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IN VITRO IMPACT OF *TENEBRIO MOLITOR* INSECT FLOUR ON HUMAN GUT MICROBIOTA

by
Nelson Fernando Mota de Carvalho

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology.

by
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[July 2018]

This thesis is dedicated to my grandmother Ana Moreira Gomes. Thanks to her, I know the importance of humility, self-pride, originality, effort and hard work. I swear to follow your advices and I promise to leave you proud wherever you are now.

When I was young, I observed that nine out of ten things I did were failures. So, I did ten times more work.
By George Bernard Shaw

I have no special talent. I am only passionately curious.
By Albert Einstein

Even without a gift, even without talent, I can be good with a lot of training. To prove my theory, I go to the end: Hard work wins the natural gift!
By Tauz

Resumo

A farinha de inseto de *Tenebrio molitor* (TMIF) é considerada um ingrediente alimentar nutritivo que ainda carece de avaliação do seu potencial nutricional na dieta humana. Um dos maiores indicadores do efeito da dieta na saúde humana é a composição da microbiota intestinal, principalmente a presença de grupos bacterianos benéficos, como as bactérias probióticas. Desta forma, foram elaborados dois modelos *in vitro* da microbiota intestinal para avaliar o efeito da TMIF na microbiota intestinal. Um dos modelos usou culturas puras de estirpes de *Lactobacillus* e *Bifidobacterium* em monoculturas e co-culturas (pares e consórcio) para avaliar o efeito da TMIF e a atividade metabólica destas bactérias. Adicionalmente, o efeito direto da farinha nas células bacterianas quando estas se encontram em stress nutritivo também foi avaliado. Posteriormente, a interação de todos os grupos presentes da microbiota intestinal bacteriana foi avaliada a partir de fezes de voluntários humanos, utilizando neste caso TMIF sujeitas ou não a uma pré-digestão. A avaliação da viabilidade celular e atividade metabólica foi realizada e comparada em ambos modelos.

No primeiro modelo com culturas puras, não foram observados efeitos negativos da TMIF na viabilidade e no crescimento das bactérias probióticas, ocorrendo um aumento do crescimento e da produção de ácidos gordos de cadeia curta (SCFA) e lactato. Durante o tempo de incubação em stress nutritivo, o número de células bacterianas viáveis foi mantido mostrando que a farinha não apresenta qualquer efeito direto tóxico nas células.

No segundo modelo, o modelo *in vitro* fecal, a TMIF digerida e não digerida demonstrou ter efeitos positivos no crescimento dos grupos bacterianos considerados benéficos (ex. *Bacteroidaceae* e *Prevotellaceae*) sem promover o crescimento significativo nos grupos com impacto negativo na saúde humana (ex. *Clostridium histolyticum*, *Desulfovibrionales* e *Desulfuromonales*). A TMIF promoveu uma produção mais elevada de ácidos orgânicos como o acetato e o propionato. Na presença de TMIF a produção de amoníaco foi na gama de concentrações consideradas não citotóxicas. Em relação ao conteúdo de aminoácidos das amostras de TMIF, a forma não digerida apresentou ter maior concentração de aminoácidos totais enquanto que a amostra digerida a maior concentração em aminoácidos livres.

Como conclusão, a TMIF pode ser um potencial substituto de carne graças ao seu conteúdo nutricional e ao impacto na microbiota intestinal.

Palavras-chaves: microbiota intestinal, modelo *in vitro*, farinha de inseto, propriedades nutricionais

Abstract

Tenebrio molitor insect flour (TMIF) is considered a nutritious food ingredient but still needs assessment of its nutritional potential in the human diet. One of the major indicators of the effect of diet on human health is the composition of the gut microbiota, especially the presence of beneficial bacterial groups, such as probiotic bacteria. In this way, two *in vitro* models of the gut microbiota were elaborated to evaluate the effect of TMIF on the gut microbiota. One of the models used pure cultures of *Lactobacillus* and *Bifidobacterium* strains in monocultures and co-cultures (pairs and consortium) to evaluate the effect of TMIF and the metabolic activity of these bacteria. Additionally, the direct effect of the flour on bacterial cells when they are in nutritive stress was also evaluated. Subsequently, the interaction of all groups present in the bacterial gut microbiota was evaluated from human volunteer faeces, using in this case TMIF subjects with or without pre-digestion. The evaluation of cell viability and metabolic activity was performed and compared in both models.

In the first model with pure cultures, no negative effects of TMIF on the viability and growth of probiotic bacteria were observed, with an increase in the growth and production of short chain fatty acids (SCFA) and lactate. During incubation time under nutritional stress, the number of viable bacterial cells was maintained showing that the flour does not have any direct toxic effect on the cells.

In the second model, the *in vitro* faecal model, digested and undigested TMIF have been shown to have positive effects on the growth of bacterial groups considered beneficial (e.g. *Bacteroidaceae* and *Prevotellaceae*) without promoting significant growth in groups with a negative impact on human health (e.g. *Clostridium histolyticum*, *Desulfovibrionales* and *Desulfuromonales*). TMIF promoted a higher production of organic acids such as acetate and propionate. In the presence of TMIF the production of ammonia was in the range of concentrations considered non-cytotoxic. Regarding the amino acid content of the TMIF samples, the undigested form presented to have higher concentration of total amino acids while the digested sample the highest concentration of free amino acids.

As a conclusion, TMIF may be a potential meat substitute because of its nutritional content and the impact on the intestinal microbiota.

Keywords: gut microbiota, *in vitro* model, insect flour, nutritional proprieties

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Last but not the least, I want to thank the person who influenced me the most during my life and who built my personality. Someone I hold very dear and who I looked up to: my grandmother Ana. Even though she is not with us anymore, I feel her in my heart and I follow by her words that have helped me so much back when she was still with us.

Contents

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Abbreviations list

BCFA- Branched chain fatty acids

CFU- Colony forming units

CHO- Carbohydrates

EDTA- Ethylenediaminetetraacetic acid

FAO- Food and Agriculture Organization of the United Nations

FCM- Flow cytometry

FISH- Fluorescence *in situ* hybridisation

FISH-FCM- Fluorescence *in situ* hybridisation combined with flow cytometry

FPLC- Fast protein liquid chromatography

g- G force

GC- Gas chromatography

HPLC- High performance liquid chromatography

IPQ- Instituto Português da Qualidade

log- Logarithm base 10

MRS- De Man, Rogosa and Sharpe

MRS-BPB- De Man, Rogosa and Sharpe agar with bromophenol blue

MTBSTFA- N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide

Nd- Not detected

OPA- Ortho-phthalaldehyde

PBS- Phosphate-buffered saline

PCA- Plate count agar

PDA- Potato dextrose agar

PFA- Paraformaldehyde

SCFA- Short chain fatty acids

SD- Standard deviation

SDS- Sodium dodecyl sulphate

SRB- Sulphate-reducing bacteria

TMIF- *Tenebrio molitor* insect flour

T2D- Type II diabetes

UK- United Kingdom

USA- United States of America

UV- Ultraviolet radiation

v- Volume

w- Weight

WHO- World Health Organization

1. Introduction

1.1. Overview

Nutrition is independent from cultural differences and has been seen as "the need to feed". Nevertheless, food habits are very different between cultures. As example, insects that are widely eaten in Africa and Asia, but not so well accepted in western countries (Shockley and Dossey, 2014).

Worldwide, it is acknowledged that nutritional disorders are major causes of death. Malnutrition is not only recognised as undernourishment, but also as a dietary disorder, such as overweight or specific nutrient shortage (WHO, 2013). Food and Agriculture Organization of the United Nations (FAO) defines undernourishment (or hunger) as an estimate of dietary energy supply below the minimum required. Studies reveal that there has been a reduction in undernourishment in several countries but, even so, these values are still not reasonable (Van Huis *et al*., 2013). Globally, a lack of vitamins and minerals affects 1.62 billion people, or 24.8% of the world's population. On other hand global financial, economic and food price crises in 2008 led many people to hunger. High food prices normally have two affects: buyer's income, leaving them with less purchasing power; and substitution, shifting to less nutritious foods (WHO, 2013).

In developed countries, besides undernourishment, one of the main concerns is malnutrition, in the sense of diet disorders. Changes in lifestyle and increased availability of energy-rich foods have been contributing to a serious threat (Tremaroli and Bäckhed, 2012).

Within these dimensions, the acknowledged "triple burden" of malnutrition is comprehensible as highlighted by FAO (2017). Consisting of undernourishment, micronutrient deficiencies, overweight and obesity, these dimensions remain a "global health emergency". It is estimated that 11 percent of the world's population starves, two billion are affected by micronutrient deficiencies and 40 percent, by 2014, of the world's population are overweight and/or obese (FAO, 2017).

In many cases, obesity is allied with gut microbiota compositional changes. This is associated with the diet, which is known to alter the gut microbiota, and has been related to changes in the energy extraction from food (Saraswati and Sitaraman, 2015, Tremaroli and Bäckhed, 2012). Gut microbiota health may be influenced by consumption of non-digestible carbohydrates, thus reaching the colon for fermentation by the gut microbiota (Scott *et al*., 2013).

Therefore, malnutrition can have an impact at the gut microbiota level. The search for a nutritional source, capable of supporting the population demands in terms of availability, nutritional values and sustainability is a health pursuit.

Emerging studies suggest that insects can, in the near future, be a solution to current demands as they are a rich source of protein, fibre and fatty acids.

1.2. Worldwide nutritional problems

In the developed world, the rate of malnutrition seems to be independent of socioeconomic inequality, but normally overweight is one of the main concerns among lower middle-income groups mainly in the Americas and European regions. Global data have shown that energy consumption rose between 1990-1992 and 2010-2012 in all regions. However, in Africa, there has been less energy consumption over the years with a lack of food availability (WHO, 2013). The possibility of achieving 9.7 billion in world population by 2050, and possible peaking up to 11 billion by the end of the century, brings a need for sustainable feed and systems that support such an endeavor in terms of impact at ecological and land pressure dimensions, production and transformation capabilities, regulations, waste and even policy coherence (FAO, 2017).

It is no more only a question of food sources, but also of policies that allow the development of new solutions, capable of responding to all requirements of the population.

1.2.1. Impact of nutritional problems in the economy and environment

Dynamically, nutritional challenges may vary and differ between and within population groups, but the main focus remains - to investigate and develop practices and methods aimed at the fulfillment of population nutritional needs worldwide, the most economical and efficiently possible. On the other hand, mainly in developed countries, where nutritional challenges are different, one new demand is for foods that are perceive as healthy (Ali *et al.*, 2015).

As it is currently, global pressure over biomass resources and land, is taking the demand into a crisis situation. Also, resources needed to cope with actual demand are starting to have their own negative effect on the planet (Premalatha *et al*., 2011). For the above, growing foods and efficiently using biomass resources is a priority, aiming for sustainable farming practices (Sun-Waterhouse *et al*., 2016).

1.3. Sustainability, economics and insect-based food

Recent studies consider a practice that has been taken on for a long time in some cultures – the *anthropoentomophagy* – which means human consumption of insects as a food source (Premalatha *et al*., 2011). Insects as food is a growing concept because of advantages towards health, the environment and livelihoods (Gmuer *et al*., 2016). Being *poikilothermic*, insects spend less energy and nutrients than livestock, they are more efficient in generating proteins

from phytomass, have a fast growth rate and have a good nutritive value. Even on the ecological side, insect species have cleaner eating habits and so they become ecosystem friendly (Premalatha *et al*., 2011). Besides their efficiently conversion from plant proteins to insect proteins, they can also be raised efficiently using bio-waste streams, making insect production economically viable with mass production satisfy many nutritional demands (Oonincx and De Boer, 2012, Oonincx *et al*., 2015).

Enlarging their economic potential, insects have short reproductive cycles, a high widespread distribution and high reproductive rates. They do not require large areas and they can be raised on a wide range of foods (Sun-Waterhouse *et al.*, 2016).

Insect farming, for nutritional intents, is therefore not only an advantage, but also has environmental and economic implications, since insects are easily maintainable, requiring less resources and at the same time have a smaller impact on the environment, mainly on the production of greenhouse gases and water consumption compared to common livestock farming (**Table 1.1**) (Oonincx *et al*., 2010, Sun-Waterhouse *et al*., 2016).

Table 1.1- Impact on environment to produce 1 Kg of protein of different sources.

Type of meat	Average Greenhouse gas (GHG) production (g)	Quantity of feed (g)	Quantity of water (L)	Arable land (m^2)
Beef	2850	10000	22000	200
Pork	1130	5000	3500	50
Chicken	300	2500	2300	45
Cricket	1	1700	1	15

Source: Van Huis *et al.* (2013) in Edible insects: future prospects for food and feed security and McGill (2015) personal communication in Entomophagy - Edibles Bugs are a Healthy and Sustainable Food.

Some surveys indicate that edible insects price exceed traditional meat products. Nevertheless, if insect production is taken to an industrial scale, along with sustainable breeding, farming and processing technologies, this may boost their market availability and at the same time lead to a sale price decrease (Sun-Waterhouse *et al.*, 2016).

1.3.1. Insects' nutritional value

Insects are sources of protein, lipids, carbohydrates and certain vitamins that may satisfy nutritious and protein demands, compared to other sources such as meat or fish. Insect proteins possess nutritional advantages compared to plant protein (Rumpold and Schlüter, 2013). Insects have also advantages compared to animal meat due to their high-quality protein content and

lipids, vitamins and minerals (Sun-Waterhouse *et al.*, 2016). Insect mass production has potential to provide animal protein to humans and livestock animals (for example poultry or pigs) through direct consumption or as food supplements (Mlcek *et al.*, 2014, Sun-Waterhouse *et al.*, 2016). This is not new, since so many nations have already used insect for these purposes, especially in tropical countries (Mlcek *et al.*, 2014, Sun-Waterhouse *et al.*, 2016).

One issue that is very important to highlight is that not all insects have the same nutritional values. At the moment, there are thousands of identified insect species considered edible (Klunder *et al*., 2012, Shockley and Dossey, 2014). The nutritional content is variable as shown in **Table 1.2**.

Table 1.2 - Nutritional values of some relevant insects regarding other high-protein foods.

Insect or Food Item	Protein (g/kg)	Fat (g/kg)	Calories (kcal/kg)	Thiamin (mg/kg)	Riboflavin (mg/kg)
Black soldier fly (*Hermetia illucens* larvae)	175	140	1994	7.7	16.2
House fly (*Musca domestica* adults)	197	19	918	11.3	77.2
House cricket (*Acheta domestica* adults)	205	68	1402	0.4	34.1
Superworm (*Zophobas morio* larvae)	197	177	2423	0.6	7.5
Mealworm (*Tenebrio molitor* larvae)	187	134	2056	2.4	8.1
Mealworm (*Tenebrio molitor* adults)	237	54	1378	1	8.5
Giant mealworm (*Tenebrio molitor* larvae)	184	168	2252	1.2	16.1
Waxworm (*Galleria mellonella* larvae)	141	249	2747	2.3	7.3
Silkworm (*Bombyx mori* larvae)	93	144	674	3.3	9.4
Beef	256	187	2776	0.5	1.8
Milk powder	265	268	4982	2.6	14.8

Source: Finke (2002) in Complete Nutrient Composition of Commercially Raised Invertebrates Used as Food for Insectivores and Shockley and Dossey (2014) in Insects for Human Consumption.

The high protein content present in insects is an indicator of a possible valuable resource for human and animal nutrition. Also, the protein content from insects is generally of good quality and highly digestible (Kouřimská and Adámková, 2016, Mlcek *et al*., 2014). The content of essential amino acids in insects is 10-30% of all amino acids (Chen *et al.*, 2009). Nevertheless, it is necessary to keep in mind that nutrient content may differ from wild to commercially farmed insect species, and that their growth stage also has an impact on the content of some substances (Klunder *et al*., 2012). For example, adults of *T. molitor* contain more protein (237 g/Kg) than their larvae (187 g/Kg) (Oonincx and Dierenfeld, 2011). The composition in amino acids is depicted in **Table 1.3** for *T. molitor* larvae. The presence of amino acids in a diet is essential. Amino acids are the basic units of proteins and contribute to food nutrition, physical

and sensorial proprieties. Amino acids are required for the biosynthesis of proteins in human metabolism and they can ensure growth, development and maintenance. From the role of amino acids, eight are considered essential because the human body cannot synthesise them. The essential amino acids are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. These amino acids can only be obtained from food sources (Van Huis *et al*., 2013).

Table 1.3 - Average content values of essential amino acids present in *Tenebrio molitor* larvae and beef. Adapted from Van Huis *et al.* (2013).

Essential amino acid	***T. molitor* (g/ kg dry matter)**	**Beef (g/ kg dry matter)**
Isoleucine	24.7	16
Leucine	52.2	42
Lysine	26.8	45
Methionine	6.3	16
Phenylalanine	17.3	24
Threonine	20.2	25
Tryptophan	3.9	---
Valine	28.9	20

By weight, yellow mealworm (*T. molitor* larvae), one of insect species commonly bred in Europe, has significantly higher contents of linoleic acid, isoleucine, leucine, valine, tyrosine, alanine and vitamins (except B12) than beef (Kouřimská and Adámková, 2016, Mlcek *et al*., 2014, Sun-Waterhouse *et al*., 2016). Insect food source, as *T. molitor,* can satisfy nutritional needs concerning essential amino acids, and in some cases, in a higher percentage compared to more "common" food sources, such as beef.

Fat content present in the edible insects is normally between 10-50% but depends on many factors such as species, habitat, diet, reproductive stages, season, age and sex (Kouřimská and Adámková, 2016, Mlcek *et al*., 2014). Insects present higher values of essential fatty acids than animal's fats, with high quality, especially long chain omega-3 fatty acids (for example α-linoleic acid) (Mlcek *et al*., 2014). The omega-3 long chain polyunsaturated fatty acids have important roles in the building of cerebral tissues (Carlson and Kingston, 2007).

Carbohydrates present in insects (6.71- 15.98%) are mostly found in the form of chitin (Raksakantong *et al*., 2010). Chitin, a poly-beta-1,4-N-acetylglucosamine, is insoluble in water and is the second most abundant polysaccharide in biomass after cellulose, and the main

component of arthropod exoskeletons. It can be a source of nitrogen as well as carbon (Hajji *et al*. 2014, Khoushab and Yamabhai, 2010). Generally, chitin represents 5-20% of dry weight of insects (Kouřimská and Adámková, 2016, Mlcek *et al*., 2014).

Recent studies show that considerable amounts of polysaccharides might improve human immune function (Mlcek *et al*., 2014). Chitin can be consumed as a form of low calorie food with high nutritional, health and medical value (Burton and Zaccone, 2007, Chen *et al*., 2009, Kouřimská and Adámková, 2016). Chitin may promote selective growth of important populations in the human gut microbiota that are responsible for maintaining the physiological state of the gut, for protection of the organism's immune system and for the efficiency of the metabolic processes (Delzenne and Cani, 2010, Geurts *et al.*, 2013, Neyrinck *et al.*, 2013). In addition, edible insects are rich in minerals elements, such as potassium, sodium, calcium, copper, iron, zinc, manganese and phosphorus, possibly due to their food sources (Kouřimská and Adámková, 2016, Mlcek *et al*., 2014). They also contain carotene and vitamins B1, B2, B6, C, D, E and K. Insects apparently may be a good sources of vitamins B, but a number of insects have low levels of thiamine (vitamin B1) (**Table 1.2)** (Mlcek *et al*., 2014). Insects are rich in proteins, fatty acids, fibres, vitamins and mineral elements. As such, they present themselves as possible nutritional sources (**Table 1.2** and **1.3)**.

1.3.2. Insect consumption hazards

Not all insects are safe to eat, and they can be vectors of zoonotic agents and carry toxins to humans. Microbiological contamination is also a main concern (Belluco *et al*., 2013, Rumpold and Schlüter, 2013). Alongside this, the dangers of consuming inappropriate developmental insect stages, of wrong culinary preparations or even of the wrong insect handling must be also considered (Mlcek *et al.*, 2014).

Allergy is a potentially life-threatening situation and a big risk when considering entomophagy practice or even just contact with insect products (Belluco *et al.*, 2013). Sensitivity to insect proteins has been observed when inhalation and/or contact occur with particulate airborne insect products (Mlcek *et al.*, 2014). Upon the idea of reported allergic reactions, those concerning chitin are significant. Although not entirely consider as a potential allergen, chitin can sometimes cause sensitisation and is a recognition element for tissue infiltration by innate cells associated with immunity. Studies showed that inhaled particulates from *T. molitor* are potent sensitisers and may lead to asthma, thus making *Tenebrionid* family beetles potentially significant allergens (Mlcek *et al.*, 2014).

Microbial safety of edible insects is relevant mainly during insect handling and/or processing. As such, in these steps the possibility of parasitic hazards that may lead to infections or even death should be considered (Pereira *et al*., 2010). Chemical hazards can include those of natural occurrence, synthesised or accumulated by insects or even those added during food processing (Sun-Waterhouse *et al.*, 2016). Finally, insects can produce toxins that may also accumulate heavy metals from the environment (Zagrobelny *et al.*, 2009).

1.3.3. Insect food acceptance by the world population

Many cultures around the world consume insects as a normal part of their diet or as a delicacy. Up to 80% of the world's nations eat insects with greater representation in countries located in the tropics. One example of this is in Africa, Ghana, where winged termites are a popular dish and are prepared in different ways.

Some researchers indicate that people are becoming increasingly aware of the many possibilities of insects as food (House, 2016, Megido *et al.*, 2014, Mlcek *et al.*, 2016). Nevertheless, many have aversion to new foods (food neophobia) making the acceptance of insects as food difficult (Verbeke, 2015). This is a big issue considering entomophagy especially in Western cultures, which have negative perceptions of insect products (Gmuer *et al*., 2016). These aversions decrease the likelihood of accepting unusual products, such as insects, for meat substitutes (Verbeke, 2015).

The aversion to insect food is then a big social-cultural challenge, especially among western communities, where acceptance of insect-based foods probably faces adaptation to flavour profiles, textures and aesthetics (Hartmann *et al.*, 2015).

1.3.4. Insect products

Insects can be used as food ingredients or even be consumed for medicinal purposes. Many insect products are already used like bee honey, food colouring, royal jelly and propolis (Schabel, 2010). Insect-based foods and its processing should, at the same time, maximise retention of nutrients and bioactives, and eliminate any human “disgusting” perception, along with potential allergens through the use of efficient technologies.

The degree of processing of insect food, making it unrecognisable, can impact on acceptance and reduce negative emotions towards entomophagy (Schouteten *et al*., 2016). The insect’s processing, making it unrecognisable, may facilitate its consumption (Mitsuhashi, 2010).

To incorporate insect flours in our daily life dishes is possible and easy to do it. It is possible to prepare cakes, muffins, cookies, smoothies, juices, protein bars, pancakes, protein shake and

other recipes. Therefore, it is not very difficult to insert insect flours in the western modern lifestyle (Wilson, 2016). However, it must be taken into consideration that insect processing affects nutritional potential, implying control of the processing conditions so as not to lose protein and vitamin (Kinyuru *et al*., 2010).

1.4. Food, digestion and gut microbiota properties and functions

The human gut microbiota has impact on health (Conlon and Bird, 2014, Barczynska *et al*., 2016). With diet influencing the human microbiota, the three dimensions correlate: diet - gut microbiota - health. As an example, obesity may be related with gut microbiota composition and compositional changes. Type II diabetes (T2D) can also be related to microbiota changes (Wang *et al*, 2017). Macronutrients provided by diet such as carbohydrates, proteins and fats have a role in shaping the composition and the activity of the complex microbial population in the gut, however the knowledge of the effect of protein and fats are less well known comparing to carbohydrates (Conlon and Bird, 2014).

1.4.1. The food, health and microbiota maintenance

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 alimentation in one's health. Demanding healthier food sources is of great importance.

The gut microbiota is affected by several factors, as health and diseases state, and most importantly, diet that can provoke dysbiosis, i.e. any change to the composition of resident commensal bacteria in the gut to the community normally found in healthy individuals and to metabolism. Hence, nowadays is of importance to evaluate impact of proteins, fatty acids, vitamins and mineral salts on the human gut microbiota (Fujimura *et al*., 2010, Ríos-Covián *et al*., 2016, Rowland *et al*., 2017). The main phylas found in the human gut are *Firmicutes* and *Bacteroidetes*, and in a smaller representation the *Proteobacteria* and *Actinobacteria* (Flint and Juge, 2015). In adult humans, some of the most common bacteria found in gut belongs to the genera *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Clostridium*, *Escherichia*, *Streptococcus* and *Ruminococcus*. About 60% of the gut microbiota bacteria are from *Bacteroidetes* or *Firmicutes* phyla and 98% are from *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria* (Conlon and Bird, 2014, Lopetuso *et al.*, 2013).

The complexity and variability of human adult gut microbiota has become more evident with recent studies during the last years. This can be related to several factors such as diet and

genetics. Composition and activity of microbial populations can vary because of life events (e.g. puberty, ovarian cycle, pregnancy and menopause) (Conlon and Bird, 2014).

1.4.2. Gut microbiota functions and processes

One of the functions of gut microbiota is the production of short chain fatty acids (SCFA) which are carboxylic acids defined by the presence of an aliphatic tail of two to six carbons. SCFA are produced through colonic fermentation, which occurs in the gut, and is a complex process that involves the interactions of many microbial species in anaerobic conditions leading to a breakdown or conversion of dietary fibre, protein and peptides into different end products (Fernandes *et al*., 2014, Ríos-Covián *et al*., 2016, Rowland *et al*., 2017, Tan *et al*., 2014). The principal end products of fermentation of dietary fibres are SCFA such as acetate, propionate and butyrate. A small amount of branched chain fatty acids (BCFA), such as isobutyrate and isovalerate, are produced from protein and peptide degradation (Barczynska et al., 2016, Fernandes et al., 2014, Ríos-Covián et al., 2016, Rowland et al., 2017, Tan *et al.*, 2014, Zhao *et al*., 2016). BCFA are mostly saturated fatty acids with a methyl branch or more on the carbon chain produced by the fermentation of branched amino acids, valine, leucine and isoleucine from indigestible protein that reaches the colon (Heimann *et al*., 2016, Ran-Ressler *et al*., 2008, Ran-Ressler *et al*., 2014, Ríos-Covián *et al*., 2016). Other studies related to BCFA showed that they induce apoptosis in human breast cancer cells, and act to inhibit tumour growth in cultured cells and in a mouse model (Ran-Ressler *et al*., 2014, Wongtangtintharn *et al*., 2004, Yang *et al*., 2000). The nutritional properties of BCFA are not fully exploited, but according to some studies, they may be important for the development and maintenance of microbiota, for enterocyte and they indicate a role against lipotoxicity, thus regulating energy homeostasis health (Heimann *et al*., 2016, Ran-Ressler *et al*., 2014).

A healthy microbiota can ferment carbohydrates and proteins that escape being absorbed in the small intestine (Ríos-Covián *et al*., 2016, Rowland *et al*., 2017, Scott *et al*., 2013). Our eating habits, meaning our diets, have effects on the composition and activity of gut microbiota, and therefore on SCFA and BCFA production (Brüssow and Parkinson, 2014, Louis *et al*., 2014, Ríos-Covián *et al*., 2016). The release of SCFA significantly reduces the prevalence of inflammatory diseases. Fermentation and SCFA production inhibit the growth of pathogenic organisms reducing luminal and faecal pH and directly promoting the growth of symbionts (Kamada *et al*., 2013, Tan *et al.*, 2014). This allows a decrease of peptide degradation and, consequently, the formation of toxic compounds such as ammonia, amines, and phenolic compounds (Slavin, 2013). SCFA are also able to regulate glucose and lipid metabolisms, to

promote mineral absorption, stimulate proliferation and differentiation of intestinal enterocytes (Barczynska *et al*., 2016). SCFA are the key to the normal function of intestine and human body (Zhao *et al.*, 2016).

1.4.3. Production of SCFA

There are several pathways to the production of SCFA (**Figure 7.1**). Most enteric bacteria produce acetate, such as acetogenic bacteria, but propionate and butyrate are specific to some genera (Rowland *et al*., 2017, Zhao *et al*., 2016).

For propionate formation, by colonic bacteria, three different pathways can be found: succinate pathway, acrylate pathway, and propanodiol pathway. Succinate is the substrate of the succinate route, for propionate formation, involving the descarboxylation of methylmalonyl-CoA to propionyl-CoA. The acrylate pathway converts lactate to propionate through the activity of the lactoyl-CoA dehydratase and downstream enzymatic reactions (Ríos-Covián *et al.*, 2016). The propanodiol pathway is characterised by the conversion of deoxy-sugars to propionate. The succinate pathway is the dominant route (Ríos-Covián *et al.*, 2016). Propionate is mainly produced by *Bacteroides* and *Propionibacterium* species by the succinate pathway (Rowland *et al.*, 2017, Zhao *et al*., 2016). As for butyrate, the predominant producers are *Firmicutes* and *Faecalibacterium prausnitzii* (Rowland *et al*., 2017). Two different pathways for butyrate production can be considered. The butyrate kinase pathway, that employs phosphotransbutyrylase and butyrate kinase enzymes, but this is not the most common. The most common pathway is the butyryl-CoA: acetate CoA-transferase pathway, in which butyryl-CoA is converted to butyrate in a single step enzymatic reaction (Ríos-Covián *et al*., 2016). Acetate, the SCFA mostly produced by the gut microbiota, has two metabolic routes to be produce (Ríos-Covián *et al*., 2016, Rowland *et al*., 2017, Scott *et al*., 2013). Most is produced through carbohydrate (CHO) fermentation by enteric bacteria. Also 1/3 of colonic acetate is from acetogenic bacteria that produces it from hydrogen and carbon dioxide or formate through the Wood-Ljungdahl pathway (Ríos-Covián *et al.*, 2016).

Another process to consider is bacterial cross-feeding, that, in a simple form consists of the use of the end products from the metabolism of a microorganism by another one (Ríos-Covián et al., 2016, Rowland et al., 2017). As this happens, there is an impact on the final balance of the production of SCFA in comparison to concentrations that can be achieved during the entire fermentation process. Studies show that cross-feeding occurs mostly from acetate to butyrate, and in a lower extent in between butyrate and propionate. Almost no metabolic flux exists between propionate and acetate (Ríos-Covián *et al*., 2016). However, some bacteria can alter

their fermentation and so produce different SCFA under different conditions (Rowland *et al*., 2017).

1.5. Regulatory restrictions

Despite the fact that the regulatory frameworks for food and feed have developed significantly in recent times, standards and regulations for the use of insects as ingredients for food and feed are rare. This happens due to the perception of many societies not regarding insects as regular food/feed product.

The present legislation referring to insects is mainly prescribing maximum limits of insect traces in foodstuffs, for example in dried products such as grains, flour, spices, etc. The lack of specific legislation derives from the very limited development of industrial insect farming in developed countries. If insects become a widely used ingredient in food and feed, an appropriate regulatory framework has to be created (Van Huis *et al*., 2013).

The Novel Food concept (food products that do not have a consumption history in the region in question) is guiding the development of rules and standards for insects as human food, mainly at national levels. The European Commission adds the obligation of such food being safe for consumers and properly labelled, to assess premarket risks to any product and gather required authorisations (Van Huis *et al*., 2013).

Efforts have to be developed in the sense of promoting standardisation. For insects as food and feed, premarket safety evaluations have to be conducted regarding the Codex Alimentarius, standards and studies are necessary in order to evaluate the impact of such sector regarding the environment and sustainability (Van Huis *et al*., 2013).

1.6. Objectives

The aim of this work was to assess the nutritional potential of *Tenebrio molitor* insect flour (TMIF) on human diet. Pursuing such main objective, two gut microbiota *in vitro* models were used, enabling the evaluation of such ingredient, in the viability and metabolic activity of the most representative bacteria groups of the gut microbiota.

2. Material and methods

2.1. Sample

2.1.1. *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 at **Table 2.1**.

Table 2.1 - Nutritional values of TMIF.

Component	Concentration
Total sugar	<0.10% (w/w)
Amino acids (ash)	5.4% (w/w)
Cholesterol	0.002 mg/100g
Fiber	3.0% (w/w)
Fat	39.4% (w/w) (saturated- 8.6% (w/w))
Carbohydrates	<0.10% (w/w)
Humidity	7.5% (w/w)
Protein	44.6% (w/w)
Sodium	142 mg/100g
Energetic value	539 kcal/100g ⇔ 2242 kJ/100g

2.1.2. Sample sterilisation

In order to guarantee that the TMIF under study was totally free of microorganisms, 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 ºC; 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 ºC 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 technique (Miles *et al*., 1938) on plate count agar (PCA), incubated at 30 ºC for 24 h and on potato dextrose agar (PDA), incubated at 30 ºC, up to five days.

2.2. Microorganisms and cultures conditions

All microorganisms used and their growing conditions are listed in the **Table 2.2**. All *Lactobacillus* and *Bifidobacterium* in the **Table 2.2** have been classified as probiotics according with their manufacturers.

Table 2.2 - Origin and growth conditions of each bacteria used in the experimental work.

Microorganism	Origin	Media	Incubation conditions
Lactobacillus casei 01	Chr. Hansen (HØrsholm, Denmark)	MRS	Aerobic, 37ºC
Lactobacillus rhamnosus R11	Lallemand (Montréal, QC, Canada)	MRS	Aerobic, 37ºC
Lactobacillus acidophilus LA-5®	Chr. Hansen (HØrsholm, Denmark)	MRS	Aerobic, 37ºC
Bifidobacterium animalis ssp. *lactis* Bb12®	Chr. Hansen (HØrsholm, Denmark)	MRS *	Anaerobic, 37ºC
Bifidobacterium animalis B_0	CSK (Ede, Netherlands)	MRS *	Anaerobic, 37ºC
Bifidobacterium longum BG3	Cell Biotech (Hellerup, Denmark)	MRS *	Anaerobic, 37ºC

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

2.3. Effect of TMIF exposure in a nutritional stress

2.3.1. Nutritive stress conditions simulation

In order to simulate a condition of nutritional stress, the inoculation of bacterial cells in 0.1% (w/v) peptone water with 0.85% (w/v) of NaCl was performed. Overnight inocula of 10% (v/v) bacteria was centrifuged during 15 min at 2,820 x g to obtain a cell pellet. The pellet was washed at least two times with 0.1% (w/v) peptone water. The pellet was resuspended in 0.1% (w/v) peptone water with 0.85% (w/v) of NaCl (HiMedia Laboratories, 2015) and TMIF was added to the media at 1% (w/v) and control was performed without TMIF. All additions and inoculations were carried out inside an anaerobic cabinet (5% H_2, 10% CO_2 and 85% N_2) to avoid any oxygen contact, mimicking colon conditions.

2.3.2. Microorganisms enumeration

At 0, 3, 6, 12, 24 and 48 h of incubation time, decimal dilutions in 0.1% (w/v) peptone water were made and plated in de Man, Rogosa and Sharpe (MRS) agar for probiotic bacterial strains counts and in PCA for bacterial contamination counts, using the Miles and Misra technique (Miles *et al*., 1938). MRS agar plates were incubated following the conditions described in **Table 2.2** while PCA plates were incubated at 30 ºC during 24 h.

2.4. Gut microbiota simulation: *in vitro* model

2.4.1. 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). The composition of this media contained 5.0 g/L trypticase soy broth (TSB) without dextrose (BBL, Lockeysville, USA), 5.0 g/L bactopeptone (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 NH_4Cl, 10.0 g/L $MgCl_2.6H_2O$ and 10.0 g/L $CaCl_2.2H_2O$), 0.2 % (v/v) of salt solution B (200.0 g/L $K_2HPO_4.3H_2O$), 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 2.2.** 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% H_2, 10% CO_2 and 85% N_2).

2.4.2. Simulation of 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 2.4.1.**.The simulation of fermentation was performed during 48 h at 37 ºC in anaerobic conditions.

2.4.3. Total viable counts enumeration

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 technique (Miles *et al.*, 1938) in MRS agar and in PCA plates. The MRS agar plates were incubated

following the conditions described in **Table 2.2** while in the case of PCA plates were incubated at 30 ºC 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) (Lee and Lee, 2008). 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.

2.4.4. 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). 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 ºC (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 sulphuric acid at a flow rate of 0.6 mL/min. The running time was 30 min, and the injection volume was 50 µL.

2.4.5. Evaluation of pH changes over time

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

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

2.5. Gut microbiota simulation: Faecal fermentations

2.5.1. Faecal microbiota

Faecal samples were obtained fresh at the premises of Department of Food and Nutritional Sciences, Reading from five healthy adult volunteers. The volunteers had a normal omnivorous diet and had not ingested any antibiotics or other medicines known to affect the microbiota for at least 6 months. Volunteers were 2 males and 3 females aged 22-37 years and were not regular consumers of prebiotics or probiotics. Samples were collected into sterile vials and kept in an anaerobic cabinet 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 stomacher

(Serward, Worthing, UK) for 2 min at 460 paddle-beats per min. This prepared was designated as faecal slurry.

2.5.2. *In vitro* gastrointestinal digestion protocol

The digestion of TMIF was performed according to Mills *et al*. (2008) with slight modifications. 20 g of TMIF were ground and dissolved in 50 mL distilled water and the mixture was put in a stomacher (Seward, Worthing, UK) during 5 min. For the oral phase, in the sample 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 that, 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. In 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) was dissolved in 41.67 mL of 0.5 M $NaHCO_3$, the pH was adjusted to 7.0 with either 6 M of HCl or NaOH and incubated at 37ºC for 3 h on a shaker. All samples were transfer 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 more 2 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 use for *in vitro* faecal fermentations. All chemicals were purchased from Sigma (St. Louis, USA).

2.5.3. Faecal batch-culture fermentation conditions

Five independent fermentation experiments were carried out. Sterile stirred batch culture fermentation vessels of 300 mL were set up and aseptically filled with 135 mL sterile basal nutrient media (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, in one vessel 1% (w/v) digested TMIF was aseptically added, in another one 1% (w/v) undigested TMIF was aseptically added, for the positive control vessel 1% (w/v) FOS (from chicory root, purity: > 95%, degree of polymerization ranging from 2 to 8) (Megazyme, Bray, Ireland) was used and for the negative control vessel no source of carbon was added. Each

vessel, with 135 mL of sterile basal nutrient media was inoculated with 15 mL of fresh faecal slurry. The batch cultures were running 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* hybridisation (FISH), analysis of SCFA, BCFA and lactate by gas chromatography (GC) and quantification of ammonia production. All media and chemicals were purchased from Oxoid (Basingstoke, UK) and Sigma (St. Louis, USA).

2.5.4. Faecal pH control

A FerMac 260 pH Controller (Electrolab Biotech Ltd., Tewkesbury, Gloucestershire, UK) was used, at 37 ºC, to maintain the pH value range for each vessel between 6.7 and 6.9 (Sánchez-Patán *et al*., 2012).

2.5.5. Bacterial enumeration by FISH-FCM

In order to evaluate differences in bacterial composition in the batch cultures, samples were analysed by fluorescence *in situ* hybridisation combined with flow cytometry (FISH-FCM). The FISH-FCM was performed according to Grimaldi *et al.* (2017) with slight modifications. From faecal batch cultures, 750 µL were centrifuged during 5 min at 17,949 x g. The supernatant was removed and pellet of the samples resuspended and homogenised in 375 µL of 1x PBS and 1,125 µL of 4% paraformaldehyde (PFA). Both PBS and PFA were stored in cold conditions (4ºC). Samples were placed at 4 ºC during a period of 4 to 8 h in order to fixing the samples. After that time, the samples were centrifuged at 17,949 x g during 5 min, then the supernatants were discarded, and the pellet of samples resuspended in 1 mL of cold 1 x PBS. The samples were again centrifuged and washed with cold 1x PBS. Again, the samples were centrifuged, supernatant was discarded and 300 µL of cold 1x PBS and 300 µL of ethanol were added, then the mixture was vortexed and stored at -20ºC until further processes. Duplicates were done for each sample. Permeabilization steps of the bacteria cell wall were performed using 150 µL of fixed batch culture samples added to 500 µL 1x PBS and centrifuged at 17,949 x g for 3 min. The supernatant was discarded, and pellets resuspended in 100 µL of filtered TE-FISH (Tris/HCl 1 M pH 8, ethylenediaminetetraacetic acid (EDTA) 0.5 M pH 8, distilled H_2O) containing lysozyme solution (1 mg/mL of 50,000 U/mg protein) and incubated for 10 min at room temperature in the dark. Then the samples were vortexed and centrifuged at 17,949 x g for 3 min. The supernatant was discarded, and pellets were washed with 500 µL 1x PBS and centrifuged at 17,949 x g for 3 min. Hybridisations steps started by discarding the supernatant of the samples and resuspending the pellets in 150 µL of filtered hybridisation buffer (5 M

NaCl, 1 M Tris/HCl pH 8, 30% formamide, distilled H_2O, 10% sodium dodecyl sulphate (SDS)), vortexed and centrifuged at 17,949 x g for 3 min. Supernatant of the samples was discarded, pellets were resuspended in 1 mL of filtered hybridisation buffer, vortexed and 50 µL aliquoted into eppendorf tubes. **Table 2.3**, 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). Non EUB338 and EUB338 I-II-III used were linked at their 5'end either to Alexa488 or Alexa647. Group-specific probes used were linked with Alexa647 at their 5'end. To each aliquoted sample, 4 µl of each probe and 4 µl of Eub338 I-II-III linked to Alexa488 was added and incubated overnight at 35 ºC in a heating block. After incubation, 125 µL of hybridisation buffer was added to the aliquot samples with the probes, vortexed and centrifuged (17,949 x g, 3 min). The supernatant was discarded, and the pellets washed with 175 µL of washing buffer (5 M NaCl, 1 M Tris/HCl pH 8, 0.5 M EDTA pH 8, distilled H_2O, 10% SDS), vortexed and incubated for 20 min at 37 ºC in a heating block. After this incubation, samples were centrifuged (17,949 x g, 3 min), supernatants were removed and sample resuspend in 300 µL of 1 x PBS. Samples were stored at 4 ºC until the time of 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 analysed by FCM.

Table 2.3 - 16 rRNA oligonucleotide probes and hybridisation conditions used in the FISH 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	*Faecalibacterium prausnitzii* and related sequences	CGCCTACCTCTGCACTAC	Devereux *et al.* (1992)
DSV687	Most *Desulfovibrionales* (excluding *Lawsonia)* and *Desulfuromonales*	TACGGATTTCACTCCT	Hold *et al.* (2003)

2.5.6. Evaluation of organic acids production by GG

To evaluate the production of organic acids by the gut microbiota, GC analysis was performed. From faecal batch cultures, 1 mL of sample of each vessel was transferred to a flat-bottomed glass tube and 50 µL of 2-ethylbutyric solution was added in 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) was 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 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 was 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.

2.5.7. Evaluation of ammonia production

The 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.

2.6. Analysis of the protein profile of digested and undigested TMIF

In order to understand the impact of the *in vitro* digestion, protein profile of TMIF was carried out. From the solid samples of undigested and digested TMIF (powder), solutions of 0.4 g/mL (concentration used in the *in vitro* digestion protocol) were prepared. The pH was adjusted to 4.0 with either 1 M of HCl or NaOH and incubated at 50 ºC for 2 h on a shaker. The solutions were centrifuged at 4,410 x g, 4 ºC for 20 min. The supernatant of each solution was transferred into another tube and centrifuged again at 4,410 x g, 4 ºC for 20 min. After this centrifugation step, the supernatant of each solution was collect and stored at 4º C.

2.6.1. Quantification of dry weight of the samples

For the dry weight analysis, 500 µL of each prepared solution were placed on a petri dish (the petri dish was weighed before and after the sample was placed in) in an oven overnight at 100 ºC. After that, petri dishes were weighed with the samples and the dry weight percentages of the samples were calculated from these results.

2.6.2. Quantification of protein of the samples

For the quantification of the protein concentration present in the samples of undigested and digested TMIF (N_{kjel}, conversion factor 6.25) a Kjeldahl method (Kjeltec system 1002 distilling unit, Tecator, Hoganas, Sweden) according to NP 1612 2006 and as described by the Instituto Português da Qualidade (IPQ) (IPQ, 2006) was used.

2.6.3. Molecular weight profiling of the samples by FPLC

Protein molecular weight profile of undigested and digested TMIF was analysed by fast protein liquid chromatography (FPLC) through injection of aliquots of 100 µL of all the final solutions and separation by gel filtration chromatography using a Superdex™ 200 10/300 GL column connected in series to Superdex Peptide 10/300 GL column (GE Healthcare Life Sciences, Freiburg, Germany), coupled to a FPLC AKTA-purifier system (GE Healthcare Life Sciences, Freiburg, Germany). The eluent used was 0.05 M phosphate buffer pH 7.0, containing 0.15 M NaCl (ionic strength) and 0.2 g/L NaN_3 (as preservative) at a flow rate of 0.5 mL/min. Elution was monitored at 280 nm and approximate molecular mass of protein solutions were determined with a high molecular weight protein kit (GE Healthcare Life Sciences, Freiburg, Germany); ovalbumin (43 kDa); carbonic anhydrase (29 KDa); ribonuclease (13.7 kDa); aprotinin (6.5 kDa) and whey peptide (1.2 kDa), were used to perform molecular weight standard curve.

2.6.4. Detection and quantification of free and total amino acids of the samples

The analysis of free and total amino acid of each sample was carried out by precolumn derivatization with ortho-phthalaldehyde (OPA) methodology. Isoindole-type fluorescent derivatives were formed in an alkaline solution (borate buffer pH 10.4) from OPA, 2-sulfanylethanol and the primary amine group of the amino acid. The derivatives were separated by HPLC (Beckman Coulter, California, USA) coupled to a fluorescence detector (Waters, Milford, USA) according to the procedure of Proestos *et al.* (2008). 100 µL of each sample, at concentration 10 mg/mL, was derivatised according described method and injection volume of derivatives was 20 µL. All analysis was made in duplicate and quantified using a calibration curve built with amino acids pure standards (Sigma – Aldrich, St. Louis, USA) and expressed as mg/g of protein content.

2.7. 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 distributions 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 in samples that did not follow normal distribution. The weight of different factors (independent variables) in the differences observed, such as bacterial type, % inocula and incubation time was evaluated by comparing the F values for the two different studies with and without TMIF. Differences between the total amino acids of digested and undigested TMIF were evaluated using an independent samples t-test as they proved to follow a normal distribution.

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 ºC for 24 h was chosen owing to the lower temperature and absence of pressure (Hammond *et al.*, 2013).

3.2. 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 3.1**), 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 3.1**, 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.

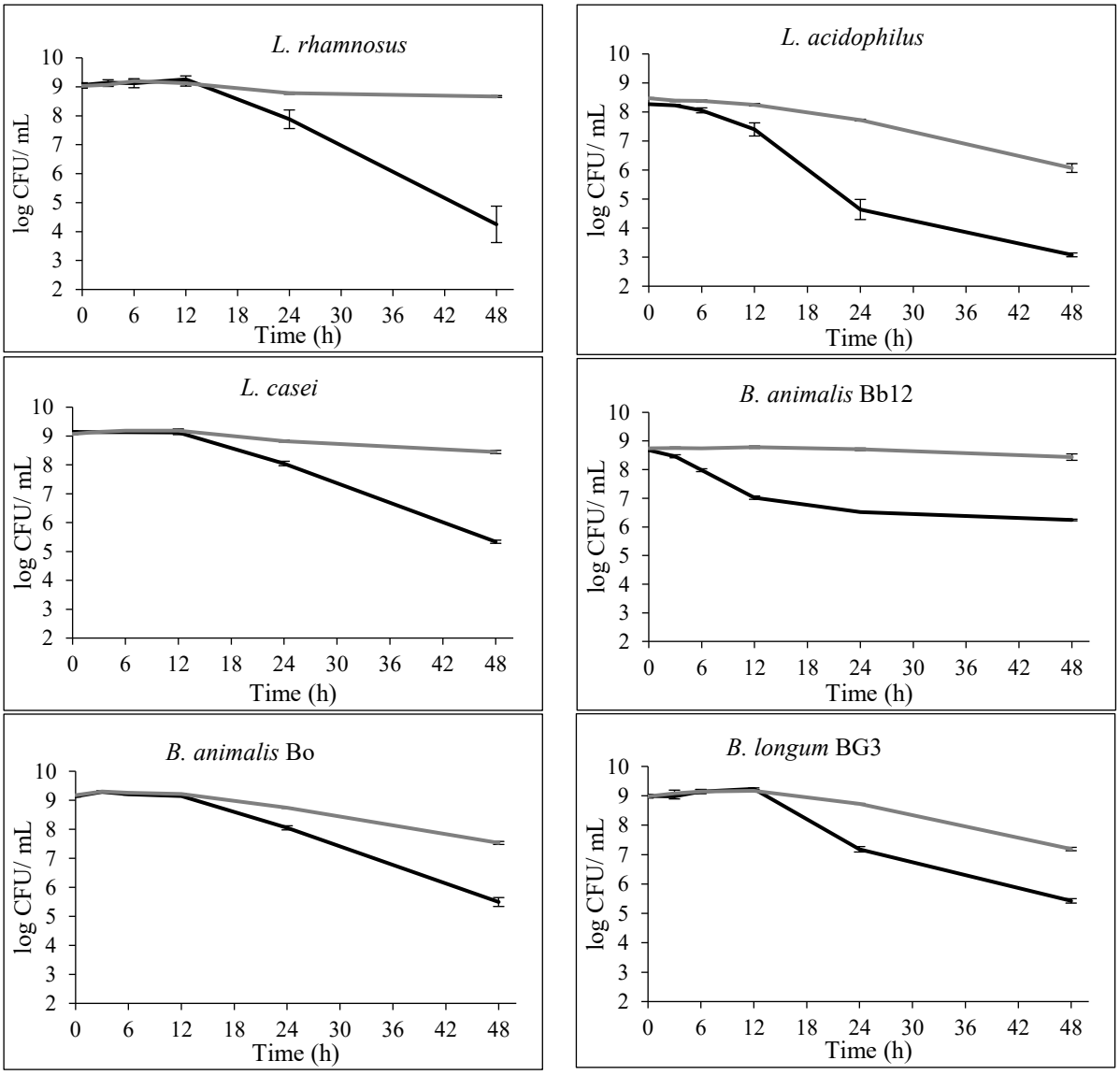

Figure 3.1- Bacterial viable cell counts (log CFU/mL, means ± SD) of *L. casei*, *L. rhamnosus*, *L. acidophilus*, *B. animalis* Bb12, *B. animalis* Bo and *B. longum* BG3 when inoculated at 10% in nutritive stress medium with (gray line) or without TMIF (black line) and incubated during 48 h at 37ºC.

3.3. Gut microbiota *in vitro* model

Recent studies have shown 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, by considering them "repulsive", their introduction on the market can be softened if they are processed in a flour form for example (Gmuer *et al.*, 2016, Verbeke, 2015, Wilson, 2016). One of the most commercialised insect flours is the one from *T. molitor*, which is a rich source of protein and an excellent source of fatty acids and fibre (Van Huis *et al.*, 2013). Insects have a high protein content, generally of good quality and highly digestible and are a good source of

essential amino acids (Chen *et al.*, 2009, Kouřimská and Adámková, 2016, Mlcek *et al*., 2014). The impact studies of such diet in humans are then particularly relevant, especially at the gut microbiota level, which is a good indicator for individual health.

The human intestine is inhabited by a highly diverse microbial ecosystem composed of hundreds of different species of bacteria. It is known that certain groups of bacteria are responsible for the metabolism of specific dietary compounds. However, microbial interactions are key in shaping the composition of the gut microbiome, where competition for nutrients has been commonly observed (Faust and Raes, 2012, Sung *et al*., 2017). Gut microbes also cooperate for resources, for example sharing macromolecule degradation products such as proteins or polysaccharides, or fermentation products such as SCFA (Abreu and Taga, 2016, Rakoff-Nahoum *et al.*, 2014). These interactions are dependent on the chemical nature of dietary compounds and could influence health through different SCFA profiles (Adamberg *et al.*, 2018, Medina *et al*., 2017; Walker *et al*., 2011). *Lactobacillus* and *Bifidobacterium* are important groups for their probiotic activities and are susceptible to the action of prebiotics (Slavin, 2013). Prebiotics, as is referred in Gibson *et al*. (2017), is "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 (Gibson *et al.*, 2017). Therefore, the consumption of TMIF, an insect product in a flour form, is prominent to provide benefits to the humans due to its characteristics, but mainly by the interaction with the beneficial microorganisms present in gut microbiota and their metabolism.

On the other hand, 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 to explore different conditions and compositions for the study, thus enabling simulation of the gut conditions (Charaslertrangsi, 2014).

3.3.1. Growth of selected probiotic bacteria

The model was used first to test monocultures, then in co-cultures as pairs and finally the use of 3 strains of each genus as a consortium. 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 (Madureira *et al.*, 2016). 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.

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 3.2**. 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 compared to initial time (0 h), but later recovered its cell concentration, as observed at time 48 h. Such behaviour may partially be explained as *L. acidophilus* is a slow acid producer (Sánchez-Zapata *et al*., 2013, Shah, 2003). The other *Lactobacillus* strains studied showed similar growth profiles along incubation times ($p>0.05$). At all fermentation times, significant differences, were only observed between the initial time (0 h) and times 24 h or 48 h. Between 24 h and 48 h there is no significant difference in terms of growth ($p>0.05$). In general, the higher cell growth was observed for 10% inocula than for 1% at the end of incubation time (48 h). In the case, of 1% of inocula the presence of TMIF positively affected *Lactobacillus* strains growth, except *L. casei* at the end of incubation time (48 h).

Lactobacillus strains are more positively affected by the presence of TMIF when compared to *Bifidobacterium*. In the case of *Bifidobacterium* strains, at the higher inocula concentration (10%) over 24 h of incubation, TMIF had no impact on the bacteria cell levels maintenance. As for the same period of time, at 1% the results are similar, with no impact, except for the *B. longum* BG3 with a small cell growth detected. Nevertheless, at 48 h the presence of TMIF showed a positive impact on *B. longum* BG3 at 10% and *B. animalis* Bo, at 1%.

Once again, the greater effect of the flour was observed after 24 h of growth, i.e. at 48 h of incubation time. This may be due to a decrease of nutritional content in the simulation basal media during the experience, since it is the most accessible nutrient source for the bacteria, and the presence of TMIF which can be used as a nutrient source for the studied bacteria (as seen in the **section 3.2.**), is consume later to help on their upkeep.

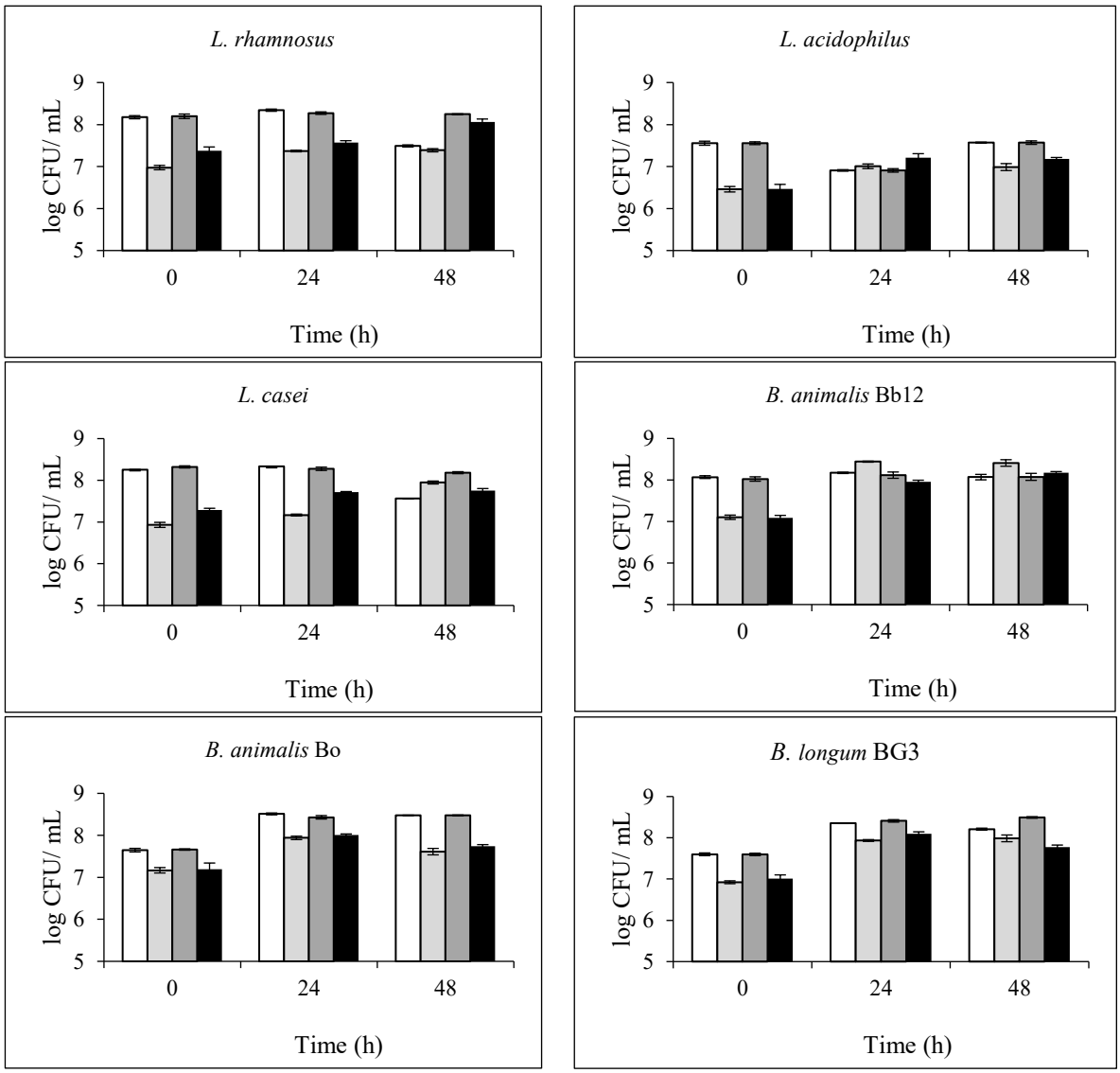

Figure 3.2- Bacterial viable cell counts (log CFU/mL, means ± SD) when inoculated at 10% and 1% in basal media without (□,□) or with TMIF (■,■) and incubated during 48 h at 37ºC.

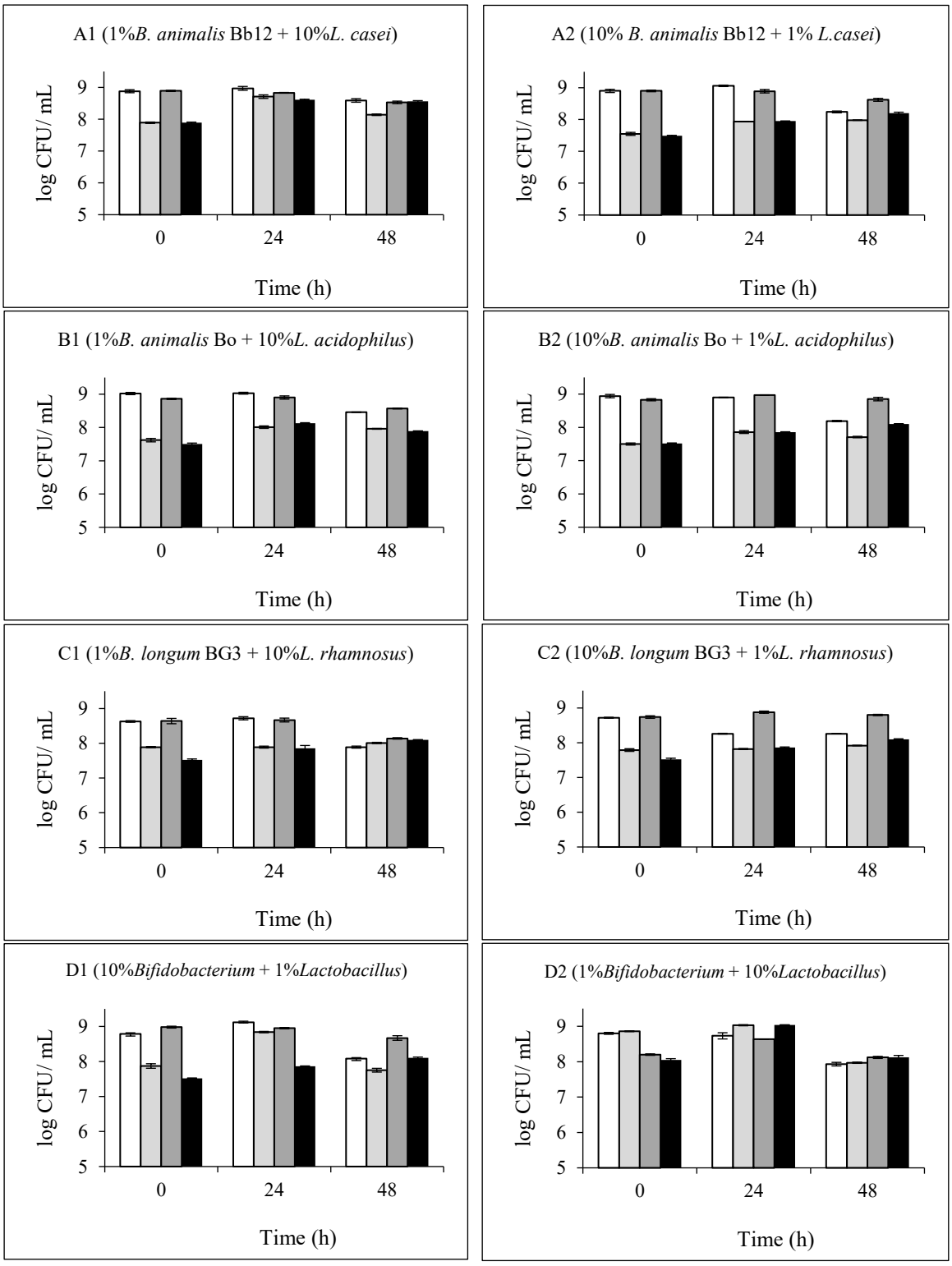

Figure 3.3- Bacterial viable cell counts (log CFU/mL, means ± SD) of co-cultures and consortium of *Bifidobacterium* and *Lactobacillus* strains in basal media without (□,□) or with TMIF (■,■) and incubated during 48 h at 37ºC.

Figure 3.3 shows the results for the growth of co-cultures, A1, A2, B1, B2, C1, C2 and consortium D1 and D2 of the studied bacteria. Overall, in the presence or not of TMIF in the media, the mixture of strains and percentage of inocula were factors that affected significantly growth of the bacteria ($p<0.05$). In terms of growth profiles, without significant differences it is possible to distinguish the consortium (D1 and D2) from the remaining mixtures. Incubation time was a factor that did not affected the growth profile ($p>0.05$), since overall, the differences observed were not statistically significant. Nevertheless, at 0 h, for the co-cultures, independently of the initial cell concentration inoculated of *Bifidobacterium/ Lactobacillus*, *Lactobacillus* was always present in lower concentrations compared to *Bifidobacterium* (Madureira *et al*., 2016).

In co-cultures, TMIF had no effect after 24 h in the majority of cases. Nevertheless, in B1 co-culture a small increase for *L. acidophilus* was detected, and in B2 a small increase for *B. animalis* Bo, both cases in the presence of TMIF. These overall results differ from 48 h 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 beneficiated 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 beneficiated 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 (Raksakantong *et al*., 2010).

Hence, such nutritional content can be used by the bacteria during fermentation, so enabling them to develop metabolism and functions (Jacobs *et al.*, 2009, Ríos-Covián *et al.*, 2016). However, the presence of fibre content, chitooligosaccharides, 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* (Fernandes *et al.*, 2012, Khoushab and Yamabhai, 2010, Yu *et al.*, 2016). It is then possible to assume that the bacterial growth enhancement maybe be possibly due to protein and peptide content present on TMIF.

3.3.2. 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**, **3.5** and **3.6**).

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 (Jacobs *et al*., 2009, LeBlanc *et al*., 2017, Ríos-Covián *et al*., 2016). Acetate and lactate were, as expected, and as found in other studies the most produced acids (Fliss *et al.*, 2010, Zalán *et al.*, 2010). 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 (Rowland *et al.*, 2017). Also, the presence of TMIF induced an increase of the production of acetate by *L. casei* at 48 h.

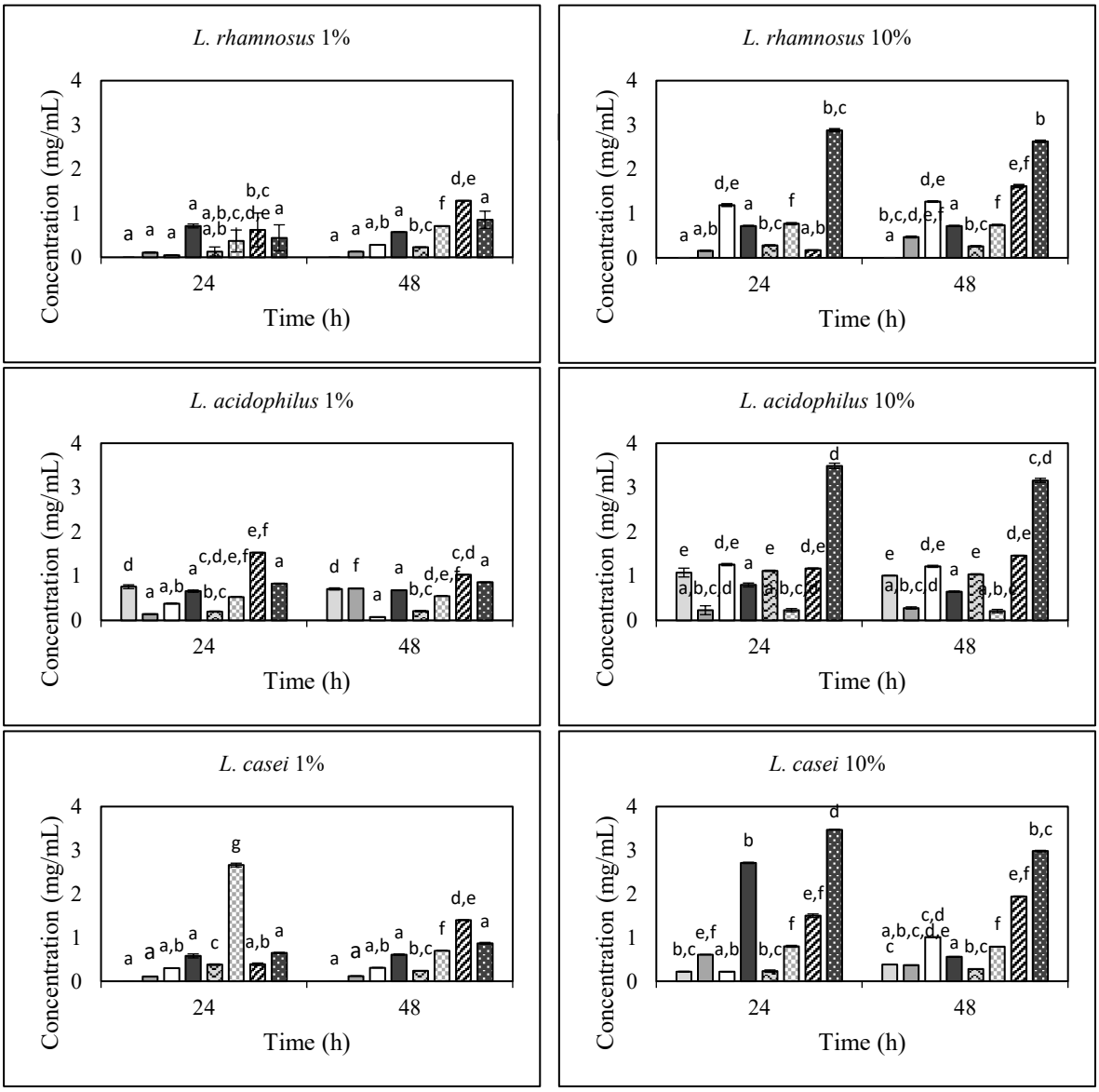

Figure 3.4- Evolution of the organic acids production (mg/mL, means ± SD) by the *Lactobacillus* strains when inoculated in basal media without (butyrate (□), propionate (■), acetate (□) and lactate (■)) or with TMIF (butyrate (▨), propionate (▨), acetate (▨) and lactate (▨)) and incubated during 48h at 37ºC. Different letters mark statistically significant ($p<0.05$) differences between samples for each compound.

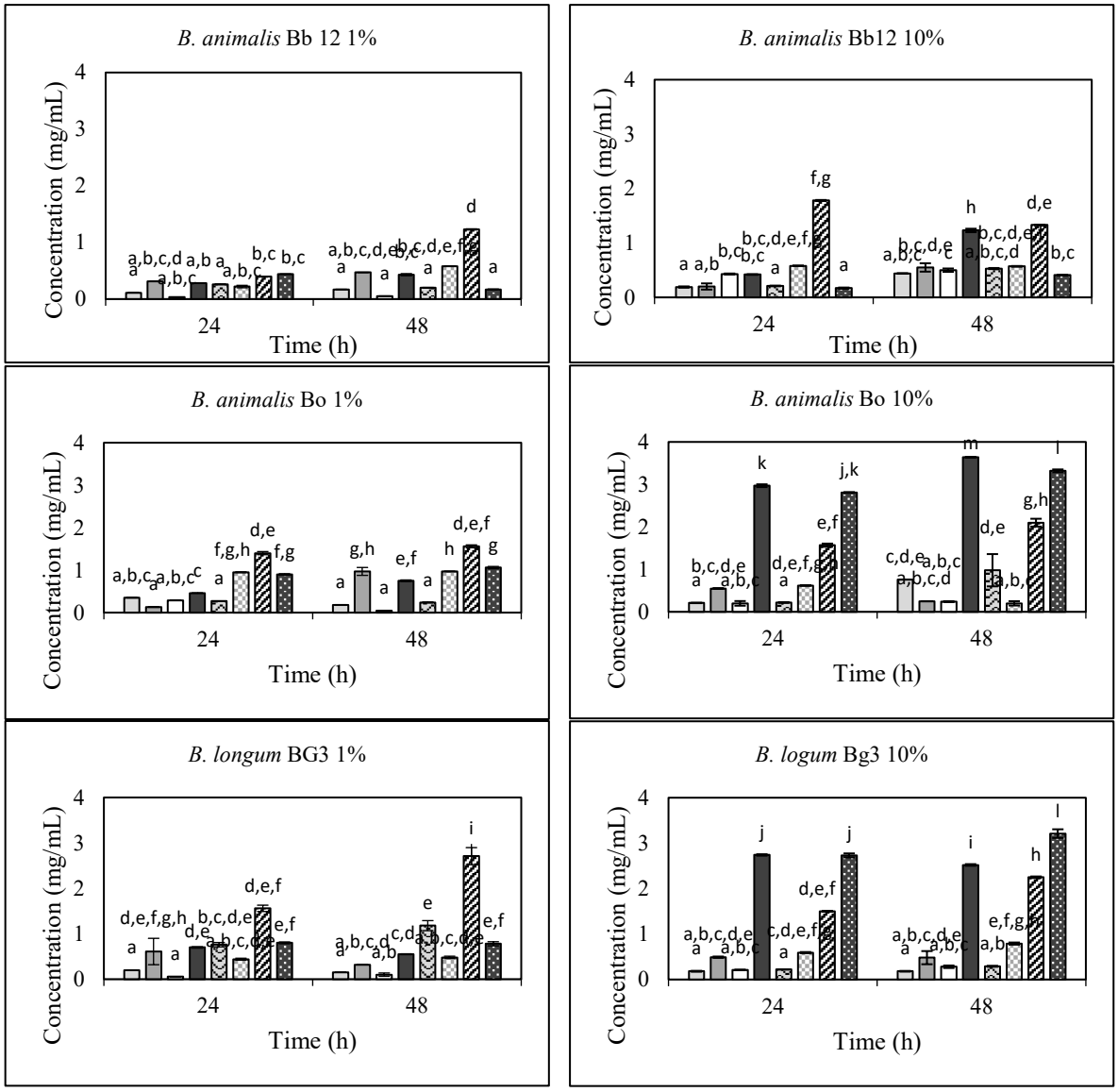

Figure 3.5 - Evolution of the organic acids production (mg/mL, means ± SD) by the *Bifidobacterium* strains when inoculated in basal media without (butyrate (□), propionate (■), acetate (□) and lactate (■)) or with TMIF (butyrate (▨), propionate (▨), acetate (▨) and lactate (▨)) and incubated during 48 h at 37ºC.Different letters mark statistically significant ($p<0.05$) differences between samples for each compound.

As shown in **Table 3.1**, 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.

Table 3.1- pH average values (± 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 (%)
L. rhamnosus	1%	5.70 ± 0.163	3.56	5.77 ± 0.193	6.12
	10%	4.60 ± 0.114	5.46	4.59 ± 0.068	2.60
L. casei	1%	5.67 ± 0.164	3.91	5.82 ± 0.175	5.77
	10%	4.49 ± 0.053	2.85	4.57 ± 0.008	0.22
L. acidophilus	1%	5.28 ± 0.341	13.19	5.44 ± 0.189	6.83
	10%	4.51 ± 0.207	10.00	4.59 ± 0.118	5.46
B. animalis Bb12	1%	5.60 ± 0.078	3.33	5.92 ± 0.114	-4.65
	10%	4.79 ± 0.167	7.57	4.89 ± 0.025	1.22
B. animalis Bo	1%	5.26 ± 0.215	6.67	5.82 ± 0.236	-10.34
	10%	4.41 ± 0.059	-0.90	4.55 ± 0.114	-5.84
B. animalis BG3	1%	5.57 ± 0.176	11.00	5.58 ± 0.156	5.00
	10%	4.69 ± 0.214	3.05	4.66 ± 0.078	-1.93

As for the production rate of SCFA in cases of co-cultures and consortium of bacteria, **Figure 3.6** 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 3.4** and **3.5**). 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 3.6**).

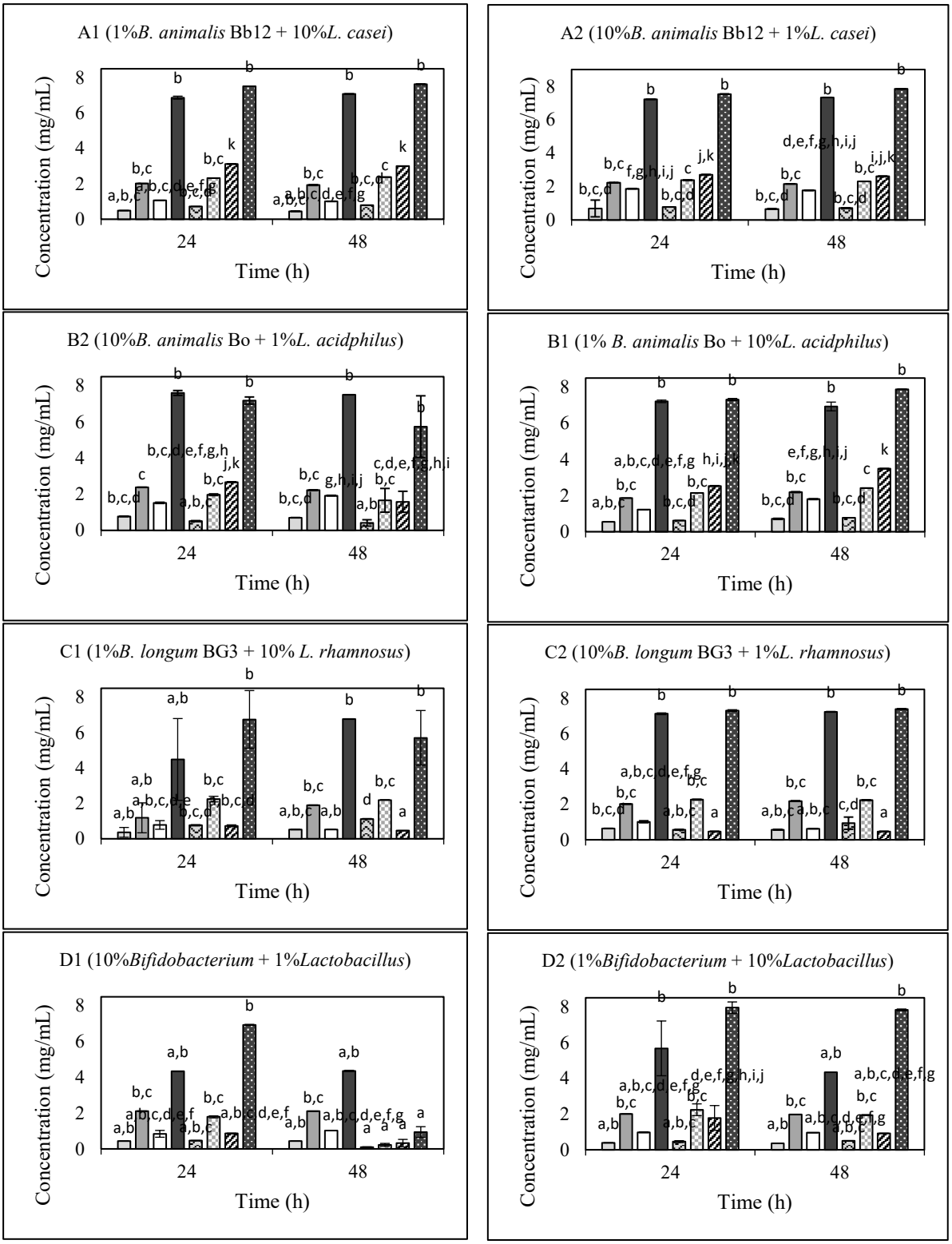

Figure 3.6- Evolution of the organic acids production (mg/mL, means ± SD) by the co-cultures and consortium strains when inoculated in basal media without (butyrate (□), propionate (■), acetate (□) and lactate (■)) or with TMIF (butyrate (▨), propionate (▨), acetate (▨) and lactate (▨)) and incubated during 48 h at 37ºC. Different letters mark statistically significant ($p<0.05$) differences between samples for each compound.

Since SCFA were being produced by the probiotics, a pH reduction on the media was expected, as a signal of its presence (**Table 3.1** and **Table 3.2**). As such, **Table 3.2** 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.

Table 3.2- pH average values (± 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 ± 0.128	5.41	4.50 ± 0.086	3.46
A2	4.39 ± 0.108	4.40	4.37 ± 0.098	5.34
B1	4.37 ± 0.049	1.58	4.43 ± 0.054	1.11
B2	4.35 ± 0.070	2.25	4.49 ± 0.067	2.40
C1	4.39 ± 0.104	3.75	4.47 ± 0.189	0.66
C2	4.36 ± 0.049	1.36	4.43 ± 0.046	0.67
D1	4.31 ± 0.034	-0.46	4.40 ± 0.025	0.46
D2	4.27 ± 0.026	1.39	4.35 ± 0.014	0.69

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 (Madureira *et al.,* 2015). At the same time, low pH values like these, inhibits 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* (Jacobs *et al.*, 2009, LeBlanc *et al.*, 2017, Morrison and Preston, 2016). This work also corroborates other findings, in that the most produced SCFA by these bacteria is acetate (den Besten *et al.*, 2013, LeBlanc *et al.*, 2017, Tan *et al.*, 2014).

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. This was the idea behind the use of an *in vitro* model, which was able to show results associated to the impact of TMIF on probiotics, primarily at monocultures, enabling an initial approach to the assessment of the individual strains interactions to the presence of TMIF.

Co-cultures and consortium studies were next due to the previous obtained results and taking in consideration that the human gut microbiota involves complex relations between several bacteria. This gave place to possible combinations and outcomes (positive, negative or neutral) of various interactions between different studied strains. With this in mind, an approach to understand how TMIF changes bacteria activity in the presence of several bacteria species was possible. At the same time, all these experiments opened a path to new study approaches, by seeking more complex impact studies of TMIF in the gut microbiota, especially when pursing other bacteria species and possible interactions. A natural evolution of these possibilities was the establishment of a faecal *in vitro* model to understand better the impact of TMIF on the human gut microbiota.

3.4. Gut microbiota effects in a faecal *in vitro* model

In order to evaluate the effect and impact of TMIF on the gut microbiota, five independent fermentation experiments were carried out on vessels with faecal samples from five healthy adult volunteers, who were not suffering from any known colonic conditions. Each donor samples were distributed by four vessels, later treated in four different conditions. Since during the digestion of the TMIF some nutrients may be lost through absorption in small intestine, on one of the vessels 1% (w/v) of digested TMIF was added and, in another vessel, 1% (w/v) of undigested TMIF. The digestion of TMIF was an *in vitro* method mimicking *in vivo* conditions (Alegría *et al*., 2015). *In vitro* models are used to assess and simulate physicochemical and physiological events on the digestive tract, allowing the studies of structural changes, bioavailability, and digestibility of foods (Hur *et al.*, 2011, Lee *et al.*, 2016). For the digestion simulation, human enzymes were used, and factors like concentration, temperature, pH and stability were taken into account (Mills *et al.*, 2008). The simulated digestion process of the TMIF also included a dialysis step to simulate the absorption in small intestine, in order to analyse the different behaviour of the bacteria present in the gut microbiota in the presence and absence of absorbed nutrients (Alegría *et al.*, 2015, Verhoeckx *et al.*, 2015). A positive control vessel had 1% (w/v) FOS added, as it is a well-established prebiotic (Oku and Nakamura, 2017, Rodrigues *et al.*, 2016, Scott *et al.*, 2015, Slavin, 2013, Yu *et al.*, 2016).

In vitro fermentations were performed in order to screen the effect of TMIF and to assess how this product can alter gut microbiota populations. The *in vitro* fermentations in this study were performed in a batch type simulator that allowed cultivation of complex intestinal microbiota from faecal samples of the donors, in anaerobic conditions.

In this work, the impact of TMIF on the gut microbiota was assessed by molecular quantitative technique, FISH-FCM, and the metabolic activity was done by GC to measure the production of SCFA, BCFA and lactate and also was quantify the amount of ammonia produced during the fermentation time.

3.4.1. Analysis of the impact on bacterial composition

For the establishment of any product as a prebiotic, or merely as safe for human consumption, it must render positive results in terms of its interaction with beneficial microbiota.

Digested and undigested TMIF results, for total bacteria, showed similar values to each other, approaching to those from the negative control. Positive control, as expected by the presence of FOS, exhibited the most significant bacterial growth throughout the study period. In general, different bacteria present in the faecal samples showed a small growth increase by 8 h of incubation and maintained the concentration of bacteria present from there until time 48 h.

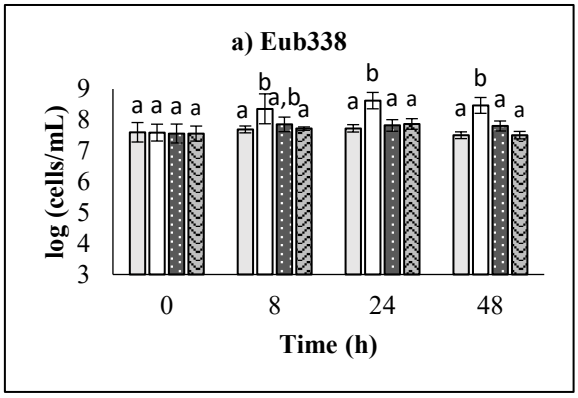

Figure 3.7- Bacterial populations (log (cells/mL), means ± SD) detected by FISH-FCM in faecal samples of 5 studied donors in 4 different conditions (negative control (□), positive control (□), undigested TMIF (▩) and digested TMIF (▨)). The used probe: a) total bacteria (Eub338). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

Bifidobacterium spp. growth showed similar behaviour over incubation time. Digested and undigested TMIF presented similar results. Negative control had approximately the same growth behaviour as the TMIF samples, as can be seen on **Figure 3.8**. Positive control showed significant increase compared to the other samples, as expected. Results obtained for *Lactobacillus spp*. showed a similar growth profile to those obtained for *Bifidobacterium*. A slight difference was observed at 8 h of incubation, where undigested TMIF results tended to get closer to those from the positive control, while at other conditions and other times, digested

and undigested TMIF tended to show growth profiles closer to the negative control values. The results showed better growth for the probiotics present in the gut microbiota (*Bifidobacterium* and *Lactobacillus*) in the presence of carbohydrates, as substrate, comparing to the two samples of TMIF (in which the main nutrient is protein). It should be highlighted the fact that the probiotics (*Lactobacillus* and *Bifidobacterium*) are in the presence of other groups of bacteria existing in the gut microbiota and, consequently, they are not expected to have significant growth, or to grow as well in the presence of TMIF as substrate as if they were alone or without other gut microbiota bacteria (as it can be seen on **Section 3.3).**

For *Bacteroidaceae* and *Prevotellaceae*, growth throughout time, in TMIF cases (digested and undigested) were similar to the positive control case. This may indicate, in this case, a positive impact of TMIF, as it partially matches FOS proprieties, in terms of bacteria upkeep, thus indicating that TMIF can be as well used as subtract as FOS, by these bacteria. *Bacteroides* are predominant proteolytic bacteria, and possess strong peptidase activity, being the main producers of propionate, and are positively associated with isovalerate and isobutyrate, which for their turn are negatively correlated to blood levels of triglycerides (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 its growth.

The growth of *Atopobium* cluster, has been reported to be increased by disaccharides, polysaccharides and long-chain inulin (Vinke *et al.*, 2017). Such facts seem to corroborate with results obtained in the positive control, where *Atopobium* cluster had significant increase at 8 h and maintained these levels throughout time. 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 differ – the sample with the undigested form maintains the *Atopobium* cluster concentration level over such periods, while, in the digested form sample, a slight concentration 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, the few studies reported a direction in which *Atopobium* presence correlates with beneficial effects in terms of cardiometabolic health (Vinke *et al.*, 2017).

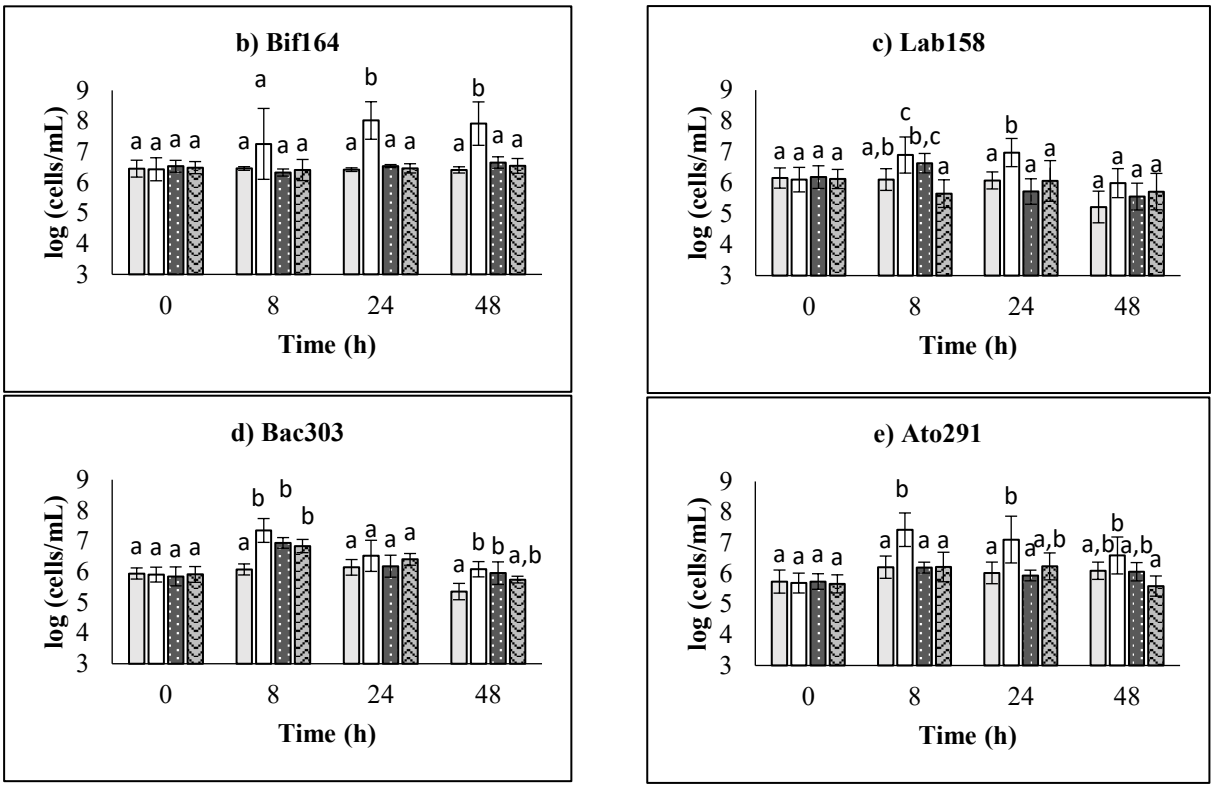

Figure 3.8- Bacterial populations (log (cells/mL), means ± SD) detected by FISH-FCM in faecal samples of 5 studied donors in 4 different conditions (negative control (□), positive control (□), undigested TMIF (▩) and digested TMIF (▨)). The used probes:
b) *Bifidobacterium* spp. (Bif164), c) *Lactobacillus* spp. (Lab158), d) most *Bacteroidaceae* and *Prevotellaceae* (Bac303) and e) *Atopobium* cluster (Ato291). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

As to the *Clostridium coccoides* / *Eubacterium rectale* group results by 8 h for the sample with digested TMIF showed a significant decrease on cells number. In the cases of undigested TMIF, positive and negative control a similar behaviour was displayed between them in every fermentation time (**Figure 3.9**). The *Clostridium coccoides* / *Eubacterium rectale*, is a group of anaerobic bacteria, well-known for butyrate production, as is *Roseburia* and *F. prausnitzii,* in the gut microbiota (Lopetuso *et al.*, 2013). The *Clostridium histolyticum* group, is a clostridia group that possesses some pathogenic species such as *Clostridium perfringens* and *Clostridium tetani.* This group showed no significant deviations between conditions at specific study times (except on positive control time 24 and 48 h). Clostridia are proteolytic bacteria and some clostridia possess weak saccharolytic activity, preferably fermenting amino acids (Rowland *et al.*, 2017, Scott *et al.*, 2013). This last characteristic may explain the significant growth of *Clostridium histolyticum* group at 24 and 48 h of incubation in the positive control

(carbohydrates), but no significant growth in the positive control on *Clostridium coccoides* / *Eubacterium rectale* group.

The *Roseburia* genera is also abundant in the intestinal microbiota, and it can produce both propionate and butyrate (Ríos-Covián *et al.*, 2016). *Roseburia* can grow in presence of carbohydrate, and some *Roseburia* species have FOS degradation genes or an inducible fructan utilisation operon (Scott *et al.*, 2013, Scott *et al.*, 2015). In this experiment, in the presence of FOS, *Roseburia* showed slight growth at 8 and 24 h. At 48 h, a major decrease in *Roseburia* was observed, in the positive control, which may be explained by the diminution of FOS, as it was being utilised along fermentation. As for the presence of TMIF, a decrease of concentration was seen, but the undigested form always maintained a higher concentration compared to the digested form. This observation is in line with other studies showing that the concentration of the *Roseburia* group is related with the concentration of carbohydrate (Rowland *et al.*, 2017, Scott *et al.*, 2014).

The *Clostridium* cluster IX belongs to the group of bacteria that mainly produce propionate in gut microbiota (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 growth profile with FOS moved toward values obtained in the samples with TMIF, and at 48 h, a decrease was observed in all samples, however in the samples with undigested TMIF such decrease was softer. Nevertheless, according to the statistical analyses, these differences are not significant, when compared between them. The findings for the *Clostridium* cluster IX seem to correlate with the scientific literature. In samples with TMIF, growth was expected, due to the presence of amino acids. As for the samples with FOS, growth was observed just up to 24 h, which must be related with cross-feeding process, as these bacteria use lactate (previously produced by other bacteria) as substrate for the production of propionate (Bernalier-Donadille, 2010, Louis and Flint, 2017), which can be related to the lactate disappearance after 8 h in the **Figure 3.10**.

Faecalibacterium prausnitzii, a strictly anaerobic bacteria, is one of the most abundant species present in healthy human microbiota. It is considered a possible next-generation probiotic and it is one of the main butyrate producers (Conlon and Bird, 2014, Scott *et al.*, 2015). In the presence of digested TMIF, there was a decrease of bacteria concentration along fermentation time, but less significant in the presence of FOS or as in the negative control. The sample with undigested TMIF, was the only that showed, at 8 h, slight growth of these bacteria.

Desulfovibrionales and *Desulfuromonales* are only found in proximally 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 H_2 or organic compounds like lactate and formate to reduce sulphate to generate hydrogen sulphide (H_2S), which has a highly toxic nature, that can have pathological consequences for the host and it has proven to be cytotoxic, genotoxic and carcinogenic in *in vitro* and animal models (Conlon and Bird, 2014, Rowland *et al.*, 2017). Several studies identified SRB in the faecal microbiota of healthy adults and despite being positively associated with inflammation, the presence of H_2S has been attributed to pro and anti-inflammatory signalling. Lactate is also a favoured 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 faecal samples of the donors, comparing to concentrations of other quantified groups. At 8 h, in all conditions, there was a small increase of *Desulfovibrionales* and *Desulfuromonales* with no significant difference between the studied conditions. 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 (**Figure 3.10**).

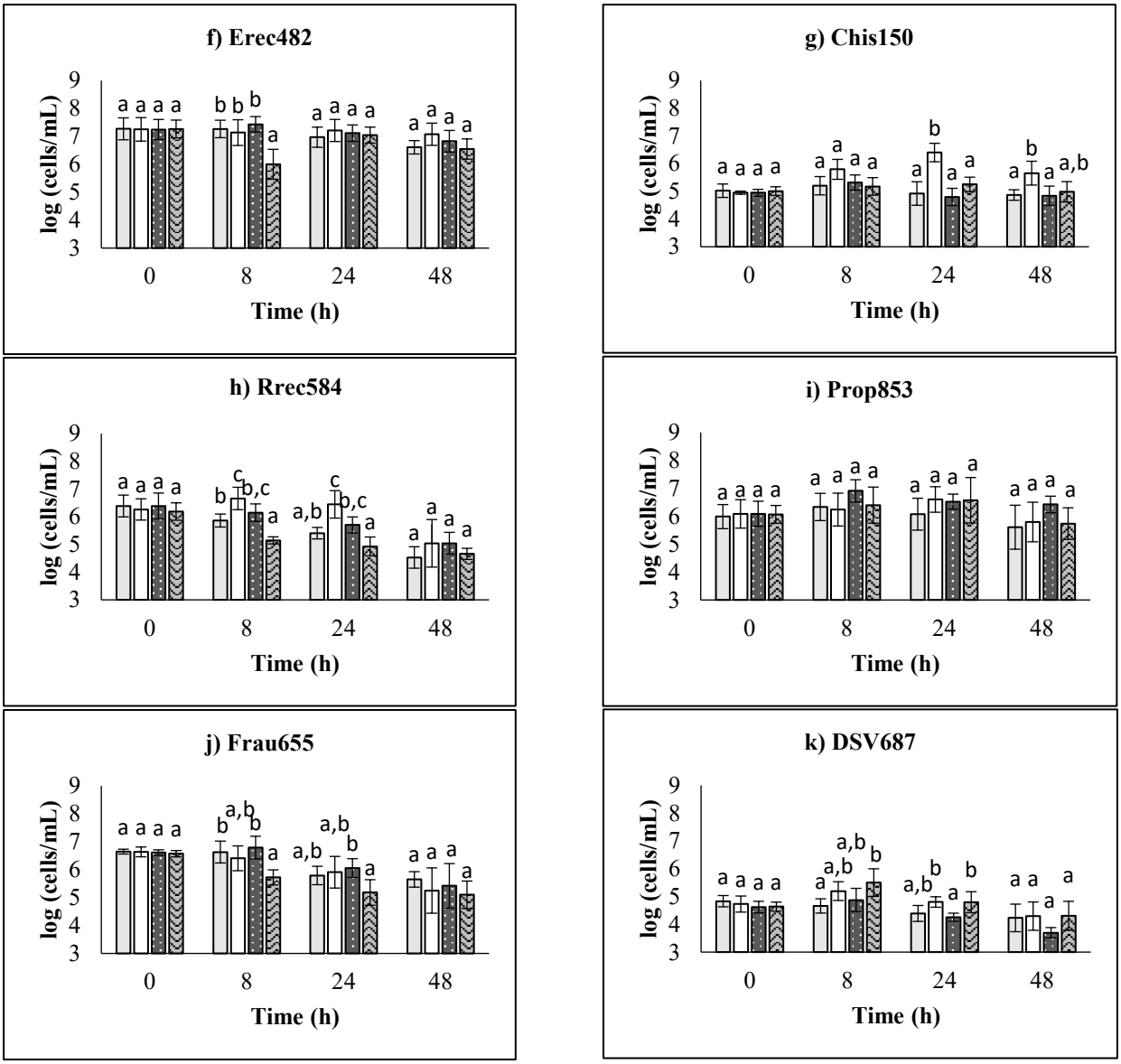

Figure 3.9- Bacterial populations (log (cells/mL), means ± SD) detected by FISH-FCM in faecal samples of 5 studied donors in 4 different conditions (negative control ( ), positive control ( ), undigested TMIF ( ) and digested TMIF ( )). The used probes: f) *Clostridium coccoides / Eubacterium rectale* group (Erec482), g) most of the *Clostridium histolyticum* group (Chis150), h) *Roseburia* subcluster (Rrec584), i) *Clostridium* cluster IX (Prop853), j) *Faecalibacterium prausnitzii* (Fprau655) and k) *Desulfovibrionales* and *Desulfuromonales* (DSV687). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

In this study it was possible to see the effect of undigested and digested TMIF through the modulation of gut bacterial population growth. The most outstanding results were found on the growth of *Bacteroidaceae* and *Prevotellaceae,* which are bacteria related to proteolytic activity

and that can confer benefits to the host through their activity (e.g. propionate production). Such result makes sense since the main nutrient on TMIF is protein. The bacteria growth seems to indicate that undigested and digested TMIF have almost the same nutrients, however 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 the concentration of these bacteria. This may be related to the fact that digested TMIF does not have so many nutrients, probably carbohydrates, which are the main substrate for their growth.

3.4.2. 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). These results were also found in this study, with the evidence of production of SCFA and BCFA, in similar proportions as those normally expected, as can be seen in **Figure 3.10** and **3.11**.

As a general overview, acetate, propionate and butyrate concentrations were higher overtime for digested and undigested TMIF in comparison to the negative control. In all cases, positive control had significant concentrations over the other trials, as expected.

Butyrate is one of the most important SCFA for human health. It provides key energy source for human colonocytes, possesses potential anti-cancer activity, by inducing apoptosis of colon cancer cells and regulating gene expression, it nourishes intestinal cells, and induces mucin production, allowing changes on bacterial adhesion and improving tight-junctions' integrity (Barczynska *et al.*, 2016, Ríos-Covián *et al.*, 2016, Rowland *et al.*, 2017). TMIF samples showed increased production of this acid (with higher production for the undigested form). Propionate acts as an energy source for the epithelial cells, has a positive effect on the growth of hepatocytes, and it 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, with high levels of this acid. Acetate, for its turn, is an essential co-factor/metabolite for the growth of other bacteria, and even in the ability of bifidobacteria to inhibit enteropathogens (Ríos-Covián *et al.*, 2016, Rowland *et al.*, 2017). Acetate was also found to reduce the appetite trough 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). This study supports the findings that acetate is one of the most abundant SCFA, as it shows high production values, and in TMIF samples such pattern also emerged, indicating that TMIF may promote the equilibrium, in terms of the production of this acid and its implications on the organism. Although for the undigested and digested TMIF, the acetate and propionate production, in each of the cases, was almost the same, in butyrate, the undigested form had higher production comparing to the digested form. These results seem promising for the TMIF, since acetate and propionate are both associated with the promotion of satiety, which is interesting for a protein enriched product whose aim is to be a substitute for meat.

Lactate was also found, produced by bacteria, such as lactic acid bacteria, 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 showed higher concentration in positive control in comparison with the other conditions. The presence of TMIF promoted this acid production in concentrations higher than the negative control, however this difference showed no significant statistical relevance. An interesting result was observed in all conditions in that there was no lactate after 8 h (in time 24 and 48 h). This is an expected result since under normal physiological conditions lactate produced in the gut by some bacteria, such as acid lactic bacteria and bifidobacterial, does not accumulate in the colon because of presence of some species that will convert into some different organic acids (Flint *et al.*, 2015, Ríos-Covián *et al.*, 2016, Rowland *et al.*, 2017). This type of relationship is known as metabolic cross-feeding, that consists in the use of end products from the metabolism of a given microorganism by another one (Ríos-Covián *et al.*, 2016, Rowland *et al.*, 2017).

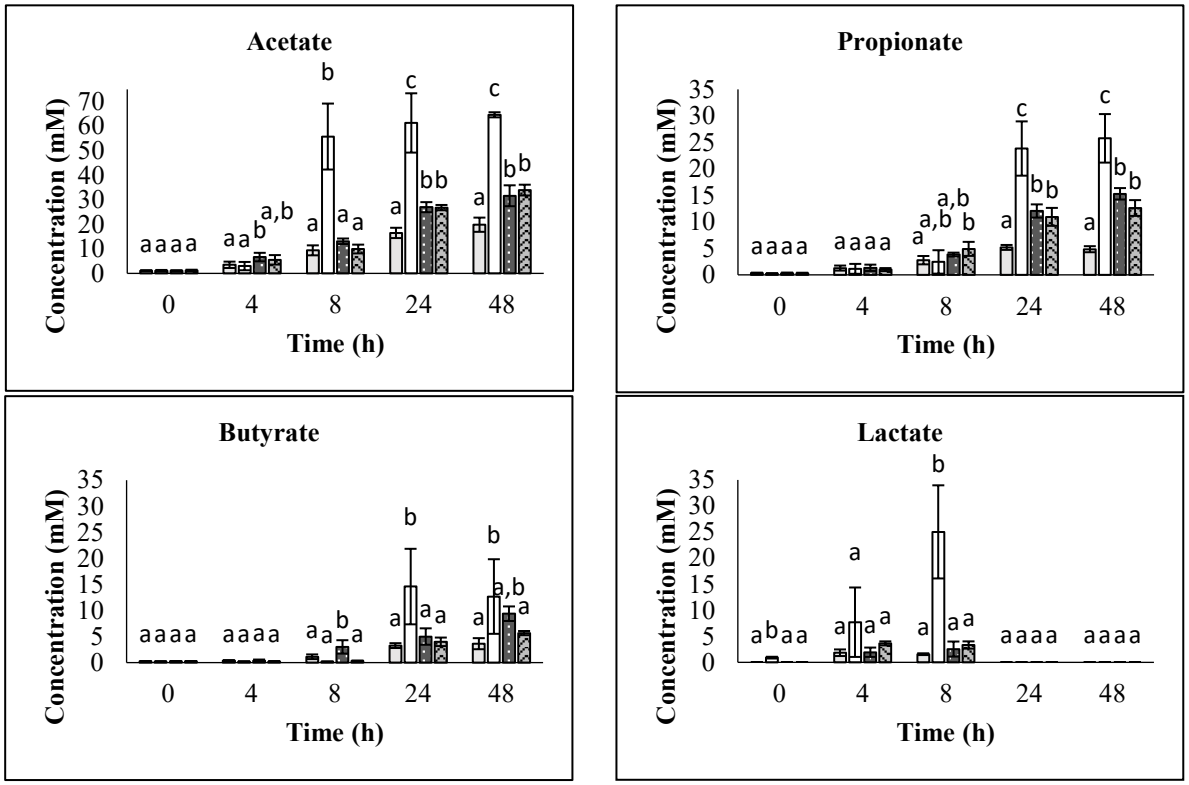

Figure 3.10- Concentration (mM, means ± SD) of the SCFA and lactate produced along fermentation time in faecal samples of 5 studied donors in 4 different conditions (negative control (□), positive control (□), undigested TMIF (▩) and digested TMIF (▩)). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

The presence of undigested TMIF showed to be the condition with the higher concentration of valerate, isobutyrate and isovalerate, especially at time 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 past 8 h. In the valerate results, the only condition that did not produce this acid was in the presence of digested TMIF. Little is known of the potential health benefit of valerate, how they are produce in the gut microbiota and what type of bacteria are these acids related (Ríos-Covián *et al.*, 2016). Further studies are required to understand the importance of valerate and how they are produce by the gut microbiota.

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 in low concentration on the human colon while acetate, propionate and butyrate are the most abundant there (90-95%) (Huda-Faujan *et al*., 2010, Ríos-Covián *et al*., 2016). The isobutyrate and isovalerate are primarily produced from the protein degradation particularly from branched

amino acids fermentation and the increase of production of these acids is observed when the presence of carbohydrates is limited (Huda-Faujan *et al*., 2010). The faecal 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). According to **Figure 3.11**, is possible to see the higher production of isobutyrate and isovalerate in the condition where undigested TMIF is present, which means that under this condition occurred the highest protein fermentation, in all tested conditions. This was expected since undigested TMIF is the substrate that contains higher indigestible protein content in contact with the faecal samples, thus inducing higher fermentation of branched amino acids present in undigested TMIF.

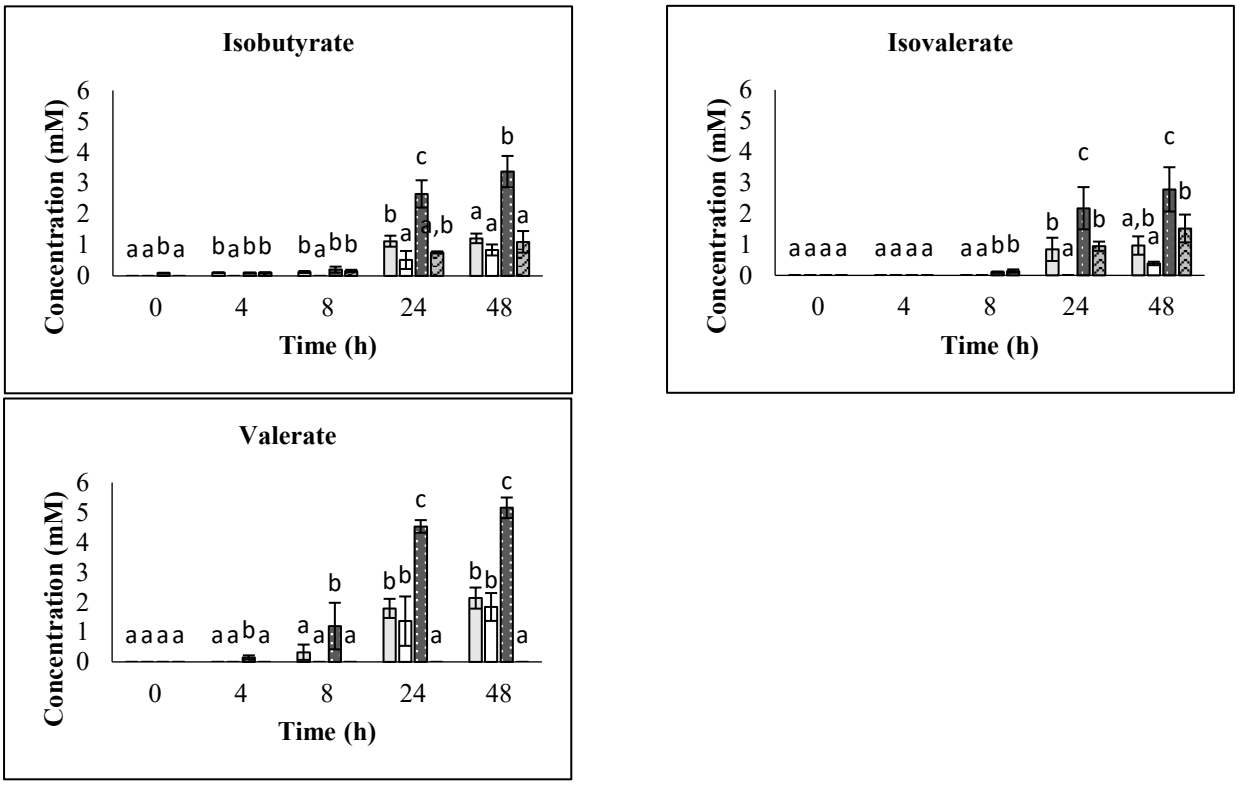

Figure 3.11- Concentration (mM, means ± SD) of BCFA and valerate produced along fermentation time in faecal samples of 5 studied donors in 4 different conditions (negative control (□), positive control (□), undigested TMIF (▩) and digested TMIF (▩)). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

The production of SCFA by the gut microbiota level is essential for the organism's well-being and healthy upkeep. TMIF, on undigested or digested form, showed a positive impact on the productions of SCFA.

3.4.3. Analysis of the impact on ammonia production

The major source of nitrogen, for the microorganisms present in the colon, are the dietary proteins. Nitrogen is used by bacteria for growth and it is also important for carbohydrate assimilation and production of beneficial compounds such as SCFA (Conlon and Bird, 2014). As the presence of SCFA and BCFA, at the gut microbiota level, is a result of the fermentation of carbohydrates and protein by the microbiota, and considering the high protein level of TMIF, a small increase of ammonia is 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 then an indicator of protein presence and degradation. The results are shown in **Figure 3.12**.

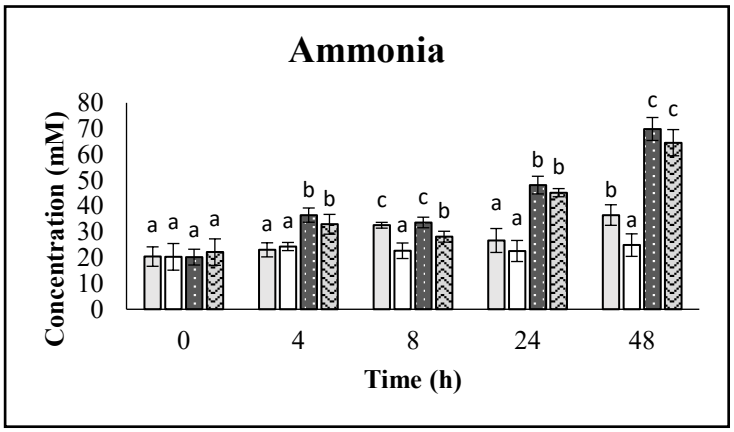

Figure 3.12- Concentration (mM, means ± SD) of ammonia produced along fermentation time in faecal samples of 5 studied donors in 4 different conditions (negative control (□), positive control (□), undigested TMIF (▩) and digested TMIF (▨)). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

In **Figure 3.12**, it is possible to assess that ammonia levels increased overtime in the presence of digested and undigested TMIF, in a similar concentration and pattern, except in time 8 h. This may be explained as the bacteria degrade the protein present in the samples, thus becoming an indicator of protein degradation, occurring along the fermentation time.

Faecal ammonia concentration in humans varies between 12 mM to 30 mM and increase with high intake of protein, which is possible to see in **Figure 3.12** (Scott *et al.*, 2013). Studies show that, up to 50 mM of NH_4Cl, there is no evidence of significant loss of membrane integrity, or alteration on pig colonic crypt cells viability, thus indicating no cytotoxic effect against colon epithelium (Leschelle *et al*., 2002); and at the same time, lesions on gastric mucosa were only

found when concentration of ammonia was superior to 125 mM (Tsujii *et al*., 1992). The results of ammonia concentration levels obtained in this study seem to be within "safety levels".
The ammonia results obtained, for the four conditions, were expected since ammonia is an end product of protein degradation and therefore the samples containing the TMIF (undigested or digested) should be those with higher concentration of ammonia comparing to the positive and negative control. It is interesting to see in the **Figure 3.12** that in both type of TMIF the concentration of ammonia is almost the same without significant differences, except at time 8 h which may mean that the protein content of both sample is the same or similar.

3.5. Protein characterisation of undigested and digested TMIF

The protein characterization of undigested and digested TMIF was performed to understand the differences between both samples in term of proteins, which is the main nutrient (44.6%). In terms of sample solubility and protein dry weight, present in **Table 3.3**, a clear difference exists in the solubility of the samples showing that, undigested TMIF could only solubilize 12.2% while the digested TMIF solubilized 48.6%. Studies show that TMIF is difficult to solubilize, and its protein solubilisation is dependent on the pH during extraction (Bußler *et al.*, 2016, Yi, 2015). For this study, pH level was set to 4, in order to solubilize the maximum of protein without interfering with its profile. This choice was due to previous studies, showing that the insect proteins had their isoelectric point around such value, and although the same works reported highest solubility at pH 10. However, this value was not pursued as it may induce changes on the free amino acid profile, altering their availability on samples (Bußler *et al.*, 2016). Even more, studies show that non-defatted TMIF decreased solubilisation of its extracted proteins, which in this study it is possible to see in **Table 3.3**. Undigested TMIF, which contains more fat than the digested TMIF, is the sample that less solubilises (Bußler *et al.*, 2016). Contrary to what happened in the previous parameter, the protein dry weight in both sample seems to be similar, between them, and it is also in the same range of protein dry weight from the scientific literature, 63-68% (Yi, 2015). That could mean that almost all the protein content present in the TMIF, may arrive at the gut after its digestion. For that reason, a FPLC was performed to see if the molecular weight of peptides of both samples were similar or not.

Table 3.3- Protein properties of undigested and digested TMIF

	Undigested TMIF	Digested TMIF
Sample solubility (%)	12.2	48.6
Protein Dry Weight (%)	67.04	66.43

As can be seen in **Figure 3.13**, the FPLC profile of both samples was similar. The molecular weight of peptides was calculated by the calibration curve obtained with the high molecular weight protein kit (y= -0.106x + 6.7794; R^2=0.9797; y-molecular weight; x- elution volume). The first absorbance peak, at 40 mL elution volume, corresponds to a peptide with 394 Da. After such elution volume, all peaks correspond to lower molecular weight compounds, which means below 191 Da (higher the elution volume, the smaller their molecular weight). So, the "real" molecular weight peptide profiles are not shown, as the obtained profile is consistent with amino acids and nitrogen compounds that solubilised. In such terms, undigested and digested TMIF, show almost no difference, as occurring differences would be expected at high molecular weight peptides, which were not possible to study since these were not soluble.

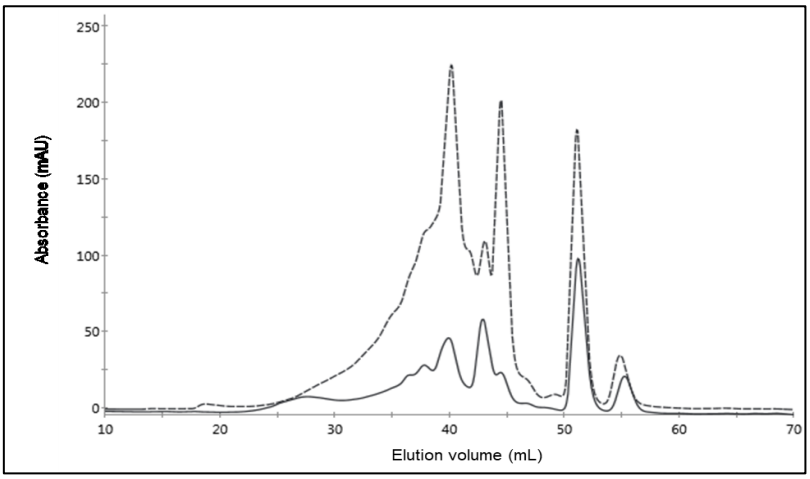

Figure 3.13- FPLC chromatogram of undigested TMIF (—) and digested TMIF (--).

For total amino acids concentration, as can been seen in **Table 3.4**, there is a slight significant difference in both samples. Undigested TMIF showed to have a significant higher total amino acids concentration, especially on isoleucine, phenylalanine, tyrosine and threonine, compared with the digested TMIF. The production of the BCFA is higher in the presence of undigested TMIF, which can be explained with the results obtained for the concentration of branched chain amino acids, which is higher in the undigested TMIF. Therefore, the results of BCFA production correlate with protein characterization of undigested and digested TMIF.

The most significant difference between undigested and digested TMIF occurred on the concentration of free amino acids (**Table 3.4**). Digested TMIF showed to have higher free amino acids content than the undigested sample.

These results were expected since one of the samples underwent a digestion protocol while the other did not. The sample that was submitted to a pre-digestion had the higher concentration of

free amino acids, due to the protein breakdown, which led to an increase in the content of free amino acids and a decrease in the molecular weight of the amino acids.

The total amino acids content present in the undigested TMIF was supposed to be higher, in comparison to the digested form. This result happened and is related to the molecular weight of the amino acids molecules and to the pore size of dialyze membrane that was used to mimic the absorption in the small intestine which allowed to lose small size amino acids (100-500 Da). The digested TMIF sample has more small size amino acids compared to the undigested TMIF sample due to the digestion protocol while, the undigested TMIF sample has molecules of amino acids with higher molecular weight. This way explaining the significant difference of total amino acids content in both samples of TMIF.

Table 3.4- Total and free amino acids concentration (mg/g, means ± SD) present in undigested and digested TMIF. * marks statistically significant ($p<0.05$) variations in amino acids content between digested and undigested samples. Nd – not detected.

	Undigested TMIF		**Digested TMIF**	
Amino acids	Free amino acids concentration (mg/g)	Total amino acids concentration (mg/g)	Free amino acids concentration (mg/g)	Total amino acids concentration (mg/g)
Aspartic acid	0.11 ± 0.001	126.90 ± 6.322	0.33 ± 0.019	112.11 ± 1.559
Glutamic acid	0.072 ± 0.00008	131.46 ± 7.032	0.32 ± 0.022	112.06 ± 1.716
Cysteine	Nd*	6.93 ± 0.580	0.038 ± 0.002*	6.37 ± 0.113
Asparagine	Nd	Nd	Nd	Nd
Serine	0.047 ± 0.0122*	46.86 ± 1.102	0.13 ± 0.007*	41.50 ± 0.505
Histidine	Nd	Nd	Nd	Nd
Glutamine	Nd*	19.04 ± 0.990	0.22 ± 0.011*	19.95 ± 0.206
Glycine	Nd*	55.39 ± 12.795	0.30 ± 0.005*	59.56 ± 2.771
Threonine	0.12 ± 0.004*	50.61 ± 0.916*	0.21 ± 0.011*	43.53 ± 0.386*
Arginine	0.86 ± 0.020*	55.04 ± 0.998	4.52 ± 0.244*	48.13 ± 0.366
Alanine	0.34 ± 0.015	63.74 ± 2.198	0.44 ± 0.007	56.20 ± 0.741
Tyrosine	0.38 ± 0.050*	65.10 ± 0.753*	2.75 ± 0.122*	54.87 ± 0.599*
Valine	0.34 ± 0.009	56.84 ± 0.801	0.49 ± 0.026	52.48 ± 0.562
Methionine	Nd	Nd	Nd	Nd
Tryptophan	Nd	Nd	Nd	Nd
Phenylalanine	0.27 ± 0.0009*	27.39 ± 0.090*	1.39 ± 0.034*	24.37 ± 0.261*
Isoleucine	Nd*	36.31 ± 0.283*	0.28 ± 0.014*	33.16 ± 0.316*
Leucine	0.044 ± 0.0045*	56.79 ± 2.051	1.43 ± 0.042*	51.39 ± 0.542

4. General conclusions

In this study, it was possible to find effects of the presence of TMIF, in gut microbiota bacteria, using *in vitro* gut microbiota models.

In the first model, the gut microbiota *in vitro* model, TMIF did not inhibit the growth of probiotic bacteria, which means that it had no antimicrobial effect. The TMIF increased the growth of almost all studied bacteria in monocultures and consequently the production of SCFA and lactate. TMIF showed a great potential for the maintenance of probiotic bacteria, especially under nutritional stress conditions.

In the second model, the gut microbiota faecal *in vitro* model, which is a model closer to reality, TMIF showed positive effect on the growth of *Bacteroidaceae* and *Prevotellaceae* but no growth was associated with *Clostridium histolyticum* group or *Desulfovibrionales* and *Desulfuromonales*. However, the probiotics growth was not significant in the presence of TMIF in this model. This may be related with the fact that, in the presence of the other bacteria, the probiotics have disadvantages for using the nutrients present in the TMIF. The production of SCFA and BCFA was highly satisfactory, especially obtaining high concentrations of acetate and propionate.

The ammonia production in the presence of TMIF was within concentration levels that do not have cytotoxic effects. Also, considering amino acids content of TMIF, it was possible to see that undigested TMIF possess higher concentration of total amino acids comparing to the digested form of TMIF, while in the content of free amino acids concentration, the digested form of TMIF achieves higher concentration of free amino acids regarding to the undigested TMIF.

Overall, these findings seem to indicate that TMIF is a potential substitute for meat, opening paths for new equilibrated, health promoting and nutritional diets. TMIF represents itself as a good food source, with high potential for the health of the organism, with its nutritional content and impact at gut microbiota level. TMIF also shows positive potential for the hosts microbiota metabolism. All this can bring great benefits to human well-being, as the found consequences will then largely improve the organism’s equilibrium.

5. Future work

This work explored the impact of TMIF on the human gut microbiota, and thanks to the achievements made, many questions arise and a wide diversity and variety of investigation on this theme can be done.

A next step on this work can be done, using the same *in vitro* faecal model, but now studying the impact of different protein sources such as beef, pork and chicken to compare with TMIF, to observe the effects of each other, on the gut microbiota and its metabolic activity (SCFA, BCFA and ammonia production). Such study could highlight the effect of the main protein source and TMIF in humans and on the gut microbiota activity, for each case, enabling the perception of which benefits the most the gut microbiota and the host.

It is important to mention that not all insects are edible, and those that are, have different nutritional values from each other, so another important study could be the impact, on the gut microbiota, of flour from different insects, aside from mealworm.

Another study that could be pursued is the impact of TMIF in humans. Human trials can be conducted, on healthy subjects, willing to introduce on their diet insect-based food, especially food with TMIF, thus substituting their usual dietary protein, by TMIF. A first approach could be done by studying the health biomarkers, acceptance and microbial effects of those products in the volunteers. This study could, at the same time, be performed with people from western culture societies, as to access their perception and disgusting factors to the consumption of insect-based foods.

6. Appendix

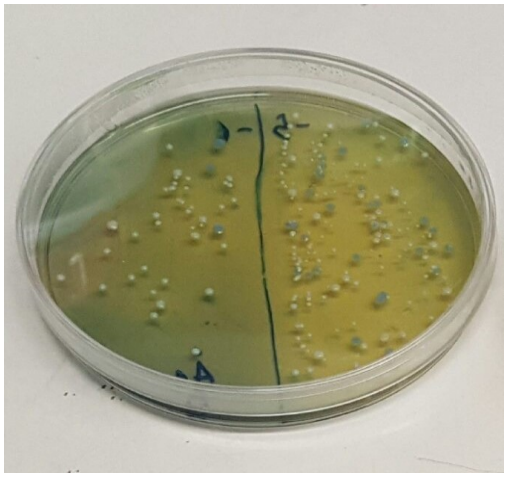

Figure 6.1- MRS-BPB with colony of *Lactobacillus* (light blue color) and *Bifidobacterium* (dark blue color).

Table 6.1- log CFU/mL (means ± SD) of the bacterial strains when in the nutritional stress conditions model with or without TMIF.

		Time (h)					
Bacterial specie (log CFU/mL)	**TMIF condition**	**0**	**3**	**6**	**12**	**24**	**48**
L. rhamnosus	Without	9.06 ± 0.082	9.15 ± 0.095	9.12 ± 0.156	9.26 ± 0.119	7.88 ± 0.321	4.25 ± 0.629
	With	9.02 ± 0.074	9.07 ± 0.055	9.20 ± 0.053	9.12 ± 0.091	8.78 ± 0.036	8.66 ± 0.032
L. casei	Without	9.14 ± 0.042	9.13 ± 0.017	9.13 ± 0.013	9.12 ± 0.076	8.05 ± 0.076	5.34 ± 0.054
	With	9.07 ± 0.023	9.14 ± 0.020	9.19 ± 0.018	9.19 ± 0.056	8.83 ± 0.032	8.45 ± 0.054
L. acidophilus	Without	8.27 ± 0.015	8.23 ± 0.023	8.06 ± 0.086	7.40 ± 0.226	4.64 ± 0.347	3.08 ± 0.066
	With	8.47 ± 0.018	8.39 ± 0.032	8.38 ± 0.035	8.25 ± 0.043	7.72 ± 0.030	6.07 ± 0.152
B. animalis Bb12	Without	8.68 ± 0.030	8.47 ± 0.053	7.98 ± 0.050	7.02 ± 0.058	6.51 ± 0.009	6.24 ± 0.027
	With	8.74 ± 0.029	8.76 ± 0.032	8.74 ± 0.017	8.78 ± 0.041	8.71 ± 0.043	8.44 ± 0.112
B. animalis Bo	Without	9.13 ± 0.021	9.29 ± 0.037	9.21 ± 0.027	9.14 ± 0.017	8.05 ± 0.071	5.49 ± 0.153
	With	9.18 ± 0.010	9.30 ± 0.024	9.26 ± 0.016	9.22 ± 0.009	8.74 ± 0.015	7.53 ± 0.052
B. longum BG3	Without	8.99 ± 0.064	8.98 ± 0.091	9.15 ± 0.070	9.24 ± 0.033	7.18 ± 0.092	5.43 ± 0.075
	With	8.98 ± 0.053	9.08 ± 0.109	9.14 ± 0.064	9.17 ± 0.040	8.73 ± 0.015	7.19 ± 0.056

Table 6.2- log CFU/mL (means ± SD) of the bacterial strains in monoculture when inoculated in basal media with or without TMIF.

			Without TMIF	**With TMIF**
Bacterial specie	**Conditions**	**Time (h)**	**Total viable cells (log CFU/mL)**	**Total viable cells (log CFU/mL)**
L. rhamnosus	1%	0	6.97 ± 0.052	7.38 ± 0.090
		24	7.37 ± 0.018	7.57 ± 0.044
		48	7.39 ± 0.038	8.06 ± 0.072
	10%	0	8.17 ± 0.038	8.20 ± 0.053
		24	8.34 ± 0.027	8.27 ± 0.030
		48	7.49 ± 0.026	8.25 ± 0.010
L. casei	1%	0	6.93 ± 0.061	7.29 ± 0.042
		24	7.17 ± 0.026	7.72 ± 0.012
		48	7.95 ± 0.036	7.75 ± 0.051
	10%	0	8.26 ± 0.030	8.32 ± 0.026
		24	8.33 ± 0.035	8.28 ± 0.040
		48	7.56 ± 0.005	8.18 ± 0.024
L. acidophilus	1%	0	6.46 ± 0.066	6.47 ± 0.104
		24	7.01 ± 0.054	7.21 ± 0.096
		48	6.99 ± 0.082	7.18 ± 0.033
	10%	0	7.50 ± 0.049	7.56 ± 0.033
		24	6.58 ± 0.018	6.91 ± 0.042
		48	7.36 ± 0.012	7.57 ± 0.041
B. animalis Bb12	1%	0	7.10 ± 0.052	7.09 ± 0.059
		24	8.44 ± 0.013	7.96 ± 0.036
		48	8.41 ± 0.078	8.17 ± 0.031
	10%	0	8.07 ± 0.033	8.02 ± 0.052
		24	8.17 ± 0.016	8.12 ± 0.076
		48	8.07 ± 0.065	8.08 ± 0.084
B. animalis Bo	1%	0	7.17 ± 0.064	7.20 ± 0.146
		24	7.94 ± 0.040	8.01 ± 0.024
		48	7.61 ± 0.077	7.74 ± 0.036
	10%	0	7.65 ± 0.043	7.66 ± 0.017
		24	8.51 ± 0.022	8.43 ± 0.040
		48	8.48 ± 0.005	8.48 ± 0.009
B. longum BG3	1%	0	6.92 ± 0.034	7.01 ± 0.087
		24	7.94 ± 0.021	8.09 ± 0.052
		48	7.99 ± 0.082	7.77 ± 0.052
	10%	0	7.60 ± 0.032	7.60 ± 0.028
		24	8.35 ± 0.007	8.41 ± 0.030
		48	8.21 ± 0.024	8.49 ± 0.019

Table 6.3- log CFU/mL (means ± SD) of the bacterial strains in consortium when inoculated in basal media with or without TMIF.

Sample	Time (h)	Lactobacilli (log CFU/mL)	Bifidobacteria (log CFU/mL)
A1	0	7.89 ± 0.022	8.88 ± 0.048
	24	8.71 ± 0.056	8.97 ± 0.060
	48	8.14 ± 0.024	8.59 ± 0.056
A2	0	7.55 ± 0.048	8.90 ± 0.051
	24	7.94 ± 0.0050	9.06 ± 0.023
	48	7.98 ± 0.006	8.24 ± 0.029
A1+TMIF	0	7.89 ± 0.021	8.89 ± 0.018
	24	8.61 ± 0.018	8.83 ± 0.011
	48	8.56 ± 0.025	8.53 ± 0.040
A2+TMIF	0	7.49 ± 0.014	8.90 ± 0.026
	24	7.95 ± 0.008	8.89 ± 0.053
	48	8.20 ± 0.032	8.62 ± 0.046
B1	0	7.62 ± 0.052	9.02 ± 0.034
	24	8.01 ± 0.038	9.03 ± 0.028
	48	7.96 ± 0.013	8.46 ± 0.006
B2	0	7.50 ± 0.032	8.94 ± 0.050
	24	7.86 ± 0.043	8.90 ± 0.0055
	48	7.71 ± 0.022	8.19 ± 0.019
B1+TMIF	0	7.50 ± 0.032	8.86 ± 0.018
	24	8.13 ± 0.014	8.90 ± 0.048
	48	7.89 ± 0.009	8.57 ± 0.012
B2+TMIF	0	7.52 ± 0.013	8.83 ± 0.032
	24	7.86 ± 0.008	8.97 ± 0.008
	48	8.10 ± 0.006	8.85 ± 0.053
C1	0	7.89 ± 0.017	8.63 ± 0.023
	24	7.89 ± 0.029	8.72 ± 0.046
	48	8.01 ± 0.018	7.89 ± 0.030
C2	0	7.79 ± 0.039	8.72 ± 0.014
	24	7.82 ± 0.015	8.26 ± 0.012
	48	7.92 ± 0.009	8.26 ± 0.0059
C1+TMIF	0	7.82 ± 0.063	8.64 ± 0.077
	24	7.93 ± 0.013	8.67 ± 0.056
	48	8.08 ± 0.034	8.14 ± 0.020
C2+TMIF	0	7.51 ± 0.034	8.74 ± 0.037
	24	8.19 ± 0.019	8.88 ± 0.032
	48	8.25 ± 0.014	8.80 ± 0.018
D1	0	7.87 ± 0.064	8.79 ± 0.028
	24	8.84 ± 0.019	9.12 ± 0.030
	48	7.75 ± 0.054	8.08 ± 0.029
D2	0	8.86 ± 0.012	8.80 ± 0.027
	24	9.03 ± 0.016	8.73 ± 0.088
	48	7.97 ± 0.018	7.93 ± 0.051
D1+TMIF	0	8.63 ± 0.010	8.98 ± 0.027
	24	8.82 ± 0.013	8.95 ± 0.019
	48	8.53 ± 0.026	8.67 ± 0.065
D2+TMIF	0	8.05 ± 0.035	8.20 ± 0.021
	24	9.04 ± 0.004	8.64 ± 0
	48	8.12 ± 0.056	8.12 ± 0.029

Table 6.4- Concentration (mg/mL, means ± SD) of the organic acids produced along fermentation time in samples with *Lactobacillus* strains. Nd – not detected. Different letters mark statistically significant ($p<0.05$) differences between samples for each compound.

Bacterial specie	Conditions	Time (h)	[Butyrate] (mg/mL)	[Propionate] (mg/mL)	[Acetate] (mg/mL)	[Lactate] (mg/mL)
L. rhamnosus	1%	24	Nd^{a}	0.11 ± 0.013^{a}	0.050 ± 0.002^{a}	0.71 ± 0.045^{a}
		48	Nd^{a}	0.13 ± 0.002^{a}	0.28 ± 0.003$^{a, b}$	0.57 ± 0.003^{a}
	10%	24	Nd^{a}	0.16 ± 0.003$^{a, b}$	1.19 ± 0.026$^{d, e}$	0.72 ± 0.006^{a}
		48	Nd^{a}	0.47 ± 0.014$^{b, c, d, e, f}$	1.27 ± 0.011$^{d, e}$	0.72 ± 0.003^{a}
	1%+TMIF	24	0.13 ± 0.104$^{a, b}$	0.37 ± 0.248$^{a, b, c, d, e}$	0.62 ± 0.381$^{b, c}$	0.44 ± 0.296^{a}
		48	0.23 ± 0.006$^{b, c}$	0.71 ± 0.002^{f}	1.28 ± 0.005$^{d, e}$	0.85 ± 0.194^{a}
	10%+TMIF	24	0.28 ± 0.003$^{b, c}$	0.77 ± 0.018^{f}	0.17 ± 0.002$^{a, b}$	2.88 ± 0.030$^{b, c}$
		48	0.26 ± 0$^{b, c}$	0.74 ± 0.010^{f}	1.62 ± 0.030$^{e, f}$	2.63 ± 0.020^{b}
L. casei	1%	24	Nd^{a}	0.11 ± 0.002^{a}	0.30 ± 0.003$^{a, b}$	0.58 ± 0.046^{a}
		48	Nd^{a}	0.12 ± 0^{a}	0.31 ± 0.006$^{a, b}$	0.61 ± 0.014^{a}
	10%	24	0.22 ± 0.007$^{b, c}$	0.61 ± 0.003$^{e, f}$	0.22 ± 0.002$^{a, b}$	2.71 ± 0.015^{b}
		48	0.38 ± 0.002^{c}	0.37 ± 0.002$^{a, b, c, d, e}$	1.01 ± 0$^{c, d}$	0.56 ± 0.006^{a}
	1%+TMIF	24	0.38 ± 0.016^{c}	2.66 ± 0.043^{g}	0.39 ± 0.028$^{a, b}$	0.65 ± 0.011^{a}
		48	0.24 ± 0.002$^{b, c}$	0.70 ± 0.002^{f}	1.40 ± 0.008$^{d, e}$	0.87 ± 0.019^{a}
	10%+TMIF	24	0.23 ± 0.027$^{b, c}$	0.80 ± 0.022^{f}	1.50 ± 0.040$^{e, f}$	3.47 ± 0.004^{d}
		48	0.28 ± 0.003$^{b, c}$	0.79 ± 0.003^{f}	1.94 ± 0.012$^{e, f}$	2.98 ± 0.017$^{b, c}$
L. acidophilus	1%	24	0.76 ± 0.044^{d}	0.14 ± 0.006^{a}	0.38 ± 0.010$^{a, b}$	0.66 ± 0.025^{a}
		48	0.71 ± 0.024^{d}	0.72 ± 0.008^{f}	0.079 ± 0.001^{a}	0.68 ± 0.008^{a}
	10%	24	1.08 ± 0.10^{e}	0.23 ± 0.10$^{a, b, c, d}$	1.26 ± 0.025$^{d, e}$	0.80 ± 0.044^{a}
		48	1.01 ± 0.002^{e}	0.28 ± 0.024$^{a, b, c, d}$	1.22 ± 0.024$^{d, e}$	0.65 ± 0.013^{a}
	1% +TMIF	24	0.20 ± 0.011$^{b, c}$	0.53 ± 0$^{c, d, e, f}$	1.53 ± 0.005$^{e, f}$	0.83 ± 0.010^{a}
		48	0.21 ± 0.013$^{b, c}$	0.55 ± 0.001$^{d, e, f}$	1.03 ± 0.002$^{c, d}$	0.86 ± 0.007^{a}
	10%+TMIF	24	1.12 ± 0.014^{e}	0.23 ± 0.036$^{a, b, c, d}$	1.17 ± 0.014$^{d, e}$	3.49 ± 0.060^{d}
		48	1.04 ± 0.013^{e}	0.21 ± 0.038$^{a, b, c}$	1.46 ± 0.010$^{d, e}$	3.16 ± 0.046$^{c, d}$

Table 6.5- Concentration (mg/mL, means ± SD) of the organic acids produced along fermentation time in samples with *Bifidobacterium* strains. Nd – not detected. Different letters mark statistically significant ($p<0.05$) differences between samples for each compound.

Bacterial specie	Conditions	Time (h)	[Butyrate] (mg/mL)	[Propionate] (mg/mL)	[Acetate] (mg/mL)	[Lactate] (mg/mL)
B. animalis Bb12	1%	24	0.11 ± 0.001^{a}	$0.31 \pm 0^{a, b, c, d}$	$0.035 \pm 0.001^{a, b, c}$	$0.28 \pm 0.001^{a, b}$
		48	0.17 ± 0.003^{a}	$0.47 \pm 0.001^{a, b, c, d, e}$	0.053 ± 0.002^{a}	$0.43 \pm 0.017^{b, c}$
	10%	24	0.19 ± 0.012^{a}	$0.20 \pm 0.057^{a, b}$	$0.43 \pm 0.009^{b, c}$	$0.42 \pm 0.006^{b, c}$
		48	$0.44 \pm 0.005^{a, b, c}$	$0.55 \pm 0.077^{b, c, d, e}$	0.50 ± 0.031^{c}	1.23 ± 0.032^{h}
	1%+TMIF	24	0.26 ± 0.006^{a}	$0.22 \pm 0.022^{a, b, c}$	$0.40 \pm 0.002^{b, c}$	$0.44 \pm 0.004^{b, c}$
		48	0.20 ± 0.005^{a}	$0.58 \pm 0.003^{b, c, d, e, f, g}$	1.23 ± 0.010^{d}	0.17 ± 0.007^{a}
	10%+TMIF	24	0.21 ± 0.006^{a}	$0.58 \pm 0^{b, c, d, e, f}$	$1.78 \pm 0.010^{f, g}$	0.17 ± 0.011^{a}
		48	$0.53 \pm 0.010^{a, b, c, d}$	$0.57 \pm 0.007^{b, c, d, e, f}$	$1.33 \pm 0.003^{d, e}$	$0.41 \pm 0.004^{b, c}$
B. animalis Bo	1%	24	$0.35 \pm 0.006^{a, b, c}$	0.13 ± 0.006^{a}	$0.29 \pm 0.004^{a, b, c}$	0.46 ± 0.002^{c}
		48	0.18 ± 0.008^{a}	$0.97 \pm 0.093^{g, h}$	0.049 ± 0.001^{a}	$0.75 \pm 0.019^{e, f}$
	10%	24	0.21 ± 0.005^{a}	$0.55 \pm 0.009^{b, c, d, e}$	$0.20 \pm 0.055^{a, b, c}$	2.97 ± 0.033^{k}
		48	$0.76 \pm 0^{c, d, e}$	$0.25 \pm 0^{a, b, c, d}$	$0.24 \pm 0^{a, b, c}$	3.64 ± 0^{m}
	1%+TMIF	24	0.27 ± 0.001^{a}	$0.95 \pm 0.009^{f, g, h}$	$1.40 \pm 0.037^{d, e}$	$0.90 \pm 0.015^{f, g}$
		48	0.24 ± 0.010^{a}	0.97 ± 0.007^{h}	$1.56 \pm 0.034^{d, e, f}$	1.06 ± 0.027^{g}
	10%+TMIF	24	0.22 ± 0^{a}	$0.62 \pm 0.005^{d, e, f, g, h}$	$1.57 \pm 0.037^{e, f}$	$2.81 \pm 0.009^{j, k}$
		48	$0.98 \pm 0.380^{d, e}$	$0.20 \pm 0.051^{a, b, c}$	$2.10 \pm 0.092^{g, h}$	3.32 ± 0.033^{l}
B. longum BG3	1%	24	0.20 ± 0.006^{a}	$0.61 \pm 0.290^{d, e, f, g, h}$	0.058 ± 0.001^{a}	$0.70 \pm 0.016^{d, e}$
		48	0.15 ± 0^{a}	$0.32 \pm 0.003^{a, b, c, d}$	$0.10 \pm 0.036^{a, b}$	$0.55 \pm 0.010^{c, d}$
	10%	24	0.18 ± 0.010^{a}	$0.49 \pm 0.015^{a, b, c, d, e}$	$0.21 \pm 0.004^{a, b, c}$	2.74 ± 0.016^{j}
		48	0.18 ± 0.008^{a}	$0.48 \pm 0.0145^{a, b, c, d, e}$	$0.28 \pm 0.028^{a, b, c}$	2.52 ± 0.018^{i}
	1%+TMIF	24	$0.76 \pm 0.048^{b, c, d, e}$	$0.44 \pm 0.022^{a, b, c, d, e}$	$1.56 \pm 0.070^{d, e, f}$	$0.80 \pm 0.020^{e, f}$
		48	1.18 ± 0.110^{e}	$0.48 \pm 0.027^{a, b, c, d, e}$	2.71 ± 0.187^{i}	$0.78 \pm 0.045^{e, f}$
	10%+TMIF	24	0.22 ± 0.002^{a}	$0.59 \pm 0.014^{c, d, e, f, g}$	$1.50 \pm 0.001^{d, e, f}$	2.73 ± 0.044^{j}
		48	$0.29 \pm 0.011^{a, b}$	$0.79 \pm 0.024^{e, f, g, h}$	2.25 ± 0.017^{h}	3.21 ± 0.095^{l}

Table 6.6- Concentration (mg/mL, means ± SD) of the organic acids produced along fermentation time in samples with co-cultures and consortium. Nd – not detected. Different letters mark statistically significant ($p<0.05$) differences between samples for each compound.

Sample	Time (h)	[Butyrate] (mg/mL)	[Propionate] (mg/mL)	[Acetate] (mg/mL)	[Lactate] (mg/mL)
A1	24	$0.49 \pm 0.014^{a, b, c}$	$2.02 \pm 0.008^{b, c}$	$1.07 \pm 0.008^{a, b, c, d, e, f, g}$	6.87 ± 0.083^{b}
	48	$0.45 \pm 0.026^{a, b, c}$	$1.93 \pm 0.036^{b, c}$	$1.01 \pm 0.023^{a, b, c, d, e, f, g}$	7.08 ± 0.023^{b}
A2	24	$0.69 \pm 0.501^{b, c, d}$	$2.23 \pm 0.002^{b, c}$	$1.87 \pm 0.014^{f, g, h, i, j}$	7.22 ± 0.007^{b}
	48	$0.66 \pm 0^{b, c, d}$	$2.16 \pm 0^{b, c}$	$1.77 \pm 0^{d, e, f, g, h, i, j}$	7.33 ± 0^{b}
A1+TMIF	24	$0.72 \pm 0.010^{b, c, d}$	$2.33 \pm 0.004^{b, c}$	3.12 ± 0.025^{k}	7.51 ± 0.002^{b}
	48	$0.79 \pm 0.015^{b, c, d}$	2.38 ± 0.007^{c}	3.00 ± 0.013^{k}	7.63 ± 0.026^{b}
A2+TMIF	24	$0.78 \pm 0.003^{b, c, d}$	2.38 ± 0.005^{c}	$2.71 \pm 0.017^{j,k}$	7.53 ± 0.013^{b}
	48	$0.71 \pm 0.003^{b, c, d}$	$2.31 \pm 0.002^{b, c}$	$2.61 \pm 0.021^{i, j, k}$	7.84 ± 0.011^{b}
B1	24	$0.55 \pm 0.005^{a, b, c}$	$1.86 \pm 0.005^{b, c}$	$1.22 \pm 0.005^{a, b, c, d, e, f, g}$	7.21 ± 0.070^{b}
	48	$0.71 \pm 0.044^{b, c, d}$	$2.19 \pm 0.039^{b, c}$	$1.80 \pm 0.034^{e, f, g, h, i, j}$	6.93 ± 0.241^{b}
B2	24	$0.77 \pm 0.009^{b, c, d}$	2.38 ± 0.008^{c}	$1.52 \pm 0.032^{b, c, d, e, f, g, h}$	7.60 ± 0.134^{b}
	48	$0.70 \pm 0.008^{b, c, d}$	$2.22 \pm 0.003^{b, c}$	$1.92 \pm 0.017^{g, h, i, j}$	7.50 ± 0.004^{b}
B1+TMIF	24	$0.62 \pm 0.012^{b, c, d}$	$2.14 \pm 0.004^{b, c}$	$2.53 \pm 0.030^{h, i, j, k}$	7.31 ± 0.062^{b}
	48	$0.78 \pm 0.007^{b, c, d}$	2.42 ± 0.008^{c}	3.49 ± 0.021^{k}	7.88 ± 0.016^{b}
B2+TMIF	24	$0.51 \pm 0.028^{a, b, c}$	$1.97 \pm 0.045^{b, c}$	$2.67 \pm 0.007^{j, k}$	7.18 ± 0.197^{b}
	48	$0.41 \pm 0.176^{a, b}$	$1.66 \pm 0.656^{b, c}$	$1.58 \pm 0.580^{c, d, , f, g, h, i}$	5.74 ± 1.702^{b}
C1	24	$0.36 \pm 0.278^{a, b}$	$1.18 \pm 0.844^{a, b}$	$0.78 \pm 0.244^{a, b, c, d, e}$	$4.48 \pm 2.31^{a, b}$
	48	$0.52 \pm 0.015^{a, b, c}$	$1.90 \pm 0.012^{b, c}$	$0.52 \pm 0.004^{a, b}$	6.76 ± 0.019^{b}
C2	24	$0.64 \pm 0.006^{b, c, d}$	$2.02 \pm 0.005^{b, c}$	$1.02 \pm 0.055^{a, b, c, d, e, f, g}$	7.12 ± 0.036^{b}
	48	$0.57 \pm 0.006^{a, b, c}$	$2.19 \pm 0.006^{b, c}$	$0.63 \pm 0.001^{a, b, c}$	7.23 ± 0.010^{b}
C1+TMIF	24	$0.76 \pm 0.035^{b, c, d}$	$2.24 \pm 0.159^{b, c}$	$0.73 \pm 0.220^{a, b, c, d}$	6.74 ± 1.629^{b}
	48	1.12 ± 0.024^{d}	$2.19 \pm 0.002^{b, c}$	0.46 ± 0.008^{a}	5.70 ± 1.543^{b}
C2+TMIF	24	$0.57 \pm 0.004^{a, b, c}$	$2.27 \pm 0.021^{b, c}$	0.47 ± 0^{a}	7.30 ± 0.035^{b}
	48	$0.94 \pm 0.345^{c, d}$	$2.24 \pm 0.010^{b, c}$	0.47 ± 0.001^{a}	7.39 ± 0.019^{b}
D1	24	$0.43 \pm 0.003^{a, b}$	$2.11 \pm 0.012^{b, c}$	$0.84 \pm 0.190^{a, b, c, d, e, f}$	$4.34 \pm 0.003^{a, b}$
	48	$0.43 \pm 0.001^{a, b}$	$2.11 \pm 0.005^{b, c}$	$1.03 \pm 0.007^{a, b, c, d, e, f, g}$	$4.36 \pm 0.033^{a, b}$
D2	24	$0.39 \pm 0.004^{a, b}$	$2.00 \pm 0.005^{b, c}$	$0.98 \pm 0.027^{a, b, c, d, e, f, g}$	5.67 ± 1.530^{b}
	48	$0.36 \pm 0.002^{a, b}$	$1.98 \pm 0.006^{b, c}$	$0.96 \pm 0^{a, b, c, d, e, f, g}$	$4.33 \pm 0^{a, b}$
D1+TMIF	24	$0.46 \pm 0.010^{a, b, c}$	$1.79 \pm 0.056^{b, c}$	$0.86 \pm 0.019^{a, b, c, d, e, f}$	6.93 ± 0.028^{b}
	48	0.091 ± 0.026^{a}	0.22 ± 0.086^{a}	0.32 ± 0.210^{a}	0.94 ± 0.301^{a}
D2+TMIF	24	$0.46 \pm 0.053^{a, b, c}$	$2.24 \pm 0.329^{b, c}$	$1.78 \pm 0.694^{d, e, f, g, h, i, j}$	7.95 ± 0.318^{b}
	48	$0.51 \pm 0.005^{a, b, c}$	$1.94 \pm 0.028^{b, c}$	$0.92 \pm 0.012^{a, b, c, d, e, f, g}$	7.82 ± 0.047^{b}

Table 6.7- Bacterial populations of total bacteria (Eub338), *Bifidobacterium* spp. (Bif164), *Lactobacillus* spp. (Lab158), most *Bacteroidaceae* and *Prevotellaceae* (Bac303), *Clostridium coccoides* / *Eubacterium rectale* group (Erec482), most of the *Clostridium histolyticum* group (Chis150) (log (cells/mL), means ± SD) detected by FISH-FCM in faecal samples of 5 studied donors in 4 different conditions (negative control, positive control, undigested TMIF and digested TMIF). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

		Bacterial enumeration (log (cells/ml))			
Probe name	**Time (h)**	**Digested TMIF**	**Undigested TMIF**	**Negative Control**	**Positive Control**
Eub338 I-II-III	0	7.56 ± 0.243^{a}	7.56 ± 0.306^{a}	7.60 ± 0.315^{a}	7.59 ± 0.272^{a}
	8	7.72 ± 0.054^{a}	$7.86 \pm 0.236^{a,b}$	7.69 ± 0.111^{a}	8.36 ± 0.484^{b}
	24	7.87 ± 0.171^{a}	7.82 ± 0.187^{a}	7.73 ± 0.120^{a}	8.62 ± 0.265^{b}
	48	7.51 ± 0.120^{a}	7.80 ± 0.162^{a}	7.50 ± 0.109^{a}	8.47 ± 0.256^{b}
Bif164	0	6.48 ± 0.197^{a}	6.52 ± 0.193^{a}	6.45 ± 0.275^{a}	6.43 ± 0.345^{a}
	8	6.40 ± 0.345^{a}	6.32 ± 0.115^{a}	6.45 ± 0.059^{a}	7.26 ± 1.156^{a}
	24	6.46 ± 0.150^{a}	6.53 ± 0.049^{a}	6.41 ± 0.059^{a}	8.02 ± 0.612^{b}
	48	6.54 ± 0.240^{a}	6.65 ± 0.186^{a}	6.41 ± 0.102^{a}	7.92 ± 0.708^{b}
Lab158	0	6.12 ± 0.310^{a}	6.18 ± 0.367^{a}	6.16 ± 0.327^{a}	6.10 ± 0.396^{a}
	8	5.65 ± 0.448^{a}	$6.64 \pm 0.314^{b,c}$	$6.11 \pm 0.351^{a,b}$	6.90 ± 0.584^{c}
	24	6.06 ± 0.658^{a}	5.72 ± 0.412^{a}	6.08 ± 0.276^{a}	6.98 ± 0.456^{b}
	48	5.72 ± 0.588^{a}	5.56 ± 0.433^{a}	5.22 ± 0.512^{a}	5.99 ± 0.470^{a}
Bac303	0	5.91 ± 0.257^{a}	5.85 ± 0.309^{a}	5.94 ± 0.182^{a}	5.91 ± 0.242^{a}
	8	6.84 ± 0.216^{b}	6.94 ± 0.174^{b}	6.07 ± 0.180^{a}	7.34 ± 0.389^{b}
	24	6.40 ± 0.191^{a}	6.18 ± 0.358^{a}	6.14 ± 0.255^{a}	6.52 ± 0.507^{a}
	48	$5.74 \pm 0.110^{a,b}$	5.95 ± 0.368^{b}	5.35 ± 0.268^{a}	6.08 ± 0.249^{b}
Erec482	0	7.26 ± 0.318^{a}	7.25 ± 0.358^{a}	7.27 ± 0.393^{a}	7.25 ± 0.421^{a}
	8	6.00 ± 0.536^{a}	7.43 ± 0.275^{b}	7.27 ± 0.313^{b}	7.13 ± 0.461^{b}
	24	7.05 ± 0.286^{a}	7.12 ± 0.294^{a}	6.97 ± 0.361^{a}	6.55 ± 0.362^{a}
	48	6.55 ± 0.362^{a}	6.82 ± 0.388^{a}	6.61 ± 0.240^{a}	7.08 ± 0.398^{a}
Chis150	0	5.01 ± 0.158^{a}	4.95 ± 0.123^{a}	5.02 ± 0.243^{a}	4.95 ± 0.057^{a}
	8	5.18 ± 0.316^{a}	5.32 ± 0.274^{a}	5.20 ± 0.330^{a}	5.79 ± 0.360^{a}
	24	5.25 ± 0.260^{a}	4.80 ± 0.311^{a}	4.92 ± 0.425^{a}	6.40 ± 0.334^{b}
	48	$4.98 \pm 0.368^{a,b}$	4.84 ± 0.347^{a}	4.87 ± 0.190^{a}	5.65 ± 0.431^{b}

Table 6.8- Bacterial populations of *Roseburia* subcluster (Rrec584), *Atopobium* spp. (Ato291), *Clostridium* cluster IX (Prop853), *Faecalibacterium prausnitzii* (Fprau655) and *Desulfovibrionales* and *Desulfuromonales* (DSV687) (log (cells/mL), means ± SD) detected by FISH-FCM in faecal samples of 5 studied donors in 4 different conditions (negative control, positive control, undigested TMIF and digested TMIF). Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

		Bacterial enumeration (log (cells/ml))			
Probe name	**Time (h)**	**Digested TMIF**	**Undigested TMIF**	**Negative Control**	**Positive Control**
Rrec584	0	6.19 ± 0.313^{a}	6.39 ± 0.467^{a}	6.39 ± 0.394^{a}	6.26 ± 0.384^{a}
	8	5.15 ± 0.127^{a}	$6.15 \pm 0.314^{b,c}$	5.86 ± 0.234^{b}	6.65 ± 0.404^{c}
	24	4.93 ± 0.341^{a}	$5.70 \pm 0.290^{b,c}$	$5.41 \pm 0.206^{a,b}$	6.45 ± 0.495^{c}
	48	4.66 ± 0.201^{a}	5.04 ± 0.394^{a}	4.53 ± 0.387^{a}	5.04 ± 0.858^{a}
Ato291	0	5.66 ± 0.295^{a}	5.74 ± 0.254^{a}	5.74 ± 0.376^{a}	5.69 ± 0.324^{a}
	8	6.21 ± 0.481^{a}	6.19 ± 0.177^{a}	6.20 ± 0.363^{a}	7.43 ± 0.546^{b}
	24	$6.23 \pm 0.439^{a,b}$	5.93 ± 0.174^{a}	6.02 ± 0.352^{a}	7.10 ± 0.762^{b}
	48	5.59 ± 0.329^{a}	$6.06 \pm 0.299^{a,b}$	$6.09 \pm 0.281^{a,b}$	6.59 ± 0.602^{b}
Prop853	0	6.06 ± 0.320^{a}	6.09 ± 0.449^{a}	5.98 ± 0.432^{a}	6.08 ± 0.509^{a}
	8	6.40 ± 0.646^{a}	6.91 ± 0.398^{a}	6.33 ± 0.488^{a}	6.24 ± 0.589^{a}
	24	6.57 ± 0.813^{a}	6.52 ± 0.268^{a}	6.07 ± 0.572^{a}	6.60 ± 0.457^{a}
	48	5.73 ± 0.566^{a}	6.42 ± 0.297^{a}	5.61 ± 0.788^{a}	5.79 ± 0.708^{a}
Fprau655	0	6.57 ± 0.114^{a}	6.61 ± 0.098^{a}	6.65 ± 0.087^{a}	6.64 ± 0.176^{a}
	8	5.72 ± 0.271^{a}	6.79 ± 0.405^{b}	6.63 ± 0.391^{b}	$6.41 \pm 0.445^{a,b}$
	24	5.18 ± 0.457^{a}	6.06 ± 0.336^{b}	$5.79 \pm 0.330^{a,b}$	$5.91 \pm 0.566^{a,b}$
	48	5.10 ± 0.492^{a}	5.42 ± 0.798^{a}	5.65 ± 0.277^{a}	5.25 ± 0.812^{a}
DSV687	0	4.64 ± 0.162^{a}	4.62 ± 0.208^{a}	4.83 ± 0.203^{a}	4.73 ± 0.289^{a}
	8	5.50 ± 0.495^{b}	$4.87 \pm 0.420^{a,b}$	4.66 ± 0.257^{a}	$5.19 \pm 0.337^{a,b}$
	24	4.79 ± 0.378^{b}	4.25 ± 0.146^{a}	$4.39 \pm 0.287^{a,b}$	4.80 ± 0.189^{b}
	48	4.30 ± 0.523^{a}	3.70 ± 0.185^{a}	4.23 ± 0.496^{a}	4.29 ± 0.510^{a}

Table 6.9- Concentration (mM, means ± SD) of SCFA produced along fermentation time in faecal samples of 5 studied donors in 4 different conditions (negative control, positive control, undigested TMIF and digested TMIF). Nd – not detected. Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

		Concentration (mM)			
Organic acid	**Time (h)**	**Digested TMIF**	**Undigested TMIF**	**Negative Control**	**Positive Control**
Acetate	0	1.20 ± 0.314^{a}	1.07 ±0.235^{a}	1.08 ± 0.238^{a}	1.06 ± 0.353^{a}
	4	5.53 ± 1.930^{a,b}	6.65 ± 1.736^{b}	3.50 ± 1.295^{a}	2.98 ± 1.693^{a}
	8	9.94 ± 1.790^{a}	13.09 ± 1.129^{a}	9.40 ± 2.029^{a}	55.77 ± 13.441^{b}
	24	26.82 ± 1.074^{b}	27.00 ± 2.051^{b}	16.50 ± 2.098^{a}	61.32 ± 12.087^{c}
	48	33.96 ± 2.194^{b}	31.64 ± 4.256^{b}	19.84 ± 2.899^{a}	64.63 ± 1.035^{c}
Propionate	0	0.29 ± 0.112^{a}	0.28 ±0.149^{a}	0.33 ± 0.085^{a}	0.23 ± 0.097^{a}
	4	0.99 ± 0.287^{a}	1.34 ± 0.596^{a}	1.28 ± 0.476^{a}	1.14 ± 0.934^{a}
	8	4.90 ± 1.323^{b}	3.83 ± 0.365^{a,b}	2.77 ± 0.786^{a}	2.44 ± 2.210^{a,b}
	24	10.95 ± 1.691^{b}	12.05 ± 1.248^{b}	5.18 ± 0.464^{a}	23.84 ± 5.121^{c}
	48	12.62 ± 1.488^{b}	15.30 ± 1.080^{b}	4.84 ± 0.572^{a}	25.76 ± 4.577^{c}
Butyrate	0	0.24 ± 0.036^{a}	0.23 ± 0.038^{a}	0.23 ± 0.034^{a}	0.21 ± 0.061^{a}
	4	0.21 ± 0.070^{a}	0.40 ± 0.189^{a}	0.36 ± 0.125^{a}	0.20 ± 0.037^{a}
	8	0.31 ± 0.093^{a}	3.02 ± 1.285^{b}	1.13 ± 0.447^{a}	0.18 ± 0.051^{a}
	24	3.99 ± 0.816^{a}	5.01 ± 1.573^{a}	3.27 ±0.463^{a}	14.59 ± 7.244^{b}
	48	5.67 ± 0.402^{a}	9.38 ± 1.397^{a,b}	3.63 ± 1.063^{a}	12.68 ± 7.157^{b}
Valerate	0	Nd^{a}	Nd^{a}	Nd^{a}	Nd^{a}
	4	Nd^{a}	0.15 ± 0.070^{b}	Nd^{a}	Nd^{a}
	8	Nd^{a}	1.20 ± 0.779^{b}	0.32 ± 0.256^{a}	Nd^{a}
	24	Nd^{a}	4.53 ± 0.211^{c}	1.79 ± 0.320^{b}	1.36 ± 0.826^{b}
	48	Nd^{a}	5.15 ± 0.346^{c}	2.13 ± 0.352^{b}	1.84 ± 0.466^{b}

Table 6.10- Concentration (mM, means ± SD) of BCFA and lactate produced along fermentation time in faecal samples of 5 studied donors in 4 different conditions (negative control, positive control, undigested TMIF and digested TMIF). Nd – not detected. Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

		Concentration (mM)			
Organic acid	**Time (h)**	**Digested TMIF**	**Undigested TMIF**	**Negative Control**	**Positive Control**
Isobutyrate	0	Nd^{a}	0.092 ± 0.0100^{b}	Nd^{a}	Nd^{a}
	4	0.098 ± 0.0209^{b}	0.10 ± 0.007^{b}	0.10 ± 0.015^{b}	Nd^{a}
	8	0.16± 0.039^{b}	0.20 ± 0.098^{b}	0.13 ± 0.030^{b}	Nd^{a}
	24	0.76 ± 0.036^{a,b}	2.65 ± 0.442^{c}	1.12 ± 0.178^{b}	0.51 ± 0.292^{a}
	48	1.10 ± 0.349^{a}	3.37 ± 0.506^{b}	1.21 ± 0.155^{a}	0.84 ± 0.178^{a}
Isovalerate	0	Nd^{a}	Nd^{a}	Nd^{a}	Nd^{a}
	4	Nd^{a}	Nd^{a}	Nd^{a}	Nd^{a}
	8	0.14 ± 0.054^{b}	0.10 ± 0.022^{b}	Nd^{a}	Nd^{a}
	24	0.94 ± 0.149^{b}	2.17 ± 0.683^{c}	0.84 ± 0.376^{b}	Nd^{a}
	48	1.52 ± 0.449^{b}	2.78 ± 0.716^{c}	0.96 ± 0.298^{a,b}	0.38 ± 0.056^{a}
Lactate	0	Nd^{a}	Nd^{a}	Nd^{a}	0.91 ± 0.166^{b}
	4	3.64 ± 0.383^{a}	1.94 ± 0.896^{a}	1.84 ± 0.634^{a}	7.70 ± 6.662^{a}
	8	3.33 ± 0.696^{a}	2.55 ± 1.443^{a}	1.57 ± 0.168^{a}	25.03 ± 8.933^{b}
	24	Nd^{a}	Nd^{a}	Nd^{a}	Nd^{a}
	48	Nd^{a}	Nd^{a}	Nd^{a}	Nd^{a}

Table 6.11- Ammonia concentration (mM, means ± SD) of ammonia produced along fermentation time in faecal samples of 5 studied donors in 4 different conditions (negative control, positive control, undigested TMIF and digested TMIF. Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

	Ammonia concentration (mM)			
Time (h)	**Digested TMIF**	**Undigested TMIF**	**Negative Control**	**Positive Control**
0	22.14 ± 5.129^{a}	20.21 ± 3.060^{a}	20.42 ± 3.767^{a}	20.32 ± 5.164^{a}
4	32.93 ± 3.837^{b}	36.49 ± 2.751^{b}	23.03 ± 2.730^{a}	24.32 ± 1.577^{a}
8	28.11 ± 2.108^{b}	33.64 ± 2.046^{c}	32.56 ± 1.120^{c}	22.68 ± 3.013^{a}
24	45.18 ± 1.577^{b}	48.11 ± 3.421^{b}	26.65 ± 4.635^{a}	22.58 ± 4.084^{a}
48	64.55 ± 5.076^{c}	69.86 ± 4.459^{c}	36.52 ± 3.984^{b}	24.86 ± 4.367^{a}

7. Annex

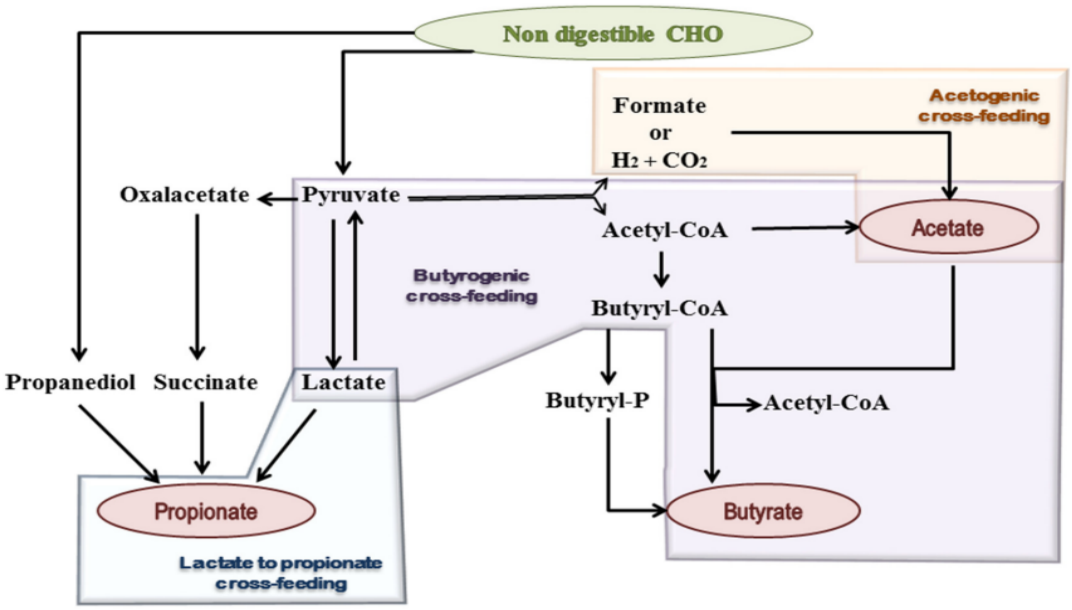

Figure 7.1- Representation of gut microbiota metabolic pathways and cross-feeding mechanisms leading to the production of SCFA. Adapted from Ríos-Covián *et al.*, 2016.

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