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

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Abstract

Human diet has evolved to include not only nutritious foods but also health-promoting ones. Moreover, there is an increasing interest in replacing animal proteins as the main protein source. Insect based foods, e.g. *Tenebrio molitor* insect flour (TMIF), show potential as alternative protein sources for the human diet. This work aims to provide insights into the effect of TMIF upon the human gut microbiota and their metabolic end products by using an *in vitro* fecal model. Digested TMIF had a positive impact on gut microbiota, observed as an increase and/or upkeep of health promoting bacterial groups and by the production of SCFA (1.7 and 2.6 times higher acetate and propionate produced respectively than in the negative control at 48 h) and BCFA. A path is opened to acknowledge TMIF as a possible healthy nutritional source for human consumption, although *in vivo* trials would be necessary to confirm this.

Keywords: *in vitro* digestion, anaerobic fecal fermentation model, insect flour, protein, gut microbiota, SCFA, BCFA

1. Introduction

Changes in consumer awareness of global food sustainability are leading to consideration of alternative protein sources. Emerging studies indicate that insects could be a good source of protein, fiber, fatty acids and amino acids (Chen et al., 2009; Kouřimská and Adámková, 2016; Mlcek et al., 2014; Premalatha et al., 2011). As such, research into the benefits of insect consumption to the consumer is required. Currently, one of the most commercially used insects

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

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

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

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

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

2. Material and methods

2.1. *Tenebrio molitor* insect flour (TMIF)

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

2.2. TMIF sterilization

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

2.3. *In vitro* gastrointestinal digestion protocol

The TMIF digestion used an *in vitro* method mimicking *in vivo* conditions as described by Mills et al. (2008) with slight modifications. 20 g of TMIF were ground and dissolved in 50 mL distilled water and the mixture put in a stomacher (Seward, Worthing, UK) for 5 min. For the oral phase, 6.66 mg of α-amylase (A 4551, Sigma) in 2.08 mL of 0.001 M $CaCl_2$ at pH 7.0 was added and incubated at 37 ºC for 30 min on a shaker. After this, 6 M HCl was used to lower the pH to 2.0. For the gastric phase, 0.9 g of pepsin (P 7000, Sigma) was dissolved in 8.33 mL of 0.1 M HCl in a volumetric flask and this pepsin solution was added to the samples and incubated at 37 ºC for 2 h on a shaker. For the small intestinal phase, a pancreatin and bile solution was prepared. For that, 186.67 mg of pancreatin (P 8096, Sigma) and 1.17 g of bile (B 8631, Sigma) were dissolved in 41.67 mL of 0.5 M $NaHCO_3$, and pH was adjusted to 7.0 with either 6 M of HCl or NaOH and incubated at 37ºC for 3 h on a shaker. The simulated digestion process of the TMIF also included a dialysis step to simulate the absorption in small intestine, in order to analyze the different behavior of bacteria present in the gut microbiota (Alegría et al., 2015; Verhoeckx et al., 2015). All samples were transferred to 100-500 Da molecular weight cut-off regenerated cellulose dialysis tubing (Spectra/Por® 6, Spectrum Europe, Netherlands) and a dialysis was performed against 1 M NaCl at 5 ºC to remove low molecular mass digestion products. After 15 h, the dialysis fluid was changed and performed for two additional hours. Afterwards, all samples were transferred to a freeze dryer (Armfield SB4 model, Ringwood,

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

2.4. Gut microbiota simulation: Fecal fermentations

2.4.1. Fecal microbiota

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

2.4.2. Fecal batch-culture fermentation conditions

Five independent fermentations were carried out using a sample from each donor. Sterile stirred batch culture fermentation vessels of 300 mL were set up and aseptically filled with 135 mL sterile basal nutrient medium (peptone water 2 g/L, yeast extract 2 g/L, NaCl 0.1 g/L, K_2HPO_4 0.04 g/L, KH_2PO_4 0.04 g/L, $MgSO_4.7H_2O$ 0.01 g/L, $CaCl_2.6H_2O$ 0.01 g/L, $NaHCO_3$ 2 g/L, 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 g/L and resazurin 4 mg/L) and gassed overnight with O_2-free N_2 with constant agitation. The temperature was kept at 37ºC. Four stirred pH-controlled batch fermenters were run in parallel: (1) 1% (w/v) digested TMIF was aseptically added; (2) 1% (w/v) undigested TMIF was aseptically added; (3) a positive control with 1% (w/v) FOS (a known prebiotic) from chicory root, purity: > 95%, degree of polymerization ranging from 2 to 8 (Megazyme, Bray, Ireland) and (4) a negative control which had no source of carbon added. Each vessel, with 135 mL of sterile basal nutrient medium was inoculated with 15 mL of fresh fecal slurry. A FerMac 260 pH Controller (Electrolab Biotech Ltd., Tewkesbury, Gloucestershire, UK) was used, at 37 ºC, to maintain pH for each vessel between 6.7 and 6.9 (Sánchez-Patán et al., 2012).The batch cultures were conducted under anaerobic conditions at 37 ºC during 48 h, in which 5 mL samples were collected from each vessel at 0, 4, 8, 24 and 48 h for bacterial enumeration by fluorescence *in situ* hybridization (FISH), analysis of SCFA, BCFA and lactate by gas

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

2.5. Bacterial enumeration by FISH-FCM

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

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

2.6. Evaluation of organic acids production by GC

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

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

2.7. Evaluation of ammonia production

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

2.8. Statistical analysis

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

3. Results and Discussion

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

3.1. Analysis of the impact on bacterial composition

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

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

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

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

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

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

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

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

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

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

Desulfovibrionales and *Desulfuromonales* are only found in approximately fifty percent of humans (Rey et al., 2013). Predominant sulphate-reducing bacteria (SRB) in human colon are members of the genus *Desulfovibrio.* They can use hydrogen or organic compounds like lactate and formate to reduce sulphate to generate hydrogen sulphide (H_2S), which has a toxic nature, that can have pathological consequences for the host (Conlon and Bird, 2014; Rowland et al., 2017). Several studies identified SRB in the fecal microbiota of healthy adults and, despite being positively correlated with inflammation, the presence of H_2S, has been attributed both to pro and anti-inflammatory signaling (Levine et al., 1998; Pitcher et al., 2000; Rey et al., 2013; Wallace et al., 2009). Lactate is also a favored co-substrate for these bacteria, forming acetate and sulphides. *Desulfovibrio* was reported to decrease in the presence of inulin, and studies showed that its lowered abundance can benefit health (Vinke et al., 2017). For this study, this group of bacteria was found in lower concentrations in the fecal samples of the donors, compared to other quantified groups. At 8 h, for all samples, there was a small increase of *Desulfovibrionales* and *Desulfuromonales* with no significant differences between the samples. After 8 h (24 and 48 h) a decline over time was observed. The small increase of these bacteria at 8 h may be correlated with the availability of lactate at that time (**Fig 1**).

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

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.

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

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

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

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

3.3. Analysis of the impact on ammonia production

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

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

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

4. Conclusions

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

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6. Conflict of interest

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

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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	*Bifidobacterium* spp.	CATCCGGCATTACCACCC	Langendijk et al. (1995)
Lab158	*Lactobacillus* spp./ *Enterococcus* spp.	GGTATTAGCAYCTGTTTCCA	Harmsen et al. (1999)
Bac303	Most *Bacteroidaceae* and *Prevotellaceae*, some *Porphyromonadaceae*	CCAATGTGGGGGACCTT	Manz et al. (1996)
Erec482	Most of the *Clostridium* coccoides/ *Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb)	GCTTCTTAGTCARGTACCG	Franks et al. (1998)
Chis150	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)	TTATGCGGTATTAATCTYCCTTT	Franks et al. (1998)
Rrec584	*Roseburia* subcluster	TCAGACTTGCCGYACCGC	Walker et al. (2005)
Ato291	*Atopobium* cluster	GGTCGGTCTCTCAACCC	Harmsen et al. (2000)
Prop853	*Clostridium* cluster IX	ATTGCGTTAACTCCGGCAC	Walker et al. (2005)
Fprau655	*Fecalibacterium prausnitzii* and related sequences	CGCCTACCTCTGCACTAC	Devereux et al. (1992)
DSV687	Most *Desulfovibrionales* (excluding *Lawsonia)* and *Desulfuromonales*	TACGGATTTCACTCCT	Hold et al. (2003)

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Figure captions:

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

Figure 2 - Concentration (mM, means ± SD) of the SCFA and lactate produced along fermentation time in Fecal samples (negative control (☐), positive control (☐), undigested TMIF (▩) and digested TMIF (▨)). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

Figure 3 - Concentration (mM, means ± SD) of BCFA and valerate produced along fermentation time in Fecal samples (negative control (☐), positive control (☐), undigested TMIF (▩) and digested TMIF (▨)). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

Figure 4 - Concentration (mM, means ± SD) of ammonia produced along fermentation time in Fecal samples (negative control (☐), positive control (☐), undigested TMIF (▩) and digested TMIF (▨)). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.