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Dynamic modelling of monogastric gut microbiota

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THE UNIVERSITY
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Declaration

I hereby declare that the thesis here presented has been composed by myself and the work has not been submitted for any other degree of professional qualification. Except from the section specified below, and when references have been cited, I herein confirm that the work submitted is my own.

I confirm that substantial contribution has been made with regards to chapter 1, in which, the mathematical model described is based on a previous work from Helen Kettle (second supervisor). The model was developed in the past for a different host (human), thus here redesigned for the porcine microbiota, reparametrized and validated through my own *in vitro* experimentation.

Salvatore Galgano, September 2019

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Acknowledgments

*"No man is an island entire of itself; every man
is a piece of the continent, a part of the main;
if a clod be washed away by the sea, Europe
is the less, as well as if a promontory were, as
well as any manner of thy friends or of thine
own were; any man's death diminishes me,
because I am involved in mankind.
And therefore never send to know for whom
the bell tolls; it tolls for thee."*

MEDITATION XVII
Devotions upon Emergent Occasions
John Donne

"As is a tale, so is life: not how long it is, but how good it is, is what matters."

Lucius Annaeus Seneca

None or a few words could describe the emotional baggage accompanying the entire process of my PhD studies, and none of my achievements could find space on this written document without the helpful input from several people met during the past three years and a half. Whilst hard is the task to thank all of them, I will attempt here to refer to the most influent and meaningful contacts, apologising in advance to the ones missing, whose interactions although omitted are not forgotten.

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Abstract

The porcine gut microbiota is composed of a considerable number of microbial species with several levels of interaction with its host, including the production of energy from sources, otherwise not digested by the animal, or the production of metabolites, such as the short chain fatty acids (SCFAs). Moreover, a subset of these species, the porcine gut core microbiota (CM), which seems not to be affected by changes in host diet or breed, has been identified by several authors. Manipulations of the whole microbiota (WM), and as an extension of the CM could achieve the production of active molecules (e.g. anti-inflammatory), and a metabolite pattern advantageous for the host health and performance. Nevertheless, the methods to explore long term perturbations are either ethically/technically demanding or expensive. Thus, mathematical modelling could provide the tools for the *in silico* experimentation able to predict changes in the metabolites due to intervention strategies.

During this project an innovative mathematical model depicting the WM dynamics was developed and validated *in vitro*, in parallel with the development of an innovative model describing the CM dynamics. A continuous fermentation experiment mimicking the porcine proximal colon was conducted to validate the WM model, which predicted well the dynamics of the major bacterial and archaeal communities and of the major SCFAs (i.e. acetate, propionate and butyrate) observed *in vitro*. De facto their modelled ratio was 47.98%, 28.06% and 23.96%, whereas analogous values observed during the experiment was 44.5%, 30.5% and 25.1%. The CM model showed a possible role of the core sub-community in forming the basis of the SCFA production, and a probable role of the core in providing acetate to the WM when compared to literature porcine microbiota studies.

Both the models were then the object of *in silico* experimentation to simulate the effects of probiotic/prebiotic therapies on the metabolite pattern, anticipating SCFA changes upon these interventions. As a result, multiple scenarios were derived from different types of intervention, for instance, a combined therapy of non-starch polysaccharides (as prebiotic) and two CM strains (as probiotic) could increase colonic butyrate concentration, suggesting a potential positive effect on the host well-being. In conclusion, the models developed during this project produce accurate estimates of the major SCFAs and bacterial communities, thus providing a valuable tool to those interested in testing the microbiota response to different starting conditions, which could serve as a step preceding the necessary *in vitro/in vivo* investigations.

Lay summary

The gut of animals, including pigs, harbours a vast population of microorganisms known as gut microbiota with various forms of relationship with its host, such as providing energy from food not already used by it, helping it regulate the immune response and preventing some diseases. The gut microbiota is composed of a dynamic population of microbes, whose composition is typical of each pig and life stage. Nevertheless, some of the microbes in the pig microbiota do not change between pigs and remain the same for most of the pig's life, these microbes form a community, part of the pig gut microbiota, called the core microbiota. Considering the level of connection between the microorganisms, mostly bacteria, and the host, it could be possible to plan different interventions aiming to achieve higher pig performance or better health status. Like all drugs, these interventions should also be tested before they are applied through standard protocols. Nevertheless, a trial would require a large number of animals, time and available funds, which must also take into consideration the possibility of studying for a long period of time an intervention that at the end would not find applications because it did not give the required results during the test.

It could be advantageous if, before such trials, there could be a way to predict if that type of intervention could lead to the hoped positive effects. A way to forecast changes in the substances that the gut bacteria produce, which are directly connected to the host health, is through mathematical modelling. A mathematical model can be seen as a tool composed by a system of equations, able to simulate the growth of microorganisms using elaborate computer software, as if it were happening in the pig intestine or during an experiment in the lab. During this PhD a mathematical model for the pig gut microbiota, and one for the core microbiota were produced. These models used a system of equation to simulate the growth of the gut bacteria, including what they eat and what they produce.

To validate the model of pig gut microbiota, an experiment was carried out and a system called a bioreactor was built and used to simulate in the lab part of the pig large intestine. Thus, the gut microorganisms, collected from a porcine faecal sample were introduced in the bioreactor and their dynamics were observed for 35 days. Afterwards, values indicative of their dynamics were used to set the model. It was observed that the model was able to predict very well the quantity of the substances produced by the bacteria. The model of the core microbiota, on the other hand, demonstrated the possible importance of the core. Finally both the model of the pig gut microbiota and the model of the pig core microbiota were used to test different types of intervention, to see if manipulation of some of the microbes or manipulations of some of the substances they feed on could achieve a pattern of substances produced, which could be related to benefits for the host. At the end of this experimentation two therapies, one for the pig microbiota and one for the core microbiota were proposed, which seemed to be able to anticipate advantages for the host health and well-being.

General Introduction

Gastrointestinal microbiota is composed of a great number of different microbial cells, which have a deep effect on host health at different levels. Before describing with more details its composition and interactions with the host, it is opportune to define the terminology used in this context. Microbiota is defined as the collection of microorganisms occurring in a defined environment, whereas the collection of genomes and gene from the members of the microbiota is defined as metagenome, thus microbiome is used to describe an entire habitat, comprised of a microbiota, its corresponding metagenome and the surrounding environment (Marchesi and Ravel, 2015). Pork is one of the most produced meat together with poultry (OECD/FAO, 2016), accounting for the 36.3% of worldwide production (Rodríguez et al., 2014). Diet interventions or microbiota manipulation are currently valid methods that benefit pig industry through increasing pig health and performance (Kenny et al., 2011). Therefore gut health and pig industry are directly related, underlying the importance of pig gut microbiota in maintaining “a state of physical and mental well-being” (Pluske et al., 2018).

Although the majority of the pig-gut microorganisms are prokaryotic (Isaacson and Kim, 2012), fungi represent a portion of the gut microbiota and have an important role in the degradation of insoluble polysaccharides, like cellulose (Hemsworth et al., 2016; Urubschurov et al., 2011). Likewise, viruses have been detected in faecal samples, which could contribute to shape the bacterial composition through strain-specific predation, horizontal gene transfer and providing nutrients after cell lysis (Devoto et al., 2019; Shan et al., 2011). Both bacterial species diversity and relative abundance differ between host breeds (Zhang et al., 2014) and can vary depending on host-internal factors such as diet and age (H. B. Kim et al., 2011; Leser et al., 2000), and on external factors, such as the environment and hygiene level (Merrifield et al., 2016; Montagne et al., 2010). Nonetheless, a core microbiota whose composition seems to remain stable and predictable, notwithstanding different external- and internal-factors, has been identified by several authors (De Rodas et al., 2018; Holman et al., 2017; Edward Alain B Pajarillo et al., 2014).

Changes in microbial communities and their molecules (i.e. substrates and metabolites), have been related to host health and performance, for instance dysbiosis connected to post-weaning diarrhoea and the importance of pig microbiota in feed efficiency (Fouhse et al., 2016). Although in some cases, it is still not clear to what extent changes in microbial populations are the direct cause or one of the consequences of the etiopathogenesis (Grimm, and Riedel, 2016). Therefore, multiple attempts have been made, in pigs (Huang et al., 2004) and other host species to modify the microbiota composition *in vivo* through prebiotic and probiotic intervention and faecal microbiota transplantation to achieve conditions positively related to the host health and performance (Fuentes and de Vos, 2016; Grimm and Riedel, 2016; Louis et al., 2016). The potential of a mathematical model, applied to human and rumen

gut microbiota has already been discussed by many authors (Bucci and Xavier, 2014; Kettle et al., 2015; Muñoz-Tamayo et al., 2010), but at the moment there is no evidence of a mathematical model describing the dynamics of the porcine gastrointestinal microbiota. *In silico* simulations are an opportunity to study this ecosystem forecasting the time evolution of microbes and metabolites before the more expensive and technical demanding *in vitro/in vivo* experimentation.

This thesis presents the first dynamic model of the entire porcine gut microbiota (WM) validated through *in vitro* experimentation, and the first dynamic model of the porcine core microbiota (CM). The models calculate and forecast the concentration over time of bacterial biomass, resource consumed (e.g. complex polysaccharides) and metabolite produced such as short chain fatty acids (SCFAs) and gases.

Anatomy and physiology of the porcine gastrointestinal tract

The main function of the porcine gastrointestinal tract (GIT) (Figure 0.1A) is the digestion of food, achieving smaller molecules which are later absorbed and then used at different levels by the host (Rowan et al., 1997). The process starts in the mouth where both mechanical forces and salivary enzymes catalyse the breakdown of large molecules. Different enzymes can be found in saliva, such as lipase, kallikrein and amylase, which mediate the hydrolysis of respectively fats, protein and starch obtaining smaller particles, such as mono- and oligo-saccharides like glucose and dextrin in case of the amylase-mediated hydrolysis (Moran, 1985). From the mouth the bolus is conveyed into the oesophagus, which transports it to the stomach through the cardiac sphincter. The glandular stomach mucosa can be divided in cardiac, gastric and pyloric, with the former occupying the major proportion of the organ. Gastric and pyloric mucosae secrete HCl, pepsinogen and chymosin (from parietal and chief cells), whereas cardiac mucosa-associated cells secrete mucus. Thus the low pH and the synergy of muscular and enzymatic actions further fragment the food material (chyme), which once past the pyloric sphincter enters the first part of the small intestine, the duodenum (Kararli, 1995).

The cylindrical lumen of the small intestine (Figure 0.1B) is surrounded by a mucosa characterised by depressions (crypts) and digital elongations (villi) (Figure 0.1C) with the function of increasing the epithelial surface area, a task enhanced by the microvilli present on the apical portion of the enterocytes (columnar cells) (Figure 0.1D) (Kararli, 1995; Mosenthin, 1998). The microvilli together with the surrounding-glycoprotein called glycocalyx form the “brush border membrane” (Mosenthin, 1998). The catalytic action started in the proximal GIT is continued in the duodenum where bile produced in the liver reaches the lumen through the sphincter of Oddi when needed for lipid digestion (Goldfarb and Sivakumar, 2012). Moreover, pancreatic α -amylase, lipases (i.e. lipase, phospholipase A2, and carboxyl ester hydrolase) and protease precursors (i.e. trypsinogen, chymotrypsinogen, proelastase,

procarboxypeptidase A and B) enter the duodenum through a minor papilla (Goldfarb and Sivakumar, 2012). From this moment the chyme is propelled through the jejunum and ileum (i.e. the other two components of the small intestine) by the peristalsis of the circular and longitudinal muscles surrounding the intestine, aided by the action of the muscularis mucosa, located between the mucosa and the submucosa. Then, from the ileocecal valve the chyme moves towards the colon, whose main function is to reabsorb water and electrolytes. The colon is arranged in a series of coils conferring it a spiral shape organised in ascending, transverse and descending sections, a series of furrows divide the lumen in different areas (Mosenthin, 1998). Just after the ileocecal valve, opposite to the first tract of the colon, the caecum can be identified as a closed appendage. Throughout the GIT, microbiota is found associated both with mucosa and lumen, which due to its role in immune and endocrine system, and in the fermentation (activity increasing towards the distal part, with peaks in caecum and colon) can be considered as a part of the GIT or as an external organ operating in symbiosis with it, as described below.

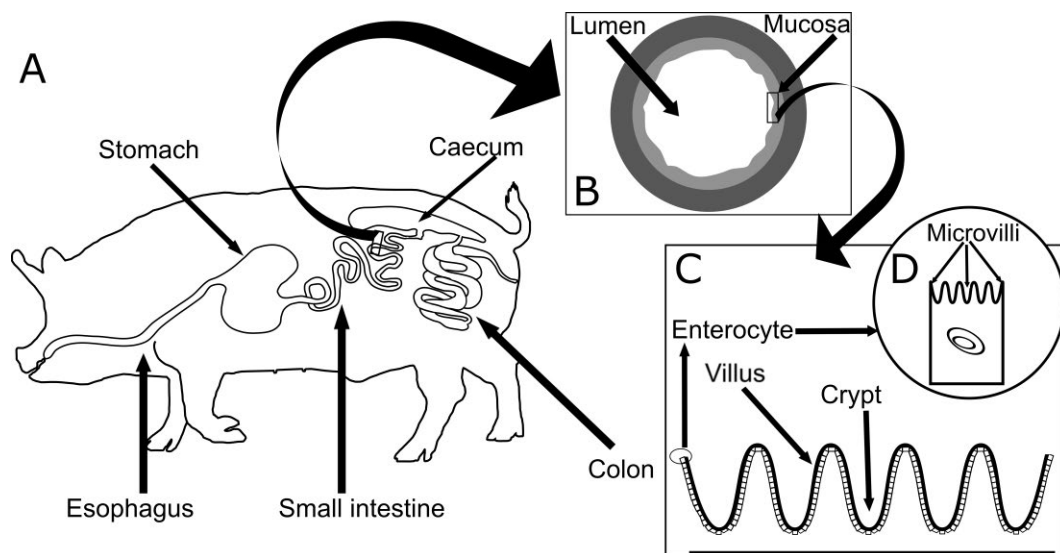


Figure 0.1. Porcine gastrointestinal tract scheme. A: structure of the GIT from the mouth to the rectum. B: small intestine transverse section in which the subdivision in lumen and mucosa can be observed. C: Magnified section of the colon mucosa showing the organisation in villi and criptae of the mucosa. The enterocytes can be found throughout the length of the mucosa, the organisation in microvilli of their apical portion can be seen in the magnification (D).

Microbiota composition

Pig gastrointestinal microbiota is an extensive ecosystem, composed by approximately 95% of bacteria, with archaea and fungi representing the minor part (Mao et al., 2011; Takahashi et al., 2014; Urubschurov et al., 2011). Moreover, the presence of a gut virome has been elucidated over the last decades, indicating how enteric viruses belonging to several families (e.g. *Picornaviridae* and *Astroviridae*) could potentially influence gut homeostasis in different hosts (Gregory et al., 2019; Keen and Dantas, 2018; Lager et al., 2012; Sachsenröder et al.,

2014). Microbiota colonisation of the porcine colonic tract is thought to happen just after birth (Konstantinov et al., 2006), due to exposure to maternal vaginal and faecal microbiota (Merrifield et al., 2016). Nevertheless, in utero fetal colonisation has been discussed in humans (Jiménez et al., 2008), therefore, considering the similarities between the two hosts (Sciascia et al., 2016), porcine in utero microbial adhesion cannot be excluded. Microbial relative abundance changes throughout life, in fact microbial succession is direct consequence of microbial adaptability to different environments, such as different diets (Hou et al., 2015a), although Bacteroidetes and Firmicutes seem to be the predominant phyla in pig faecal sample at any age (H. B. Kim et al., 2011).

Pre-weaning

Maternal contribution on piglet microbiota via gestation and feeding is evident, especially when comparing normal birth weight and intrauterine growth restricted piglets (Jiang et al., 2019). At phylum level, Firmicutes and Proteobacteria seemed to be predominant in ileal digesta of 2 day-old piglets (Konstantinov et al., 2006), confirmed by Illumina sequencing of faecal samples from 1-day-old piglets, which also showed how Proteobacteria decreased significantly towards weaning, whilst Bacteroidetes gradually increased (Chen et al., 2018). At family level, *Clostridiaceae*, *Streptococcaceae* and *Enterococcaceae* seemed to be the most abundant families immediately after birth, whose relative abundance seemed to be decreasing towards weaning, whilst *Lactobacillaceae*, *Ruminococcaceae* and *Lachnospiraceae* increased during the same period (Petri et al., 2010). These findings seemed to be consistent with compositional analysis at genus level from another study, which showed that *Clostridium sensu strictu*, *Escherichia-Shigella* and *Halomonas* were predominant at day 1, and whilst their abundance decreased towards ~day 20, *Bacteroides* and *Lactobacillus* numbers increased (Chen et al., 2018). In agreement, another study found that the most abundant species observed in ileal content of young piglets were *Escherichia coli*, *Shigella flexneri* followed by *Lactobacillus sobrius*, *Lactobacillus acidophilus* and *Lactobacillus reuteri* (Konstantinov et al., 2006). It should be noted that high number of *Shigella* clones found in those samples could be due to the fact that the 16S rRNA sequencing analysis performed was not discriminative of closely related genera such as *Shigella* and *Escherichia* (Konstantinov et al., 2006). Immediately before weaning (day 21) Bacteroidetes (~44%) and Firmicutes (~41%) seemed to be the most abundant phyla in rectum samples, followed by Spirochaetes, Proteobacteria and Tenericutes (Guevarra et al., 2018). At family level *Ruminococcaceae*, *Prevotellaceae* and *Spirochaetaceae* appear to be the predominant ones, whilst *Bacteroides* was shown to be the most abundant genus in pre-weaning pigs (Guevarra et al., 2018; Lu et al., 2014). Some studies on the other hand, reported that Firmicutes (~50%) was the predominant phylum in pre-weaning piglets, followed by Bacteroidetes (~40%), Proteobacteria, Spirochaetes and Tenericutes (Kim and Isaacson, 2015).

Weaning

The abrupt passage from sow milk to plant-based commercial feed greatly affects porcine gut microbial communities (Guevarra et al., 2019), and represent a major source of stress for piglets, which usually experience poor growth performance or post-weaning diarrhoea (Heo et al., 2013). According to a study from 2104, at phylum level, Bacteroidetes appeared to be the most abundant in faecal samples of pigs immediately after weaning (~60%), followed by Firmicutes, whose abundance seems to decrease notably after weaning, then Spirochaetes, Proteobacteria and Tenericutes (Edward Alain B. Pajarillo et al., 2014). Prevotellaceae is the most predominant family with *Prevotella* being the most abundant genus (Guevarra et al., 2018). Relative abundance of *Prevotella* and *Clostridium* increases after weaning, whilst *Bacteroides* tended to decrease at the same time (Edward Alain B. Pajarillo et al., 2014), also confirmed by evidences of *Clostridium spp.* increasing abundance towards day 23 in weaned piglets (Konstantinov et al., 2006). Another study showed a sudden increase of *Prevotella* and a decrease of *Bacteroides* following weaning, which also was accompanied by a general decrease in *Lactobacillaceae* and by an increase in *Clostridium* and *Ruminococcus* (De Rodas et al., 2018). The abundance of *Prevotella* after weaning has been associated with its ability to degrade complex plant saccharides, in contrast with tendency of *Bacteroides* to prefer mono and oligosaccharides, more abundant during pre-weaning diet (Kim and Isaacson, 2015).

Post-weaning

A study from 2011 demonstrated that at phylum level Firmicutes increased from week 10 to week 22 of age becoming the most dominant, whilst the percentage of Bacteroidetes decreased, and Spirochaetes, Proteobacteria and Actinobacteria were still detectable at lower percentage (H. B. Kim et al., 2011). On the other hand, a study from 2018 showed that Firmicutes and Bacteroidetes had almost the same abundance in both proximal and distal colon of 17-week old pigs, whilst Proteobacteria and Spirochetes were still represented at lower percentage (Crespo-Piazuelo et al., 2018). These authors also showed that Firmicutes was the most abundant phylum throughout the small intestine, followed by Proteobacteria and then Bacteroidetes (Crespo-Piazuelo et al., 2018), also confirmed in 22-weeks old pigs (De Rodas et al., 2018) and ~40-week old pigs (Yang et al., 2016). These same authors also confirmed the predominance of Firmicutes in the lower gut, (i.e. 51.7% in the caecum), followed by Bacteroidetes (37.6%) and Spirochaetes (2.7%) (Yang et al., 2016). Firmicutes was shown to be the most abundant phylum in both caecum and colon of 22-week old pigs, followed by Proteobacteria, Tenericutes and Spirochaetes (De Rodas et al., 2018). Remarkably, Firmicutes was found to be the most abundant phylum in both caecum and colon of ~4-year old Gottingen mini-pigs, followed by Bacteroidetes Spirochaetes, Tenericutes and Proteobacteria (Pedersen et al., 2013).

At family level, differences have been reported in the colon content of two different 4-year-pig-obesity profiles, indicating that amongst all the samples analysed *Prevotellaceae* was the most abundant one, followed by *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Lactobacillaceae*, *Spirochaetaceae*, *Bacteroidaceae*, *Veillonellaceae*, and *Enterobacteriaceae* (Pedersen et al., 2013).

At genus level *Prevotella* was reported to be the most abundant one at week 10, although differently from what shown in other studies, its abundance was found to be decreasing over time, and at week 22 *Anaerobacter* seemed to be predominant amongst the classifiable sequences, whilst genera like *Streptococcus*, *Lactobacillus*, *Megasphaera*, *Faecalibacterium* and *Blautia* were amongst the most abundant over the 12 weeks analysed (H. B. Kim et al., 2011). The abundance of *Prevotella* in both proximal and distal colon was confirmed by another study, which also pointed towards compositional differences between gut locations in 17-week old pigs, as expected, indicating that in duodenum and jejunum the most abundant genera were *Lactobacillus* and *Clostridium*, whereas *Streptococcus* and an unspecified genus belonging to *Clostridiaceae* family were predominant in the ileum. (Crespo-Piazuelo et al., 2018). Whilst *Prevotella* was found the most abundant also in caecal samples from ~40-old pigs, *Lactobacillus* and *Escherichia* seemed also to be amongst the most abundant ones in the same gut location (Yang et al., 2016). The colonic relative abundance of genera like *Prevotella*, *Coprococcus*, *Blautia*, *Roseburia*, *Lactobacillus*, *Campylobacter*, and *Faecalibacterium* was reported to increase significantly at day 33 compared with samples collected at day 2 (De Rodas et al., 2018). *Prevotella* was reported to be ~10% of normalised reads in caecum and colon of ~4-year old Gottingen mini-pigs (Pedersen et al., 2013).

Porcine core microbiota

Considering the fluctuations of microbiota composition throughout the pig's life and amongst different animals, the concept of a core microbiota could be used to group those organism that appear to be consistently abundant in the same host species (Thursby and Juge, 2017). It is unclear whether the core microbiota has defined rules to include a determinate percentage of species based on their recurrence in the same environment. Moreover, although some authors consider it a robust approach in the comparison of different microbiota from the same type of environment, others underline how the functional similarities are more consistent than the taxonomical ones when defining the core (Astudillo-García et al., 2017; Thursby and Juge, 2017). Therefore, in some cases a functional core microbiota has been defined instead of a taxonomical one, identifying a set of microbial genes observed in a recurrent way in the same group of individuals (Hernandez-Agreda et al., 2017; Lemanceau et al., 2017). Thus, although some studies suggest to focus the attention on defining core microbiota, there is a number of studies that tried to identify the core component species in several hosts (Holman et al., 2017; Shade and Handelsman, 2012; Turnbaugh et al., 2009). In pigs up to twenty genera have been identified to be present in 90% of the animals during a metanalysis, with relative

abundance typical of the gut location, identifying *Prevotella* as the most abundant one followed by *Bacteroides* and *Clostridium* (Holman et al., 2017).

Substrate availability

Molecules that escape host digestion becoming potential bacterial substrate can provide up to 30% of the energy requirements of a growing pig thanks to bacterial fermentation (Metzler and Mosenthin, 2008). Host diet, health and digestion rate can influence microbial substrate availability in the lower gut (Varel, 1987), which can be summarised in:

i. dietary-derived, i.e. resistant starch (RS), plant cell wall-polysaccharides and other non-starch polysaccharides (NSP), together with a small amount of simple sugars and disaccharides (Pokusaeva et al., 2011; Varel, 1987).

ii. host-derived, such as glycoproteins from saliva, gastric juice, mucinous secretions and intestinal mucosa (Pokusaeva et al., 2011; Varel, 1987).

iii. bacterial-derived, such as bacterial fermentation products, which can be effectively metabolised by other species in the context of cross-feeding reactions (Pokusaeva et al., 2011; Van Immerseel et al., 2010; Varel, 1987).

Microbial substrate degradation and ability to shift between different resources are key features for bacterial survival, conferred by proteolytic enzymes and carbohydrate active enzymes (CAZymes) (Cantarel et al., 2009; El Kaoutari et al., 2013; Flint et al., 2012; Gibson et al., 1989). The CAZymes database (<http://www.cazy.org/>) describes the families of enzymes that degrade, modify, or create glycosidic bonds (Lombard et al., 2014). At the time of writing, it identified 156 families of glycoside hydrolases (GH), 29 of polysaccharide lyases (PL) and 16 of carbohydrate esterases (CE) (Lombard et al., 2014). The same database summarised 15 families of auxiliary activities (AA) acting in conjunction with CAZymes, 84 families of carbohydrate binding molecules (CBMs) and 106 of glycosyl transferases (GT) involved in the creation of bonds with and within carbohydrates during biosynthesis and redox reactions. Different bacteria possess different sets of CAZymes targeting different substrates, and as a consequence, a map of bacterial CAZymes throughout the porcine digestive tract has been described showing differences in microbial substrate utilisation throughout different gut locations (Yang et al., 2016). The following paragraphs describe in more detail the main bacterial substrates used in the models and their degradation.

Resistant Starch degradation

Resistant starch (RS), defined as the sum of starch and products of its degradation not absorbed in the small intestine of a healthy human, is present in large amounts in pigs' diet in the form of complex polysaccharide (Bach Knudsen et al., 2006; Leszczynski, 2004). It is composed by two glucose-polymers α -amylose and amylopectin, and due to physical or chemical characteristics can resist the host digestion becoming available as microbial substrate (Sajilata et al., 2006) (Table 0.1). Many microorganisms within the porcine gut possess amylolytic enzymes, thus potentially able to hydrolyse starch, although the presence

of specific enzymes does not indicate necessarily that the microorganism is a starch-degrader (Cockburn et al., 2015; Flint, 2013; Macfarlane and Gibson, 1997; Ze et al., 2012). Starch utilization system (Sus) of *Bacteroides thetaiotaomicron* is a well characterised example, its locus belongs to the class of the polysaccharide utilization loci (PULs) and comprises 8 genes (Sus R, A, B, C, D, E, F and G) (Foley et al., 2016). Their corresponding proteins are organised between the two membranes to tether, degrade and transport starch molecules from the cell surface into the periplasm as maltooligosaccharides, where they are further cleaved in simpler sugar molecules and carried into the cytoplasm. Sus-like systems are expressed by all the mammalian gut *Bacteroidetes* and are able to mediate the uptake of different carbohydrates (Foley et al., 2016; Koropatkin et al., 2008; Xu and Gordon, 2003).

Non starch polysaccharides and non-digestible oligosaccharides degradation

Non-starch polysaccharides (NSP) are a heterogeneous group of molecules found in plant cells with structural or non-structural roles, whose monomer concentration and type vary among different plant species (Choct, 1997). NSP include soluble and insoluble (apart from lignin) dietary fibres, which usually cannot be digested by monogastric animals such as pigs (Căpriță et al., 2010). Thanks to this attribute NSP can potentially be used as prebiotics in pigs (Metzler-Zebeli et al., 2010), in line with the definition of prebiotic as “selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health.” (de Vrese and Schrezenmeir, 2008). NSP be divided in the three big groups of cellulose, non-cellulosic polymers and pectic polysaccharides (Choct, 1997), or according to their solubility in soluble and non-soluble NSP (Căpriță et al., 2010). Table 0.2 resumes the most common NSP that can be found associated with pig nutrition, whereas more details about main classes and compounds are provided below.

Cellulose is the most common complex polysaccharide in land plants, is a high molecular weight non-soluble linear homopolymer of (1→4)- β -glucose units, whose hydroxyl groups allow bonds with oxygen residues on the same or adjacent chains (Choct, 1997; Sinha et al., 2011). Cellulose chains are usually organised in parallel microfibrils in the primary cell wall, and their concentration is directly related to cell wall thickness conferring structural advantages to the plant (Bayer et al., 1998; Sinha et al., 2011). Apart from cellulose, hemicelluloses and pectins are the major classes of polysaccharides in land plants (Yokoyama et al., 2015). Hemicelluloses include xyloglucans, xylans, mannans, glucomannans, arabinoxylans and β -(1→3,1→4)-glucans, whose structure, like in the cellulose microfibrils, is based on a β -D-pyranosyl backbone, which also allows hydrogen bonds with the amorphous cellulose regions (Scheller and Ulvskov, 2010; Yokoyama et al., 2015). Galactans, arabinans, and arabinogalactans are also usually included amongst the hemicelluloses although some authors consider them as part of pectins due to the absence of an equatorial β -(1→4)-linked central structure (Bakker et al., 1998; Scheller and Ulvskov, 2010; Yokoyama et al., 2015).

Pectin is a heterogeneous family of hydrophilic polysaccharides with multiple roles in plant growth and development (Ridley et al., 2001). Main pectin sugar component generally include arabinose, galactose, galacturonic acid and rhamnose, whereas the main pectic domains are homogalacturonan, rhamnogalacturonan I and II (Ridley et al., 2001; Yokoyama et al., 2015).

Apart from the NSPs, some small oligosaccharides can also escape host digestion becoming available substrate for resident bacteria, i.e. non-digestible oligosaccharides (NDO) (Houdijk et al., 2002). As per definition, oligosaccharides are usually composed by 2 to 20 monomeric units (Roberfroid and Slavin, 2000), although according to IUB-IUPAC nomenclature they contain between 3 and 10 sugar moieties (Mussatto and Mancilha, 2007). Fructose, glucose, galactose and xylose usually represent the main NDO-composing monosaccharide units (Mussatto and Mancilha, 2007). Thanks to their low host-driven hydrolysis rate, NDO such as galactooligosaccharides (GOS), fructooligosaccharides (FOS) and glucooligosaccharides are actively used as prebiotics (Swennen et al., 2006). According to the nature of their glycosidic bond, GOS can be found in the two conformations α and β which require different enzymes for their biotransformation (Bruno-Barcena and Azcarate-Perli, 2015). Some NDO such as FOS and transgalactooligosaccharides (TOS) have been shown to interact with pH, SCFA and bacterial profiles in both ilea and faeces of pigs (Houdijk et al., 2002). Some of the NDO that can be found associated with pig nutrition or used as prebiotics can be found summarised in Table 0.2.

Some of the resident bacteria found throughout the gut, especially in the lower intestine, are able to utilise both NSP and NDO, whose fermentation products can provide energy to the host (Flint et al., 2012). Different polysaccharides degradation strategies can be observed amongst microbes (Flint and Bayer, 2008), and a few representative examples are described as follows. For instance, the aforementioned class of PULs in *Bacteroidetes* delineates clusters of colocalised and coregulated genes, whose products direct binding, degradation and transport of complex polysaccharide molecules (Grondin et al., 2017). The xus cluster of *Prevotella ruminicola*, which allows xylans and pectin degradation is an example belonging to the PULs class (Onumpai et al., 2011; Zorec et al., 2014). Apart from PULs, other enzymes can be found involved with NSP microbial degradation, such as cellulases, capable of hydrolyse the β -1,4 glycosidic bonds of cellulose (Bayer et al., 1998). The complex crystalline structure of cellulose often requires that cellulases are organised in supramolecular complexes, called cellulosomes (Bayer et al., 1998). Cellulolytic bacteria are found in different environments such as rumen (*Ruminococcus flavefaciens*), human gut (*Ruminococcus champanellensis*), horse manure (*Clostridium thermocellum*) and many others, and in some cases their cellulolytic activity make soluble saccharides available to “surrounding” non-cellulolytic bacteria (Artzi et al., 2017).

The two main cellulosome components are (Fontes and Gilbert, 2010):

- structural proteins (i.e. scaffoldins), usually found compounded with the protein complexes cohesins;
- enzymes usually found compounded with the protein domains dockerins, which allow binding with cohesins, and carbohydrate binding domains (CBM), which allow the bonding with polysaccharides.

Whilst dockerin-cohesin non-covalent interaction is central in the cellulosome structure and fundamental for the final cellulosome assembling, CBM can be also found throughout primary scaffoldins, such as the family 3 CBM (CBM3) (Artzi et al., 2017; Doi and Kosugi, 2004).

Host-derived substrates and cross-feeding

About 44% of the Sus-like PUL molecules of *B. thetaiotaomicron* target the degradation of host-derived glycans underlying the importance of this class of substrates for the gut-bacterial metabolism (Flint et al., 2012). Amongst them, mucin is probably the most relevant, being the major glycoprotein overlapping the porcine intestinal mucosa and accomplishing protective functions towards the entire luminal area (Fogg et al., 1996; Stanley et al., 1986). Microbial degradation of mucin could be an advantageous trait when competing for the same substrate typologies in the gut, which could lead to a diet-independent increasing of metabolite production, and therefore the host diet-unrelated energy intake. This has been confirmed by a metagenomic study aiming to identify mucin-degrader bacteria in pig gut, and describing butyrate producers between them (Levine et al., 2013). Protein is also an important class of host-derived substrates, and the total amount available for microbial degradation can be both exogenous and endogenous, such as host-derived enzymes and sloughed cells (Varel, 1987). Although most of the exogenous protein are digested and absorbed by the host in the upper gut, excessive protein intake or unbalanced host physiology can lead to their luminal increment becoming substrate for the microbial fermentation. In this case the metabolic end-products could include potentially harmful molecules, such as biogenic amines, ammonia, hydrogen sulphide and phenolic compounds (Pieper et al., 2016; Rist et al., 2013). Luminal carbohydrate concentration and pH seem to influence the protein bacterial toxigenic effect of these compounds, affecting the gut microbial ecology and opening the way to possible strategies to reduce the microbial protein degradation (Barker et al., 1982; Gerhardt et al., 2000; Pieper et al., 2016).

The utilisation of microbial-derived metabolites by other bacteria, termed cross-feeding, covers an important role in the gut ecology, including the uptake of substances potentially dangerous for the host, like lactate and the uptake of hydrogen, which prevent a drastic decrease of the luminal pH (Duncan et al., 2004; Sergeant et al., 2014). *Megasphaera elsdenii* is the main lactate utiliser in the pig intestine, which would convert it in acetate and propionate. Other genera sharing the same pathway and harboured in the pig gut are *Selenomonas* and *Veillonella* (Counotte et al., 1981; Duncan et al., 2004). Lactate and acetate are also used in

butyrogenic pathways, like in the case of *Faecalibacterium prausnitzii*, that uses acetate as a secondary resource (Duncan et al., 2002b; Kettle et al., 2015) and *Eubacterium hallii* that uses lactate to produce butyrate (Belenguer et al., 2006; Duncan et al., 2002b; Muñoz-Tamayo et al., 2011).

Table 0.1. The four forms of resistant starch.

Resistant starch 1	Plant-cell wall polymers protection from the host enzymatic digestion.
Resistant starch 2	Compact, radial-patterned granular structure, probably decreases the host-enzymes adsorption.
Resistant starch 3	Formed when amylose and amylopectin are heated before temperature decreases again in the presence of water in a process called retrogradation.
Resistant starch 4	Chemical or physical modifications.

Table 0.2. Most common NSP and NDO which can be found in (or that can be supplemented to) pig nutrition.

	Soluble	Non-soluble	References
		Cellulose	(Bayer et al., 1998)
		Hemicelluloses	(Scheller and Ulvskov, 2010)
Non-starch polysaccharides	Xyloglucans	Xylans (solubility can increase reducing molecule degree of polymerisation)	(Piqué et al. 2018) (Bonechi et al., 2017)
	Mannans		(Sharma, 2010)
	Glucomannans		(Keithley and Swanson, 2005)
		β -(1→3,1→4)-glucans	(Bin-fen et al., 2008; Jamar et al., 2011)
	Pectins Homogalacturonan Rhamnogalacturonan I Substituted galacturonans (including rhamnogalacturonan II)		(Raj et al., 2012; Ridley et al., 2001)
Non-digestible oligosaccharides	Galactooligosaccharides Fructooligosaccharides Palatinose (Isomaltulose) Isomaltoligosaccharides Lactosucrose Xylooligosaccharides Glycosylsucrose Cyclodextrins		(Mussatto and Mancilha, 2007; Roberfroid and Slavin, 2000)

Microbial metabolites

The scarcity of oxygen as an electron acceptor induces gut-bacteria to base their metabolism on fermentative pathways, whose main end-products are short chain fatty acids (SCFAs), organic acids, such as formate, lactate and some gaseous molecules such as CO₂, H₂, and CH₄ (Mosenthin, 1998). Whilst microbial protein fermentation contributes to acetate and propionate production, the main products of saccharolytic fermentation are acetate, butyrate, propionate formate and lactate (Morrison and Preston, 2016). The different bacterial fermentative metabolic pathways, together with the mostly diet-dependent substrate availability, and gut physiological factors (e.g. pH), can determine concentration and type of microbial metabolites in the gut (Louis et al., 2014), underlying a clear connection between gut concentrations of soluble/insoluble fibre and SCFAs (Flint et al., 2015; Haenen et al., 2013). Microbial SCFAs affect host metabolism at different levels, for example acetate contributes to muscle energy reserve, propionate is converted to glucose in the liver (Giuberti et al., 2013) and formate concentration seems to be linked to inflammation (Morrison and Preston, 2016). Butyrate is the main energy source for the colonic cells, it is involved in oxidative stress regulation (e.g. H₂O₂-DNA damage reduction), it is involved in colonic defence barrier mechanisms and intestinal epithelial permeability, has an effect on host satiety, it has been shown to have direct anti-inflammatory effect and has been linked to carcinogenesis (Hamer et al., 2008; Pryde et al., 2002; Scheppach, 1994; Van Immerseel et al., 2010). Therefore, modulation of colonic butyrate to improve pig health and performance through probiotic, probiotic and diet interventions has been the main aim of numerous studies (Hold et al., 2003; Kien CL, 2008; Louis et al., 2010; Trachsel et al., 2016; Vital et al., 2014). The main metabolic pathways used by porcine gut microbes are described in the following sections with more detail.

Acetate

Acetate is the most abundant fatty acid in pig hindgut (Suarez-Belloch et al., 2013) and is produced by most enteric bacteria from pyruvate via acetyl-CoA (Louis and Flint, 2017), yielding 3 molecules of acetate for each molecule of glucose (Louis et al., 2014). Acetic acid can be also formed from H₂ and CO₂ or from formate by acetogenic bacteria via the *Wood-Ljungdahl* pathway (Louis et al., 2014). During the latter, pyruvate derived from *Embden-Meyerhof-Parnas* pathway is oxidised into two molecules of CO₂ via pyruvate:ferredoxin oxidoreductase, thereafter reduced to a molecule of acetate ($2\text{CO}_2 + 4\text{H}_2 + n\text{ADP} + n\text{P}_i \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O} + n\text{ATP}$) (Müller, 2003). During the first reaction of the *Wood-Ljungdahl* pathway CO₂ is reduced to formate via formate dehydrogenase (Ragsdale and Pierce, 2008).

Propionate

Three main fermentative pathways are described amongst gut bacteria to produce propionate, which are the *succinate* pathway, the *acrylate* pathway and the *propanediol* pathway

(Reichardt et al., 2014). Succinate pathway; common in *Bacteroidetes* and some *Negativicutes* is used to convert most hexose and pentose (Louis and Flint, 2017), whereas lactate is usually metabolised via the *acrylate* pathway. Although lactate can be converted to propionate either through succinate (e.g. *Veillonella*) or acrylate pathway (e.g. *Megasphaera*) (Louis and Flint, 2017). Fucose and rhamnose are degraded via *propanediol* pathway; by some Firmicutes (Louis et al., 2014). Some species can also convert succinate to propionate, such as *Phascolarctobacterium succinatutens* (Watanabe et al., 2011). Certain Lachnospiraceae can convert rhamnose and fucose in propionate and propanol via the *propanediol* pathway (Louis and Flint, 2017), and the intermediate 1,2-propanediol can be used by other species, such as *Eubacterium hallii* as a substrate for the glycerol/diol dehydratase PduCDE enzyme to produce propionate instead of deoxy sugars (Engels et al., 2016). Moreover, microbial degradation of some amino acids through propionyl-CoA can lead to propionate production in some species (Gonzalez-Garcia et al., 2017).

Succinate

Succinate is mainly produced by members of Bacteroidetes phylum (Connors et al., 2019), and is the main product accumulated via the *succinate* pathway by some bacteria such as *P. ruminicola* (Fernández-Veledo and Vendrell, 2019).

Butyrate

Two different pathways can be undertaken during the final step of butyrate production from butyryl-CoA, once the latter is reduced from acetoacetyl-CoA formed by two molecule of acetyl-CoA (Louis and Flint, 2017). Butyryl-CoA can be either enter the butyryl-phosphate pathway, through phosphotransbutyrylase and butyrate kinase enzymes or butyryl-CoA:acetate CoA transferase pathway which requires acetate as a co-substrate (Louis and Flint, 2009). The latter seems to be the most common amongst porcine gut bacteria (Levine et al., 2013). Butyrate can be also produced from lactate by some species such as *Eubacterium hallii* when in presence of acetate (Duncan et al., 2004). The majority of the butyrate producing bacteria isolated from pig intestinal content and pig faecal samples are found to be members of Clostridia class, such as *Lachnospiraceae*, *Eubacteriaceae*, *Clostridiaceae* and *Ruminococcaceae* (Li et al., 2016). Moreover, *Acidaminococcus fermentans* and *Megasphaera elsdenii* were also found isolated as butyrate producers from pig gut content in high percentage (Levine et al., 2013; Li et al., 2016). *M. elsdenii* is a lactate utiliser (both D- and L-lactate) usually rather abundant in pig gut (Kajihara et al., 2017) and it should be noted that propionate/butyrate ratio produced depends on environmental characteristics such as enantiomeric D/L lactate ratio (Kajihara et al., 2017) or sugar concentration (Prabhu et al., 2012). *M. elsdenii* converts lactate to propionate via *acrylate* pathway, using acrylyl coenzyme A (Prabhu et al., 2012), or to butyrate (Bourriaud et al., 2005), requiring acetate for the conversion (Prabhu et al., 2012).

Lactate and ethanol

Lactic acid bacteria play an important role in gut homeostasis, as demonstrated by their prominent use as probiotics (Yang et al., 2015) and by the lactate key-role in cross-feeding reactions (Duncan et al., 2004; Ouwerkerk et al., 2002; Pham et al., 2017). The most abundant lactate producers detected in pig faeces are *Streptococcus* and *Lactobacillus* (H. B. Kim et al., 2011; Yang et al., 2015), whose metabolic pathways allow the degradation of hexoses and pentose through either homolactic or heterotactic fermentation (Pessione, 2012). Generally, homofermentative hexose metabolism yields 2 molecules of lactate via Emden–Meyerhof pathway, whereas pentose phosphate pathway normally yields 5 molecules of lactate per pentose (Gänzle, 2015). Hexose and pentose that are metabolised through heterofermentative reactions by lactic acid bacteria enter the phosphoketolase pathway yielding lactate and ethanol, or lactate and acetate respectively (Gänzle, 2015). Ethanol can be also generated directly from pyruvate, via conversion of acetaldehyde (Louis et al., 2014).

Formate

Formate is a direct product of pyruvate metabolism, which in absence of oxygen as an electron acceptor can be cleaved by pyruvate-formate-lyase producing formate and acetyl-CoA (Leonhartsberger et al., 2002). Formate is an important metabolite in cross-feeding reactions, as seen before, it can contribute to the formation of acetate, or it can be used by methanogenic archaea to produce methane (Oliphant and Allen-Vercoe, 2019).

H₂ and CO₂

As seen above, porcine gut bacteria possess different fermentative pathways which lead to the production of different metabolites, including H₂ and CO₂. (Louis and Flint, 2009)

Methane

Methanogens archaea, such as *Methanobrevibacter*, which represent a small portion of porcine microbiota (Mao et al., 2011), are able to use CO₂ and H₂ or formate to produce CH₄ (Oliphant and Allen-Vercoe, 2019). Most methanogens can convert CO₂ in CH₄ and 2H₂O using 4H₂ as electron donor, moreover many hydrogenotrophic methanogens are also able to oxidise 4 molecules of formate to 4CO₂ via formate dehydrogenase, with one CO₂ further converted in CH₄ (final stoichiometry: 4 formate→3CO₂ + CH₄ + 2H₂O) (Liu and Whitman, 2008).

Other microbial metabolites, such as phenylacetic acid, phenols, indoles, and p-cresol product of aromatic amino acids fermentation by several *Bacteroides spp.* and some Firmicutes, and hydrogen sulphide produced by *Desulfovibrio spp* using lactate as a co-substrate (Louis et al., 2014) are not described here because not included amongst the metabolites of the dynamics model of the pig gut microbiota.

Microbiota-host interactions

The absence of GIT illness, good physiological functionality, effective immune status and absence of gut microbiota dysbiosis have been related to the regulation of the epithelial and immune functions, general health status, performance, and development of behaviour (Pluske et al., 2018). The main consequence of the pig gut microbiota activity is to increase the animal's energy intake from substrates otherwise undigested by the host, thus microbiota succession can influence the animal's ability to using different types of substrates (Metzler and Mosenthin, 2008). During lactation, pigs with higher relative abundance of *Bacteroides*, *Oscillibacter* and *Clostridium XIVa* were thought to be more able to digest free-milk oligosaccharides, and after weaning, *Prevotella* was positively associated with increased performances, as members of this genus might provide essential help breaking down complex dietary polysaccharides (Mach et al., 2015). These findings were also confirmed by a study analysing the relationship between different enterotypes and host performance, associating the enterotype-like group dominated by *Prevotella* and *Mitsuokella* with host higher body weight and average daily gain (Ramayo-Caldas et al., 2016). The connection between microbiota and feed efficiency could be also seen in obese pigs, which seemed to have a high abundance of *Firmicutes* (Lawlor et al., 2017). Postweaning dysbiosis characterised by a decrease of *Lactobacillus* and of the microbial diversity in general could lead to diarrhoea (Gresse et al., 2017), moreover, the disruption of the gut microbiota could cause inflammatory bowel disease or be the cause of opportunistic infections like in the case of *Clostridium difficile* (Fouhse et al., 2016; Sun and Jia, 2018). Physiological changes in microbiota composition have been related to immune system variations and its maturation, in fact not only has a connection been demonstrated between IgA production and microbiota in axenic mice, but the same immunoglobulin class luminal secreted could coat the microbes creating a non-inflammatory host-microbiota relationship (Den Hartog et al., 2016; Mach et al., 2015). Furthermore, a brain-gut microbiome axis can be demonstrated through the several microbial-derived neuroactive molecules like tryptophan metabolites, through the microbiota-activation of the hypothalamic-pituitary-adrenal axis, and through the regulation the host serotonin biosynthesis (Farzi et al., 2018; Winek et al., 2016; Yano et al., 2015). Thus, microbiota has been directly linked to metabolic disorders (e.g. food addiction), and psychiatric and neurological disorders such as depression and anxiety (Winek et al., 2016).

In vitro investigation of the gut microbiota

The study of microorganisms can be based on both culture-based methods and on culture-independent methods. The first approach is required for instance to elucidate the microbial roles related to healthy and disease status, whereas the second one could allow to retrieve more information to be retrieved on the communities, identifying the species that cannot be cultivated (Lau et al., 2016).

Culture-based methods

Culture techniques are valid research approaches in different cases in the study of the gut microbiota, this methods can have many advantages, such as allowing the identification of new species and to test the bacteria in different environments (Lagier et al., 2015). Generally, in order to be able to cultivate the gut-microorganisms it is necessary to simulate the environmental conditions in which those microbes are usually found (Lagier et al., 2015). With reference to the gut microbiota it is essential to ensure a temperature similar to the one found *in vivo*, but also oxygen level and pH are important factors to control (Bindelle et al., 2007). The complex medium used must be based on the composition of the chyme typical of the gut section simulated, comprising carbon- and nitrogen-based resources, host-derived molecules such as mucins, minerals and vitamins (Goodman et al., 2011). These parameters are a necessary requirement not only to allow the proliferation of the single species, but also to ensure that the microbial community dynamics are similar to those found *in vivo* (Ito et al., 2019; Stewart, 2012). There are several systems employed to set up these types of fermentation, from batch to continuous cultures, with a different level of complexity allowing control and maintenance of the mentioned parameters for long periods of time (Crowther et al., 2016; Difilippo et al., 2016; Sommer, 2015; Tanner et al., 2014; Walker et al., 2005). Although still not used to cultivate the microbiota, novel types of bioreactors have been designed to allow the 3D cultivation of intestinal cells or to reproduce artificial intestinal portion *in vitro* (Costello et al., 2017; Zhou et al., 2018), which could be applied in the future for the study of the exchanges between the microbial and eukaryotic cells and to test the impact of the immune system on the microbiota through co-cultures.

Culture-independent methods

Metataxonomics is the high-throughput process employed to analyse an entire microbiota creating a metataxonomic tree allowing to explore the composition of a microbiota independently of cultural methods (Marchesi and Ravel, 2015). The metataxonomic study of the bacterial phylogeny and taxonomy uses species-specific sequences present in the 16S ribosomal RNA gene (Chakravorty et al., 2007). This is a 1500 bp length gene composed by 9 hypervariable regions interspersed by conserved regions. Punctiform mutations appear in a proportional fashion to the taxonomical distance between two species, which can be used to assign differences amongst the microbial populations (Chakravorty et al., 2007; Janda and Abbott, 2007). High-throughput systems, such as Illumina, which uses labelled nucleotide incorporated with the DNA generating ~20 million sequencing reads, can be used to sequence the 16S rRNA gene (Jo et al., 2016). Thus the data can be analysed with bioinformatic software which compare them to a database to build a taxonomic table with the qualification of the community present in a sample on in a certain time (Caporaso et al., 2011a). Different strategies have been designed which use different combination of hypervariable regions and depend on different factors, such as the type of sample from which the total DNA is extracted

or preferences in the way exploring the bacterial community (Chakravorty et al., 2007; Jo et al., 2016).

Mathematical modelling

The typical bacterial growth curve observed in batch cultures is composed of a lag phase, in which the growth rate is equal to 0, succeeded by the acceleration and then increasing growth rate (characterised by an increasing growth rate) which usually decreases during the retardation phase, stops during the stationary phase and is negative in the following decline phase (Monod, 1949) (Figure 0.2). Mathematical modelling represents a reliable tool to describe the microbial growth *in silico*, which could also be used to anticipate changes due to manipulation of the microbial growth curve (Esser et al., 2015). Different approaches have been described to model microbial communities, especially in relation to their environment and the amount of information theorised (Bucci and Xavier, 2014; Kettle et al., 2015; Montoliu et al., 2016; Stein et al., 2013). Thus, several examples can be found in literature describing the dynamics of the gut microbes, their substrates and metabolites (Gerritse et al., 1992; Kettle et al., 2015; Lewis and McGlone, 2008; Lin et al., 2000; Muñoz-Tamayo et al., 2011, 2010; Shashkova et al., 2016). When modelling such a complex community like the GIT microbiota, a mathematical model should take into account the governing physical laws, such as the conservation of mass, which could be summarised by the study of the microbial metabolic pathways, stoichiometry and the biomass yield from a generic substrate (Panikov, 1991). Moreover, the summarisation of the microbial complexity is pivotal to easily represent the microbial dynamics in the most concise way possible. To describe the behaviour of the variables modelled, a set of parameters is used, which can define different aspects of the microbial replication, such as the ability to generate biomass or metabolites when the microorganisms ferment different substrates (Stein et al., 2013).

Both the models generated during this PhD were knowledge-based considering the physical and biological laws existing between microorganisms and their related molecules in the gastrointestinal tract of pigs. A detailed description of the model for the entire porcine gut microbiota is the object of the first chapter whereas the porcine core microbiota model is described in the fourth chapter.

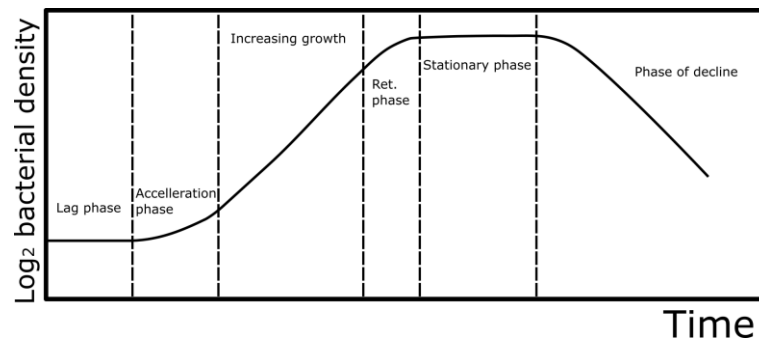


Figure 0.2. Different phases of bacterial growth, picture edited from Monod, 1949. The typical growth of the microorganisms as it could be observed in a batch culture is composed by a lag phase, with growth rate= 0, succeeded by the acceleration and then increasing growth rate, which usually decreases during the retardation phase, stops during the stationary phase and is negative in the decline phase.

Hypothesis

The entire PhD project was developed to test the hypothesis: “A mathematical model can represent the dynamics of the porcine gut microbiota and could be used to forecast the possible effects of different intervention types”. Therefore, this PhD was developed via the following stages:

1. Development of the first mathematical model of the entire porcine gut microbiota starting from the structure of a previous model developed for the human gut microbiota.
2. Setting up of an *in vitro* experimentation simulating the porcine proximal colon dynamics, therefore, reproducing the environment computed by the mathematical model.
3. Parametrisation and validation of the model using data collected during the *in vitro* experimentation.
4. Development of the first mathematical model forecasting the dynamics of the porcine core microbiota.
5. Simulations of probiotic/prebiotic therapies through *in silico* experimentation using both the models developed.

Consequently, this thesis has been structured through five chapters following the different stages above. After a general introduction of the pig gut microbiota, its composition and its relationship with the host, together with a description of the mathematical modelling applied to this type of environment, the first chapter of the thesis will focus on the structure and formulation of the mathematical model of the entire porcine gut microbiota. Afterwards, a detailed description of the *in vitro* experimentation will be the object of the second chapter, in which all the findings observed during this stage will be fully explained. The third chapter is an explanation of the parametrisation process, which used data from the *in vitro* work to fit the model in such a way that it described the dynamics observed when simulating a faecal inoculum growing in a proximal colon-like environment. The fourth chapter focuses on the second model developed during this PhD, which describes the dynamics of the porcine core microbiota, and the *in silico* findings, suggesting how the core is possibly responsible for shaping the fermentation pattern of the entire community. Thus, the fifth chapter describes the *in silico* experimentation, during which simulations of prebiotic and probiotic interventions assess the effects on the modelled variables using both the entire- and the core-microbiota models. Finally, the last chapter of this thesis resumes, in the form of a general discussion, the main findings generated during this PhD.

1 Dynamic model of the porcine proximal colon microbiota

1.1 Introduction

Microbial ecology models can represent a valuable tool to predict the microbial dynamics in a certain environment or the impact of interventions aiming the improvement of the general environmental conditions (Bucci and Xavier, 2014). Several can be the approaches to model microbial communities, including conceptual models, mathematical or equation-based models, purely phenomenological or mechanistic characterised by the presence of informative parameters (Esser et al., 2015). The level of complexity usually depends on the type of ecosystem considered and on the amount of information available and/or included in the model. Nevertheless, in general a successful predictive model must benefit from having deterministic dynamics, and from a well characterised conditions (D'hoë et al., 2018).

The application of microbial ecology models are different, such as food safety research, wastewater treatment systems and host-associated ecosystems like the gut microbiota to mention only a few examples, (Bucci and Xavier, 2014; Esser et al., 2015). In wastewater treatment typically a bacterial community is responsible for reducing the carbon content through different bacterial metabolic pathways, which can be simulated by a model using several parameters like the water flow and composition together with the microbial growth (Bucci and Xavier, 2014; Esser et al., 2015). Like for the wastewater treatment, the models of the gut microbiota can assume a mechanistic approach by the analysis of the component microbial communities and the relationships between them, which can lead to summarise the complexity of the populations through different methods. Thus different models have been developed for several ecosystem types, such as bioreactors, or colonic carbohydrate degradation (Esser et al., 2015; Muñoz-Tamayo et al., 2016, 2010; Panikov, 1996; Wang et al., 2016). Gut microbiota is a relatively closed environment constituted by different microbial species in connection with the host, and different parameters (e.g. maximum growth rate or biomass yield) can describe the rate of change of the microbial cells (Kettle et al., 2015; Lin et al., 2000; Longhi et al., 2017). Nevertheless, sometimes the lack of information with regards to the mentioned parameters could decrease the accuracy of a model.

In this chapter the first dynamic model of the entire porcine gut microbiota is presented, it was based on the structure of a previous model developed for the human gut microbiota (Kettle et al., 2015), whose main differences with the model here presented were the selection of the parameters descriptive of the microbial dynamics and the validation/parameter-fitting process undertaken. The model simulated an *in vitro* bioreactor, due to the *in silico* absence of biotic

factors, such as the effect of the immune system on microbiota or the host-driven SCFA depletion. This chapter describes in detail the model equations and the data frames summarising the porcine gut microbial community. Moreover, it presents a reliability test of the model predictions when simulating a fermentation study found in literature starting with two different microbial communities. The model described in this chapter was accurate in terms of metabolite concentration and relative abundance, although *in silico* dynamics were slower than *in vitro* observations. Moreover, lack of precise microbial dynamics data throughout the *in vitro* fermentation from literature did not allow a direct comparison between simulations and observations. Nevertheless, as explained in detail in Chapter 2 and 3, *in vitro* porcine colon content fermentation experiment was carried out to perform further parameter fitting and model comparison with observed data.

1.2 Model description

1.2.1 Structure of the pig-microbiota model considering its derivation from the previous human-microbiota model (Kettle et al., 2015)

The model of the entire pig gut microbiota developed and presented here was based on a human colonic microbiota model developed previously (Kettle et al., 2015). Nevertheless, some dissimilarities are acknowledged between the two environments, thus the following sections describe intestinal microbiota in the two hosts and how functional groups composing the human model were adapted when used to summarise porcine gut microbes.

1.2.1.1 Intestinal microbial composition in humans and pigs

Anatomical and physiological similarities between pigs and humans make the former an excellent model for human research (Baker, 2008), furthermore similar nutritional requirements translate in the development of a very similar intestinal microbiota in the two hosts (Heinritz et al., 2013). As stated in [General Introduction](#), pig gut microbiota experiences both temporal and longitudinal changes (Hou et al., 2015a), although *Bacteroidetes* and *Firmicutes* seem to be the predominant phyla in pig faecal sample at any age (H. B. Kim et al., 2011). Interestingly, this also seems to be the case in humans, in which these two phyla represent 90% of the total followed by *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (Rinninella et al., 2019). Although with lower abundance than what observed in pig's intestine, *Bacteroides* and *Prevotella* are predominant amongst *Bacteroidetes* phylum in humans (Rinninella et al., 2019). The two species are also highly represented in pigs, with *Prevotella ruminicola* being the most abundant species especially in some part of the animal's life (Crespo-Piazuelo et al., 2018). Amongst the phylum *Firmicutes*, genera like *Clostridium*, *Enterococcus*, and *Ruminococcus* are common in humans (Rinninella et al., 2019) and pigs (H. B. Kim et al., 2011), although *Clostridium* can represent 95% of the phylum in humans (Rinninella et al., 2019) but not in pigs, and *Eubacterium* is more represented in humans than pigs (Heinritz et al., 2013). Further differences can be found in the higher relative abundance of *Lactobacillus*, *Streptococcus* and *Megasphaera* in pigs (H. B. Kim et al., 2011; Yang et al., 2015), compared with humans (Thursby and Juge, 2017), whilst *Bifidobacterium*, within *Actinobacteria* phylum can represents ~4% of human gut microbiota (Rinninella et al., 2019), whereas its numbers are much lower in pigs (Heinritz et al., 2013).

1.2.1.2 Functional groups differences between dynamic models of human and porcine colonic microbiota

Within the human model, gut microbial complexity was summarised through 10 bacterial functional groups (MFGs) (Kettle et al., 2015), later redefined as microbial functional groups (MFGs) considering that some of them were not bacteria (i.e. archaea) (Kettle et al., 2018). Human colonic-microbiota members were thus grouped according to their metabolic

pathways, substrate preferences and metabolite production, generally identifying propionate-, butyrate-, succinate-, lactate-producing bacteria, acetogens and methanogens (Kettle et al., 2015). Butyrate producers were further subdivided in three different groups to take into account of the two different enzymes/pathways, butyrate kinase and butyryl coenzyme A (CoA):acetate CoA transferase distributed amongst gut microbes (Kettle et al., 2015; Louis et al., 2004), and the ability of some species to produce butyrate from lactate (Duncan et al., 2004). As described in more detail in the following paragraphs, MFGs were modelled through data frames containing stoichiometry of their metabolic pathway(s), dynamic parameter values and information on optimal pH. Representative species from each MFGs were used to inform such data frames and therefore several considerations were made when those were adapted to describe pig colonic microbiota. The main differences between the two models can be summarised as follow.

Assumptions for Acetate/succinate/propionate producers (MFG1) for the pig model were based on *Prevotella ruminicola* due to its prevalence in pigs (H. B. Kim et al., 2011; H. Yang et al., 2017), whereas observations of *Bacteroides thetaiotaomicron*, *B. ovatus* and *B. fragilis* were used in the human model to describe the stoichiometry of this group. *Ruminococcus* spp. that can use prevalently resistant starch (MFG2) or non-starch polysaccharides (MFG3) were considered in both models to parametrise these data frames, based on compositional similarities between the two hosts (Tiwari et al., 2019; Ze et al., 2012). Moreover, information on *Succinivibrio* spp. was added in the pig model to take into consideration its contribution to MFG3 in pigs differently from humans (O'Herrin and Kenealy, 1993; Robert and Bernalier-Donadille, 2003; Tanner et al., 2014; Varel and Yen, 1997). Lactate producers (MFG4) were modelled based largely on *Bifidobacterium* spp. in the human model, although the abundance of this species within pig's gut is negligible (Heinritz et al., 2013), therefore parametrisation of this group was based on *Lactobacillus reuteri* and *Streptococcus* spp., which are the most abundant lactate producers within pig gut (H. B. Kim et al., 2011; Yang et al., 2015). With regards to butyrate producers (MFG5, MFG6), the distribution of the abovementioned pathways is similar in pigs and humans (Levine et al., 2013; Louis et al., 2004; Trachsel et al., 2016), albeit relative abundances of belonging species could differ between the hosts. Therefore, although data frames were parametrised using assumptions based on the same species as the ones used in the human model, their final relative abundance in the assembled model was based on their quantities in pigs, as described later in the text.

Apart from using simple/complex carbohydrates, members of propionate producers functional group (MFG7) can also grow on lactate, producing mainly propionate and acetate through *acrylate* or succinate pathway (Louis and Flint, 2017). The data frame of this group was modelled in both humans and pigs basing assumptions on observations for *Megasphaera* spp. (Kettle et al., 2015), considering that especially *M. elsdenii* can represent a big fraction of the pig gut microbiota (Levine et al., 2013). It should be noted that the organism is known to

use lactate to produce propionate and butyrate (Levine et al., 2013), although propionate/butyrate ratio depends on environmental characteristics such as enantiomeric D/L lactate ratio (Kajihara et al., 2017), sugar concentration (Prabhu et al., 2012) or pH (Chen et al., 2019). A mixture of propionate and acetate was reported when the species was growing in the presence of excess lactate (Prabhu et al., 2012), and in continuous culture the bacterium seemed to produce higher percentage of propionate and negligible amount of butyrate when growing in a chemostat on either 15mM or 30mM lactate (Chen et al., 2019). The last butyrate producers' group (MFG8) is composed of microorganisms that can use either carbon based substrates or lactate to produce mainly butyrate, this data frame was based on organisms related to *E. hallii* in the human model (Kettle et al., 2015). Production of butyrate from hexoses was based on organisms related to *E. hallii* in the pig model as it was for the human one (Kettle et al., 2015). Whereas, stoichiometry for lactate fermentation was based on butyrate kinase pathway of *M. elsdenii* as observed in continuous-culture (Chen et al., 2019) to acknowledge both *M. elsdenii* prevalence compared to *E. hallii* and the former's possible contribution to butyrate production in pigs. As detailed later in the text, when data frames were assembled to form the porcine microbiota model, starting bacterial (i.e. functional groups) concentration was established based on previous *in vitro* study from literature ([Chapter 1](#)) or on *in vitro* fermentation experiments conducted during this PhD project ([Chapter 3](#)). As discussed below in the thesis, *Megasphaera elsdenii* was assigned to both groups in different proportions ([Chapter 3](#)).

Acetogens and methanogens, MFG9 and MFG10 respectively, represent a small percentage of pig's gut microbiota (H. B. Kim et al., 2011; Mao et al., 2011), although taxonomical similarities between members from the two groups amongst human and pig intestines led to base the assumptions for stoichiometry and parameters on *Blautia spp.* and *Methanococcus spp.* to summarise the dynamics in the two data frames respectively (Eren et al., 2015; Mi et al., 2019).

1.2.2 Environment modelled

Biotic factors such as the effect of the immune system on microbiota or the host-driven SCFA depletion were not included into the dynamic model of the whole porcine gut microbiota (WM) here developed. Therefore, the model described an *in vitro* experimentation, such as a bioreactor, rather than the porcine proximal colon, which simulated the physio-chemical characteristics of the porcine proximal colon in terms of pH, temperature, retention time and microbiota composition. The choice of excluding the role of the host, especially with regards to the SCFA absorption was made because it could have been technically demanding to include this aspect in the *in vitro* experimentation used to proceed with the parameter fitting (Chapter 2 and Chapter 3). Thus, the model simulates a continuous fermentation experiment in which an inoculum constituted by gut-derived faecal material (assumed to represent the colon microbiota) grew in a complex medium subjected to a flow-in and -out simulating the

movement of digesta through a peristaltic pump (**Error! Reference source not found.**). The model was developed and solved using the package `microPop` in R (<https://CRAN.R-project.org/package=microPop>) (Kettle et al., 2018), which solves a system of ordinary differential equations (ODEs) and allows simulation of the dynamics and interactions of multiple microbial populations. The rates of change of bacterial biomass, substrates and metabolites are the variable output of the main function '`microPopModel()`'. To summarise the microbial complexity the bacterial populations were modelled by 10 different microbial groups according to their substrate preferences and metabolic pathways, and the main substrates were summarised in four classes, with some of the bacterial metabolite becoming cross-feeding substrates for other microbes (Kettle et al., 2015). The kinetic parameters and metabolic stoichiometry were summarised in data frames, likewise, the environmental conditions (e.g. inflow/outflow rates, pH, starting concentrations etc.) were input through two different data frames.

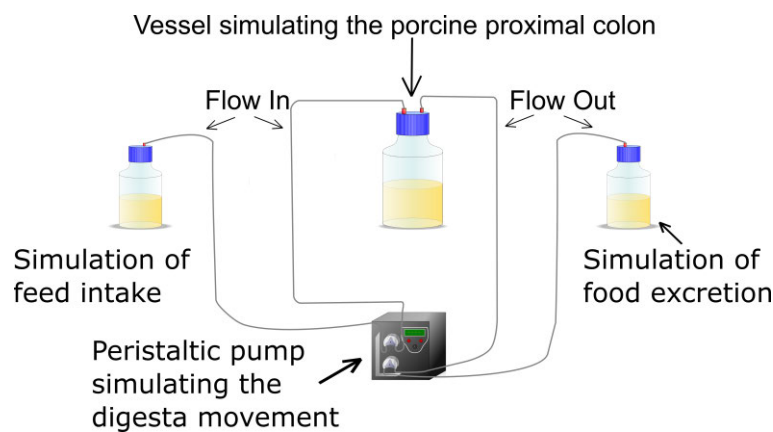


Figure 1.1. Schematic of a simple bioreactor simulating the porcine proximal colon. A faecal inoculum is grown in the main vessel, in the central part of the picture, which simulate *in vitro* the porcine proximal colon. A flow-in (i.e. feed intake *in vitro* simulation) and a flow-out (i.e. food excretion *in vitro* simulation) are connected to the main vessel through a peristaltic pump. The latter allows to simulate *in vitro* the passage of digesta through the proximal colon.

1.2.3 Modelling the substrates and metabolites

To simplify the complexity of molecular interactions found *in vivo*, four substrates were included in the model, i.e. resistant starch (RS), non-starch polysaccharides (NSP), proteins and sugars (Kettle et al., 2015). RS comprised all starch types available for bacterial degradation that can be found in the colon, NSP included all the dietary fibres apart from starch, whereas sugars summarised all the mono- and poly-saccharides that were not included in NSP or RS. These substrates had an assumed modelled molecular weight of 180.16 g/mol, based on the molecular weight of glucose. Protein categorised all the aminoacidic substrates available for bacterial consumption without differentiating between the different types of

peptides (modelled molecular weight: 111 g/mol). Other substrates were not included in the model at this stage, such as mucins and endogenous secretions in general, saccharides derived from bacterial degradation of complex polysaccharides or non-digestible oligosaccharides. Moreover, the model so far developed does not differentiate between different polysaccharides amongst RS or NSP as described in the [General Introduction](#). As already recognised for the human model (Kettle et al., 2015), modelling complex categories within the already modelled substrate would represent a further refinement of the model, although it would require an expansion of MFG and therefore computing power demand. The current model architecture, adapted from the human model, allows to depict well microbial and molecular dynamics as showed later, and although improvements could be planned for further developments, they were not considered during the length of this PhD project, mostly due to time constraints.

The metabolites modelled were 11 in total, i.e. H_2 , CO_2 , CH_4 , H_2O , acetate, propionate, succinate, lactate, formate, ethanol and butyrate, with some of them being also a substrate for other bacteria according to cross-feeding pathways as specified below.

1.2.4 Modelling microbial communities

Ten microbial functional groups (MFGs) were used to model the bacterial and archaeal populations found in the porcine proximal colon, according to the type of enzymes allowing them to use different substrates through one or more metabolic pathways (Kettle et al., 2015). The MFGs can be modelled in microPop as a single entity or as a community composed by different strains belonging to the same MFG. Indeed, multiple strains can be assigned to each group, which would have stochastically generated parameters for the distinctive traits starting from the values assigned as described in the following section. The strains will thus compete for the same set of resources allowing the community to reach a new equilibrium targeting a balanced composition. The dynamic of the community could be easily followed by studying the behaviour of the mean trait value (i.e. a weighted average of the strain concentration considering the trait value), with a net increase in biomass in those groups with higher capability to adapt.

A detailed description of the MFG data frames will follow the definition of the parameters used in the model.

1.2.5 Mathematical formulation

Due to the low redox potential in the gut, bacterial metabolism is mainly characterised by fermentation, where substrates are used by bacteria to produce energy and bacterial biomass (releasing metabolites as a waste product). Therefore, the state variables in the system of ordinary differential equations (ODEs) shown below are the concentrations of the MFGs, resources and metabolites. These are to the ODEs used in the package microPop (Kettle et al., 2017).

According to the Monod equation for the growth of microorganisms (Monod, 1949), the growth rate (μ) of the MFG j using the substrate i is defined as:

$$\mu_{ij}(t) = \mu_{ij}^{max} \frac{R_i(t)}{R_i(t) + K_{ij}} , \quad (1-1)$$

where μ_{ij}^{max} is the maximum growth rate of the MFG j consuming the substrate i with concentration R . K is the half saturation of the MFG j , which has concentration X_j , on the substrate i , defined as the concentration of substrate present when the microbes reach half of their μ_{max} (Monod, 1949). To model the generation of new bacterial biomass the parameter yield (Y) is defined as the grams of MFG j produced using 1 gram of substrate i :

$$Y_{ij} = \frac{X_j}{R_i} , \quad (1-2)$$

Therefore, K , Y and μ_{max} values, together with the stoichiometry and the substrate type must be assigned to each bacterial functional group in order to implement the model in microPop. Data found in literature were used to assign values to Y and μ_{max} , according to cultural experiments of reference species for each MFG. Nevertheless, little information was found for the half saturation constant, which therefore maintained the highest degree of uncertainty in the model. Thus, to attribute its value, a double step process was undertaken. First, an *in silico*-empirically determined value was assigned to each group, aiming to achieve a reasonable correlation between the MFG/SCFA model outputs and their analogue values from *in vitro* continuous fermentation data found in literature. Thereupon, as shown in Chapter 3, using the data observed during the *in vitro* 35 day continuous fermentation (described in detail Chapter 2), a new set of K values was stochastically generated and assigned to an increased number of strains in a 300 run parameter fitting process.

1.2.5.1 Dilution rate

The model mimicked porcine proximal colon environment, whose passage of digesta throughout was assumed to be mostly constant, although *in vivo* it can be affected by factors like diet, substrate type and health of the animal (Wilfart et al., 2007). The time that the food is retained in a gut compartment is determined by different factors, such as physiological-anatomical animal characteristics and diet content (Hilton et al., 1998; Wilfart et al., 2007). The porcine proximal colon retention (RT) time was assumed to be 9 hours (Latymer et al., 1990; Tanner et al., 2014), and it was modelled through the dilution rate (D), as a reciprocal of RT , according to the following equation:

$$D = \frac{1}{RT} = \frac{Flow_{out}}{working\ volume} , \quad (1-3)$$

where, the working volume refers to the volume of the main vessel, used to simulate the porcine proximal colon.

1.2.5.2 Bacterial specific growth rate

The rate of change of the MFG j ($\text{gl}^{-1}\text{h}^{-1}$) with concentration X_j (gl^{-1}) that grows on the resource i with concentration R (gl^{-1}), at time t (h) is modelled with the ODE:

$$\frac{dX_j(t)}{dt} = D_{X_j}^{in} X_j^{in}(t) + X_j \mu_{ij}(t) - D_{X_j}^{out} X_j(t) . \quad (1-4)$$

The first and the third terms on the right-hand side of Eq. (1-4) are respectively the flow-in and the flow-out, where $D_{X_j}^{in}$ and $D_{X_j}^{out}$ are the dilution rates applied to the MFG j respectively through the flow-in and -out. X_j^{in} represents the MFG j immitted in the system through the flow-in. Whereas $\mu_{ij}(t)$ is the specific growth rate (h^{-1}) of the strain j on the substrate i at time t . The equation for μ_{ij} , which follows the dynamics described by Monod equation for the growth of the microorganisms, changes according to the substrate type, and was shown previously in its simplest form in Equation 1. The death rate of microorganism was assumed as negligible compared with the flow out rate when modelling the rate of change of X_j . In microPop, the resources are classified according to their relationship with the bacteria in essential (Se), perfectly substitutable (S), and boosting resources (Sb) as applied later. Water is classified in microPop as substrate, “Sw”, and considered not limiting to the growth. Thus Equation 1 is adapted to each substrate type as described below.

When a substrate is pivotal for the growth of the MFG j , it is defined as an essential resource (Se), and the specific growth rate is modelled as:

$$\mu_{ij}(t) = \mu_{ij}^{max} \frac{R_{Se}(t)}{(R_{Se}(t) + K_{Se,j})} \frac{S_i(t)}{(S_i(t) + K_{ij})} , \quad (1-5)$$

therefore, even if a generic substrate i , with concentration S , is present, growth can occur only when $R_{Se} > 0$.

Perfectly substitutable resources (S) have the same contribution to the bacterial growth, which is modelled according to Ballyk and Wolkowicz, (1993). Therefore, the bacteria can use the different substitutable resources in the system, although there is a limit on the total number of resources that can be consumed given for example by the saturation of the transport system of the bacterial cells. Thus, the specific growth of the MFG j is given by:

$$\mu_{ij}(t) = \mu_{ij}^{max} \frac{R_i(t)/K_{ij}}{1 + \sum_{s=1}^{N_R} \frac{R_s(t)}{K_{Sj}}} . \quad (1-6)$$

Then N_R (number of resources)= 1, Eq. (1-6) reduce to the Monod equation (Eq. (1-1)).

As shown in Equation (1-7) below, the boosting substrate S_b enhances the growth of the MFG j , which could not proliferate using S_b only as it still needs i for its growth, such as for example acetate used in the butyryl-CoA:acetate CoA-transferase to produce butyrate (Duncan et al., 2002a):

$$\mu_{ij}(t) = \mu_{ij}^{max} \left(F_b + (1 - F_b) \frac{R_{Sb}(t)}{R_{Sb}(t) + K_{Sb,j}} \right) \frac{R_i(t)}{R_i(t) + K_{ij}} , \quad (1-7)$$

where F_b is the fraction of maximum growth of MFG j on R_i when S_b is absent. Thus F_b , with values $0 \leq F_b < 1$, indicates the ratio at which S_b enhances the growth, i.e. $F_b=1$ in Equation (1-7) means that the growth is possible only on i , thus the equation reduces to Eq. (1-1).

1.2.5.3 Modelling the effect of pH on bacterial growth

The ability of each MFG to grow optimally only in a limited range of pH values is modelled in microPop through the argument “pHcorners” in each MFG data frame. The four pH corner points are used to define the x axis of a trapezium (pH default function in the R package), whose y axis points (i.e. 0, 1, 1, 0) describe the limitation. Therefore, the output from the pH function, called H_i (i.e. limiting pH function H acting on the growth of the MFG j) with values in the range $0 < H_i < 1$ scales μ_{ij} according to the pH corners described in each MFG data frame as follow:

$$\mu_{ij}(t) = H_i \mu_{ij}^{max} \left(F_b + (1 - F_b) \frac{R_{Sb}(t)}{R_{Sb}(t) + K_{Sb,j}} \right) \frac{R_i(t)}{R_i(t) + K_{ij}} . \quad (1-8)$$

1.2.5.4 Modelling the resource uptake and the metabolite production

The rate of the uptake (U_{ji}) ($\text{gl}^{-1}\text{h}^{-1}$) of R_i by X_j is equal to:

$$U_{ji} = \frac{\mu_{ij}}{Y_{ij}} X_j , \quad (1-9)$$

where Y_{ij} is defined in Equation 2. Thus, the substrate (i) concentration rate of change ($\text{gl}^{-1}\text{h}^{-1}$) moving through the system and consumed by MFG j , is modelled using the following ordinary differential equation:

$$\frac{dR_{ij}(t)}{dt} = D_{S_i}^{in} R_i^{in}(t) - U_{ij}(t) - D_{S_i}^{out} R_i(t) . \quad (1-10)$$

As described above, $D_{S_i}^{in}$ and $D_{S_i}^{out}$ are the dilution rates applied to the substrate i respectively through the flow-in and -out, and R_i^{in} represents the substrate i immitted in the system through

the flow-in. Since the model depicts a scenario in which multiple types of resources are used by different MFGs, Eqs. (1-4) and (1-10) become part of a large system representing several pathways and different equations

To model the metabolite production rate, microPop uses stoichiometry ratios, however the microbial biomass growth is not included in the stoichiometry in the case of substitutable resources. Therefore, according to the equation used in the R package, the production of the metabolite z , in a system in which there are N_z metabolites and N_i substrates, the production rate M ($\text{gl}^{-1}\text{h}^{-1}$) of z by the MFG j is given by:

$$M_{jz} = \frac{m_z n_z}{\sum_{k=1}^{N_z} m_k n_k} \left(\sum_{i=1}^{N_i} U_{ji} + U_{jw} - \mu_{ji}(t) \right), \quad (1-11)$$

where m_k is the molecular mass and n_k is the number of molecules of product k . In this way, the microbial growth rate (μ_{ij}) is subtracted to conserve mass, computing also the uptake of water (U_w). Thus, the general equation which models the rate of change ($\text{gl}^{-1}\text{h}^{-1}$) of the metabolite z , with concentration P , produced by j at time t is:

$$\frac{dP_{zj}(t)}{dt} = D_{P_z}^{in} P_z^{in}(t) + M_{zj}(t) - D_{P_z}^{out} P_z. \quad (1-12)$$

1.2.6 Microbial functional group data frames

The following is a description of all the MFG data frames, with details on parameter values, substrate types, stoichiometry, and pH corners.

As described above, the final values for the half saturation constant were output of an empirical determination, whose final values will be described in Chapter 3, following reparameterization using *in vitro* observations. The values depicted in the following MFG data frames are empirically derived using metabolite observation from literature (Tanner et al., 2014) whilst comparing the *in silico* model outputs.

1.2.6.1 Microbial functional group 1

Members of MFG1 are able to grow on protein- and carbohydrate-based resources producing mainly acetate, propionate, succinate (Kettle et al., 2015). The group comprises mostly bacteria belonging to *Bacteroidetes* phylum, one of the most abundant in colon of adult pigs (H. B. Kim et al., 2011). Amongst the phylum members, *Prevotella* is one of the most abundant genera in the porcine colon throughout pigs' life (Crespo-Piazuelo et al., 2018), with *P. ruminicola* being one of the most abundant species (H. Yang et al., 2017). Therefore, observations of *P. ruminicola* in culture-based experiments have been used to parametrise MFG1 data frame (Table 1.1). Succinate pathway is the main propionate production route from hexose and pentose found in *Bacteroidetes* (Louis and Flint, 2017), and the main propionate

pathway in both *Bacteroides spp.* and *Prevotella spp.* (Connors et al., 2019). Both *Bacteroides spp.* and *Prevotella ruminicola* have been shown to produce more succinate than propionate under certain growing conditions (Fernández-Veledo and Vendrell, 2019). For example, acetate and succinate are the major fermentation products of *P. ruminicola* 23 growing in a low B12 concentration environment (Strobel, 1992).

Stoichiometry assumption for NSP, RS and sugars was based on fermentation pattern analysis of *Prevotella ruminicola* growing on different substrates (4g/l) (Marounek and Dušková, 1999). *P. ruminicola* has been shown to growth efficiently on peptides but not amino acids (J. N. Kim et al., 2017), although due to lack metabolite analysis, the assumption for the stoichiometry for protein degradation was based on the same functional group from the previous model of human colonic microbiota, which was based on *Bacteroides spp.* (Kettle et al., 2018, 2015). As depicted in Table 1.1, “other” amongst the metabolites in the second pathway indicates other metabolites typical of protein degradation which are not included in the present model, but that are included in the stoichiometry to maintain a balanced equation (Kettle et al., 2018, 2015)

Maximum growth rate (μ_{max})

Values for μ_{max} on RS and NSP were based on observations of *Prevotella ruminicola* AR29 growing on different substrates (Kovar et al., 1999). It should be noted that data for xylose were used to describe NSP dynamics, which were assumed to approximate growth on hydrolysis products derived from plant polysaccharides. Moreover, μ_{max} on RS and NSP was assumed to have the same value, theorising the same dynamics when growing on complex polysaccharides. Values for μ_{max} for Pr were derived from observations of *P. ruminicola* 23 growing on peptides (J. N. Kim et al., 2017) (Table 1.1).

Yield (Y)

Assumption for yield on NSP and RS and sugars was based on batch cultures of *P. ruminicola* growing on different substrates (Kovar et al., 1999; Marounek and Dušková, 1999). It must be acknowledged that batch culture-based experiments are not representative of gut environment, due to medium physiological changes and resource dynamics. Nevertheless, the values assumed here for carbohydrate-based resources were rather close to theoretical considerations on energy gain (Kettle et al., 2018, 2015; Russell and Cook, 1995) and therefore considered a good approximation of actual gut dynamics. Assumption for yield on Pr was based on theoretical energy gain as found for the same functional group during the previous model of human colonic microbiota (Kettle et al., 2018, 2015).

pH corners

Assumption for pH corners were based on observations of *Bacteroides ruminicola* (basonym) growing at different pH values (Russell and Dombrowski, 1980)

Table 1.1. MFG1 data frame, stoichiometry is reported in brackets for substrates and metabolites, resource type is specified in each case following the model description ([1.2.3.3 Bacterial specific growth rate](#)). *K* value parametrisation process is detailed in [1.2.3.1 Mathematical formulation](#).

	1 st pathway	2 nd pathway	References
Substrates	NSP; S (6), RS; S (6); Sugars (6); H ₂ O (Sw) (2)	Pr; S (6)	(Kettle et al., 2018, 2015; Marounek and Dušková, 1999)
Metabolites	Acetate (4), lactate (2) succinate (4), propionate (1), H ₂ (9), CO ₂ (3)	Acetate (2), propionate (1), succinate (1), H ₂ (2), CO ₂ (1), other (7)	(Kettle et al., 2018, 2015; Marounek and Dušková, 1999)
μ_{max} (d ⁻¹)	6.48 (NSP), 6.48 (RS), 9.6 (Sugars)	5.76 (Pr)	(J. N. Kim et al., 2017; Kovar et al., 1999)
<i>K</i> (g/l)	0.000812 (NSP), 0.000501 (RS), 0.000184 (Sugars)	0.00138 (Pr)	This study
<i>Y</i> (gl ⁻¹ / gl ⁻¹)	0.287 (NSP), 0.271 (RS), 0.3 (Sugars)	0.2 (Pr)	(Kettle et al., 2018, 2015; Kovar et al., 1999)
pH corners	5.25, 6.0, 6.5, 7.0		(Russell and Dombrowski, 1980)

1.2.6.2 Microbial functional group 2

This functional group is composed mainly by species belonging to *Ruminococcus* genus that can ferment mainly starch, but in minor way also NSP to produce principally acetate. It is well recognised how an increased concentration of RS in the diet of pigs can lead to an increase in the relative abundance of *R. bromii* (Tiwari et al., 2019), whose importance as a starch degrader is also recognised within human gut microbiota (Ze et al., 2012). The assumption for stoichiometry was based on *R. bromii* (Moore et al., 1972) continuous monocultures growing on 0.5% starch (Laverde Gomez et al., 2019) (Table 1.2).

Maximum growth rate (μ_{max}) and Yield (Y)

Assumption for μ_{max} and Y was also based on theoretical consideration, as per description of the same group in the human model, i.e. between 2.8 and 4 ATP molecules generated per hexose unit consumed (Kettle et al., 2018, 2015)

pH corners

Assumption for pH limitation was based on observation of *R. bromii* growing in YCFAG medium (Duncan et al., 2009), and considerations taken for the same group for the human model (Kettle et al., 2018, 2015)

Table 1.2. MFG2 data frame, stoichiometry is reported in brackets for substrates and metabolites, resource type is specified in each case following the model description ([1.2.3.3 Bacterial specific growth rate](#)). K value parametrisation process is detailed in [1.2.3.1 Mathematical formulation](#).

	1 st pathway	References
Substrates	NSP; S (2), RS; S (2), H ₂ O; Sw (2)	(Laverde Gomez et al., 2019)
Metabolites	Acetate (2), ethanol (2), formate (2), H ₂ (2), CO ₂ (2)	(Laverde Gomez et al., 2019)
μ_{max} (d ⁻¹)	14.4 (RS); 3.6 (NSP)	(Kettle et al., 2018, 2015)
K (g/l)	0.000517(NSP), 0.000346 (RS)	This study
Y (gl ⁻¹ / gl ⁻¹)	0.333 (RS), 0.286 (NSP)	(Kettle et al., 2018, 2015)
pH corners	5.5, 6.25, 6.71, 8.35	(Duncan et al., 2009; Kettle et al., 2018, 2015)

1.2.6.3 Microbial functional group 3

Members of this functional group are able to use particularly NSP producing mainly acetate and succinate. Some *Ruminococcaceae*, such as *Ruminococcus flavefaciens* produce succinate instead of propionate through succinate pathway (Louis and Flint, 2017). This microorganism is a predominant cellulolytic bacterium in rumen (Helaszek and White, 1991) and is present in lower abundance in pig intestine together with other cellulolytic bacteria such as *Succinivibrio* spp. (O'Herrin and Kenealy, 1993; Tanner et al., 2014; Varel and Yen, 1997). The assumption for stoichiometry was based on observations of *R. flavefaciens* isolated from human colon growing in pure culture (Kettle et al., 2018, 2015; Robert and Bernalier-Donadille, 2003). Substrates RS and sugars were added in the stoichiometry to accommodate other members of this group present in pig intestine apart from *R. flavefaciens*, such as *Succinivibrio* (Bryant, 2007; O'Herrin and Kenealy, 1993) (Table 1.3).

Maximum growth rate (μ_{max})

The assumption for μ_{max} , for NSP was based on observations of *R. flavefaciens* growing (Wachenheim and Hespell, 1985), whereas assumptions for RS and sugars were based on observations for *Succinivibrio dextrinosolvens* growing in chemostat under glucose limiting conditions and from the genus description (Bryant, 2007; O'Herrin and Kenealy, 1993).

Yield (Y)

Assumption for Y for NSP was based on *R. flavefaciens* growing in continuous culture (Shi and Weimer, 1992) at a dilution rate similar to what found in the proximal colon of pigs (see [Chapter 3](#)) (Latymer et al., 1990; Tanner et al., 2014). Energetical considerations were taken into account to base the assumption for RS and sugars on the stoichiometry shown for *S. dextrinosolvens* (O'Herrin and Kenealy, 1993)

pH corners

Assumption for optimal pH was based on observations of *R. flavefaciens* growing in a chemostat on cellobiose (0.614 g/l) (Russell and Dombrowski, 1980)

Table 1.3. MFG3 data frame, stoichiometry is reported in brackets for substrates and metabolites, resource type is specified in each case following the model description ([1.2.3.3 Bacterial specific growth rate](#)). K value parametrisation process is detailed in [1.2.3.1 Mathematical formulation](#).

	1 st pathway	References
Substrates	NSP; S (1), RS; S (1), Sugars; S (1)	(Kettle et al., 2018, 2015; Robert and Bernalier-Donadille, 2003)
Metabolites	Acetate (1), succinate (1), H ₂ (1)	(Kettle et al., 2018, 2015; Robert and Bernalier-Donadille, 2003)
μ_{max} (d ⁻¹)	9.12 (NSP); 2.808 (RS), 3.936 (Sugars)	(Bryant, 2007; Wachenheim and Hespell, 1985)
K (g/l)	0.00001 (NSP), 0.000017(RS); 0.00012 (Sugars)	This study
Y (gl ⁻¹ / gl ⁻¹)	0.189 (NSP); 0.333 (RS); 0.333 (Sugars)	(Bryant, 2007; Kettle et al., 2018, 2015; O'Herrin and Kenealy, 1993; Shi and Weimer, 1992)
pH corners	6.0, 6.25, 6.5, 7.0	(Russell and Dombrowski, 1980)

1.2.6.4 Microbial functional group 4

This microbial functional group is represented by lactate producers, saccharolytic bacteria that produce principally lactate, although different lactic acid bacteria could produce several different metabolites (Feiner, 2006). The most abundant MFG4 members detected in pig faeces are related to *Lactobacillus* spp. and *Streptococcus* spp. (H. B. Kim et al., 2011; Yang et al., 2015). According to the sequencing analysis performed in this study ([Chapter 2](#)) *L. mucosae*, *L. pontis* and *L. reuteri* were the most abundant species in pooled faecal samples of 20-week old pigs, whereas *Streptococcus* genus was unclassified at species level. *L. mucosae* has been previously isolated from pig intestine and has been described as an obligate heterofermentative producing both D- and L-lactate from non-complex saccharides (Roos et al., 2000). On the other hand, *L. pontis* has been described to produce lactate, acetate, ethanol and CO₂ from fermentation of maltose or fructose (Vogel et al., 1994), similar to what described for the obligate heterofermentative *L. reuteri* (Pancheniak et al., 2012), which is found as endogenous in pigs (Hou et al., 2015b).

MFG4 stoichiometry assumption is based on *L. reuteri* growing in a chemostat with 20 mM glucose (Burgé et al., 2015; El-Ziney et al., 1998) with theoretical considerations for acetate production since its production depend on environmental conditions (Årsköld et al., 2008) and cannot be excluded in the gut: $2C_6H_{12}O_6 \rightarrow CO_2 + 3CH_3COOH + C_2H_5OH + C_3H_6O_3$. Amylase activity has been reported in several species of *Lactobacillus*, which are also able to obtain energy by oligosaccharide degradation (Gänzle and Follador, 2012), and possibly NSP (Stewart et al., 2009). Hexose and pentose metabolised through heterofermentative reactions in lactic acid bacteria enter phosphoketolase pathway yielding lactate and ethanol, or lactate and acetate respectively (Gänzle, 2015). It is acknowledged that in some cases, some species (e.g. different *L. reuteri* strains) can convert carbohydrates into lactate through homofermentative pathways using the Embden-Meyerhof pathway (EMP) (Burgé et al., 2015). Nevertheless, MGF4 stoichiometry is based on heterofermentative reactions through phosphoketolase pathway of *L. reuteri*, since it represent its main source of energy (Årsköld et al., 2008), and as stated above, it seems to be the most diffused amongst pig gut species (Pancheniak et al., 2012; Roos et al., 2000; Vogel et al., 1994). NSP and RS are included as substrates in the data frame, to take into consideration of the ability of some species found in pig (e.g. *S. hyointestinalis* and *S. thermophilus*) to use them as a substrate (Chen et al., 2016).

Maximum growth rate (μ_{max})

Assumption for μ_{max} has been based on *L. reuteri* growing in a chemostat at fixed pH of 6.5 and at 37°C (Pancheniak et al., 2012). Although *Lactobacillus* spp. has been described to growth on simple sugars rather than complex carbohydrates, the data frame include NSP and RS as substrate to acknowledge the substrate preferences of some gut species of *Streptococcus* (Chen et al., 2016).

Yield (Y)

Assumption for Y was based on *L. reuteri* growing on simple sugars (2.5% p/v) in a bioreactor for 28 hours at 37°C with a constant pH of 6.5 (Pancheniak et al., 2012).

pH corners

Assumption for pH corners for MFG4 was based on observations of *L. reuteri* growing at different pH values (Klantschitsch et al., 1996; Palmfeldt and Hahn-Hägerdal, 2000).

Table 1.4. MFG4 data frame, stoichiometry is reported in brackets for substrates and metabolites, resource type is specified in each case following the model description ([1.2.3.3 Bacterial specific growth rate](#)). K value parametrisation process is detailed in [1.2.3.1 Mathematical formulation](#).

	1 st pathway	References
Substrates	NSP; S (2), RS; S (2), Sugars; S (2)	(Årsköld et al., 2008; Burgé et al., 2015; Chen et al., 2016; El-Ziney et al., 1998)
Metabolites	Acetate (3); Lactate (1), Ethanol (1), CO ₂ (1)	(Årsköld et al., 2008; Burgé et al., 2015; El-Ziney et al., 1998)
μ_{max} (d ⁻¹)	2.5 (NSP), 2.5 (RS), 7.248 (Sugars)	(Chen et al., 2016; Pancheniak et al., 2012)
K (g/l)	0.000061 (NSP), 0.000038 (RS), 0.000023 (Sugars)	This study
Y (gl ⁻¹ / gl ⁻¹)	0.189(NSP); 0.333 (RS); 0.18 (Sugars)	(Kettle et al., 2018, 2015; Pancheniak et al., 2012)
pH corners	4, 6, 6.8, 7.5	(Klantschitsch et al., 1996; Palmfeldt and Hahn-Hägerdal, 2000)

1.2.6.5 Microbial functional group 5

To this group belong mainly members of *Lachnospiraceae* able to produce butyrate H_2 and CO_2 from both complex and simple carbohydrates, which at same time can use acetate as a semi-essential substrate ([1.2.3.3 Bacterial specific growth rate](#)), i.e. increasing MFG growth rate according to the *Fb* fraction but bacteria could not growth on this substrate alone (Kettle et al., 2015). Members of this group are bacteria related to *Roseburia spp.* and *Eubacterium rectale* which represent a part of butyrate producer bacteria of pig gut microbiota (Li et al., 2016) (Table 1.5).

Maximum growth rate (μ_{max})

μ_{max} assumption was based on the observations reported for *R. inulinivorans* and *E. rectale* growing on starch, inulin and glucose (basal YCFA medium with glucose 5g/l) (Scott et al., 2014)

Yield (Y)

Assumption for Y was based on values reported for the same group for the model of human colonic microbiota (Kettle et al., 2018, 2015).

pH corners

Assumption based on observations of *R. intestinalis* growing on glucose and β -mannans (La Rosa et al., 2019).

Table 1.5. MFG5 data frame, stoichiometry is reported in brackets for substrates and metabolites, resource type is specified in each case following the model description ([1.2.3.3 Bacterial specific growth rate](#)). K value parametrisation process is detailed in [1.2.3.1 Mathematical formulation](#).

	1 st pathway	References
Substrates	NSP; S (2), RS; S (2); Sugars; S (2), Acetate; Sb ($F_b=0.75$) (2)	(Kettle et al., 2018, 2015)
Metabolites	Butyrate (3), H_2 (2), CO_2 (4) H_2O (2)	(Kettle et al., 2018, 2015)
μ_{max} (d ⁻¹)	9.6 (RS), 10.14 (NSP), 9.96 (sugars)	(Scott et al., 2014)
K (g/l)	0.000756 (NSP), 0.000397 (RS), 0.000198 (Sugars)	This study
Y (gl ⁻¹ / gl ⁻¹)	0.286 (NSP); 0.333 (RS); 0.333 (Sugars)	(Kettle et al., 2018, 2015)
pH corners	5.5, 6.2, 6.5, 7	(La Rosa et al., 2019)

1.2.6.6 Microbial functional group 6

Members of this functional group produce mainly butyrate, together with lactate, formate and CO₂, they can use acetate as a semi-essential resource ([1.2.3.3 Bacterial specific growth rate](#)) (Kettle et al., 2015). Stoichiometry assumption is based on bacteria related to *Faecalibacterium prausnitzii* (Duncan et al., 2002b; Kettle et al., 2015). Similarly to humans, *F. prausnitzii* appear to have a wide distribution amongst gut microbes in pigs (Miquel et al., 2014), and seems to be one of the dominant butyrate producers associated with certain phases of pigs life (Massacci et al., 2020). This bacterium produces butyrate via butyryl-CoA:acetate CoA-transferase with net consumption of acetate, whose stoichiometry is shown in Table 1.6 (Duncan et al., 2002b; Louis and Flint, 2009).

Maximum growth rate (μ_{max})

Assumption for μ_{max} on sugars was based on evidence of *F. prausnitzii* strain A2-165 growing on YCFA medium (5g/l glucose) and acetate 33mM. Assumption for growth on NSP and RS were based on observation of *F. prausnitzii* growing on different substrates (Lopez-Siles et al., 2012).

Yield (Y)

Assumption for yield was based on theoretical considerations based on the assumed ATP produced by *F. prausnitzii* during glucose fermentation (Duncan et al., 2002b; Kettle et al., 2015) coupled with the theoretical amount of bacterial cells produced per mol of ATP (Russell and Cook, 1995). Due to high similarity of theoretical values achieved during this study with the ones described previously for the same functional group for the model of the human colonic microbiota, the latter have been used to parametrise this data base (Kettle et al., 2018, 2015).

pH corners

Assumption for pH corners was based on description of *F. prausnitzii* (Foditsch et al., 2014; Lopez-Siles et al., 2017).

Table 1.6. MFG6 data frame, stoichiometry is reported in brackets for substrates and metabolites, resource type is specified in each case following the model description ([1.2.3.3 Bacterial specific growth rate](#)). K value parametrisation process is detailed in [1.2.3.1 Mathematical formulation](#).

	Pathway	References
Substrates	NSP; S (6), RS; S (6), Sugars; S (6), Acetate; Sb (Fb=0.1) (4)	(Duncan et al., 2002b; Kettle et al., 2015)
Metabolites	Butyrate (7), Lactate (2), Formate (6), CO ₂ (4), H ₂ O (4)	(Duncan et al., 2002b; Kettle et al., 2015)
μ_{max} (d ⁻¹)	6.72 (NSP); 0.672 (RS); 7.68 (Sugars)	(Duncan et al., 2002b; Lopez-Siles et al., 2012)
K (g/l)	0.000276 (NSP), 0.000209 (RS), 0.000119 (Sugars)	
Y (g l ⁻¹ / g l ⁻¹)	0.286 (NSP); 0.333 (RS); 0.333 (Sugars)	(Kettle et al., 2018, 2015)
pH corners	4.6, 5.7, 6.7, 7	(Foditsch et al., 2014; Lopez-Siles et al., 2017)

1.2.6.7 Microbial functional group 7

Members of MFG7 are propionate producer microorganisms, such as *Veillonella spp.* or *Megasphaera spp.*. They can ferment both carbohydrates and lactate producing propionate as main metabolite, together with acetate, CO₂ and H₂O (Prins, 1977). According to sequencing data carried out during this project, *M. elsdenii* is the most abundant amongst members of MFG7 in ~20-week old faecal sample pooled from 5 different animals, therefore the assumption for the stoichiometry is based on findings Gottschalk (1979) and Prins (1977), for bacteria related to *Megasphaera* growing on glucose or lactate, as described for the model of the human gut microbiota (Kettle et al., 2015). Lactate is usually metabolised via the *acrylate* pathway by MFG7 bacteria, although it can be converted to propionate either through succinate (e.g. *Veillonella*) or acrylate pathway (e.g. *Megasphaera*) (Louis and Flint, 2017). Moreover, microbial degradation of some amino acids through propionyl-CoA can lead to propionate production in some species (Gonzalez-Garcia et al., 2017). *M. elsdenii* converts lactate to propionate via *acrylate* pathway, using acrylyl coenzyme A (Prabhu et al., 2012), or to butyrate (Bourriaud et al., 2005), requiring acetate for the conversion (Prabhu et al., 2012). It should be noted that propionate/butyrate ratio produced by *M. elsdenii* depends on environmental characteristics such as enantiomeric D/L lactate ratio (Kajihara et al., 2017) or sugar concentration (Prabhu et al., 2012). A mixture of propionate and acetate was reported when the species was growing in the presence of excess lactate (Prabhu et al., 2012) (Table 1.7).

Maximum growth rate (μ_{max})

Assumption for μ_{max} has been based on evidences shown for *M. elsdenii* growing on in chemostat with single (non-limiting) substrates (Theodorou and France, 2005) and on growth on lactate (Russell and Baldwin, 1978). Has been shown that the species weakly degrade starch and NSP (Marounek et al., 1989), although in order to include in the group substrate preferences from different species, values for μ_{max} for those substrate were theorised based on information for bacterial growth due to lack of specific data.

Yield (Y)

Assumption for Y was based on values reported for the same group for the model of human colonic microbiota (Kettle et al., 2018, 2015).

pH corners

Assumption for pH corners has been based on description of *M. elsdenii* (Helene Marchandin et al., 2009) and continuous culture experiment with *M. elsdenii* growing at different pH values (Chen et al., 2019).

Table 1.7. MFG7 data frame, stoichiometry is reported in brackets for substrates and metabolites, resource type is specified in each case following the model description ([1.2.3.3 Bacterial specific growth rate](#)). K value parametrisation process is detailed in [1.2.3.1 Mathematical formulation](#).

	1 st pathway	2 nd pathway	References
Substrates	NSP; S (3), RS; S (3), Sugars; S (3)	Lactate; Se (3)	(Kettle et al., 2015; Seeliger et al., 2002)
Metabolites	Propionate (4), acetate (2), CO ₂ (2), H ₂ O (2)	Propionate (2), acetate (1), CO ₂ (1), H ₂ O (1)	(Kettle et al., 2015; Seeliger et al., 2002)
μ_{max} (d ⁻¹)	12 (Sugars); 7 (RS); 7 (NSP)	5.04	(Russell and Baldwin, 1978; Theodorou and France, 2005)
K (g/l)	0.000495 (NSP), 0.000324 (RS), 0.000400 (Sugars)	0.00081	This study
Y (g l ⁻¹ / g l ⁻¹)	0.286 (NSP); 0.333 (RS); 0.333 (Sugars)	0.111	(Kettle et al., 2018, 2015).
pH corners	4.6, 5.5, 6.05, 7.8		(Helene Marchandin et al., 2009)

1.2.6.8 Microbial functional group 8

The third group of butyrate producers include but is not limited to bacteria such as *Clostridiales* related to *E. hallii* that can use either carbon based substrates or lactate to produce mainly butyrate (Kettle et al., 2015). The data frame has two pathways (Table 1.8), stoichiometry assumption for hexoses fermentation was based on organisms related to *E. hallii*. On the other hand, a different assumption was made for lactate fermentation. To acknowledge both *M. elsdenii* prevalence compared to *E. hallii* and the former's possible contribution to butyrate production in pigs, the second pathway of this functional group was based on butyrate kinase pathway of *M. elsdenii* and the stoichiometry assumption was based on the organism growth on lactate in continuous-culture (Chen et al., 2019). The choice to represent this group with pathways representative of taxonomically unrelated bacteria underlines both their enzymatic similarities and the trait of all the MFG described to be composed by taxonomically diverse species.

Maximum growth rate (μ_{max})

Assumption for μ_{max} on lactate was based on observations for *M. elsdenii* (Russell and Baldwin, 1978), whereas NSP μ_{max} was based on *E. hallii* growing on high performance inulin (Scott et al., 2014). RS is usually not fermented by *E. hallii* therefore observations for *M. elsdenii* were taken into consideration instead (Russell and Baldwin, 1978; Theodorou and France, 2005) as described for MFG7.

Yield (Y)

Assumption for Y was based on values reported for the same group for the model of human colonic microbiota (Kettle et al., 2018, 2015).

pH corners

Optimal pH assumption was based on observation for *Anaerobutyricum hallii* (*E. hallii* basonym) and species description (Holdeman and Moore, 1974; Shetty et al., 2018)

Table 1.8. MFG8 data frame, stoichiometry is reported in brackets for substrates and metabolites, resource type is specified in each case following the model description ([1.2.3.3 Bacterial specific growth rate](#)). K value parametrisation process is detailed in [1.2.3.1 Mathematical formulation](#).

	1 st pathway	2 nd pathway	References
Substrates	NSP; S (10), RS; S (10), Sugars; S (10) H ₂ O Sw (2)	Lactate; Se (5)	(Chen et al., 2019; Kettle et al., 2018, 2015)
Metabolites	Acetate (2), Butyrate (9), Formate (12), H ₂ (10), CO ₂ (8)	Butyrate (1), Acetate (5) CO ₂ (1), H ₂ O (1)	(Chen et al., 2019; Kettle et al., 2018, 2015)
μ_{max} (d ⁻¹)	11.04 (NSP); 12(Sugars); 7 (RS)	5.04 (lactate)	(Russell and Baldwin, 1978; Scott et al., 2014; Theodorou and France, 2005)
K (g/l)	0.001056 (NSP), 0.000311 (RS), 0.000390 (Sugars)	0.00081	This study
Y (g l ⁻¹ / g l ⁻¹)	0.286 (NSP); 0.333 (RS); 0.333 (Sugars)	0.111 (lactate)	(Kettle et al., 2018, 2015)
pH corners	5.5, 6.0, 7.5, 8.0		(Holdeman and Moore, 1974; Shetty et al., 2018)

1.2.6.9 Microbial functional group 9

MFG9 group is a phylogenetically diverse group composed of strictly anaerobic acetogenic bacteria, able to use acetyl-CoA (Wood-Ljungdahl) pathway as a terminal electron-accepting, energy-conserving, CO₂-fixing process (Drake et al., 2008). The group data frame (Table 1.9) described three pathways, indeed acetogenic bacteria can produce 3 molecules of acetate from 1 molecule of glucose (Louis et al., 2014), they able to covert, H₂ and CO₂ into acetate (Müller, 2003) or degrade formate according to the equation: $4\text{CH}_2\text{O}_2 \rightarrow \text{CH}_3\text{COOH} + 4\text{H}_2 + 2\text{CO}_2$ (Kamlage et al., 1997). According to the 16S rRNA gene sequencing analysis performed during this study species *Blautia* spp. and *Terrisporobacter* spp. represented the majority of acetogens present in faeces of ~20-week old pigs when pooling together a faecal sample from 5 different animals ([Chapter 2](#)).

Maximum growth rate ($\mu_{\max}$)

The assumption for $\mu_{\max}$ on sugars was based on acetogens growing on DSMZ₁₀₄ medium, which contains glucose 5g/l (Groher and Weuster-Botz, 2016). Assumption for growth on CO₂ and H₂ is based on acetogens growing on medium specifically studied for acetogens (Groher and Weuster-Botz, 2016). The growth rate for complex polysaccharides was assumed to be equal to the average of the two values found, as theoretically cells would proliferate better on simple sugars (Kettle et al., 2015).

Yield (Y)

Yield assumption for growth on glucose and growth on CO₂ + H₂ was based on theoretical considerations on energy gain. Formation of acetate from hexose is coupled with the formation of 4 molecules of ATP (Müller, 2003), whereas Wood- Ljungdahl pathway allows the synthesis of 1 to 2 mol of ATP" (Müller, 2003). Theoretical yield from molecule of ATP assumed to be 10.5 grams of cells/mol of ATP (Russell and Cook, 1995).

pH corners

Assumption for pH corners was based on description of *Blautia hydrogenotrophica* (Liu et al., 2008) and the basonym *Ruminococcus hydrogenotrophicus* (Bernalier et al., 1996)

Table 1.9. MFG9 data frame, stoichiometry is reported in brackets for substrates and metabolites, resource type is specified in each case following the model description ([1.2.3.3 Bacterial specific growth rate](#)). *K* value parametrisation process is detailed in [1.2.3.1 Mathematical formulation](#).

	1 st pathway	2 nd pathway	3 rd pathway	References
Substrates	NSP; S (1), RS; S (1), Sugars; S (1)	CO ₂ ; Se (2), H ₂ ; Se (4)	Formate; Se (2), NSP; S (1), RS; S (1), sugars; S (1)	(Drake et al., 2008; Kamlage et al., 1997; Kettle et al., 2018, 2015; Louis et al., 2014; Müller, 2003)
Metabolites	Acetate (3)	Acetate (1), H ₂ O (2)	Acetate (3), H ₂ (2), CO ₂ (2)	
μ_{max} (d ⁻¹)	4.00(NSP and RS) 5.00 (Sugars)	3.00 (CO ₂)	4.00 (NSP and RS) 5.00 (Sugars)	(Groher and Weuster-Botz, 2016)
<i>K</i> (g/l)	0.000057 (NSP), 0.000035 (RS), 0.000070 (Sugars)	0.00001	0.000057 (NSP), 0.000035 (RS), 0.000070 (Sugars)	This study
<i>Y</i> (g l ⁻¹ / g l ⁻¹)	0.200 (NSP and RS), 0.233 (Sugars)	0.179 (CO ₂)	0.200 (NSP and RS), 0.233 (Sugars)	(Müller, 2003; Russell and Cook, 1995)
pH corners		6, 6.5, 6.6, 7		(Bernalier et al., 1996; Liu et al., 2008)

1.2.6.10 Microbial functional group 10

This group encompasses methanogenic archaea, which represent a small percentage of the pig gut microbial species (Mao et al., 2011) and can degrade H_2 and CO_2 or formate to produce mainly CH_4 (Oliphant and Allen-Vercoe, 2019). The majority of MFG10 members can produce CH_4 and $2H_2O$ from CO_2 using $4H_2$ as electron donor, whereas hydrogenotrophic MFG10 species can oxidise 4 molecules of formate to $4CO_2$ via formate dehydrogenase, with one CO_2 further converted in CH_4 (final stoichiometry: $4 \text{ formate} \rightarrow 3CO_2 + CH_4 + 2H_2O$) (Liu and Whitman, 2008). These two pathways were used to derive the stoichiometry used in the data frame shown in Table 1.10 to summarise MFG10 metabolic activity.

According to 16S rRNA gene sequencing data performed during this project ([Chapter 2](#)), the most common genera amongst the methanogens found in ~5-month-old male pigs' faeces are: *Methanobrevibacter*, *Methylophilaceae*, *Methanosphaera*, therefore the assumption for the parameters in this data frame has been based on *Methanobrevibacter smithii*.

Maximum growth rate (μ_{max})

In a batch culture experiment, Archer et al., found that *M. smithii* had μ_{max} of $2.28 \text{ (d}^{-1}\text{)}$ when growing at 37°C on $H_2 + CO_2$ (Table 1.10). Growth on formate on pathway 2 in Table 1.10 has been assumed the same as the one on H_2 , based on observations from another study, in which no differences in the growth rate of *M. maripaludis* were found using either $H_2 + CO_2$ or formate (Long et al., 2017).

Yield (Y)

Theoretical consideration for different substrate were taken into consideration, as per the previous model for the human gut microbiota (Kettle et al., 2018, 2015).

pH corners

Optimal pH values assumed from *Methanobrevibacter spp.* were consider to base the assumption of pH corners (Taubner et al., 2015)

Table 1.10. MFG10 data frame, stoichiometry is reported in brackets for substrates and metabolites, resource type is specified in each case following the model description ([1.2.3.3 Bacterial specific growth rate](#)). *K* value parametrisation process is detailed in [1.2.3.1 Mathematical formulation](#).

1 st pathway		2 nd pathway	References
Substrates	H ₂ ; Se (4), CO ₂ ; Se (1)	Formate; Se (4)	(Kettle et al., 2018, 2015; Liu and Whitman, 2008)
Metabolites	CH ₄ (1), H ₂ O (2)	CO ₂ (3), CH ₄ (1), H ₂ O (2)	(Kettle et al., 2018, 2015; Liu and Whitman, 2008)
μ_{max} (d ⁻¹)	4.07	4.07	(Archer and Powell, 1985)
<i>K</i> (g/l)	0.00021 (H ₂), 0.00021 (CO ₂)	0.00021 (Formate)	This study
<i>Y</i> (g l ⁻¹ / g l ⁻¹)	0.03	0.00724	(Kettle et al., 2018, 2015)
pH corners	5.5, 7.0, 8.0, 10.0		(Taubner et al., 2015)

1.3 Simulating porcine colonic microbiota under continuous fermentation conditions.

The model described up to this point was used to simulate the growth of a bacterial community representative of porcine colonic microbiota allowing evaluation of metabolite production and microbial dynamics *in silico*. Two different sets of simulations were performed, the first one based on observations found in literature describing a faecal inoculum growing in a continuous fermentation experiment (Tanner et al., 2014). Faecal bacterial (i.e. genus level) and medium composition as described in such study were used to inform model MFG and substrate starting conditions respectively. Therefore, model forecasts were compared with observations from the study, especially in terms of short chain fatty acid (SCFA) and microbial dynamics. Nevertheless, considering the low resolution of sequencing data found in the article, a further data set at genus level (H. B. Kim et al., 2011) was used to derive MFG starting conditions. These settings were used to simulate growth of such community in a chemostat and SCFA outputs were compared with *in vivo* expectations for the same variables.

Finally, using the latter dataset (H. B. Kim et al., 2011), strain number within each MFG was increased from 1 as described so far, to 5. As detailed below, this was done in MicroPop varying parameter values according to a range appropriate for each MFG. Therefore, when the simulation started, 5 strains for each MFG were generated (50 in total), with all of them having slightly different parameter values. This approach allowed two different types of analysis; first community dynamics were assessed for an increased number of simulated microbes having somewhat different traits between each other. This is similar to what found *in vivo*, where theoretically, each MFG is composed by a number of different species, which although share the same pathways have slightly different growth characteristics. On the other hand, frequency distribution analysis of the main SCFAs was assessed. Acknowledging uncertainty that accompanies parameter values chosen due to lack of specific cultural data, this type of analysis allowed to assess how variations in parameter values affected modelled final output. The following sections describe experimental conditions found in literature and how those were simulated *in silico*, together with the methodology behind the settings input into the model. Following, results of comparisons between *in silico* and *in vivo* scenarios are described, accompanied by details on SCFA frequency distribution when changing parameter values for 5 strain per MFG (instead of 1).

1.3.1 Materials and methods

1.3.1.1 1st simulation: continuous culture system (Tanner et al., 2014)

The authors described a novel *in vitro* continuous fermentation system, in which a bead-based inoculum derived from faecal sample (5-month old sow) was grown for 54 days in a medium simulating ileal chyme. Flow-out was maintained constant at 26 ml/h in the paper and considering working volume of 234 ml for the main vessel in the manuscript, dilution rate was modelled as 2.67 d^{-1} (1-3).

Substrate and MFG starting concentrations are depicted in Table 1.11. Substrate starting and continuous flow-in concentrations were based on medium composition described in the paper. MFG values were calculated from genus relative abundance observed in the faecal samples. In particular, as shown in Table 1.12 *Prevotella* was considered as part of MFG1 (Marounek and Dušková, 1999), while *Ruminococcus* relative abundance was divided between MFG2 and MFG3, due to lack of information at species level. *Lactobacillus* and *Turicibacter* were both considered as part of MFG4. *Clostridium* genus is composed by a multitude of species, and many of them are found in gut of healthy pigs (Rainey et al., 2009). Some of the species, such as *C. butyricum* are butyrate producers, which can also ferment lactate in presence of acetate to butyrate (Detman et al., 2019), and therefore assigned to MFG8 (De Vos et al., 2009a). *Roseburia* was assumed to be part of MFG5 while *Blautia* was considered to be part of the MFG9. A total of 54.37% of total sequences were unclassified. Thus, as an assumption, this amount was divided equally amongst the MFGs, including MFG7. This was not done for methanogens (MFG10) since their concentration was not contemplated in sequencing study. It is acknowledged here that assigning unidentified sequences to all the MFGs does not necessarily reflect the real distribution that would have been observed if MFG assignment were based on more comprehensive data at species level. Nevertheless, the aim of this specific part of the chapter is to evaluate model behaviour in terms of metabolite and microbial dynamics. Therefore, it was considered that limiting substrates have more effect on the steady state values than the starting MFG conditions, thus allowing analysis of model dynamics even acknowledging that most likely real starting microbial conditions in the paper were somewhat different than what assumed in this model.

Table 1.11. Starting conditions modelled used to simulate the continuous fermentation experiment as described in the text. Starting inoculum concentration was 60g/l in the manuscript, thus MFG concentration was derived by relative abundance of genera in faecal sample applied to inoculum concentration.

MFG	Starting concentration (g/l)	Resource	Starting concentration (g/l)
1	5.46	NSP	7
2	3.76	RS	4.32
3	3.93	Sugars	2
4	13.42	Protein	15
5	3.71		
6	3.62	pH (constant value)	
7	3.62	6.00	
8	11.63	Dilution rate	
9	10.84	2.67d ⁻¹	
10	0.00		

Table 1.12. Genus relative abundance described in the paper and MFG assignment

Genus	Relative abundance (%)	MFG
<i>Prevotella</i>	3.06	1
<i>Succinivibrio</i>	0.29	3
<i>Lactobacillus</i>	12.39	4
<i>Turicibacter</i>	3.94	4
<i>Oscillibacter</i>	1.02	8
<i>Roseburia</i>	0.15	5
<i>Blautia</i>	0.29	9
<i>Ruminococcus</i>	0.44	2 and 3
<i>Clostridium</i>	24.05	8 and 9
<i>Unassigned</i>	54.37	
Tot.	100.00	

1.3.1.2 2nd simulation: simulation of faecal microbial population (H. B. Kim et al., 2011) growing in a chemostat

During the second simulation, the model was used to simulate the growth of a porcine faecal inoculum under continuous culture conditions for 54 days at constant temperature and pH. Due to the lack of resolution of sequencing data at genus level associated with the 1st simulation, a further data set was used (H. B. Kim et al., 2011) to derive starting MFG concentration of the modelled faecal inoculum. In their manuscript, the authors described faecal microbiota composition from animals housed in two different farms at different ages, producing an extensive dataset with microbial information at genus level. Average data for 22-week old pigs from both farms were used for the comparison in this chapter, due to similar age of pigs from both the first simulation described above and the *in vitro* experimentation carried out during this PhD project.

Kim et al, (2011) found 73 different genera when analysing V3 hypervariable region of the 16S rRNA gene through a Roche 454 GS-FLX sequencer (454 Life Sciences, CT, USA). Therefore, bacterial genera were assigned to different MFGs according to their substrate preferences and metabolic pathways, as described in the sections above. MFG relative abundance derived in this way was used to inform MFG starting concentration in the model, whereas substrate initial concentration and flow-in concentration were the same as the ones described for the first simulation due to their derivation from soybean based feed for pigs (Tanner et al., 2014). The simulation was carried out for a period of 54 days, thus SCFA final concentrations were compared with *in vivo* expectations (Tanner et al., 2014). The extensive list generated for the alignment of microbial genera to MFGs is not shown here for sake of brevity, although MFG starting conditions are summarised in Table 1.13, together with substrate concentration, pH and dilution rate set in the model. It must be noted that Table 1.13 also depicts the abundance of sequences that were either not classified through bioinformatic analysis following 16S rRNA gene sequencing in the manuscript or unassigned considering that MFG in the porcine model at this stage are not inclusive of all the microbial species found in pig gut ([1.3.3 Discussion](#)), such as for example lipolytic bacteria like *Anaerovibrio spp* (Strömpl and Jarvis, 2009).

Table 1.13. Starting conditions characterising the 2nd simulation, which modelled a faecal inoculum, whose MFG abundance was derived from a literature dataset (H. B. Kim et al., 2011), was grown in under continuous culture conditions as described in the text.

MFG	Starting concentration (%)	Resource	Starting concentration (g/l)
1	4.07	NSP	7
2	7.62	RS	4.32
3	2.12	Sugars	2
4	9.79	Protein	15
5	4.31		
6	6.71	pH (constant value)	
7	0.33		6.00
8	28.13	Dilution rate	
9	2.70		2.67d ⁻¹
10	0.00		
Unassigned/unclassified	34.23		

1.3.1.3 Simulation of multiple strains for each MFG

MicroPop enables to increase number of strains per MFG and to generate stochastic parameter values for each of them. This was used here to analyse variations of model outputs when changing parameter values in a range compatible with each MFG, and to observe dynamics of a more complex community representative of *in vivo* conditions. Practically, stochastic values for μ_{max} , K , Y and pH trait were generated for 5 strain per MFG (50 in total) at the beginning of each simulation for a total of 100 simulations. This was done by setting input “numStrains” and “percentTraitRange” to the “strainOptions” argument in the “microPopModel” function to 5 and 20 respectively (Figure 1.2). The effect of these new settings was an increase in strains number to 5 and the generation of parameter values in a $\pm 20\%$ range from their original average value. Stochastic variation at the beginning of each single simulation was allowed via the argument “strainOptions\$randomParams” in the “microPopModel” function, setting seed value through “strainOptions\$randomParams\$Seed” (Figure 1.2). Finally, acetate, propionate, butyrate and lactate frequency distribution at steady state were analysed.

```
startRun=1
numRuns=100
numHours=54
timeInt=1

out<-microPopModel(

  microbeNames = c('MFG1','MFG2','MFG3','MFG4','MFG5',
                   'MFG6','MFG7','MFG8','MFG9','MFG10'),

  times=seq(0,numHours,timeInt),
  resourceSysInfo = data.frame(PigCompleteRmat,stringsAsFactors = FALSE),
  microbeSysInfo = data.frame(PiCompleteMmat,stringsAsFactors = FALSE),
  numStrains=5,
  pHLimit=TRUE,
  pHVal=6,

  strainOptions = list(randomParams=c('halfSat','maxGrowthRate','yield','pHtrait'),
                      seed=run,
                      distribution='uniform',
                      percentTraitRange=20,
                      tradeOffParams=c('maxGrowthRate','halfSat'),applyTradeOffs=TRUE),
  checkingOptions = list(reBalanceStoichiom=TRUE),
  plotOptions = list(plotFig=FALSE,sumOverStrains=FALSE,
                    ylabel='Concentration (g/L)',xlabel='Time (h)')
)
```

Figure 1.2. Programming code used to generate 100 simulations varying the number of strains to 5 and all the parameter values $\pm 20\%$ of their usual average values. The R code shows the function “microPopModel” of the package microPop with the several editable arguments; “strainOptions” was used to generate the parameter values at the beginning of each run associating the seed to the run value.

1.3.2 Results

1.3.2.1 1st simulation: continuous culture system (Tanner et al., 2014)

Acetate:butyrate:propionate ratio predicted by the model at day 54 of the simulation was 64:13:23: (Figure 1.3), rather similar to the one observed *in vitro* of 61:12:27 as described by the authors at the end of the fermentation study. SCFA data in the paper were characterised by several fluctuations and a “pseudo” steady state seemed to be reached at day 54. After a rapid increase in acetate concentration ~90mM at day 11, its values remained somewhat stable *in vitro* until the end of the experiment. Propionate and butyrate followed a similar trend *in vitro* with values stabilised at ~40mM and 20mM respectively. The model able to depict the same acetate pick in the first week of simulation, followed by a decrease towards steady state, with its value at day 54 being 71.88mM. Very interestingly, also propionate and butyrate followed the same trends as the ones observed *in vitro* by the authors of the manuscript, although propionate had in general lower values *in silico*, with steady state values of 26.52mM and 14.82mM for propionate and butyrate respectively.

MFG *in silico* (Figure 1.4) followed a similar evolution to the microbial populations observed in the paper, although a comparison was not always easy to accomplish due to the low clarity of sequencing data at genus level in the paper. The model predicted an evolution of microbial species according to which MFG1 became the most dominant one at day 54 (75%), which was also observed *in vitro* with *Prevotella spp.* becoming the most abundant by the end of the experiment. MFG2 and MFG3 had very different evolutions *in silico*, with the former increasing up to 8.60% at day 54 and the latter tending to 0% at steady state. *Turicibacter spp.* and *Lactobacillus spp.* were decreasing *in vitro* throughout the fermentation, confirmed by the model, which forecasted an increase in MFG4 relative abundance at day 4, followed by low value a steady state (0.13%). The main differences between the model and the paper were about MFG5, MFG6 and MFG8 (butyrate producers), whose relative abundance decreased *in vitro*, and although the model predicted a decrease in their relative abundance they were still present at day 54. *Blautia* concentration tended to increase *in vitro* while MFG9 concentration passed from 18.07 % at day 0 to 0% at day 54. It must be noted that Figure 1.4 depicts 100% total relative abundance, which means that it does not include the wide amount of unclassified species as described above. This method of representing MFG data was chosen due to more clarity of graphs, although it must be acknowledged that the model population is not representative of the whole due to lack of data.

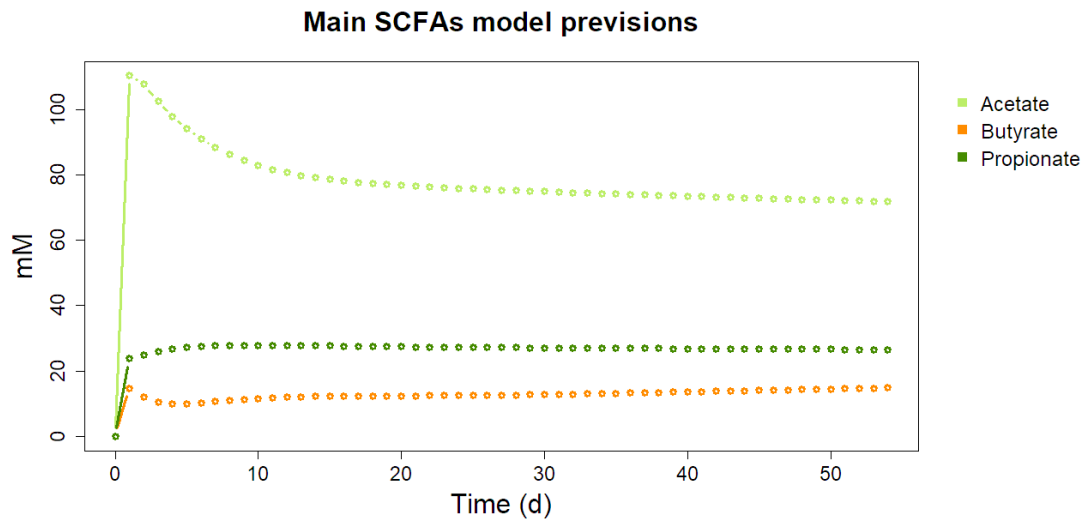


Figure 1.3. Concentration of acetate, propionate and butyrate predicted by the WM model simulating experimental conditions described by Tanner et al., 2014, where a faecal inoculum was grown for 54 days in a continuous fermentation experiment. Concentrations of acetate, propionate and butyrate at day 54 71.88mM, 26.52mM and 14.81mM, respectively.

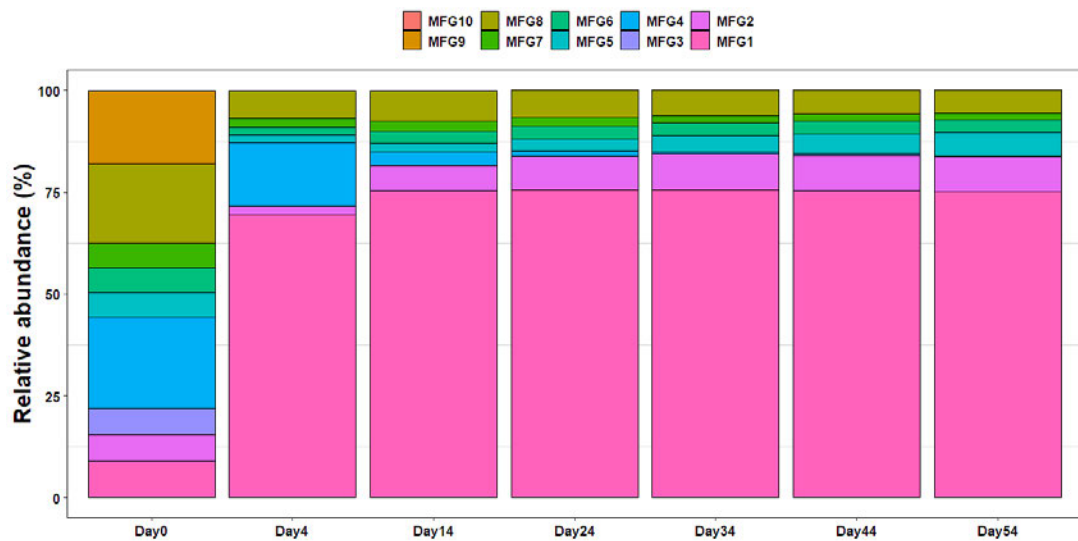


Figure 1.4. MFG dynamics predicted by the model simulating the experimental conditions described by Tanner et al., 2014 where a faecal inoculum was grow in a continuous fermentation experiment for 54 days.

1.3.2.2 2nd simulation: simulation of faecal microbial population (H. B. Kim et al., 2011) growing in a chemostat

A total of 73 genera (and 34.10% unclassified genera) were found in faecal samples of 22-week old pigs housed in two different farms, and as described in the [General introduction](#), the authors found that although *Prevotella spp.* was the most abundant genus in younger pigs (~25% at week 10) its abundance decreased towards week 22 reaching ~4%. *Anaerobacter*, on the other hand, was the most abundant genus (~24%) on the 22nd week, followed by *Sporacetigenium* (~7%), *Coprococcus* (~4.5%), *Lactobacillus* (~4%). Butyrate producers in the faecal sample (MFG5, MFG6 and MFG8) represented the most abundant groups in the faecal samples, followed by lactate producers (MFG4), MFG2 and MFG1. Interestingly, the model seemed to predict an initial increase in butyrate followed by stable values ~16mM around day 54. Interestingly, at the end of the simulation, acetate:propionate:butyrate was 65:21:15, which is extremely similar to 60:25:15 and 63:25:12 for *in vivo* caecal and faecal observation (Tanner et al., 2014) (Figure 1.5).

Observing the predicted microbial dynamics (Figure 1.6), the model predicted an increase in MFG1 which was the dominant group at day 54, followed by MFG2, MFG5, MFG8 and MFG6. MFG4 and MFG8 relative abundance was predicted decrease towards 0 throughout the simulation, although a comparison in this case is not possible due to the lack of data.

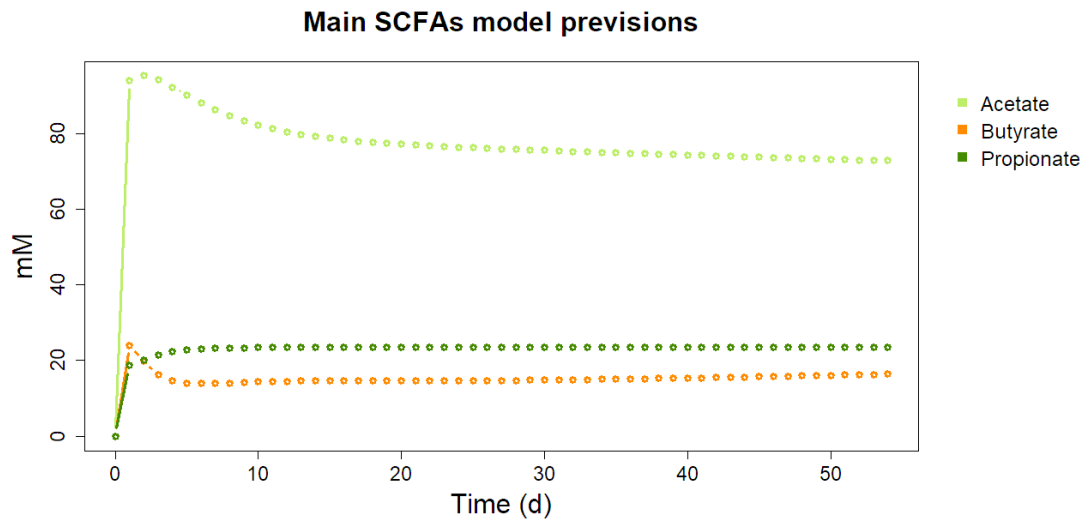


Figure 1.5. Concentration of acetate, propionate and butyrate predicted by the porcine model simulating growth of faecal inoculum under continuous culture conditions. Concentrations of acetate, propionate and butyrate at day 54 were 72.77mM, 23.49mM and 16.37mM, respectively.

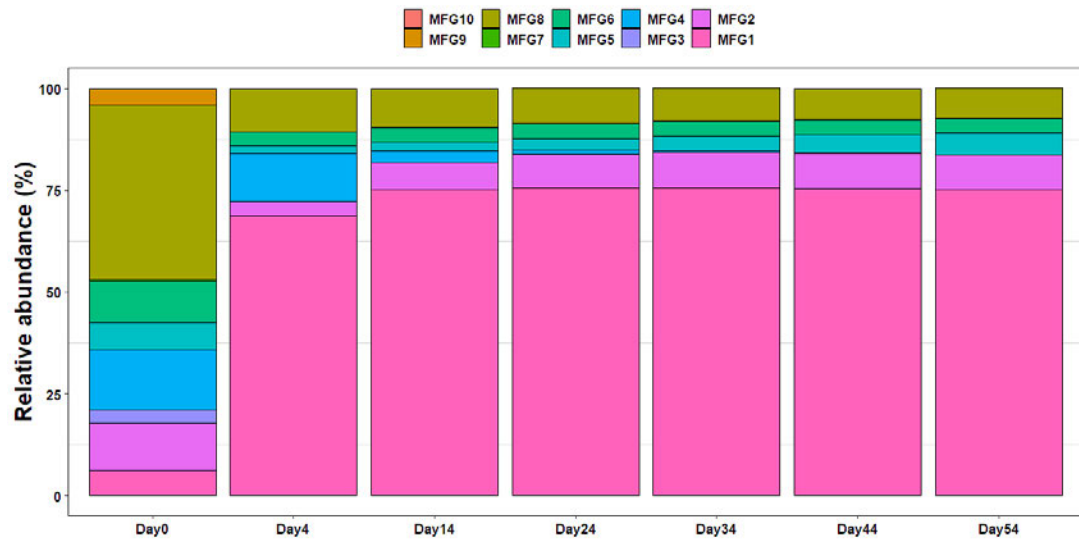


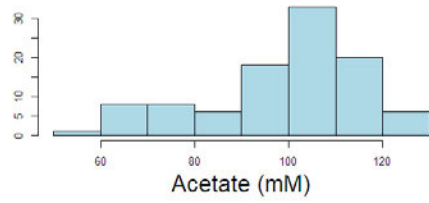
Figure 1.6. MFG dynamics predicted by the model simulating growth of faecal inoculum under continuous culture conditions for 54 days.

1.3.2.3 Simulation of multiple strains for each MFG

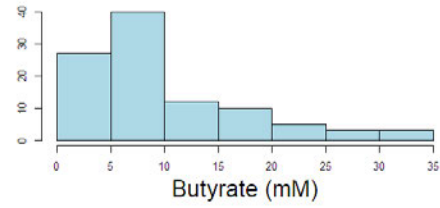
In vivo, MFGs are theoretically composed by several species, which albeit share metabolic pathways are different in terms of growth traits (i.e. parameter values). The model used to generate the 2nd simulation described above (H. B. Kim et al., 2011) was used here to generate 5 strain per MFG (50 in total), each of them having slightly different parameters in a range compatible with every MFG. Therefore, this model with an increased strain number was run for 100 times and at the beginning of each simulation, stochastic values for μ_{max} , K , Y and pH trait were generated for each strain. This analysis allowed to observe dynamics of a more complex community, representative of *in vivo* conditions, and to assess eventual variations in model outputs when changing parameter values in a range compatible with each MFG. A frequency distribution analysis of acetate, propionate, butyrate and lactate steady-state values from the 100 simulations was performed, indicating how these model outputs fluctuated when changing model parameters, especially considering the uncertainty that accompanies some of them.

As shown in Figure 1.7 this modelling approach had a different effects on the four variables. Acetate was the most impacted metabolite, indeed mean of observations was higher than 74mM, as observed *in silico* at day 54 before. Most of the observations were found to be in the range between ~70mM and 110mM, although the wide distribution of values amongst the 100 simulations was characterised by a standard deviation of 17. Propionate and butyrate steady-state values did not seem to be greatly affected by changes in parameter values for 5 strains per MFG. In fact, most of their observations were found in a range compatible with the previous model output and most importantly with *in vivo* expectations (Tanner et al., 2014). Lactate was the molecule with the apparent least level of variation upon the stochastic fluctuation, although it must be noted that whilst it seemed to follow a somewhat normal distribution, all the steady-state values modelled were rather close to 0mM.

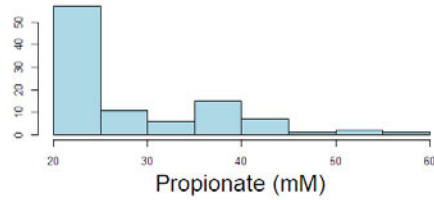
mean = 99.2 sd = 17 min. = 55.4 max. = 127.3



mean = 9.4 sd = 7.8 min. = 0 max. = 32.2



mean = 28.2 sd = 8.6 min. = 20.3 max. = 58.3



mean = 0 sd = 0 min. = 0 max. = 0

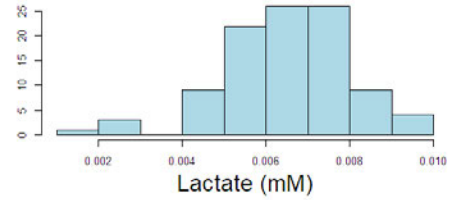


Figure 1.7. Frequency distribution analysis of the main SCFAs and lactate predicted by the model simulating a faecal inoculum (H. B. Kim et al., 2011) growing under continuous-culture conditions. The histograms collect values from 100 simulations , each of them having stochastically generated values for μ_{max} K , Y and pH trait $\pm 20\%$ of their mean value for 5 strains per MFG.

1.4 Discussion

Pig gastrointestinal microbiota is largely composed by prokaryotes, whose metabolic dynamics and succession greatly affects host health and well-being at different levels, hence several papers focus their attention, in elucidating microbial-host interactions through probiotic and/or prebiotic interventions (Angelakis, 2017; Haenen et al., 2013). This chapter presented the steps in the development of the first dynamic model of the porcine colon microbiota, which was used here to simulate two different continuous-culture experiments. Following the structure of a previous human model (Kettle et al., 2015), microbial community was summarised in 10 MFGs in the porcine model, whose stoichiometry and parameter assumptions were largely based on culture-based experiment for predominant species in pigs. Nevertheless, due to the lack of data, a certain amount of uncertainty accompanied the several dynamic parameters, especially K , which was derived empirically here based on model comparison with *in vitro* SCFA observations. Therefore, further model validation through *in vitro* experimentation and parameter fitting will be detailed in [Chapter 2](#) and [Chapter 3](#). Although comprehensive of most gut species, MFGs in the porcine model did not accommodate the totality of microbes that can be found in the porcine colon. Lipolytic or asaccharolytic genera like *Anaerovibrio* (Strömpl and Jarvis, 2009) or *Adlercreutzia* (Maruo et al., 2008), or H_2 depletion by sulphate-reducing bacteria (Rey et al., 2010) were not considered in the groups. To limit the amount of computing power required by the model, substrates were summarised in the four categories of RS, NSP, protein and sugars, which did not differentiate between different composing polysaccharides. Moreover, resources such as mucins and endogenous secretions in general, saccharides derived from bacterial degradation of complex polysaccharides, non-digestible oligosaccharides, or lipids were not included in the model. It was already stated for the human model (Kettle et al., 2015) that, modelling complex categories within the already modelled substrates would represent a further refinement, although it would require an expansion of MFGs and therefore computing power demand.

Two different simulations were performed at this stage, the model was first used to simulate an *in vitro* continuous fermentation found in literature mimicking the porcine physiological colonic conditions (e.g. dilution rate, temperature, pH and anaerobiosis), and then a wider dataset at genus level was used to simulate the composition of a faecal inoculum growing in a chemostat. The model was rather successful in predicting very well SCFA dynamics, which had both same evolution as observed *in vitro* and ratio as observed both *in vitro* and *in vivo* in porcine colon and faeces (Tanner et al., 2014). As observed *in vitro* (Tanner et al., 2014) new microbial dynamics characterised chemostat experiments starting from the faecal inoculum, possibly due to most microbes inability of growing *in vitro*. This was represented very well by the model, in which MFG1 became the most abundant group as it was observed for *Prevotella* *in vitro*. *Lactobacillus* was observed to decrease towards 0 at the end of the experiment, which was represented very well by the model in regard to MFG4 dynamics. Butyrate producers *in*

vitro also had a good correlation with MFG5, MFG6 and MFG8 *in silico*. Nevertheless, due to lack of resolution of sequencing data in the manuscript used for the 1st simulation, a further dataset describing 73 genera in faecal samples from 22-week old pigs (H. B. Kim et al., 2011) was used to inform starting conditions of a faecal inoculum simulated in a second continuous culture. In this case, although microbial dynamics were confirmed to appear the same as the ones observed during the first simulation, lack of cultural data made a comparison impossible, hence the need of compare (and fit) the model with own *in vitro* experimentation, as detailed in the following chapters.

The model was then used to increase strain number to 5 per MFG and attribute stochastically different parameter values to each of them. Moreover, this new model setup was used in 100 simulations, allowing the determination of the weight of those changes on the modelled SCFAs. Interestingly, the only metabolite that seemed to be majorly affected was acetate, whose values ranged from 60mM to 130mM over the 100 simulations. Nevertheless, both propionate and butyrate seemed to remain rather stable around values recorded during the two simulations. The fluctuations of the major SCFA concentrations suggested that the parameters chosen influenced the modelled variables. Therefore, although the model was able to simulate the results from the literature fermentation study and *in vivo* expectations, a new parametrisation through model fitting was carried out through comparison with *in vitro* continuous fermentation study ([Chapter 3](#)).

2 In vitro experimentation

2.1 Introduction

Study of microbiota, such as compositional or microbial interaction analyses can be carried out applying both cultural and non-cultural methods (Hiergeist et al., 2015). Cultural-based techniques allow exploration of microbial dynamics and interactions, e.g. substrate consumption, metabolite production and drug kinetics (Sommer, 2015). Amongst those techniques, batch and continuous systems can be used, offering different levels of complexity largely dependent on research needs (Costello et al., 2017). Examples range from growth of one or more microbial species in a relatively simple environment, to elaborate systems enabling simulation of interactions between prokaryotic and eukaryotic cells as observed *in vivo* (Costello et al., 2017; Lin et al., 2011; Liu et al., 2018; Walker et al., 2005; Zhou et al., 2018). In most cases, the intent of *in vitro* continuous systems is to mimic the gastrointestinal environmental conditions through the modulation of medium composition, pH, temperature and retention time (Difilippo et al., 2016).

On the other hand, molecular approaches are based on the analysis of specific genes allowing microbial compositional and/or functional investigation whilst bypassing cultural-based approaches (Goodrich et al., 2014). Example of such technical approaches could be microbial quantification through quantitative polymerase chain reaction (qPCR) or high throughput next generation sequencing (Chaloemnon et al., 2016; Pedersen et al., 2013). One of the main targets in this case, especially for compositional analysis and functional inferences is the 16S rRNA gene due to its ubiquity amongst bacteria combined with gene's species-specific sequences (Crespo-Piazuelo et al., 2018). The structure of the ~15000 bp 16S gene is characterised by 9 hypervariable regions (HRs) distributed through conserved regions (CRs) (Zhang et al., 2018). This allows to design primers aligning to CRs whilst producing amplicons capturing HRs interspecies variability, whose sequence can be aligned to a particular taxonomical species (Janda and Abbott, 2007; Woese and Fox, 1977). Targeting a specific HR during a study is mostly dependent on sample type and targeted microbial community, indeed analysis of different HRs could lead to somewhat different levels of taxonomical details indeed (Bukin et al., 2019; Chakravorty et al., 2007; Zhang et al., 2018). For example, region V4 was sequenced during the 16S rRNA gene analysis performed during this PhD project, due to high quality outputs reported when analysing gut microbial diversity through this region (De Rodas et al., 2018; Mejía-León et al., 2014; Zhang et al., 2018; Zhu et al., 2016).

The environment described by the model developed and presented in [Chapter 1](#) was recreated *in vitro* through a 35-day continuous fermentation experiment. This chapter describes in detail both cultural-based and molecular approaches carried out in the context of this *in vitro*

experimentation simulating porcine proximal colon dynamics. The main aim of the fermentation study was to observe microbial community- and metabolite-dynamics in a controlled environment. This allowed to generate data, such as MFG or SCFA concentration over time that could be compared with the model described earlier ([Chapter 1](#)) in order to perform parameter fitting and assess model reliability, as later described in [Chapter 3](#). In summary, main physiological conditions found in pig proximal colon were maintained *in vitro* by modulating pH, temperature, dilution rate and medium composition in a triplicate tailor made bioreactor enclosed in a tailor made incubator. A pooled faecal sample collected from ~5 month old male pigs (total= five animals) was used as inoculum. Thus, fermentation was maintained for 35 days and samples collected periodically and analysed for SCFAs and microbial community, whose values throughout the fermentation were used to compare analogue outputs from model simulations.

2.2 Materials and methods

2.2.1 Continuous fermentation

The 35 day continuous fermentation experiment was carried out in triplicate in a bespoke continuous fermentation set-up. A porcine faecal inoculum was grown in a complex medium in a home-made bioreactor enclosed in a home-made incubator. The experimental conditions applied mimicked the porcine proximal colon, thus the medium used to grow the inoculum was a representation of the ileal chyme of pigs fed on a soya bean meal based ration (Tanner et al., 2014). The temperature was constant at 38 °C and anaerobiosis was maintained throughout the experiment, pH and retention were typical of the porcine proximal colon (Latymer et al., 1990). Samples were collected 4 times during the first four days, twice a day the following two days and daily from the 7th day to the end of the experiment. Short chain fatty acids were measured through gas chromatography-mass spectrometry, whereas the microbial population was assessed qualitatively through 16S rRNA gene sequencing and quantitatively through qPCR.

2.2.2 Sample collection and inoculum preparation

Faecal samples naturally excreted were collected from five ~ 5-month-old male apparently healthy pigs fed on a standard commercial diet and pooled together in sterile 50 ml Falcon tubes. The samples were stored in an anaerobic jar containing two anaerobic bags and rapidly transported to the lab, where they were transferred to an anaerobic chamber (Whitley MG 500 + MG Airlock Anaerobic Workstation, Don Whitley Scientific Limited, UK) for the preparation of the inoculum, while an aliquot (~5 ml) was stored at -80° C until further analysis. To prepare the inoculum in the anaerobic chamber, 80 g of pooled faeces were homogenised in 320 ml of the medium used during the continuous fermentation (described below), obtaining a faecal slurry that was sieved through a 100 µm cell strainer (Corning, UK) to separate eventual food residues. Finally, 56.16 ml of the sieved slurry were used to inoculate 177.84 ml of medium in the main vessel of each of the three bioreactors (final volume 234 ml), obtaining a final inoculum concentration of 60g/l. All the media used up to this stage were previously stored in the anaerobic chamber for two days to remove the dissolved oxygen before the inoculation.

2.2.3 Bioreactor

A homemade bioreactor was designed and used to carry out the 5 week continuous fermentation in triplicate. The main parts of the bioreactor are depicted by the schematic in Figure 2.1, and a picture of the triplicate system is shown in Figure 2.2. The faecal slurry described in the previous section was inoculated in the main vessel, which simulated the condition within the porcine proximal colon. The medium composition simulated the ileal chyme content of a pig fed on a soya bean based diet (Tanner et al., 2014) and contained carbon based substrates (i.e. resistant starch, non-starch polysaccharides, oligo- and mono-saccharides), ammonium-based compound, salts and vitamins and tween was used to

stimulate G⁺ anaerobes growth and to avoid bacterial clumping (Bourriaud et al., 2005) (Table 2.1 and Table 2.2). Resazurin salt was also added to the medium to detect the presence of oxygen, and NaOH 2M was used to regulate the pH, which was maintained in the 6.0 ≤ pH ≤ 6.8 range (Kararli, 1995; Tanner et al., 2014). The medium was sterilised by autoclave at 120 °C for 15 minutes, and then preincubated at 38°C while being connected to a CO₂ canister through a PVFE membrane filter (0.2 µm; Cole Parmer). This treatment allowed to remove the oxygen present, which was assessed through the resazurin reaction, which became colourless when no oxygen was detected in the medium (Figure 2.3). A peristaltic pump was used to connect the main vessel to the flow-in and the flow-out vessels, representing respectively the feed intake and the excretion. The dilution rate (*D*) of 0.11 h⁻¹ was used as the reciprocal of the porcine proximal colon transit time of 9 hours (Tanner et al., 2014) according to:

$$D = \frac{Flow_{out}}{volume\ of\ the\ main\ vessel} \quad (2-1)$$

Thus, considering the main vessel volume of 234 ml, the pump was set at 26 ml/h. Anaerobiosis was ensured throughout the experiment connecting both the main vessel and the flow-in vessels to an anaerobic jar in which an anaerobic bag depleted the system from the eventual residual O₂. A resazurin strip was also included in the jar to detect the eventual presence of atmospheric O₂. The gases produced during bacterial fermentation were collected in a 10 l gas bag connected to the anaerobic vessel, which also ensured a constant pressure in the system. Clamps were present at every vessel connection, so all the sections of the system could be immediately isolated and replaced, in case of oxygen detection by the resazurin strips and resazurin salt. Oxygen was not detected in the system throughout the experiment. Both the main and the flow-in vessels were continuously stirred through a magnetic stirrer, and the temperature was maintained at 38°C, enclosing the bioreactor in a homemade incubator, described in the following section.

Table 2.1. Vitamin solution used in the complex medium (B.-S. Kim et al., 2011).

Component	Concentration (mg/l)
Menadione	1.0
Biotin	2.0
Pantothenate	10.0
Nicotinamide	5.0
Vitamin B ₁₂	0.5
Thiamine	4.0
P-Aminobenzoic acid	5.0

Table 2.2. Medium composition (Tanner et al., 2014).

Component	Concentration (g/l)
Corn starch	4.32
Pectin (citrus)	2
Xylan (corn core)	2
Arabinogalactan (beechwood)	2
Guar gum	2
Soy peptone	13
Yeast extract	4.50
Mucin (from porcine stomach)	4.00
L-cysteine HCL monohydrate	0.8
Vitamin solution	1 ml
Resazurin (0.01%)	1 ml

Component	Concentration (g/l)
Bile extract porcine	0.4
KH ₂ PO ₄	0.5
NaHCO ₃	1.5
NaCl	4.5
KCl	4.5
MgSO ₄	0.64
CaCl ₂ · 2H ₂ O	0.15
MnCl ₂ · 4H ₂ O	0.20
Hemin solution (0.05g/ml)	0.05
Tween 80	1.00

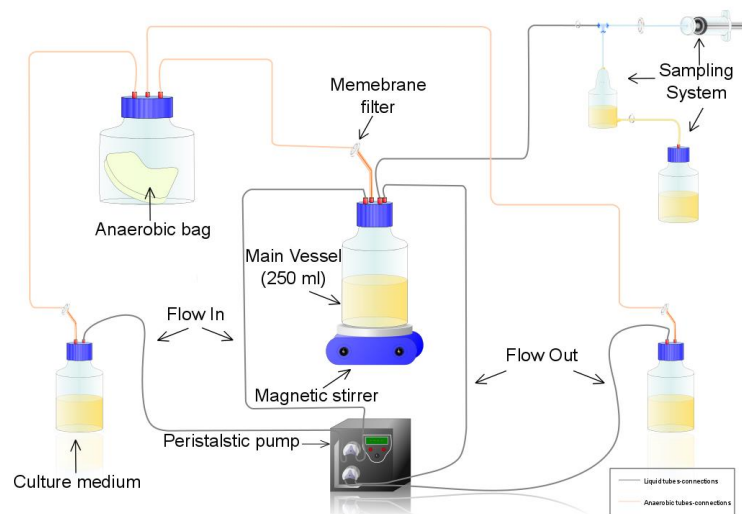


Figure 2.1. Bioreactor schematic representing a system composed by only one main vessel mimicking the porcine proximal colon. The main vessel is connected to a flow-in (simulating the feed intake) and a flow-out (simulating the excretion) through a peristaltic pump representing the retention time. The anaerobiosis was maintained connecting the vessels to an anaerobic jar containing an anaerobic bag.

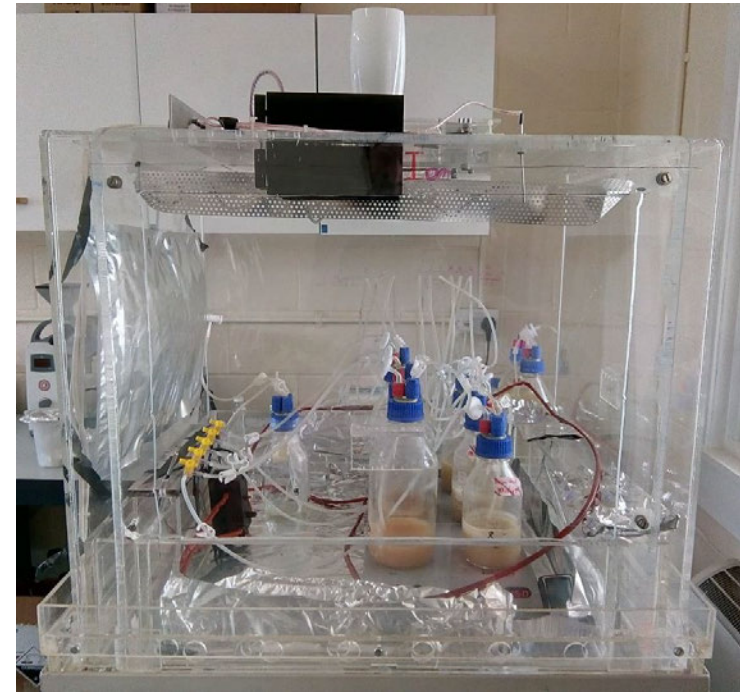


Figure 2.2. Home-made bioreactor used to perform the 35 day fermentation experiment in triplicate. The pig faecal inoculum was grown in the main vessel containing a medium simulating the ileal chyme content. The medium was provided constantly by a flow-in vessel through a peristaltic pump mimicking the proximal-colon retention time. All the vessels were connected to both an anaerobic jar, ensuring anaerobiosis, and to a gas bag used to regularise the pressure inside the system. Resazurin-strips (in the anaerobic jar) and -salt in the medium were used to detect the eventual presence of oxygen. The bioreactor was enclosed in a home-made incubator to ensure a constant temperature throughout the experiment.

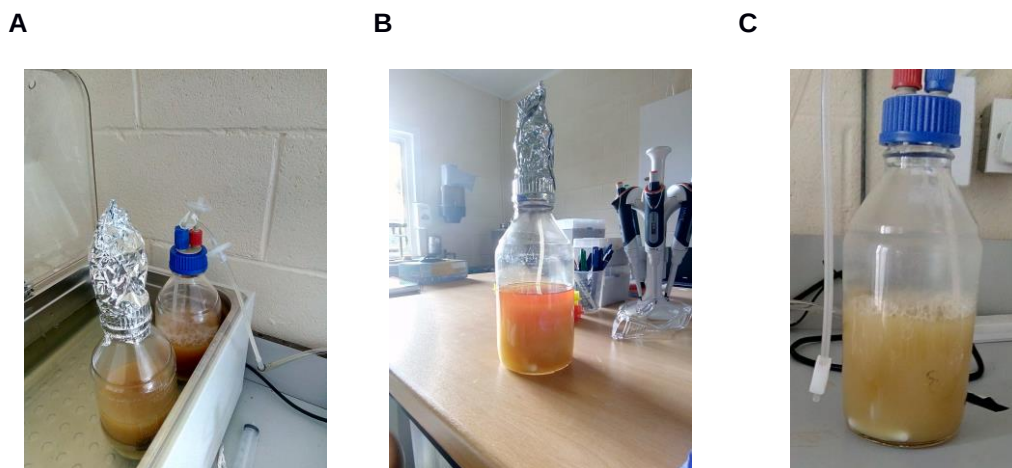


Figure 2.3. A: medium CO₂ treatment in a water bath (38°C), the autoclaved medium was connected to a CO₂ canister through a sterilised silicon tube and a PVFE 0.2 µm membrane to avoid contamination; B medium before CO₂ treatment (pink resazurin reaction due to the O₂ presence); C medium CO₂ treated (colourless resazurin reaction).

2.2.4 Incubator

To maintain the temperature constant at 38°C during the whole experiment, a home-made incubator was assembled using an acrylate box mounting two heating cables, one on the top and one on the bottom, both connected to a temperature controller (Inkbird ITC-310T-B) as shown in Figure 2.4. To ensure air circulation four fans were mounted on the top part of the incubator, and to regulate the temperature, in particular to avoid overheating of the system the fans were enclosed in an electric metal plate connected to a second temperature sensor and to a magnetic lid, which opened in case of high temperature (Figure 2.5). An opening cut in the front section allowed the manipulation of the bioreactor throughout the experiment minimising the lowering of the temperature.

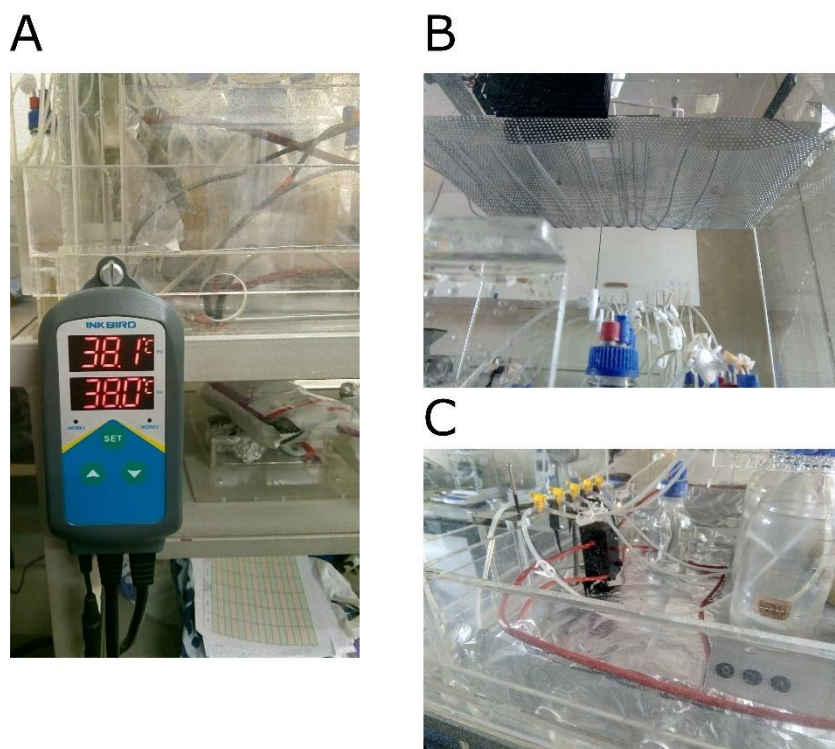


Figure 2.4. A: temperature controller used to maintain the temperature constant at 38°C, which was connected to two heating cables in the top (B) and the lower part (C) of the incubator

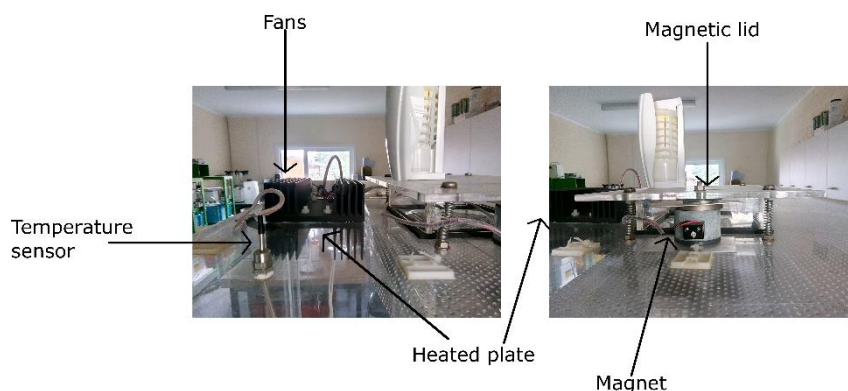


Figure 2.5. Top section of the incubator showing the fans used to allow air circulation inside the incubator and the magnetic lid connected to a temperature sensor which opened automatically in case of overheating.

2.2.5 pH and optical densitometry₆₀₀ (OD) measurement

pH was measured on bioreactor samples' aliquots using Thermo Scientific™ Orion™ 2-Star Benchtop pH Meter after calibration with both pH 4 and 7 solutions. To measure OD₆₀₀, single beam UV visible spectrophotometer LT-291(Labtronics, India) was calibrated with blank (i.e. same medium used during the fermentation). Thus, OD₆₀₀ was measured on samples diluted

(in double-distilled H₂O) 1:20 for the first ~2 days. Following, samples were diluted 1:40 in ddH₂O, to avoid OD₆₀₀ poor quality reads ≥ 1 , due to increased microbial concentration.

Data analysis was performed as follows. In order to improve visualisation of OD₆₀₀ measurements from the two different dilutions using the same graphic scheme, raw data were normalised through the following formula:

$$\frac{OD_{600t} - OD_{600}^m}{OD_{600}^M - OD_{600}^m}, \quad (2-2)$$

where, OD_{600t} was the OD₆₀₀ value recorded at time t during the fermentation, OD_{600}^m and OD_{600}^M were the minimum and the maximum OD₆₀₀ values recorded throughout the fermentation, respectively. Furthermore, to plot pH and OD₆₀₀ data on the same graph, equation (2-2) was adapted as follows to normalise pH measurements:

$$\frac{pH_t - pH^m}{pH^M - pH^m}, \quad (2-3)$$

where, similarly to equation (2-2), pH_t was the value of pH recorded at time t during the fermentation, whilst pH^m and pH^M were minimum and maximum pH values, respectively as observed throughout the fermentation.

This type of data analysis allowed the direct comparison of pH and OD₆₀₀ behaviours on the same graph, further allowing potential inference of their possible interactions during the *in vitro* fermentation study.

2.2.6 Gas chromatography-mass spectrometry

GC-MS was used to assess the SCFA concentration. Bioreactor samples destined to GC-MS were centrifuged at 14,000 rpm for 10 minutes at room temperature to separate the microbial cells from the metabolites. The supernatant was filtered through 0.2 μ m PVDF sterile membrane filters (Whatman puradisc, 13 mm), and 2.5 μ l of HCl were added to 100 μ l of the filtered supernatant (FS) to reduce the pH allowing the molecule to stabilise in their non-ionic form, more suitable for the downstream analysis. To maximise their final concentration, the SCFAs and lactate in the FS were twice extracted with diethyl ether (EE). Thus 500 μ l of EE were spiked with the internal standard 2-ethylbutyric acid (C₆H₁₂O₂) (5mM final concentration) and then added to 102.5 μ l of FS plus HCl, which were vortexed for 15 minutes and centrifuged for 3 minutes at 1000 g. Thus, 430 μ l of this supernatant were stored in a fresh vial while the pellet went through the second extraction with 500 μ l of EE without internal standard, proceeding with the same vortex/centrifuge protocol as described for the first extraction. The two supernatants from the extractions were pooled together and 500 μ l were transferred to the GC-MS instrument (GC/Q-TOF model 7200 A) and derivatised with 50 μ l of N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (Sigma-Aldrich, UK) with an automated protocol

composed of: vortex for 15 minutes and incubation at room temperature for 15 minutes repeated twice before the injection (protocol modified from Moreau et al., 2003).

GC-MS was carried on at the EdinOmics (Waddington Building, King's buildings, The University of Edinburgh), with the following conditions: inlet temperature: 250 °C, oven temperature 250-300 °C; column (Agilent DB-5ms, 10 meters guard + 30 meters x 250 µm ID x 0.2 µm stationary phase): 70°C for 3 minutes; 20 °C/minute up to 160 °C; 30 °C/minute up to 300 °C and held for 3 minutes. The liner used for the injection of the sample was the ultra-inert liner universal, low PSI drop, wool (5190-2295 Agilent). The mass spectrometry (GC/Q-TOF model 7200 A) source temperature was 230 °C, quad temperature was 150 °C, filament current was 35.10 µA, and emission current was 70 eV. Metabolite concentration was calculated from raw data using the MassHunter Workstation Software, Quantitative Analysis, version B.07.01/Build 7.1.524.0 for GC-MS (Agilent Technologies, Inc. 2008).

2.2.7 DNA isolation

A 250 µl aliquot of the bioreactor samples, as well as 250 µg of faeces for inoculum preparation were collected to isolate total DNA using the QIAamp PowerFecal DNA Kit (QIAGEN, UK), following the manufacturer protocol. The isolated DNA was stored at -80°C until further analysis. The DNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

2.2.8 16S rRNA gene sequencing

A total of 99 amplicon libraries were pooled together in a single ready-to-sequence sample and sequenced using MiSeq apparatus (MiSeq v2, 2 x 250 bp paired-end reads, Illumina, UK) at Edinburgh Genomics (King's Buildings, The University of Edinburgh). The amplicon libraries were prepared amplifying the V4 region of the 16S rRNA gene using the Earth Microbiome Project primers (Caporaso et al., 2011b; Parada et al., 2016), characterised by the presence of a sample-specific 12 bp Golay barcode associated with the reverse primer (Caporaso et al., 2012). The use of the V4 region was based on its successful previous applications in the exploration of gut microbiota diversity (Moreau et al., 2014; Parada et al., 2016; Thompson et al., 2017; Zhang et al., 2018). Thus, a PCR reaction was carried out to generate the V4 amplicons, which were then purified from gel using the Wizard® SV Gel and PCR Clean-Up System (Promega, UK) following the manufacturer protocol. A PicoGreen assay was performed to quantify the purified amplicons before an equal concentration of amplicon was used to generate the single ready-to-sequence pool.

2.2.8.1 Amplification of the 16S rRNA V4 region

A ~350 bp amplicon was generated through PCR reaction targeting the V4 region of the 16S rRNA gene using the primers depicted in Table 2.3. The PCR reactions were prepared in a laminar flow PCR cabinet (Labcaire System, UK), where a 15 minute UV light (254nm) treatment was applied to the empty tubes first, and a second time after PCR grade nuclease-

free H₂O (Sigma), buffer and MgCl₂ were added to eliminate any contaminant DNA. The reaction was carried out in 25 µl containing 1X PCR buffer (NEB ThermoPol® Buffer), 1 mM MgCl₂, 200 µM of dNTP mix, 0.025 U/µl of Taq DNA polymerase (M0267S; New England BioLabs), 250 nM of both forward and reverse (barcoded) primers, 1 µl of template DNA and nuclease-free PCR grade H₂O up to 25 µl. The template DNA was added on the bench outside the cabinet once the mix was prepared. The reactions were then transferred to a thermocycler (Labnet MultiGene OptiMAX Thermal Cycler) where 94°C for 3 minutes were applied to achieve an initial denaturation of the DNA before proceeding with 25 cycles of denaturation at 94 °C for 45 seconds, annealing at 50 °C for 60 seconds and extension at 72 °C for 90 seconds, finally, a final extension cycle at 72 °C for 10 minutes was performed. Agarose gel (1.5 %) electrophoresis was used to separate the amplicons (Figure 2.6), which were then cut and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) following the manufacturer protocol.

Table 2.3. Primers used during the PCR reaction (5'→3') for the amplification of the V4 region of the 16S rRNA gene.

Forward primer (5' → 3') (Parada et al., 2016)	
Illumina adapter	AATGATACGGCGACCACCGAGATCTACACGCT
forward primer pad	TATGGTAATT
forward primer linker	GT
forward primer (a515)	GTGYCAGCMGCCGCGGTAA
Reverse primer (5' → 3') (Caporaso et al., 2011b)	
Illumina adapter	CAAGCAGAAGACGGCATACGAGAT
Golay barcode	XXXXXXXXXXXX (sample-specific)
Reverse primer pad	AGTCAGTCAG
Reverse primer linker	CC
Reverse primer (806R)	GGACTACHVGGGTWTCTAAT

100bpNeg B30C30

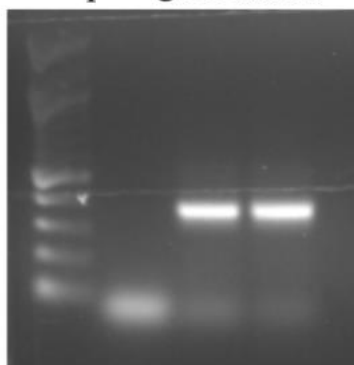


Figure 2.6. Example of amplicon separation through 1.5 % agarose gel electrophoresis. B30 and C30 are samples from reactor B and C at the time point 30, whereas 100 bp represent the molecular ladder used for size comparison.

2.2.8.2 Quantification and normalisation

Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific) was used to quantify the purified amplicons. A standard curve was built with five serial dilutions of λ DNA (Table 2.4) and used to calculate the concentration of 1 μ l of amplicon DNA. PicoGreen fluorescence was then calculated through excitation: 480 nm and emission: 520 nm using a plate reader (Spectra MAX GEMINI XS, Molecular Devices) and actual DNA concentration was calculated comparing their fluorescence with the regression standard curve. Finally, the samples were pooled together normalising their concentration to 3.28 nM, so that the final concentration of the pool was 2.16 ng/ μ l.

Table 2.4. λ DNA serial dilution used to build the standard curve during the PicoGreen assay.

Standard	Concentration
Standard 1	1000 ng/ml
Standard 2	100 ng/ml
Standard 3	10 ng/ml
Standard 4	1 ng/ml
Standard 5	0 ng/ml

2.2.8.3 Bioinformatic analysis

A total of 11,066,443 FASTQ paired-end demultiplexed reads (~ 110,000 sequences/sample) were imported as a '.qza' artifact in the QIIME2 pipeline (Bolyen et al., 2018; Caporaso et al.,

2010) (Figure 2.7) using the manifest method (Bolyen et al., 2018). A total of 88.8 % of the reads were retained after using the “join-pairs” command of VSEARCH (Rognes et al., 2016), which paired the reads decreasing their total number to ~90,000 per sample. This was possibly due to the sub-optimal quality observed through the final length of the reverse reads despite an appropriate level of quality, especially in the forward reads (Figure 2.8). The quality score of the joined reads was ~40 throughout their sequence length (Figure 2.9). Following, the quality-filtering was carried out with minimum Phred score of 20 (Bokulich et al., 2013; McKinney, 2010). Deblur was used to denoise the filtered joined reads, which were first trimmed at 250 bp, then, based on their Hamming distance, all the predicted error-derived reads were subtracted from the neighbouring ones, after being sorted according to their abundance. Therefore, all the non-valid sequences, i.e. the ones with abundance equal to 0 after the subtraction, were removed. Ultimately, singletons, spurious non-16S amplicon sequences were removed using a built-in algorithm that truncated all the reads with a high error derived from technical sequencing biases before removing PCR chimeras (Amir et al., 2017).

The taxonomy was assigned using the q2-feature-classifier plugin, i.e. a Naïve Bayes classifier was trained based on the primers used and the last release of the Silva data base (132, 99% of similarities) (Bokulich et al., 2018; Pedregosa et al., 2011; Pruesse et al., 2007). Both phylogenetic and non-phylogenetic metrics were applied to measure the community α and β diversity. Samples were rarefied to simulate an even number of sequences per sample using a minimum per-sample sequence depth of 15669. This value was chosen in order to retain the majority of sequences per samples excluding the samples with a lower number of sequences (i.e. control samples). The within-sample diversity (α -diversity) was calculated through the Faith's phylogenetic diversity (species richness based on cladistic information) and the Pielou's evenness index (determination of the species number closeness) and estimating the Shannon's diversity index, which accounts for both abundance and evenness (Anderson, 2001; Faith, 1992; B. R. Kim et al., 2017). The significance of the sample type (bioreactor replicate) and of the sample collection time (longitudinal analysis) on the α -diversity was measured through the Kruskal-Wallis test, which computed the one-way ANOVA in a non-parametric way according to the method used by Benjamini and Hochberg (Benjamini and Hochberg, 1995; Kruskal and Wallis, 1952). The between-sample diversity (β -diversity) was measured through the calculation of the Bray-Curtis dissimilarities and the weighted UniFrac distances. The first one measured the compositional dissimilarities amongst different samples, whereas the weighted UniFrac also incorporated phylogenetical information accounting for the abundance of the observed species (Bray and Curtis, 1957; Lozupone et al., 2011). The permutational multivariate analysis of variance (PERMANOVA) was used to assess the significance of the distance between samples when evaluating the β -diversity (Anderson, 2001). The R packages qiime2R (<https://github.com/jbisanz/qiime2R>) and MicrobeR

(<https://jbisanz.github.io/MicrobeR/>) were used to graphically represent the 30 most abundant genera in the three reactors and the Shannon's index throughout the experiment.

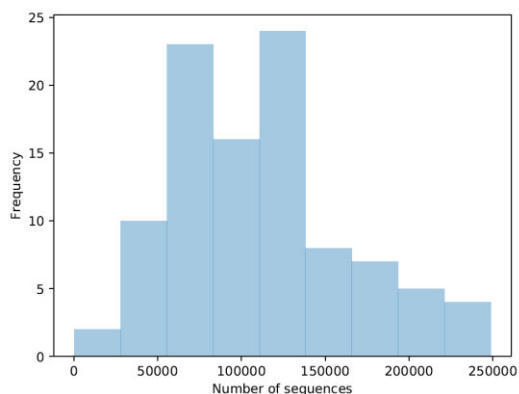


Figure 2.7. Demultiplexed sequence count distribution before quality-filtering and denoising.

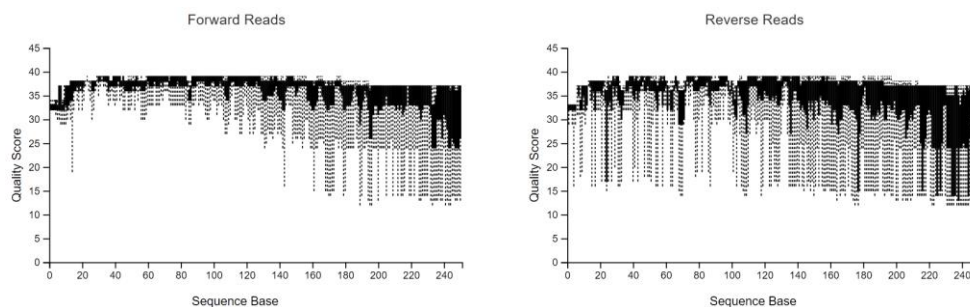


Figure 2.8. Boxplots of the quality score showing the distribution of sequence quality at each position throughout the sequence data. The position in the sequence is indicated in the x-axis, whereas the y-axis indicates the quality score, which represents the probability of a base of being incorrect. The probability to find an incorrect base decrease with high quality score values. A different quality boxplot depicts the distribution of the quality values for that determinate sequence base, the black line represents the interquartile ranges, whereas the grey lines represent the whiskers. Outliers are not shown in the graph for clarity.

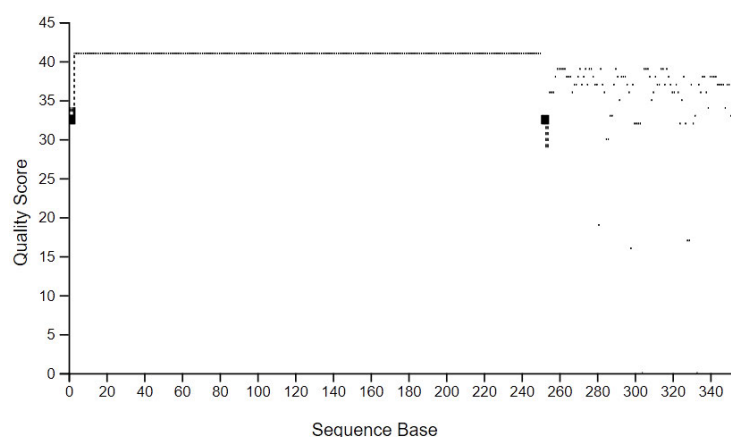


Figure 2.9. Boxplot quality graph showing the quality score of the joined reads for each position in the sequence base. The overall quality was ~40, meaning that the probability to find an incorrect base was 1 in 10,000 (base call accuracy: 99.99 %) About ~120 over the 10,000 sequences sampled were found to have length ≥ 253 , which were then discarded through Deblur denoising.

2.2.8.4 Assigning 16S rRNA gene sequencing taxonomy to MFGs

Bacterial species relative abundance output of bioinformatic taxonomical analysis as described above was used here to infer MFG relative abundance in the faecal sample and throughout the fermentation. Taxonomical data at species level were used to assign bacteria to the ten modelled MFGs and when information at species level was missing due to poor resolution after bioinformatic alignment, higher taxonomical hierarchy (i.e. genus level) was used to infer functional groups relative abundance throughout the fermentation study.

In general, species enzymatic and pathway patterns together with substrate requirements and metabolite production were considered to assign bacteria to the different MFGs.

To mention some examples, species belonging to *Prevotella*, and *Bacteroides* were assigned to MFG1 as described in the previous chapter. *Muribaculaceae* is a relatively novel described family amongst *Bacteroidetes* phylum, which includes several genera such as *Duncaniella* or *Muribaculum* (Lagkouvardos et al., 2019). Although cultural based data are still missing, this family has been significantly associated with propionate production (Smith et al., 2019), thus considering this and taxonomical similarity with *Prevotellaceae* and *Bacteroidaceae*, genera related to this family found in the analysed samples were assumed to be part of MFG1. Species related to *Lactobacillus* and *Streptococcus* genera were assigned to MFG4 according to the model description. Species related to genus *Ruminococcus* were assigned mainly to MFG2 (e.g. *R. bromii* or *R. gausvreauii*) (Domingo et al., 2008) and MFG3 (e.g. *R. flavefaciens* or *R. albus*), whereas species belonging to Archaea domain were assigned to MFG10. As anticipated in the first chapter MFG7 and MFG8 both acknowledged *Megasphaera elsdenii* ability to produce propionate and butyrate from lactate, respectively (Bourriaud et al., 2005; Prabhu et al., 2012; Xiao-lin et al., 2016b). The bacterium was therefore assigned to both groups at a ratio established through several *in silico* experiments simulating *in vitro* batch and

continuous cultures (data not shown). Since the model in its current form does not include the totality of gut species, unclassified genera/species such as for example the asaccharolytic *Mogibacterium* (De Vos et al., 2009b) or *Peptococcus* (Ezaki, 2009a) represented ~20% of the total in the faecal sample.

2.2.9 Quantitative polymerase chain reaction

Region V3 of the 16S rRNA gene and *McrA* gene were quantified through qPCR using standard curve method (absolute quantification) and SYBR green detection. Two different 8-point standard curves for each gene analysed were built through serial dilutions of linearised plasmid DNA, whose inserts were the target amplicons. The following sections describe in more detail the several steps of the analysis.

2.2.9.1 Standard curves preparation

Standard curve was built using linearised plasmid containing the same target amplified during the qPCR. Two different plasmids were used to quantify bacteria and archaea, respectively. Total DNA isolated from porcine faecal sample was used as a template in two different PCR reactions using 341F/543R and *mcrA* primers respectively (Table 2.5). Resulting amplicons were then cloned into pCR2.1 plasmid vectors using the TA Cloning™ Kit and One Shot INVαF' Chemically Competent *E. coli* (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

In detail, PCR reactions were carried out in 50 µl containing 0.25 µl (1.25 units) of *Taq* DNA polymerase (M0267S; New England BioLabs, Ipswich, MA, USA), 0.2 µM of each primer, dNTPs (200 µM), 1X buffer (ThermoPol® Buffer; New England BioLabs). PCR conditions were 95 °C initial denaturation for 1 minute, 30 cycles of denaturation at 95 °C, annealing at amplicon-specific temperature as depicted in Table 2.5, and extension at 68 °C, with a final extension cycle at 68 °C for 5 minutes. Amplicon bands excised from agarose gel (1.5 %) after electrophoresis run were purified using the QIAquick gel extraction kit (QIAGEN, UK) and cloned into the pCR2.1 vector (Thermo Fisher Scientific, Waltham, Massachusetts, United States) following manufacturer protocols. Ligase reaction was carried out for one hour at room temperature with a 1:1 insert to vector concentration ratio. Thus, 5µl of plasmid/insert were transformed into chemically competent One shot® INVαF' *E. coli* cells (Thermo Fisher Scientific, Waltham, Massachusetts, United States) by heat shock.

After selection of plasmid/containing colonies through X-gal selection, plasmids were isolated from pelleted cells using the QIAprep Miniprep kit as per manufacturer instructions (QIAGEN, UK). *EcoRI* restriction enzyme digestion followed by Sanger sequencing were used to assess insert presence and orientation. Endonuclease digestion was carried out using 20 U of *EcoRI* HF enzyme (New England BioLabs) incubated with 90 ng (average value) of plasmid DNA, together with 10 X CutSmart® buffer (New England BioLabs) and H₂O in 20 µl total volume for 2 hours at 37 °C. Sanger sequencing was carried out by DNA Sequencing and Services,

Medical Sciences Institute, School of Life Sciences, University of Dundee, where primer M13F (plasmid position 389-404) and M13R (plasmid position 205-221) were used to sequence the plasmid before the lacZ α gene and the T7 Promoter operon. The expected amplicon sequences, taking into account the degenerate nature of the primers, were checked using BLAST.

Plasmids were then linearised through endonuclease digestion with 5 units of HindIII (New England BioLabs), using 10 X CutSmart[®] buffer (New England BioLabs), ~150 ng of plasmid DNA and H₂O up to 50 μ l of volume. Two parallel reactions were carried out for each plasmid including a non-cut control, which were then run through 1% agarose electrophoresis (Figure 2.10) Thus, linear-plasmid was excised and purified using the QIAquick gel extraction kit (QIAGEN, UK). Finally, linearised-plasmid concentration (ng/ μ l) was measured in triplicate with NanoDrop TM 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and used to calculate linear-plasmid copy number (CN). Linearised-plasmid concentration (ng/ μ l) was measured in triplicate with NanoDrop TM 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and used to calculate linear-plasmid copy number (CN). All the reactions were performed in triplicate. Serial linear-plasmid dilutions were thus used in an eight-point standard curve during the qPCR reactions.

Table 2.5. Primers used to amplify V3 region of the 16S rRNA gene and mcrA gene during qPCR.

Target	Primer	Sequence (5'→3')	Annealing temp. (°C)	Reference
V3	341F	CCTACGGGAGGCAGCAG	65	(Muyzer et al., 1993)
hypervariable region	518R	ATTACCGCGGCTGCTGG	65	
mcrA (methyl coenzyme-M reductase, sub. α)	488	GGTGGTGTMGATTACACARTAYGCWAC AGC	55	(Luton et al., 2002)
	491	TTCATTGCRTAGTTWGGRTAGTT	55	

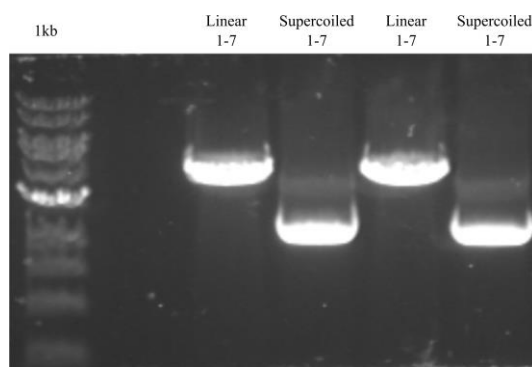


Figure 2.10. Example of an agarose gel electrophoresis showing the endonuclease reaction carried out with HindIII to linearise the plasmid DNA (1-7 in the example). Each reaction was performed and run in duplicate, including a non-cut control, which generated a supercoiled plasmid as visible on the gel. The linear plasmid was then isolated from the gel and used to build the standard curve.

2.2.9.2 Real time PCR and data analysis

Quantitative PCR reactions were carried out in 20 µl containing (final concentration) 1X Takyon qPCR MasterMix with Low Rox (Eurogentec, Liège, Belgium), 50 nM of each primer (Table 2.5), ~10 ng of DNA template and nuclease-free water (QIAGEN, Hilden, Germany). Cycling conditions (Mx3000 thermocycler, Agilent Technologies, Santa Clara, California, USA) were 95 °C for 3 minutes (denaturation and Taq Polymerase activation) followed by 40 cycles at 95 °C for 5 seconds (denaturation) and amplicon-specific annealing temperature (Table 2.5) for 35 seconds. Qualitative template control was performed through melting curve analysis, all the reactions were run in triplicate. Efficiency of the reactions based on standard curve analysis was in the range between 99% and 106%, with slope range between -3.27 and -3.4.

Data analysis was performed as follows. Gene copy number per reaction output of the qPCR was converted in cell number per ml of sample. To achieve this, 16S copy number from qPCR was first converted to cell number, normalising the former to the average number of copies of this gene present in a single bacterial cell, i.e. 5 at the time of writing (Stoddard et al., 2015). Successively, bacterial cell number per qPCR reaction calculated so far was converted to bacterial cells per ml of sample through the following formula:

$$\frac{\left(\left(\frac{CN}{5}\right) \cdot C \cdot D\right)}{(S \cdot V)} \quad (\text{Singh et al., 2014}); \quad (2-4)$$

where, C and D were respectively concentration and dilution volume of extracted DNA, S was the amount of DNA subjected to qPCR and V was the amount of sample used to isolate DNA (Singh et al., 2014). This allowed to infer number of bacterial cells per ml of sample. On the other hand, number of archaeal cells per ml was calculated using mcrA copy number per

qPCR reaction directly in the above formula, assuming that only one copy of this gene was present in a single archaeal cell.

2.2.10 Combining qPCR and sequencing data to infer MFG concentration throughout the fermentation study (inferring observation used for model comparison)

MFG concentration predicted by the model was expressed in g/l, therefore in order to compare predictions and observations, similar variables had to be produced both *in silico* and *in vitro*. This was achieved combining MFG relative abundance derived from 16S rRNA gene analysis and bacterial/archaeal concentration derived from qPCR as described below.

As detailed above, copy number output of qPCR was initially transformed in cell number per ml. The latter was further converted to grams/ml normalising cell number to bacterial mass, whose assumption was based on average mass of bacterial cells in stool as 4.6pg (<https://bionumbers.hms.harvard.edu/bionumber.aspx?id=112998&ver=5&trm=mass+bacterium&org=Bionumbers> Average mass of bacterium in stool) (Sender et al., 2016). Due to lack of specific information, mass of archaea was assumed to be the same as the one assumed for bacterial cells. This assumption was likely to lead to a slight underestimation of MFG10 concentration, although considering their low relative abundance compared to the remaining MFGs this underestimation was considered negligible. Consequentially, MFG relative abundance from bioinformatic analysis was applied to bacterial and archaeal concentration (g/l) inferred up to this point. This allowed to derive specific concentration of each MFG (g/l) throughout the experiment, which was the variable compared to model predictions as described in later [Chapter 3](#). Part of microbiota present in both faecal and bioreactor samples remained unassigned, due to the model described so far being not inclusive of all the microbial species. Therefore, Unassigned concentration (g/l) was part of the output of this analysis.

2.3 Results

2.3.1 *pH and OD₆₀₀*

As depicted in Figure 2.11, OD₆₀₀ measurements throughout the experiment were rather comparable amongst the three reactors. A decrement in microbial densitometry characterised the very first hours after inoculation, which was expected due to microbial adaptation into the “new environment”. Afterwards (~day 1), a positive response of the inoculum was registered that led the species to stabilise to a fluctuating constant value until day ~10. Thereafter, OD₆₀₀ values decreased in the same fashion in the three reactors after day 12, whilst being characterised by the same fluctuation already observed through the fermentation.

A major effort was applied to maintain pH constant in a range compatible with values typical of porcine proximal colon ($6.0 \leq \text{pH} \leq 6.8$) (Kararli, 1995; Tanner et al., 2014). An initial sudden drop to ~5.2, was observed soon after inoculation, although pH was promptly adjusted to ~6.2 as recorded after two hours post-inoculation. In general, pH was characterised by lower values during the first 12 days of the experiment. As described above, pH was regulated manually through NaOH 2M, and it was rather technically demanding to balance both microbial growth and CO₂ negative effect on pH. (Cole and Prairie, 2009). From day 12 onwards until the end of the experiment, a better understanding of such effects allowed to maintain pH to values closer to 6.6.

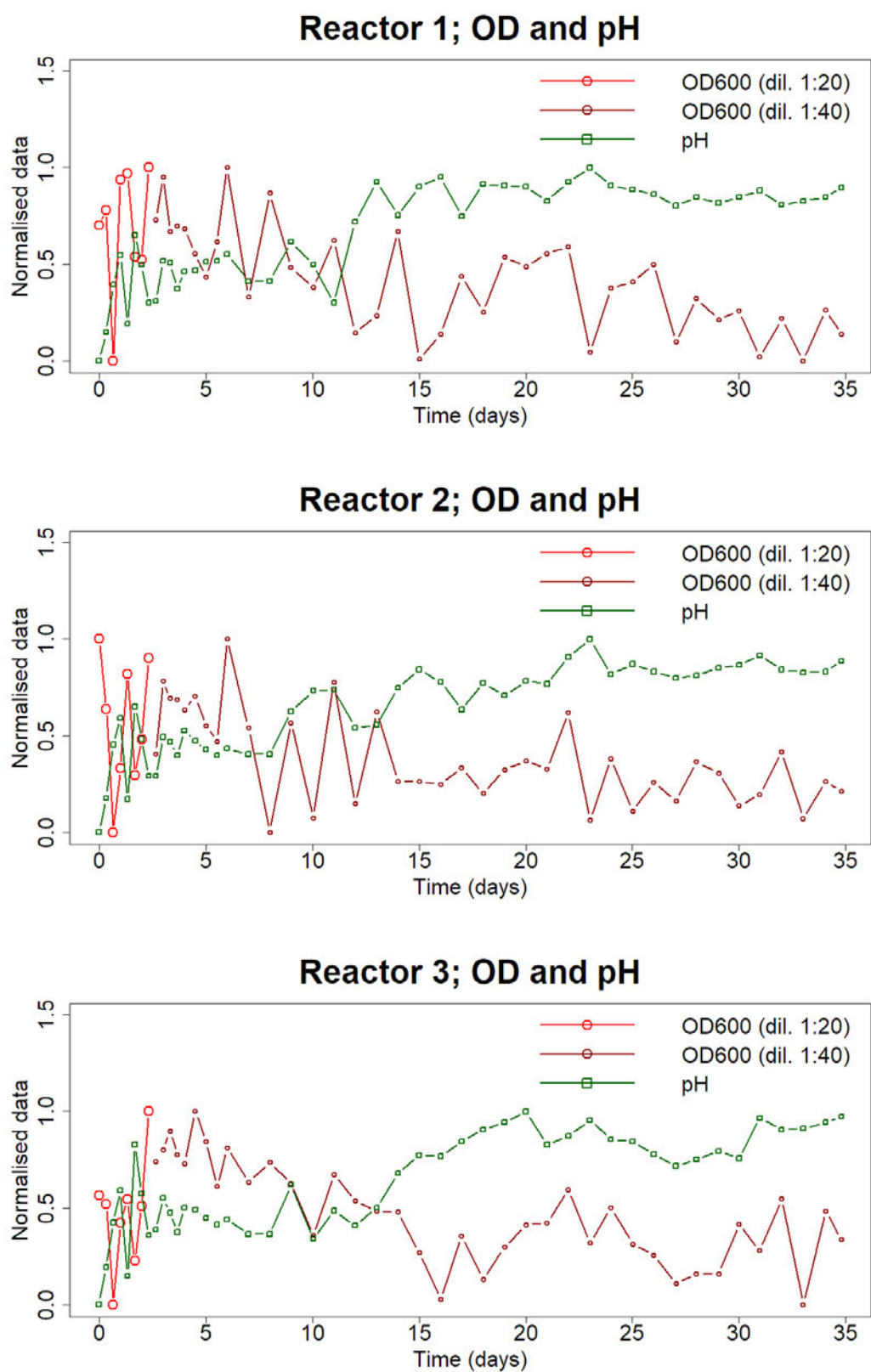


Figure 2.11. OD₆₀₀ and pH as observed throughout the *in vitro* fermentation in the three reactors. Raw data were normalised according to Eq. (2-2) and Eq. (2-3) as detailed in the text.

2.3.2 Gas chromatography-mass spectrometry

GC-MS was used to measure acetate, propionate and butyrate concentration in the bioreactor samples. As depicted in Figure 2.12, sampling frequency was higher in the first five days of the fermentation, which was arranged to accommodate model predictions ([Chapter 1](#)) according to which most of the SCFA fluctuations would be observed during the first days. Indeed, this was the case, especially for reactors 2 and 3 (Figure 2.12). The three molecules appeared to have a similar behaviour amongst the three reactors, with acetate being the most abundant one in all of them.

Acetate concentration was higher during the first five days with an average of 66 mM, although a decrease of its concentrations was observed in the three replicates towards day 35 (average of 50 mM). Propionate concentration was rather constant although also in this case a decrease was noticed, especially in the second reactor, with average concentrations before and after the fifth day equal to 42.5 mM and 33 mM, respectively. Butyrate was lower in the first part of the experiment (average of 24 mM), but it tended towards 28 mM after day 5. Generally, stable dynamics with rather constant concentrations were observed from day 5 to day 35 of the fermentation, although a pick of the three metabolite was noticed at day 20 in reactor 1. Nevertheless, after this time point concentration of acetate, propionate and butyrate tended to values close to the ones observed prior day 20 in reactor 1, which were rather similar to values observed in the other two reactors. all. In all the reactors, propionate concentration seemed to increase at day 20, whilst ratio between butyrate and propionate seemed to decrease. In general, average metabolite ratio (acetate:propionate:butyrate) was 49:31:20, and ratio observed at day 35 was 44.46:30.46:25.07.

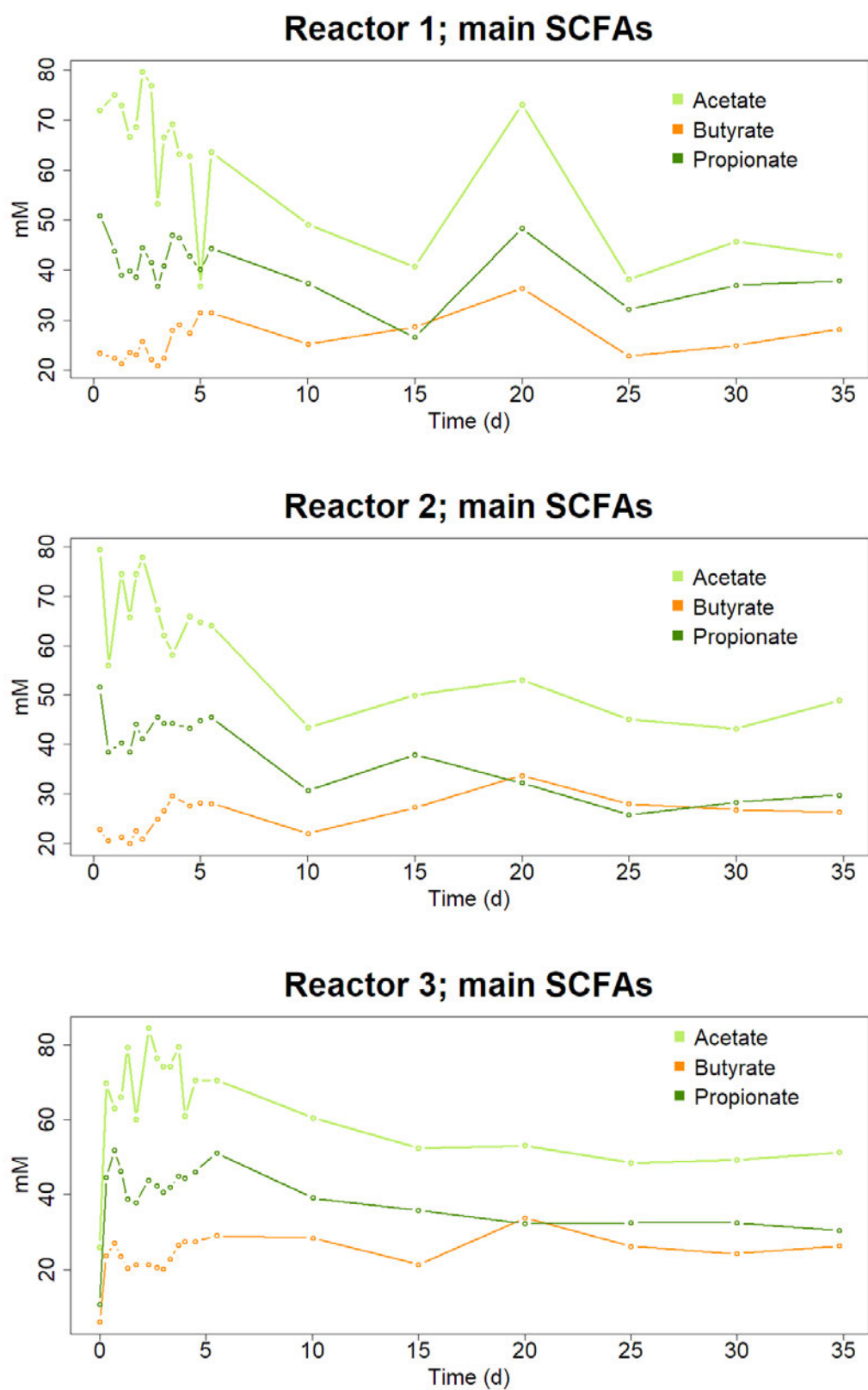


Figure 2.12. Main SCFA concentration observed in the three reactors throughout the experiment.

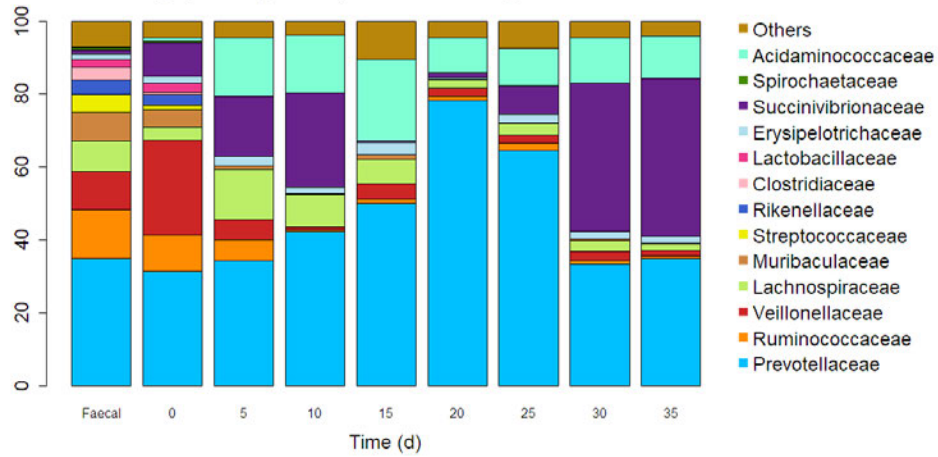
2.3.3 Sequencing

2.3.3.1 Taxonomy

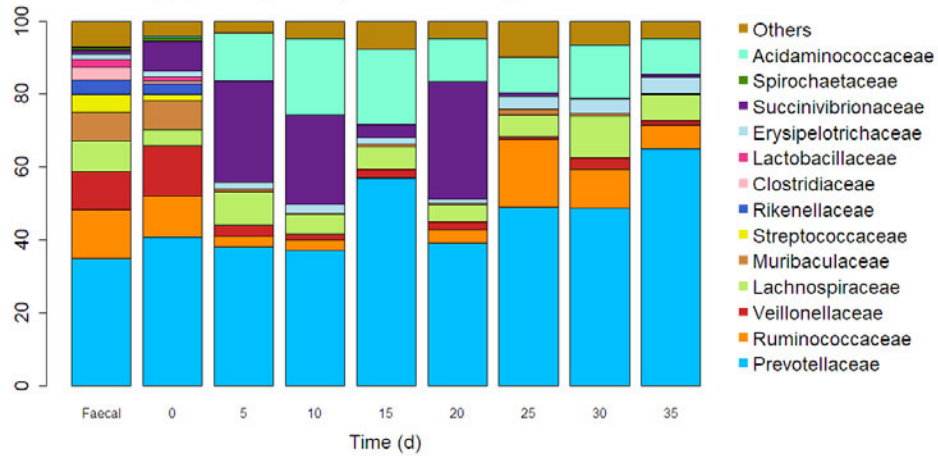
As expected, the complexity and richness observed in the faecal sample decreased during the first days of the experiments, supporting the view that not all the microorganisms can be maintained *in vitro*. The most abundant family both in the faecal sample (35 %) and during the continuous fermentation was *Prevotellaceae*, with a mean relative abundance amongst the triplicates going from 33.2 % at time 0 to 46.4 % at day 35 (Figure 2.13). As shown in Figure 2.13, *Ruminococcaceae*, *Veillonellaceae*, *Lachnospiraceae*, and *Muribaculaceae* were highly represented in the faecal sample (i.e. mean values amongst the reactors respectively 13 %, 10.5 %, 8.5 % and 7.6 %). While *Lachnospiraceae* concentration remained somewhat constant in the three reactors throughout the experiment, both *Ruminococcaceae* and *Veillonellaceae* concentration decreased, whereas *Muribaculaceae* almost disappeared after day 5. The same was observed for *Lactobacillaceae*, *Clostridiaceae* and *Spirochetaceae*, which were not detected in any of the triplicates from ~ day 5. *Succinivibrionaceae* and *Acidaminococcaceae* apparently adapted rather well to the conditions applied during the fermentation and were found in major proportions after the inoculation. *Archaea* represented ~0.2 % of the microbes in the faecal sample (data not shown), composed principally by *Methanobacteriaceae* and *Methanomethylophilaceae*, with the majority of them no longer detected after the fifth day. Nevertheless, some members of the family *Methanomethylophilaceae* were detected again around day 30 in all the reactors (data not shown).

As expected, some differences were found in the microbial dynamics amongst the biological replicates (Figure 2.13), in particular, it was noticed a peculiar change in relative abundance at day 20 in reactor 1, also observed during the diversity analysis, as described in the following section. At this time point (reactor 1) *Prevotellaceae* reached a peak in their concentration (42.2 %), while the other microorganisms' percentage drastically decreased, before *Succinivibrionaceae* concentration expanded again and was found to be one of the most abundant families at the end of the fermentation (43.6 %). This was not observed in the other two reactors, whose microbial dynamics were somewhat constant throughout the experiment, although *Succinivibrionaceae* concentration decreased after day 20 in reactor 2, and after day 30 in reactor 3.

Taxonomy (Family level)- Faecal sample and reactor 1



Taxonomy (Family level)- Faecal sample and reactor 2



Taxonomy (Family level)- Faecal sample and reactor 3

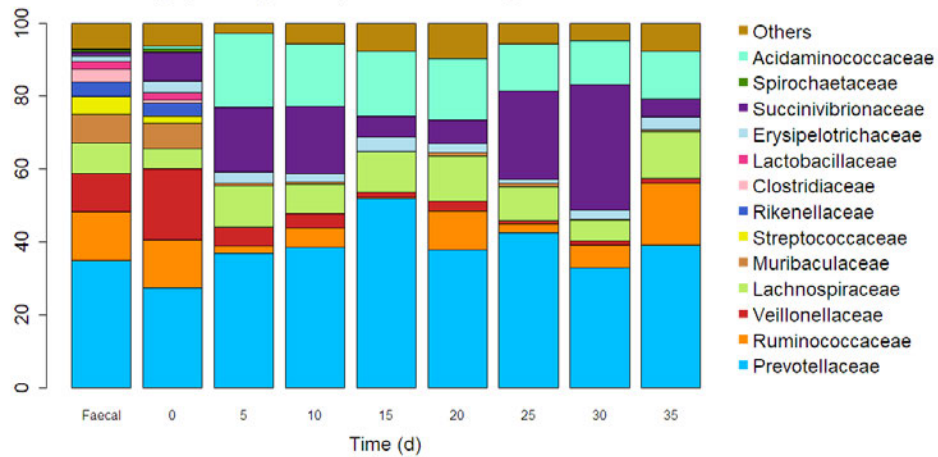


Figure 2.13. Taxa bar plot showing the microbial relative abundance in the faecal sample and in the three reactors throughout the experiment. Microorganisms with $\leq 0.5\%$ were grouped as "Others".

2.3.3.2 Diversity analysis; alpha-diversity

The Shannon's index (species richness and relative abundance) decreased after the inoculation of three bioreactors from ~6 measured from the faecal sample to ~3.5 (Figure 2.14). According to the Kruskal-Wallis pairwise test the sample type effect was not significant (Table 2.6), nevertheless, the presence of only one replicate for the faecal sample (32 in the reactors) most probably affected the analysis output, possibly indicating that the richness and evenness in the three reactors were not statistically different while being different from the faecal sample. The Spearman test was used to analyse the statistical dependence between the α diversity indexes (i.e. Shannon's index, Faith's phylogenetic diversity (pd) and the Pielou's evenness index) and the time-points throughout the experiment. The test showed that the effect of time on the within-sample diversity was strongly statistically significant (Table 2.7). From Figure 2.15 it can be seen that in all the cases time had a significant negative effect on the α -diversity metrics, as richness and evenness decreased from the faecal sample to the first days of the experiment before they stabilised in a "pseudo-steady state" from day 5 until the end of the fermentation. It was interesting to note that at day 20 the Shannon's index dropped in one of the reactors (reactor 1), which was confirmed to be due to a drop in the evenness but not of the richness (Figure 2.15).

Table 2.6. Kruskal-Wallis pairwise significance of the Shannon's index measuring the α -diversity between the sample type. The test shows that the difference in both species richness and relative abundance was not significant amongst the different type of samples.

Kruskal-Wallis (pairwise)		H	p-value
Faecal sample	Reactor A	2.8	0.09
	Reactor B	2.8	0.09
	Reactor C	2.8	0.09
Reactor A	Reactor B	0.5	0.485
	Reactor C	1.4	0.243
Reactor B	Reactor C	0.3	0.582

Table 2.7. Spearman test to assess the significance of the time correlated with the richness and evenness of the species present in the samples (α -diversity metrics). The results show that the time had a strong statistically significant effect on the within-sample diversity.

Spearman test on the different α -metrics	Test statistic	p-value
Shannon's index	-0.734	0.0000
Faith's pd	-0.8	0.0000
Pielou's evenness index	-0.34	0.0006

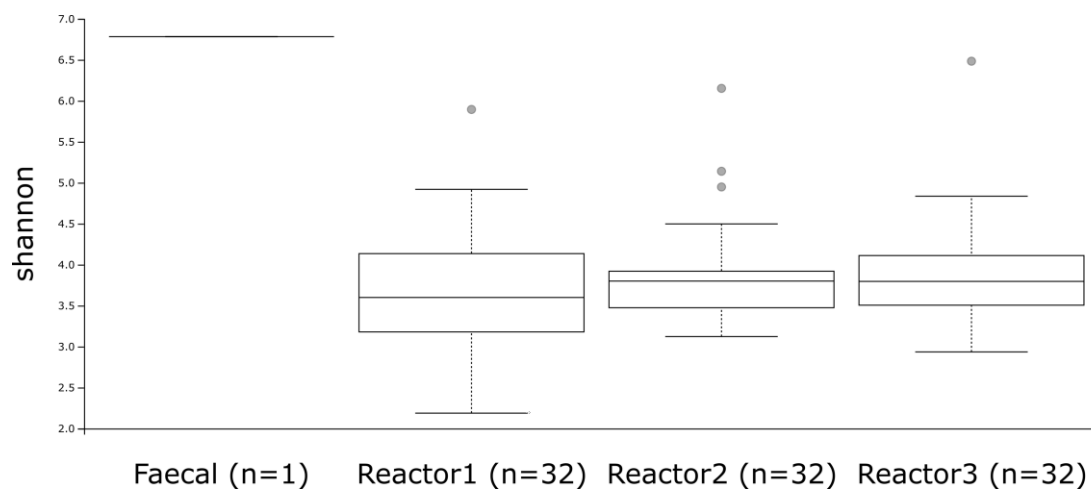
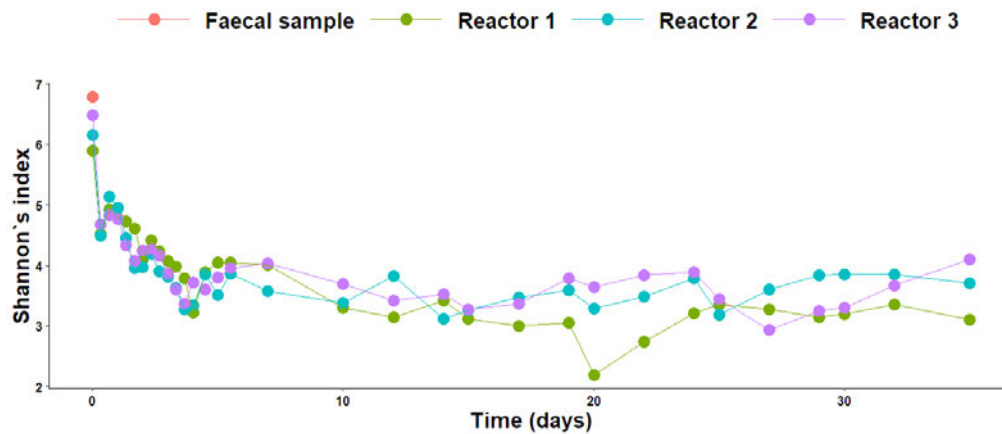
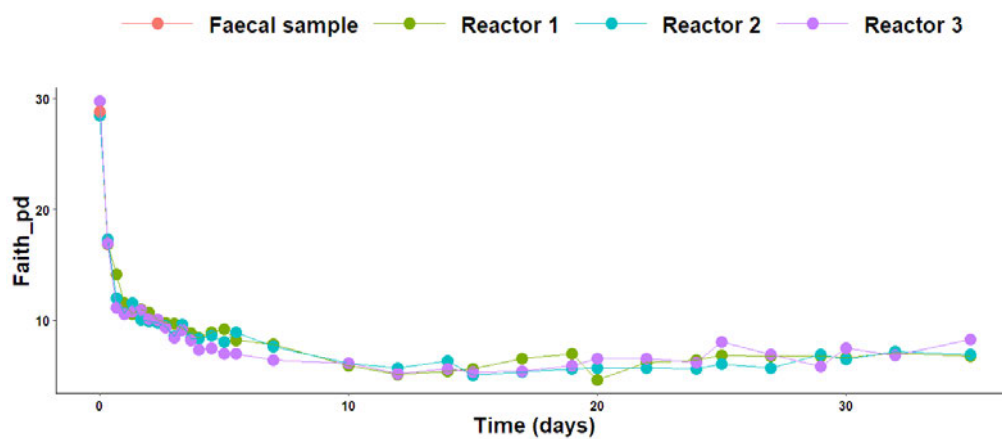


Figure 2.14. Association between the Shannon's index (measure of community richness and relative abundance) and the sample type (i.e. the faecal sample and the three reactors). The numbers in brackets indicate the number of comparisons used to analyse the significance. According to the Kruskal-Wallis (pairwise) test there was not statistically significant difference between the different samples, although this was considered an artifact in the case of the faecal sample, due to the difference in the number of replicates.

Shannon's index



Faith's phylogenetic diversity



Pielou's evenness index

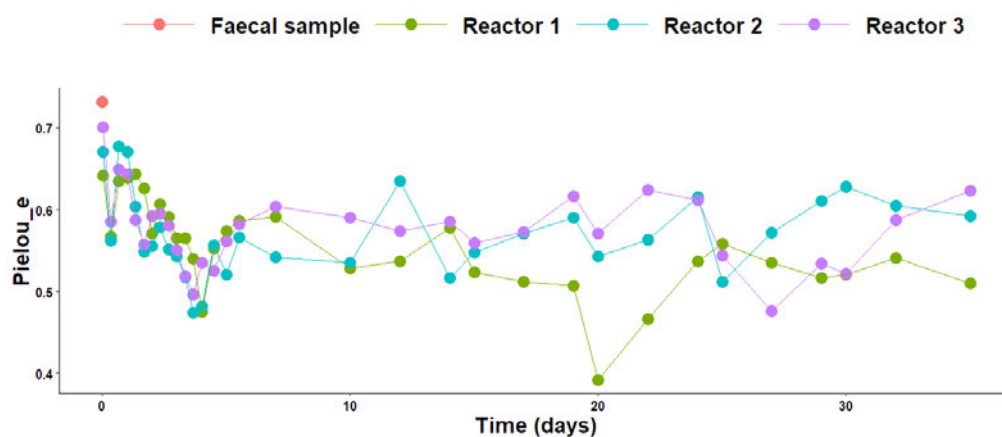


Figure 2.15. . Alpha diversity analysis; correlation between the sampling time and the three different α -diversity metrics. Shannon's index (measure of community richness and relative abundance); Faith's phylogenetic diversity (measure of community richness incorporating phylogenetic relationships between the features); Pielou's evenness index (species closeness in quantitative terms).

2.3.3.3 Diversity analysis; beta-diversity

Bray-Curtis dissimilarities and the weighted UniFrac distances were used to represent the between-sample diversity (β -diversity), and data were also represented through principal coordinates analysis (PCoA), which visualised data dissimilarities through summarising the variability in the dataset on the most significant orthogonal axes. Figure 2.16 and Figure 2.17 depict the β -diversity according to the Bray-Curtis dissimilarities, which quantify the compositional dissimilarities amongst different samples. In this case the PCoA plot in Figure 2.16 confirmed that the sampling time was an important correlation factor in shaping the structure of the community. This was also observed from the PCoA plot showing the weighted UniFrac, which also incorporated phylogenetic distances between the different species (Figure 2.18). The latter graph also delineated wider distances amongst the samples compared to the Bray-Curtis dissimilarities PCoA plot. In both the cases, the pairwise PERMANOVA test carried out showed no statistical differences between the reactor biological replicates (Figure 2.17, Figure 2.19 and Table 2.8), demonstrating that the three reactors behaved in a very similar way. As shown in Table 2.8, the difference between the faecal sample and the three reactors was statistically significant (p-value: ≤ 0.035) showing that, as expected, the microbial composition changed after the inoculation, but remained stable throughout the experiment.

Table 2.8. Pairwise permanova calculating the significance of the correlation between the distances from the Bray-Curtis dissimilarities and the weighted UniFrac and the different samples. The results show that the between-sample diversity was significant between the faecal sample and the reactor triplicates, but not between the three bioreactors, validating the biological triplicate as experimental method.

Sample		Pairwise permanova			
		Bray-Curtis		Weighted UniFrac	
		pseudo-F	p-value	pseudo-F	p-value
Faecal sample	Reactor A	3.47	0.035	3.94	0.035
	Reactor B	3.73	0.033	4.99	0.029
	Reactor C	4.13	0.025	5.78	0.03
Reactor A	Reactor B	0.84	0.469	0.92	0.433
	Reactor C	1.25	0.247	2.5	0.051
Reactor B	Reactor C	0.77	0.524	0.91	0.464

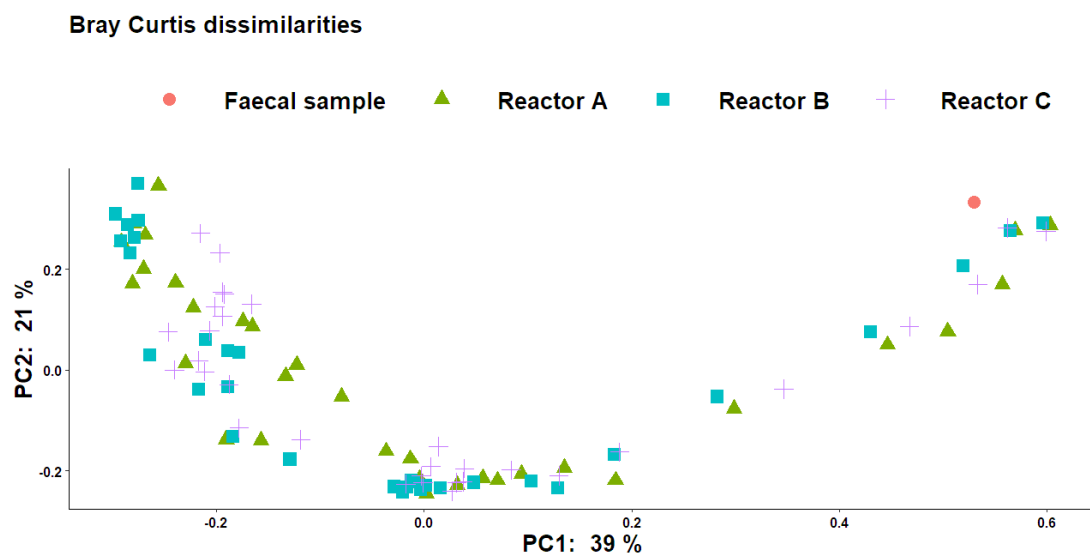


Figure 2.16. Principal coordinates analysis (PCoA) plot visualising the Bray-Curtis dissimilarities. Each axis indicates the amount of variation in it.

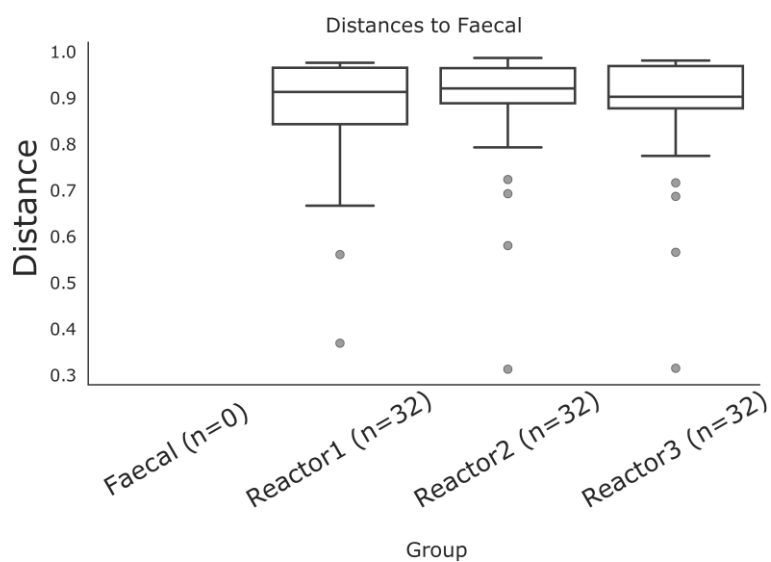


Figure 2.17. Distances between the different reactor replicates and the faecal samples according to the Bray-Curtis dissimilarities. Pairwise permanova was applied to test the significance of the distances here represented, which indicated significant differences between the faecal sample and the reactors, but not between the reactors.

Weighted UniFrac dissimilarities

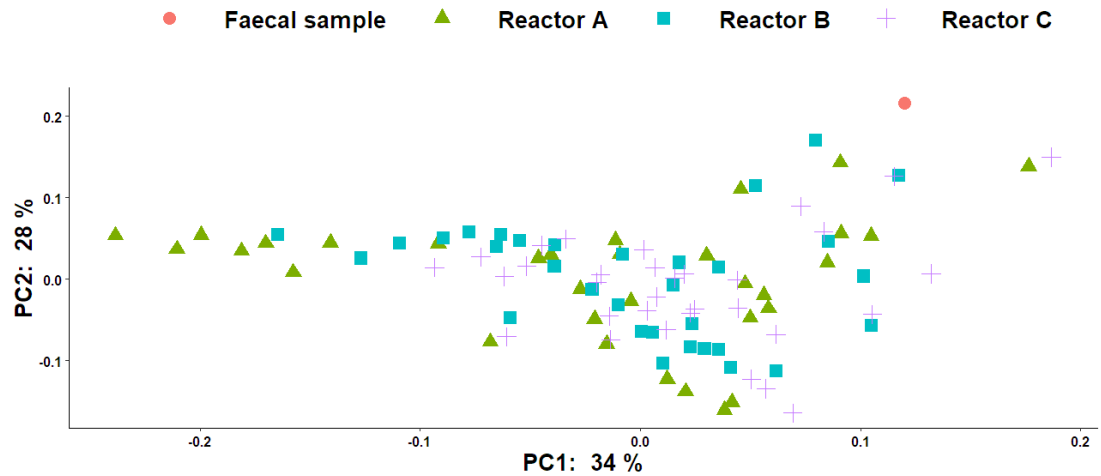


Figure 2.18. Principal coordinates analysis (PCoA) plot showing the weighted UniFrac dissimilarities, which took into account of the microbial communities' quantitative information and relatedness.

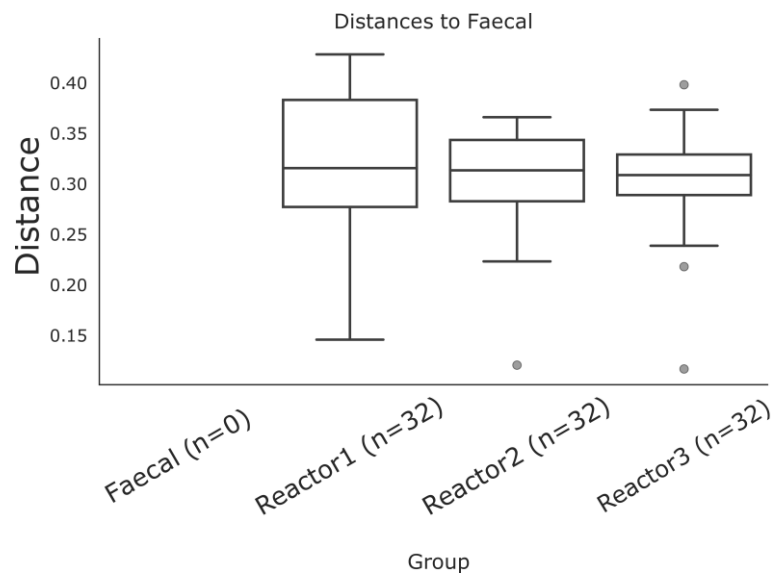
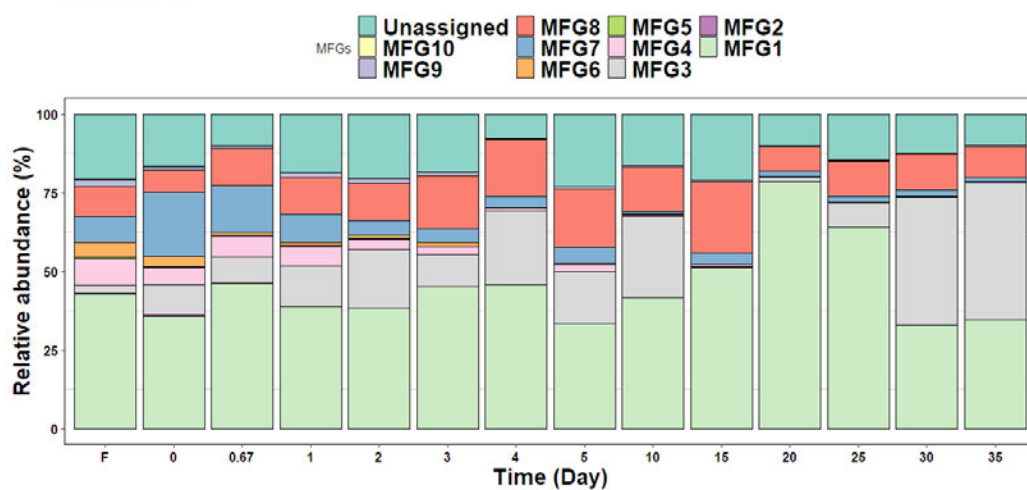


Figure 2.19. Distances between the faecal sample and the reactor replicates according to the weighted UniFrac. Pairwise permanova was applied to test the significance of the distances here represented, which indicated significant differences between the faecal sample and the reactors, but not between the reactors.

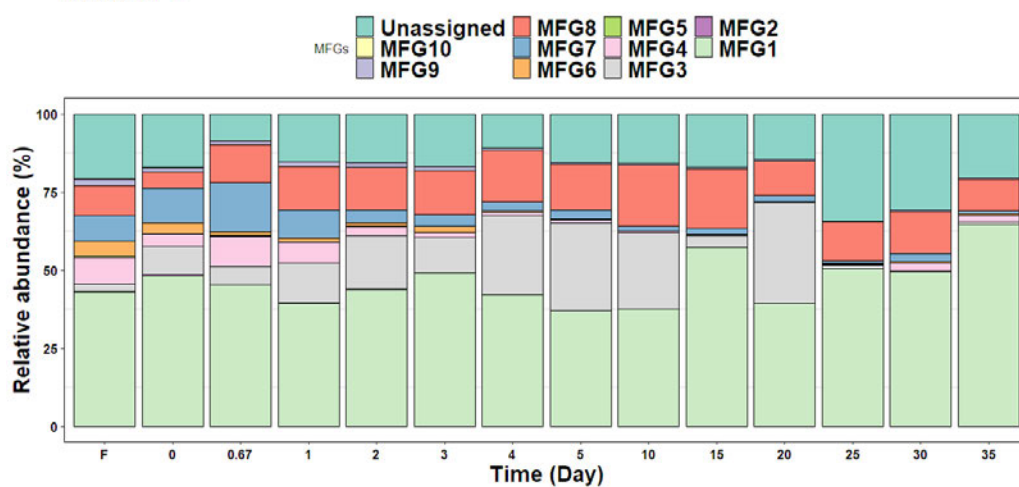
2.3.3.4 Assigning 16S rRNA gene sequencing taxonomy to MFGs

Figure 2.20 and Figure 2.21 depict MFG relative abundance inferred by assigning taxonomical sequencing data at species level (i.e. genus level when species level information was missing) to the 10 MFGs according to bacterial enzymatic pattern, substrate demand and metabolite production. The dominant group in the faecal sample was MFG (43%), followed by MFG8 (9.5%) MFG7 (8.4%), MFG4 (8%) and MFG6 (4.5%), MFG3 (2.3%), MFG9 (2%), MFG5 (0.6%), MFG2 (0.3%) and MFG10 (0.2%). Moreover 20% of species remained unassigned since the model developed so far did not include the totality of the gut species. On average (Figure 2.21), MFG1 remained the predominant group throughout the fermentation study, and whilst a pick was observed between day 15 and day 25 its average abundance was 46% at day 35. Most likely the average pick observed was due to a pick observed in reactor 1 during the same time interval. Indeed, MFG1 was 79% at day 20 in reactor 1 principally followed by MFG8 (8%) MFG7 (2%) and MFG3 (1.4%). It is worth to notice that whilst in reactor 1 at day 35 MFG3 abundance increased almost at the same level of MFG1, this was not observed in the other two reactors, where MFG3 abundance was ~4%. Apart from MFG5 abundance which increased towards day 35 in reactor 3, the behaviour of the remaining groups was similar in the three reactors. On average, at day 35 MFG1 was still the predominant group followed by MFG3 as seen above, MFG8 (11%), MFG5 (2.5%), MFG4 (1.4%), MFG7 (1%), MFG6 (0.76%), MFG9 (0.47%), MFG10 (0.07%) and MFG2 (0.00%). Average unclassified abundance at day 35 was 19.9%.

Reactor 1



Reactor 2



Reactor 3

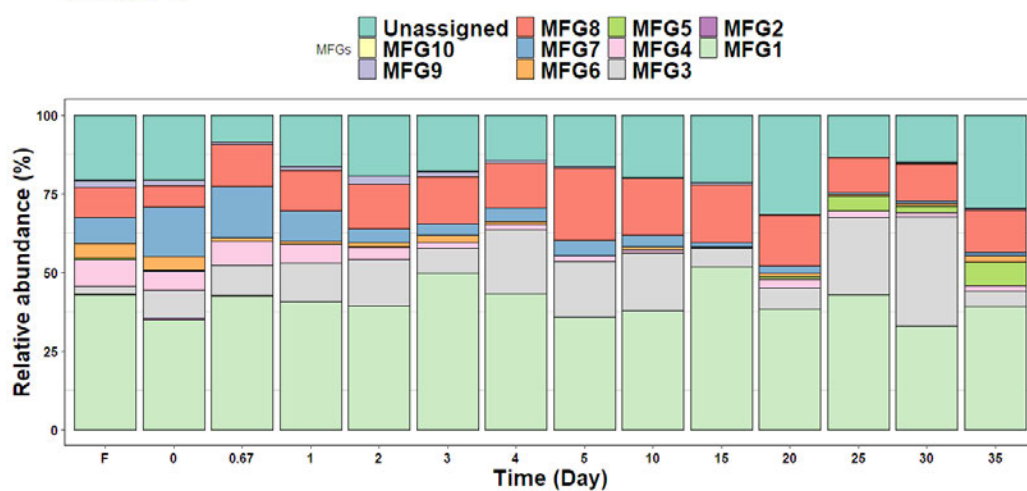


Figure 2.20. MFG relative abundance inferred by assigning sequencing data at species (or genus) level to the 10 MFGs according to bacterial enzymatic pattern, substrate demand and metabolite production. Unassigned species are depicted since the model developed so far does not include the totality of gut species. Data are presented individually for the three reactors in the three plots, respectively.

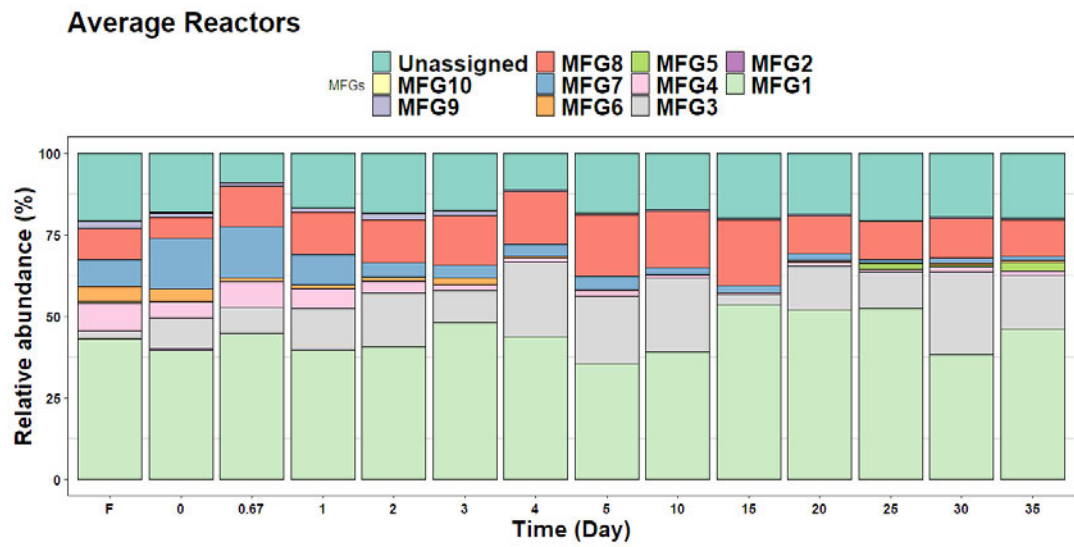


Figure 2.21. MFG relative abundance inferred by assigning sequencing data at species (or genus) level to the 10 MFGs according to bacterial enzymatic pattern, substrate demand and metabolite production. Unassigned species are depicted since the model developed so far does not include the totality of gut species. Data in the plot are presented as the average of the relative abundance calculated for the three reactors.

2.3.4 Quantitative polymerase chain reaction

Total bacterial and archaeal number was assessed through absolute quantification in qPCR using primer sets specific for the V3 region of the 16S rRNA gene or *mcrA* gene, respectively. Faecal sample and samples collected at days 0, 0.67, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30 and 35 of the fermentation were analysed. Higher sampling frequency during the first part of the fermentation was due to variations anticipated during the first five days based on WM model simulations ([Chapter 1](#)).

As depicted in Figure 2.22 and Figure 2.23 Log₁₀ cell number per ml of sample throughout the fermentation was characterised by important fluctuations throughout the fermentation for both bacteria and archaea. Bacterial Log₁₀ cell number per ml had a rather similar behaviour in the three reactors (Figure 2.22). Indeed, logarithmic bacterial concentration was 9.67 in the faecal sample and after an initial decrease in all reactors towards ~9.22, it showed a positive trend remaining constant, albeit mentioned fluctuations, to an average value of ~9.5 throughout the fermentation. Bacterial concentration in reactor 1 was somewhat lower than in the other two reactors, in fact it seemed to decrease differently from the rest of the replicates after day 3 and more evidently after day 20 towards the end of the fermentation.

Archaea had as expected lower concentration than bacteria both in the faecal sample and throughout the fermentation (Figure 2.23). Although values fluctuated throughout the *in vitro* fermentation as described above, archaeal concentration showed generally a negative trend in the first 15 days, passing from 4.7 Log₁₀ cell number in the faecal sample to average 3.9 at day five, with lowest value observed in reactor 3 of 3.32. Interestingly, after reaching its lowest average point (3.57), archaeal concentration increased from day 15 towards the end of the experiment with final Log₁₀ concentration of 5.35. Moreover, a pick at day 25 in reactor 1 of 5.9 was observed, differently from what observed in the other two reactors where the logarithmic concentration remained somewhat constant at 4.3.

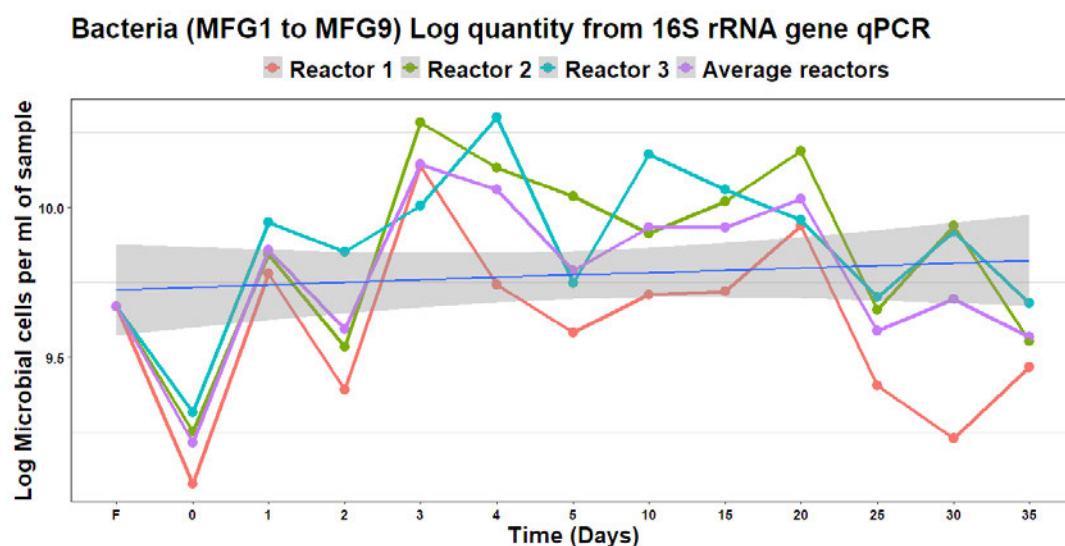


Figure 2.22. Log bacterial cell number per ml of sample as quantified through qPCR after comparison with an 8-point standard curve. Data from the three reactors and average observations for faecal and bioreactor samples are depicted in different colours. Linear regression calculated for average observations is depicted by a blue continuous line, whilst the grey area surrounding it represents the 95% confidence interval.

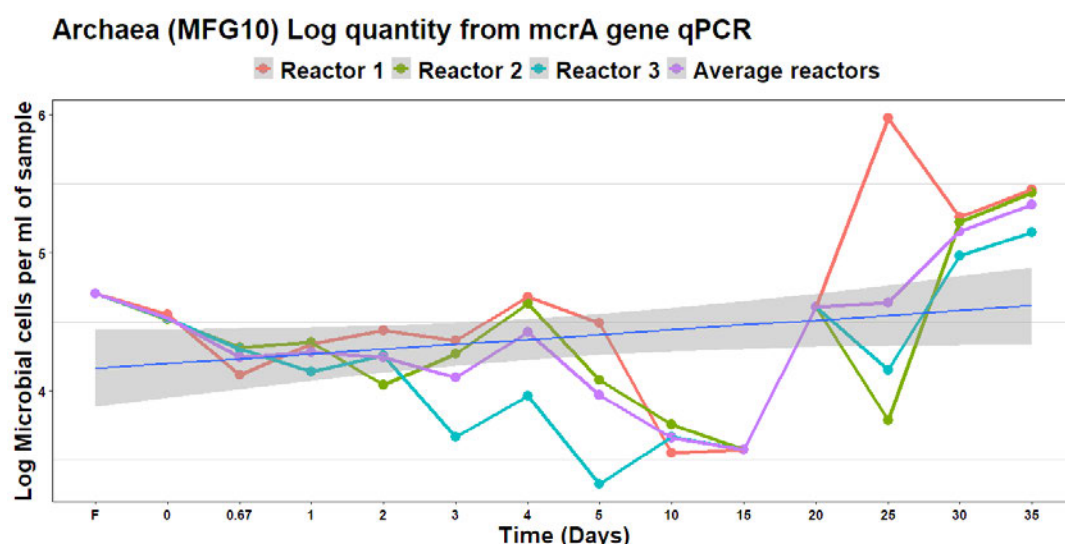


Figure 2.23. Log archaeal cell number per ml of sample as quantified through qPCR after comparison with an 8-point standard curve. Data from the three reactors and average observations for faecal and bioreactor samples are depicted in different colours. Linear regression calculated for average observations is depicted by a blue continuous line, whilst the grey area surrounding it represents the 95% confidence interval.

2.3.5 Combining qPCR and sequencing data to infer MFG concentration throughout the fermentation study

The main aim of the *in vitro* continuous fermentation described in this chapter was to generate observations later used to compare model predictions ([Chapter 3](#)). MFG relative abundance and total bacterial/archaeal concentration were calculated as shown above, using sequencing analysis and qPCR. Here, in order to generate similar output to the model, MFG abundance inferred through bioinformatic analysis was used to calculate MFG concentration (g/l) taking into consideration total bacterial or archaeal concentration (g/l) inferred from qPCR.

Figure 2.24 depict MFG inferred concentration in the faecal sample and in the three reactors throughout the fermentation, whereas Figure 2.25 shows average MFG inferred concentration. Total MFG concentration in the faecal sample was 21.4 g/l, which also included 4.43 g/l of unassigned species, as described above. Variations between MFG and concentration were the same as the ones described above. In summary, total MFG concentration fluctuated in a particular way in the three reactors, with a rapid decrease soon after inoculation (total 7.75 g/l at day 0). A pick in concentration seemed to be observed in all reactor at day 3 and 4, during which average concentration was ~65 g/l. Again, lower total MFG concentration was noticed in reactor 1 from day 25 to day 35.

In general, MFG1 was predominant in the faecal sample (9.25 g/l), whilst MFG8, MFG7 and MFG4 had a concentration of 2.04g/l, 1.81g/l and 1.80g/l, respectively. MFG6 (0.96g/l), MFG3 (0.49g/l), MFG9 (0.44g/l), MFG5 (0.14g/l), and MFG2 (0.06g/l) were less present in the faecal sample. As described above, each MFG followed particular dynamics during the fermentation and at day 35, MFG1 was still the most abundant one with an average concentration of 8g/l. On average at day 35, concentration of MFG3, MFG4, MFG5, MFG6, MFG7, MFG8 and MFG9 was 2.85g/l, 0.25g/l, 0.44g/l, 0.13g/l, 0.20g/l, 1.93g/l and 0.08g/l, and whilst MFG2 tended to 0g/l, MFG10 concentration was 0.09g/l.

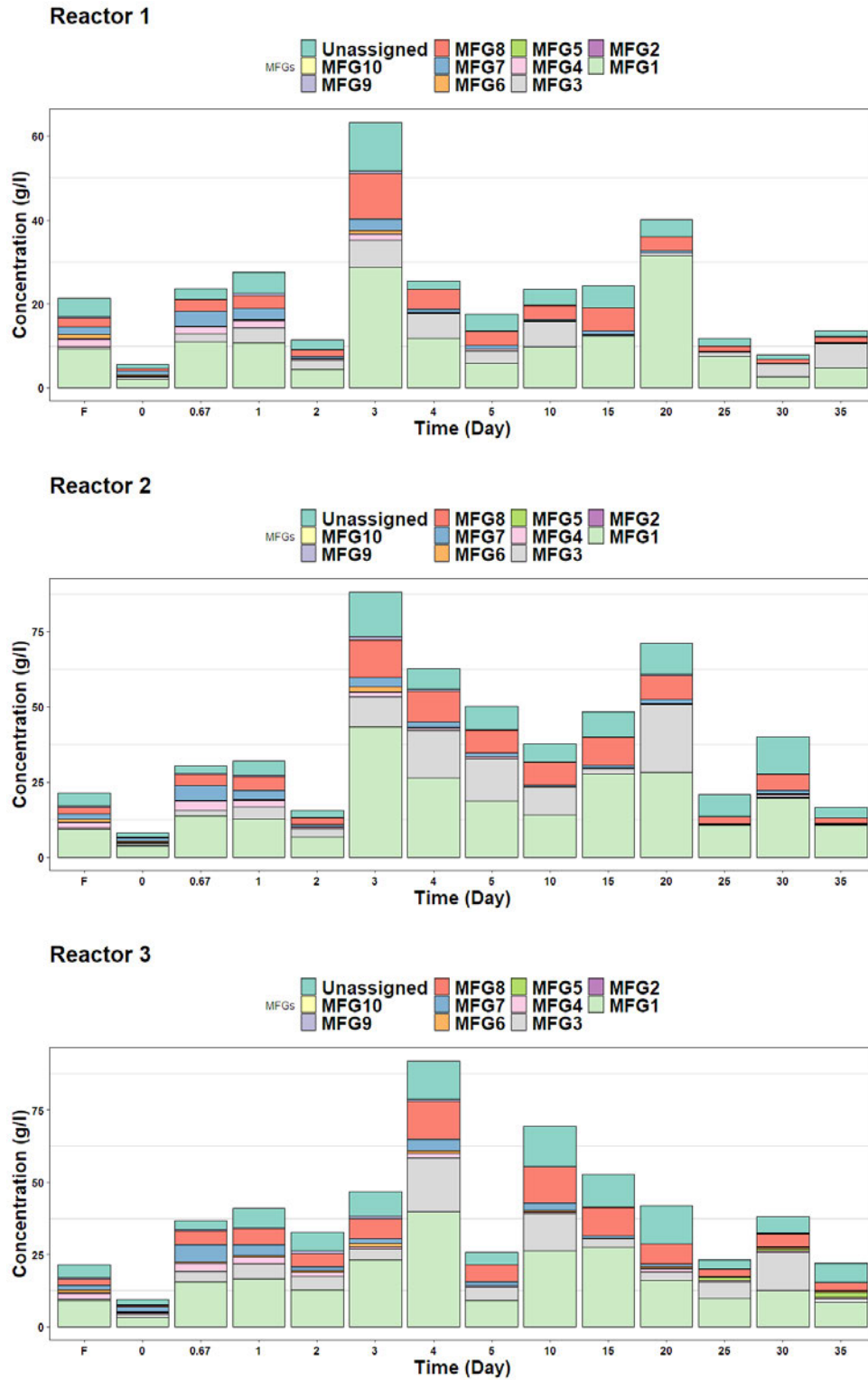


Figure 2.24. MFG concentration (g/l) observed in the faecal sample and in the three reactors throughout the *in vitro* fermentation. Concentration values were inferred calculating the percentage abundance of each MFG on the total bacterial or archaeal concentration. MFG1 to MFG9 concentrations were based on bacterial qPCR, whereas MFG10 concentration was based on archaea qPCR. The model developed so far does not include the totality of the gut species, therefore a certain concentration of unassigned bacteria is depicted in the graphs.

Average reactors

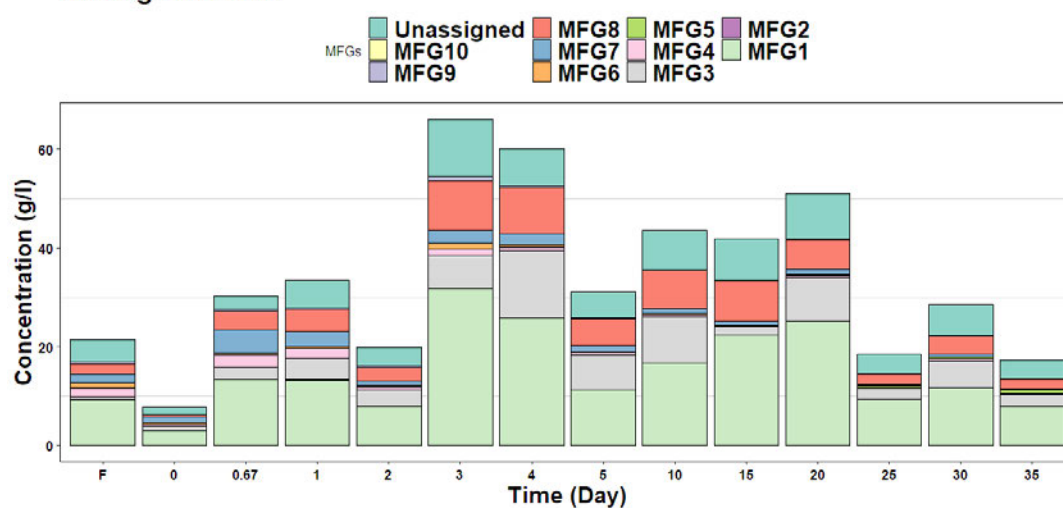


Figure 2.25. Average MFG concentration (g/l) observed in the faecal sample and in the three reactors throughout the *in vitro* fermentation. Concentration values were inferred calculating the percentage abundance of each MFG on the total bacterial or archaeal concentration. MFG1 to MFG9 concentrations were based on bacterial qPCR, whereas MFG10 concentration was based on archaea qPCR. The model developed so far does not include the totality of the gut species, therefore a certain concentration of unassigned bacteria is depicted in the graphs.

2.4 Discussion

A continuous fermentation experiment was set up triplicate and run for 35 days simulating porcine proximal colon pH, temperature, content composition and dilution rate. The main aim of this *in vitro* experimentation was to reproduce the environment modelled in [Chapter 1](#) and therefore generate observations, i.e. both SCFA and MFG concentration over time that were later used to compare and validate the model in [Chapter 3](#). Both the bioreactor and incubator were designed and assembled specifically for this project and were rather stable throughout the *in vitro* fermentation, ensuring constant temperature, constant dilution rate, constant pressure and anaerobiosis.

The bioreactors were inoculated with a faecal sample pooled from five different 5-month old animals housed in the same pen. It is acknowledged here that the pooled sample could have led to an artificial increase in microbial diversity, and although it could have biased the elucidation of microbial composition at an individual animal level, microbial ecology of individual pig-microbiota was not the first focus of this project. Indeed, the main aim of this PhD project to develop a mathematical model of the porcine gut microbiota, whose microbial starting composition can be set at every simulation as described in the previous chapter. The fermentation experiment presented here was used to recreate *in vitro*, microbial dynamics of a population which could be found in the porcine gut, and although possibly not representative of the diversity of a singular animal was still assumed to be representative of porcine faecal microbial species. The choice to produce a pooled faecal sample was due to the amount of faecal material needed and the collection procedure. To avoid contamination with environmental microbes, only the top portion of naturally excreted faeces was collected, using a sterile spoon, directly from the pen. Due to the sampling procedure, only a certain amount of faecal material could be collected from each animal, hence the need to pool more samples together to produce a quantitatively functional inoculum. Future studies using the same model could advantage by being based on microbial communities derived from samples collected by one animal, should this be required. In general, microbial composition of faecal sample was found to be in line to what described in literature (De Rodas et al., 2018; H. B. Kim et al., 2011; Kubasova et al., 2018; Tanner et al., 2014).

OD₆₀₀ and pH were measured daily and although technically demanding due to lack of automatization, pH was generally maintained in a range comprised between 6.0 and 6.8, as observed in the porcine proximal colon *in vivo* (Kararli, 1995; Tanner et al., 2014). pH was more stable especially after day 12 due to a better management of pCO₂ and bacterial growth effect on H⁺ concentration. Indeed, pH values were rather constant in the three reactors between day 12 and day 35, whilst more variations tending towards lower values were observed in the first 12 days. OD₆₀₀ showed constant lower values after day 12, which were not confirmed by findings from sequencing and qPCR. Indeed, Shannon's index was rather constant from day 5 onwards showing a pseudo-steady state, whilst qPCR showed that

bacterial communities proliferated better after day ~10. It is worth to notice that higher pH values from day 12 seemed to be correlated to lower OD₆₀₀, although results from other analyses during the same interval indicated that the former could have been due to a technical artifact. Whether pH had a positive influence on bacterial growth during this part of the experimentation was hard to establish. Nevertheless, a direct relationship between more stable and optimal pH of predominant genera like *Prevotella* from day 12 to day 35 cannot be excluded.

OD₆₀₀, α - and β -diversity, inferred MFG concentration and qPCR confirmed an initial decrease of bacterial concentration soon after inoculation, which was expected due to part of the microbial community not being able to proliferate *in vitro*. Although not statistically significant according to Kruskal-Wallis test performed on α -diversity metrics, ~40 % reduction observed from faecal to bioreactor samples could be argued as a biologically relevant due to differences in replicate number between the reactors and the faecal sample. On the other hand, β -diversity analysis revealed statistically significant differences between faecal sample and reactors but not between the three reactors, meaning that microbial community varied quantitatively and qualitatively from the inoculum towards the end of the experiment, whilst changing in a consistent way in the three biological replicates. The prevalence of some microbial species due to the new *in vitro* dynamics was expected, in fact for example species with growth rate lower than the dilution rate would have been washed out from the system. Data also supported the view that species able to consume protein were more likely to predominate *in vitro* (e.g. MFG1).

Observed acetate, propionate, and butyrate most likely reflected changes observed in the microbial communities throughout the experiment. Indeed, generally higher values were observed during the first five days for the three metabolites, whilst their concentration was somewhat stable from day 10 until day 35. Generally, acetate was the most abundant molecule, as expected due to its presence as a metabolite amongst most MFG metabolic pathways. Groups like MFG1 and MFG7 were likely to directly influence propionate concentration, which was almost always higher than butyrate concentration, due to the less abundant MFG5, MFG6 and MFG8. Acetate peak (~80mM) in all the reactors at day ~3 was most likely due to MFG1, MFG3 and MFG9 increased relative abundance observed during the same interval. MFG1 together with MFG7 abundance could also explain propionate peak observed around day 3. Despite to an almost constant ratio, butyrate concentration was observed to be higher than propionate at day 15 in reactor 1, from day 20 to day 25 in reactor 2, and at day 20 in reactor 3. It is interesting to note that during the same time points it was observed an increased butyrate producers' abundance, especially MFG8 and partially MFG5. Whereas MFG1 and MFG7 concentration decreased at the same time, possibly connected to the drop in propionate concentration observed.

All analyses performed seemed to indicate a sudden change in dynamics at day 20, especially in reactor 1. In particular, diversity and more evidently evenness index decreased importantly at day 20 in reactor 1, indicating the likelihood proliferation of dominant species. Inferred MFG concentration and relative abundance seemed to confirm this, in fact albeit total bacterial concentration remained similar to the other two reactors, MFG1 reached ~75% of the total amount followed by MFG8, MFG7 and MFG3, which were 8%, 1,7% and 1.4% of the total, respectively. This was different from the other two reactors, in which evenness index remained somewhat constant to 0.6. Acetate, propionate and in minor part butyrate had a pick at day 20 in reactor 1, reflecting the findings described above. Whether this was caused by local differences in temperature, pH or medium composition is hard to establish, nevertheless, it was worth to notice how the resilience of the community led to a normalisation of the microbial species, which could be observed by day 25 of the experiment.

Part of the microbial gut species remained unassigned due to the structure of the model developed so far, and therefore their concentration and abundance was reported throughout the fermentation. Amongst the groups that tended to 0 at the end of the experiment MFG4 could be explained by *Streptococcus spp.* and *Lactobacillus spp.* concentration which tended to zero according to 16S rRNA gene sequencing. *Lactobacillus spp.* seems to be ubiquitous *in vivo* (Holman et al., 2017; H. B. Kim et al., 2011), although its absence *in vitro* at the end of the fermentation could be explained with competition with members of other MFGs and also with the fact that *in vivo* the genus is probably supplied from the stomach, whose contribution was obviously missing *in vitro* (Xiao et al., 2018). Acetate:propionate:butyrate ratio detected during this study (49:31:20) was found to be somewhat different from what usually observed *in vivo* (60:25:15) (Tanner et al., 2014), especially with regards to the lower acetate concentration in the reactors. However, similar ratios were described by other authors using different kind of substrates, with both colon or faecal inocula (Ding et al., 2015; Lin et al., 2011; Tanner et al., 2014). In some cases, differences in SCFA pattern could be explained with different microbial ratio in the inoculum, although due to lack of information this is not always comparable with literature data. For example, different microbial dynamics were observed in their experiment by tanner et al. (2014), where *Acidaminococcaceae*, *Spirochaetaceae*, *Erysipelotrichaceae*, and *Veillonellaceae* concentration were lower than what found in the microbial population described here.

In conclusion, an *in vitro* continuous fermentation system was designed, built and used to mimic porcine proximal colon dynamics, in order to reproduce the environment and predictions simulated by the model presented in the previous chapter. The system allowed constant sample collection and analysis of the bacterial communities and their metabolites, which had a similar trend in the three biological replicates used *in vitro*. The concentration of the major metabolites and the inferred MFG concentration over time were used to compare the model outputs and to proceed with the parameter fitting as described in the following chapter.

3 Parameter fitting and model comparison with observations from *in vitro* continuous fermentation

3.1 Introduction

Model validation is the process of determining the degree to which a model is an accurate representation of the real world, which usually relies on the comparison between the model predictions and the observations of a system mimicking the same environment described by that model (Urbina et al., 2005). Uncertainty is part of the modelling process as in most cases the phenomena described *in silico* are largely unexplored (i.e. measured) in reality (Chatfield, 1995). The constant parameters on which a mathematical model is based are most of the times unknown or difficult to measure. Thus, one essential step in the modelling process is model calibration, which employs fitting model to data to achieve parameter estimation (Soetaert and Petzoldt, 2010; Vyazovkin and Wight, 1999). The validation process could comprise the four steps of validation, calibration, comparison and prediction, during which data from an *in vitro/in vivo* experimentation could be used in a reverse modelling procedure (Soetaert and Petzoldt, 2010; Urbina et al., 2005). To fit the model to data during the validation, it is pivotal that the observations are collected from a system sharing the same experimental conditions modelled, and that the state variables are accurately quantified in order to achieve the best comparison (Stein et al., 2013). Thus, a major effort was employed during this project to recreate the same environment found in the porcine proximal colon, as described in [Chapter 2](#).

The model of the whole pig gut microbiota (WM) presented in [Chapter 1](#) was able to simulate fermentation studies found in literature, and to reproduce the SCFA pattern observed *in vivo* and *in vitro* when starting microbial concentration was set according to literature data. This chapter presents three different analysis:

- Comparison between model predictions (i.e. as parametrised in [Chapter 1](#)) and *in vitro* observations (i.e. from [Chapter 2](#) fermentation). MFG and SCFA concentrations over time collected through the *in vitro* continuous fermentation presented in [Chapter 2](#) were compared to model outputs. Thus, closeness between observations and predictions was quantified through root mean square error (RMSE), which served as an indication of model performance.
- Re-parametrisation through reverse modelling based on *in vitro* observations from [Chapter 2](#) and RMSE assessment comparing re-parametrised model predictions to *in*

vitro observations. To improve performance, *in vitro* observations were used through reverse modelling to assign new values to K (half saturation constant), being the parameter with the highest level of uncertainty. RMSE was calculated again assessing closeness between re-parametrised model predictions and *in vitro* observations from [Chapter 2](#).

- Multiple simulations (i.e. 100) with 5 strains per MFG with stochastically varying parameter values for all strains in each simulation. This analysis allowed to assess SCFA distribution analysis amongst the 100 simulations, RMSE between best-fit simulation predictions and *in vitro* observations, mean trait value to analyse strain dynamics through the study of their parameter dynamics.

In general, model performance and accuracy in depicting *in vitro* values increased after re-parametrisation. Indeed, the re-parametrised model described accurately the dynamics of the MFGs and the main metabolites.

3.2 Materials and methods

3.2.1 Comparison between model predictions before re-parametrisation and observations from *in vitro* continuous fermentation ([Chapter 2](#))

The model developed so far and described in [Chapter 1](#) was used to simulate the continuous fermentation experiment described in [Chapter 2](#). As described in the previous chapter, microbial and therefore MFG concentration in both faecal and bioreactor samples was inferred by combining qPCR and 16S rRNA gene sequencing data analysis. Therefore, as depicted in Table 3.1, MFGs starting concentration was modelled based on MFG concentration inferred for the faecal sample. It is important to notice that Unassigned concentration (4.43g/l in the faecal sample) was not included in the model albeit being part of the total bacterial concentration in the faecal sample (21.43g/l) as inferred in the previous chapter. The *in vitro* continuous fermentation was carried out for 35 days and therefore the *in silico* simulations were also carried out for a period of 35 days. The *in vitro* flow out set for the peristaltic pump was 26 ml/h, whereas the volume of the main vessel was 234ml, therefore the *in silico* dilution rate (D) was set to 2.67 d^{-1} in the model. D settings were based on porcine proximal colon retention time of 0.375 days (Latymer et al., 1990; Tanner et al., 2014), with D being the reciprocal of the retention time. Substrate starting concentration was modelled based on medium composition used *in vitro*, whereas pH was set to 6.25 as the average pH value recorded throughout the *in vitro* continuous fermentation (Table 3.1). The model was solved using the package microPop in R (<https://CRAN.R-project.org/package=microPop>) (Kettle et al., 2018). Data frames 'Rmat' (for resources) and 'Mmat' (for microorganisms) were implemented in microPop with values depicted in Table 3.1. Both H_2O and CO_2 were considered not limiting in the model, due their role in anaerobic media preparation.

Closeness between observations and predictions was calculated using the RMSE as follows:

$$\sqrt{\frac{\sum_{v=1}^n (x_v - y_v)^2}{n}}, \quad (3-1)$$

With v being the various data points *in silico* or *in vitro* and x and y being predictions and observations, respectively.

Table 3.1. Starting conditions modelled to simulate a continuous fermentation experiment.

MFG	Starting concentration (g/l)	Resource	Starting concentration (g/l)
1	9.25	NSP	7
2	0.06	RS	4.32
3	0.49	Sugars	2
4	1.80	Protein	15
5	0.14		
6	0.96		
7	1.81		
8	2.04		
9	0.44		
10	0.00233		
		pH (constant value <i>in silico</i>)	
		6.25	

3.2.2 Re-parametrisation and comparison between re-parametrised model predictions and *in vitro* observations

The model described in the previous section (3.2.1) with settings depicted in Table 3.1 was run 300 times with 10 strains per MFG. At the beginning of each simulation K value for each of the strains amongst the 10 MFGs was stochastically changed in a biologically compatible manner (Kovarova-Kovar and Egli, 1998) through the R code depicted in Figure 3.1. This code generated 300 model outputs, which albeit had the same starting concentrations, had different SCFA and MFG output concentrations due to different K values for the composing strains.

RMSE was measured between SCFA and MFG predictions from each of the 300 runs and observations from the *in vitro* continuous fermentation, which allowed to identify a best-fit simulation. The latter had lowest average RMSE value for the measured variables, indicating high level of similarity between *in silico* predictions and *in vitro* observations. Thus, K values for the most abundant strains amongst the 10 MFGs in the best-fit simulation were used to re-parametrise the model (Table 3.2). As a result, the re-parametrised model had new K values for MFG3, MFG4, MFG5, MFG7 and MFG8, whereas K did not change for the remaining MFGs due to negligible differences between old and new values before and after re-parametrisation through reverse modelling using 300 simulations.

The re-parametrised model was further used to simulate again the *in vitro* fermentation (i.e. one-strain simulation) using the previously described settings (Table 3.1), in this way RMSE was calculated again assessing closeness between observations and predictions of the re-parametrised model.

Table 3.2. *K* values before and after parameter fitting through reverse modelling as detailed in the text. The table depicts parameter values only for MFG whose *K* changed after re-parametrisation. As described in the text, after reverse modelling *K* values changes for other MFG was negligible and therefore it was not changed in the re-parameterised model.

MFG3					
Old <i>K</i> values (g/l)			New <i>K</i> values (g/l)		
NSP	RS	Sugars	NSP	RS	Sugars
0.00001	0.000017	0.00012	0.000265	0.000171	0.000115
MFG4					
Old <i>K</i> values (g/l)			New <i>K</i> values (g/l)		
NSP	RS	Sugars	NSP	RS	Sugars
0.000061	0.000038	0.000023	0.000122	0.000125	0.000230
MFG5					
Old <i>K</i> values (g/l)			New <i>K</i> values (g/l)		
NSP	RS	Sugars	NSP	RS	Sugars
0.000276	0.000209	0.000119	0.000907	0.000795	0.000198
MFG7					
Old <i>K</i> values (g/l)			New <i>K</i> values (g/l)		
NSP	RS	Sugars	NSP	RS	Sugars
0.000495	0.000324	0.000400	0.000165	0.000324	0.0004
MFG8					
Old <i>K</i> values (g/l)			New <i>K</i> values (g/l)		
NSP	RS	Sugars	NSP	RS	Sugars
0.001056	0.000311	0.000390	0.000391	0.000466	0.00039

```

startRun=1
numRuns=300

oumicroPopModel(
  microbeNames = c('MFG1','MFG2','MFG3','MFG4','MFG5',
                    'MFG6','MFG7','MFG8','MFG9','MFG10'),
  times=seq(0,35,1),
  resourceSysInfo = data.frame(PigCompleteRmat,stringsAsFactors = FALSE),
  microbeSysInfo = data.frame(PiCompleteMmat,stringsAsFactors = FALSE),
  numStrains=10,
  #oneStrainRandomParams=TRUE ,
  pHLimit=TRUE,
  pHVal=6.25,
  strainOptions = list(randomParams=c('halfSat'),
                        seed=run,
                        distribution='uniform',percentTraitRange=20,
                        tradeOffParams=c('halfSat'),applyTradeOffs=FALSE),
  checkingOptions = list(reBalanceStoichiom=TRUE),
  plotOptions = list(plotFig=FALSE,sumOverStrains=FALSE,
                      yLabel='Concentration (g/L)',xLabel='Time (h)')
)

```

Figure 3.1. Programming code used to generate 300 simulations in R. Each of the simulations had 10 strains per MFG, and at the beginning of each of them K values stochastically varied in a biologically compatible range (Kovarova-Kovar and Egli, 1998).

3.2.3 Multiple simulations with five strains per MFG

After the single-strain comparison between observations and predictions, as described in the previous section, the re-parametrised model was used in 100 simulations, with each of them having 5 strains per MFG and stochastically generated parameter values at the beginning of each simulation. The code used to generate 100 simulation is depicted in Figure 3.2, where the input arguments “numStrains”, “percentTraitRange” in the “microPopModel” function, were set to respectively 5 and 20, to increase the number of strains accordingly and to randomly generate the μ_{max} , K , Y and pH trait values in the $\pm 20\%$ range from their original average value. The latter was based on biological compatible parameter values (Kovarova-Kovar and Egli, 1998). The 100 5-strain simulations were used here to assess the impact of parameter variation on main metabolite outputs through distribution analysis, to measure the performance of a 5-strain model through RMSE assessment and to analyse competition dynamics between different strains through mean trait value assessment.

3.2.3.1 Main metabolite distribution analysis

Acetate, propionate, butyrate and lactate concentrations output of the model from each of the 100 simulations were used to calculate distribution through “hist()” function in R.

3.2.3.2 RMSE assessment between best-fit simulation predictions and in vitro observations

RMSE between observations and best-fit simulation predictions was calculated using Equation (3-1).

3.2.3.3 Mean trait value assessment

Mean trait value (z^{av}) was calculated as follows:

$$z^{av} = \frac{\sum_{j=1}^{N_s} X_j Z_j}{\sum_{j=1}^{N_s} X_j}, \quad (3-2)$$

where, X_j is the concentration of the MFG j associated with its trait Z_j .

```
startRun=1
numRuns=100

out<-microPopModel(
  microbeNames = c('MFG1','MFG2','MFG3','MFG4','MFG5',
                   'MFG6','MFG7','MFG8','MFG9','MFG10'),
  times=seq(0,35,1),
  resourceSysInfo = data.frame(PigCompleteRmat,stringsAsFactors = FALSE),
  microbeSysInfo = data.frame(PiCompleteMmat,stringsAsFactors = FALSE),
  numStrains=5,
  #oneStrainRandomParams=TRUE ,
  pHLimit=TRUE,
  pHVal=6.25,
  strainOptions = list(randomParams=c('halfSat','maxGrowthRate','yield','pHtrait'),
                       seed=run,
                       distribution='uniform',percentTraitRange=20,
                       tradeOffParams=c('maxGrowthRate','halfSat'),applyTradeoffs=TRUE),
  checkingOptions = list(reBalanceStoichiom=TRUE),
  plotOptions = list(plotFig=FALSE,sumOverStrains=FALSE,
                    yLabel='Concentration (g/L)',xLabel='Time (h)')
)
```

Figure 3.2. . Programming code used to generate 100 simulations in R. Each of the simulations had 5 strains per MFG, and at the beginning of each of them μ_{max} , K , Y and pH preference were stochastically varied in a biologically compatible range (Kovarova-Kovar and Egli, 1998).

3.3 Results

3.3.1 Comparison between model predictions before re-parametrisation and observations from *in vitro* continuous fermentation ([Chapter 2](#))

The model developed so far and described in [Chapter 1](#) was used to simulate the continuous fermentation experiment described in [Chapter 2](#) using the experimental conditions summarised in Table 3.1. Acetate, propionate, butyrate concentration, together with the concentration of the 10 MFGs predicted throughout the simulation were compared with the observations collected during the *in vitro* experimentation and their closeness was calculated through RMSE using Eq. (3-1).

In general, predictions generated by the model at this stage were not entirely in line with the observations collected during the *in vitro* continuous fermentation (Figure 3.3 and Figure 3.4). The least level of similarity between predictions and observations was noticed for butyrate producers and therefore for butyrate. Indeed, MFG5 and MFG6 seemed to decrease towards 0 at ~day 2 in the model whilst their concentration was almost constant apart for a MFG6 pick at day3 throughout the *in vitro* fermentation. Although slightly higher, predicted MFG8 concentration was still lower than observations. RMSE confirmed the dissimilarities between predictions and observations with a value of ~18 for butyrate producers (Table 3.3). Consequently, RMSE for butyrate was 26.34, underlying differences between *in vitro* and *in silico* data, which were characterised by a rapid decrease in butyrate concentration towards 0mM. *In silico* propionate contribution was mainly due to MFG1, which had the lowest RMSE measured at this point, together with MFG3. MFG7 concentration predicted by the model was ~0 at steady state, contrarily to what observed *in vitro*. Nevertheless, thanks to MFG1 similarities with the continuous fermentation, predicted propionate dynamics were similar to observations, with the lowest RMSE amongst the measured metabolites. The remaining MFGs tended to 0g/l at steady state, in fact the dominant groups at day 35 *in silico* were MFG1 and MFG3. On the other hand, acetate concentration was overestimated by the model (~90mM Vs ~50mM), which resulted in a RMSE of 40. Since the model developed so far did not include all species present in the porcine gut, unassigned concentration was detected throughout the *in vitro* observations ([Chapter 2](#)), although unassigned were not included in the model and therefore their concentration remained 0 throughout the *in silico* experimentation (Figure 3.4).

Table 3.3. RMSE (Equation (3-1) calculated between observations collected during the *in vitro* continuous fermentation ([Chapter 2](#)) and predictions generated by the model parametrised so far ([Chapter 1](#)).

SCFA	RMSE	MFG	RMSE
Acetate	39.48	1	15.28
Propionate	17.42	2	18.09
Butyrate	26.34	3	15.35
		4	17.98
		5	18.08
		6	18.04
		7	18
		8	17.96
		9	18.06
		10	18.09

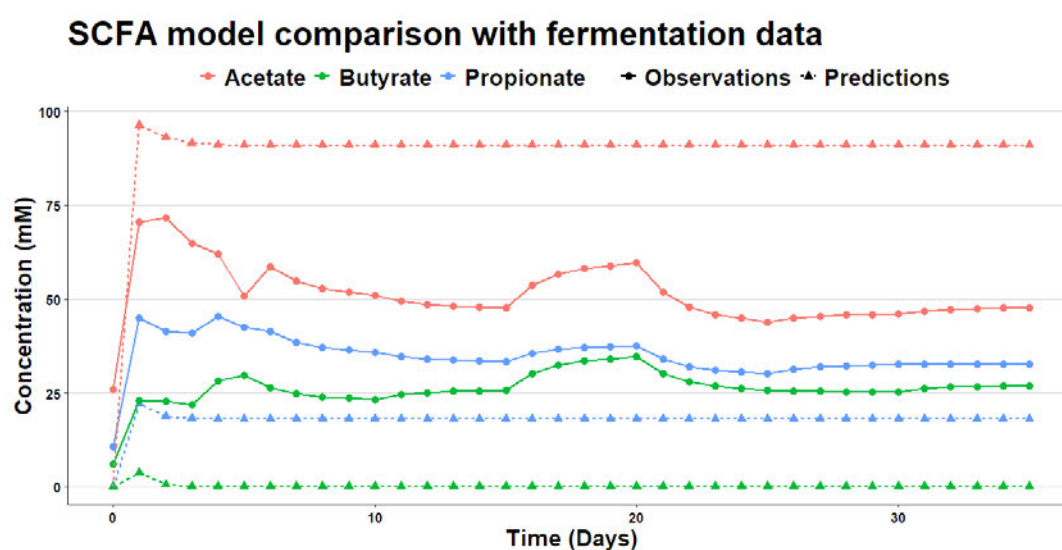


Figure 3.3. Main SCFAs observed *in vitro* (continuous lines) and predicted by the model (dotted lines) presented [Chapter 1](#). Observed SCFAs depicted here represent average concentration measured in the three reactors.

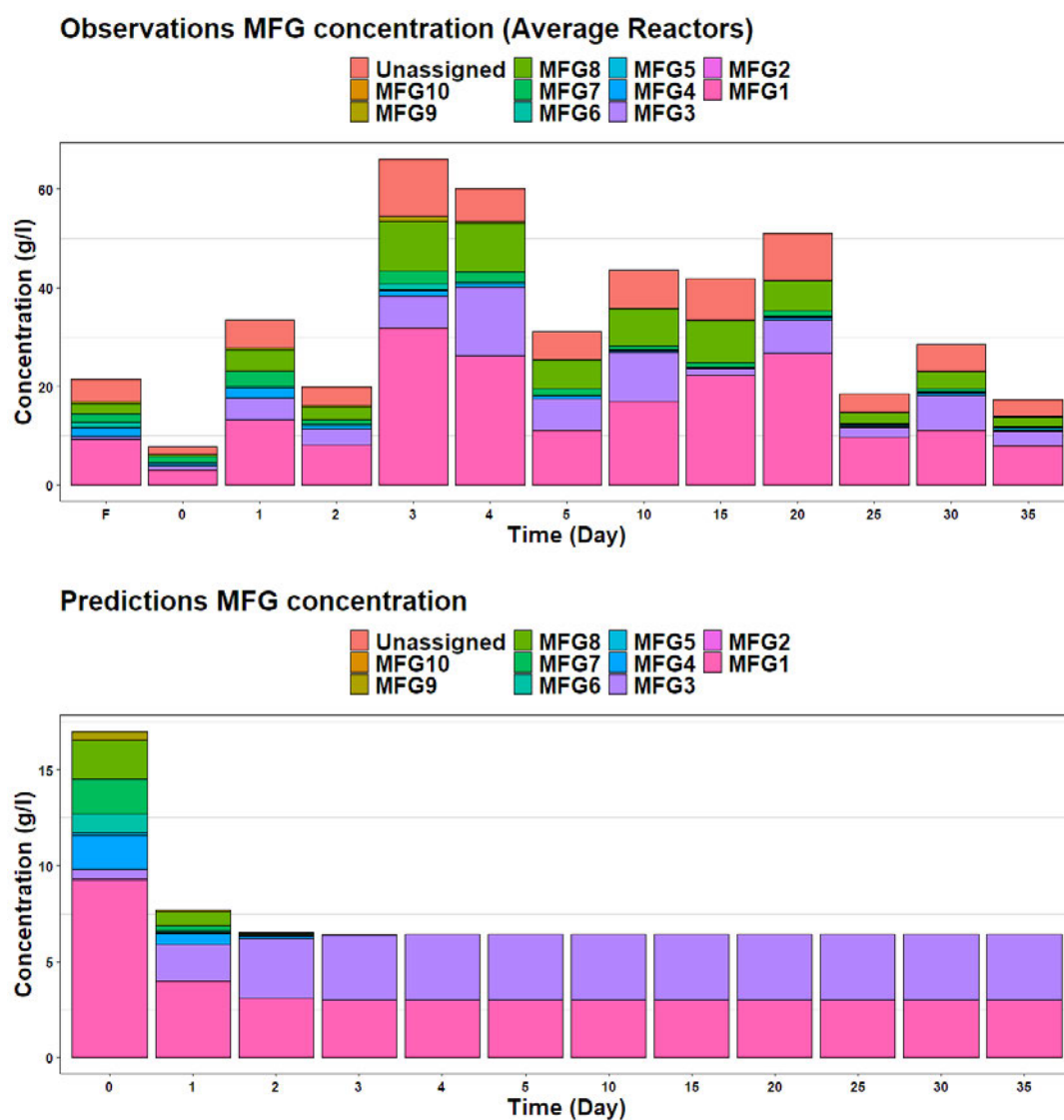


Figure 3.4. MFG concentration observed *in vitro* and predicted by the model parametrised so far as described in [Chapter 1](#). Observed MFGs depicted here represent the average concentration measured in the three reactors.

3.3.2 Re-parametrisation and comparison between re-parametrised model predictions and *in vitro* observations

As seen in the previous section, predictions generated by the model developed so far were not in line with *in vitro* observations, therefore parameter fitting through reverse modelling targeting K was performed through 300 multiple-strain simulations and comparison with *in vitro* observations. After parameter fitting, the re-parametrised model was used to simulate again the *in vitro* conditions (Table 3.1) through a one-strain simulation to assess whether model performance had improved following assignment of new K values for some of the groups. Therefore, RMSE was calculated again as a measure of the closeness between observations and predictions generated by the re-parametrised model.

As depicted in Figure 3.5 and Figure 3.6, re-parametrised model predictions were closer to *in vitro* observations, indicating that after K fitting, the model was able to depict rather optimally the dynamics observed through the 35 days of fermentation. This was also demonstrated by lower RMSE values for the measured variables, especially regarding main SCFAs (Table 3.4). In detail, predicted acetate, propionate and butyrate concentration at steady state were ~65mM, ~32mM and 23mM, respectively, rather close to day 35 *in vitro* findings of ~50mM, ~33mM and 27mM, respectively. Closeness between predictions and observations was detected by RMSE, which was 25, 6 and 11 for acetate, propionate, and butyrate, respectively.

Total MFG concentration was lower in the model than what observed *in vitro*, although the model was able to predict well the dynamics of the dominant groups MFG1, MFG3, MFG7 and MFG8. MFG1 and MFG7 predicted concentration and dynamics were rather similar to what observed *in vitro*, although higher levels of fluctuations and higher values were registered during some of the time points *in vitro*. MFG8 concentration and dynamics were almost identical *in vitro* and *in silico* with steady state values of 1.93g/l and 1.97g/l, respectively, on the other hand predicted MFG3 concentration was lower than observations at day 35, i.e. 0.17g/l and 2.8g/l. The remaining MFGs seemed not able to compete *in silico*, in fact MFG2, MFG4, MFG5, MFG6, MFG9 and MFG10 concentration tended to 0 at steady state in the model, similar to what observed *in vitro* for MFG2, MFG9 and MFG10. Nevertheless, although MFG4, MFG5 and MFG6 concentration was below 0.5g/l, was still detectable at day 35 *in vitro*.

Table 3.4. RMSE (Equation (3-1)) calculated between observations collected during the *in vitro* continuous fermentation ([Chapter 2](#)) and predictions generated by the re-parametrised model.

SCFA	RMSE	MFG	RMSE
Acetate	25.44	1	14.28
Propionate	5.65	2	18.08
Butyrate	11.28	3	17.81
		4	17.57
		5	18.08
		6	18.02
		7	17.56
		8	17.1
		9	18.06
		10	18.09

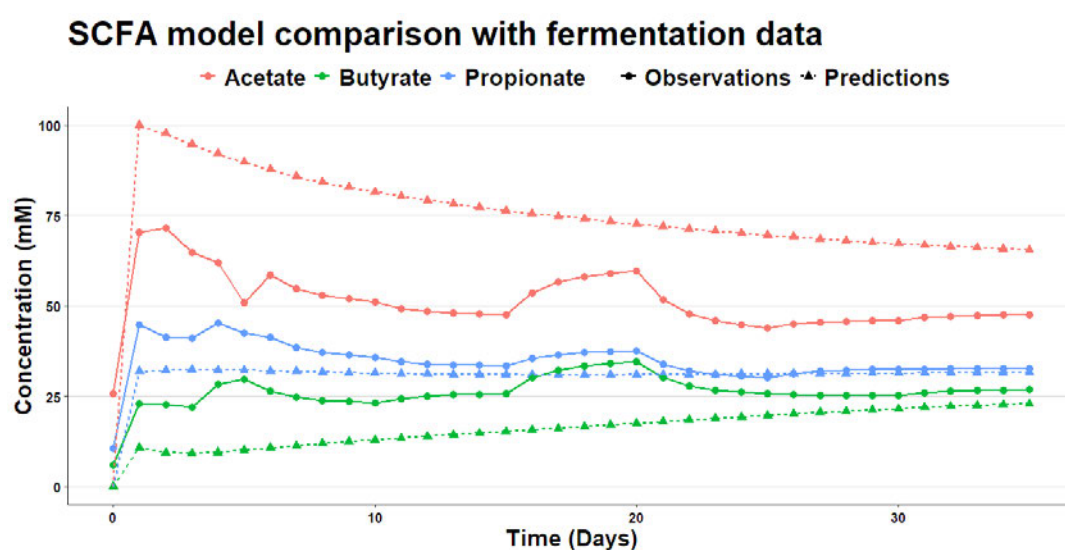
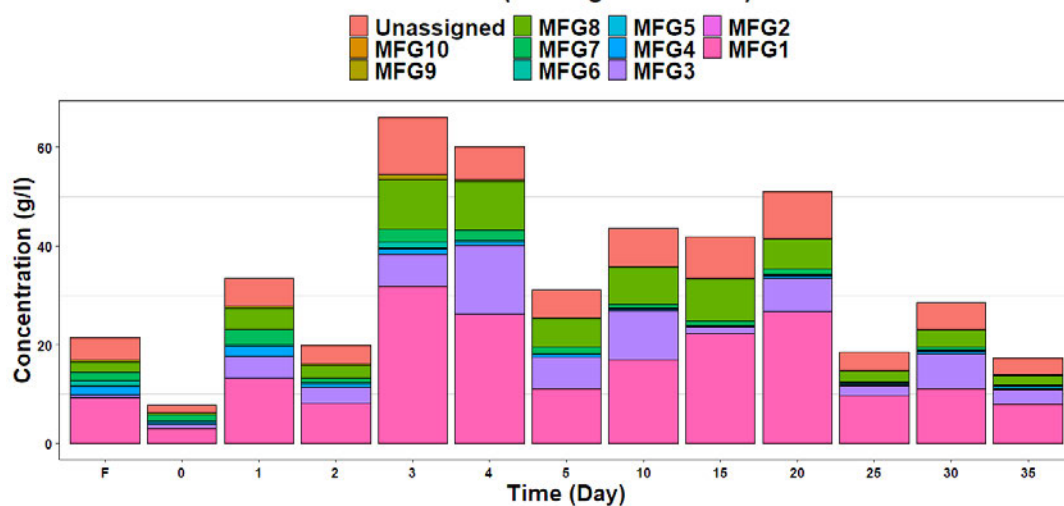


Figure 3.5. Main SCFAs observed (continuous lines) during the *in vitro* continuous fermentation ([Chapter 2](#)) and predicted by the re-parametrised model (dotted lines). Observed SCFAs depicted here represent average concentration measured in the three reactors.

Observations MFG concentration (Average Reactors)



Predictions MFG concentration

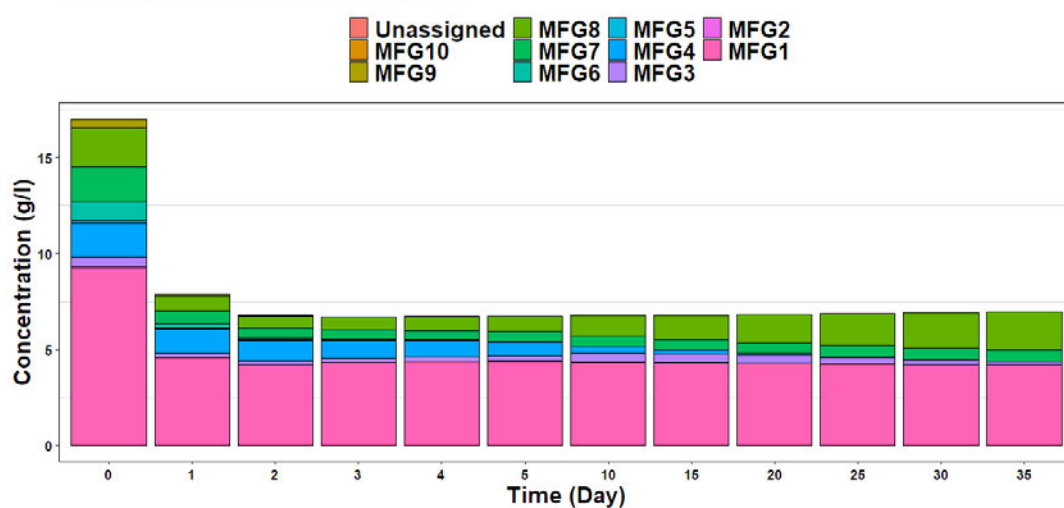


Figure 3.6. MFG concentration observed *in vitro* and predicted by the re-parametrised model. Observed MFGs depicted here represent the average concentration measured in the three reactors.

3.3.3 Multiple simulations with five strains per MFG

Following the comparison between the single-strain re-parametrised model and *in vitro* observations shown in the previous section (Figure 3.5 and Figure 3.6), the re-parametrised model was used here in 100 simulations with 5 strains per MFG and stochastically generated μ_{max} , K , Y and pH preferences. These 100 multiple-strain simulations allowed to:

- assess how parameter variations in a biological compatible range (Kovarova-Kovar and Egli, 1998) affected main metabolite concentrations. Indeed, frequency distribution of main metabolites amongst the 100 runs was studied, allowing to assess the type of metabolite fluctuations predicted when changing parameter values. This informed impact of parameters selected on final outputs.
- assess RMSE between predictions of the best fit simulation (amongst the 100) and *in vitro* observations, informing whether five-strain model (i.e. more similar simulation of *in vivo* conditions) also improved model performance.
- compare main treat value calculated for best-fit simulation to main treat value calculated for one-strain simulation (i.e. [Section 3.3.2](#), Figure 3.5 and Figure 3.6). This served as an indication of microbial dynamics variation when increasing model complexity.

The following sections describe in detail the finding for each analysis.

3.3.3.1 Main metabolite distribution analysis

In general, as depicted in Figure 3.7 main metabolites seemed to be rather sensitive to changes in parameter values. Acetate mean concentration from the 100 runs was 131.6mM, which was widely different from 65mM predicted during the previous single-strain simulation. Moreover, most of the predicted acetate seemed to be in the range between 120mM and 160mM, higher than what previously registered. Similarly, propionate and butyrate seemed to be likewise sensitive to parameter variations, with average values of 22.3mM and 5.4mM lower than single-strain predictions. Lactate concentration was the least affected by stochastic parameter generation with the majority of the steady state values collected from the 100 runs being ~0mM.

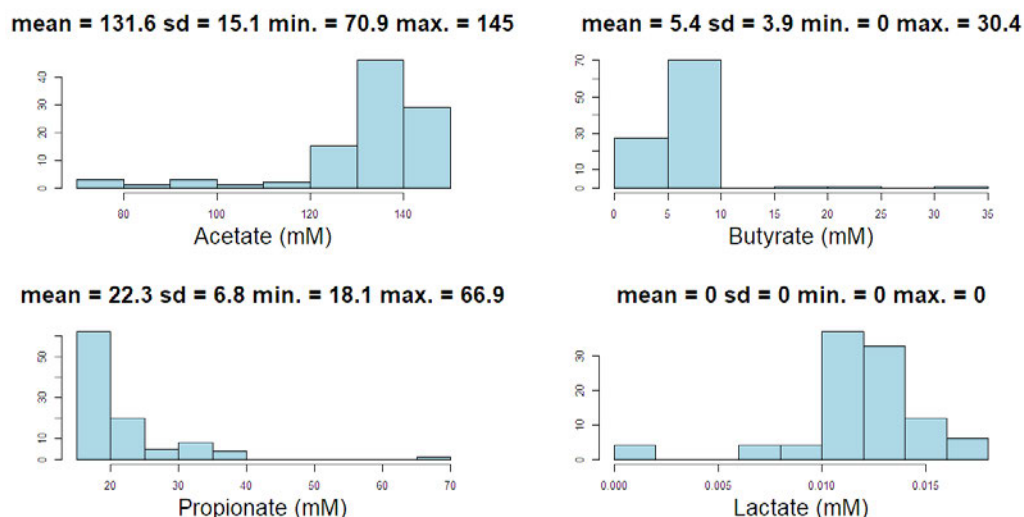


Figure 3.7. Frequency distribution analysis of the main SCFAs and lactate predicted by the re-parametrised model simulating *in vitro* conditions (Table 3.1) for 100 times with stochastically μ_{max} , K , Y and pH trait and 5 strains per MFG.

3.3.3.2 RMSE assessment between best-fit simulation predictions and *in vitro* observations

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Best-fit simulation amongst the 100 was identified calculating RMSE between metabolite and main SCFAs observed and predicted. Specific RMSE values for main SCFA and the 10 MFGs are depicted in Table 3.5, whilst graphic comparison between main SCFAs and MFGs predicted and observed are depicted in Figure 3.8 and Figure 3.9. The closest metabolite dynamics predicted were for propionate followed butyrate and acetate, which was still overestimated by the best-fit simulation. Indeed, predicted acetate steady state concentration was ~75mM, whilst its concentration at day 35 of the fermentation was ~50mM. Propionate and butyrate were still slightly underestimated *in silico*, although their general dynamics were rather close to observations. Predominant MFGs at day 35 *in silico* were MFG1, MFG8, MFG7, MFG4 and MFG3.

Table 3.5. RMSE (Equation (3-1) calculated between observations collected during the *in vitro* continuous fermentation ([Chapter 2](#)) and predictions generated by the best-fit simulation of the re-parametrised model amongst the 100.

SCFA	RMSE	MFG	RMSE
Acetate	26.24	1	13.97
Propionate	7.11	2	18.08
Butyrate	10.44	3	17.88
		4	17.62
		5	18.08
		6	18.01
		7	17.64
		8	16.98
		9	18.06
		10	18.09

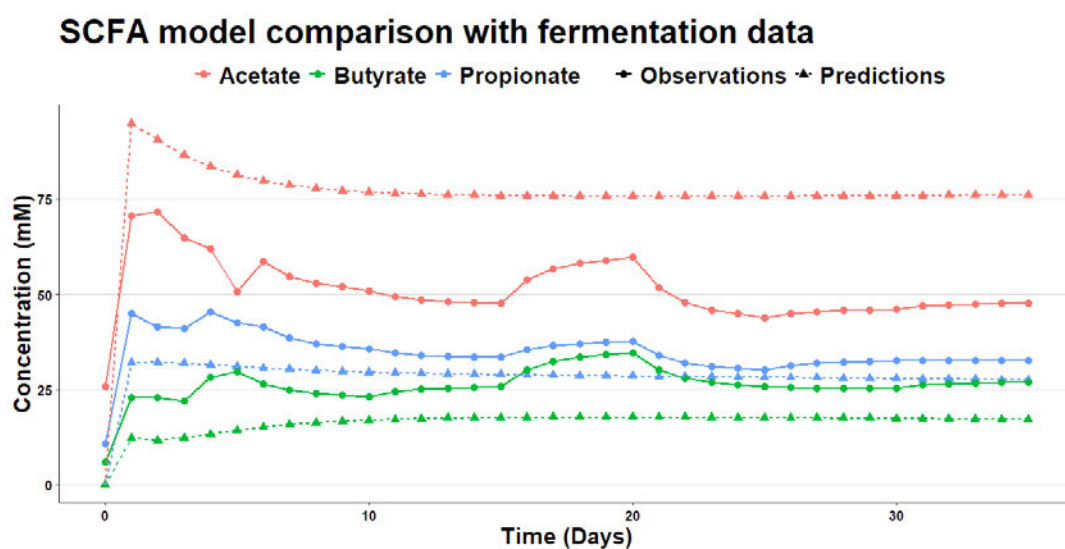
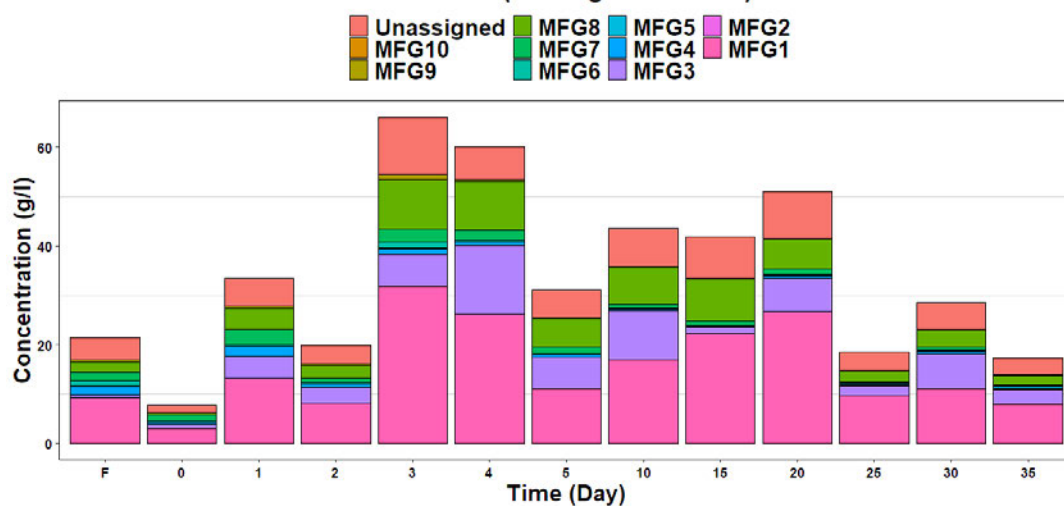


Figure 3.8. Main SCFAs observed (continuous lines) during the *in vitro* continuous fermentation ([Chapter 2](#)) and predicted by the best fit simulation of the re-parametrised model amongst the 100 (dotted lines). Observed SCFAs depicted here represent average concentration measured in the three reactors.

Observations MFG concentration (Average Reactors)



Predictions MFG concentration

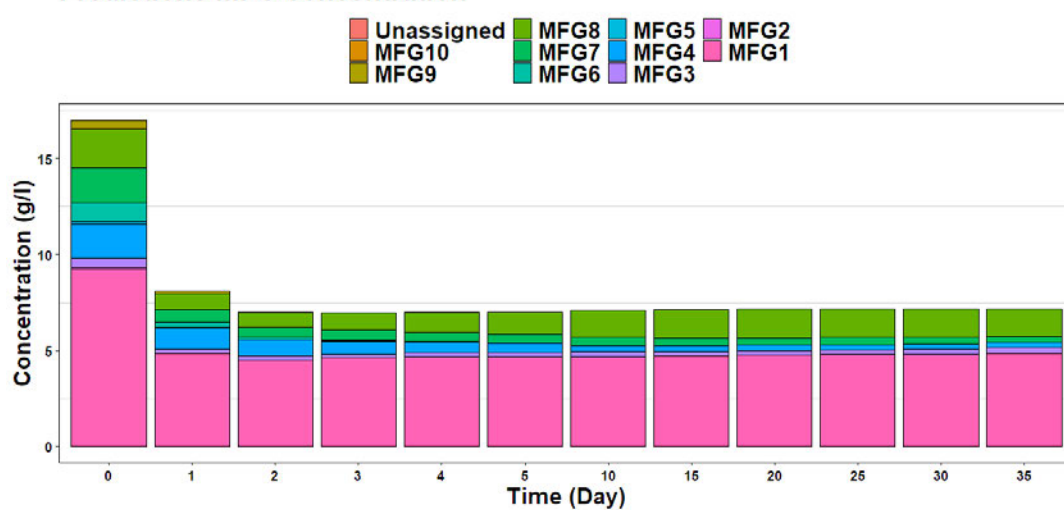


Figure 3.9. MFG concentration observed *in vitro* and predicted by the best fit simulation of the re-parametrised model amongst the 100. Observed MFGs depicted here represent the average concentration measured in the three reactors. Predicted strains amongst the different MFGs are summarised together under the main MFG to which they belong

3.3.3.3 Mean trait value assessment

The increased number of strains using the re-parametrised model led to new dynamics within each single microbial functional group, during which the competition for the same number of substrates determined the whole community to stabilise and to achieve adaptation. The new dynamics were followed by studying the behaviour of the mean trait value (z^{av}), as the weighted average of the strain concentration considering a specific trait value, such as for example K (Eq. (3-2)). The z^{av} would be expected to increase in those groups with higher capability to adapt, and consequently, the more resilient strains within a group would outcompete the others throughout the simulation. Mean trait value was calculated using Equation (3-2), therefore its values calculated for the best-fit multi-strain simulation of the re-parametrised model and the single-strain simulation of the re-parametrised model were compared here (Table 3.6 and Figure 3.10). The average pH trait is not reported in Table 3.6, given that the differences between the two model designs were hardly noticeable.

In all the cases the z^{av} for the three parameters did not follow a specific trend (Figure 3.10). In general, a high μ_{max}/k ratio would positively influence the competition in a low-resource scenario, whereas when the concentration of resource is not limiting a high μ_{max} value would guarantee the major survival chances (Kettle et al., 2015). The z_K^{av} for K decreased for MFG1, while showing an increment for all the other MFGs, especially for MFG2, MFG3, MFG 7 and MFG10. On the other hand, z_{μ}^{av} for μ_{max} decreased only for MFG5, MFG 6 and MFG9, while maintaining a positive percentage change for the other functional groups. The z^{av} for Y had a negative trend for the majority of the MFGs apart from MFG3, MFG4, MFG6 and MFG8. The variable increase in z^{av} for K and μ_{max} corresponded sometimes to an increased biomass in the best-fit run, such as in the case of MFG1, MFG3, MFG8.

Table 3.6. Average trait values calculated for the re-parametrised model when performing a single- or multiple-strain simulations. In the latter case z^{av} was calculated on the best fit simulation amongst the 100 performed.

MFG	$z^{av} (K)$		$z^{av} (\mu_{max})$		$z^{av} (Y)$	
	Single-strain	Multiple-strain (Best-fit simulation)	Single-strain	Multiple-strain (Best-fit simulation)	Single-strain	Multiple-strain (Best-fit simulation)
1	0.00072	0.00070	7.08	7.98	0.26	0.26
2	0.00043	0.00047	9.00	10.32	0.31	0.27
3	0.00018	0.00021	5.29	6.11	0.29	0.31
4	0.00002	0.00002	4.08	4.60	0.23	0.25
5	0.00052	0.00053	9.90	9.12	0.32	0.30
6	0.00018	0.00018	5.02	4.87	0.32	0.33
7	0.00042	0.00047	7.76	9.03	0.27	0.26
8	0.00051	0.00052	8.77	9.31	0.27	0.27
9	0.00004	0.00004	4.14	3.94	0.21	0.20
10	0.00021	0.00024	4.07	4.72	0.02	0.02

Average trait percentage change

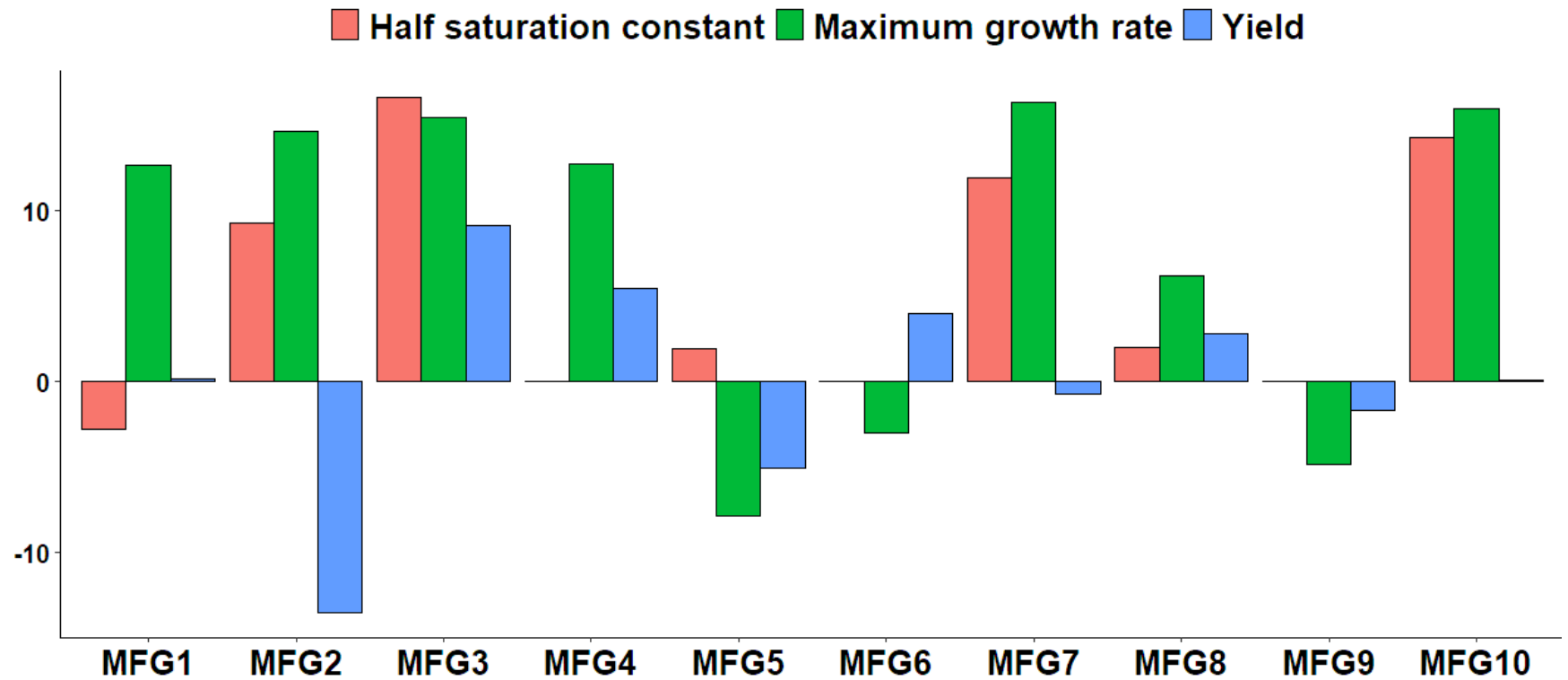


Figure 3.10. Average trait percentage change between the best-fit simulation of the re-parametrised model (five-strain simulation) and the single-strain simulation of the re-parametrised model.

3.4 Discussion

The first mathematical model of the entire pig gut microbiota (WM) presented in [Chapter 1](#) was first used to simulate the 35-day continuous fermentation described in [Chapter 2](#) through setting *in silico* starting conditions to mimic *in vitro* retention time, temperature, pH, and medium/inoculum composition. Model predictions were somewhat distant from *in vitro* observations, especially regarding butyrate producers and therefore butyrate produced throughout the simulation. Therefore, the model was re-parametrised targeting K through a reverse modelling protocol, for which 300 multiple-strain model outputs with stochastic K values were generated. Thus, best-fit simulation was identified through RMSE, and new K values were selected and fitted into the model data frames based on K of the most abundant strains from the best-fit simulation. Consequently, the re-parametrised model was used to simulate the *in vitro* continuous fermentation through a single-strain simulation first and a 5-strain simulation in a second moment. RMSE increased noticeably, especially for main SCFA dynamics after re-parametrisation, indicating an increase in model performance, which was observed with both the single- and multiple-strain design. Acetate, propionate, and butyrate predicted concentrations over time were considerably closer to the observations after parameter fitting, nevertheless acetate remained overestimated by the model. This could have been due to the absence in the model of further acetate-uptake pathways, which could otherwise be found amongst gut microbes.

Although RMSE remained somewhat unaltered after re-parametrisation, a net change in predicted microbial dynamics was noticed. Indeed, more microbial groups were able to survive using the re-parametrised model, indicating different competition dynamics, which were more similar to observations. Moreover, as discussed previously in the text, the model developed so far was not inclusive of all the microbial species found in the porcine gut, therefore some of the differences found between observation and predictions could have been due to the intrinsic roles of unassigned species which were reported amongst the observations.

Beyond the comparisons described so far, the multiple-strain simulations of the re-parametrised model gave the opportunity to study both the effect of parameter values on main metabolite through distribution analysis and microbial dynamics through the study of the average trait value. Indeed, the study of the mean trait value could provide useful information about the new microbial dynamics, such as for example, an increase in biomass would be observed in groups with higher capability to adapt. Main metabolite distribution analysis indicated that model outputs were rather sensitive to changes in K , μ_{max} and Y . This was already clear when analysing data during reverse modelling to re-parametrise K , which was not surprising, considering their role in the ODEs. The study of the microbial dynamics through z^{av} led to contrasting results, and although further studies focusing on its correlation to steady state biomass values would probably lead to more clarification, this would represent an entirely different area of research and therefore was not possible to be included in this PhD. Such

follow-up study could advance the hypothesis that further intra-group strain competitions could be directly affected by changes in the average trait, therefore theorising new competitions between MFGs determining their new dynamics and their final relative abundance.

To conclude, the first mathematical model of the entire pig gut microbiota was developed and validated through *in vitro* experimentation. Model performance greatly increased after re-parametrisation and the re-parametrised model was able to anticipate the dynamics of the main SCFAs and of the major MFGs as observed *in vitro*. Although not meant to be a substitute of *in vitro/in vivo* experimentation, it represents the development of a new tool to assist research on porcine gastrointestinal microbiota and other biological ecosystems.

4 Dynamic model of the porcine core gut microbiota

4.1 Introduction

Core gut microbiota (CM) is a bacterial population enclosed in the whole gut microbiota, whose main feature is its redundancy amongst the same host species independently of, under certain extents, external or internal factors, such as diet or host breed, whilst its relative abundance can fluctuate due to environmental changes (Astudillo-García et al., 2017; Li et al., 2017; Roeselers et al., 2011; Tap et al., 2009; Turnbaugh et al., 2009). As described in the previous chapters porcine gut microbiota composition can variate amongst hosts, especially in terms of relative abundance, thus representing some disadvantages when planning specific interventions (e.g. diet or microbial pathological marker detection) (Lane et al., 2017; Schaubek et al., 2016). The identification of a core microbiota and its related function(s) could contribute to direct targeted manipulations of core species to improve host health and performance (Shade and Handelsman, 2012). Indeed, CM could represent a significant therapeutic target due to positive interactions between CM and host, likely due to their coevolution and the somewhat consistent core microbial composition (Salonen et al., 2012). Nevertheless, planning long-lasting interventions through CM manipulations would require a thorough understanding of its role in metabolite production, interaction with both host and non-CM bacteria. CM composition is usually assessed by microbial grouping through Venn diagram, with overlapping areas defining the CM species (Shade and Handelsman, 2012), or through meta-analysis targeting redundant species (CM) in different studies (Holman et al., 2017). Indeed, a recent study from Holman et al. (2017) showed that 20 genera were shared between more than 90% of pigs in twenty different literature studies analysed, whose relative abundance fluctuated from the gastric mucosa to faeces (Holman et al., 2017).

Here the first dynamic model of the porcine colon CM is presented, based on the meta-analysis developed by Holman et al. (2017), which was used *in silico* to predict possible CM metabolite pattern. The second aim of the chapter was the comparison of colon CM model with literature studies. Therefore, CM model was used to simulate three different literature studies, and model outputs were compared with *in vitro* observations (Ding et al., 2015; Lin et al., 2011; Tanner et al., 2014). Based on the fermentation pattern predicted *in silico*, a possible CM role in forming the basis of the global SCFA production was theorised.

At the time of writing, no *in vitro* or *in silico* experimentation using CM species was found in literature, thus a dynamic model like the one presented here, forecasting the rate of change of bacterial biomass, and both related substrates and metabolites could represent the first step

in unravelling the porcine colonic CM role. The model features editable starting conditions allowing *in silico* experimentation of CM interventions, such as prebiotic or probiotic therapies, as shown in [Chapter 5](#). In the following sections a description of the model development and its architecture will precede the explanation of the composing model units, together with the description of the parametrisation process undertaken.

4.2 Model development

4.2.1 Model resolution

As described for the model of the entire pig gut microbiota (WM) (Chapter 1), microPop package (<https://CRAN.R-project.org/package=microPop>) (Kettle et al., 2018) has been used in R to develop and solve the core microbiota model. The main differences between the two models are the model design (i.e. microbial community summarisation) and the half saturation constant parameterisation, which are described in the following sections.

4.2.2 Model design

Following the description from Holman et al. (2017), CM associated with porcine colon lumen was composed of 19 different genera, whose total abundance was ~50% of the total colonic species (Table 4.1). Similarly to the MFGs described in [Chapter 1](#), each CM genus was modelled as a bacterial unit (BU) (Figure 4.1). Therefore, 19 different data frames were built summarising dynamic parameters, metabolic pathways, substrate preferences, stoichiometry and pH preferences for each BU. As detailed in the WM model ([Chapter 1](#)), the main dynamic parameters of the ODEs for the CM model were μ_{max} , K and Y . Values for K for the 19 BUs were empirically determined during the development of the CM model, whilst values for μ_{max} , and Y were based on theoretical considerations as previously performed by different authors during the development of a model for the human colonic microbiota (Kettle et al., 2018, 2015). When cultural-based data were available, assumptions for data frames were based on representative species for each genus (i.e. modelled as a BU), taking into consideration bacteria isolated in the porcine gut. When insufficient or no cultural information was available, theoretical description of representative species, with preference for those originally isolated from porcine gut was taken into consideration.

Due to its architecture the CM model maintained a certain degree of uncertainty based both on the unknown parameters and because the stoichiometry cannot depict the complexity of some genera composed by a great number of species. Nevertheless, considering both the closeness between BU *in silico* predictions and *in vitro* observations for some of the representative species and the fact that BUs data frames were based on metabolic pathways for representative species, it was not unreasonable to assume that when the BUs data frames were assembled in a CM model, *in silico* SCFA pattern could be comparable to the one obtained by a similar bacterial population *in vitro* (Van Wey et al., 2014).

Table 4.1. Genus relative abundance of core microbiota associated with porcine colonic lumen, as derived from Holman et al., 2017. Total colonic CM abundance was 49% according to the meta-analysis on which this CM model is based and therefore CM genera (BUs) relative abundance reflect CM abundance relative to the whole microbiota (Holman et al., 2017).

#	Genus (BU)	Relative abundance (%) in colon lumen
1	<i>Pseudobutyrvibrio</i>	1.07
2	<i>Parabacteroides</i>	1.65
3	<i>Phascolarctobacterium</i>	0.88
4	<i>Ruminococcus</i>	0.78
5	<i>Megasphaera</i>	1.84
6	<i>Sarcina</i>	0.49
7	<i>Faecalibacterium</i>	1.65
8	<i>Escherichia-Shigella</i>	0.39
9	<i>Bacteroides</i>	5.06
10	<i>Streptococcus</i>	1.26
11	<i>Blautia</i>	1.46
12	<i>RC9 gut group</i>	1.65
13	<i>Alloprevotella</i>	3.21
14	<i>Succinivibrio</i>	0.88
15	<i>Treponema</i>	1.84
16	<i>Clostridium</i>	6.32
17	<i>Lactobacillus</i>	5.54
18	<i>Prevotella</i>	12.35
19	<i>Turicibacter</i>	0.68
TOT		49

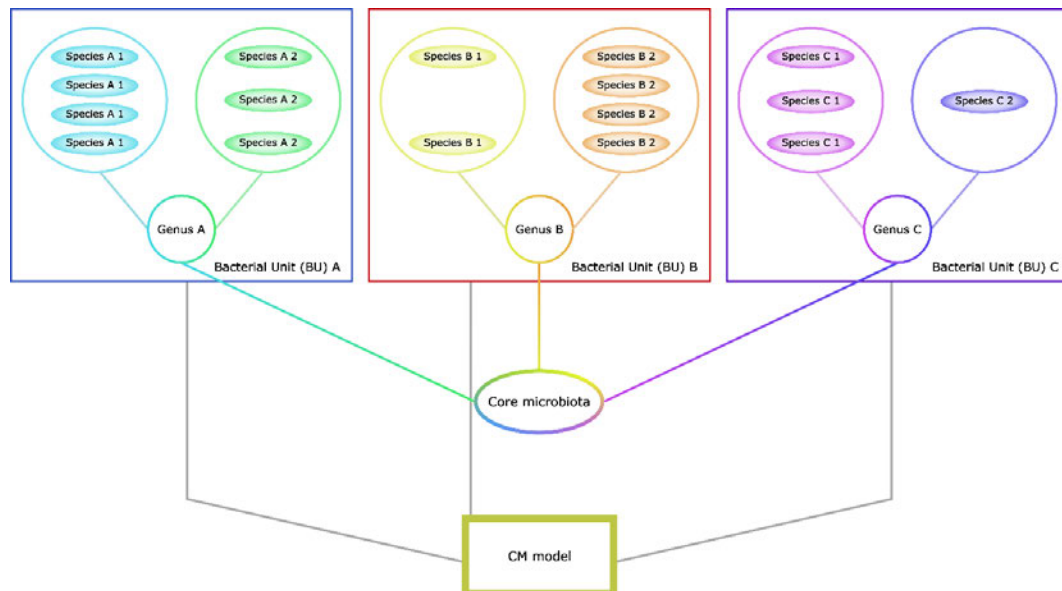


Figure 4.1. Schematic example of a model for a core microbiota composed by only three genera. Each BU simulates a genus with its species diversity, and a CM model is developed merging the data frames of the composing BUs.

4.2.3 Model structure

Following the structure of the whole microbiota model ([Chapter 1](#)) and the previous model, on which the WM model was based (Kettle et al., 2015), microbial substrates were grouped in four categories:

1. resistant starch (RS), all the types available for bacterial degradation;
2. non-starch polysaccharides (NSP);
3. sugars (S), mono- and poly-saccharides;
4. proteins (Pr).

The resources were modelled as essential (Se), semi-essential (Sb) and substitutable (S) (Kettle et al., 2015) as described in [Chapter 1](#). The 10 metabolites modelled were acetate, propionate, butyrate, succinate, formate, lactate, ethanol, H₂O, H₂, and CO₂, with some of them being fermented by other BUs during cross-feeding. As a result, a model describing the dynamics of the CM colonic lumen (CI-CM) was developed, which simulated the rate of change over time of four substrates, ten metabolites and nineteen genera modelled as BUs. The model did not consider biotic factor due to host contribution (e.g. SCFAs colonic absorption), therefore as seen in [Chapter 1](#), the environment modelled was an *in vitro* fermentation. The dilution rate, D (h⁻¹), was considered as the inverse of retention time assumed for the whole colon of 30 hours (Latymer et al., 1990), whilst pH was assumed to have a constant value of 6.5 (Kararli, 1995; Tanner et al., 2014).

4.2.4 Bacterial unit description

To implement CI-CM model in microPop (Kettle et al., 2018) parameter values, stoichiometry and pH preferences for each BU were assigned through 19 data frames. The following sections ([4.2.4.1](#) through [4.2.4.19](#)) describe BU data frames, including, when possible due to *in vitro* data availability, a comparison between observed and predicted outcomes, which were used as a measure of model accuracy. Values for μ_{max} and Y were derived from theoretical consideration as described by other authors during the development of a previous model of the human gut microbiota (Kettle et al., 2018, 2015). Values for K were derived empirically through the development of the CM model during this study. Similar methodologies were used for each of the nineteen BUs, which inevitably did results in significant repetition, but for completeness is reported here in full.

4.2.4.1 *Pseudobutyrvibrio* BU

Pseudobutyrvibrio BU data frame was based on the two classified species of this genus *P. ruminis* (Van Gylswyk et al., 1996) and *P. xylanivorans* (Kopečný et al., 2003), originally isolated from rumen of cows and from rumen of sheep and cows, respectively. Therefore, two pathways represented the dynamics of these two species (Table 4.2). Assumption for μ_{max} was on functionally similar bacteria (Kettle et al., 2018, 2015). Assumption for stoichiometry was based on cultural-based data (Kopečný et al., 2003; Van Gylswyk et al., 1996), thus a comparison between predictions and observations is shown in Figure 4.2.

Table 4.2. *Pseudobutyrvibrio* BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	1st pathway based on <i>P. ruminis</i>	2nd pathway based on <i>P. xylanivorans</i>	Reference
Substrates	NSP; S (7.912), Sugars; S (7.912), H ₂ O; Sw (2)	NSP; S (6.174), Sugars; S (6.174), Acetate; Sb (Fb=0.1) (4)	(Kettle et al., 2018, 2015; Kopečný et al., 2003; Van Gylswyk et al., 1996)
Metabolites	Formate (5), butyrate (4), lactate (8), acetate (0.1), CO ₂ (3), H ₂ (10)	Formate (5.2), butyrate (6.5), lactate (3), succinate (0.07), ethanol (0.3), CO ₂ (4), H ₂ O (4)	(Kettle et al., 2018, 2015; Kopečný et al., 2003; Van Gylswyk et al., 1996)
μ_{max} (h⁻¹)	0.6 (NSP), 1 (Sugars)		(Kettle et al., 2018, 2015)
K (g/l)	0.001		Empirically determined during this study
y (g/g)	0.286 (NSP), 0.333 (Sugars)		(Kettle et al., 2018, 2015)
pH corners	5.0, 5.5, 5.6, 6.8		(Rainey, 2009)

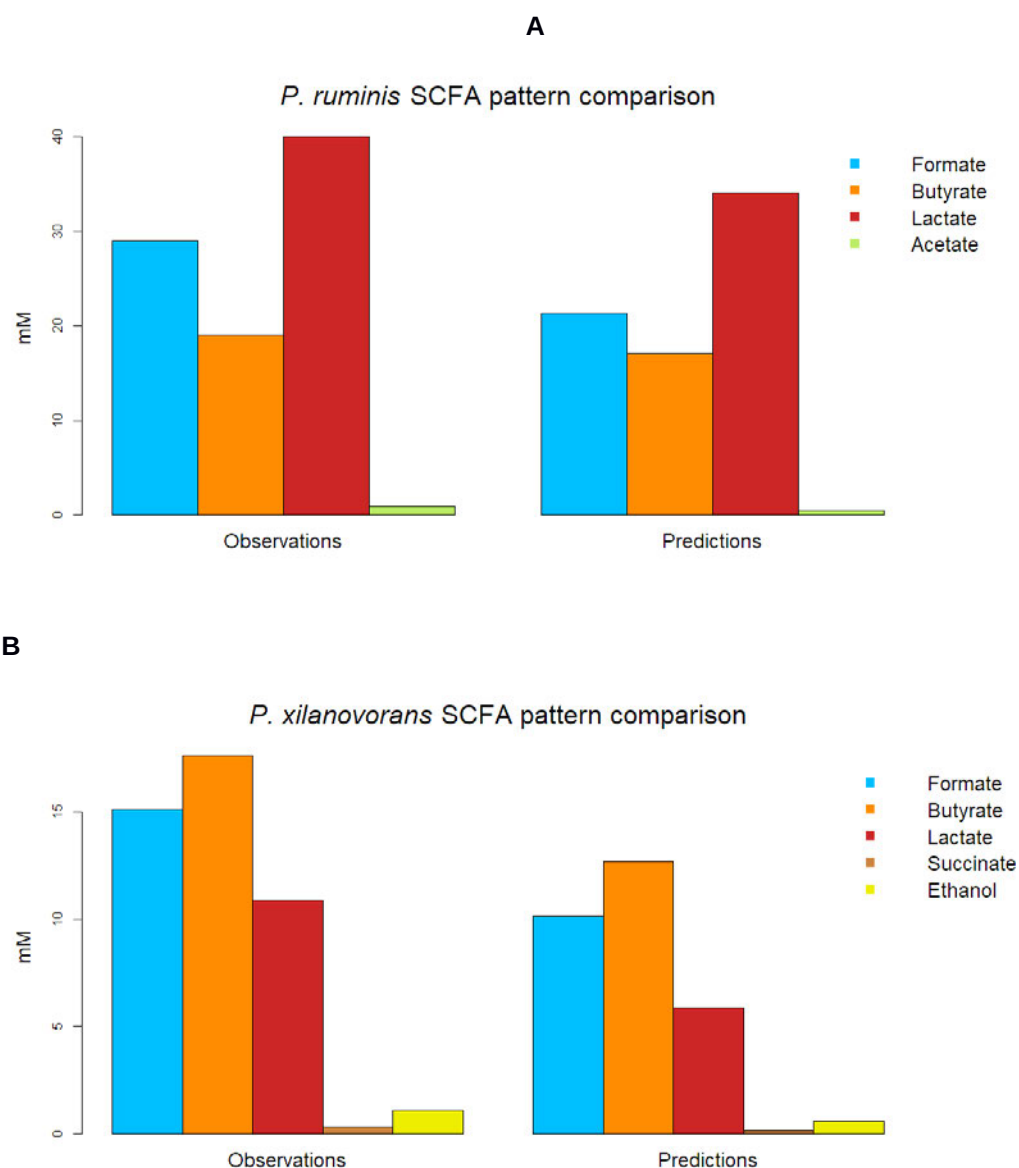


Figure 4.2 (A) Observations vs predictions when simulating *Pseudobutyrvibrio ruminis* growing in a batch culture; starting concentration: glucose (modelled as sugars), 9g/l, bacterium, 0.1g/l (Rainey, 2009). B Observations vs predictions when simulating *Pseudobutyrvibrio xylanovorans* growing in a batch culture; starting concentration: glucose, 3.96g/l (modelled as sugars), bacterium, 0.1g/l (Rainey, 2009). *In silico* time scale was 35 days in order to allow the system to reach steady state, whilst no information were found on the duration of the *in vitro* experiments which were batch systems.

4.2.4.2 Parabacteroides BU

Amongst the fifteen described species of *Parabacteroides* genus, 11 have been isolated from human intestinal or faecal samples, and whilst none of them have been directly isolated from porcine gut (Table 4.3) their presence amongst pig gut species has been documented (Tan et al., 2019).

Stoichiometry assumption was based on succinate pathway and species descriptions (Sakamoto and Benno, 2006), whilst parameter values were based on *Bacteroides spp* as in human model (Kettle et al., 2018, 2015) as depicted in Table 4.4. Assumption for pH corners was based on *P. acidifaciens* (Wang et al., 2019).

Table 4.3. List of classified *Parabacteroides* species

#	Species	Isolated from	Reference
1	<i>Parabacteroides acidifaciens</i>	Human faeces	(Wang et al., 2019)
2	<i>Parabacteroides bouchedurhonensis</i>	Human faeces	(Yimagou et al., 2020)
3	<i>Parabacteroides chartae</i>	Paper mill wastewater	(Tan et al., 2012)
4	<i>Parabacteroides chinchilla</i>	Chinchilla (faeces)	(Kitahara et al., 2013)
5	<i>Parabacteroides chongii</i>	Human blood	(Kim et al., 2018)
6	<i>Parabacteroides distasonis</i>	Human faeces	(Sakamoto and Benno, 2006)
7	<i>Parabacteroides faecis</i>	Human faeces	(Sakamoto et al., 2015)
8	<i>Parabacteroides goldsteinii</i>	Human Intestine	(Song et al., 2005)
9	<i>Parabacteroides gordonii</i>	Human blood	(Sakamoto et al., 2009)
10	<i>Parabacteroides johnsonii</i>	Human faeces	(Sakamoto et al., 2007)
11	<i>Parabacteroides massiliensis</i>	Human faeces	(Bellali et al., 2019)
12	<i>Parabacteroides merdae</i>	Human faeces	(Johnson et al., 1986)
13	<i>Parabacteroides pacaensis</i>	Human gut	(Benabdelkader et al., 2020)
14	<i>Parabacteroides provencensis</i>	Human gut	(Benabdelkader et al., 2020)
15	<i>Parabacteroides timonensis</i>	Human faeces	(Bilen et al., 2016)

Table 4.4. Parabacteroides BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	1 st pathway	2 nd pathway	Reference
Substrate	Sugars; S (1.918), NSP; S (1.918) and RS; S (1.918)	Protein; Se (3.112)	(Kettle et al., 2018, 2015; Sakamoto and Benno, 2006)
Metabolites	Succinate (1), acetate (2) propionate (0.8), H ₂ (2) and CO ₂ (1)	Acetate (2), propionate (0.8), succinate (1) H ₂ (2) and CO ₂ (1)	
μ_{max} (h⁻¹)	0.5 (NSP); 1 (RS); 1 (Sugars); 1 (Protein)		(Kettle et al., 2018, 2015)
K (g/l)	0.001		Empirically determined during this study
y (g/g)	0.286 (NSP), 0.333 (RS), 0.333 (Sugars), 0.200 (Protein)		(Kettle et al., 2018, 2015)
pH corners	6.0, 6.5, 7.5, 10		(Wang et al., 2019)

4.2.4.3 *Phascolarctobacterium* BU

Three species were described at the time of writing for *Phascolarctobacterium* genus, *P. faecium* (Del Dot et al., 1993), *P. succinatutens* (Watanabe et al., 2011), and *P. wakonense* (Shigeno et al., 2019), isolated from koala faeces, human faeces and marmoset faeces, respectively.

Unfortunately no cultural-based data were found, therefore *Phascolarctobacterium* BU data frame was developed through assumptions based on genus description, which typically consume succinate producing propionate (Del Dot et al., 1993), as shown in Table 4.5. Assumption for μ_{max} was based on growth on organic acids as reported for the human model (Kettle et al., 2018, 2015), whilst assumption for Y was based on theoretical consideration of 0.3 to 0.88 molecules of ATP produced per consumption of succinate (Kettle et al., 2018, 2015). Assumption for pH corners was due to theoretical considerations based on the presence of this genus in the gastrointestinal tract of humans and other species.

Table 4.5. *Phascolarctobacterium* BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	<i>Phascolarctobacterium</i> BU pathway	Reference
Substrates	Succinate; Se (2)	(Del Dot et al., 1993)
Metabolites	Propionate (2) and CO ₂ (2)	
μ_{max} (h ⁻¹)	0.2	(Kettle et al., 2018, 2015)
K (g/l)	0.001	Empirically determined during this study
y (g/g)	0.08	(Kettle et al., 2018, 2015)
pH corners	6, 6.5, 7, 8	Theoretical considerations

4.2.4.4 *Ruminococcus* BU

Ruminococcus BU data frame summarised the metabolism of the 13 described species of this genus (Ezaki, 2009b) through two pathways. (Table 4.6). The assumption for stoichiometry for the first pathway was based on continuous monocultures of *R. bromii*, originally isolated from human faeces growing on 0.5% starch (Laverde Gomez et al., 2019). The assumption for stoichiometry for the second pathway was based on observations of *R. flavefaciens* isolated from human colon growing in pure culture (Kettle et al., 2018, 2015; Robert and Bernalier-Donadille, 2003).

Table 4.6. *Ruminococcus* BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	1 st pathway	2 nd pathway	Reference
Substrates	NSP; S (1), RS; S (1)	NSP; S (2), RS; S (2), H ₂ O; Sw (2)	(Kettle et al., 2018, 2015; Laverde Gomez et al., 2019; Robert and Bernalier-Donadille, 2003)
Metabolites	Acetate (1), succinate (1), H ₂ (1)	Acetate (2), ethanol (2), formate (2), H ₂ (2), CO ₂ (2)	(Kettle et al., 2018, 2015; Laverde Gomez et al., 2019; Robert and Bernalier-Donadille, 2003)
μ_{max} (h⁻¹)	0.7 (NSP), 0.15 (RS)	0.6 (RS), 0.15 (NSP)	(Kettle et al., 2018, 2015)
K (g/l)		0.001	Empirically determined during this study
y (g/g)	0.286 (NSP)	0.333 (RS)	(Kettle et al., 2018, 2015)
pH corners		6.0, 6.25, 6.5, 7.0	(Russell and Dombrowski, 1980)

4.2.4.5 *Megasphaera BU*

Amongst the species in this genus, *M. elsdenii*, originally isolated from rumen plays an important role in pig gut (Kajihara et al., 2017; Hélène Marchandin et al., 2009). The assumption for the stoichiometry of this data frame was therefore based on *M. elsdenii* growing on batch culture on lactate (2.25g/l) and glucose (4.5g/l) as shown in Table 4.7 (Kettle et al., 2015; Hélène Marchandin et al., 2009; Xiao-lin et al., 2016b).

Table 4.7. *Megasphaera BU* data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	1 st pathway	2 nd pathway	Reference
Substrates	NSP; S (0.208), RS; S (0.208), Sugars; S (0.208)	Lactate; Se (0.416)	(Kettle et al., 2015; Hélène Marchandin et al., 2009; Xiao-lin et al., 2016a)
Metabolites	Propionate (0.06), butyrate (0.06), acetate (0.08), CO ₂ (0.5), H ₂ (0.5)	Propionate (0.06), butyrate (0.06), acetate (0.08), CO ₂ (0.5), H ₂ (0.5)	(Kettle et al., 2015; Hélène Marchandin et al., 2009; Xiao-lin et al., 2016a)
μ_{max} (h⁻¹)	0.3 (NSP), 0.3 (RS), 1 (Sugars) 0.2 (Lactate)		(Kettle et al., 2018, 2015)
K (g/l)	0.001		Empirically determined during this study
y (g/g)	0.286 (NSP), 0.333 (RS), 0.333 (Sugars), 0.11 (Lactate)		(Kettle et al., 2018, 2015)
pH corners	4.6, 5.5, 6.05, 7.8		(Hélène Marchandin et al., 2009)

4.2.4.6 *Sarcina BU*

The data frame of *Sarcina BU* was based on *S. ventriculi* (Table 4.8), which although was firstly isolated from stomach content of diseased humans and other animals, it was also later isolated from faeces of healthy humans (Crowther, 1971) and found in caecum and colon of healthy pigs (Yu et al., 2016). *S. ventriculi* is an acetate producer which can grow on carbohydrates but not RS and can also use formate, as seen for MFG9 in [Chapter 1](#) (Canale-Parola, 2009). Stoichiometry assumption was therefore based on cultural-based observations of *S. ventriculi* growing in batch cultures (Finn et al., 1984) and formate hydrogen-lyase pathway (Kamlage et al., 1997). It is worth to notice that although in the second pathway formate is necessary for the growth, this would not be possible without fermentable carbohydrates.

A comparison between the observed SCFA pattern (Finn et al., 1984) and a BU model simulating the same experimental conditions is depicted in Figure 4.3.

Table 4.8. *Sarcina BU* data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	1 st pathway	2 nd pathway	Reference
Substrates	NSP; S (0.8), Sugars; S (0.8)	Formate; Se (2), NSP; S (1) sugars; S (1)	(Canale-Parola, 2009; Finn et al., 1984; Kamlage et al., 1997)
Metabolites	Acetate (0.589), ethanol (0.891), H ₂ (1.178), CO ₂ (1.486)	Acetate (3), H ₂ (2), CO ₂ (2)	
μ_{max} (h⁻¹)	0.3 (NSP), 1 (sugars)		(Kettle et al., 2018, 2015)
K (g/l)	0.001		Empirically determined during this study
y (g/g)	0.286 (NSP), 0.333 (sugars)		(Kettle et al., 2018, 2015)
pH corners	3, 6, 7, 8		(Lowe et al., 1989)

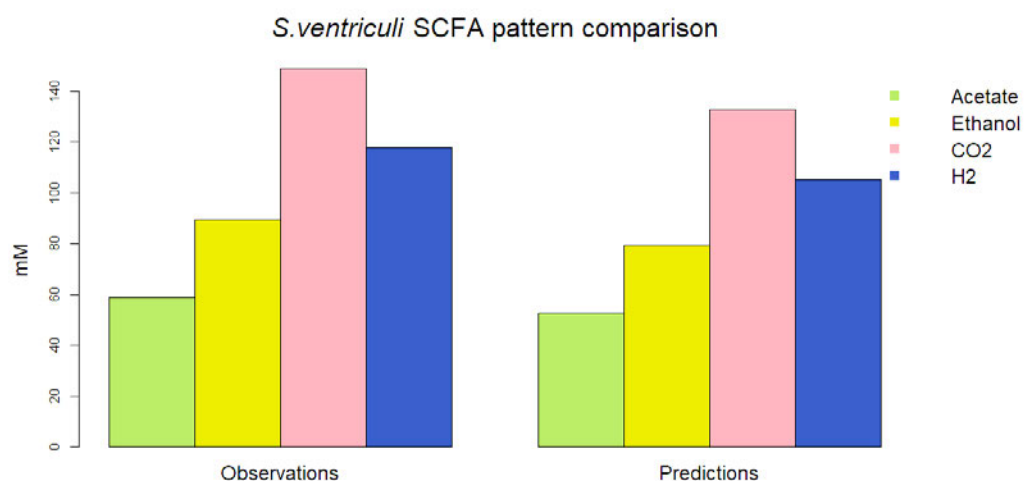


Figure 4.3. Comparison between observed and predicted SCFA pattern when *Sarcina* BU was used to simulate the experimental conditions described by Finn et al (1984). In the *in vitro* experiment *S. ventriculi* was grown in a batch culture ($D=0$) for 24 hours on 18.4g/l of arabinose. The model simulating the experiment had 18.4g/l of sugars as starting conditions considering the monosaccharide used by the authors (Finn et al., 1984).

4.2.4.7 *Faecalibacterium* BU

As seen in [Chapter 1](#), *Faecalibacterium* spp. produce butyrate, lactate, formate and CO₂, through net consumption of acetate (Duncan et al., 2002b; Louis and Flint, 2009) as depicted in Table 4.9. The BU data frame was used to simulate a culture-based experiment in which *F. prausnitzii* strain A2-165 was grown for 24 hours in a batch culture in YCFAG medium containing glucose (10mM) and acetate (33mM) (Duncan et al., 2002a). A comparison between these observation and the prediction of the model simulating the *in vitro* starting conditions (i.e. sugars: 1.8g/l, acetate: 1.99g/l, D=0) for 24 hours is depicted in Figure 4.4.

Table 4.9. *Faecalibacterium* BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	<i>Faecalibacterium</i> BU pathway	Reference
Substrates	NSP; S (6), RS; S (6), Sugars; S (6), Acetate; Sb (Fb=0.1) (4)	(Duncan et al., 2002b; Kettle et al., 2015)
Metabolites	Butyrate (7), Lactate (2), Formate (6), CO ₂ (4), H ₂ O (4)	
μ_{max} (h⁻¹)	0.6 (NSP), 0.3 (RS), 1 (sugars)	(Kettle et al., 2018, 2015)
K (g/l)	0.002	Empirically determined during this study
y (g/g)	0.285 (NSP), 0.333 (RS), 0.333 (sugars)	(Kettle et al., 2018, 2015)
pH corners	4.6, 5.7, 6.7, 7	(Foditsch et al., 2014; Lopez-Siles et al., 2017)

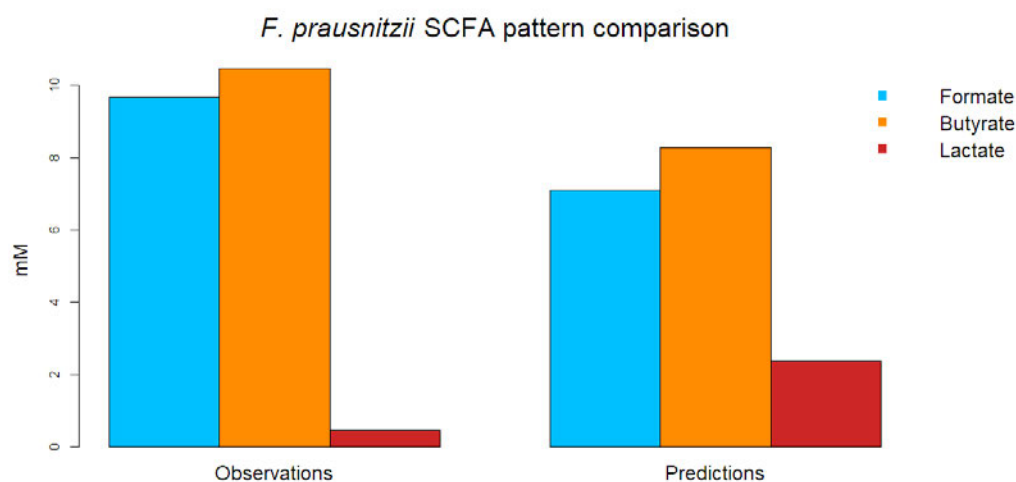


Figure 4.4. Comparison between *F. prausnitzii* A2-165 observations from growth in YCFAG medium containing glucose (10mM) and acetate (33mM) for 24 hours (Duncan et al., 2002a) and prediction generated modelling the same starting conditions through *Faecalibacterium* BU.

4.2.4.8 *Escherichia* BU

This BU data frame was based on the fermentative metabolism of *Escherichia coli*. Some of the strains belonging to *E. coli* have been classified as commensals in pigs (Moodley and Guardabassi, 2009), although they can be cause of colibacillosis due to different reasons, such as microbial stress due to weaning (Heo et al., 2013; Rasko et al., 2008). *E. coli* is a facultative anaerobe, which in absence of oxygen can ferment sugars through mixed acid fermentation (Alam and Clark, 1989), whose fermentation balance (Clark, 1989) and observations from batch cultures (Nutting and Carson, 1952) were used to derive stoichiometry assumptions. The assumption for μ_{max} was based on batch culture observations of strain ATCC11303 growing on glucose (Sauer et al., 1999), whilst yield and K followed the same theoretical and empirical considerations as per the rest of the modelled BUs (Table 4.10).

Observations for *E. coli* growing on xylose (1mM) at pH 6.5 (Nutting and Carson, 1952) were simulated using *Escherichia* BU data frame and a comparison between observation and predictions is depicted in Figure 4.5 (starting conditions were 0.18g/l of sugars, pH 6.5, whilst the model simulated the batch culture for 24 hours).

Table 4.10. *Escherichia* BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	Metabolic pathway	Reference
Substrates	Sugars; S (0.873)	
Metabolites	Acetate (0.47), ethanol (0.31), lactate (0.73), formate (0.91), succinate (0.06)	(Clark, 1989; Nutting and Carson, 1952)
μ_{max} (h ⁻¹)	0.65	(Sauer et al., 1999)
K (g/l)	0.001	Empirically determined during this study
y (g/g)	0.333 (sugars)	(Kettle et al., 2018, 2015)
pH corners	5.5, 6, 7.5, 8	(Adler and Templeton, 1967)

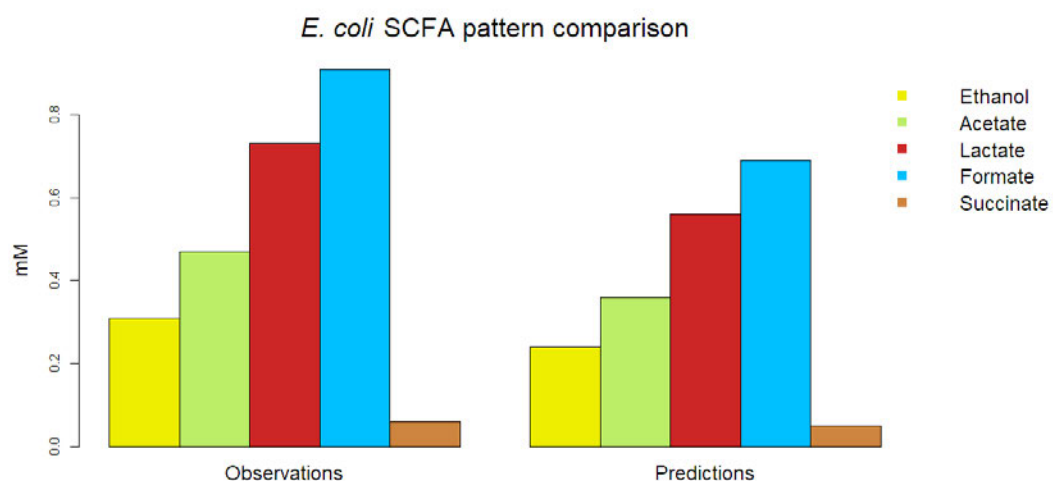


Figure 4.5. Comparison between observations for *E. coli* growing on xylose (1mM) at pH 6.5 (Nutting and Carson, 1952) and predictions using *Escherichia* BU data frame (starting conditions were 0.18g/l of sugars, pH 6.5, whilst the model simulated the batch culture for 24 hours).

4.2.4.9 *Bacteroides* BU

Bacteroides BU data frame was modelled through two pathways, for growth on carbohydrates (Frantz and McCallum, 1979) and on protein (Kettle et al., 2018, 2015), respectively (Table 4.11). The data frame was used to model the experimental conditions described by from Frantz and McCallum (1979), in which SCFAs from *B. fragilis*, originally isolated from gut abscess, growing on glucose (modelled as sugars, 44.84mM) in batch culture (D= 0ml/h) for ~20 hours were measured. A comparison between observations and predictions is depicted in Figure 4.6.

Table 4.11. *Bacteroides* BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	1 st pathway	2 nd pathway	Reference
Substrates	NSP; S (0.93), RS; S (0.93), Sugars; S (0.93)	Proteins; Se (2)	(Eggerth and Gagnon, 1933;
Metabolites	Acetate (0.511), propionate (0.551), succinate (0.458), ethanol (0.025), lactate (0.452)	Acetate (2), propionate (1), succinate (1), CO ₂ (1), H ₂ (2);	Frantz and McCallum, 1979; Kettle et al., 2015)
μ_{max} (h⁻¹)	0.5 (NSP), 1 (RS), 1 (sugars), 1 (protein)		(Kettle et al., 2018, 2015)
K (g/l)	0.001		Empirically determined during this study
y (g/g)	0.286 (NSP), 0.333 (RS), 0.333 (sugars), 0.2 (protein)		(Kettle et al., 2018, 2015)
pH corners	6, 6.5, 7, 8.5		(Allison and Macfarlane, 1989; Song et al., 2015)

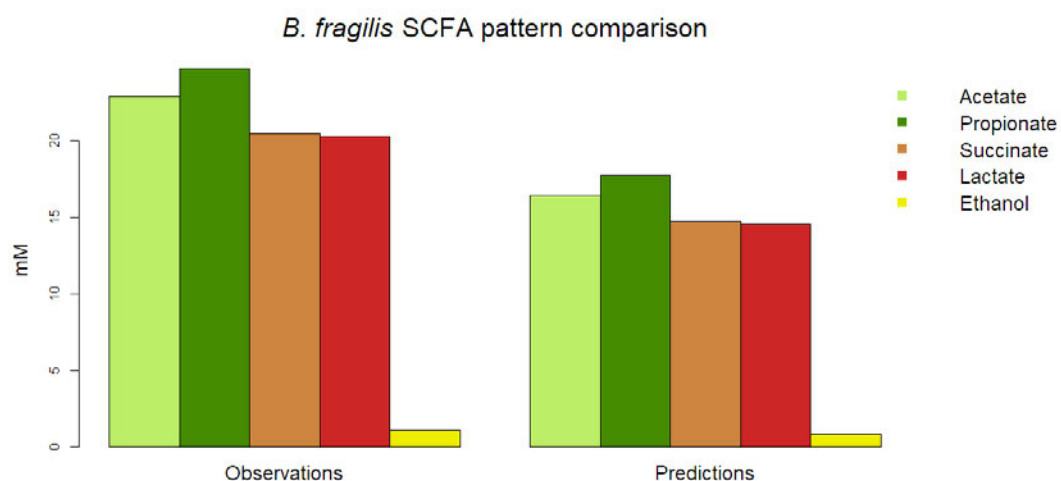


Figure 4.6. Comparison between observations of *B. fragilis* growing on glucose 44.84 mmol (Frantz and McCallum, 1979) and predictions of *Bacteroides* BU model simulating the same experimental conditions. Please see text for starting conditions.

4.2.4.10 *Streptococcus* BU

Streptococcus BU data frame was based on *S. hyointestinalis* found in pig intestine (Thanantong et al., 2006), whose acid production was reported from glucose, fructose, mannose, galactose, lactose, N-acetylglucosamine, arbutin, salicin, maltose, saccharose, trehalose, and starch (Devriese et al., 1988). Stoichiometry was based on heterofermentative metabolism following genus description, according to which all streptococci ferment carbohydrates, producing predominantly lactic acid although minor amounts of acetic and formic acids, ethanol, and CO₂ may also be produced. (Whiley and Hardie, 2015) (Table 4.12).

Table 4.12. *Streptococcus* BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	Metabolic pathway	Reference
Substrates	RS; S (3), Sugars; S (3), H ₂ O;Sw (1)	(Devriese et al., 1988;
Metabolites	Lactate (1), acetate (4), formate (2), ethanol (2) and CO ₂ (1)	Whiley and Hardie, 2015)
μ_{max} (h⁻¹)	0.25 (RS), 1 (Sugars)	(Kettle et al., 2018, 2015)
K (g/l)	0.001	Empirically determined during this study
y (g/g)	0.333 (RS), 0.333 (Sugars)	(Kettle et al., 2018, 2015)
pH corners	5, 5.5, 6.5, 7	(Beal et al., 1989)

4.2.4.11 *Blautia* BU

Although no cultural-based data were found, *Blautia* BU data frame was developed through three pathways (Table 4.13), based on the genus description and on MFG9 data frame, since both were based on *Blautia* spp., such as *B. massiliensis* originally isolated from human faecal samples (Durand et al., 2017; Kamlage et al., 1997; Kettle et al., 2015; Park et al., 2013).

Table 4.13. *Blautia* BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	1 st pathway	2 nd pathway	3 rd pathway	References
Substrates	NSP; S (1), RS; S (1), Sugars; S (1)	CO ₂ ; Se (2), H ₂ ; Se (4)	Formate; Se (2), NSP; S (1), RS; S (1), sugars; S (1)	(Drake et al., 2008; Kamlage et al., 1997; Kettle et al., 2018, 2015; Louis et al., 2014; Müller, 2003)
Metabolites	Acetate (3)	Acetate (1), H ₂ O (2)	Acetate (3), H ₂ (2), CO ₂ (2)	
μ_{max} (d⁻¹)	0.3 (NSP), 0.3 (RS), 1 (sugars), 0.1 (CO ₂)			(Kettle et al., 2018, 2015)
K (g/l)	0.001			Empirically determined during this study
Y (g l⁻¹/ g l⁻¹)	0.286 (NSP), 0.333 (RS), 0.333 (sugars), 0.03 (CO ₂)			(Kettle et al., 2018, 2015)
pH corners	6, 6.5, 6.6, 7			(Bernalier et al., 1996; Liu et al., 2008)

4.2.4.12 RC9 BU

The RC9 gut group belongs to the *Rikenellaceae* family, therefore the data frame is based on the characteristic of the two genera of this family, *Rikenella* and *Alistipes*, whose metabolism relies mainly on fermentation of few carbohydrates, mostly simple sugars, whilst producing mainly succinate, propionate and trace of acetate (Krieg et al., 2015) (Table 4.14). The stoichiometry is an estimated guess, based on the family description.

Table 4.14. RC9 BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	RC9 BU pathway	Reference
Substrates	Sugars; Se (3)	
Metabolites	Propionate (2), succinate (2), acetate (2), H ₂ O (2)	(Krieg et al., 2015)
μ_{max} (h⁻¹)	1 (sugars)	(Kettle et al., 2018, 2015)
K (g/l)	0.001	Empirically determined during this study
y (g/g)	0.333 (sugars)	(Kettle et al., 2018, 2015)
pH corners	6, 6.5, 7, 8.5	(Allison and Macfarlane, 1989; Song et al., 2015)

4.2.4.13 Alloprevotella BU

Alloprevotella spp. are moderately saccharolytic bacteria, whose main products of fermentation are acetate and succinate (Downes et al., 2013). The BU data frame is based on the description of the basonym *P. originally tanneriae* isolated from human oral cavity (consequently reclassified as *A. tanneriae*) (Downes et al., 2013; Moore et al., 1994), Whilst dynamic parameters values were based on *Prevotella* due to the close taxonomy between the two genera (Table 4.15).

Table 4.15. *Alloprevotella* BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	<i>Alloprevotella</i> BU pathway	Reference
Substrates	RS; S (1) Sugars; S (1), CO ₂ ; S (1)	
Metabolites	Succinate (1), acetate (1), formate (1)	(Downes et al., 2013)
μ_{max} (h⁻¹)	1 (RS), 1 (sugars)	(Kettle et al., 2018, 2015)
K (g/l)	0.001	Empirically determined during this study
y (g/g)	0.333 (RS); 0.333 (sugars)	(Kettle et al., 2018, 2015)
pH corners	5, 6.5, 7, 8	(Downes et al., 2013)

4.2.4.14 *Succinivibrio BU*

At the time of writing only one species, *S. dextrinosolvens* was described as belonging to this genus and therefore *Succinivibrio BU* data frame was developed through one pathway based on genus description (Bryant, 2007; O'Herrin and Kenealy, 1993) (Table 4.16). *S. dextrinosolvens* are strictly anaerobic, Gram negative rods, whose metabolism relies mainly on carbohydrate fermentation, with glucose, D-xylose, maltose, galactose, dextrin and pectin being the main substrate used, and whilst some strains use cellobiose as a resource none of them seem to be able to use starch directly (Bryant and Small, 1955). Stoichiometry assumption was based on metabolite pattern reported for *S. dextrinosolvens* growing in rumen fluid-glucose medium (Bryant and Small, 1955), whilst dynamic parameters were due to theoretical considerations as per MFG3 from previous model of human gut microbiota (Kettle et al., 2018, 2015) due to similarities in metabolite pattern and substrate demand.

Table 4.16. *Succinivibrio BU* data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	<i>Succinivibrio BU</i> pathway	Reference
Substrates	Sugars; S (1), NSP; S (1), CO ₂ ; S (1)	(Bryant and Small, 1955)
Metabolites	Succinate (1), acetate (1), formate (1)	
μ_{max} (h⁻¹)	0.7 (NSP), 1 (sugars)	(Kettle et al., 2018, 2015)
K (g/l)	0.001	Empirically determined during this study
y (g/g)	0.286 (NSP), 0.333 (sugars)	(Kettle et al., 2018, 2015)
pH corners	6, 6.8, 6.9, 8	(Hailemariam et al., 2020)

4.2.4.15 *Treponema* BU

Amongst the 31 species of *Treponema* described at the time of writing, only the microorganisms isolated from the porcine gastrointestinal tract were considered to build *Treponema* BU data frame. Nevertheless, no cultural information was found for *T. berlinense* and *T. porcinum*, isolated from the swine gut (Nordhoff et al., 2005), thus the stoichiometry assumption was based on the metabolisms of *T. succinifaciens*, isolated from colon of pigs (Cwyk and Canale-Parola, 1979) (Table 4.17). *T. succinifaciens* ferments carbohydrates but not protein and requires CO₂ for growth, whilst main metabolites produced are acetate, formate, succinate and lactate (Cwyk and Canale-Parola, 1979). Whilst *K* and yield values followed the same theoretical considerations adopted for the rest of the BUs, μ_{max} and pH corners were assigned after empirical and theoretical considerations during this study.

Table 4.17. *Treponema* BU data frame. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	<i>Treponema</i> BU pathway	Reference
Substrates	RS; S (3), Sugars; S (3), CO ₂ ; Se (2)	
Metabolites	Acetate (2), formate (2), succinate (2) and lactate (2)	(Cwyk and Canale-Parola, 1979)
μ_{max} (h⁻¹)	0.5 (RS), 1 (sugars)	Empirically determined during this study
<i>K</i> (g/l)	0.001	Empirically determined during this study
<i>y</i> (g/g)	0.333 (RS), 0.333 (sugars)	(Kettle et al., 2018, 2015)
pH corners	6, 6.2, 6.5, 7	This study (theoretical considerations)

4.2.4.16 *Clostridium* BU

Assumptions used to develop this data frame were based on the type species *C. butyricum* (Rainey et al., 2009), due to its documented presence in gut of healthy animals, which also explain its importance as a probiotic in pigs (Sato et al., 2019). The stoichiometry assumption was based on butyrate-kinase pathway of *C. butyricum* (Guo et al., 2020). This species can ferment most carbohydrates, including RS and some NSP such as pectin for example, whilst main metabolites are butyrate, acetate, formate, with minor amount of lactate and succinate, H₂ and CO₂ are also sometimes detected (Rainey et al., 2009). The stoichiometry of the first pathway of this data frame was based on cultural-based observations from Schink et al. (1981), which analysed pectin consumption of *C. butyricum*. Interestingly, it has been recently demonstrated that *C. butyricum* can convert lactate in butyrate, when in presence of acetate, which represented the assumption for the second pathway of this data frame (Detman et al., 2019). Kinetic parameters were based on same theoretical considerations as in the previous model for the human gut microbiota for bacteria belonging to the same functional group (Kettle et al., 2018, 2015) (Table 4.18).

Table 4.18. *Clostridium* BU pathways. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	1 st pathway	2 nd pathway	Reference
Substrates	Sugars; S (2), RS; S (2), NSP; S (2)	Lactate: S (4), acetate: Se (1)	(Detman et al., 2019; Rainey et al., 2009; Schink et al., 1981)
Metabolites	Butyrate (1), acetate (3), formate (2).	Butyrate (2), Ethanol (1), CO ₂ (4), H ₂ (2), H ₂ O (1)	
μ_{max} (h⁻¹)	0.3 (NSP), 0.3 (RS), 1 (sugars), 0.2 (lactate)		(Kettle et al., 2018, 2015)
K (g/l)	0.001		Empirically determined during this study
y (g/g)	0.286 (NSP), 0.333 (RS), 0.333 (sugars), 0.11 (lactate)		(Kettle et al., 2018, 2015)
pH corners	6.0, 6.5, 6.6, 7.5		(He et al., 2005)

4.2.4.17 *Lactobacillus* BU

As seen in Chapter 1, amongst species belonging to this genus found in the porcine gut, *L. mucosae* has been previously isolated from pig intestine and has been described as an obligate heterofermentative producing both D- and L-lactate from non-complex saccharides (Roos et al., 2000). On the other hand, *L. pontis* has been described to produce lactate, acetate, ethanol and CO₂ from fermentation of maltose or fructose (Vogel et al., 1994), similar to what described for the obligate heterofermentative *L. reuteri* (Pancheniak et al., 2012), which is found as endogenous in pigs (Hou et al., 2015b). Therefore, as seen for MFG4 stoichiometry assumption for *Lactobacillus* BU was based on *L. reuteri* growing in a chemostat with 20 mM glucose (Burgé et al., 2015; El-Ziney et al., 1998) with theoretical considerations for acetate production since its production depend on environmental conditions (Årsköld et al., 2008) and cannot be excluded in the gut: $2C_6H_{12}O_6 \rightarrow CO_2 + 3CH_3COOH + C_2H_5OH + C_3H_6O_3$ (Table 4.19).

Hexose and pentose metabolised through heterofermentative reactions in lactic acid bacteria enter phosphoketolase pathway yielding lactate and ethanol, or lactate and acetate respectively (Gänzle, 2015). It is acknowledged that in some cases, some species (e.g. different *L. reuteri* strains) can convert carbohydrates into lactate through homofermentative pathways using the Embden-Meyerhof pathway (EMP) (Burgé et al., 2015). Nevertheless, as detailed for MGF4, this BU stoichiometry is based on heterofermentative reactions through phosphoketolase pathway of *L. reuteri*, since it represent its main source of energy (Årsköld et al., 2008), and as stated above, it seems to be the most diffused amongst pig gut species (Pancheniak et al., 2012; Roos et al., 2000; Vogel et al., 1994).

Assumption for μ_{max} and Y was based on theoretical consideration as performed by other authors a previous model for the human gut microbiota (Kettle et al., 2018, 2015), whilst for pH corners were based on observations of *L. reuteri* growing at different pH values (Klantschitsch et al., 1996; Palmfeldt and Hahn-Hägerdal, 2000).

Table 4.19. *Lactobacillus* BU pathway. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	<i>Lactobacillus</i> BU pathway	Reference
Substrates	Sugars; S (2)	(Årsköld et al., 2008; Burgé et al., 2015; Chen et al., 2016; El-Ziney et al., 1998)
Metabolites	Acetate (3); Lactate (1), Ethanol (1), CO ₂ (1)	
μ_{max} (h ⁻¹)	1	(Kettle et al., 2018, 2015)
K (g/l)	0.001	Empirically determined during this study
y (g/g)	0.333	(Kettle et al., 2018, 2015)
pH corners	4, 6, 6.8, 7.5	(Klantschitsch et al., 1996; Palmfeldt and Hahn-Hägerdal, 2000)

4.2.4.18 *Prevotella* BU

As seen in Chapter 1 for members of MFG1, *Prevotella* is one of the most abundant genera in the porcine colon throughout pigs' life (Crespo-Piazuelo et al., 2018), with *P. ruminicola* being one of the most abundant species (H. Yang et al., 2017). Therefore, as detailed earlier in the text, observations of *P. ruminicola* from culture-based experiments have been used to parametrise this BU. Stoichiometry assumption for NSP, RS and sugars was based on fermentation pattern analysis of *Prevotella ruminicola* growing on different substrates (4g/l) (Marounek and Dušková, 1999). *P. ruminicola* has been shown to growth efficiently on peptides but not amino acids (J. N. Kim et al., 2017), although due to lack metabolite analysis, the assumption for the stoichiometry for protein degradation was based on the same functional group from the previous model of human colonic microbiota, which was based on *Bacteroides spp.*, whose theoretical consideration for μ_{max} and Y were also used here to parametrise this data frame (Kettle et al., 2018, 2015). Assumption for pH corners were based on observations of *Bacteroides ruminicola* (basonym) growing at different pH values (Russell and Dombrowski, 1980) (Table 4.20).

Table 4.20. *Prevotella* BU pathways. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	1 st pathway	2 nd pathway	References
Substrates	NSP; S (6), RS; S (6); Sugars (6); H ₂ O (Sw) (2)	Pr; S (6)	(Kettle et al., 2018, 2015; Marounek and Dušková, 1999)
Metabolites	Acetate (4), lactate (2) succinate (4), propionate (1), , H ₂ (9), CO ₂ (3)	Acetate (2), propionate (1), succinate (1), H ₂ (2), CO ₂ (1), other (7)	(Kettle et al., 2018, 2015; Marounek and Dušková, 1999)
μ_{max} (d⁻¹)	0.5 (NSP), 1(RS), 1 (sugars)	1 (Protein)	(Kettle et al., 2018, 2015)
K (g/l)	0.003	0.003	Empirically determined during this study
Y (g l⁻¹/ g l⁻¹)	0.286 (NSP), 0.333 (RS), 0.333 (sugars)	0.2 (Protein)	(Kettle et al., 2018, 2015; Kovar et al., 1999)(Kettle et al., 2018, 2015; Kovar et al., 1999)
pH corners	5.25, 6.0, 6.5, 7.0		(Russell and Dombrowski, 1980)

4.2.4.19 *Turicibacter* BU

The BU is based on the genus description, with reference to the type species, *T. sanguinis*, which grows in meat chopped carbohydrates medium using proteins and few sugars only (Bosshard et al., 2002a, 2002b). The two BU pathways described in the data frame in Table 4.21 were based on the pyruvate formate lyase and the lactate dehydrogenase pathways, due to few information retrieved about *Turicibacter* (Bosshard et al., 2002a, 2002b). Formate production was not reported for the type species, thus it was assumed that the latter was completely transformed in H₂ and CO₂, therefore the reaction catalysed by the formic hydrogen-lyase was modelled as a part of the pyruvate formate lyase pathway. Kinetic parameter values were based on theoretical considerations, based on the approach taken for the model for the human colonic microbiota as described earlier in the text (Kettle et al., 2018, 2015).

Table 4.21. *Turicibacter* BU pathways. Stoichiometry is depicted in brackets for both substrates and metabolites, whilst substrate type is indicated each time (S: substitutable; Se: essential; Sb: boosting; Sw: water).

	1 st pathway	2 nd pathway	References
Substrates	Sugars; S (2), H ₂ O; Sw (1)	Protein; S (3.246), H ₂ O; Sw (1)	(Bosshard et al., 2002a, 2002b)
Metabolites	Lactate (3), Acetate (1), H ₂ (2), CO ₂ (1)	Lactate (3), Acetate (1), H ₂ (2), CO ₂ (1)	
μ_{max} (h ⁻¹)	1 (sugars), 1 (protein)		(Kettle et al., 2018, 2015)
<i>K</i> (g/l)	0.001		Empirically determined during this study
<i>y</i> (g/g)	0.333 (sugars), 0.2 (protein)		(Kettle et al., 2018, 2015)
pH corners	6.4, 7.0 ,7.5, 8.1		(Bosshard et al., 2002a, 2002b)

4.3 Model comparison with literature data

In order to explore the CM model dynamics, the 19 BU data frames were assembled in a similar way to what described in Chapter 1 for the MFGs, to form a CM model. Such CM model was thus composed by 19 BUs and was set up and solved through the same system of ODEs described in details previously in [Chapter 1](#) (Kettle et al., 2018, 2015). Once solved, the ODEs returned the rate of change of BUs, substrate and metabolite over time. Moreover, model settings applied allowed to simulate *in silico*, an *in vitro* fermentation experiment, which was thus compared to 2 different literature studies. In such way, outputs from the studies and from the CM model were compared looking for differences in fermentation pattern between what observed for the whole microbiota in literature and what forecasted *in silico* for the CM. Table 4.22 depicts a summary of different experimental conditions and principal findings throughout the studies used for the comparisons, whereas a detailed description of the findings from each comparison is given in the following sections.

Table 4.22. Description of the experiments used to compare the CM models. IR: Inoculum reactor; CR: Control reactor.

	#1 (Lin et al., 2011)	#2 (Ding et al., 2015)
Aim	Batch culture to assess the microbial degradation of three different types of fibres	Batch culture to assess the effect of different structures of flaxseed dietary fibres on the microbial community.
Sample used	Inoculum from pig caecum digesta; Inoculum from pig colon digesta	Pig colon digesta (Sample frozen before starting the culture)
Starting conditions	Inoculum: 13.89 g/l; NSP: 2.425 g/l; Proteins: 0.00875 g/l	Inoculum: 100 g/l; NSP: 10 g/l
Data collected	Relative abundance (%) of the major SCFAs	Concentration (mM) and relative abundance (%) of the main SCFAs

4.3.1 1st simulation; colon and caecum digesta batch cultures (Lin et al., 2011).

Lin et al. (2011) assessed microbial ability to degrade three different types of fibres (i.e. β -glucan, flaxseed gum and fenugreek gum) through carrying out different batch fermentations experiments. In their *in vitro* batch cultures, the authors grew two different inocula from caecum and colon digesta, respectively, whilst analysing the relative abundance of the main SCFAs and lactate. The inoculum (0.25 g) previously stored at -20°C, was prepared in 1.8 ml anaerobic incubation medium (AIM), and then 0.5 ml of this mixture was used in a batch culture ($D = 0 \text{ h}^{-1}$) of 4.5 ml of AIM containing the tested fibre. The inoculum starting concentration was modelled as 13.89 g/l and the tested fibre concentration (0.25 % W/V in the study) as 2.5 g/l, of which 97 % carbohydrates and 0.35 % of protein. In the study, main SCFAs and lactate concentration was measured 96 hours after the batch fermentation started and expressed as a percentage value. After this time-step, an inoculum (10 % of the current culture) was transferred in a fresh medium vessel, maintaining the medium composition. Two further inoculations with 10% of the previous culture were performed every fourth day, measuring the SCFAs concentration at each time-step. For the present *in silico* comparison, only the results of the first cultural time step (0h to 96h) when testing the microbial fermentation of the β -glucan fibres were considered. The SCFA pattern observed *in vitro* from the colon digesta inoculum was compared with the colon lumen-CM. The study showed also the results for iso-butyrate, but since this is not one of the modelled metabolites its data were not considered in the comparison and the SCFAs relative abundance was adjusted consequently. Colonic pH was modelled as 6.35 as the average of pH values normally recorded throughout the colon (Merchant et al., 2011).

4.3.1.1 Results

The SCFA pattern predicted by CM model compared to colon digesta observations was somewhat similar to the *in vitro* observations (Figure 4.7). In detail, predicted and observed acetate relative abundance was 48.70% and 56.15%, respectively, whilst predicted and observed butyrate relative abundance was 14.29% and 17.14%, respectively. Less identical values were predicted for propionate, whose observed abundance in the study was 26.72%, whilst *in silico* abundance was predicted as 37.01% after 96 hours simulation. Lactate was not detected in the simulations, confirming what also observed *in vitro* (Figure 4.7).

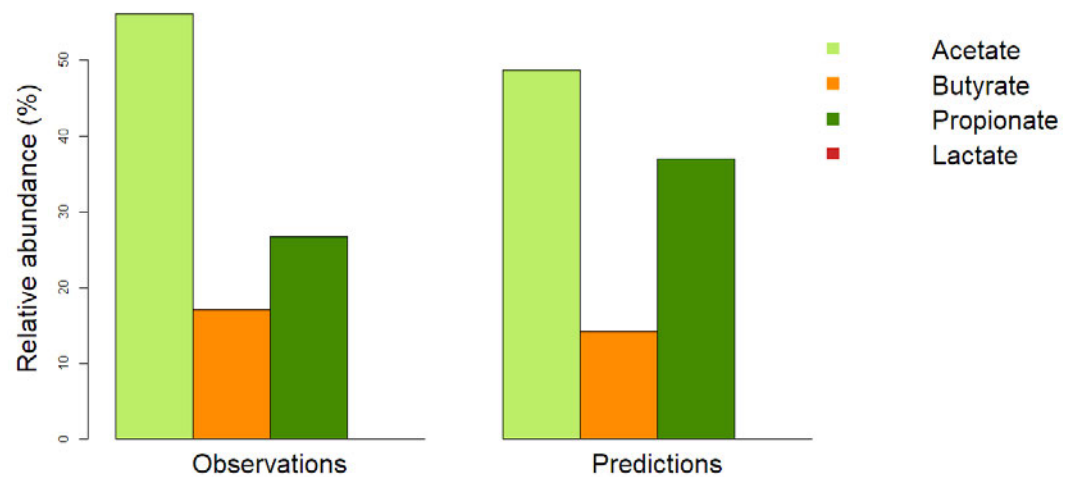


Figure 4.7. Comparison between the colon digesta batch culture (Lin et al., 2011) and the model outputs.

4.3.2 2nd simulation; pig colon digesta batch culture (Ding et al., 2015).

In this study, main SCFA concentration was investigated in relation to microbiota fermentation of different structures of flaxseed dietary fibres (Ding et al., 2015). Therefore, an inoculum derived from colon digesta (10%, w/v) was grown in batch culture for 72 hours in a medium (total volume of 6ml) containing 1% (w/v final concentration) of the studied fibre as a sole carbon source. Starting conditions modelled were 100 g/l for the inoculum, 10 g/l for NSP (considering psyllium arabinoxylans as a substrate), 0 h⁻¹ for D, and 6.35 for pH considering average values observed throughout colon *in vivo* (Merchant et al., 2011).

4.3.2.1 Results

The CM model predicted a lower concentration of acetate (35.38mM) after 72 hours simulation when compared with 56.16mM observed in the study after the same period of time through which the batch fermentation was carried out (Figure 4.8). On the other hand, both propionate and butyrate predicted concentrations were higher than what observed *in vitro* by the authors of the study used for this comparison. Indeed, propionate concentration predicted was 26.93mM, whilst propionate concentration observed was 7.08mM. At the same time butyrate concentrations observed and predicted were 1.80mM and 10.48mM, respectively.

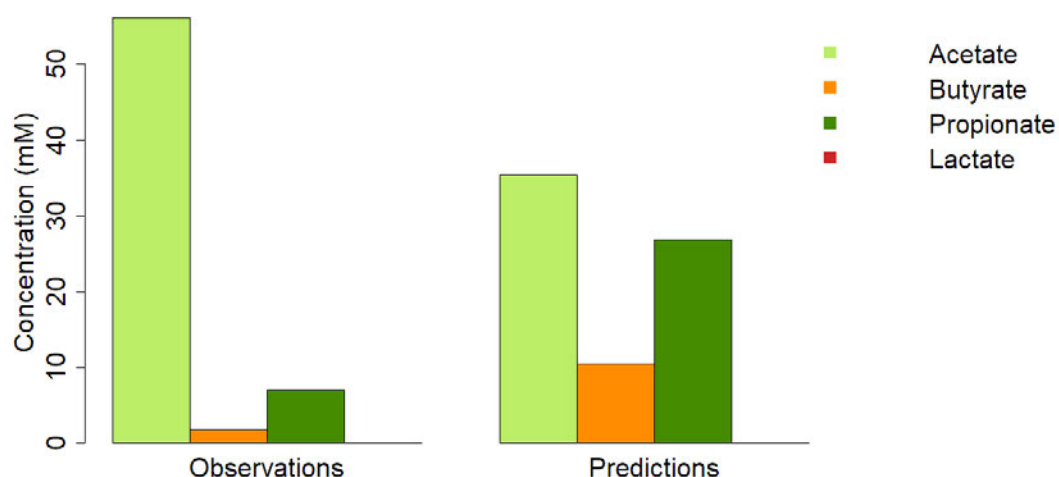


Figure 4.8. Comparison between the pig colonic digesta batch culture observations (Ding et al., 2015) and the CM model predictions when simulating the same experimental conditions.

4.4 Discussion

Following a meta-analysis study (Holman et al., 2017), the first dynamic model of the porcine colonic CM was developed and presented here. Such meta-analysis compared and found similarities between microbiota composition through 20 published studies, identifying 19 bacterial genera composing the colonic CM. During the course of this thesis, these CM genera were modelled as bacterial units, taking into account metabolic pathways and substrate preferences of representative species, when possible originally isolated from porcine gut. In this way 19 BU data frames were generated, one for each CM genus identified in the colon. These data frames were based on empirically determined half saturation constant, whilst parameters for yield and maximum growth rate were established through theoretical considerations as previously performed by other authors when developing a model for the colon human microbiota (Kettle et al., 2018, 2015). Stoichiometry in the 19 data frames was modelled according to information on metabolic pathways of the representative species, and when available on culture-based studies on the same representative species. Despite the accompanying grade of unavoidable uncertainty, the CM model, built assembling the 19 data frames in microPop in R (Kettle et al., 2018), was assumed to represent the dynamics of a similar bacterial community growing *in vitro*.

When using the CM model to simulate literature studies exploring microbiota fermentation activities, it was noticed that in general, the CM model tended to overestimate the total concentration of propionate whilst underestimating total acetate concentration. On the other hand, predicted butyrate concentration was lower than observations at the end of one of the simulations and higher than observed values in the second simulation. Interestingly, both simulations predicted close-to-zero lactate concentration, which is in accordance to what usually observed *in vivo* and *in vitro* (Roberfroid et al., 2010). The differences between observed and predicted SCFA values could be due the choices made during the construction of the BU data frames, which could indicate that either the dynamic parameters selected, or the stoichiometry could have been partly representative of CM-genera dynamics, since data frames were indeed based on representative species only. On the other hand, it must be noticed that the total concentration of colonic CM species was reported as ~50% of the total microbiota (Holman et al., 2017), therefore difference between predictions and observations could possibly indicate that functionally important genera were likely missing when modelling only CM genera as BUs. By contrast, some similarities were noticed in the SCFA patterns predicted and observed, which could possibly indicate that CM could have a role in forming the basis for the SCFA production *in vivo*.

Whether the predictions simulated here are effectively compatible with the dynamics of a similar bacterial community *in vitro* needs further investigations. The present study could indeed lay the basis for a future *in vitro* experimentation, during which the dynamics and metabolite production of an inoculum composed of CM genera could be grown under specific

conditions *in vitro*. In conclusion, the present chapter presents the first dynamic model of the colonic core microbiota, based on its composition as described during a recent meta-analysis. The CM model was further used to analyse the CM metabolite pattern when compared to literature studies describing whole colonic microbiota fermentation. The model designed, developed and solved in this chapter could provide the basis for possible future studies on CM role within the whole microbiota, whilst predicting an eventual SCFA pattern according to the experimental design applied.

5 In silico experimentation: probiotic and prebiotic simulations

5.1 Introduction

Pig gut microbiota relates to the host at several levels, and to some extent, it has been considered as another organ due to its chemical and physical interaction with it (Hooper et al., 2001). Apart from being responsible for supplying about 30% of the energy requirements for a growing pig through metabolising molecules otherwise undigested by the host (Metzler and Mosenthin, 2008), gut microbiota is connected to the maturation of the immune system through the selection of the host humoral immunity (L. Yang et al., 2017). There is a clear link between mucosal microbiota composition and inflammatory bowel disease, as changes in the microbial communities has been reported during mucosal inflammation (Walker et al., 2011). Moreover, apart from being associated with Chron's disease (Schaubeck et al., 2016) and obesity (Neis et al., 2015), gut microbiota has been also been proposed as a therapeutic target for central nervous diseases (Galland, 2014; Martin et al., 2018; Winek et al., 2016). Due to its multiple interactions with the host, manipulations, such as modification of its composition and therefore of the microbial metabolic pattern could influence host health and performance (Fouhse et al., 2016). There are currently many strategies targeting to modify the microbiota, such as probiotic, prebiotic interventions and faecal transplantation (Fuentes S. and de Vos W. M., 2016; Roberfroid et al., 2010; Sakata et al., 2003).

According to the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), probiotics are defined as “live micro-organisms which when administered in adequate amounts confer a health benefit on the host” (FAO and WHO, 2001). Moreover, the main features of a probiotic applied to pig nutrition can be summarised as 1) ability to colonise the gut while being metabolically active; 2) ability to improve host health either directly or indirectly; 3) ability to be produced at industrial level and 4) ability to be safely used in the host without being toxic and/or pathogenic and without transmission of antimicrobial resistance genes (Gaggia et al., 2010). Applications of probiotic therapies can occur at different stages of the animal life, such as in sows to enhance their health and performance increasing their feed consumption or increasing their body conditions at the end of the lactation. Weaning piglets could benefit from probiotic to ameliorate the mucosa response to the change towards a solid diet, finishing pigs could exploit probiotic therapies as antibiotic alternatives (Barba-Vidal et al., 2019).

Dietary prebiotics are defined as “selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring

benefit(s) upon host health” (Gibson et al., 2010). The main action of prebiotics would thus be to promote the proliferation of “beneficial” microbiota members, which could be maximised selecting dietary molecules that usually escape the host digestion becoming the main bacterial metabolic substrate (Zimmermann et al., 2001). Several plant derived substances have been proposed and tested as a prebiotic, such as resistant starch or small undigested oligosaccharides, which seemed to affect the caecal concentration of the main bacterial short-chain fatty acids (SCFAs) (Pan et al., 2009; Topping et al., 2003). The possible prebiotic applications are the same as the ones described for the probiotics, and a combination of both has also been described to enhance their beneficial effects (Zimmermann et al., 2001).

It is clear how the possible advantages that could be derived from those therapies are directly related to the knowledge of the microbiota and its relationship with the host (Barba-Vidal et al., 2019). Moreover, further studies are required to gain a solid understanding of the effect of these interventions (Barba-Vidal et al., 2019). Mathematical modelling could be a valid way of studying changes in the SCFA pattern due to probiotic or prebiotic therapies. Thus, the two models developed and presented in the previous chapters could represent reliable tools to explore such changes. The main feature of both the entire pig gut microbiota (whole microbiota) model (WM) (Chapter 3) and the porcine core microbiota (CM) model (Chapter 4) is that starting conditions are fully editable. In this way, instead of simulating a conventional diet/microbial relative abundance, the models could simulate enhancement in either substrates (prebiotic interventions) or microbial groups (probiotic interventions), or in fact both. Therefore, in this chapter *in silico* probiotic and prebiotic interventions are studied through performing different set of simulations. First, in order to assess whether both the models were reliable to anticipate the same changes observed *in vitro/in vivo*, a literature study exploring the probiotic effect of *L. casei* on SCFAs was simulated. In the two models, the microbial communities were summarised either in bacterial functional groups (MFGs) for the WM or in bacterial units (BUs) for the CM model. Thus, *in silico* MFG or BU enhancement (probiotic simulation) and then *in silico* substrate enhancement (prebiotic simulation) were performed in further two sets of simulations to study their possible effect on the SCFA production. Finally, a combined therapy using both probiotic and prebiotic, based on the results of the previous simulations, was used to explore the relative effect on the major metabolites inferring the possible positive effects on host health. The different *in silico* studies are fully described in the following sections.

5.2 Simulation of probiotic treatment found in literature

To assess the reliability of the models in predicting microbiota interventions and further investigate *in silico* possible strategies, the study from Ohashi et al. (2004) was simulated, and the observations from the study were compared with the model predictions. In their work the authors tested the effect of *L. casei* strain Shirota (LCS) administration on acetate, propionate and butyrate. Three pigs were used during a 32 day experiment, divided in a 16 day pre-administration (pigs fed on a normal diet) and a 16 day administration period (normal diet plus probiotic). Each period was composed by 14 days of adaptation and 2 days of sampling. During the administration time, at least 10^{10} colony-forming units (CFU) of LCS/day were provided to the animals (contained in Yakult fermented milk, Yakult, Japan), while they were being fed on a commercial diet (Nikuton VV) containing crude protein, 13.6%; crude fat, 1.7%; crude fibre, 2.6%; and crude ash, 2.8% (Tsukahara et al., 2014). The same diet was used during the pre-administration period. On the 16th day of each period the SCFA concentration was measured and ANOVA test used to assess the effect of the probiotic treatment on the metabolites. The authors did not find any significant difference in the caecal acetate, butyrate and propionate concentrations between the two diets (i.e. with and without probiotic).

5.2.1 Model environment

Both the WM and the CM were used to simulate the work from Ohashi et al. (2018). The experimental conditions described in the study were assumed to be similar to the porcine proximal colon, thus retention time (9h) (Tanner et al., 2014), pH (6.25) (Kararli, 1995; Tanner et al., 2014) and bacterial relative abundance were simulated accordingly. The colon-lumen CM model (Chapter 4) and the MFG faecal composition (Chapter 3) were used to simulate the microbial abundance, assuming the faecal composition used to model the WM an approximation of the colonic microbiota. Also, as specified in Chapter 1, the biotic factor, such as the SCFA absorption from the colon mucosa, were not included in the models. The models were developed and solved in R using the package *microPop* (Kettle et al., 2018), which uses data frame to summarise the bacterial metabolism/substrate preferences and to summarise the starting conditions (i.e. bacterial relative abundance and substrate concentration in the system). As described in detail in Chapter 1, *microPop* uses the function *microPopModel* to apply the conditions specified in the data frames in the context of a system of ordinary differential equations that simulate the microbial growth, the substrate consumption and the metabolite production returning their concentration at a given point in time. The dilution rate was considered as the reciprocal of the 9h retention time (Tanner et al., 2014) (0.11/h; 2.67/d), whereas to model the substrate concentration (Table 5.1) the digestion indexes (DI) of protein, NSP and sugars was considered. The NSP DI was assumed to be 60% considering the variability among the plant species and processing methods (Jha and Berrocso, 2015; Kerr and Shurson, 2013), whereas the protein DI was assumed as 75% (van der Wielen et al., 2017). Colonic sugar concentration was assumed to be 1g/l taking into account dietary sugars

DI, which would not be normally found in the colon and endogenous secretions (e.g. from mucins). The probiotic intervention was modelled as a separate BU or MFG for the CM or the WM model respectively. Therefore, LCS was considered either a *Lactobacillus* BU or a MFG4 (lactate producer bacteria), in the CM or the WM respectively, and had a dedicated data frame with a particular relative abundance, which was equal to 0 during the simulation of the pre-administration time and equal to 0.07 g/l/d during the administration time. In their work, Ohashi et al. (2004) used 10^{10} CFU per day of probiotic, thus, to model the starting conditions as g/l, the dry weight of *E. coli* of 0.95 pg was considered, assuming the latter as a close approximation to the LCS dry weight as follows:

$$10^{10} \text{CFU in } 130 \text{ ml} = 7.69 \cdot 10^{10} \text{ CFU in } 1 \text{ l} \quad (5-1)$$

$$(7.69 \cdot 10^{10}) \cdot 0.95 \text{ pg} = 7.31 \cdot 10^{10} \text{ pg/l} \quad (5-2)$$

$$7.31 \cdot 10^{10} \text{ pg/l} = 0.07 \text{ g/l} \quad (5-3)$$

To simulate the different animals used during the study each MFG/BU was assigned 5 strains with a 10% variation in the parameter values (*numStrains*=5 and *percentTraitRange*=10 in the *microPopModel* function). Three different runs were used to generate stochastic parameters for five strains instead of one at the beginning of each run, simulating three different pigs with similar microbiota having equal relative abundance but somewhat different parameters. It is important to note that although, in order to mimic the experimental design from the paper, three different runs were used in this context to simulate three similar microbiota in three different animals, they were not comparable with the individual pigs used in the study. A t-test analysis was conducted to assess first the difference between the observations and the predictions and afterwards to confirm the findings in the literature, comparing the steady state SCFA concentrations between the simulated pre- and administration periods. The t-test was preferred to the ANOVA test in this case because only two vectors were compared each time (e.g. acetate observed versus acetate predicted). In the following sections, the results from the comparison first with the WM model and then with the CM model will be fully described.

Table 5.1. Substrate starting concentration modelled during the simulation of the probiotic treatment with *L. casei* strain Shirota (Ohashi et al., 2004)

Protein	34 g/l
NSP	15.6 g/l
Sugars	1 g/l

5.2.1.1 Pig gut microbiota

T-test conducted between observations and the showed that the porcine gut microbiota model was successful in predicting results observed during both experimental periods, especially for propionate, although it was less precise in predicting acetate and butyrate concentration both before and after probiotic administration (Table 5.2). In particular, the model tended to overestimate acetate, whilst underestimating butyrate (Figure 5.1). The model predicted an slight increase in butyrate when simulating the probiotic treatment, (Figure 5.1).

Table 5.2. P-value calculated through the T-test between the observations from literature (Ohashi et al., 2004), i.e. caecal SCFA concentration amongst the three pigs, and the predictions from the WM model, i.e. steady-state SCFA concentration output of the three runs. The table shows the p-values calculated for both the pre-administration and the administration periods.

	Pre-administration	Administration
Acetate	0.05	0.05
Propionate	0.13	0.18
Butyrate	0.02	0.05

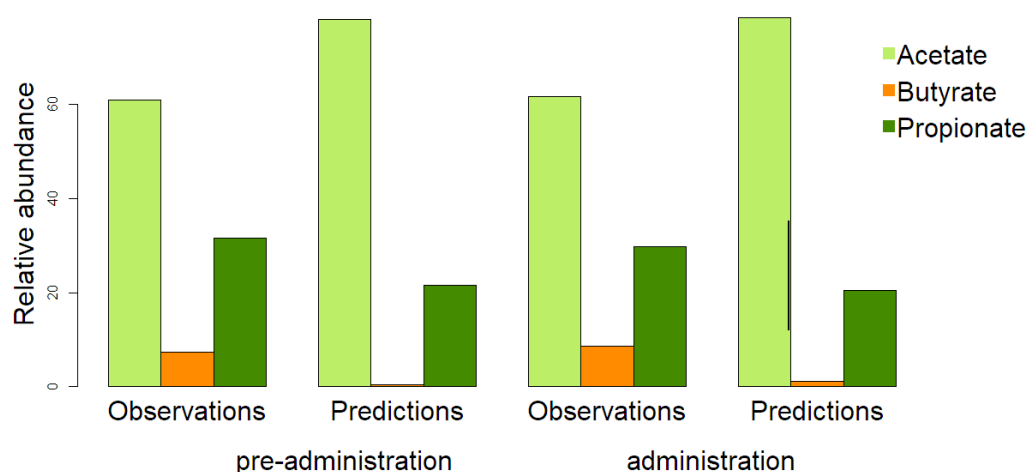


Figure 5.1. Major SCFA relative abundance observed in the study by Ohashi et al. (2004) and predicted by the WM model comparing the pre-administration and the administration periods. The results depicted here for the predictions resemble the value simulated when the model had just one strain and without generating random parameters, therefore they represent the value predicted from only one of the three pig used in total for the comparison.

5.2.1.2 Porcine core microbiota

Observations from the study and colon lumen CM model predictions were rather similar when the latter was used to simulate the experimental conditions found in literature (Figure 5.2), although butyrate was underestimated by the model. Indeed, amongst the three SCFAs, only butyrate showed differences between *in-silico* and *in-vivo* values (Table 5.3). Predicted relative abundance was almost identical between *in-silico* pre-administration and *in-silico* administration periods, confirming that the probiotic intervention did not affect major metabolite concentration, as found in the literature study.

Table 5.3. P-value calculated through the T-test between the observations from literature (Ohashi et al., 2004), i.e. caecal SCFA concentration amongst the three pigs, and the predictions from the CM model, i.e. steady-state SCFA concentration output of the three runs. The table shows the p-values calculated for both the pre-administration and the administration periods.

	Pre-administration	Administration
Acetate	0.69	0.82
Propionate	0.39	0.34
Butyrate	0.02	0.05

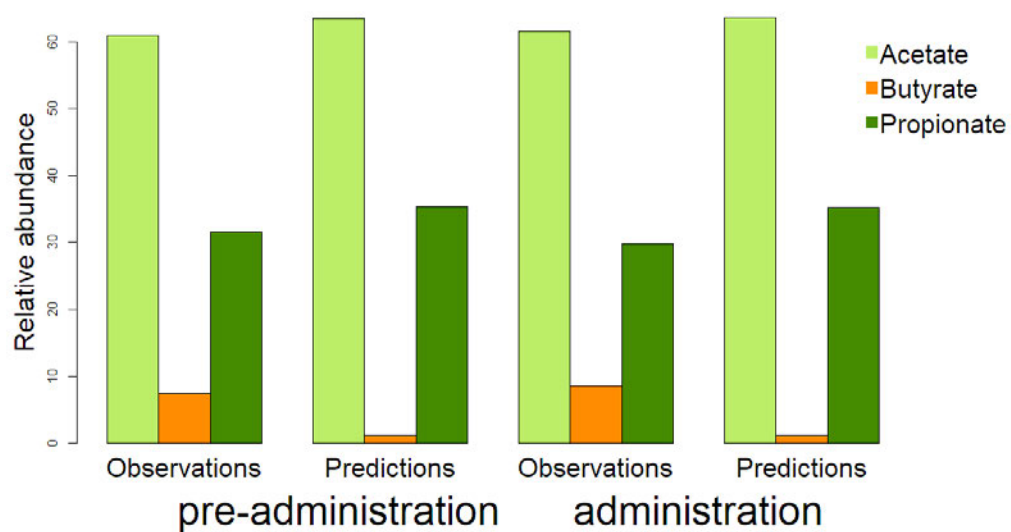


Figure 5.2. Major SCFA relative abundance observed in the study by (Ohashi et al., 2004) and predicted by the model of the colon-lumen CM, comparing the pre-administration and the administration periods. The results depicted here for the predictions resemble the value simulated when the model had just one strain and without generating random parameters, therefore they represent the value predicted from only one of the three pig used in total for the comparison.

5.3 Probiotic simulation

Both models were used to simulate the effect of probiotic interventions *in silico*, which were measured in terms of main SCFAs concentration. Therefore, both models were used to simulate multiple 32-day *in vitro* continuous fermentation experiments, in which microbial groups (i.e. MFGs or BUs) had an increased starting concentration and an increased flow-in concentration. In summary, the models simulated an *in vitro* fermentation, during which a specific microbial group amongst the ones already present in that community had an enhanced concentration. In this way, SCFA concentrations from simulation of probiotic intervention were compared with a simulation of the same model without probiotic enhancements. In each case, both starting concentration and in-flow concentration of MFGs/BUs were increased twofold compared to their concentration in the non-enhanced simulation, whilst substrate concentration and dilution rate remained the same (Table 5.4).

In the following sections the results for the probiotic simulation using the pig gut microbiota model and then the CM model are described in detail. The comparison between the probiotic simulation and a simulation without probiotic enhancements (from hereafter referred to as “normal simulation”) are therefore expressed according to the following:

$$\left(\frac{[SCFA]_{probiotic\ simulation} - [SCFA]_{normal\ simulation}}{[SCFA]_{normal\ simulation}} \right) \cdot 100 \quad (5-4)$$

Table 5.4. Substrate and dilution rate starting condition applied during the probiotic enhancement simulation in both the models.

	Substrates	Dilution rate
Pig gut microbiota	NSP= 7 g/l; Protein= 15 g/l; RS= 4.32 g/l; Sugars= 2 g/l	2.67 /d
Porcine core microbiota	NSP= 7 g/l; Protein= 15 g/l; RS= 4.32 g/l; Sugars= 2 g/l	

5.3.1 Pig gut microbiota

As depicted in Table 5.5 and in Figure 5.3 each MFG enhancement had a characteristic effect on the metabolite concentrations. In general, increasing two times the starting and flow-in concentration of MFG1 (acetate-succinate-propionate produces), MFG2 and MFG3 (fibre degraders), MFG4 (lactate producers), MFG7 (propionate producers) and MFG 9 (acetogens) led to a decreased steady state butyrate concentration, which increased when the probiotic groups simulated were MFG 5, MFG 6, MFG 8 (butyrate producers). Acetate increased due to probiotic simulations of MFG1, MFG2, MFG3, MFG4, MFG7 and MFG9, whilst propionate visibly increased only when simulating a probiotic intervention with MFG7. No changes in the steady state SCFA concentration was reported when simulating a probiotic intervention with MFG10.

Table 5.5. Effect on acetate, propionate and butyrate due to the 2x MFG enhancement. Normal indicates a simulation in which none of the MFGs had an increased concentration while running on the same experimental conditions as described in Table 5.4.

	Acetate (mM)	Propionate (mM)	Butyrate (mM)
Normal	66.46	31.54	22.19
2XMFG1	87.18	26.86	3.7
2XMFG2	87.36	22.35	4.34
2XMFG3	90.99	19.9	0.91
2XMFG4	145.93	18.04	5.05
2XMFG5	66.4	21.2	22.25
2XMFG6	61.22	27.4	25.55
2XMFG7	70.71	82.64	0.00
2XMFG8	49.3	18.65	43.43
2XMFG9	134.71	21.28	2.63
2XMFG10	66.46	31.54	22.19

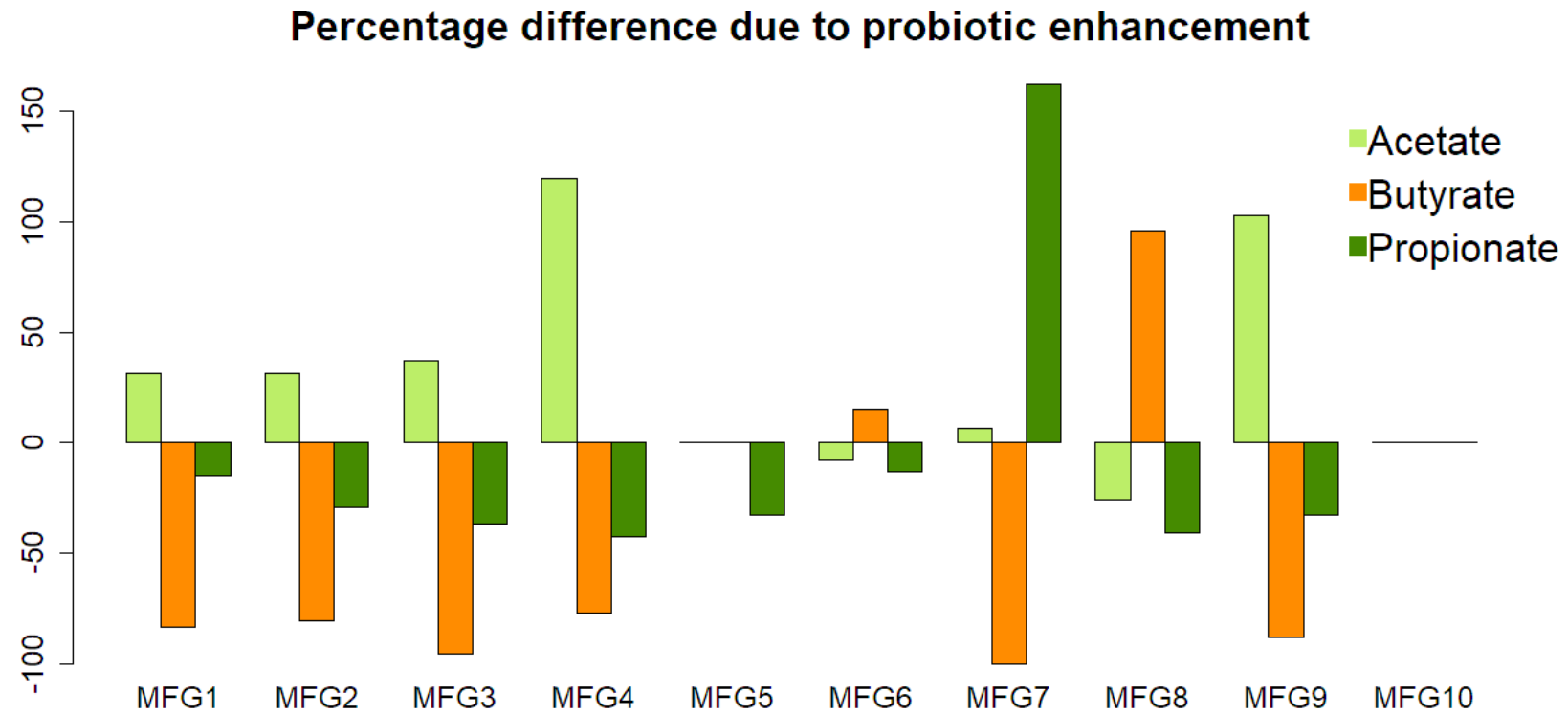


Figure 5.3. Percentage difference (Equation (5-4) between the simulations in which the MFG starting and flow-in concentrations were enhanced two times and a simulation (normal) in which none of the MFG concentrations were increased.

5.3.2 Porcine core microbiota

Similar to that observed for the pig gut microbiota, a twofold BU enhancement produced a characteristic effect on the major metabolites depending on which BU was used as a probiotic when using the porcine core microbiota model (Table 5.6 and Figure 5.4). *Pseudobutyrvibrio*, *Faecalibacterium*, *Turicibacter* and *Clostridium* BU probiotic interventions had a similar effect increasing the steady-state butyrate concentration, with the major effect on the molecule due to *Clostridium* BU enhancement. At the same time these probiotics had a negative effect on both acetate and propionate. *Clostridium* had a positive effect on acetate and a negative effect on propionate. Almost an unnoticeable effect on the metabolic pattern analysed was reported when simulating probiotic interventions using the rest of the BUs.

Table 5.6. Effect on acetate, propionate and butyrate due to the 2x BU enhancement (CM model). Normal indicates a simulation in which none of the BUs had an increased concentration while running on the same experimental conditions as described in Table 5.4

	Acetate (mM)	Propionate (mM)	Butyrate (mM)
Normal	76.06	92.39	7.27
Pseudobutyrvibrio BU	49.7	69.53	29.45
Parabacteroides BU	122.8	105.6	0.00
Phascolarctobacterium BU	76.06	92.4	7.27
Ruminococcus BU	83.25	86.48	4.61
Megasphaera BU	72.65	72.07	9.79
Sarcina BU	77.07	82.97	5.9
Faecalibacterium BU	38.91	68.04	36.88
Turicibacter BU	62.67	66.44	30.93
Escherichia BU	74.84	87.15	8.26
Bacteroides BU	60.45	88.68	9.44
Streptococcus BU	81.01	85.09	7.15
Blautia BU	147.75	67.23	4.42
RC9 BU	76.18	93.58	6.66
Alloprevotella BU	87.74	92.21	3.74
Succinivibrio BU	86.48	92.01	4.14
Treponema BU	77.31	87.76	8.65
Clostridium BU	85.03	0.14	71.25
Lactobacillus BU	80.77	86.53	7.54
Prevotella BU	68.09	75.4	6.6

Percentage difference due to probiotic enhancement

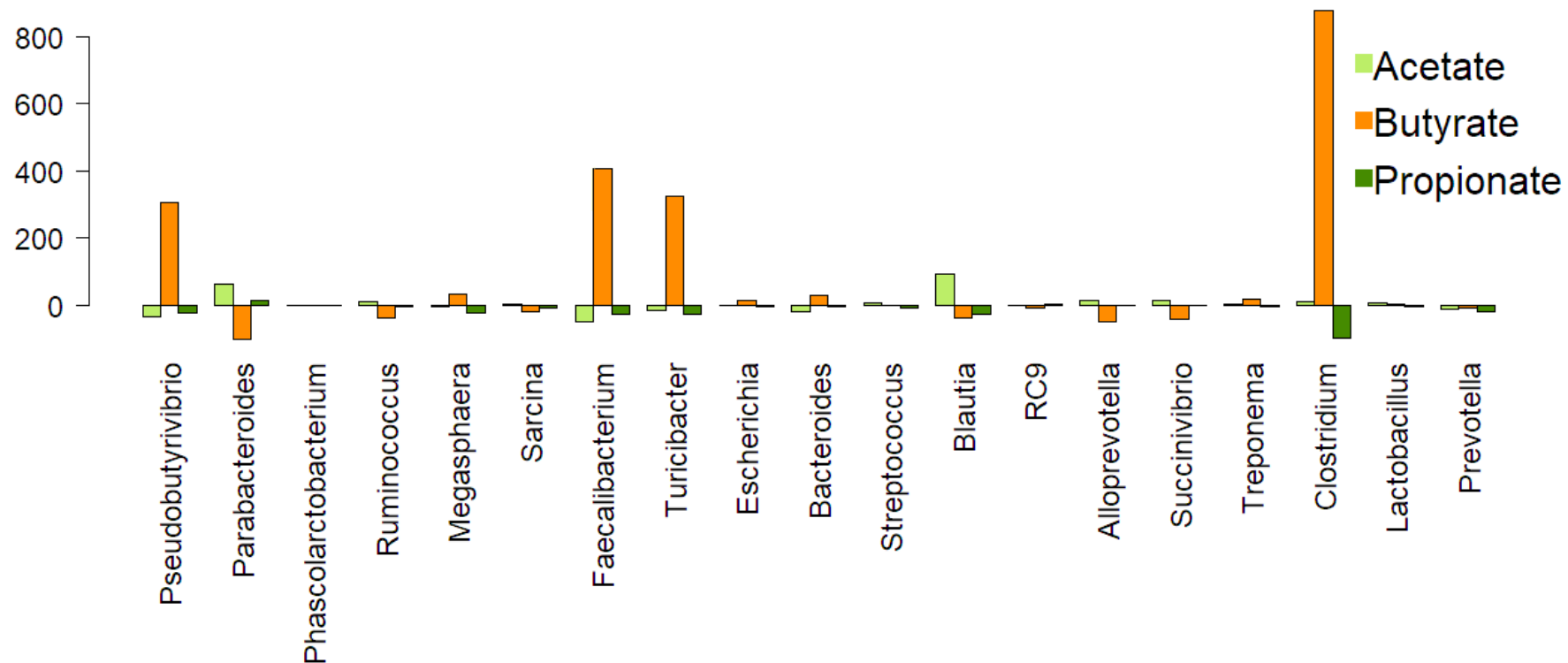


Figure 5.4. Percentage difference (5-4) between the simulations in which the BU starting and flow-in concentrations were enhanced two times and a simulation (normal) in which none of the MFG concentrations were increased

5.4 Prebiotic simulation

At this point both models were used to simulate prebiotic interventions and *in silico* effects on main SCFAs were quantified through calculating percentage difference (Eq. (5-4) between steady-state predictions of prebiotic simulation and steady-state SCFA predictions of a normal simulation (i.e. simulation in which no prebiotic effect was modelled). The environment simulated to carry out prebiotic *in silico* tests was once again a 32-day bioreactor fermentation in which microbial communities (i.e. either MFGs or BUs) were growing under controlled conditions. Since the aim was to represent an experimental environment similar to the porcine colon, pH was modelled as constant at 6.25, whilst dilution rate was modelled as constant at 2.67 d^{-1} , as detailed previously in this chapter. As depicted in Table 5.7, NSP, protein, RS and sugars starting conditions for the normal simulation were 7g/l, 15g/l, 4.32g/l and 2g/l, respectively. To simulate prebiotic interventions a series of simulations were run (i.e. four prebiotic simulations in total for each model), during each of them the concentration of a substrate at the time was increased 100% (Table 5.7). Moreover, to simulate a continuous intervention, substrate concentration was increased two-fold both as a starting value and as a flow-in value. The following sections describe in detail the results from simulations of prebiotic interventions using the model of the whole porcine gut microbiota first and then using the model of the colon lumen core microbiota.

Table 5.7. Starting substrate concentration at the beginning of both the normal simulation and each of the four prebiotic simulations carried out. Substrate enhanced to simulate a specific intervention is highlighted in bold in each column of the table. It is important to notice that in order to simulate a continuous intervention the enhanced substrate was also associated with the flow-in in the prebiotic simulations.

Substrate	Normal simulation	1 st Prebiotic simulation	2 nd Prebiotic simulation	3 rd Prebiotic simulation	4 th Prebiotic simulation
NSP	7 g/l	14 g/l	7 g/l	7 g/l	7 g/l
Protein	15 g/l	15 g/l	30 g/l	15 g/l	15 g/l
RS	4.32 g/l	4.32 g/l	4.32 g/l	8.64 g/l	4.32 g/l
Sugars	2 g/l	2 g/l	2 g/l	2 g/l	4 g/l

5.4.1 Pig gut microbiota

The four prebiotic simulations predicted a decrease in butyrate production when compared to the normal simulation, with the least negative effect on this metabolite due to NSP intervention (Table 5.8 and Figure 5.5). On the other hand, acetate concentration was constantly higher as a result of all the prebiotic interventions simulated whilst NSP and sugars had a negative effect on propionate, whose concentration was increased by interventions with protein and RS.

Table 5.8. Effect on acetate, propionate and butyrate due to the 2x substrate enhancement when using the WM model. Normal indicates a simulation in which none of the substrates had an increased concentration while running on the same experimental conditions as described in Table 5.4.

	Acetate (mM)	Propionate (mM)	Butyrate (mM)
Normal	66.46	31.54	22.19
Protein	123.97	41.41	3.82
NSP	102.19	29.6	17.53
RS	80.07	72.22	10.76
Sugars	109.45	22.87	11.6

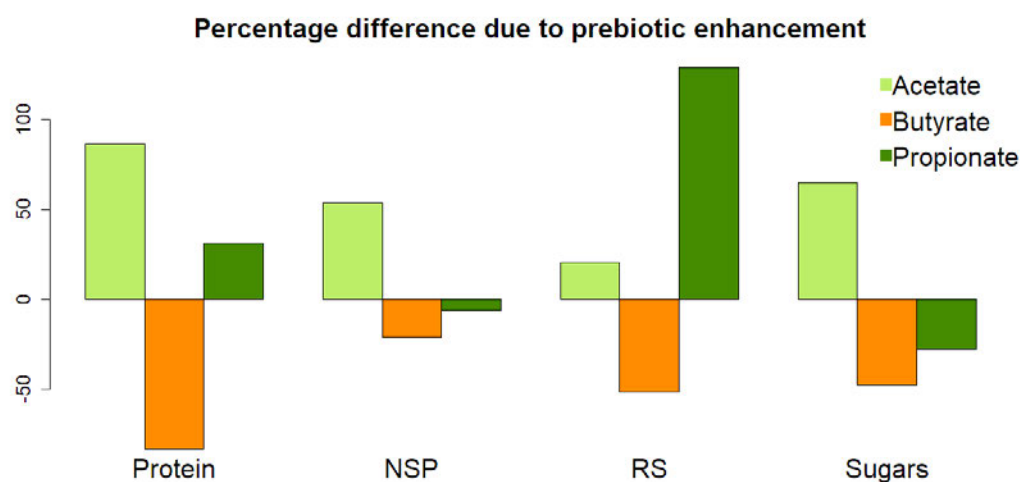


Figure 5.5. Percentage difference (Equation (5-4)) between the pig gut microbiota simulations in which the substrate starting and flow-in concentrations were enhanced two times and a simulation (normal) in which none of the substrate concentrations were increased.

5.4.2 Porcine core microbiota

In silico prebiotic interventions using the CM model showed similar effects on the major metabolites dependent on the substrate used for the simulated interventions (Table 5.9, Figure 5.6). Protein had the major positive effect on both acetate and propionate, increasing acetate concentration by ~60% and propionate by ~40% compared to the “normal” simulation. RS, and sugars had the most noticeable positive effect on butyrate, i.e., ~30% and ~20%, respectively more than the normal simulation. Acetate was increased of ~40% when using NSP, of ~10% when using RS and of ~5% when using sugars as a prebiotic.

Table 5.9. Effect on acetate, propionate and butyrate due to the 2x substrate enhancement when using the model of porcine core microbiota. Normal indicates a simulation in which none of the substrates had an increased concentration while running on the same experimental conditions as described in Table 5.4.

	Acetate (mM)	Propionate (mM)	Butyrate (mM)
Normal	76.06	92.39	7.27
Protein	119.85	132.78	7.76
NSP	104.40	120.04	7.77
RS	85.91	108.65	9.51
Sugars	80.98	99.68	8.32

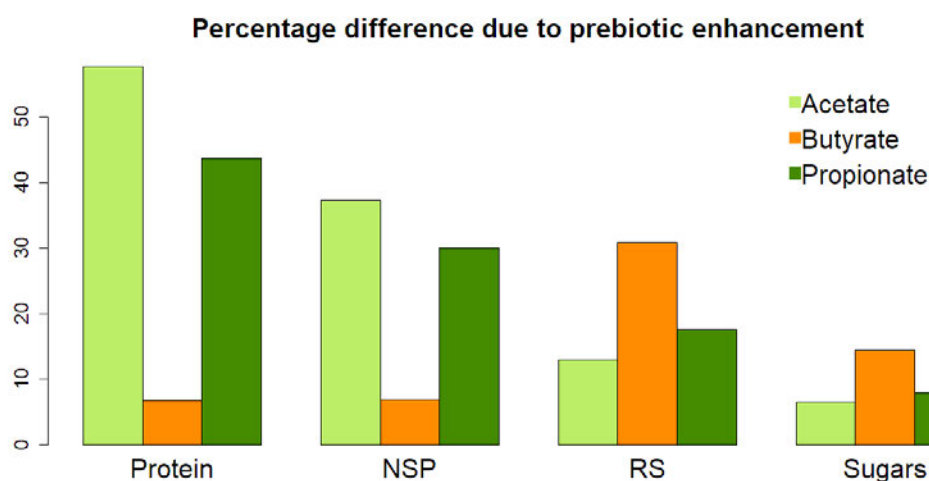


Figure 5.6. Percentage difference (Equation (5-4) between the porcine core microbiota simulations in which the substrate starting and flow-in concentrations were enhanced two times and a simulation (normal) in which none of the substrate concentrations were increased.

5.5 Combined therapy

The synergy between probiotic and prebiotic interventions might lead to a considerable effect on the fermentation pattern. Therefore, in this section the *in silico* effects of a combined probiotic/prebiotic intervention on the main SCFAs were assessed. During these simulations particular attention was given to monitoring predicted steady state butyrate concentration, due to its beneficial effects on the host health (Hamer et al., 2008). Whilst both the WM and the CM models were used to run simulations of a combined intervention, a complete description of model setup and rationales, as well as of the relative SCFA changes achieved is given in the following sections.

5.5.1 Pig gut microbiota

5.5.1.1 Model setup

As seen previously in this chapter, NSP had the least noticeable negative effect on butyrate production (Figure 5.5), whereas butyrate producers (MFG5, MFG6 and MFG8) had the most important effect on butyrate. Therefore, the combined intervention simulated here used MFG6 and NSP testing *in silico* the possible effect of their combined continuous administration. The choice towards MFG6 was due proven use as probiotics of some members of this group such as *F. prausnitzii* (Scott et al., 2014). The environment simulated to test the combined therapy *in silico* was the same as the one described earlier (i.e., pH of 6.25, D of 2.67d^{-1} and normal starting conditions as described in Table 5.7), whilst simulating a 32days continuous fermentation. To mimic a chronic intervention, two-fold NSP increase (14 g/l/d) and a MFG6 of 1g/l/d were simulated both as starting conditions and inflow concentrations.

5.5.1.2 Results

The combined probiotic/prebiotic intervention targeting the colonic porcine gut microbiota through modifications of MFG conditions had rather noticeable effect on butyrate, whose steady-state concentration had a ~20% increase when compared to predictions of a normal simulation. Acetate concentration was also increased, by ~10% whilst propionate concentration was ~5% less than what predicted without combined intervention (Table 5.10 and Figure 5.7).

Table 5.10. Comparison between the steady-state values of acetate, butyrate and propionate between the normal simulation (without enhancements) and a simulation of the combined therapy using 14 g/l/d of NSP and 0.07 g/l/d of MFG6 for 32 days targeting the entire pig gut microbiota.

	Acetate (mM)	Butyrate (mM)	Propionate (mM)
Normal	66.46	31.54	22.19
Combined therapy	74.69	37.88	21.61

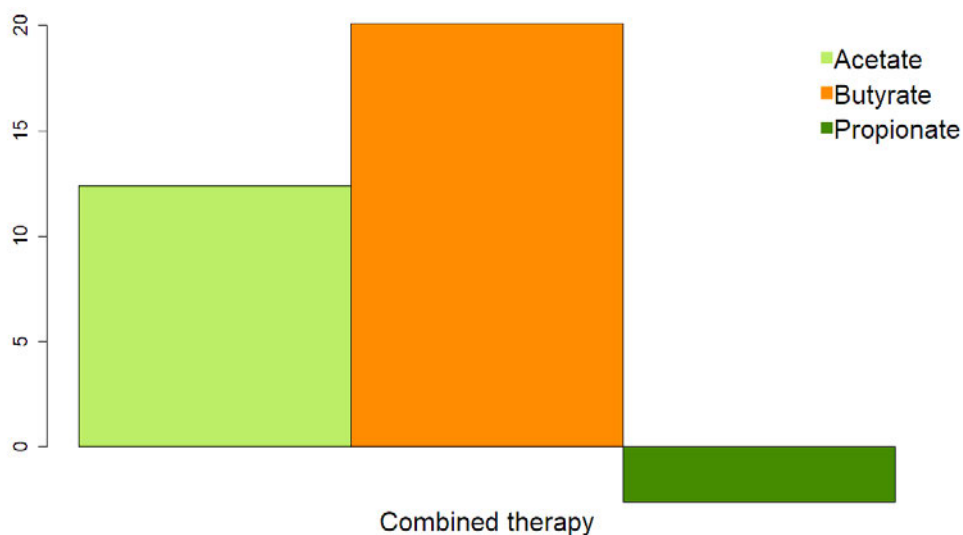


Figure 5.7. Difference percentage (5-4) between the acetate, butyrate and propionate steady-state concentrations retrieved between the simulation of a combined therapy using NSP, 14 g/l/d and MFG6, 1g/l/d targeting the entire pig gut microbiota compared with the same values predicted by a "normal" simulation sharing the same experimental conditions (Table 5.4) but without any enhancements.

5.5.2 Porcine core microbiota

5.5.2.1 Model setup

The combined intervention simulated targeting the CM model used *Faecalibacterium* BU, 1g/l/d (acetate consumers and butyrate producers and RS, 9g/l/d, which had the most noticeable metabolic effect on butyrate during the previous. Therefore, as described earlier both BU and substrate concentrations were enhanced as starting concentration and flow-in concentration.

5.5.2.2 Results

As depicted in Table 5.11 and Figure 5.8 the combined therapy had a rather important effect of butyrate. Indeed, butyrate concentration predicted at the end of the probiotic/prebiotic simulation was ~150% higher than the concentration of butyrate predicted without interventions. Propionate concentration was also increased at the end of the combined intervention increasing, although to a much lesser extent (i.e., ~10%), whilst acetate concentration decreased by ~5% according to the prediction of the combined intervention.

Table 5.11. Comparison between the steady-state values of acetate, butyrate and propionate between the normal simulation (without enhancements) and a simulation of the combined therapy using 14 g/l/d of NSP and 0.07 g/l/d of MFG6 for 32 days targeting the porcine core microbiota.

	Acetate (mM)	Butyrate (mM)	Propionate (mM)
Normal	76.07	7.27	92.39
Combined therapy	73.65	20.1	101.47

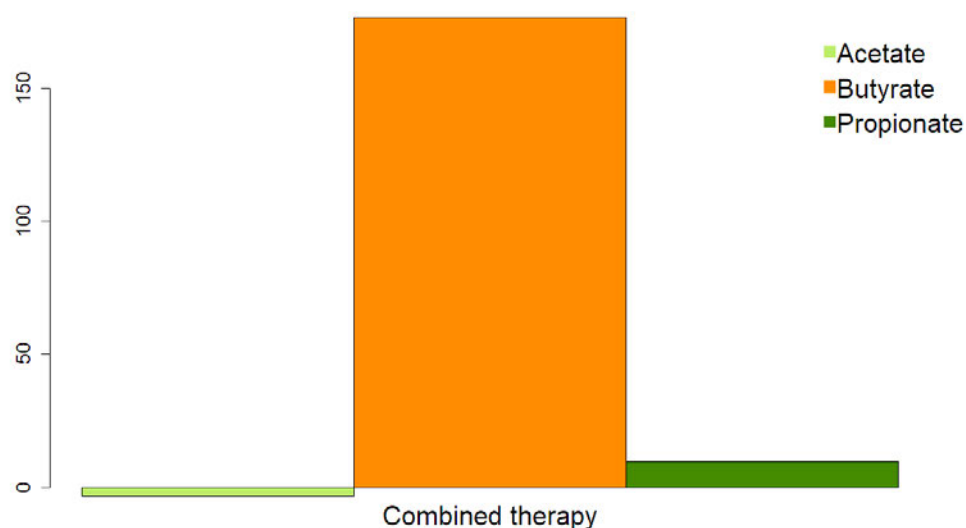


Figure 5.8. Difference percentage (5-4) between the acetate, butyrate and propionate steady-state concentrations retrieved between the simulation of a combined therapy using NSP, 14 g/l/d and MFG6, 0.07 g/l/d targeting the porcine core microbiota compared with the same values predicted by a "normal" simulation sharing the same experimental conditions (Table 5.4) but without any enhancements

5.6 Discussion

Probiotic and prebiotic interventions have been used in pigs trying to achieve a microbial environment or a fermentation pattern favourable for the health of the host (Heinritz et al., 2013; Herfel et al., 2013). Although there is still the need to clarify the mechanism of action, these kinds of therapies have been demonstrated able to modify the microbiota in such a way to achieve a reduction of pathogenesis (Liu et al., 2014). After the development and validation of the pig gut microbiota model (Chapter 3) and the development and comparison with studies of the porcine core microbiota model (Chapter 4), here *in silico* experimentation was undertaken to explore the possible effects of intervention strategies using probiotics, prebiotics and a combination of probiotics and prebiotics targeting the gut communities. In the first place, it was important to evaluate whether the models would be able to reproduce changes in the metabolite pattern due to external intervention. Thus, a study found in literature, during which *L. casei* strain Shirota was used in three pigs was used to assess the eventual statistical significance of such changes (Ohashi et al., 2004). In that case the authors did not find a statistical significance in the changes observed due to the probiotic intervention used. It is worth noting that only three pigs were used during that study, therefore, the results may have been different with a wider number of animals. The WM model depicted rather optimally the changes in the metabolite concentrations when compared to the values observed in the study, although butyrate was underestimated by the model, which could be possibly due to differences in starting microbial community. The CM model produced also similar results to the study in terms of main SCFA concentration, corroborating the findings of the previous chapter according to which the CM could be important in forming the basis for the SCFA production observed in the colon. This supports the view that the core microbiota is rather stable between hosts (Salonen et al., 2012; Yang et al., 2018), thus therapies targeting this communities could be used to plan both prebiotic and probiotic interventions.

The models were thus used to assess possible effect of probiotic and prebiotic interventions. It was observed that the effect on the major SCFAs was strictly dependant on the bacterial group enhanced, as expected increasing the concentration of one of the groups led to new substrate competition dynamics with changes in the fermentation. As expected, increasing the concentration of the substrate simulating a chronic prebiotic intervention altered the SCFA production when compared to a simulation of the same experimental conditions without any enhancements. Interestingly, when targeting the WM (Figure 5.5) the different prebiotics had a different effect on the metabolites, possibly depending on the selection towards those MFGs having major affinity with the prebiotic used. On the other hand, all the substrates used as a prebiotic had a similar effect on the metabolite, although at different magnitudes, with the protein producing the most noticeable changes, possibly due to its higher starting concentration. Based on the results achieved so far, a combined therapy was proposed and tested through a series of *in silico* experimentations targeting both the models. Using both

NSP and *F. prausnitzii* (MFG6) with the WM model achieved a ~20% increase in butyrate. The porcine core microbiota model was also used to test *in silico* a combined therapy using RS, and *Faecalibacterium* BU, showing a rather important increase in butyrate concentration due to the combined intervention simulated.

In conclusion, a number of therapeutic strategies targeting the WM and the colonic CM were here tested *in silico* to modify the final fermentation pattern. These strategies might represent the first step in the study of the role of these interventions in terms of fermentation pattern, which could be ultimately related to the health of the host.

6 General discussion

6.1 Mathematical model of the entire porcine gut microbiota

The main hypothesis behind this PhD was:

“A mathematical model can represent the dynamics of the porcine gut microbiota and could be used to forecast the possible effects of different intervention types”.

The first mathematical model of the whole pig gut microbiota (WM) was developed and validated through *in vitro* experimentation. Based on a previous model developed for the human gut microbiota (Kettle et al., 2015), the WM model summarised microbial populations through ten bacterial functional groups (MFGs). The porcine model described in this thesis was based on a functional classification of the microorganisms, which led to a subdivision of the different groups based on substrate-demand and metabolite consumption, which were due to characteristic microbial pathways and metabolic enzymes. Many other different strategies have been adopted by other authors when modelling different complex ecosystems. An example can be found in the spatially explicit individual-based model of bacterial growth, called BacSim (Kreft et al., 2000), with which the authors were able to model bacterial growth on a microscopic level, anticipating substrate demand and competition on 2-dimension. In another study, carbohydrate degradation by the human colonic microbiota was modelled considering the different compartments in which hydrolysis takes place (Muñoz-Tamayo et al., 2010), and although this model was also based on the Monod equation for the growth of microorganisms, the dynamics derived from such subdivision allowed the authors to integrate microbial growth with intestinal physiology and biological reactions and transport between lumen and mucus. The main differences between the WM and the human models were the environment modelled and the new parameterisation of the data frames. After re-parametrisation through reverse modelling and comparison with experimental data, the model produced a good representation of the environment modelled.

Continuous fermentation experiments are usually applied for the study of complex microbial ecosystems or to investigate microbial dynamics of one or few species under controlled conditions. As seen in Chapter 2, a tailor made triplicate bioreactor and tailor made incubator were designed and built to mimic the porcine proximal colon conditions and therefore carry out a 35-day continuous fermentation growing an inoculum derived from pig faeces. A few challenges accompany these type of experiments, whose management and maintenance are not always effortless, especially when lacking of automation. Concentration of H^+ in the main-vessel was continuously affected by microbial activity, therefore pH was promptly buffered using NaOH and maintained constant in an appropriate range throughout the fermentation.

Several systems are described in literature which use sensor-connected pumps (Tanner et al., 2014; Walker et al., 2005), or sparging gasses (Hoshan et al., 2019), allowing constant control of pH. Whilst such alternatives would have likely represented an automatic alternative to achieve constant pH, such option would have not suited the design of the vessels used due to the particular set up presented in this thesis. It is known that a shift in pH could favour the growth of specific bacterial population (Walker et al., 2005), nevertheless alpha-diversity analysis in particular, showed that pH did not seem to be correlated to changes in microbial diversity during the *in vitro* study carried out. Possibly, further experimentation with longer fermentation time and varying the pH value over a wider range might allow a better understanding of the relationship between pH and bacterial/metabolite dynamics. In general, the bioreactors performed according to expectations (i.e. not dissimilar from literature data), although acetate concentration was constantly lower than literature data when comparing similar medium compositions (e.g. Tanner *et al.* 2014). Although it is difficult to identify a specific cause, the lower acetate values could be explained by the pH range used, e.g. lower pH could have caused a higher butyrogenic fermentation (Walker et al., 2005); or the inoculum (and/or subsequent) microbiota composition, e.g. due to pigs' diet prior inoculation.

MFG relative abundance and concentration were inferred through calculation from data obtained from both 16S rRNA gene sequencing and qPCR, whereas GC-MS was used to quantify SCFA concentration. Whilst the analysis presented here allowed to infer MFG concentration, ideally a new set of primers could have also been generated and validated during this study to attain information on MFG concentration. Such set of primers could have targeted both 16S rRNA and metabolic genes, specific for the different MFGs. Nevertheless, such set of primers would have likely been extensive and required validation, due to lack of supporting literature in some of the cases. Therefore, such approach was unfortunately not considered due to time and fund constrains. Bioinformatic taxonomical analysis, such as the one performed here through QIIME2, was based on microbial 16S data bases, to which sample-16S sequences were aligned to establish taxonomy. Such data bases are continuously updated over time, in parallel with the discovery and classification of new species, therefore it is possible to assume that taxonomical analysis could acquire more definition (i.e., show more species) if aligned, in the future, to a more comprehensive data base. It could be interesting to infer MFG concentration again in the future, beyond the purpose of this PhD, in order to compare whether higher definition of bioinformatic data bases could affect MFG relative abundance as presented here.

Microbial diversity changed, as expected, from the faecal sample towards the final day of the experiment, confirming that not all the species were cultivable *in vitro*. Anaerobiosis was maintained throughout the fermentation as confirmed by the strictly anaerobic bacteria found at the last day of the experiment according to sequencing data. The three replicates performed similarly, although a peculiar change of both microbial and metabolite dynamics was noticed

around the twentieth day especially in one of the reactors. Whether this was due to local changes in pH, temperature or medium composition is hard to establish, but it is important to note that according to the data, the system apparently re-established an equilibrium by the next time point, illustrating resilience of microbial communities to temporary perturbation.

Inverse modelling was applied for model fitting during reparameterization, as seen in Chapter 3. Deriving dynamic parameters from experimental data that represent the modelled environment have been applied in several context (Stern and Coe, 1984) and for different model equations (Chiou and Wang, 2001). Thus, inverse modelling provide an extensive tool when experimental data are available (Soetaert and Petzoldt, 2010), Data collected during the triplicate continuous fermentation were used to compare the WM model, evaluating the closeness between observations and predictions through root mean square error (RMSE) calculation (Chapter 3). The observations were also employed in a parameter fitting process targeting the half saturation constant, which was used in combination with the multiple-strain generation ability of the model, allowing stochastic parameters generation assigning new K values to MFGs. The model accuracy in predicting SCFAs increased, according to RMSE, after parameter fitting.

As noticed for the human model, the accuracy of the predicted SCFA concentration, especially for butyrate and propionate reflected the detailed knowledge of MFG (Kettle et al., 2015). The human model overestimated formate concentration, which in that case was thought to be due either to a direct overestimation of formate production or to an underestimation of its conversion to H_2 and CO_2 or CH_4 by other community members (Kettle et al., 2015). Nevertheless, although formate concentration was unfortunately technically impossible to measure throughout the *in vitro* fermentation undertaken here, it was reported as not detected during an experiment using a similar *in vitro* set up (Tanner et al., 2014), whereas its predicted value was ~3 g/l at steady state, possibly confirming its overestimation by the WM model.

6.2 Mathematical model of the porcine core microbiota

The second output of this PhD was the first mathematical model of the porcine core microbiota (CM). Although the definition of a core microbiota is still debated in the literature, the CM model was based on a meta-analysis identifying up to nineteen bacterial genera in the porcine colon lumen (Holman et al., 2017). The microbial communities were summarised following a taxonomical approach, thus each of them was modelled as a bacterial unit (BU). After comparison with two different literature studies the outcomes of the CM model were thought to show *in silico* a possible CM role of forming the basis for the SCFA production, due to similarities with the WM metabolite pattern observed in these studies. The closeness between observations and predictions could indicate in this case that possibly non-CM members do not majorly contribute to SCFA production, or due to the majority of the functional genes being shared amongst the CM members only.

6.3 *In silico* experimentation

Both the models were used to first simulate a probiotic-intervention study, in which *L. casei* strain Shirota was administrated to three pigs. The models were both found to be able to reproduce the results of the study, thus they were then used to simulate an increased inflow concentration of the single MFGs/BUs (i.e. probiotic simulation) or of the single substrates (i.e. prebiotic simulations). Both when using the WM and the CM model it was demonstrated, *in silico*, that manipulations of bacteria/substrate influenced metabolites, according to the intervention type. Thus, in order to increase the final butyrate concentration due to its beneficial effects on the host health (Hamer et al., 2008), two different therapies were proposed, showing that the administration of both NSP or resistant starch (prebiotic) and WM/CM members (probiotic) had a significant effect on the steady state metabolite pattern.

6.4 Final remarks

During this PhD two different models were developed for porcine gastrointestinal microbiota, representing the WM and the CM dynamics respectively. Given the assumptions made for its development and validation, the WM model may be a reliable tool in predicting both metabolic and microbial dynamics, based on *in vitro* data collected during the 35-day continuous fermentation carried out. The CM model was used *in silico* to theorise a possible role for the CM. Such model could represent a valid tool when designing interventions targeting CM genera or their substrates. Both the models were able to anticipate different types of intervention. The final product of this PhD can be thus summarised in two *in silico* tools, ready to be used by those interested in planning *in vitro* or *in vivo* interventions targeting the porcine gut microbiota. Moreover, the integration of empirical data with mathematical modelling has been defined as a crucial “missing link” in current microbial ecology (Widder et al., 2016). Therefore, the validation approach used here for the WM model, i.e. based on observations generated throughout the length of the fermentation, might represent a good example for the development of future microbial ecology models. The incorporation of experimental data into mathematical models might lead to more accurate predictions of microbial dynamics, which could be applied to different environments beyond the porcine gastrointestinal tract, such as waste water treatment or food industry (Widder et al., 2016).

6.5 Future studies

This final section of the thesis summarises possible developments of the project here undertaken, such as the use of the models during the planning phase of research studies and/or experiments to implement the model complexity.

- It would be interesting to test probiotic and prebiotic interventions using an *in vitro* system while ensuring the fully automated control of all the environmental parameters, e.g. pH, temperature or anaerobiosis. Such an automated bioreactor would allow the precise regulation of the experimental conditions reducing the risk of technical biases. The results from such experimentation could be used to validate similar approaches *in silico*, giving the model a higher reliability during the simulation of probiotic/prebiotic interventions.
- It would be useful to test the effect of the pH on the pig-gut microbial communities in order to better understand and align the link between pH variation and SCFA/microbial profile.
- It would be considered a great step forward in the development of this model if biotic factors could be included in the system of ordinary differential equations. The host effect on SCFA and the bacterial community could be validated by the set-up of a sophisticated *in vitro* experimentation which would combine the use of for example semipermeable membranes on which colonic eukaryotic cells would grow in contact with luminal content collected from pigs.
- An *in vitro* experimentation using the CM communities could greatly assist the validation of the CM model.
- The use of both the models to plan *in vivo* interventions targeting the pig gut microbiota would help to reduce the total experimental costs by ruling out approaches shown not to have an effect *in silico*. Related, outcome of experiment that are carried out informed by the models can be used to improve the performance of the model (e.g. by re-parametrisation), according to the idea that the model developed and presented here is an “open-loop” that can be consistently improved.

7 References

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