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Potential prebiotic activity of *Tenebrio molitor* insect flour using an optimized *in vitro* gut microbiota model

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Abstract

The *Tenebrio molitor* insect flour (TMIF) is considered a nutritive food ingredient, but its impact at the gut microbiota level and its potential prebiotic activity still need be assessed. For such studies, an *in vitro* simulation model of gut microbiota was optimized. Pure cultures of *Lactobacillus* and *Bifidobacterium* strains in monocultures and co-cultures (pairs and consortium) were used on this model to evaluate the effect of TMIF on the viability and metabolic activity of those bacteria. The optimization of the *in vitro* model of the gut microbiota was successful, and growth of the most important groups of bacteria in the gut microbiota was observed. As so this model can be used to study the effects of other ingredients at that level. It also enabled to pinpoint prebiotic effects of the studied TMIF suggesting possible symbiotic interactions. Additionally, the direct effect of the TMIF on bacterial cells, when in nutritive stress was also evaluated. In terms of TMIF effects on probiotic bacteria viability and growth, no negative effects were observed, and even an enhancement of growth and an increase of production of short chain fatty acids (SCFA) and lactate, in most of the cases, was observed. Also, this study showed that TMIF help maintaining the viability of bacteria during incubation time when these are under nutritional stress conditions. A potential prebiotic effect of TMIF is then predictable with this study, opening a path to new researches on this subject.

Key words: gut microbiota; *in vitro* study; *Tenebrio molitor* insect flour; probiotics; prebiotic effect

1. Introduction

Health can be sometimes described as an obsession, but it can never be forgotten or disregarded, no matter the cultural beliefs and/or social-economic status. Regardless to say, health is the key to survival.

Therefore, enhancing our health, or simply maintaining it at minimal desirable levels, is and has been, a crucial requirement for humanity. Related to health promoting practices, health care and healthy lifestyle, is the idea of equilibrated eating habits. Studies show the huge impact of diet patterns on health. So, today demanding healthier food sources is of great importance.

Recent studies have been showing the relevance of insects as food source due to their nutritional content, and despite some difficulties on introducing them to some cultures and their eating habits, owing to the “repulsive” factor, their introduction on the market can be softened if they are processed in a flour form for example.^{1,2} One of the most commercialised insect flours is the one from yellow mealworm larvae (*Tenebrio molitor*), which is a rich source of protein and an excellent source of fatty acids and fibre.³ Insects have a high protein content, generally of good quality and highly digestible and are a good source of essential amino acids.⁴⁻⁶ The impact studies of such diet in the human organism is then particularly relevant, especially at the gut microbiota level, which is a good indicator for individual health. Gut microbiota is the name given to the microbe population living in the intestine, especially at colon. The main phyla found in the human gut are Firmicutes (where *Lactobacillus* are included), Bacteroidetes, and in a smaller representation the Proteobacteria and Actinobacteria (where it can be found *Bifidobacterium*).⁷ These bacteria are of primordial importance for the well-being of the individual, as in the case of disease, or even in the maintenance of the immunological activity.⁸ Hence, nowadays it is important to evaluate impact of proteins, fatty acids, vitamins and mineral salts in gut microbiota.⁹⁻¹¹

The *in vitro* simulation of the gut microbiota is a method to reduce the use of *in vivo* models, and it is useful to set-up and explore different conditions and compositions for the study, thus enabling the simulation of the gut conditions.¹² In gut microbiota, *Lactobacillus* and *Bifidobacterium*, the most important genera of gut for their probiotic activities, are susceptible to the action of prebiotics.¹³ Prebiotics, as is referred in Gibson *et al.* (2017)¹⁴, is seen as “a substrate that is selectively utilised by host microorganisms conferring a health benefit”. The expected health effects of prebiotics include benefits, to the gastrointestinal tract, to the cardiometabolism, to mental health, bone, and other.¹⁴

One of the functions of gut microbiota is the production of short chain fatty acids (SCFA). A healthy microbiota significantly reduces the prevalence of inflammatory diseases, notably through the release of SCFA. Fermentation and SCFA production inhibit the growth of pathogenic organisms reducing the luminal and faecal pH and directly promoting the growth of symbionts.¹⁵⁻¹⁷ This allows a decrease of peptide degradation, and, consequently, of the formation of toxic compounds such as ammonia, amines, and phenolic compounds, and decreases the activity of undesirable bacterial enzymes.¹³

With enhancement of indigenous bifidobacteria and lactobacilli, with proven prebiotic and probiotic properties, increasing survival and activity of an organism, a definition of synbiotics is achieved.^{18, 19} On a daily basis, humans already consume foods that have proven to enhance the activity of indigenous bacteria in the human gut microbiota. Fermented foods and beverages are examples of well-established products (e.g. fermented milk, processed cheeses and yogurts) on the human diet that support the delivery of probiotics to the gastrointestinal tract.^{20, 21} At the human gut microbiota level insect food, in the form of insect flour, is also prominent to achieve benefits, due to all its characteristics mainly by interacting with probiotics and even changing their metabolism. Nevertheless, nutritional challenges offer themselves not only in the form malnutrition, but also as lack of nutrient, and the impact of a food source, as insect flour, at the gut microbiota level when the most representative genera find themselves in a nutritional stress environment is also a factor in consideration.

This study aimed to find and access the possible prebiotic effect of *Tenebrio molitor* insect flour (TMIF) on probiotic bacteria, at the gut microbiota level through the optimization of an *in vitro* model. The assessment of the impact of TMIF was focused on probiotic bacteria *Lactobacillus* and *Bifidobacterium* strains. Within the obtained results and the research track, it also broadened its objectives to evaluate those same parameters in terms of nutritional and survival behaviour of the studied bacteria, when in nutritional stress conditions.

2. Materials and Methods

2.1. *Tenebrio molitor* insect flour (TMIF) and sterilisation process

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 at **Table 1**. In order to guarantee that the TMIF under study was free of microorganisms (below of the quantification limit, 2.88 log CFU/mL) , different heating/UV processes were selected assuming an efficient microbial elimination and the lowest impact on flour quality in terms of protein denaturation: 1) UV exposure (laminar flow

chamber) for 50 min; 2) drying for 24 h in an incubator at 40 °C; 3) drying for 24 h in an incubator at 40 °C followed by radiation UV exposure for 50 min; 4) dissolution of TMIF in nutrient broth (Biokar Diagnostics, Pantin, France) and classic low temperature and time pasteurisation for 30 min at 65 °C; 5) dissolution of the TMIF in the nutrient broth (Biokar Diagnostics, Pantin, France) and pasteurisation for 30 min at 80 °C; 6) drying the TMIF at 100 °C for 24 h and 7) sterilisation at 121°C at 20 min in the autoclave. TMIF was added to the nutrient broth (Biokar Diagnostics, Pantin, France) at 1% (w/v), and then incubated for 24 h at 37 °C under aerobic conditions. At 0 and 24 h of incubation time, decimal dilutions in 0.1% (w/v) peptone water were made and plated using the Miles and Misra technique²² on plate count agar (PCA), incubated at 30 °C for 24 h and on potato dextrose agar (PDA), incubated at 30 °C, up to five days.

2.2. Microorganisms and cultures conditions

All microorganisms used and their growing conditions are listed in the **Table 2**. All *Lactobacillus* and *Bifidobacterium* in the **Table 2** have been classified as probiotics according with their manufacturers.

2.3. Chemical and biological simulation of gut conditions

Gut bacterial growth media simulation was performed in terms of nutrients, salts, substrates and pH level according to Madureira *et al.* (2016)²³. The composition of this media contained 5.0 g/L trypticase soy broth (TSB) without dextrose (BBL, Lockesville, USA), 5.0 g/L bactopectone (Amersham, Buckinghamshire, UK), 5.0 g/L yeast nitrogen base (BD, Wokingham, UK), 1.0% (v/v) of salt solution A (100.0 g/L NH₄Cl, 10.0 g/L MgCl₂.6H₂O and 10.0 g/L CaCl₂.2H₂O), 0.2 % (v/v) of salt solution B (200.0 g/L K₂HPO₄.3H₂O), 0.2% (v/v) of 0.5 g/L resazurin solution, 10.0 mL/L trace mineral supplement (ATCC, Virginia, USA) and prepared in distilled water. All probiotic bacteria mentioned in **Table 2**. were used as monocultures, co-cultures (paired) or as consortium (three cultures). Overnight inocula were added to the simulation media at two testing cell concentrations, 1 and 10% (v/v) and incubated for 48 h under anaerobic conditions. Individual cultures as well the mixtures were tested in duplicate and designated with the following abbreviations: A1–1% *Bifidobacterium animalis* ssp. *lactis* Bb12[®] + 10% *Lactobacillus casei* 01; A2–10% *B. animalis* ssp. *lactis* Bb12[®] + 1% *L. casei* 01; B1–1% *Bifidobacterium animalis* Bo + 10% *Lactobacillus acidophilus* LA-5[®]; B2- 10% *B. animalis* Bo + 1% *L. acidophilus* LA-5[®]; C1-1%

Bifidobacterium longum BG3 + 10% *Lactobacillus rhamonosus* R11; C2- 10% *B. longum* BG3 + 1% *L. rhamonosus* R11; D1- 3.33% *Bifidobacterium animalis* ssp. *lactis* Bb12® + 3.33% *B. animalis* Bo + 3.33% *B. longum* BG3 + 0.33% *L. casei* 01 + 0.33% *L. acidophilus* LA-5® + 0.33% *L. rhamonosus* R11; D2- 0.33% *B. animalis* ssp. *lactis* Bb12® + 0.33% *B. animalis* Bo + 0.33% *B. longum* BG3 + 3.33% *L. casei* 01 + 3.33% *L. acidophilus* LA-5® + 3.33% *L. rhamonosus* R11. Controls were made only using the basal media without bacteria. All additions and inoculations were carried out inside an anaerobic cabinet (5% H₂, 10% CO₂ and 85% N₂).

2.4. TMIF fermentation conditions

TMIF was added to the simulation media at 1% (w/v) and the gut microbiota model was used as described previously in the **section 2.3**. The simulation of fermentation was performed during 48 h at 37 °C in anaerobic conditions. Microorganisms enumeration was made at 0, 24 and 48 h of incubation time, by performing decimal dilutions in 0.1% (w/v) peptone water and plated using the Miles and Misra technique²² in de Man, Rogosa and Sharpe (MRS) agar and in PCA plates. The MRS agar plates were incubated following the conditions described in **Table 2** while in the case of PCA plates were incubated at 30 °C during 24 h. To distinguish *Bifidobacterium* and *Lactobacillus* colonies growth in MRS agar media, a dye, bromophenol blue was added to the agar media at 0.002% (MRS-BPB) as described in Lee and Lee (2008).²⁴ In this media, in anaerobic conditions, *Lactobacillus* acquires a light blue colour, while *Bifidobacterium* grows dark blue and in smaller dimensions allowing colony selective enumeration.

2.5. Evaluation of organic acids production by HPLC

Aliquots of each sample were taken at times 24 and 48 h and centrifuged at 20,817 x g for 15 min at room temperature. After centrifugation, the supernatant of each sample was transferred to vials and analysed by high performance liquid chromatography (HPLC) as described in Sousa *et al.* (2015)²⁵ with slight modifications. Conditions for the HPLC system consisted of a LaChrom L-7100 pump (Merck-Hitachi, Germany), an ion exchange Aminex HPX-87H Column (300 x 7.8 mm) (Bio-Rad), which was maintained at 65 °C (L-7350 Column Oven; LaChrom, Merck-Hitachi); and one detector, spectrophotometry to analyse organic acids (220 nm) (L-7400 UV Detector; LaChrom, Merck-Hitachi). The mobile phase used was 13 mM

168 sulphuric acid at a flow rate of 0.6 mL/min. The running time was 30 min, and the injection
169 volume was 50 μ L.

171 2.6. Evaluation of pH changes over time

172 Changes in pH were followed with a Crison microPH 2002 pH reader (Crison Instruments, S.
173 A., Barcelona, Spain). The pH evaluation over the incubation time was evaluated by the
174 average pH at time 0, 24 and 48 h and by the pH reduction rate obtained using the following
175 equation:

$$176 \quad pH \text{ reduction rate} = \left(\frac{pH \text{ time } 0 \text{ h} - pH \text{ time } 48 \text{ h}}{pH \text{ time } 0 \text{ h}} \right) \times 100 \%$$

178 2.7. Nutritive stress conditions simulation

179 In order to simulate a condition of nutritional stress, the inoculation of bacterial cells in 0.1%
180 (w/v) peptone water with 0.85% (w/v) of NaCl was performed. Overnight inocula of 10%
181 (v/v) bacteria was centrifuged during 15 min at 2,820 x g to obtain a cell pellet. The pellet
182 was washed at least two times with 0.1% (w/v) peptone water. The pellet was resuspended in
183 0.1% (w/v) peptone water with 0.85% (w/v) of NaCl²⁶ and TMIF was added to the media at
184 1% (w/v) and control was performed without TMIF. All additions and inoculations were
185 carried out inside an anaerobic cabinet (5% H₂, 10% CO₂ and 85% N₂) to avoid any oxygen
186 contact, mimicking colon conditions. Microorganisms enumeration was made at 0, 3, 6, 12,
187 24 and 48 h of incubation time, by performing decimal dilutions in 0.1% (w/v) peptone water
188 were made and plated in de MRS agar for probiotic bacterial strains counts and in PCA for
189 bacterial contamination counts, using the Miles and Misra technique.²² MRS agar plates were
190 incubated following the conditions described in **Table 2** while PCA plates were incubated at
191 30 °C during 24 h.

193 2.8. Statistical analysis

194 Statistical analysis of the results was carried out using IBM SPSS software (24.0.0.0, IBM,
195 Chicago, USA). The normality of the distributions was evaluated using Kolmogorov-Smirne's
196 test. As the samples followed normal distributions the means were compared, considering
197 95% confidence interval, using one-way ANOVA coupled with Tukey's post-hoc test. The
198 weight of the different factors (independent variables) in the differences observed, such as
199 bacteria strain, % inocula and incubation time was evaluated by comparing the F values for
200 the two different studies with and without TMIF.

3. Results and Discussion

3.1. TMIF sterilisation

Different sterilisation processes of TMIF were tested to obtain a flour free of contaminants. Nevertheless, some careful attention was taken to the stability of the protein present in the flour by FPLC (results not shown). In terms, of the microbiological analyses, no viable cell numbers were obtained in PCA for two of the tested TMIF sterilisation processes: drying at 100 °C for 24 h in the incubator and sterilisation at 121 °C for 20 min in an autoclave. As the results were the same for both methods, the drying process at 100 °C for 24 h was chosen owing to the lower temperature and absence of pressure.²⁷

3.2. Gut microbiota *in vitro* model

Since gut microbiota is composed by several bacterial strains, this model was developed first by testing the use of monocultures, then in co-cultures as pairs and finally the use of 3 strains of each genus in order to closely simulate a possible consortium of probiotic bacteria existent at human gut. Two percentages of inocula (1 and 10%) were used since *Lactobacillus* and *Bifidobacterium* can be found with differences of 1 log or more in gut microbiota and that these numbers differ from individual to individual to individual.²³ In order to study the impact of TMIF on selected gut microbiota bacteria strains, samples with TMIF were compared with controls (without TMIF). The results were expressed in bacterial growth (log CFU/ml) over time (0, 24 and 48 h). The statistical differences of growth of the same strain or mixture observed during incubation time were evaluated.

3.3. Effect of TMIF in the viability of the probiotic bacteria

Growth of probiotic bacteria inoculated at two different percentages (1 and 10%) and with or without TMIF along fermentation time of 48 h is shown in **Figure 1**. The percentage of inocula, incubation time and bacteria affected the growth profile of the tested bacteria ($p<0.05$), with exception of *B. animalis* BG3 and *B. animalis* Bo ($p>0.05$). In the presence of TMIF, all the factors such as bacteria strain, incubation time and percentage of inocula affected the growth of bacteria along the fermentation time, and the percentage of inocula was the most significant factor ($p<0.05$; $F=381$). In presence of TMIF, the *Lactobacillus* strains, at the highest concentration (10%), seem to have a better upkeep. *Lactobacillus acidophilus* behaved differently from all the other *Lactobacillus* strains ($p<0.05$). This strain, at 10%, showed a growth reduction at 24 h

234 compared to initial time (0 h), but later recovered its cell concentration, as observed at time 48
235 h. Such behaviour may partially be explained as *L. acidophilus* is a slow acid producer.^{28, 29}
236 The other *Lactobacillus* strains studied showed similar growth profiles along incubation times
237 ($p>0.05$). At all fermentation times, significant differences, were only observed between the
238 initial time (0 h) and times 24 h or 48 h. Between 24 h and 48 h there is no significant
239 difference in terms of growth ($p>0.05$). In general, the higher cell growth was observed for
240 10% inocula than for 1% at the end of incubation time (48 h). In the case, of 1% of inocula
241 the presence of TMIF positively affected *Lactobacillus* strains growth, except *L. casei* at the
242 end of incubation time (48 h).
243 *Lactobacillus* strains are more positively affected by the presence of TMIF when compared to
244 *Bifidobacterium*. In the case of *Bifidobacterium* strains, at the higher inocula concentration
245 (10%) over 24 h of incubation, TMIF had no impact on the bacteria cell levels maintenance.
246 As for the same period of time, at 1% the results are similar, with no impact, except for the *B.*
247 *longum* BG3 with a small cell growth detected. Nevertheless, at 48 h the presence of TMIF
248 showed a positive impact on *B. longum* BG3 at 10% and *B. animalis* Bo, at 1%.
249 Once again, the greater effect of the flour was observed after 24 h of growth, i.e. at 48 h of
250 incubation time. This may be due to a decrease of nutritional content in the simulation basal
251 media during the experience, since it is the most accessible nutrient source for the bacteria,
252 and the presence of TMIF which can be used as a nutrient source for the studied bacteria, is
253 consume later to help on their upkeep.
254 **Figure 2** shows the results for the growth of co-cultures, A1, A2, B1, B2, C1, C2 and
255 consortium D1 and D2 of the studied bacteria. Overall, in the presence or not of TMIF in the
256 media, the mixture of strains and percentage of inocula were factors that affected significantly
257 growth of the bacteria ($p<0.05$). In terms of growth profiles, without significant differences it
258 is possible to distinguish the consortium (D1 and D2) from the remaining mixtures.
259 Incubation time was a factor that did not affected the growth profile ($p>0.05$), since overall,
260 the differences observed were not statistically significant. Nevertheless, at 0 h, for the co-
261 cultures, independently of the initial cell concentration inoculated of *Bifidobacterium*/
262 *Lactobacillus*, *Lactobacillus* was always present in lower concentrations compared to
263 *Bifidobacterium*.²³
264 In co-cultures, TMIF had no effect after 24 h in the majority of cases. Nevertheless, in B1 co-
265 culture a small increase for *L. acidophilus* was detected, and in B2 a small increase for *B.*
266 *animalis* Bo, both cases in the presence of TMIF. These overall results differ from 48 h
267 fermentation time, where the presence of TMIF positively affects all cases except in co-

culture A1 for the *B. animalis* Bb12 and in B1 for *L. acidophilus*. As for the consortium for D1 and D2, again at 24 h no major impact was found for the presence of TMIF, except on the consortium D1 in case of lactobacilli, where it had a negative impact. Similar behaviour of the mixtures occurs at 48 h, and the presence of TMIF showed a positive impact on the consortium D1 and D2.

In sum, TMIF shows positive impact on the bacteria growth of the studied monocultures. In those cases, *Lactobacillus* appears to be the most benefited genera, when compared to the studied *Bifidobacterium* strains. As for co-cultures and consortium, it seems that a dynamic equilibrium is achieved between both genera, with a relative concentration being similarly maintained between them, no matter the presence or absence of TMIF, or even the percentage of inocula for each one (*Bifidobacterium* being the most representative specie in every cases). Nevertheless, *Bifidobacterium* strains seem to be the most benefited for the co-culture and consortium relationship, as in most cases they are the ones that show some growth, in the presence of TMIF. About the percentage of inocula, results show no significant impact of the presence of TMIF on the bacteria growth profile as monocultures, co-cultures and consortium. In what concerns with the incubation time, TMIF seems to have better and more noticeable impact on the bacteria with the passage of time of the experience comparing to the controls. Results show positive effect of TMIF on the growth and/or upkeep of the bacterial strains. *T. molitor*, in dehydrated form, shows high nutritional value, with high protein and unsaturated fat percentage, and the presence of fibre content mostly from chitin.³⁰ Hence, such nutritional content can be used by the bacteria during fermentation, so enabling them to develop metabolism and functions.^{10, 31} However, the presence of fibre content, chitoooligosaccharides, substrates derived from the degradation of chitosan (deacetylated form of chitin) and chitin, are not expected to stimulate the growth of some strains of *Lactobacillus* and *Bifidobacterium*, while other studies showed that whey peptide extracts 1% (w/v) have the capacity to stimulate the growth of some strains of *Lactobacillus* and *Bifidobacterium*.^{17, 32, 33} It is then possible to assume that the bacterial growth enhancement maybe be possibly due to protein and peptide content present on TMIF.

Studies in mice reveal that the gut bacteria alter the distribution of free amino acids in the gastrointestinal tract, and the gut microbiota also affects the bioavailability of amino acids in the host. *Lactobacillus* are amino acid fermentation bacteria and, as precursors for SCFA synthesis, amino acids play an important role in the homeostasis of the host.³⁴ *Lactobacillus* and *Bifidobacterium* are also able to secrete proteolytic enzymes.¹⁷ *Tenebrio molitor* is rich in essential amino acids (e.g. leucine, valine, lysine and isoleucine) among other amino acids, as

seen in Van Huis *et al.*, 2013³. Most of those amino acids have already proven to be preferred substrates for colonic bacteria, therefore enhancing the role played by amino acids in the modulation of the intestinal microbiota.³⁴

3.4. Effect of TMIF in the metabolic activity of the probiotic bacteria

The HPLC was performed in order to evaluate the concentration of organic acids produced (SCFA and lactate) in samples (Figures 3, 4 and 5).

In general, the percentage of inocula used, had an effect on concentration of acids produced. The use of 10% inocula promoted a higher production of acids from both bacteria genera. The organic acids acetate, propionate, butyrate and lactate produced by selected probiotics throughout fermentation were detected at both times of incubation, according the expected for these strains.^{10, 31, 35} Acetate and lactate were, as expected, and as found in other studies the most produced acids.^{36, 37} In terms of TMIF for all strains from both genera, its presence promoted a higher production of SCFA (especially acetate) and lactate. In general, at 1% of inocula, and in presence of TMIF, lactobacilli and bifidobacteria, produced higher concentrations of acetate with exception of 1% *L. casei* at time 24 h, in which, propionate was the one that showed major increment ($p < 0.05$). At 10% of inocula for both genera, in the presence of TMIF, the production of lactate was more notorious.

Lactobacilli produced acetate and lactate in higher concentration in most cases independent of the presence of TMIF. In the case of *Bifidobacterium*, without TMIF, the most produced organic acid was lactate. But this behaviour was strain dependent.

In the absence of TMIF there were almost no significant differences in the concentration of acids between both times of incubation (24 and 48 h), in contrast when in the presence of TMIF, which at time 48 h it can be observed an increment of acids concentration. In the presence of TMIF, higher concentrations of SCFA and lactate were also found for both periods of time.

In the case of lactobacilli, *L. rhamnosus* produced butyrate only in the presence of TMIF, which differentiates this strain from the other two studied lactobacilli. This may be explained, since only certain *Firmicutes* species can produce butyrate, from peptide and amino acid fermentation, reinforcing the hypothesis of protein and peptide from TMIF be the most important mechanism concerning its impact on gut microbiota. Nevertheless, it is important to keep in mind that some bacteria can change their metabolic profile and so produce different SCFA in different growth conditions.¹¹ Also, the presence of TMIF induced an increase of the production of acetate by *L. casei* at 48 h.

As shown in **Table 3**, during fermentation, for most cases, there was a pH reduction along time, with and without the presence of TMIF for both inocula concentrations. Average pH values, with and without TMIF, are similar within the same species and inocula percentage. Also, pH values for 10% inocula, for the same species were lower compared to 1% concentration. However, values tend to be approximately analogous between the different genera and species, in the same conditions. This seems to indicate that the major factor of influence in the pH value was the percentage of inocula. Therefore, this pH reduction along fermentation time is an indicator of SCFA production.

As for the production rate of SCFA in cases of co-cultures and consortium of bacteria, **Figure 5** shows that for the co-cultures and consortium with the addition of TMIF, there was a significant effect mainly in the production of lactate and acetate, obtaining higher concentration values for both cases. Also, the presence of TMIF had no negative impact on the production of the studied organic acids. In the mixtures in pairs and consortiums, all acids were produced at higher concentrations compared to those obtained for monocultures (**Figure 3 and 4**). This may be relating to the presence of a higher bacteria percentage present in the inocula. In the presence of TMIF, there is a small increase of butyrate production in some samples, and also a small increase of production of propionate, for most cases. In addition, generally, the major production of the acids was observed during the first 24 h.

The consortium of 3 strains of bifidobacteria plus 3 strains of lactobacilli, produced the same acid types as the ones produced by monocultures and co-cultures. In sample D1, the presence of TMIF had positive impacts up to 24 h for butyrate, acetate and lactate, but showed a decrease of concentration at time 48 h in comparison to those without TMIF. Generally, sample D2 produced more acids in the presence of TMIF, mainly propionate, acetate and lactate (**Figure 5**).

Since SCFA were being produced by the probiotics, a pH reduction on the media was expected, as a signal of its presence (**Table 3 and Table 4**). As such, **Table 4** confirms that, for the samples without TMIF, in most cases, there was a decrease in pH overtime for each case (bacterial species and associated percentage condition) and this also happened, in the same way, for all the cases with TMIF. For samples with and without TMIF, pH values were approximately the same between all cases.

The low pH level obtained, establishes an ideal condition for the bacteria growth and to their metabolism. In fact, the decrease of such values along time, within acceptable values (near or above 5), helps the upkeep of those probiotics, especially *Bifidobacterium*, whose growth is

retarded when pH is below 4.³⁸ At the same time, low pH values like these, inhibit the growth of pathogenic bacteria and retards peptide degradation.

Most of the obtained results are consistent with other studies showing a representative production of lactate and SCFA that derive from the process of dietary fibre fermentation by specific colonic anaerobic bacteria, in this case *Bifidobacterium* and *Lactobacillus*.^{31, 35, 39} This work also corroborates other findings, in that the most produced SCFA by these bacteria is acetate.^{16, 35, 40} Studies show that SCFA have impact at cellular and molecular levels, being butyrate a key promoter of colonic health and integrity.¹⁶ Still there is lack of data on actual fluxes of SCFA and metabolic processes regulated by them.^{39, 40}

The results obtained with TMIF, both at the probiotic growth and at organic acid production level, are in the same register of foods that are considered prebiotic (e.g. fructooligosaccharides (FOS) and galactooligosaccharides (GOS)), i.e., they promote the growth of probiotics and stimulate production of SCFA that can lead to health benefits to the host.^{41, 42} There are several studies on prebiotic effect and the impact of certain foods on the gut microbiota and probiotics present in the intestinal microbiota such as *Aloe Vera* mucilage, “horchata” co-products, seaweeds and mushrooms extract and whey peptide that can promote the growth of the probiotics or have prebiotic potential.^{17, 28, 43, 44} It can be seen from the current studies that the potential of proteins / peptides that promote probiotic growth and metabolic enhancement has not yet been fully explored. This may be related to the fact that the majority of the substances identified as prebiotics are currently non-digestible oligosaccharides.¹⁷ TMIF is a product with a high percentage of protein content which may be related to the fact that this substrate promotes the growth of studied probiotics as well as increased organic acids production of them.

This study pursues, for the first time, the hypothesis of a relevant role of insect protein on the promotion of *Lactobacillus* and *Bifidobacterium*, thus seeking such implications on the gut microbiota functions, upkeep, and metabolic role, exploring possible interactions with human health status.

3.5. Effect of TMIF in bacteria viability at nutritive stress conditions

This study aimed to evaluate the effect of TMIF when probiotics were under nutritive stress i.e. without any type of nutrient present in the growth media and with the bacterial cells under osmotic pressure. With this study, the direct effect of the flour in the bacterial cells could be evaluated (e.g. inhibitory effect). Nevertheless, the presence of TMIF positively affected all bacteria strains in this study (**Figure 6**), since in the absence of TMIF, bacteria viability

tended to reduce sooner and more rapidly, compared to the bacteria viability in the presence of TMIF. Most *Lactobacillus* cases showed a significant decrease in culture upkeep without TMIF after 24 h in comparison with TMIF cultures. For the *Bifidobacterium* strains, significant differences between with and without TMIF cultures started after 12 h (except the case of *B. animalis* Bb12 that started sooner comparing to the other two species of *Bifidobacterium*). Results indicate no antimicrobial effect of TMIF on the studied strains of *Lactobacillus* and *Bifidobacterium*. *Bifidobacterium* strains seem to better cope with nutritional stress as they tended to show less accentuated decrease overtime, compared to *Lactobacillus* strains. According to **Figure 6**, TMIF had no negative effects on the cellular viability of the studied bacteria, and that *Lactobacillus* and *Bifidobacterium* can use TMIF as a substrate to survive when under the previously mentioned conditions.

3.6. In vitro methods and food ingredients impact studies

The optimized *in vitro* gut microbiota simulation model presented itself as an essential tool to screen proprieties of substrates, as dietary ingredients, in this case TMIF, enabling the assessment on how they impact on gastrointestinal environments and bacteria populations, as *Lactobacillus* and *Bifidobacterium*, particularly for this study objectives. This simple and costless method, as compared to others, may be seen as a precursor to complex methods, showing first hand, valuable results in term of what can be expected from the impact or interaction of a food or food ingredient with the gut microbiota, in terms of bacterial monocultures, or even in complex bacteria interactions by the establishment of consortiums, enabling the comprehension of bacteria behaviour changes in different conditions. This work leads the path to understanding results that pinpoint possible metabolic responses to specific ingredients or food, open the path to later studies, on fields such as faecal sample studies or even later *in vivo* trials. The *in vitro* gut microbiota simulation model allows to cultivate complex intestinal microbiota bacteria, in controlled conditions, to study their metabolism and reaction to a specific ingredient, with the possibility of being adjusted according to each study aims.

4. Conclusion

In this study, it was possible to find effects from the presence of TMIF on the tested probiotics using a simple *in vitro* gut microbiota simulation model.

436 TMIF did not inhibit the growth of probiotic bacteria in the studied gut microbiota model, which means that it had no antimicrobial effect. The TMIF increased the growth of almost all
437 studied bacteria in monocultures and consequently the production of SCFA and lactate with a
438 decrease of pH in the media.

440 Also, TMIF showed a great potential for the maintenance of probiotic bacteria, especially
441 under nutritional stress conditions. *Lactobacillus* are the most benefited, in terms of
442 maintenance and growth, from the presence of TMIF, in comparison with *Bifidobacterium*.

443 This study then indicates that TMIF shows potential prebiotic activity interacting with
444 probiotics, improving the organism's metabolic activity, mostly increasing at long term (48 h
445 in this study) the survival of the bacteria, and promoting SCFA and lactate production, thus
446 indicating the possibility of being considered a symbiotic relation.

447 TMIF represents itself as a good food source and a potential meat substitute, with high
448 potential on the health of the organism, with its nutritional content and its impact on the
449 probiotics present in gut microbiota level show the potential for the hosts microbiota
450 metabolism, even when compared to other sources from other studies (e.g. FOS, GOS, *Aloe*
451 *vera* mucilage, etc.).

452 This work presents the possibility to evaluate the possible pinpoints of TMIF as a safe food
453 source with great benefits to human well-being, as its findings show the potential to largely
454 improve the organism's equilibrium with prebiotic effects. However, this cannot be claimed
455 without further works, including human studies, which are essential to prove such possibility.

457 **Conflict of interest**

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

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List of Tables:

Table 1 - Nutritional values of TMIF (per 100 g).

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

Table 2- Origin and growth conditions of each bacteria used in the experimental work

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Microorganism	Origin	Media	Incubation conditions
<i>Lactobacillus casei</i> 01	Chr. Hansen (Hørsholm, Denmark)	MRS	Aerobic, 37°C
<i>Lactobacillus rhamnosus</i> R11	Lallemand (Montréal, QC, Canada)	MRS	Aerobic, 37°C
<i>Lactobacillus acidophilus</i> LA-5®	Chr. Hansen (Hørsholm, Denmark)	MRS	Aerobic, 37°C
<i>Bifidobacterium animalis</i> ssp. <i>lactis</i> Bb12®	Chr. Hansen (Hørsholm, Denmark)	MRS *	Anaerobic, 37°C
<i>Bifidobacterium animalis</i> B ₀	CSK (Ede, Netherlands)	MRS *	Anaerobic, 37°C
<i>Bifidobacterium longum</i> BG3	Cell Biotech (Hellerup, Denmark)	MRS *	Anaerobic, 37°C

*supplemented with 0.05 % (w/v) L-Cysteine-HCl.

Table 3- pH average values (\pm SD) and acidification rates in basal medium with and without TMIF inoculated with *Lactobacillus* and *Bifidobacterium* strains.

Bacterial specie	Condition	Average pH along fermentation without TMIF	pH reduction rate without TMIF (%)	Average pH along fermentation with TMIF	pH reduction rate with TMIF (%)
<i>L. rhamnosus</i>	1%	5.70 \pm 0.163	3.56	5.77 \pm 0.193	6.12
	10%	4.60 \pm 0.114	5.46	4.59 \pm 0.068	2.60
<i>L. casei</i>	1%	5.67 \pm 0.164	3.91	5.82 \pm 0.175	5.77
	10%	4.49 \pm 0.053	2.85	4.57 \pm 0.008	0.22
<i>L. acidophilus</i>	1%	5.28 \pm 0.341	13.19	5.44 \pm 0.189	6.83
	10%	4.51 \pm 0.207	10.00	4.59 \pm 0.118	5.46
<i>B. animalis</i> Bb12	1%	5.60 \pm 0.078	3.33	5.92 \pm 0.114	-4.65
	10%	4.79 \pm 0.167	7.57	4.89 \pm 0.025	1.22
<i>B. animalis</i> Bo	1%	5.26 \pm 0.215	6.67	5.82 \pm 0.236	-10.34
	10%	4.41 \pm 0.059	-0.90	4.55 \pm 0.114	-5.84
<i>B. animalis</i> BG3	1%	5.57 \pm 0.176	11.00	5.58 \pm 0.156	5.00
	10%	4.69 \pm 0.214	3.05	4.66 \pm 0.078	-1.93

Table 4- pH average values (\pm SD) and acidification rate in basal medium with and without TMIF inoculated with the co-cultures and consortium.

Bacterial specie	Average pH along fermentation without TMIF	pH reduction rate without TMIF (%)	Average pH along fermentation with TMIF	pH reduction rate with TMIF (%)
A1	4.44 \pm 0.128	5.41	4.50 \pm 0.086	3.46
A2	4.39 \pm 0.108	4.40	4.37 \pm 0.098	5.34
B1	4.37 \pm 0.049	1.58	4.43 \pm 0.054	1.11
B2	4.35 \pm 0.070	2.25	4.49 \pm 0.067	2.40
C1	4.39 \pm 0.104	3.75	4.47 \pm 0.189	0.66
C2	4.36 \pm 0.049	1.36	4.43 \pm 0.046	0.67
D1	4.31 \pm 0.034	-0.46	4.40 \pm 0.025	0.46
D2	4.27 \pm 0.026	1.39	4.35 \pm 0.014	0.69

Figure captions:

Figure 1- Bacterial viable cell counts (log CFU/mL, means \pm SD) when inoculated at 10% and 1% in basal media without (□, □) or with TMIF (■, ■) and incubated during 48 h at 37°C.

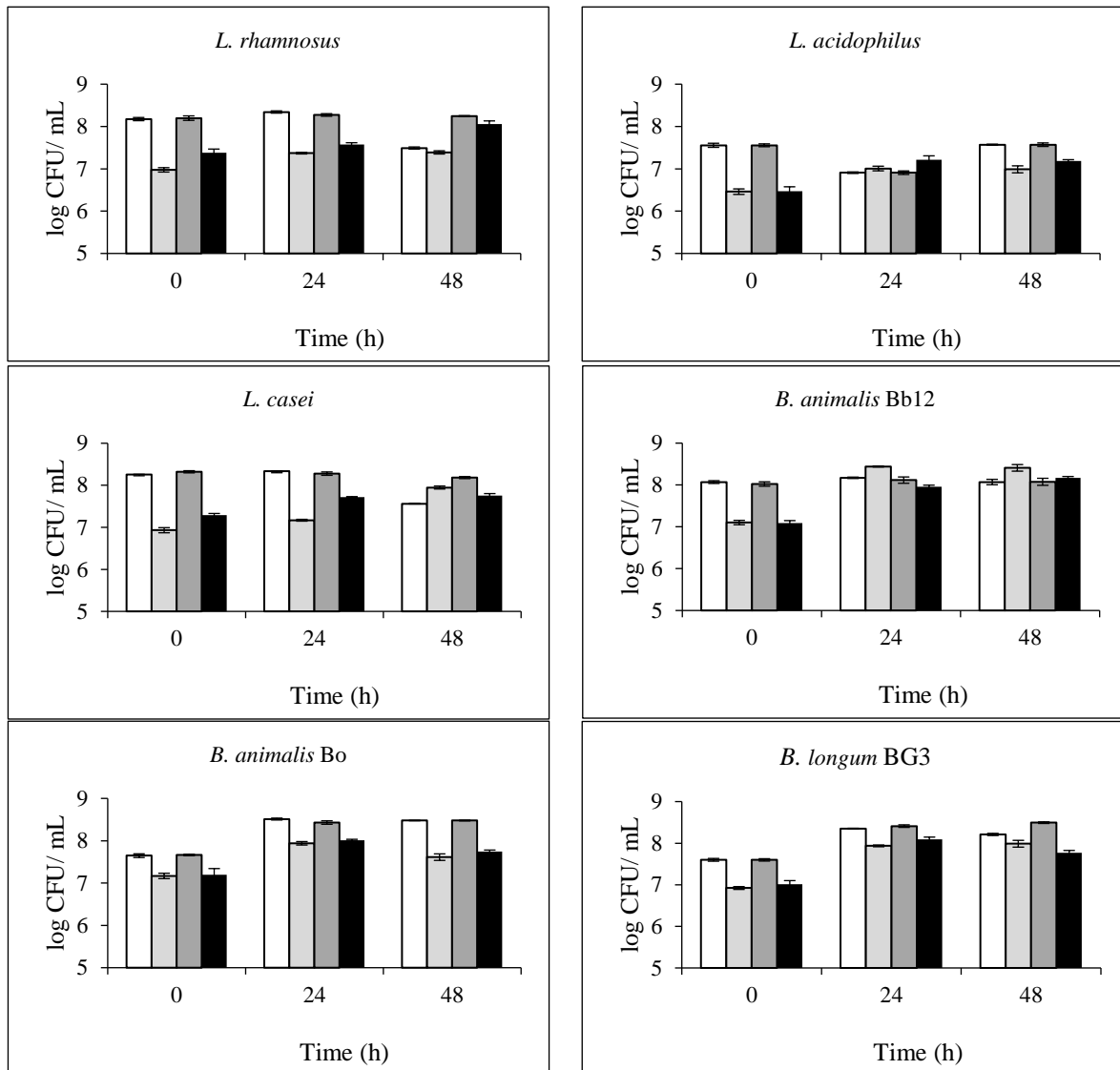
Figure 2- Bacterial viable cell counts (log CFU/mL, means \pm SD) of co-cultures and consortium of *Bifidobacterium* and *Lactobacillus* strains in basal media without (□, □) or with TMIF (■, ■) and incubated during 48 h at 37°C.

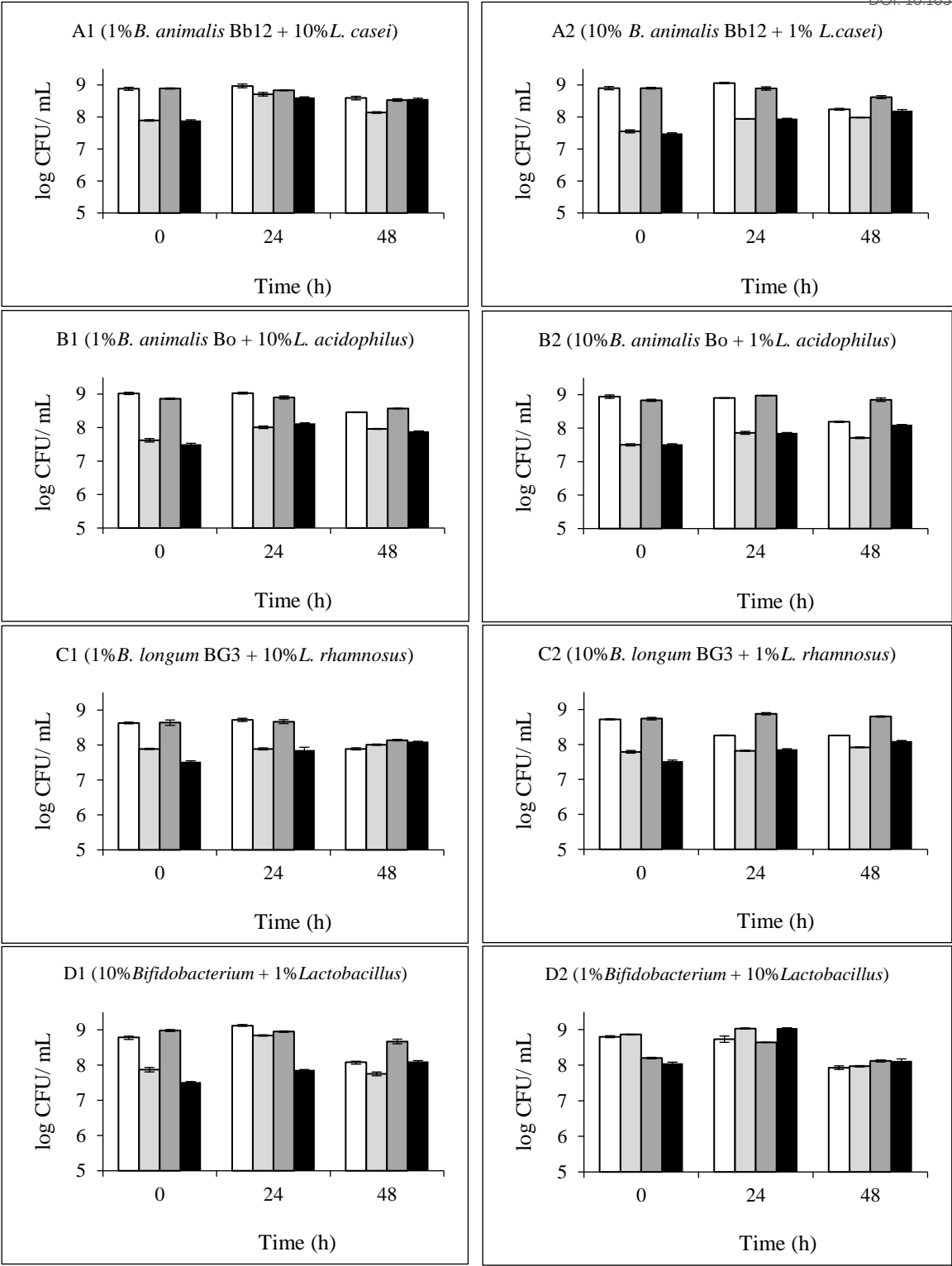
Figure 3- Evolution of the organic acids production (mg/mL, means \pm SD) by the *Lactobacillus* strains when inoculated in basal media without (butyrate (□), propionate (■), acetate (□) and lactate (■)) or with TMIF (butyrate (▨), propionate (▩), acetate (▧) and lactate (▦)) and incubated during 48 h at 37°C. Different letters mark statistically significant ($p < 0.05$) differences between samples for each compound.

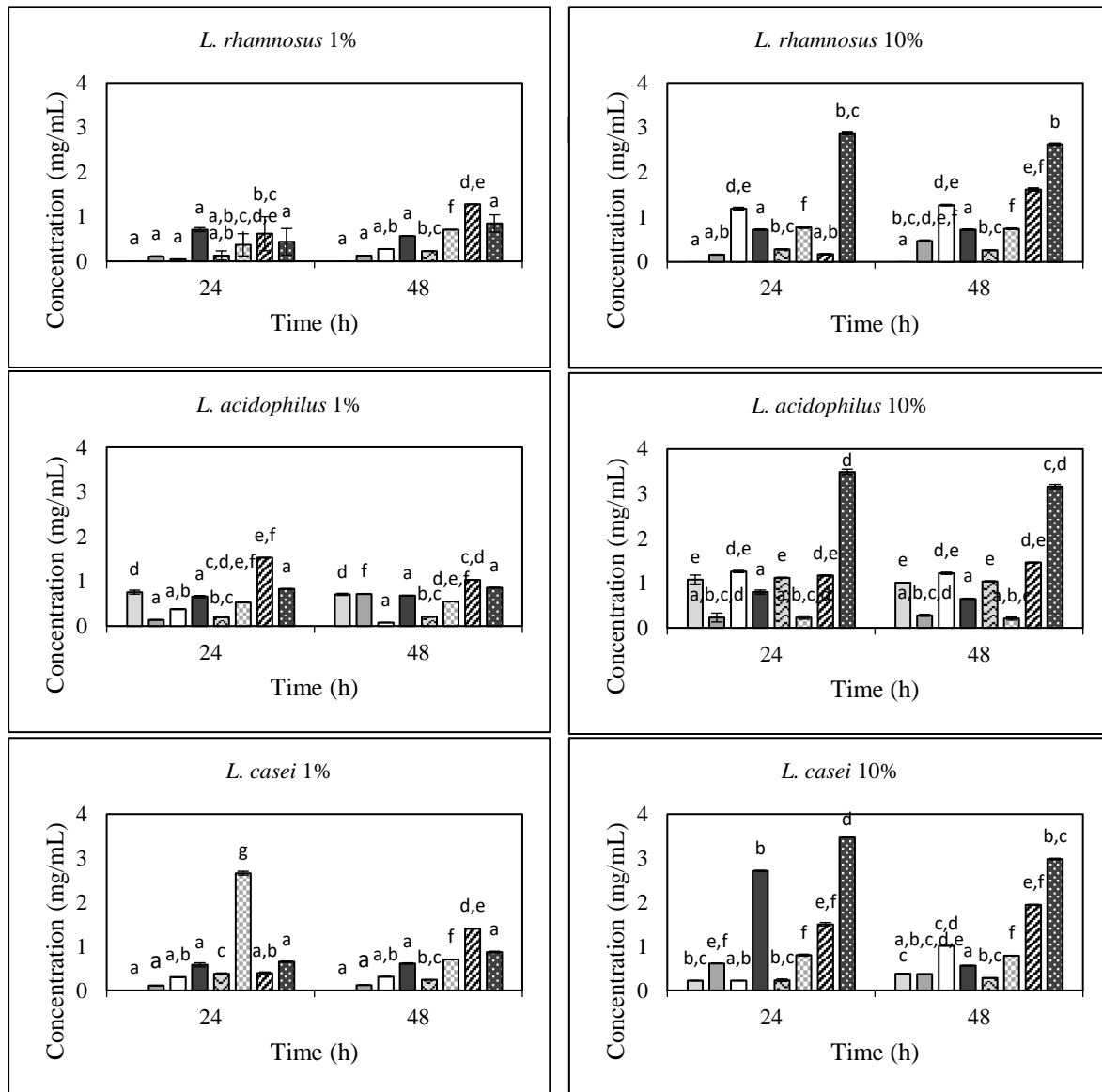
Figure 4- Evolution of the organic acids production (mg/mL, means \pm SD) by the *Bifidobacterium* strains when inoculated in basal media without (butyrate (□), propionate (■), acetate (□) and lactate (■)) or with TMIF (butyrate (▨), propionate (▩), acetate (▧) and lactate (▦)) and incubated during 48 h at 37°C. Different letters mark statistically significant ($p < 0.05$) differences between samples for each compound.

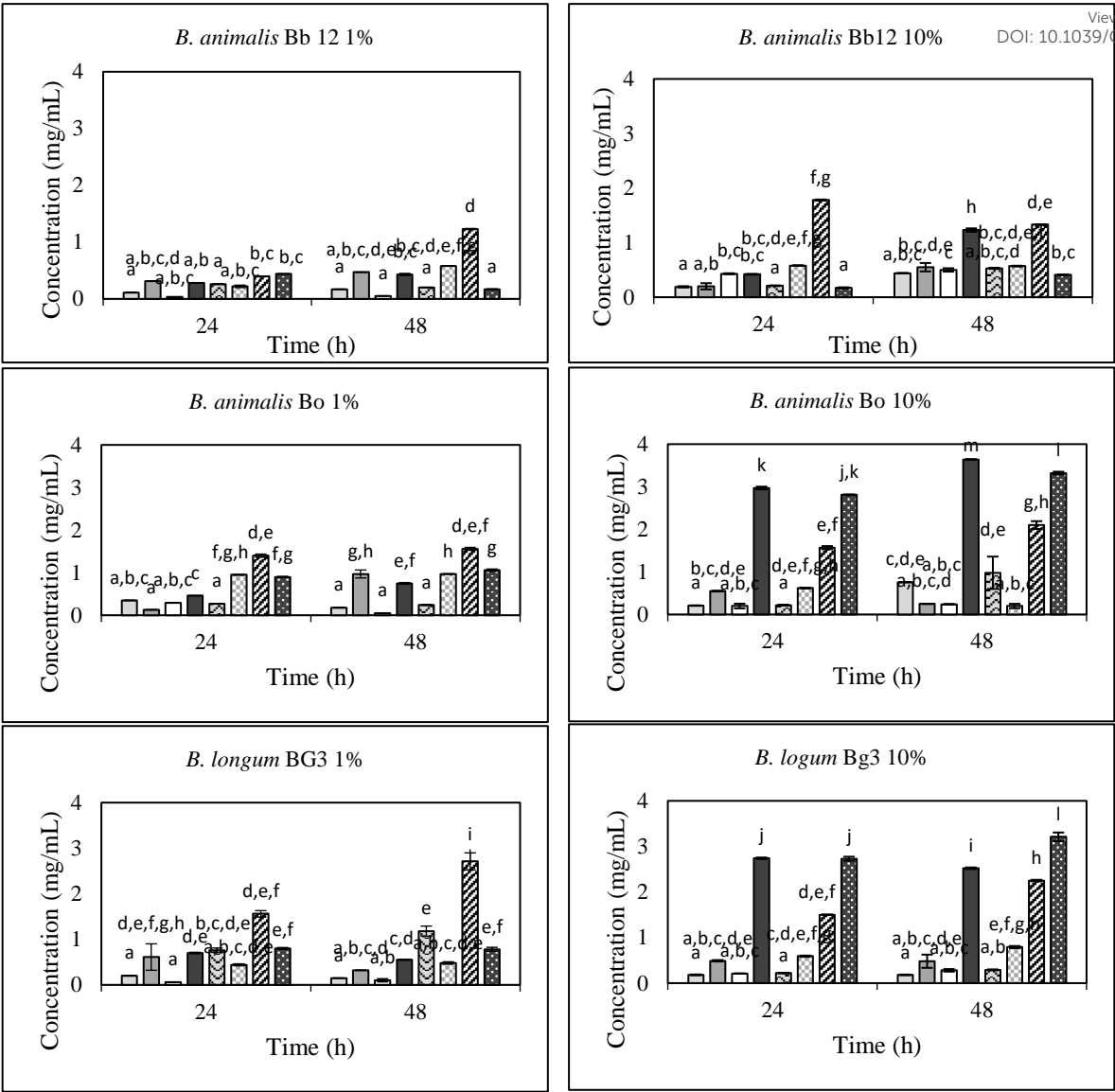
Figure 5- Evolution of the organic acids production (mg/mL, means \pm SD) by the co-cultures and consortium strains when inoculated in basal media without (butyrate (□), propionate (■), acetate (□) and lactate (■)) or with TMIF (butyrate (▨), propionate (▩), acetate (▧) and lactate (▦)) and incubated during 48 h at 37°C. Different letters mark statistically significant ($p < 0.05$) differences between samples for each compound.

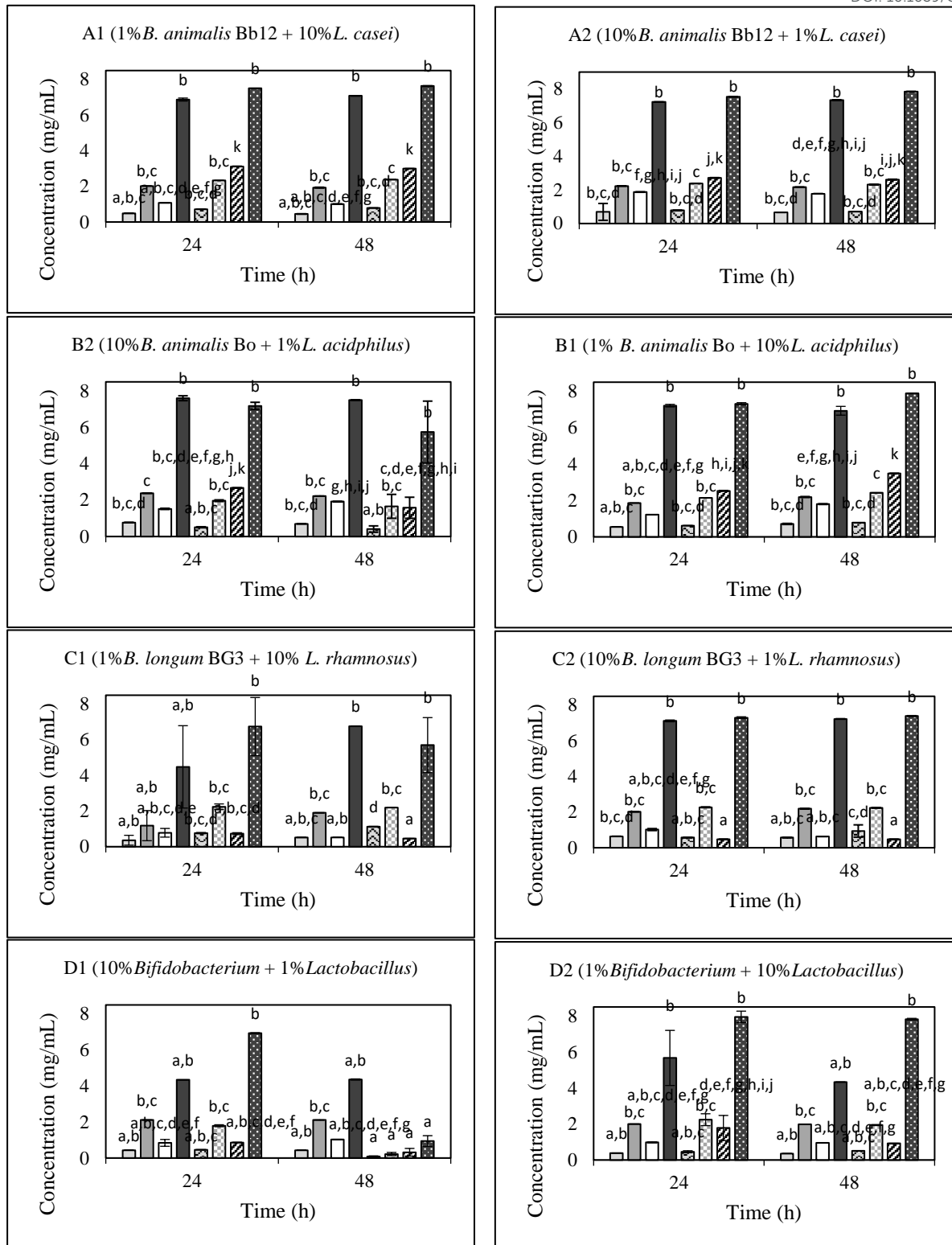
Figure 6- Bacterial viable cell counts (log CFU/mL, means \pm SD) of *L. casei*, *L. rhamnosus*, *L. acidophilus*, *B. animalis* Bb12, *B. animalis* Bo and *B. longum* BG3 when inoculated at 10% in nutritive stress medium with (gray line) or without TMIF (black line) and incubated during 48 h at 37°C.

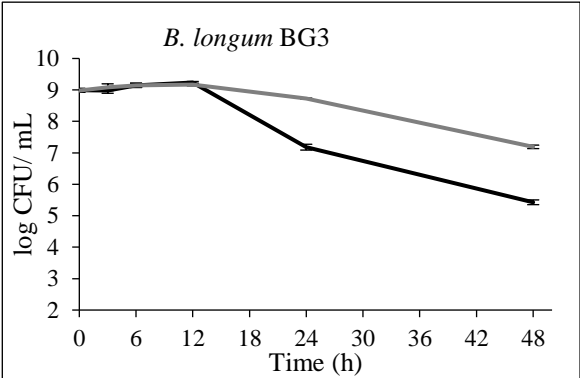
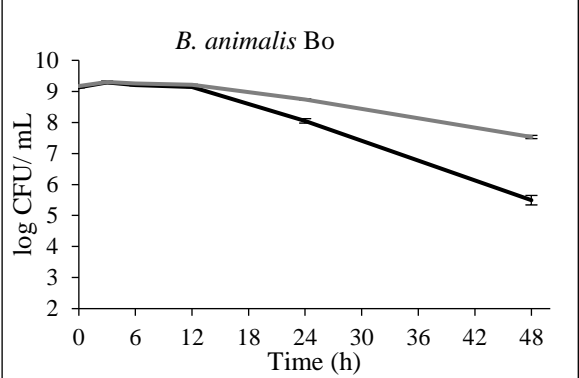
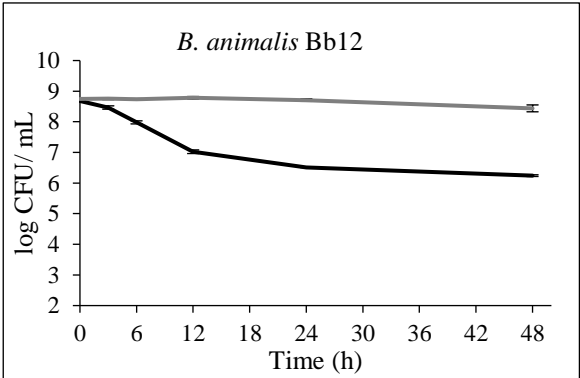
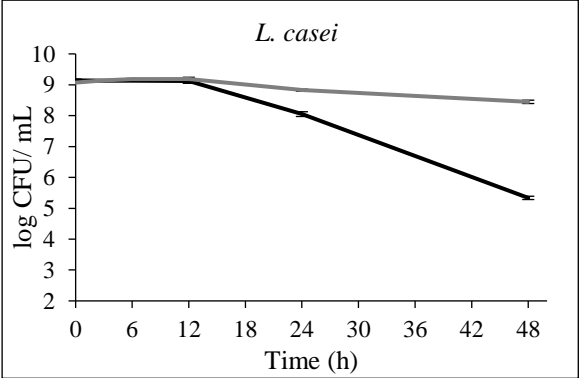
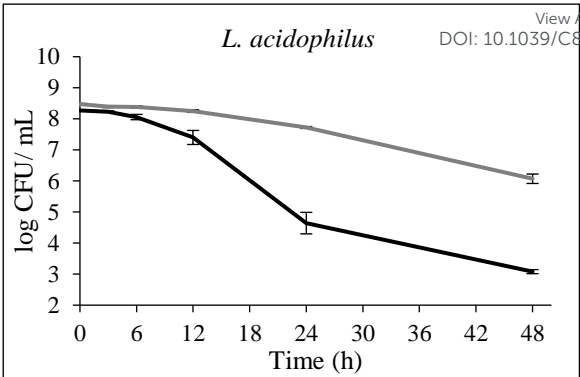
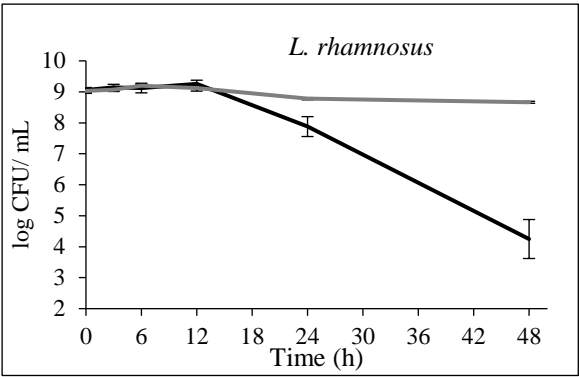


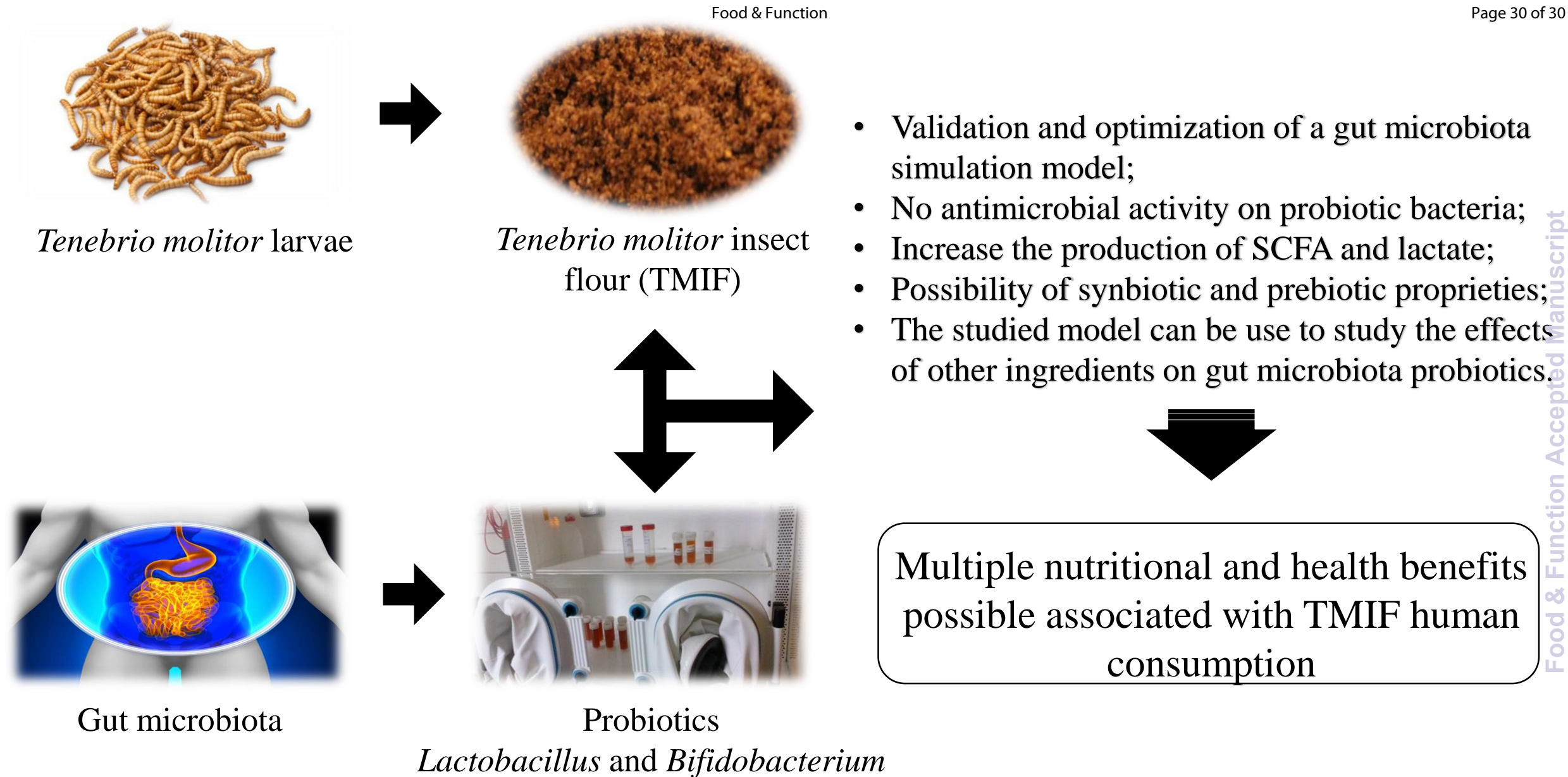












Legend: Potential prebiotic activity of *Tenebrio molitor* insect flour using an optimized *in vitro* gut microbiota model