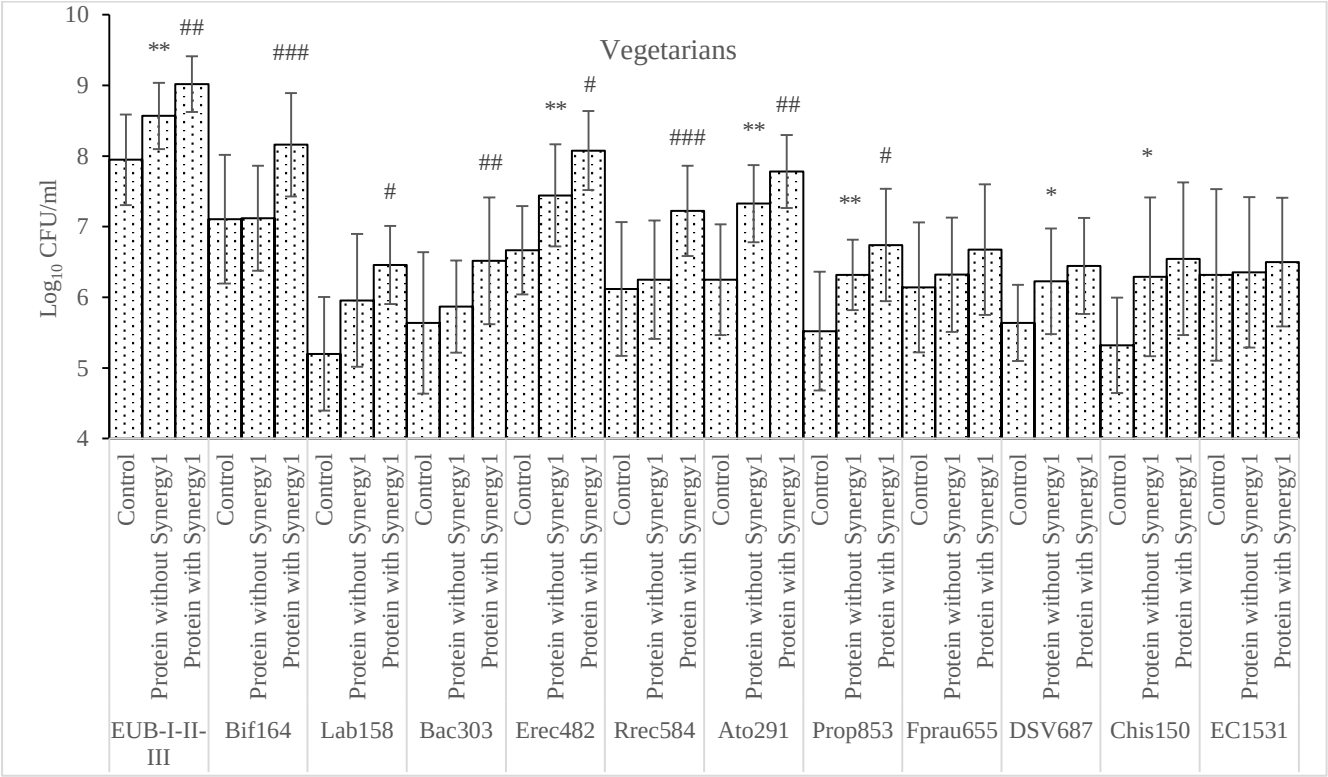

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646 **Figure 2** Bacterial counts as $\log_{10}$ CFU/mL in the single stage batch culture as analysed by FISH. Values are mean values at 24 and 48 hours

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

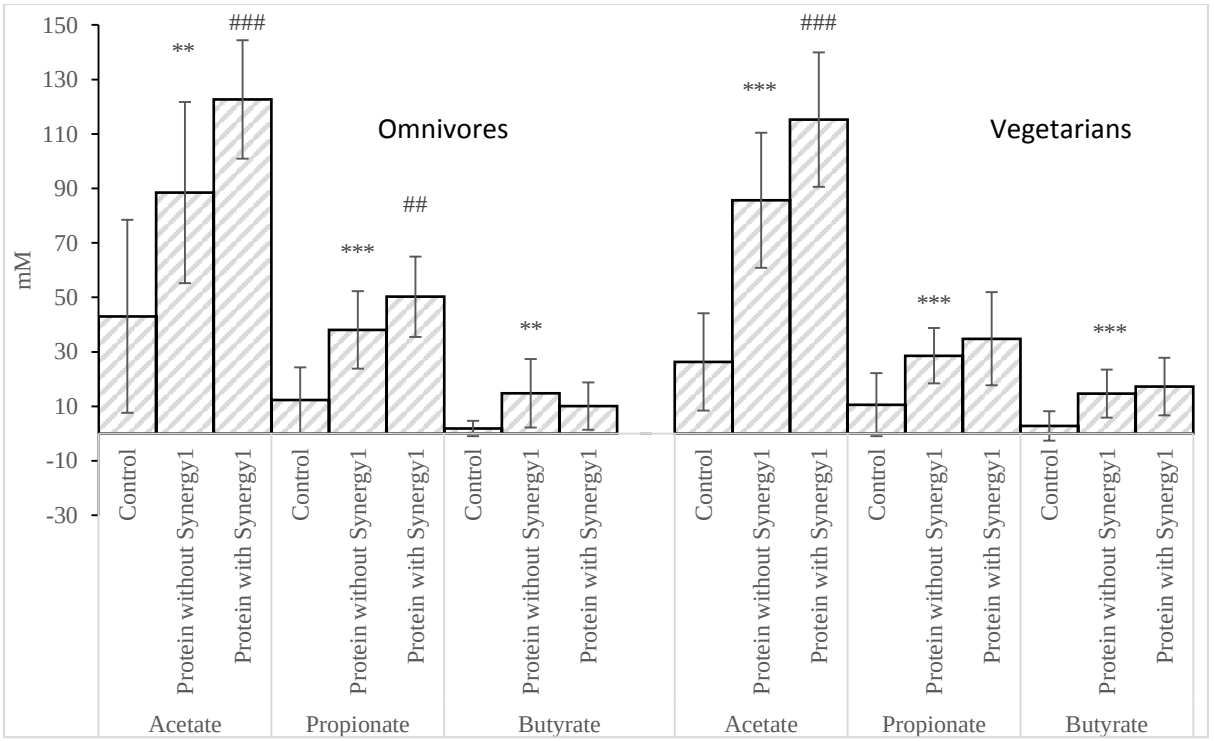

Figure 3 SCFA differences between sample with and without protein as mM in the single stage batch culture. Values are mean values at 24 and 48 hours fermentation from 3 omnivores' microbiota and 3 vegetarians' microbiota ± standard deviation. * Mean values were significantly different between control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

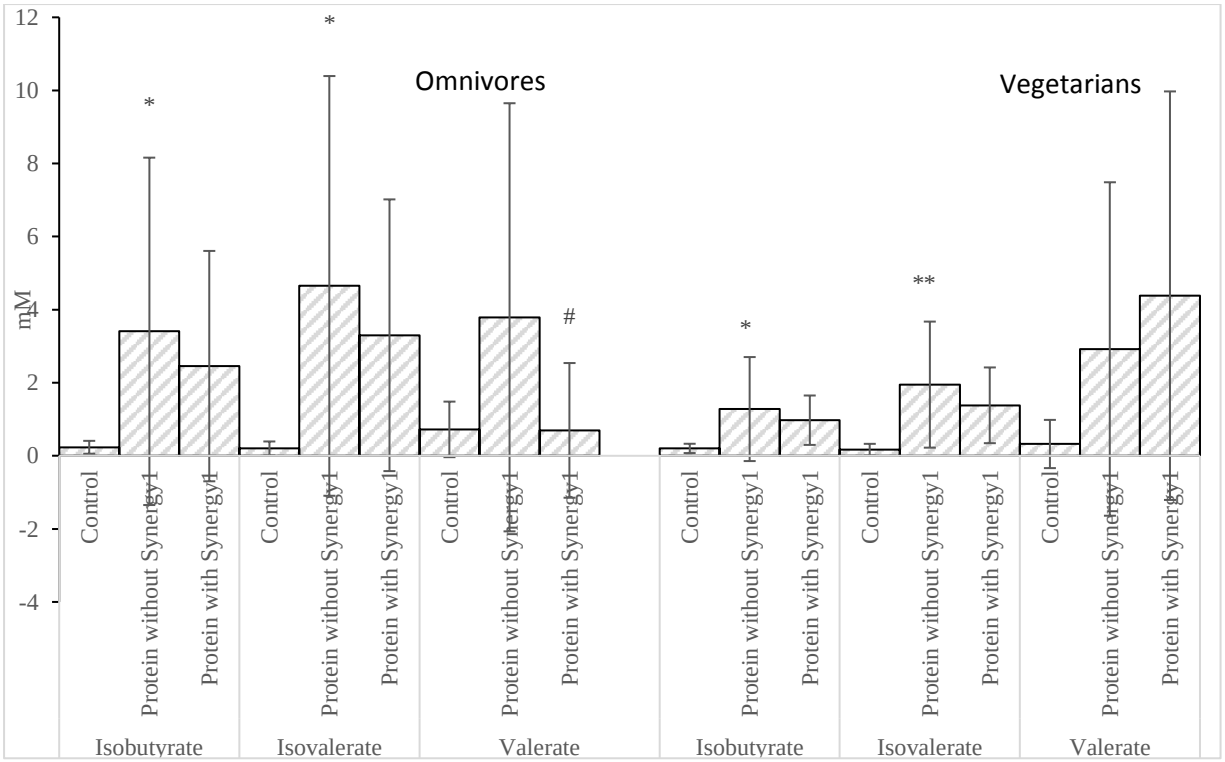

Figure 4 BCFA and valerate differences between sample with and without protein as mM in the single stage batch culture. Values are mean values at 24 and 48 hours fermentation from 3 omnivores' microbiota and 3 vegetarians' microbiota ± standard deviation. * Mean values were significantly different between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

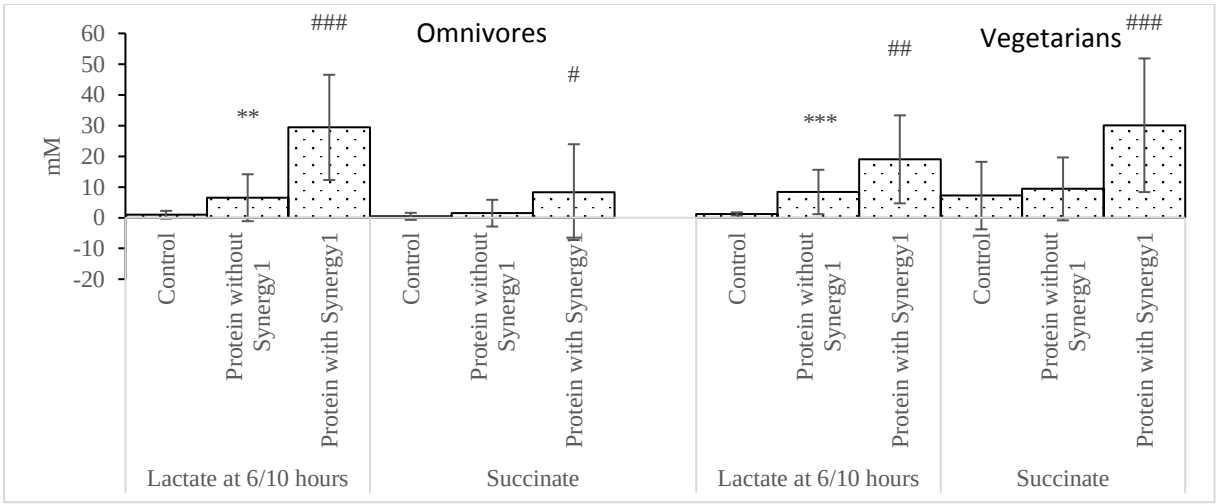

Figure 5 Lactate and succinate differences between sample with and without protein as mM in the single stage batch culture. Values are mean values at 24 and 48 hours fermentation unless specified from 3 omnivores' microbiota and 3 vegetarians' microbiota ± standard deviation. a: * Mean values were significantly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

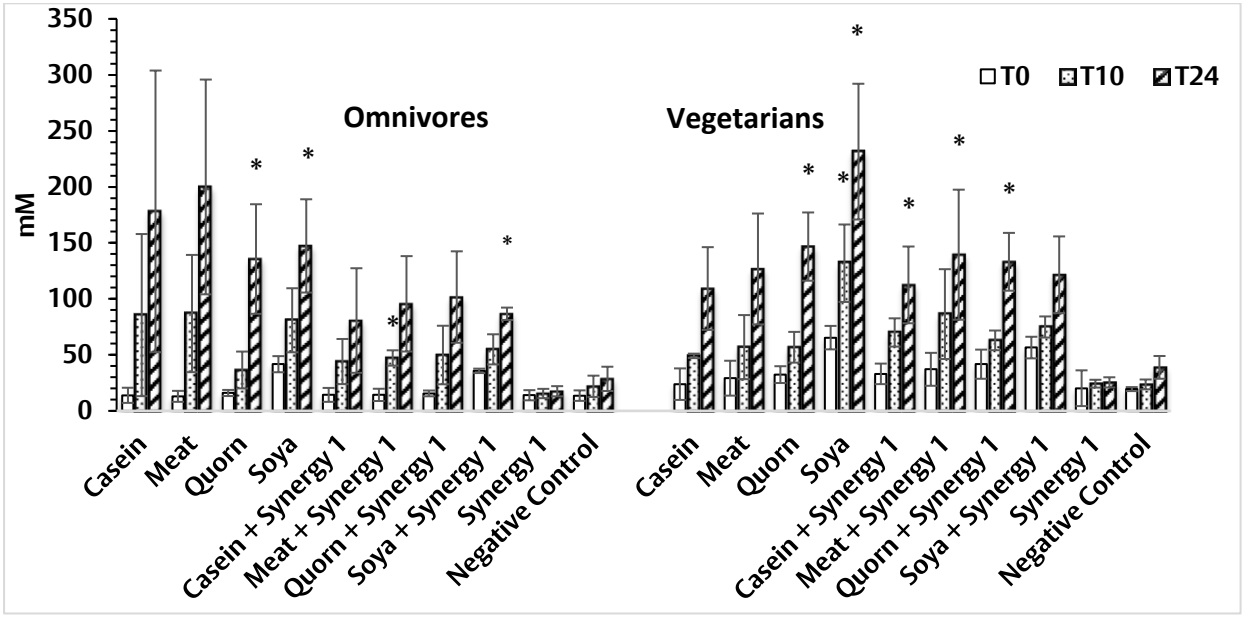

Figure 6 Changes in ammonia concentration (mM) of batch culture sample over time. Values are mean values at three time points from 3 omnivore and 3 vegetarian faecal donor's ± standard deviation. * Mean values were significantly different from 0 hour fermentation samples ($p<0.05$).

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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

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

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)

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

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

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

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	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	(57)
Eub338	GCTGCCTCCCGTAGGAGT	Most Bacteria	(58)
Eub338II	GCAGCCACCCGTAGGTGT	Planctomycetales	(59)
Eub338III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	(59)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	(60)

Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*	(61)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(62)
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa and XIVb)	(63)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus	(64)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(65)
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX	(64)
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives	(66)

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

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