

Study of in vitro digestion of Tenebrio molitor flour for evaluation of its impact on the human gut microbiota

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34 is the mealworm, *Tenebrio molitor*, which can be used and consumed in a flour form (Van Huis
35 et al., 2013).

36 Any non-digested portion of foods may provide an energy source for gut microbiota, residing
37 mainly in the colon. This microbial community can be manipulated by diet and is known to
38 have an impact on health. As such, an important perspective for the impact of food ingredients
39 on the consumer can be obtained by investigating effects on host gut microbiota.

40 The microbiota includes pathogenic and non-pathogenic microorganisms, as well as eukaryotic
41 and prokaryotic cells and viruses. This microbial consortium plays a key role in the breakdown
42 of dietary fiber, vitamin synthesis, nutrient cycling and energy metabolism. It also acts as a
43 physical barrier to prevent pathogens from colonizing the GIT through competitive exclusion
44 (Charaslertrangsi, 2014). In an adult, the dominant phyla include Firmicutes, Bacteroidetes,
45 Proteobacteria and Actinobacteria (Charaslertrangsi, 2014; Moon et al., 2016; Thursby and
46 Juge, 2017). These bacteria are involved in immunological activity, energy consumption,
47 intestinal permeability, intestinal motility, and even effects in the enteric nervous system and
48 brain activities (Barczynska et al., 2016; Bull and Plummer, 2014; Carabotti et al., 2015; Conlon
49 and Bird, 2014; Derrien and van Hylckama Vlieg, 2015; Fung et al., 2017; Kim et al., 2016;
50 Mayer et al., 2015; Powell et al., 2017). Some of these actions are driven by the ability of the
51 microbiota to produce short-chain fatty acids (SCFA) (Brüssow and Parkinson, 2014; Louis et
52 al., 2014; Ríos-Covián et al., 2016). As such, SCFA are important end products of microbial
53 fermentation and their production is highly dependent on substrates reaching the large intestine.
54 The understanding of host-microbiota-food component interactions is of major significance,
55 and for that, simulation models can help elucidate this complex relationship. *In vitro* digestion
56 models are used to assess and simulate physicochemical and physiological events of the
57 digestive tract, allowing studies of structural changes, bioavailability and digestibility of foods
58 when they arrive at the colon (Hur et al., 2011; Lee et al., 2016). As such, *in vitro* fermentation
59 models are useful tools to screen substances, from dietary ingredients to pathogens and to assess
60 how they alter or are altered by gastrointestinal environments and microbial populations
61 (Verhoeckx et al., 2015). *In vitro* fermentation allows cultivation of complex intestinal
62 microbiota, in controlled conditions, to study metabolism (Moon et al., 2016).

63

64 **The aim of this work was to evaluate the impact of an edible insect, *Tenebrio molitor*, on the**
65 **human gut microbiota using an *in-vitro* digestion model. This is a very novel study, only one**
66 **similar previous study has been reported by Stull et al. 2018, where the impact of edible cricket**
67 **consumption on human gut microbiota was investigated in an *in vivo* study. Two samples of**

68 the insect in flour form (TMIF) were investigated, digested and undigested and anaerobic fecal
69 fermentation models were used. The evaluation of the impact of the samples on the gut
70 microbiota was carried out based on measurements of bacterial composition and short fatty acid
71 production during a time course. This enabled assessment of the potential of TMIF to impact
72 on the microbial community and its metabolites.

73

74 **2. Material and methods**

75 **2.1. *Tenebrio molitor* insect flour (TMIF)**

76 *Tenebrio molitor* insect flour (TMIF) was purchased from Insagri company, Málaga, Spain and
77 kindly offered by Frulact company, Maia, Portugal. The composition and nutritional
78 information of TMIF is shown in **Table 1**.

79

80 **2.2. TMIF sterilization**

81 To ensure TMIF was free of microorganisms, the sample was dried at 100 °C for 24 hours.

82

83 **2.3. *In vitro* gastrointestinal digestion protocol**

84 The TMIF digestion used an *in vitro* method mimicking *in vivo* conditions as described by Mills
85 et al. (2008) with slight modifications. 20 g of TMIF were ground and dissolved in 50 mL
86 distilled water and the mixture put in a stomacher (Seward, Worthing, UK) for 5 min. For the
87 oral phase, 6.66 mg of α -amylase (A 4551, Sigma) in 2.08 mL of 0.001 M CaCl₂ at pH 7.0 was
88 added and incubated at 37 °C for 30 min on a shaker. After this, 6 M HCl was used to lower the
89 pH to 2.0. For the gastric phase, 0.9 g of pepsin (P 7000, Sigma) was dissolved in 8.33 mL of
90 0.1 M HCl in a volumetric flask and this pepsin solution was added to the samples and incubated
91 at 37 °C for 2 h on a shaker. For the small intestinal phase, a pancreatin and bile solution was
92 prepared. For that, 186.67 mg of pancreatin (P 8096, Sigma) and 1.17 g of bile (B 8631, Sigma)
93 were dissolved in 41.67 mL of 0.5 M NaHCO₃, and pH was adjusted to 7.0 with either 6 M of
94 HCl or NaOH and incubated at 37°C for 3 h on a shaker. The simulated digestion process of the
95 TMIF also included a dialysis step to simulate the absorption in small intestine, in order to
96 analyze the different behavior of bacteria present in the gut microbiota (Alegría et al., 2015;
97 Verhoeckx et al., 2015). All samples were transferred to 100-500 Da molecular weight cut-off
98 regenerated cellulose dialysis tubing (Spectra/Por® 6, Spectrum Europe, Netherlands) and a
99 dialysis was performed against 1 M NaCl at 5 °C to remove low molecular mass digestion
100 products. After 15 h, the dialysis fluid was changed and performed for two additional hours.
101 Afterwards, all samples were transferred to a freeze dryer (Armfield SB4 model, Ringwood,

102 UK) in order to obtain a powder (digested TMIF) to be used for *in vitro* fecal fermentations.
103 All chemicals were purchased from Sigma (St. Louis, USA).

104

105 **2.4. Gut microbiota simulation: Fecal fermentations**

106 **2.4.1. Fecal microbiota**

107 Fecal samples were obtained fresh at the Department of Food and Nutritional Sciences, Reading
108 from five healthy adult volunteers. The volunteers had normal omnivorous diets and had not
109 ingested any antibiotics or other medicines known to affect the microbiota for at least 6 months
110 prior to the study. Volunteers were 2 males and 3 females aged 22-37 years and were not regular
111 consumers of prebiotics or probiotics. Samples were collected into clean containers and
112 immediately placed in an anaerobic cabinet (nitrogen 80%, carbon dioxide 10%, hydrogen
113 10%) (Don Whitley, UK) and used within 1 h of collection. A 10% (w/w) dilution in 0.1 M
114 phosphate-buffered saline pH 7.4 (PBS) solution was prepared and homogenized using a
115 stomacher (Serward, Worthing, UK) for 2 min at 460 paddle-beats per min. This produced a
116 fecal slurry.

117

118 **2.4.2. Fecal batch-culture fermentation conditions**

119 Five independent fermentations were carried out using a sample from each donor. Sterile stirred
120 batch culture fermentation vessels of 300 mL were set up and aseptically filled with 135 mL
121 sterile basal nutrient medium (peptone water 2 g/L, yeast extract 2 g/L, NaCl 0.1 g/L, K₂HPO₄
122 0.04 g/L, KH₂PO₄ 0.04 g/L, MgSO₄·7H₂O 0.01 g/L, CaCl₂·6H₂O 0.01 g/L, NaHCO₃ 2 g/L,
123 Tween 80 2 mL/L, hemin 0.05 g/L, vitamin K 10 µL/L, L-cysteine HCl 0.5 g/L, bile salts 0.5
124 g/L and resazurin 4 mg/L) and gassed overnight with O₂-free N₂ with constant agitation. The
125 temperature was kept at 37°C. Four stirred pH-controlled batch fermenters were run in parallel:
126 (1) 1% (w/v) digested TMIF was aseptically added; (2) 1% (w/v) undigested TMIF was
127 aseptically added; (3) a positive control with 1% (w/v) FOS (a known prebiotic) from chicory
128 root, purity: > 95%, degree of polymerization ranging from 2 to 8 (Megazyme, Bray, Ireland)
129 and (4) a negative control which had no source of carbon added. Each vessel, with 135 mL of
130 sterile basal nutrient medium was inoculated with 15 mL of fresh fecal slurry. A FerMac 260
131 pH Controller (Electrolab Biotech Ltd., Tewkesbury, Gloucestershire, UK) was used, at 37 °C,
132 to maintain pH for each vessel between 6.7 and 6.9 (Sánchez-Patán et al., 2012). The batch
133 cultures were conducted under anaerobic conditions at 37 °C during 48 h, in which 5 mL
134 samples were collected from each vessel at 0, 4, 8, 24 and 48 h for bacterial enumeration by
135 fluorescence *in situ* hybridization (FISH), analysis of SCFA, BCFA and lactate by gas

136 chromatography (GC) and quantification of ammonia with 53659-FluoroSelect™ Ammonia
137 Kit (Sigma-Aldrich, Gillingham, Dorset, UK). All media and chemicals were purchased from
138 Oxoid (Basingstoke, UK) and Sigma (St. Louis, USA).

139

140 **2.5. Bacterial enumeration by FISH-FCM**

141 Samples were analyzed by fluorescence *in situ* hybridization combined with flow cytometry
142 (FISH-FCM) in order to determine differences in bacterial composition in the batch cultures.
143 The FISH-FCM was performed according to the protocol used by Grimaldi et al. (2017) with
144 slight modifications on volumes used on the permeabilization steps where, in this case, 150 µL
145 of fixed batch culture samples were added to 500 µL 1x PBS.

146 **Table 2** shows the probes used (Eurofins Genomics, Ebersberg, Germany) in this protocol
147 (Daims et al., 1999; Devereux et al., 1992; Franks et al., 1998; Harmsen et al., 1999; Harmsen
148 et al., 2000; Hold et al., 2003; Langendijk et al., 1995; Manz et al., 1996; Walker et al., 2005;
149 Wallner et al., 1993). Samples were stored at 4 °C until flow cytometry (FCM) analysis by a
150 BD Accuri™ C6 Cytometer (BD, Womersley, Wokingham, UK). Numbers of specific and total
151 bacteria were determined considering the dilution factor, calculated from different volumes
152 used in the different steps of the preparation of the samples, and events/µL obtained from Non
153 Eub338 and Eub338 I-II-III probes analyzed by FCM.

154

155 **2.6. Evaluation of organic acids production by GC**

156 GC analysis was performed to evaluate the production of organic acids by the gut microbiota.
157 From fecal batch cultures, 1 mL of sample of each vessel was transferred to a flat-bottomed
158 glass tube and 50 µL of 2-ethylbutyric solution added to each tube. In the fume hood, 500 µL
159 of concentrated HCl and 3 mL diethyl ether was added and vortexed. The tubes were centrifuged
160 at 720 x g for 10 minutes at room temperature (18 °C). The tubes went back again into the fume
161 hood, where 400 µL upper layer of the tubes were transferred into GC-vials and 50 µL of N-
162 tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) added to each GC-vial. The
163 vials were left at room temperature for at least 72 hours before conducting GC analysis.
164 Production of the SCFA, BCFA (branched chain fatty acids) and lactate was determined by an
165 Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS 30 m×0.25 mm
166 column with a 0.25 µm coating (Crosslinked (5%-Phenyl)-methylpolysiloxane) (Hewlett
167 Packard, UK). Temperatures of injector and detector were 275 °C, with the column programmed
168 from 63 °C for initial time (0 minutes) to 190 °C at 15 °C min⁻¹ and held at 190 °C for 3 min.
169 Helium was the carrier gas (flow rate 1.7 mL/min; head pressure 133 KPa). A split ratio of

170 100:1 was used. Peaks were integrated using Agilent ChemStation software (Agilent
171 Technologies, Oxford, UK) and organic acids content quantified by multiple-point internal
172 standard method (12.5, 25, 50, 75 and 100 mM). Peak identity and internal response factors
173 were determined using 0.1 mM calibration cocktail including acetate, propionate, isobutyrate,
174 butyrate, isovalerate, valerate and lactate.

175

176 **2.7. Evaluation of ammonia production**

177 Quantification of ammonia present in the studied samples was performed with 53659-
178 FluoroSelect™ Ammonia Kit (Sigma-Aldrich, Gillingham, Dorset, UK) following the protocol
179 that was provided by the kit. Briefly *o*-phthalaldehyde reagent was reacted with an amino acid
180 solution and sulfite (reagent A) resulting in a color change in the presence of ammonia. As such
181 a calibration curve of ammonia concentration was constructed alongside samples by recording
182 fluorescence intensity at a wavelength of 360/460nm using a Tecan plate reader (Tecan Genios,
183 Switzerland).

184

185 **2.8. Statistical analysis**

186 Statistical analysis of the results was carried out using IBM SPSS software (24.0.0.0, IBM,
187 Chicago, USA). Normality of the distributions was evaluated using Shapiro-Wilk's test. As the
188 samples followed normal distribution, means were compared, considering a 95% confidence
189 interval, using One-way ANOVA coupled with Tukey's post-hoc test. The non-parametric
190 Mann-Whitney test was used when the results did not follow a normal distribution. Differences
191 between the total amino acids of digested and undigested TMIF were evaluated using an
192 independent sample t-test as they proved to follow a normal distribution.

193

194 **3. Results and Discussion**

195

196 The impact of TMIF on the gut microbiota was assessed based on determination of bacterial
197 composition by a molecular quantitative technique, FISH-FCM. Moreover, metabolic activity
198 was determined based on the production of SCFA, BCFA and lactate; the amount of ammonia
199 produced during fermentation was also determined.

200

201 **3.1. Analysis of the impact on bacterial composition**

202 Digested and undigested TMIF results for total bacteria were not significantly different and
203 were very similar to those of the negative control. The digested TMIF sample should be richer
204 in end-products (e.g. small peptides, amino acids) than the undigested TMIF upon simulated
205 digestion. However, both products were subjected to a final dialysis step which should remove
206 all small molecular products such as, free fatty acids, small peptides and amino acids. This
207 would explain why no differences were observed in total bacteria between the two TMIF
208 samples.

209 Positive control (FOS) as expected, exhibited the most significant bacterial growth throughout
210 the study period. In general, at all conditions, the concentration of different bacteria present in
211 the fermentation vessels increased during the first 8 h of incubation.

212 Results of bacterial composition are shown in **Fig 1**. *Bifidobacterium* spp. growth showed
213 similar outcomes over incubation time for the digested and undigested TMIF. *Bifidobacterium*
214 spp. on the positive control showed significant growth increase compared to the other
215 conditions. Results obtained for *Lactobacillus* spp. showed a similar growth profile to those
216 obtained for *Bifidobacterium* spp. Overall, better growth of *Bifidobacterium* spp. and
217 *Lactobacillus* spp. was observed for samples with carbohydrates as substrate than with the two
218 samples of TMIF which are predominantly protein.

219 Growth of *Bacteroidaceae* and *Prevotellaceae* with TMIF samples (digested and undigested)
220 were similar to the positive control. This may indicate, in this case, a positive impact of TMIF,
221 as it partially matches the effect of FOS, in terms of bacteria upkeep, thus indicating that TMIF
222 can be used as substrate by these bacteria. *Bacteroides* spp. possess strong peptidase activity
223 and are associated with isovalerate and isobutyrate production (Scott et al., 2013; Zhao et al.,
224 2016). This means that *Bacteroidaceae* and *Prevotellaceae*, depending on the type of substrate,
225 can utilize effectively both its saccharolytic and proteolytic pathways for growth.

226 The growth of *Atopobium* cluster, has been reported to be increased by disaccharides,
227 polysaccharides and long-chain inulin (Vinke et al., 2017). This seems to agree with results
228 obtained with the positive control, where *Atopobium* cluster showed a significant increase at 8
229 h and maintained these levels up to 48 h. In the presence of both forms of TMIF at 8 h, small
230 and similar growth was observed. In the same samples, at 24 and 48 h, the results differed – the
231 sample with the undigested form maintained the *Atopobium* cluster concentration level over
232 such periods, while, in the digested form sample, a slight increase was observed, later followed
233 by a decrease at 48 h. *Atopobium* cluster is relatively unresearched, and very few studies
234 demonstrate a correlation between its presence and human health. Nevertheless, it has been

235 reported that the presence of *Atopobium* correlated with beneficial effects in terms of
236 cardiometabolic health (Vinke et al., 2017).

237 Regarding the *Clostridium coccooides* / *Eubacterium rectale* group at 8 h, the sample with
238 digested TMIF showed a significant decrease in cell numbers. In the case of undigested TMIF,
239 positive and negative control no significant deviation was seen in their growth profile (**Fig 1**).
240 The *Clostridium coccooides* / *Eubacterium rectale*, is a group of anaerobic bacteria, well-known
241 for butyrate production, as are *Roseburia* and *F. prausnitzii*, in the gut microbiota (Lopetuso et
242 al., 2013). The *Clostridium histolyticum* group, is a clostridial group that possesses some
243 pathogenic species such as *Clostridium perfringens* and *Clostridium tetani*. This group showed
244 no significant deviations between samples at specific study times, except for positive control at
245 24 and 48 h. Clostridia are proteolytic bacteria and some clostridia possess saccharolytic
246 activity, preferably fermenting amino acids (Rowland et al., 2017; Scott et al., 2013).

247 The *Roseburia* genera is also abundant in the intestinal microbiota, and it can produce both
248 propionate and butyrate (Ríos-Covián et al., 2016). *Roseburia* spp. can grow in presence of
249 carbohydrate, and some *Roseburia* spp. have FOS degradation genes or an inducible fructan
250 utilization operon (Scott et al., 2013; Scott et al., 2015). In this work, *Roseburia* showed slight
251 growth in the presence of FOS at 8 and 24 h (**Fig 1**). At 48 h, a major decrease in *Roseburia*
252 was observed, in the positive control, which may be explained by a decrease in FOS availability
253 as it was being utilized during fermentation. In the presence of TMIF, a decrease was observed
254 but the undigested form always maintained a higher concentration compared to the digested
255 form.

256 The *Clostridium* cluster IX belongs to the group of bacteria that mainly produce propionate in
257 gut microbiota and use amino acids as main source of energy (Bernalier-Donadille, 2010;
258 Tottey et al., 2017; Van den Abbeele et al., 2010). At 8 h, growth of this cluster was observed
259 in the presence of TMIF, mostly in the undigested form sample. At 24 h the bacteria growth
260 profile on FOS and TMIF samples were similar and at 48 h, a decrease was observed in all
261 samples however, in the samples with undigested TMIF such a decrease was lower;
262 nevertheless, these differences were not significant. In samples with TMIF, growth was
263 expected, due to the presence of amino acids. As for the samples with FOS, growth was
264 observed up to 24 h, which must be related with cross-feeding process, as these bacteria use
265 lactate (previously produced by other bacteria) as substrate to produce propionate (Bernalier-
266 Donadille, 2010; Louis and Flint, 2017), which can be related to the lactate disappearance after
267 8 h in the **Fig 1**. The cross feeding process may also explain the *Clostridium histolyticum* group
268 growth in presence of FOS, up to the same study time (24 h).

269 *Faecalibacterium prausnitzii*, a strictly anaerobic bacterium, is one of the most abundant
270 species present in healthy human microbiota. It is one of the main butyrate producers (Conlon
271 and Bird, 2014; Scott et al., 2015). In the presence of digested TMIF, bacteria growth showed
272 a decrease over time. For the positive and negative control was also observed a decreasing
273 growth profile over time. The sample with undigested TMIF, was the only one that showed, at
274 8 h, slight growth of these bacteria.

275 *Desulfovibrionales* and *Desulfuromonales* are only found in approximately fifty percent of
276 humans (Rey et al., 2013). Predominant sulphate-reducing bacteria (SRB) in human colon are
277 members of the genus *Desulfovibrio*. They can use hydrogen or organic compounds like lactate
278 and formate to reduce sulphate to generate hydrogen sulphide (H₂S), which has a toxic nature,
279 that can have pathological consequences for the host (Conlon and Bird, 2014; Rowland et al.,
280 2017). Several studies identified SRB in the fecal microbiota of healthy adults and, despite
281 being positively correlated with inflammation, the presence of H₂S, has been attributed both to
282 pro and anti-inflammatory signaling (Levine et al., 1998; Pitcher et al., 2000; Rey et al., 2013;
283 Wallace et al., 2009). Lactate is also a favored co-substrate for these bacteria, forming acetate
284 and sulphides. *Desulfovibrio* was reported to decrease in the presence of inulin, and studies
285 showed that its lowered abundance can benefit health (Vinke et al., 2017). For this study, this
286 group of bacteria was found in lower concentrations in the fecal samples of the donors,
287 compared to other quantified groups. At 8 h, for all samples, there was a small increase of
288 *Desulfovibrionales* and *Desulfuromonales* with no significant differences between the samples.
289 After 8 h (24 and 48 h) a decline over time was observed. The small increase of these bacteria
290 at 8 h may be correlated with the availability of lactate at that time (**Fig 1**).

291 In this study, it was possible to see the effect of undigested and digested TMIF through
292 modulation of gut bacterial populations. The most marked results were found on the growth of
293 *Bacteroidaceae* and *Prevotellaceae*, which are bacteria related to proteolytic and saccharolytic
294 activity conferring benefits to the host through their activity (e.g. propionate production). Such
295 results make sense since TMIF is predominantly protein. It is important to highlight the fact
296 that digested TMIF did not promote the growth of butyrate producers during the fermentation,
297 such as *Clostridium coccooides* / *Eubacterium rectale* group, *Roseburia* subcluster and
298 *Faecalibacterium prausnitzii*, while undigested TMIF promoted growth or maintained these
299 bacteria. This study indicates an influence of TMIF on bacterial populations of the human gut
300 microbiota however, *in vivo* studies must be carried out in order to evaluate the impact of such
301 bacterial group variations on humans.

302

3.2. Analysis of the impact on SCFA, BCFA and lactate production

Acetate, propionate and butyrate are the most abundant SCFA existing in the colon, normally present in molar ratios ranging from 3:1:1 to 10:2:1 (Ríos-Covián et al., 2016; Rowland et al., 2017; Scott et al., 2013; Tan et al., 2014). In this study SCFA and BCFA were found (Fig 2 and 3).

As a general overview, acetate, propionate and butyrate concentrations were significantly higher overtime for digested and undigested TMIF in comparison to the negative control (Fig 2). In all cases, the positive control sample had significantly higher concentrations than the other samples.

Butyrate is an important SCFA for human health. It provides an energy source for human colonocytes, possesses potential anti-cancer activities by inducing apoptosis of colon cancer cells and regulating gene expression, it nourishes intestinal cells and induces mucin production allowing changes in bacterial adhesion and improving tight-junction integrity (Barczynska et al., 2016; Ríos-Covián et al., 2016; Rowland et al., 2017). TMIF samples showed increased production of this SCFA (with higher production for the undigested form). Propionate acts as an energy source for epithelial cells, has a positive effect on the growth of hepatocytes, and also plays a role in gluconeogenesis in the liver (Barczynska et al., 2016; Ríos-Covián et al., 2016; Rowland et al., 2017). Propionate is also correlated with the promotion of satiety and with the reduction of cholesterol (Louis and Flint, 2017). This study showed an increased production of this SCFA over time and higher production in TMIF samples than in negative control (Fig 2). Acetate, which can be produced by bifidobacteria is an essential co-factor/metabolite for the growth of other bacteria, and even to inhibit enteropathogens (Ríos-Covián et al., 2016; Rowland et al., 2017). Acetate was also found to reduce the appetite through the interaction with the central nervous system (Ríos-Covián et al., 2016). Acetate is used by the human body in cholesterol metabolism and lipogenesis (Rowland et al., 2017). **The present study supports the findings that acetate is one of the most abundant SCFA, as it shows high production values particularly after 8 hours (Fig 2). This was also observed in the TMIF samples where acetate concentration was higher than in the negative control sample, indicating that TMIF fermentation resulted in production of this acid. Although for the undigested and digested TMIF the acetate and propionate production were almost the same, the undigested sample had higher production of butyrate compared to the digested form. Overall these results are promising for the application of TMIF as a substitute of animal derived proteins in foods since acetate and propionate are both associated with the promotion of satiety.**

336 Lactate is also produced by bacteria, such as bifidobacteria and proteobacteria, despite not being
337 a SCFA. In addition, lactate can also be used by butyrate and propionate producing bacteria,
338 avoiding accumulation and metabolic acidosis (Flint et al., 2015; Ríos-Covián et al., 2016).
339 Lactate production was higher in fermentation of FOS than in the other samples. No significant
340 difference was found between the negative control and TMIF samples. An interesting result
341 was observed in all samples as there was no lactate after 8 h (at 24 and 48 h). This is an expected
342 result since, under normal physiological conditions, lactate does not accumulate in the colon
343 because of its conversion into different organic acids through metabolic cross-feeding (Flint et
344 al., 2015; Ríos-Covián et al., 2016; Rowland et al., 2017).

345 TMIF samples produced higher concentration of BCFA than both the negative and positive
346 controls particularly after 24 hours (**Fig 3**). The undigested TMIF sample was the one with the
347 highest concentration of valerate, isobutyrate and isovalerate, especially at 24 and 48 h.
348 Concentrations of these acids at 0 h and 4 h are null or very low for most trials, and significant
349 levels appear mainly after 8 h. Moreover, digested TMIF was the only sample to show no
350 valerate production. Little is known of the potential health benefit of valeric acids, how they
351 are produced in the gut microbiota and what type of bacteria are these acids related to (Ríos-
352 Covián et al., 2016).

353 These results showed a major impact of TMIF in the undigested form, especially over 8 h, on
354 the production of the valerate, isobutyrate and isovalerate, which are normally present at low
355 concentration in the human colon while acetate, propionate and butyrate are the most abundant
356 (90-95%) (Huda-Faujan et al., 2010; Ríos-Covián et al., 2016). Isobutyrate and isovalerate are
357 primarily produced from the protein degradation particularly, from branched amino acids
358 fermentation and an increase in production of these acids maybe observed when the presence
359 of carbohydrate is limited (Huda-Faujan et al., 2010). Fecal concentrations of BCFA are
360 markers for bacterial protein fermentation, and not actual indicators of colonic health
361 (Bernalier-Donadille, 2010; Scott et al., 2013; Verbeke et al., 2015).

362 In summary, TMIF, in undigested or digested form, showed a positive impact on the production
363 of SCFA and BCFA. The production of these by the gut microbiota may contribute to the host's
364 well-being.

365

366 **3.3. Analysis of the impact on ammonia production**

367 Considering the high protein level of TMIF, a small increase of ammonia may be expected with
368 the fermentation of this substrate, as ammonia forms from the deamination of amino acids

369 (Conlon and Bird, 2014; Davila et al., 2013; Ríos-Covián et al., 2016; Rowland et al., 2017;
370 Scott et al., 2013). The presence of ammonia is an indicator of protein presence and degradation.
371 Ammonia levels increased overtime in the presence of digested and undigested TMIF, in a
372 similar concentration and pattern, except at 8 h (**Fig 4**). Bacteria degrade the protein present in
373 the samples, thus becoming an indicator of protein degradation, occurring along the
374 fermentation time.

375 Fecal ammonia concentration in humans varies between 12 mM to 30 mM and increase with
376 high intakes of protein (Scott et al., 2013). Higher levels can be considered negative for
377 colonocytes, however, ammonia concentration levels obtained in this study seem to be within
378 “safe levels” (up to 70 mM) (Leschelle et al., 2002; Tsujii et al. 1992).

379

380 **4. Conclusions**

381 According to the gut microbiota fecal *in vitro* model, TMIF had a positive impact as it promoted
382 the growth of *Bacteroidaceae* and *Prevotellaceae* but not of *Clostridium histolyticum* group or
383 *Desulfovibrionales* and *Desulfuromonales*. Also, TMIF showed a positive impact on the
384 production of SCFA especially acetate and propionate and on the production of BCFA. The
385 ammonia production in the TMIF samples was within concentration levels that are considered
386 to have no cytotoxic effects. Therefore, TMIF shows potential as a protein source for human
387 consumption due to its nutritional content and SCFA generating properties. Moreover, TMIF
388 resulted in an increase in the production of acetate and propionate, these compounds have been
389 associated with promotion of satiety (Louis and Flint, 2017; Ríos-Covián et al., 2016). This also
390 opens the possibility for a protein enriched product without animal derived proteins and
391 possibly additional functionalities. Human trials will be required to prove the additional
392 functionalities.

393

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396 running of the *in vitro* model.

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399

400 **6. Conflict of interest**

401 The authors have no financial or other type of relationship with insect industry that would
402 present a conflict of interest.

403

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List of tables:

Table 1 - Nutritional composition of TMIF (per 100g), **as provided by the manufacturer.**

Component	Concentration
Total sugar	<0.10 g
Amino acids	5.4 g
Cholesterol	0.002 mg
Fiber	3.0 g
Fat	39.4 g (saturated- 8.6g)
Carbohydrates	<0.10 g
Humidity	7.5 g
Protein	44.6 g
Sodium	142 mg
Energetic value	539 kcal ⇔ 2242 kJ

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631 **Table 2** - 16 rRNA oligonucleotide probes and hybridisation conditions used in the FISH
 632 analysis. *These probes were used together in equimolar concentration of 50 ng/μL.

Probe name	Specificity	Sequence (5' - 3')	Reference
Non Eub338	---	ACTCCTACGGGAGGCAGC	Wallner et al. (1993)
Eub338 I-II-III*	Members of the domain Bacteria	GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT	Daims et al. (1999)
Bif164	<i>Bifidobacterium</i> spp.	CATCCGGCATTACCACCC	Langendijk et al. (1995)
Lab158	<i>Lactobacillus</i> spp./ <i>Enterococcus</i> spp.	GGTATTAGCAYCTGTTTCCA	Harmsen et al. (1999)
Bac303	Most <i>Bacteroidaceae</i> and <i>Prevotellaceae</i> , some <i>Porphyromonadaceae</i>	CCAATGTGGGGGACCTT	Manz et al. (1996)
Erec482	Most of the <i>Clostridium</i> coccoides/ <i>Eubacterium</i> <i>rectale</i> group (<i>Clostridium</i> cluster XIVa and XIVb)	GCTTCTTAGTCARGTACCG	Franks et al. (1998)
Chis150	Most of the <i>Clostridium</i> <i>histolyticum</i> group (<i>Clostridium</i> cluster I and II)	TTATGCGGTATTAATCTYCCTT	Franks et al. (1998)
Rrec584	<i>Roseburia</i> subcluster	TCAGACTTGCCGYACCGC	Walker et al. (2005)
Ato291	<i>Atopobium</i> cluster	GGTCGGTCTCTCAACCC	Harmsen et al. (2000)
Prop853	<i>Clostridium</i> cluster IX	ATTGCGTAACTCCGGCAC	Walker et al. (2005)
Fprau655	<i>Fecalibacterium prausnitzii</i> and related sequences	CGCCTACCTCTGCACTAC	Devereux et al. (1992)
DSV687	Most <i>Desulfovibrionales</i> (excluding <i>Lawsonia</i>) and <i>Desulfuromonales</i>	TACGGATTTCACTCCT	Hold et al. (2003)

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638 **Figure captions:**

639 **Figure 1** - Bacterial populations (log (cells/mL), means \pm SD) detected by FISH-FCM in Fecal
640 samples (negative control (□), positive control (□), undigested TMIF (■) and digested
641 TMIF (▨)). The used probes: I) total bacteria (Eub338), II) *Bifidobacterium* spp. (Bif164),
642 III) *Lactobacillus* spp. (Lab158), IV) most *Bacteroidaceae* and *Prevotellaceae* (Bac303) and
643 V) *Atopobium* cluster (Ato291), VI) *Clostridium coccooides* / *Eubacterium rectale* group
644 (Erec482), VII) most of the *Clostridium histolyticum* group (Chis150), VIII) *Roseburia*
645 subcluster (Rrec584), IX) *Clostridium* cluster IX (Prop853), X) *Faecalibacterium prausnitzii*
646 (Fprau655) and XI) *Desulfovibrionales* and *Desulphuromonales* (DSV687). Different letters
647 mark statistically significant ($p < 0.05$) differences between samples at each sampling point.

648

649 **Figure 2** - Concentration (mM, means \pm SD) of the SCFA and lactate produced along
650 fermentation time in Fecal samples (negative control (□), positive control (□), undigested
651 TMIF (■) and digested TMIF (▨)). Different letters mark statistically significant ($p < 0.05$)
652 differences between samples at each sampling point.

653

654 **Figure 3** - Concentration (mM, means \pm SD) of BCFA and valerate produced along
655 fermentation time in Fecal samples (negative control (□), positive control (□), undigested
656 TMIF (■) and digested TMIF (▨)). Different letters mark statistically significant ($p < 0.05$)
657 differences between samples at each sampling point.

658

659 **Figure 4** - Concentration (mM, means \pm SD) of ammonia produced along fermentation time in
660 Fecal samples (negative control (□), positive control (□), undigested TMIF (■) and
661 digested TMIF (▨)). Different letters mark statistically significant ($p < 0.05$) differences
662 between samples at each sampling point.

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