IN VITRO MODELLING OF COLONIC FERMENTATION

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ABSTRACT

The in vitro gut models have proved to be useful to study the effects of food, probiotics and pharmaceutical components on gut microbiota composition. In vitro models vary from simple batch fermenters using anaerobic conditions to more complex dynamic multi-vessel bioreactors. Their common purpose is to cultivate a complex intestinal microbiota under controlled environmental conditions for carrying out microbial modulation and metabolism studies. The appropriate selection of the model depends on the objective of the research and the advantages and limitations of each model.

In this thesis there is presented the main types of in vitro systems used nowadays to simulate the colon fermentation, as well as a research on previous designed models to summarize the best conditions to mimic the gut environment. Moreover, Minifors 2 (Infors HT) bioreactor is used to study the microbial dynamics of one single strain of *Lactobacillus* sp*.* The biomass generated was tested after the process of lyophilization and the storage in both room temperature and in the refrigerator, and it survived successfully. Apart from that, this thesis includes the design of an in vitro model of colonic fermentation, based on a previous bibliographic research, and its establishment using a complex microbiota representation from human fecal samples. There is characterized the presence of *Enterobacteriaceae*, *Clostridium* and *Lactobacilli* species This model will be appropriate for further studies on the effects of any bioactive compound, such as polyphenols, in the gut microbiome.

Key words: gut, microbiota, *in vitro*, colon, fermentation, bioreactor

TABLE OF CONTENTS

1. INTRODUCTION

In vitro models are the starting point in biological and medical research. With scientific progress and emerge of different in vitro models, it has been possible to gain a great knowledge of the entire human organism. They have been used for many segments of the organism, but there is one that has created a lot of interests on scientists: the gut microbiome.

The human gastrointestinal tract is colonized by trillions of microbes to form a gut microbiota that has an important influence on the nutritional and health status of the host. It carries approximately 150 times more genes than are found in the entire human genome[1]. Recent studies of human gut microbes and their interactions with hosts have uncovered their key roles in human health and disease. Human microbiota has an important role in basic biological processes and in the development and progression of major human diseases such as infectious diseases, liver diseases, gastrointestinal cancers, metabolic diseases, respiratory diseases, mental or psychological diseases, and autoimmune diseases.

It has long been known that food components ingested by the hosts influence their gut microbiota, and as a consequence, various food components such as prebiotics and probiotics have attracted interest as potential ways to manipulate the gut microbiota and to improve human health. Dietary fibres can be considered key ancestral compounds that preserve gut ecology, especially regulating macronutrients and host physiology.

Usually, the functionality of such food components has been evaluated by human intervention trials or animal-feeding trials. However, animal models have the limitation that their microbiota composition is different from the human, so the obtained results from animal trials are not totally translatable to humans[1]. For human trials, they are often constrained by ethical considerations. Therefore, in vitro models that mimic the complex gut environment are needed, as they allow to simulate the human colonic microbiota not only regarding the composition of microbiota but also the metabolism. Such in vitro models offer several advantages, including dynamic sampling over time and high reproducibility, without the ethical issues that can arise in clinical contexts[2].

The objective of our research group is to develop an in vitro model of colonic fermentation using the Minifors 2 bioreactor system (Infors HT). In this thesis, first different types of in vitro models are reviewed, and a bibliographic research is done in order to gain a wide background about previously designed models regarding colonic fermentation. Second, some experimental trials with a single microorganism (*Lactobacillus sp.*) are done to check the bioreactor work and stability and gain a better

understanding of microbial dynamics. Additionally, the survival of the biomass of *Lactobacillus sp.* produced is tested after lyophilization and under different storage conditions. Finally, the microbial model with a complex microbial community where different bioactive compounds can be tested, such as dietary fibres or polyphenols, is stablished.

1.1 HUMAN GASTROINTESTINAL TRACT

The mechanical and digestive processes have one goal: to convert food into molecules small enough to be absorbed by the epithelial cells of the intestinal villi. The organ system responsible for this in humans and other animals is the gastrointestinal tract (GIT). The GIT includes all structures between the mouth, and it can be divided into two parts: the upper and the lower tract. The upper gastrointestinal tract consists of the mouth, pharynx, oesophagus, stomach and the duodenum, jejunum, and ileum, which represents the small intestine. The lower tract includes the large intestine, which comprises the cecum, colon, rectum, and anal canal (*Figure 1*).

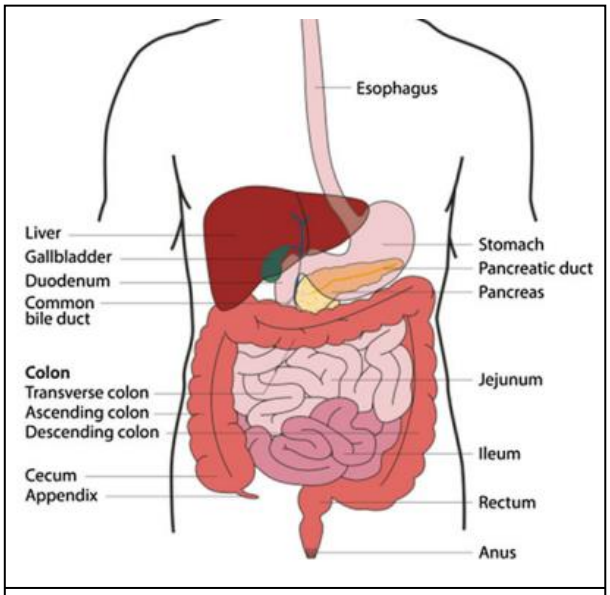


Figure 1. *Parts of the human gastrointestinal tract. Obtained from Kleiveland et al (2015)[3].*

During the process of food digestion, each part of the GIT plays a specific role. The food bolus formed in the mouth is broken down into chyme, which is gradually transported to the small intestine. In the duodenum, the low pH of the stomach is neutralised by bicarbonate and digestive juices from the pancreas. Then, maltase, sucrose-isomaltase, lactase and peptidases from the membrane of the small intestinal enterocyte digest dietary carbohydrates and proteins and permit the absorption of the nutrients produced by the enterocytes of the jejunum and in the ileum[3]. Once here, the process continues in the large intestine, where mainly water is adsorbed.

The most significant fact of the large intestine is that it contains a large microbial population, which contribute to the digestion of food components, including prebiotics such as complex polysaccharides which cannot be digested by humans[3].

1.2 GUT MICROBIOME

The human body is colonized by a vast number of microbes, collectively referred as the human microbiota. It consists of about 10^{14} bacteria that play an important role in human health and disease. The human digestive tract microbiome is a complex ecosystem that harbours a diversity of microorganisms, including *Bacteria*, *Archaea*, viruses, and *Eukarya*, being *Bacteria* the most dominant group in the community. Predominant genera in the human intestinal tract are *Bacteroides, Lactobacillus, Clostridium, Escherichia and Bifidobacterium*[4]. Together with yeasts and other microorganisms it contains in total more than 1000 species and carries 150 times more microbial genes than are found in the entire human genome. However, the composition of microbiota is unique and different for every individual, and it changes during the stages of life, but retains a stable function[4].

The gut microbiota is involved in the metabolism of key bioactive compounds such as short-chain fatty acid (SCFAs), branched chain fatty acids (BCFAs), branched chain amino acids (BCAAs), biogenic amines, vitamins, bile acids (BAs), and xenobiotics, as well as production of gases. Many of these compounds take part in maintenance of barrier function, modulation of cellular growth and immune system regulation[5].

Foods digested by intestinal microbiota are primarily dietary fibres and can be digested by a specific species of *Bacteroides*. Other non-digestible fibres, such as fructooligosaccharides and oligosaccharides, can be utilized by *Lactobacillus* and *Bifidobacterium*[1]. The normal gut microbiome produces short-chain fatty acids (SCFAs), such as acetic, propionic, and butyric acids, and serves as an energy source to the host intestinal epithelium. These SCFAs can be quickly absorbed to participate in regulating gut motility, inflammation, glucose homeostasis, and energy harvesting. Moreover, the gut microbiota has been shown to deliver vitamins to the host, such as folates, vitamin K, biotin, riboflavin (B2), cobalamin (B12), and possibly other B vitamins[1].

The microbiota is essential for correct body growth, the development of immunity and nutrition. As a result, it clearly influences the health of its host. It provides crucial benefits in the form of immune system development, prevention of infections, nutrient acquisition, and even brain and nervous system functionality[6]. In addition, animal models of human diseases have evidenced the significant role of the microbiome in the development of several pathologies, such as obesity, autoimmune diseases, and neurological diseases[3]. Moreover, microbiome assembly can be disrupted by environmental factors what can lead to a disease. Some human diseases are often

associated with a "dysbiosis" of the gut microbiota, meaning an altered composition or functionality[6].

Another possibility to influence the composition of the gut microbiota is by direct oral administration of health-promoting bacteria, so-called probiotics. *Table 1* shows the taxonomic composition of the gastrointestinal microbiome upon ingestion of bacteria, the total number of resident bacteria and the predicted transit time taking into account the volume and population densities along the gastrointestinal tract[7].

Table 1. *Bacterial community along the human GI tract. Table obtained from Derrien et al (2015)[7].*

	Taxonomy (phylum level)	Resident bacteria (number/ml or g)	Transit time[a]	Relative abundance of ingested bacteria compared to resident bacteria[b]
Stomach[c]		10^2–10^4	15 min–3 h	100 to 10 000-fold
Small intestine (ileum)[d]		10^6–10^8	2–5 h	0.01 to 1-fold
Colon (feces)[e]		10^{10}–10^{11}	12–24 h	0.0001 to 0.00001-fold

Due to the complexity of microbial gut community, its metabolic function, and the variety of genes in bacterial genome, the relation between the gut microbiome and diet in the field of health-disease is still unclear. For this reason, in vitro models such as the presented in this thesis are needed.

1.3 PROBIOTICS

Probiotics are defined as *"live microorganisms which when administered in adequate amounts confer a health benefit on the host"*[8]. Human probiotic microorganisms belong mostly to the following genera: *Lactobacillus, Bifidobacterium*, and *Lactococcus*, *Streptococcus, Enterococcus*. Moreover, strains of Gram-positive bacteria belonging to the genus *Bacillus* and some yeast strains belonging to the genus *Saccharomyces* are commonly used in probiotic products. Lactic acid bacteria have been mostly selected as probiotics due to their health-promoting properties and technological suitability and are claimed for the development of functional food products that they improve gut health and consumer well-being[9]. *Table 2* summarizes the main probiotic microorganisms used in human nutrition (food additives or pharmaceutical products).

Most probiotic bacteria have their source either from the mammalian gut or from traditional milk fermented products such as yogurt, from fermented fruits and vegetable, like sauerkraut and kimchi (from cabbage) or they can also be isolated from rice, wheat and rye[9]. The abundance of orally ingested strains from fermented foods and probiotics ranges between 10^8 and 10^{12} CFU per day, being fermented food and beverages the major sources of those environmental bacteria that enter the gastrointestinal tract[7].

Table 2. *Principal probiotic microorganisms used in human nutrition. Table obtained from Markowiak et al (2017)* [9].

Type *Lactobacillus*		**Type *Bifidobacterium***	**Other Lactic Acid Bacteria**	**Other Microorganisms**
L. acidophilus	*L. johnsonii*	*B. adolescentis*	*Enterococcus faecium*	*Bacillus clausii*
L. amylovorus	*L. petosus*	*B. bifidum*	*Lactococcus lactis*	*Escherichia coli Niselle 1917*
L. casei	*L. plantarum*	*B. breve*	*Streptococcus thermophilus*	*Saccharomyces cerevisiae (boulardi)*
L. gasseri	*L. reuteri*	*B. infantis*		
L. helveticus	*L. rhamnosus*	*B. longum*		

Probiotics exert their beneficial effects through various mechanisms, including lowering intestinal pH, decreasing colonization and invasion by pathogenic organisms, and modifying the host immune response[10]. Some advantageous functions in human organisms are the development of the microbiota inhabiting the organisms by ensuring the proper balance between pathogens and ordinary bacteria in the gut and the restoration of natural microbiota after antibiotic therapy. Moreover, probiotics can inhibit the development of some pathogens, such as: *Clostridium perfringens, Campylobacter jejuni, Salmonella Enteritidis* and *Escherichia coli.* Finally, they are able to produce group B vitamins, enzymes, co-enzymes and antibiotics, and can also Increase the efficiency of the immunological system, enhance the absorption of vitamins and mineral compounds, and stimulate the generation of organic acids and amino acids[9,10].

The European Food Safety Authority (EFSA) has well-stablished the process of safety evaluation of probiotics before being granted the so-called qualified presumption of safety (QPS) status. The QPS process assess taxonomic identity, body of knowledge, safety and antimicrobial resistance of biological agents. It is necessary to conduct in vitro test to characterize probiotics functionality and to assess the safety of probiotic microbes (*Table 3*). As for EFSA guidelines, health benefits of proposed probiotic candidates should be confirmed in animal models, before allotting them probiotic status. Then probiotics effectiveness must be validated in human studies. Finally, when the new probiotic food is approved, the following information is recommended to be included in

the label: contents (genus, species and strain designation), minimum numbers of viable bacteria at end of shelf-life, proper storage conditions and corporate contact details for consumer information.

Table 3. *Parameters for probiotic functionality and safety characterization. Table adapted from Wilkins et al (2017)[10].*

Probiotic attributes	Safety parameters
Resistance to gastric acidity	Antibiotic susceptibility
Bile acid resistance	Stability of antibiotic resistance genes
Adherence to mucus and/or human epithelial cells and cell lines	Haemolytic activity
Antimicrobial activity against potentially pathogenic bacteria	Hydrogen peroxide production
Ability to reduce pathogen adhesion to surfaces	Biogenic amine production
Bile salt hydrolase activity	Mucin degradation

1.4 PREBIOTICS

Prebiotics can be defined as non-digestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract. However, its definition has been a trouble for scientists during the last decades. In 2015, Bindels *et al (2015)*[11] published the consensus definition of prebiotic: "*a nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host*".

Given the proposed definitions already described, as well as other, the need for a consensus definition was evident. The current ISAPP consensus panel defines a prebiotic as: "*a substrate that is selectively utilized by host microorganisms conferring a health benefit*"[12]. Substrates that affect composition of the microbiota through mechanisms not involving selective utilization by host microorganisms are not prebiotics. These substrates would include antibiotics, minerals, vitamins and bacteriophages, which are not growth substrates, even though their intake might alter microbiota and metabolic composition[13].

Potential sources of prebiotics are tomatoes, artichokes, bananas, asparagus, berries, garlic, onions, chicory, green vegetables, legumes, as well as oats, linseed,

barley, and wheat. Moreover, prebiotics can be artificially produced, such as: lactulose, galactooligosaccharides (GOS), fructooligosaccharides (FOS), maltooligosaccharides (MOS), cyclodextrins, and lactosaccharose. The purpose of adding prebiotics to food is to improve their nutritional and health value. Several fermentable carbohydrates have been reported to convey a prebiotic effect, but the dietary prebiotics most extensively documented to have health benefits in humans are the non--digestible oligosaccharides fructans and galactans[14]. According with the consensus definition, prebiotics can be classified as shown in *Figure 2*.

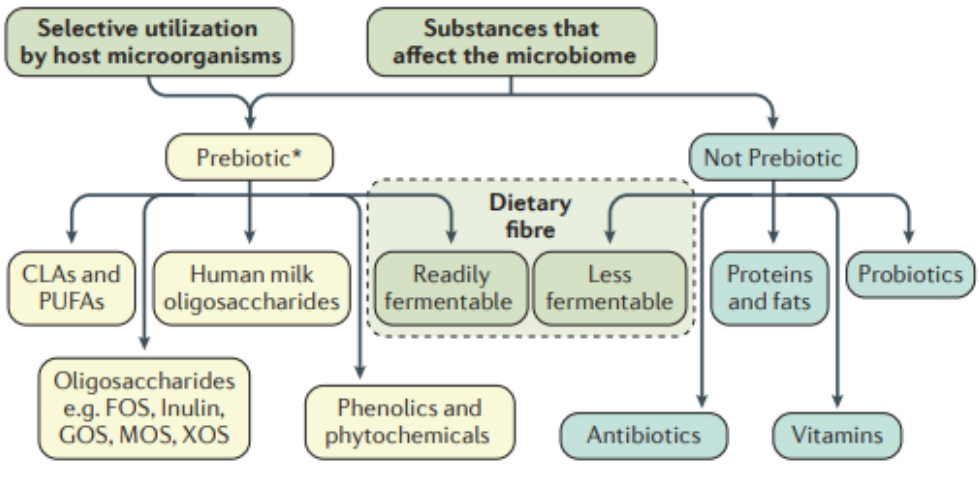


Figure 2. *Distinction of prebiotic according to the consensus definition. Obtained from He et al (2017)[13].*

Dietary fibre and prebiotics are very related terms used for food components that are not digested in the intestine. While prebiotics are fermented by defined and specific microorganisms, dietary fibre is used by most of the microbiota. So, it can be said that prebiotic may be a dietary fibre, but dietary fibre is not always a prebiotic.

Consumption of prebiotics is a dietary strategy by which the gastrointestinal microbiota can be modified for health benefit. This means that is perfectly possible to modulate specific gut bacteria (using adequate substrate) aiming to produce metabolites or regulating bacterial population size (nutrient competition and/or changes in pH) which can be useful to maintain, restore or improve host health. As a result, the SCFAs production may also be changed[10,13]. Nevertheless, prebiotics are able to withstand food processing conditions and remain unchanged, non-degraded or chemically modified, so they are always available for bacterial metabolism in the gut[10].

1.4.1 Dietary fibres

As mentioned, prebiotic carbohydrates are dietary fibres, but not all dietary fibres are considered prebiotics. The most widely definition was publicized in 2009 by the Codex Alimentarius Commission[15], a joint principal branch of the Food and Agriculture Organization and the World Health Organization, which defines dietary fibre as "*polymers 1 with 10 or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories:*

1. Edible carbohydrate polymers naturally occurring in the food as consumed.

2. Carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities,

3. Synthetic carbohydrate polymers, which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities."

Dietary fibres can be found in plants, bacteria, and fungi, and can also be chemically synthesized. According to this, dietary fibres can be classified depending on several parameters, including their origin, their chemical structure, and their physicochemical properties (*Figure 3*).

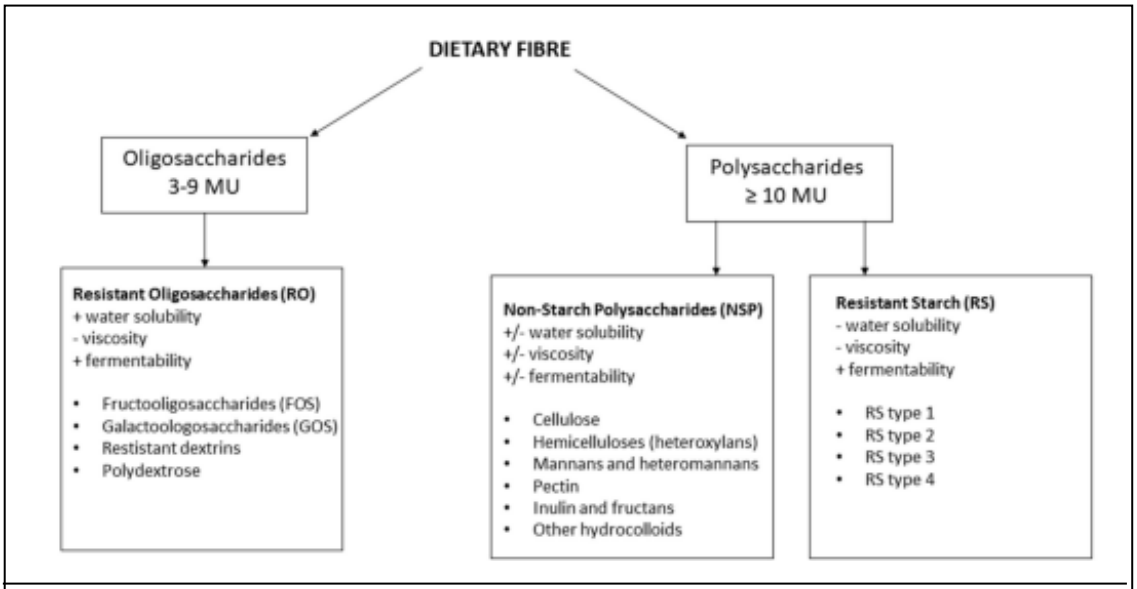


Figure 3. *Classification of dietary fibres. Obtained from Deehan et al (2017)[16].*

Dietary fibres are key candidate in facilitating changes in the gut microbiota as they escape digestion by the host in the small intestine and pass into the colon, where

they are available to the microbial community. Species belonging to *Firmicutes* and *Actinobacteria* are the main responders to dietary fibre although they contain relatively few fibre-metabolizing enzymes per organism[17].

The three major SCFAs produced from the fermentation of dietary fibres by gut microbiota, coming the majority from resistant starches, are acetate, propionate, and butyrate, but also gases as CO_2 and H_2. Its production is determined by several factors, including the amount and types of microflora present in the colon, substrate source and gut transit time. The principal site of the fermentation of dietary fibres is the cecum and proximal colon, for this reason this fermentation is also called colonic fermentation. The distal colon is carbohydrate and water depleted[18]. It is important to highlight that the bacterial fermentation of polysaccharides results also in a decrease of the colonic pH, which directly affects the colonic microflora. It decreases the solubility of bile acids, increases absorption of minerals, and reduces the ammonia absorption by the protonic dissociation of ammonia and other amines. More acidic conditions may lead the growth of butyrate-producing *Firmicutes*, while reducing the proliferation *Bacteriodes*, which are sensitive to these conditions[17,18].

1.4.2 Phenolic compounds

Phenolic compounds are plant secondary metabolites characterized by the presence of at least one phenolic ring in their structure. This family of compounds is involved in several aspects of the plants such as morphological development, physiological functions (colouring for camouflage, defence against herbivores, antibacterial, antimicrobial and antifungal activities, UV protectants) and reproduction (incorporating attractive substances to accelerate pollination and seed-dispersing animals). In addition, they give bitterness, astringency, colour, flavour, odour and oxidative stability to plants and foods that contain them. They represent wide variety of compounds that occur in fruits, vegetables, wine, tea, extra virgin olive oil, chocolate, and other cocoa products. The content of phenolic compounds varies according to several factors such as the genotype, type of culture, climatic factors and agricultural factors. Moreover, the daily intake of polyphenols fluctuates due to environmental conditions, storage and the processing of the foods[19].

The biological activity of dietary polyphenols results on a wide range of healthy properties acting in different fields, such as: antioxidant activity, cardioprotection activity, anticancer activity, anti-inflammation activity, anti-neurodegenerative action, antimicrobial effect and anti-ageing effect[20]. The gut microbiota plays a key role in modulating the production and bioavailability of phenolic metabolites, but there is also

evidence on the activity of dietary polyphenols on the modulation of the colonic microbial population composition and activity. Recent studies have suggested that both the phenolic substrates supplied to the gut bacteria through different patterns of dietary intake and the aromatic metabolites produced may in turn modulate the composition of the microflora populations through selective prebiotic effects and antimicrobial activities against gut pathogenic bacteria[21].

1.5 THE NEED OF IN VITRO FERMENTATION MODELS

Industrial fermentation is the process that has been used in the production of a variety of bio-products that have a broad area of applications. The main objective of bioreactors is to produce biomass or cell-derived metabolite, such as enzymes, proteins, or antibodies. A company's success at producing specific products depends in part on its ability to control various parameters. The high-specificity and controlling of the conditions allows a successful growth of microorganisms and cells during a short period of time and in a high quantity. Once the bioprocess is done, it is possible to remove all the media from the bioreactor and separate from there the substrate of interest.

In vitro colon modelling allows to simulate the fermentation in the human colon, and it can provide better understanding of the interactions between diet and microbiota in the colon. They allow the screening of a large number of substances, from dietary ingredients to pathogens, bioactive compounds or drugs, to assess how they modify and are modified by the microbial populations and its environment. Such in vitro models offer several advantages, including dynamic sampling over time and high reproducibility, without the ethical issues that can arise in clinical contexts. Therefore, studies of this kind are non-invasive, controllable, fast and samples can be collected throughout the experiments.

The main purpose of in vitro colonic fermentation models is to cultivate a complex intestinal microbiota under controlled environmental conditions to ensure the growth and development of these microorganisms. The models vary from simple batch incubators in anaerobic conditions to more complex continuous models of one or multiple connected vessels representing different parts of the human colon. Models should be appropriately selected according to the objectives of the study and their advantages and limitations. In addition, all models are able to control the conditions and to perfectly simulate the fermentation to carry out microbial modulation and metabolism studies.

2. HYPOTHESIS AND OBJECTIVES

Given the background of all factors related to the gastrointestinal human tract, the hypothesis of this thesis is that in vitro fermentation systems can be optimized for the study of the human gut microbiome in different conditions and for different aims of study.

Therefore, the general aim of this work is to design an in vitro model of human colonic fermentation using a batch bioreactor system. The specific objectives of this work are:

1. To do a bibliographic research to compare different types of in vitro models and to determine the optimal conditions to simulate the human colonic environment
2. To assess the model to study the microbial dynamics in the bioreactor using one single bacterial specie (*Lactobacillus sp.).*
   As an additional objective, the biomass produced will be used to study its survival after lyophilization and under different storage conditions.
3. To evaluate the colon model with a complex microbial community to study the effect of the polyphenolic compound Schisandrin B

3. THEORICAL PART: IN VITRO FERMENTATION MODELS

In order to design a successful in vitro model to simulate the fermentation in the human colon, it is necessary to have a great understanding and background about the different types of fermentation models that have been previously designed, its characteristics, advantages and limitations and the different conditions considered to run different types of studies. Therefore, this section includes a bibliographic research of different outlined studies in colonic fermentation and summarizes the main factors to consider.

3.1 BATCH FERMENTATION MODELS

Batch fermentation models are the simplest types of simulators of colonic fermentation for short-term studies under anaerobic conditions. These fermenters are usually sealed bottles with pure cultures, defined mixed cultures or faecal slurry and they model a specific part of the gastrointestinal tract. They are mainly used to study the effects of substrates, such as dietary fibres, on the gut microbiota, and viceversa. This effects are evaluated by molecular quantitative and qualitative techniques and the impact on the metabolic activity is evaluated measuring the formation of SCFAs or other metabolites, as they provide a first assessment of the types of microbial metabolites formed and help to elucidate the pathways involved[2].

During the entire batch process, nutrients, gases, acids and bases can be added to maintain the conditions. This strategy is suitable for rapid experiments such as strain characterization or the optimization of the nutrient medium. The disadvantage of this method is that the biomass and product yields are limited. Since the carbon source and/or oxygen transfer are usually the limiting factor, the microorganisms are not in the exponential growth phase for long[22].

After the end of a bioprocess run in batch mode, only the biomass or medium is then harvested and appropriately processed to obtain the desired product. From the reactor point of view, the process is repeatedly interrupted by cleaning and sterilization steps, and the biomass is only produced in stages.

Most batch fermentation systems used to model the human colonic flora have been adapted from a system described by Wang & Gibson in 1993[23]. They studied the effects of the fermentation of oligofructose and inulin in batch culture fermenters of 280 mL. The fermenters were inoculated with mixed faecal slurries and carbohydrates at 37ºC for 48 hours, with oxygen-free conditions and maintained at a pH of 7. Samples were removed every 3 hours to proceed with fermentation product analysis and to evaluate the biomass production of selected bacteria. Results in this study suggest that the addition for oligofructose or inulin to the diet may cause an improvement in the composition of the gut microflora.

This first batch model has been widely adapted over the years to study different effects on the gut microbiota. As an example, it has been assessed whether the different cooking methods of food may affect somehow the structure and functionality of the gut population. In one study conducted by Pérez-Burillo *et al* (2018)[24], furosine and furfural were used as Maillard reaction indicators to control the heat treatment in five foods, which were submitted to in vitro digestion-fermentation. They designed a batch fermentation model for this study, which was carried out with mixed human faecal samples. They outlined that intense cooking technologies, such as roasting and grilling, increased the abundance of beneficial bacteria like *Ruminococcus spp.* or *Bifidobacterium spp.* compared to milder treatments like boiling.

Similarly, probiotic effects on the gut microbiome have also been studied in in vitro batch models. The study of the probiotic's ability to affect the redox status in the gut lumen of healthy subject or those with gastrointestinal disorders have recently caused many interests. In Gaisawat *et al* (2019)[25] research it was assessed the ability of single strain and multispecies probiotic supplementation to cause a change in the redox status of normal faecal water and in *Clostridium difficile*-infected faecal water using a

simulated gastrointestinal model of batch fermentation. In this model it was used a filter-sterilized gastrointestinal food culture medium, previously optimized by Molly *et al* (1993)[37] (*Table 16, Annex 9.1*).

The simulation of the human small intestinal and large intestinal environments has also been useful to evaluate the influence of antibiotics on the viability of gut microbiome. Oliphant *et al* (2020)[26] evaluate how antibiotic rifaximin pre-treatment influenced the effectiveness of faecal microbiota transplantation (FMT) in an ulcerative colitis-derived defined microbial community. FMT is the process of implanting intestinal microbiota from a healthy donor into the gastrointestinal tract of the recipient, so the intestinal flora is restored, and it increases the bacterial diversity. They used Minifors (Infors HT) bioreactor system as an in vitro batch model of the distal human gut at the following conditions: 37ºC, pH 7.0, retention time of 24 hours (feed rate of 400 mL/day) and anaerobic conditions by sparging N_2 gas. Three replicate bioreactors each were assigned to the conditions of control and treatment in a randomized fashion, for a total of six. They were inoculated with a defined microbial community of 24 bacterial strains, characteristic of the disease. The SIEM media was used (*Table 15, Annex 9.1*), replacing the component xylan by xylooligosaccharide. The treatment bioreactors were inoculated with a dosage of rifaximin of 200 mg/day. They found out that the effect of the antibiotic on the relative species abundances in the ulcerative model was minimal, but it was observed a change in few several metabolite concentrations.

Moreover studies related to the effect of different conditions in the bacterial gut metabolism, such as SCFA production and gas profiles, have been the most studied in batch fermentation models, but it is also possible to make this kind of studies in anaerobic chambers. Anaerobic chambers are not bioreactors and less sophisticated but mimic the anaerobic environment of the colon. For example, it has been studied whether different sizes of wheat bran fractions may be fermented differently by gut microbiota, which would lead to size-dependent differences in metabolic products and community structure. Tuncil *et al* (2018)[27] performed in vitro fermentation assays of 5 different size fractions of wheat bran with faecal microbiota from healthy donors in an anaerobic chamber (BACONTREX). SCFA production, measured by gas chromatography, uncovered size fraction-dependent relationships between total SCFAs produced, and 16S rRNA sequencing revealed that these outcomes were accompanied by the development of divergent microbial community structures.

Finally, researchers have developed a prototype in vitro parallel gut microbial fermentation screening tool with a working volume of only 5 mL consisting of five parallel reactor units that can be expanded with multiples of five to increase throughput, and

called it Copenhagen MiniGut (CoMiniGut) (Wiese *et al,* 2018)[28]. To study its viability, they proceed with stirred batch fermentations of inulin and lactulose with 4.5 mL of adapted SIEM medium at 37ºC. pH was set to increase from 5.7 to 6.0 during the first 8 h of fermentation simulating pH conditions prevalent in the proximal colon, followed by an 8 h pH increment from pH 6.0 to 6.5 representing the pH conditions in the transverse colon and finally a pH increment from 6.5 to 6.9 for distal colon.

3.2 DYNAMIC FERMENTATION MODELS

Batch cultures are usually employed before conducting more lengthy multivessel continuous fermentation experiments. These dynamic systems simulate the gut on both structural and functional levels.

3.2.1 Three-stage colon models

Bibliographic research shows up that most of the designed in vitro models of the colon for more complex studies are based, and so do their conditions, in the three-stage continuous system first developed in 1988 by Macfarlane *et al (1988)*[29] for the study of the effects of mucin on the activities of intestinal sulphate-reducing bacteria and methanogenic bacteria. The vessel that mimics the proximal colon is 0.3 L and a pH of 5.5, the one mimicking the transverse is 0,5 L and a pH of 6.2, and the one for the distal colon is 0.8 L and a pH of 6.8. Each fermenter is magnetically stirred and maintained under an atmosphere of CO_2. Vessel 1 is fed with the medium from a reservoir (R1) and can also receive mucin from reservoir R2 (*Figure 4*). The model can operate at different retention times to assess the effect of colonic transit on carbohydrate utilization, metabolite formation, and the ecology of numerically important bacterial populations.

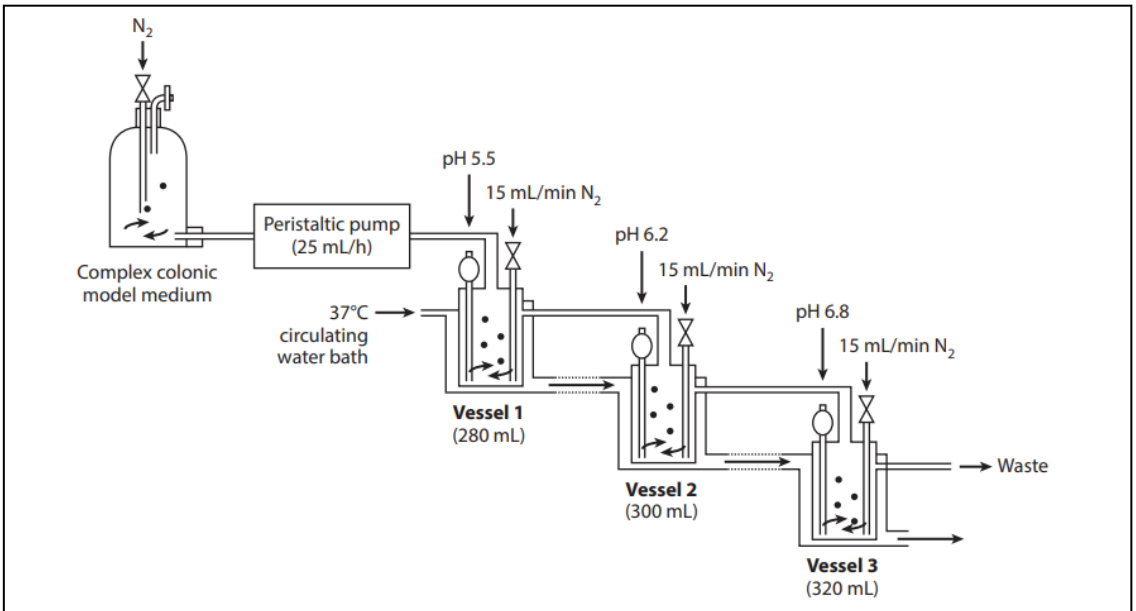


Figure 4*. Three-stage continuous colonic model System. Figure obtained from Macfarlane et al (1988)*[29]

Thus, the first vessel has a high availability of substrate, representing a rapid bacterial growth rate and is operatic at an acidic pH, similar to the events that happen in the proximal colon. In contrast, to achieve the characteristics of the distal regions of the colon, the final vessel has a neutral pH, leading to a slow bacterial growth and low substrate availability. For the culture medium, the authors developed nutritious medium that has been extensively used in both batch and dynamic fermentation models, known as simulated ileal environment media (SIEM) (*Table 14, Annex 9.1*). This medium has been adapted and modified in further studies.

In the next years, this model was widely validated and redesigned in different experiments, so it has provided a great better understanding of the human colon and the microbial activity in it. For example, studies of the metabolism related to the composition of the bacterial families in the gut. In the study conducted by Bircher *et al* (2018)[30], two independent continuous fermentation systems (Sixfors, Infors HT) were used to evaluate the impact of two cryoprotectives, glycerol (15% v/v) and inulin (5% w/v), alone and in combination, in preserving short-chain fatty acid formation and recovery of major butyrate-producing bacteria in three artificial microbiota during cryopreservation for 3 months at 80°C. After 24 hours of anaerobic fermentation of the preserved microbiota, butyrate and propionate production were maintained when glycerol was used as cryoprotectant, while acetate and butyrate were formed more rapidly with glycerol in combination with inulin.

Continuous fermentation models have also been used to investigate the patterns and timescales of microbiota variation in an artificial human gut (Silverman *et al,* 2018)[31]. A four-vessel continuous artificial gut system (Multifors 2, Infors HT) was used to culture gut microbiota seeded from human stool and 300 mL of McDonald Gut Media (MGM)[32] were used as medium (*Table 17, Annex 9.1*). Additionally, dynamic models have demonstrated to be suitable for mimicking infants gut microbiome. It has been discovered that the infant microbiota is particular and specific in the early stages of human lives, and it develops during the years until it constitutes the characteristic microbiota of an individual adult. Therefore, when designing a model of fermentation of an infant colon, it is important to consider the different conditions of it. Researchers have put a lot of interest on studying aspects of the infant gut metabolism and so they have modulated different in vitro models. Medina *et al* (2018)[33] studied the impact of a dietary switch from fructooligosaccharides (FOS) to 2-fucosyllactose (2FL) in a continuous culture. They used two independent continuous fermentations in a 250 mL bioreactor with automatic control (Minibio 250). The media specific for infant microbiome was mZMB, an adaptation of the ZMB-1 medium (*Medina et al,* 2017)[34].

To conclude with the general overview of all aspects that in vitro dynamic models can allow to study, it is important to mention those studies on viruses and fungi. *Candida albicans* is well known as a major human fungal pathogen, but it is also a permanent resident of healthy gastrointestinal tracts. Some of the bioactive molecules of the human gut microbial metabolome may have useful antivirulence activities, as it has been demonstrated that they can inhibit the growth of *C. albicans* and other opportunistic yeasts (García *et al*, 2017)[35]. Many are bacteriophages and their role in driving bacterial diversity have been evaluated with the use of chemostat culture systems that can reproduce human microbial communities[36].

3.2.2 SHIME

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) is one of the few gut models that mimics the entire gastrointestinal tract incorporating stomach, small intestine and different regions of the colon. It is a highly flexible experimental setup, so it is easy to modify to target digestive conditions of interest. It was developed by Molly *et al* (1993)[37] in order to overcome the problem that single-stage bioreactors are only useful for limited periods of time as the conditions constantly change. SHIME mimics the conditions in the ascending, transverse and descending colon regions. It incorporates the upper digestive tract conditions, leading to a succession of five compartments simulating the upper (stomach, small intestine) and the lower (ascending, transverse and descending colon) digestive tract (*Figure 5*). Upon digestion in the gastric and intestine compartments, the slurry is pumped in the ascending colon vessel where colon digestion is initiated. According to the conditions in the intestine, temperature is set at 37ºC. The pH of the colon compartments is controlled between 5.6 and 5.9 in the ascending, 6.1–6.4 in the transverse and 6.6–6.9 in the descending colon. Mixing of the digestive slurry in the respective compartments is obtained with magnetic stir bars. The entire SHIME system is kept anaerobic by daily flushing the headspace of the respective compartments with N_2 gas or a 90/10 % N_2/CO_2 gas mixture. Mixing of the digestive slurry in the respective compartments is obtained with magnetic stir bars. The medium used in SHIME is composed of complex carbohydrate and protein sources with addition of mucins and a minerals and vitamin mix.

The main features of this model are[38]:

- it aims to allow a suitable adaptation period for the faecal microbiome to adapt to the conditions that prevail in the respective colon compartments
- it is typically inoculated with the faecal microbiome derived from one individual and succeeds in maintaining the microbial metabolic phenotype

- it has been optimized for mimicking mucosal microbial colonization by incorporation of mucin-covered microcosm
- it is flexible, its compartments can be added or left away easily
- it gives the possibility of simulating the microbiome from different human target groups as well as the simulation of animal microbiome

A recent study of the effects of olive and pomegranate by-products on human microbiota used SHIME with the same conditions described above (Giuliani *et al*, 2019)[39]. The ability of these products (with high phenolic composition and polysaccharides) to modulate the microbial community was studied simulating a daily intake for nine days. Results of quantitative analysis of SCFA and NH_4^+ showed that microbial functionality was stable during the treatment and Illumina sequencing demonstrated that no significant changes occurred to microbial composition. Therefore, they could conclude that olive and pomegranate can be used as new food ingredients without risk for the microbiota.

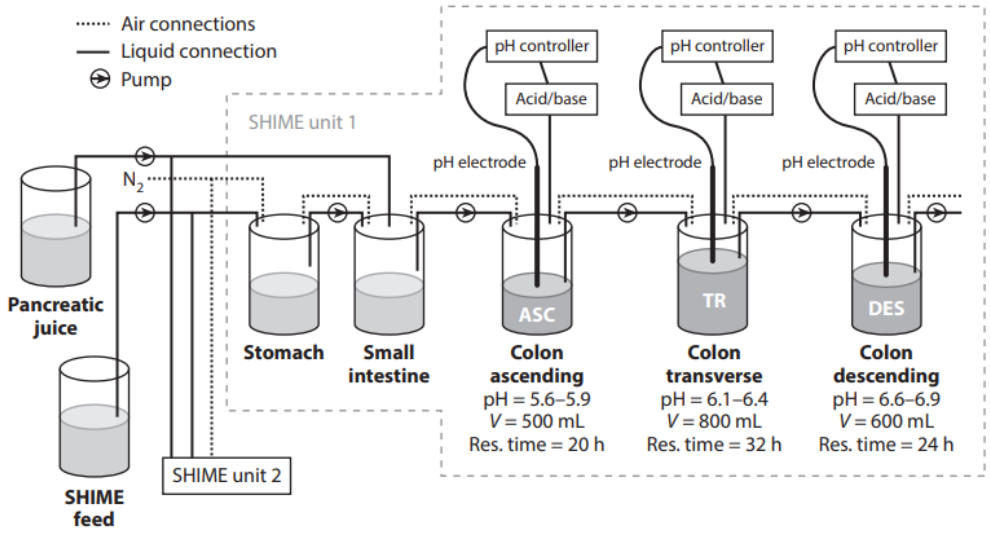


Figure 5. *SHIME model setup. Obtained from Williams et al (2015)* [38]

3.2.3 TIM-2

The TNO computer-controlled dynamic in vitro gastro-intestinal model of the colon (TIM-2) was developed by Minekus *et al* in 1999[40]. This system combines removal of metabolites and water with peristaltic mixing to obtain and handle physiological concentrations of microorganisms, dry matter and microbial metabolites.

In comparison to other in vitro models mimicking the colon, TIM-2 has many unique features[3]:

- the peristaltic movements of the flexible membrane give a better mixing and movement of components through the entire model than would be accomplished by stirring (in a fermenter) or shaking (on a rocking-platform or otherwise)
- there is no phase-separation of solids and liquids, so viscous or insoluble components can be used
- a single parameter in the system can be changed, and the effect of that single parameter on microbiota activity can be studied.
- the dialysis system maintains a highly active microbiota with a similar density as that found in the human large intestine and allows TIM-2 to mimic better the physiological situation in the large intestine.
- all metabolites can be collected and measured, so a complete mass-balance can be made

For all of this advantages, TIM-2 model is, together with SHIME, the most used model nowadays. It has been recently used to study the effect of commercial potato fiber (FiberBind 400) on the survival of probiotic *Lactobacillus* strains (Larsen *et al* 2019)[41]. Results of 16S DNA sequencing and analysis of SCFAs showed that commercial potato fiber had an ability to improve survival of the four tested strains and its fermentation resulted in more diverse microbial communities compared to starches.

3.2.4 PolyFermS

All models, from simple batch to the more complex continuous models, have the aim of stable cultivation an intestinal microbiota for a defined period of time while preserving the activities of the predominant microbial groups. However, they are not able to ensure a complete establishment of microbial communities as they are inoculated with liquid faecal samples, so there is the lack of biofilm-associated states of bacterial populations. To solve this, researchers use immobilized faecal microbiota in mixed xanthan-gellan gum gel beads, so the microbial diversity is maintained longer time.

Apart from that, reproducibility and functional stability of the microbiota in gut fermentation models is often questioned, so Polyfermentor Intestinal Model (PolyFermS) was designed in 2013 (Zihler *et al*, 2013)[42] to solve all these problems. Compared to other intestinal fermentation models, this model is characterized by the advantageous possibility to cultivate complex intestinal communities in multiple reactors allowing studying in parallel the impact of many different treatments (dietary compounds, antibiotics and drugs, environmental parameters, etc.) compared to a control reactor[3,42].

The setup of PolyFermS (*Figure 6*) consists of a first-stage continuous inoculum reactor (IR) containing immobilized faecal microbiota and mimicking the upper proximal

colon and a set of set of second-stage control (CR) and test (TR) reactors operated in parallel with conditions of the proximal colon. Effluents of IR are used to continuously feed the CR and TR reactors[42].

Whatsmore, not only three-stage fermentation models are able to simulate the infant gut environment, but also PolyFermS. The metabolism of lactate impacts infants gut health and may lead to the accumulation of lactate or H_2, which is associated with pain and crying of colicky infants. In a study of Pham *et al* (2019)[43], they used the continuous colonic fermentation model PolyfermS, inoculated with immobilized faecal microbiota, which mimics the proximal colon of infants. The effects of pH and retention time on lactate metabolism and H_2 production of lactate-utilizing bacteria were studied. They could observe that a decrease in pH from 6.0 to 5.0 raised the number of lactate-producing bacteria and decreased lactate-utilizing bacteria. For this experiment, fermentation medium was based on the composition designed previously to mimic the chyme entering the colon of 6-month-old infants (Le blay *et al*, 2010)[47] (*Table 18, Annex 9.1*).

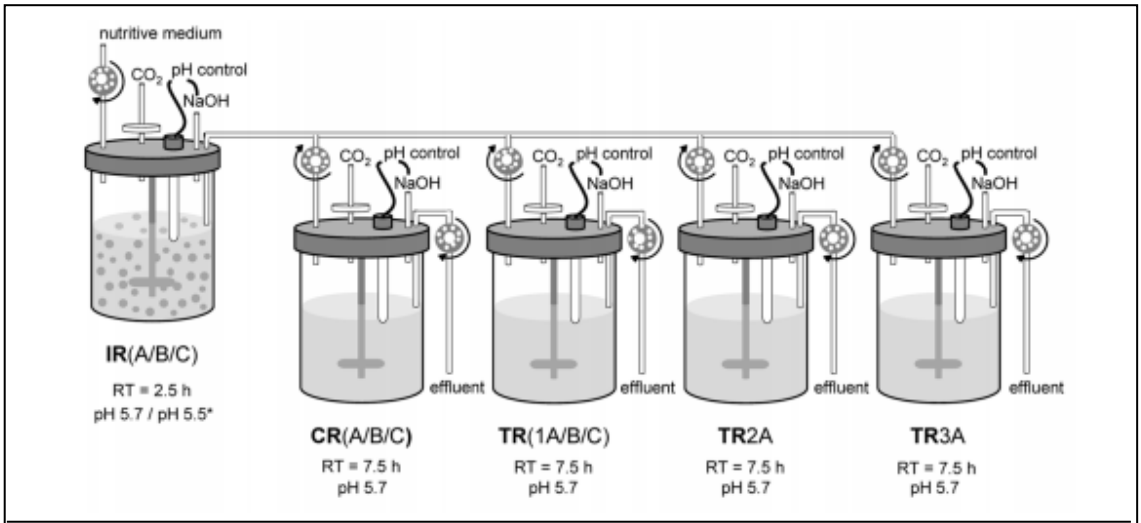


Figure 6. *Setup of the PolyFermS model. Obtained from Pham et al (2019)[42].*

3.3 COMPARISON OF IN VITRO MODELS

In vitro batch models of fermentation explain the effects of food matrix on release of components or bioconversion of food components to their microbial metabolites, but they are not able to mimic the changes in the pH, the adsorption of the nutrients or predict the bioavailability. On the other hand, they are easy to operate and maintain under anaerobic conditions and allow to monitor the pH of the incubation and to stir the culture medium to enhance enzyme-substrate interactions. They allow to predict qualitatively bioavailable metabolites with distinctive structural changes from dietary precursors and

the role of microbiota in the nutritional metabolomics. Moreover, its data analysis and statistics is quite easy thanks to non-targeted metabolomics coupled with bioinformatics and compound libraries. For short term screening and interindividual variability studies, batch models are further recommended as they are easy to use and more cost-effective but require accuracy at all stages of the process.

Dynamic models allow the control of the environmental parameters and are capable to mimic specific regions of the colon. It is also possible to simulate metabolite absorption, peristalsis and the intestinal mucus with some dynamic models, and also to make quantitative analysis of human metabolism, but they cannot mimic the host functionality. Additionally, there are not feed-back mechanisms in the system (apart from volume and pH), so the experiments will always be at most an indication of what may occur in real life, and the results need to be interpreted with care. *Table 4* compares the different parameters employed in the above presented gut model systems.

Table 4. *Comparison of parameters employed in various in vitro gut model systems*

In vitro model	Target organ	Vessel volumes	pH	Running time	Peristaltic pumping	Absorption	Mucus	Cell Line
Batch fermenter	Any region of GIT (usually distal)	Varies	Usually 6.8	≤ 48 h	No	No	No	No
Three-stage continuous	Proximal (V1), Transverse (V2), Distal (V3)	80 mL (V1), 100 mL (V2), 120 mL (V3)	5.5 (V1), 6.2 (V2), 6.8 (V3)	16 days to steady state	No	No	No	No
SHIME	Stomach to colon	300-1600 mL	2.0-7.0	30 days per cycle	No	Yes	No	No
TIM-2	Proximal colon	200 mL	5.8	3 days	Yes	Yes	No	No
PolyFermS	Proximal colon	300 mL	5.5	6 days	Yes	No	No	No

3.4 GENERAL BIOREACTOR PROTOCOL

3.4.1 Stabilization

This process is also known as the cultivation period and it is one of the most crucial steps during a bioprocess. Following the inoculation of the culture in the bioreactor, cells first need to adjust to their new environmental conditions. During this period, agitation, pH, temperature and pO_2 are monitored and controlled in real time via the bioprocess control software. In this stage it is possible to take culture sample to analyse the biomass of the bacteria or the concentration of metabolites. The aim of this is to reach the best of the four growth phases of the culture, depending on the experiment objectives: latency, exponential, stationery and death phase.

3.4.2 Run batch

Once the bioreactor is prepared, the conditions are set up and the bacteria has been inoculated, it is time to start running the batch fermentation, which will simulate and mimic the process and the environment desired. This process will vary depending on the aim of the study, but there are some common issues important to consider in order to proceed a successful bioprocess with the bioreactor:

- **Prevent contamination.** The most frequent cause of contamination is from the starter culture: improper handling, insufficient autoclaving culture vessels or components of the media, etc. It is important to make sure that the temperature is maintained long enough during the autoclaving process and that all the parts of the bioreactor are previous cleaned carefully. This includes the disinfection of the rings with 70% ethanol, which have to be in perfect conditions.
- **Maximize the growth.** Depending on the requirements of the microorganisms, the amount of dissolved oxygen must be individually configured and controlled by various parameters, such as the total air flow, the stirrer speed and the gas mix. Oxygen control cascades for microbial processes generally include the stirrer, since higher stirring speeds improve oxygenation, and the gas flow rate es most often used for controlling oxygen concentration. Apart from oxygen, if any nutrient has a limiting effect, the growth of the bacteria will be limited.
- **Maintain the culture volume.** It has been reported that when autoclaving the medium in the bioreactor, 10% of it is lost[45]. This can be prevented by adding some sterile water before inoculating the reactor, so the medium does not become too concentrated. In addition, when taking samples during the batch fermentation, this may not be over than 10 mL.

- **Prevent foam formation.** Foam formation occur in protein-rich media with higher gassing rates and/or stirrer speeds. This can damage the microorganisms, the final product and disrupt the hole process. One of the best methods to avoid this is to add a small portion of antifoam agent to the medium from the start.
- **Constant pump speed.** It is important to make sure that all supple tubes, which acid, base, antifoam and the feed, are neither kinked or squashes on their way to cover the plate of the bioreactor. The flow rate may vary with the viscosity of the liquid in each pump tube, so this is something important to consider.

3.4.3 Conditions

Bioreactors allow for the creation of optimal environmental conditions for the growth of cells or microbes, like incubators and shakers. The main difference between them is how these conditions are established[46]. *Table 19* in *Annex 9.1* shows the conditions of the systems used in the different studies that mimic colon fermentation mentioned in this thesis.

- **Stirring and culture mixing.** If the nutrients in the bioreactor are not dispersed well enough, conditions in certain parts of the bioreactor will deviate significantly from the ideal. Without uniform stirring, the microorganisms along the edge of the vessel will literally be boiled, while those in the middle get cold feet. In a bioreactor, instead of mixing by shaking, the culture is stirred with an impeller, which is connected to a motor. The typical stirring speed varies depending on the cultivated organism.
- **Temperature.** Microorganisms and cell cultures have enzymes that need certain temperature and pH ranges. If conditions fall outside of these ranges, the desired bioprocess will proceed much more slowly, because growth and metabolic performance are highly dependent on these enzymes.
- **pH.** The bioreactor is equipped with a pH sensor known as a single-rod measuring cell for pH, so it can correct any deviations in the pH; for this purpose, an acid and/or an alkaline solution is made available and connected to the culture vessel via tubes and pumps. Depending on the need, the pumps feed the culture media. The concentration of the acid and base must be skilfully selected.
- **Adding nutrients.** A batch operation run is when everything is made available to the microorganisms all at once and feeding is discontinued by pump addition. For this reason, the carbon source of nutrients is added gradually during the bioprocess.
- **Gassing.** Oxygen is important for culture growth as it establishes the aerobic or anaerobic conditions in the bioreactor. The bioreactor can feed a sterile gas mixture such as air into the culture medium. The minimum and maximum values of agitation, gas flow rate and oxygen concentration can be optimized depending on the

organisms and process needs. The amount of oxygen dissolved in the medium is continuously measured with a pO_2 sensor. Constant stirring not only distributes the nutrients but also reduces the size of the gas bubbles that arise in the culture vessel, thus efficiently releasing oxygen into the nutrient solution. The gassing rate is usually measured in litres per minute

- **Pressure.** The higher pressure is the vessel, the more oxygen is dissolved. Culture vessels made of glass are frequently only approved for a pressure of up to 0,5 bar. At a higher operating pressure, slightly damaged culture vessels made of glass can burst, which not only can ruin the experiment, but is also a safety risk.
- **Foam formation.** Foam forms at the interface between the liquid and gas phase in the culture vessel and can quickly finds its way up under the lid. In the worst case scenario, in then blocks the exhaust filter, which in turn blocks the flow of gas. A typical antifoam control system consists of a sensor installed at a specific height in the culture vessel. If the foam height reaches the sensor, and antifoam agent is pumped from a reservoir into the culture vessel.

3.5 FERMENTATION ANALYSES

The products of the fermentation are tested to obtain the characterization of the fermentation. The relevant parameters to be evaluated are:

- **Physic-chemicals.** pH values, gas production, SCFAs production, etc.
- **Gas chromatography.** Allows to analyse the SCFA production as well as the gases resulted in the fermentation process (H_2 and CO_2).
- **Biomass production.** This procedure consists on the final colony count of the bacteria after a fermentation process. Bacterial growth can be tested during the experiment in order to evaluate the survival, and total biomass production is estimated by removing the total volume in the vessel of the bioreactor and centrifuging it to obtain the bacterial pellets, so the amount of bacteria is tested.
- **Microbial population and changes**. Composition and distribution analysis.
    - Bacterial culturing for survival and growth evaluation
    - Bacterial selective count of different samples of the fermentation liquid plated in different selective media (BSM, BBE, LAMVAB, etc.), so different species of microorganisms can be isolated and counted.
    - qPCR: amplification of taxonomic or functional markers of genes in DNA to quantify the number of microorganisms in a sample, and can identify different families, genera or species based on the specificity of the marker.

- 16S sequencing: identification of sequence differences (polymorphisms) in the hypervariable regions of the 16S rRNA gene, so bacteria population can be identified and characterized in genera and specie.

4. EXPERIMENTAL PART: DESIGN OF IN VITRO MODEL OF COLONIC FERMENTATION

OBJECTIVE

The objectives of this study are, firstly, evaluate microbial dynamics of *Lactobacillus sp.* in the bioreactor and produce biomass for its later lyophilization and testing in two different storage conditions. Moreover, this study will provide a better understanding of the Minifors 2 bioreactor system and a good training for further experiments. To achieve this, four different batch trials were run in sequence to prove and improve the conditions for *Lactobacillus sp.* growth in a single-batch bioreactor, based on previous designed models searched in bibliographic work. *Lactobacillus sp.* was chosen as it is a typical beneficial component of gut microbiota and it is easy to monitor as single species.

Secondly, evaluate the in vitro colon model with a complex microbial community. The gut environment was simulated in the in vitro model system using human faecal samples, which will be used to study the effect of polyphenol Schisandrin B in gut microbiota. In this thesis there is included the experiment to determine the stabilization period needed by the complex microbial population and the one control experiment, which will be useful for future studies with the polyphenol Schisandrin B.

MATERIALS AND METHODS

The single-batch fermentation system

Operations of the single-batch fermentation system were performed using the Minifors 2 bioreactor (Infors HT). The fermenter consists of a 3 L-vessel located on a compact base unit with four freely configurable pumps, pH and pO_2 sensors, two fully automatic gas lines with mass flow controllers and a touch screen control unit. Media feet rate, oxygen, pH, temperature and stir rate were controlled by the eve® software (Infors HT). Oxygen concentration in the vessel was kept at 21 % via positive nitrogen pressure and continuously measured using pO2

Figure 7. *Minifors 2 bioreactor setup. Obtained from Minifors 2 Cookbook[45].*

probe (Hamilton®). The oxygen probes were calibrated using a two-point calibration with 0% of oxygen as the zero-point calibration and 21% of oxygen as the 100% calibration point. pH was maintained at 6 using 20% H_3PO_4 solution and 25% NH_3 solution. pH was measured continuously with a pH probe (Hamilton®). pH probes were calibrated with a two-point calibration with standardized pH buffers at 4 and 7. Vessels were maintained at 37º via the Infors onboard temperature control system. Vessels were continuously stirred using magnetic impeller stir-shafts. The fermentation system with the culture medium and the reagents were autoclaved at 121ºC for 1 hour. See *Figure 8* for bioreactor setup.

Medium and substrates

To evaluate *Lactobacillus sp.* microbial dynamics, 1,5 L of MRS culture media were used. For the simulation of the human gut environment, medium in the batch fermentation system consisted in 600 mL of adapted SIEM medium designed by Neunen *et al* (2003)[47] (*Table 5*).

Reagents in each pump bottle were as follows:

- 200 mL 20% H_3PO_4 (pump 1 acid)
- 200 mL 25% NH_3 (pump 2, base)
- 200 mL 50% glucose (pump 3, feed)
- 200 mL SE-15 (pump 4, antifoam)

Table 5. *SIEM culture medium.*

Reagent	Weight (g/L)
Pectin	4.7
Xylan	4.7
Arabinogalactan	4.7
Amylopectin	4.7
Casein	23.5
Starch	39.2
Tween 80	17
Bactopeptone	23.5
Bile	0.4

***Lactobacillus sp.* strain preparation and fermentation process**

Lactobacillus sp. was provided by Innolact Oy and it is under patentation. Colonies were inoculated in 9 mL MRS broth at 37ºC for 24 hours. 9mL of the preculture were centrifuged at 4000 rpm for 3 minutes at room temperature and resuspend in 5 mL MRS broth. 100 µL were taken for serial dilution (10^{-1} to 10^{-9}) and plating on MRS plates in duplicates for bacterial count. Plates were incubated at 37ºC.

The four fermentations were initiated by inoculation of 4 mL of precultured *Lactobacillus sp.* strain to 1.5 L of culture medium. Aliquots of the fermentation cultures were sampled without disturbing the internal anaerobic conditions. Running batch time and sampling for colony counting vary in each trial as best conditions were studied (see *Table 6* for conditions in each trial).

Table 6. *Conditions set in each batch trial. Arrows indicate a change of the value during the process*

	TRIAL 1	TRIAL 2	TRIAL 3	TRIAL 4
pH	6±0.2	5	6±0.2	6±0.2
Temperature	37 ºC	37 ºC	37 ºC	37 ºC
Stirring	200 → 500 min^{-1}	100 → 500 min^{-1}	500 min^{-1}	500 min^{-1}
pO_2	21%	21%	21%	21%
Air flow	0-0.3 $L \cdot min^{-1}$	0-0.3 $L \cdot min^{-1}$	0-0.3 $L \cdot min^{-1}$	0-0.3 $L \cdot min^{-1}$
Batch time	24 h	48 h	-	24 h
Sampling time	24 h	6, 24 ,29, 48 h	0, 6, 24 h	0, 6, 24 h

Faecal samples preparation and fermentation process

Faecal samples were obtained from three healthy human male volunteers who had not been treated with antibiotics and were selected randomly from the youth to middle aged people. Human faecal samples were handled, and a written informed consent was obtained from every volunteer. A questionnaire was asked to be filled to report the main factors of volunteer's diet and daily routine for further conclusions (*see Annex 9.2*). After collection, faecal samples were immediately placed under anaerobic conditions using anaerobic boxes. Each faecal sample was weighed to 1 g and diluted in 5 mL of saline (0.9% NaCl) and 555 µL of glycerol prior to freezing in -80C to preserve the viability of the microbiota. As it is difficult to add glycerol to the samples as it is very viscous, the saline and glycerol were mixed before autoclave. This procedure was done in Sololab anaerobic environment (see *Annex 9.3*). Before each inoculation, faecal slurry is mixed and centrifuged at 600rpm for 3 minutes and supernatant is removed for inoculation.

Fermentation was conducted under anaerobic conditions of the by purging 0.4 L N_2 per hour. Each fermentation was performed at 37ºC with magnetic stirring at 100 rpm and continuous pH monitoring, adjusted to 6±0.2 by addition of to mimic the pH of the colon of a healthy adult. Feed is added as 0.1% after hour 19. Conditions for colonic fermentation are based on the previous research. Each fermentation was run at a 600 mL working volume per vessel. For the stabilization period test, 10 mL of faecal sample, previously centrifuged, were added to 600 mL of MRS medium in the bioreactor vessel and samples were taken at 0, 17, 20, 24 and 48 hours after the initiation of fermentation.

For the control trial, fermentation was initiated by the inoculation of 10 mL of faecal suspension to 600 mL of SIEM medium and as tested before, a period of 18 hours were necessary for stabilization of the microbial community. At that time, 1.5 mL of DMSO where added as the control. Samples were taken at 0, 2, 4, 8, 12 and 24 hours.

Lactobacillus colony counting and biomass production

2mL of batch culture was collected at each sampling time using the Super Safe Sampler. 100uL of dilutions were plated on MRS plates and incubated at 37ºC during 48 hours for colony counting. After batch was completed, batch culture was collected in 50mL Falcon tubes, 40mL each. The Falcon tubes were labelled 1-47 beforehand and their weights were recorded. The filled tubes were centrifuged at 3000 rpm for 3 minutes at room temperature to precipitate bacteria and the supernatant was discarded. The biomass in each tube was calculated by subtracting the weight of the empty Falcon tube from the weight of bacteria and the Falcon tube. The weight of bacteria in all the tubes were summed up to conclude the total biomass generated from the batch-culture. Sterilized 10% skimmed milk was added to the biomass in 1:1 ratio (w/v).

Lyophilization of *Lactobacillus*

Each trial was proceeded with lyophilization. The bacteria/skimmed milk mixture was aliquoted to 2mL vials (500uL each) to proceed with lyophilization, which was done by the services of University of eastern Finland. Primary drying at -37ºC and secondary drying at 4ºC. Pressure in primary and secondary drying was 100 mTorr for all samples. Bacteria from trials 2 and 4 where lyophilized together. Lyophilized bacteria were resuspended in peptone water in 1:10 ratio (w/v). Dilutions 10^{-2} to 10^{-7} were plated in duplicates on MRS agar plates. Colony formation unit was calculated until four weeks after lyophilization so bacterial survival was tested. Lyophilized bacterial survival from trials 2 and 4 was also tested under two conditions: room temperature and refrigerator (8ºC).

Colony counting and selective growth of the complex microbial community

100 µL of the samples were plated in different dilutions on PCA plates and incubated at 37ºC during 24 hours for colony counting, both in anaerobiosis and aerobiosis. Samples were also plated in selective media to characterize bacterial genera during the control experiment (*Table 7*).

Table 7. *Selective media.*

Selective media	Selective bacterial growth
Agar BSM	*Bifidobacteria*
Agar BBE	*Bacteriodes*
Agar LAMVAB	*Lactobacilli*
Agar BBL	*Clostridium*
Agar VRBG	*Enterobacteriaceae*
Agar SDA	*Funghi*

RESULTS AND DISCUSSION

- Evaluation of the bioreactor microbial dynamics with a single bacterial specie:

Lactobacillus colony count in four batch trials

Batch time in Trial 1 was run for 24 hours and only after batch competition samples were plated in MRS broth from dilution 10^{-5} to 10^{-7}. Colonies were counted at dilution plate -6 as to $3.30 \cdot 10^{-7}$ CFU/mL. Stirring was changed from 200 to 500 min^{-1} during batch run.

For Trial 2, which lasted for 48 hours, pH was lowered to 5. Samples were taken at time 6, 24, 29 and 48 hours. Dilutions for sample at 6 hours where not high enough so there were too many colonies to count. At 24 hours, there were $6.75 \cdot 10^6$ CFU/mL and at batch competition, $1.90 \cdot 10^6$ CFU/mL.

Trial 3 was planned to run the batch for 24 hours, but it was not successful. At first stages of the procedure, where 3 to 4 drops of antifoam have to be added, there were added 6 mL approximately. It is suspected that the antifoam can clump so it is needed to shake the bottle before starting the batch. Due to this, results from this trial are no longer considered.

As seen in trials 1 and 2, 24 hours of batch seemed to be enough as it was presumed that *Lactobacillus sp.* could reach its stationary phase at this time. Trial 4 was a replicate of trial 1, but in this case inoculum concentration was analysed. Inoculum concentration was equal to $1.21 \cdot 10^8$ CFU/mL, sampling at time 6 was $2.12 \cdot 10^6$ CFU/mL and at 24 hours $1.81 \cdot 10^8$. All the results are summarized in *Table 8*.

Table 8. *Inoculum concentration and colonies formed per millilitre of each sampling time*

	TRIAL 1	**TRIAL 2**				**TRIAL 4**		
Inoculum (CFU/mL)	N/A	N/A				$1.21 \cdot 10^8$		
Sampling time (h)	24	6	24	29	48	0	6	24
CFU/mL	$3.30 \cdot 10^7$	$>10^7$	$6.75 \cdot 10^6$	$3.50 \cdot 10^6$	$1.90 \cdot 10^6$	$>10^7$	$2.12 \cdot 10^6$	$1.81 \cdot 10^8$

Biomass production and resuspension in 10% of skimmed milk

Lactobacillus sp. biomass production in each trial was nearly 20 g, with a concentration of approximately $1 \cdot 10^{-8}$ CFU/mL after resuspension in 10% of skimmed milk, as shown in *Table 9*.

Table 9. *Biomass production and concentration after resuspention in 10% of skimmed milk*

	TRIAL 1	TRIAL 2	TRIAL 3	TRIAL 4
CFU/mL	$1.58 \cdot 10^{-9}$	$1.20 \cdot 10^{-8}$		$7.15 \cdot 10^{-8}$
Biomass production (g)	18.9903	19.2147	N/A	20.7179

N/A: not available

Lactobacillus survives the process of lyophilization

Lactobacillus sp. survived to lyophilization as there has not been a significant decrease in bacterial growth after the lyophilization ($4.89 \cdot 10^8$ CFU/mL as the average of all trials). Referring to the results showed in *Table 10*, the survival is slightly decreased one week after for those lyophilized bacteria stored at room temperature ($9.43 \cdot 10^7$ CFU/mL), but it stabilizes at weeks 3 and 4 and stops decreasing, keeping the concentration at the order of 10^5 CFU/mL. For those lyophilized bacteria stored at the refrigerator at 8ºC (*Table 11*), they behave similarly as the ones kept at room temperature: the viability decreases during the first week but seems to keep stable during the following weeks, reaching a concentration average at week 4 of $3.81 \cdot 10^6$ CFU/mL. Results from week 2 show an increased concentration in the viability to the order of 10^8, but then the following weeks it maintains lower, at the order of 10^6. These results are not taken into consideration as they may be a contamination from another bacterial species during the plate culturing. Therefore, it is possible to conclude that *Lactobacillus sp.* survives successfully to the process of lyophilization and it decreases during the next four weeks till it stabilizes, and that storage at the refrigerator is more favourable to its survival than storage at room temperature.

Table 10. *Lactobacillus sp. concentration (CFU/mL) until 4 weeks after lyophilization stored at room temperature*

	Week 0 (before lyophilization)	Week 0 (after lyophilization)	Week 1	Week 2	Week 3	Week 4
Trial 1	$1.58 \cdot 10^9$	$1.10 \cdot 10^9$	$1.54 \cdot 10^7$	$1.42 \cdot 10^7$	-	
Trial 2	$1.20 \cdot 10^8$	$1.83 \cdot 10^8$	$1.07 \cdot 10^8$	$3.01 \cdot 10^6$	$2.35 \cdot 10^5$	$7.40 \cdot 10^5$
Replicates	$7.15 \cdot 10^8$	$1.83 \cdot 10^8$	$1.61 \cdot 10^8$	$3.46 \cdot 10^6$	$1.50 \cdot 10^5$	$8.20 \cdot 10^5$
Average	$8.05 \cdot 10^8$	$4.89 \cdot 10^8$	$9.43 \cdot 10^7$	$6.89 \cdot 10^6$	$1.93 \cdot 10^5$	$7.80 \cdot 10^5$

Table 11. *Lactobacillus sp. concentration (CFU/mL) until 4 weeks after lyophilization stored at 8ºC (refrigerator)*

	Week 0 (before lyophilization)	Week 0 (after lyophilization)	Week 1	Week 2	Week 3	Week 4
Trial 1	$1.58 \cdot 10^9$	$1.10 \cdot 10^9$	-	-	-	-
Trial 2	$1.20 \cdot 10^8$	$1.83 \cdot 10^8$	$1.35 \cdot 10^7$	$8.31 \cdot 10^8$	$9.55 \cdot 10^5$	$5.20 \cdot 10^5$
Replicates	$7.15 \cdot 10^8$	$1.83 \cdot 10^8$	$9.35 \cdot 10^6$	$4.84 \cdot 10^8$	$8.40 \cdot 10^6$	$7.10 \cdot 10^5$
Average	$8.05 \cdot 10^8$	$4.89 \cdot 10^8$	$1.14 \cdot 10^7$	$6.58 \cdot 10^8$	$4.68 \cdot 10^6$	$3.81 \cdot 10^6$

In this first experiment, the bioreactor Minifors 2 was tested under different conditions in order to get a better understanding of its working protocol and *Lactobacillus sp.* growth was measured for this aim. A temperature of 37ºC, pH 6±0.2, 500 rpm of stirring, 21% of O_2 environment with an air flow of 0.3 L/min are considered good conditions to get *Lactobacillus sp.* growth up to 24 hours of batch process and to study its dynamics. In all trials, the biomass produced was approximately 20 g. This biomass was lyophilized and it survived the procedure successfully, and it was shown that its survival is better if the storage is in the refrigerator at 8ºC than at room temperature, although more replicate should be done to obtain a statistical analysis.

During the several batch trials, some problems appeared to be significant for the results and to be considered for further experiments. One of the main procedures where most uncertain fact happened was the autoclaving. Glucose used to escape the filter during autoclave in all the trials although the tubes where checked to be correctly cleaved. Similarly, antifoam bottle appeared with some brown liquid after autoclave, which suggest that some media culture from the vessel could go into the bottle. Apart from this, autoclave was considered successful as no signs of contamination where noticed. Related to the measurement of bacterial growth, the OD sensor never worked properly so it was not possible to measure the OD properly. Related to EVE program and the bioreactor software, a training session was done in order to solve doubts and problems.

Finally, the most time limiting factor was the calibration of the pH and pO_2 probes. Depending on the trial, pH could last from 20 to 40 minutes and pO_2 calibration was also very long lasting and its procedure was quite not accurate. Nevertheless, pO_2 sensor

need to be calibrated twice a year, but is it something to be considered to improve for further experiments.

After this experiment it can be concluded that the Minifors 2 biroeactor is a successful system to simulate the gut environment and will be used in next studies. The four trials have been a great training to learn how the bioreactor works, and together with a good bibliographic research, it will be possible to simulate any type of environment for microbial growth.

- Evaluation of the in vitro colon model with a complex microbial community:

Stabilization period of 18 hours for complex microbial community

According to the volume of medium (600 mL) and the amount of faecal sample, the bacterial community showed that 18 hours would be sufficient to get stabilized in the environment. Both aerobes and anaerobes plated in PCA plates showed an exponential growth till 17 hours, when the growth stopped. In *Table 12* it can be seen how bacterial growth starts to stabilize at time 17 of the experiment. These results were also contrasted with the OD graph obtained from EVE program® (*Graphic 1, Annex 9.4*).

Table 12. *Inoculum concentration and colonies formed per millilitre of each sampling during stabilization test*

	Aerobes				**Anaerobes**			
Inoculum (CFU/mL)	-				$2.8 \cdot 10^2$			
Sampling time (h)	17	20	24	48	17	20	24	48
CFU/mL	$1.1 \cdot 10^9$	$2.05 \cdot 10^9$	$1.4 \cdot 10^9$	$1.25 \cdot 10^8$	$7.5 \cdot 10^8$	$2.95 \cdot 10^9$	$3.05 \cdot 10^9$	$1.45 \cdot 10^8$

Control batch experiment

Plate Count Agar shows anaerobes and aerobes up to 10^9 CFU/mL

Colony count on PCA plates showed the different communities of bacteria in the control experiment. It can be seen that bacterial concentration kept in the order of 10^9 during time 0, 2, 4, 8, 12 and 24 hours after the control inoculation (after 18 hours of stabilization), both for aerobes and anaerobes (*Table 13*).

Table 13. *Inoculum concentration and colonies formed per millilitre of each sampling time during control test*

PCA aerobes						
Inoculum (CFU/mL)	$1.25 \cdot 10^3$					
Sampling time (h)	0	2	4	8	12	24
CFU/mL	$3.19 \cdot 10^9$	$3.20 \cdot 10^9$	$2.60 \cdot 10^9$	TNTC	$3.65 \cdot 10^9$	$1.53 \cdot 10^9$
PCA anaerobes						
Inoculum (CFU/mL)	$6.80 \cdot 10^4$					
Sampling time (h)	0	2	4	8	12	24
CFU/mL	$3.01 \cdot 10^9$	$2.68 \cdot 10^9$	$2.33 \cdot 10^9$	TNTC	$3.65 \cdot 10^9$	$1.39 \cdot 10^9$

Comparing to the graph obtained from EVE program® that shows the variation of OD and pH, it can be seen that OD experiences changes during the bioprocess due to the growth of the different bacterial species present in the medium, and as a result, pH also varies for each community growth (*Graphic 2, Annex 9.4*).

Selective growth of different microbial species

Selective colony counting showed that the predominant bacterial species are *Enterobacteriaceae* and *Clostridium*, while *Lactobacilli* are also present but in a lower concentration (*Table 14 and Images 1,2 and 3 in Annex 9.5*). There were no colonies formed in the plates for *Funghi* and *Bidifobacteria.* It is important to mention that this control was done with the faecal sample of just one donor and the microbial communities can vary from one donor to another.

Table 14. *Inoculum concentration and colonies formed per millilitre of Enterobacteriaceae (VRGBA), Clostridium (BBL) and Lactobacilli (LAMVAB)*

VRGBA						
Inoculum (CFU/mL)	$9.50 \cdot 10^2$					
Sampling time (h)	0	2	4	8	12	24
CFU/mL	$6.85 \cdot 10^8$	$6.85 \cdot 10^8$	TNTC	TNTC	TNTC	$1.53 \cdot 10^9$
BBL						
Inoculum (CFU/mL)	$1.45 \cdot 10^3$					
Sampling time (h)	0	2	4	8	12	24
CFU/mL	$5.67 \cdot 10^8$	$1.29 \cdot 10^9$	TNTC	TNTC	TNTC	$1.22 \cdot 10^9$

LAMVAB						
Inoculum (CFU/mL)	$2.05 \cdot 10^2$					
Sampling time (h)	0	2	4	8	12	24
CFU/mL	$1{,}02 \cdot 10^6$	$8{,}20 \cdot 10^5$	$1{,}84 \cdot 10^5$	$1{,}25 \cdot 10^4$	$6{,}00 \cdot 10^3$	0

These results, together with those in previous references mentioned in this thesis, show that it is possible to simulate the adult gut environment using an in vitro model system. In this case, the conditions chosen, based on the bibliographic research, were successful to ensure that a complex microbial community could be mimicked in the bioreactor using human faecal samples.

The stabilization experiment showed that 18 hours would be sufficient to ensure that all microbial communities adapt to the environment in the model. Therefore, after this stabilization period it is possible to inoculate any bioactive compound that is wanted to be studied. Here DMSO was added as the control and the bacterial community variations were studied using one donor's faecal sample. It could be only seen a presence of *Enterobacteriaceae*, *Clostridium* and *Lactobacilli.* It has been mentioned in this thesis that the microbial diversity varies from each individual donor as it can be modulated with the diet or antibiotics consumption. For this reason, two more controls with other human faecal samples are going to be done in the future to get a wide conception of the different microbial communities.

These results will be useful for future experiments, where polyphenol Schisandrin B will be added in different concentrations and its interactions with the gut microbiota will be studied. Moreover, DNA extraction from the samples will be proceeded to do DNA sequencing to obtain a more accurate view of the different bacterial communities, and also qPCR will allow to get a quantitative result of the bacterial species.

5. GENERAL DISCUSSION

At the end of this research work, it has been shown how the design of an in vitro model of colonic fermentation could be used in different applications, such as the biomass production of a single species of *Lactobacillus* and the evaluation of the effect of a polyphenolic compound in a complex microbial community. These results are consistent with previous results of other studies mentioned in this thesis and show the possibility to cultivate a complex intestinal microbiota under controlled environmental conditions to ensure the growth and development of these microorganisms.

In vitro gut fermentation models can be used to complement human and animal studies to overcome some of the limitations of in vivo models. The key objective of these models is to culture stable, reproducible, and complex microbial communities in a highly controlled environment. Compared with animal studies, microbial communities cultured in gut fermentation models can be sampled frequently, with less ethical and technical constraints.

There are a variety of gut fermentation models described, each of which varies in their design and complexity. The different in vitro models available to researchers consist on single or multiple vessels that are inoculated with fresh human faeces or a defined microbial community. These vessels are operated under anaerobic conditions and microbial communities are grown using a temperature, pH, growth medium, and transit time set to mimic a specific intestinal segment. High stability and reproducibility of microbial communities cultured in gut fermentation models are achieved, because researchers can tightly control the experimental parameters. These models simulate different spatial, temporal, nutritional, and physiochemical properties of different gut segments. Each model has their own advantages and disadvantages, so model selection depends on the objective of the study. These models have been reviewed in detail in this thesis.

Gut microbiome studies have been gaining popularity over the years, especially with the development of new technologies (metataxonomics, metagenomics and metabolomics) that makes it easier for researchers to characterize the composition and functionality of these complex microbial communities. The goal of these studies is to identify a microorganism, group of microbes, or microbial metabolite which correlates with a disease state. Gut fermentation models are the ideal systems to study the direct effects of interventions on the gut microbiota, as changes in the gut microbiota can be measured on the host. They have been used to study changes in the gut microbiota in

response to many interventions, including dietary substrates, antibiotics, steroids, bile acids, xenobiotic compounds, probiotics, prebiotics, and pathogens.

In vitro gut fermentation models, animal models, and human studies should be used together to fully understand the mechanism of an intervention or disease. The ideal method to study the effects of various interventions on the gut microbiota is to start from simplified models and work up to more complex models, for example, starting out by testing an intervention in batch culture, then in chemostat systems, then in animal models, then in humans. Alternatively, in cases where human studies have hinted that an intervention may affect the gut microbiota, researchers should return to in vitro models to confirm the causality of these observations.

Due to the COVID-19 pandemic, this study was paused for two months and it was not possible to include all the validation trials of the methods in this thesis as only some experiments could be performed during the internship. As future perspectives, this work would be continued with the complete study of the effects of polyphenol Schisandrin B on gut microbiome. Moreover, the designed model will be useful to test other bioactive compounds such as dietary fibres and to mimic other parts of the human. This would be related with studies of the interactions between food and gut microbiota, or the effect of other bioactive compounds. Moreover, it could be also used to produce specific metabolites produced by some bacterial communities or to produce biomass of a probiotic of interest.

6. CONCLUSIONS

Summing up the results presented, we may conclude that:

1. Both batch and dynamic fermentation models present advantages and limitations that are important to consider depending on the aim of the study. Dynamic models are able to simulate metabolite absorption, peristalsis and the intestinal mucus with some dynamic models, and also to make quantitative analysis of human metabolism. Nevertheless, batch models are good to conduct short-term studies and they are easy-to-use, cost-effective, and able to monitor the different batch conditions. They are good enough to conduct studies of biomass production, qualitative prediction of bioavailable metabolites and microbial population.

The optimal conditions to simulate the human colonic environment have been determined according to previous references and have been tested in Minifors 2 bioreactor system (Infors HT). Therefore, it has been possible to design an in vitro model for human colonic fermentation which would be used in future experiments to study the effect of a bioactive compound on gut microbiota.

2. Microbial dynamics of one single bacterial specie of *Lactobacillus* were studied in the bioreactor and it was possible to obtain biomass to lyophilize this bacterium. The survival after lyophilization was positive, and the storage in the refrigerator seemed to be more favorable than at room temperature, tested till 4 weeks after the process.

3. The designed in vitro model for human colon was evaluated with a complex microbial community using human fecal samples. The microbial community's stabilization period was determined, and the different species where characterized in selective growth plates, so these results will be useful in future experiments to test the effect of the polyphenolic compound Schisandrin B on the microbiome.

Both the bibliographic research of different in vitro models and the experimental part of this thesis will provide future researchers a good knowledge of the need to use in vitro models, its possible applications in human studies and an example of a successful in vitro model of human colonic fermentation.

7. REFERENCES

1. Wang B, Yao M, Lv L, Ling Z, Li L. The Human Microbiota in Health and Disease. Vol. 3, Engineering. Elsevier Ltd; 2017. p. 71–82.

2. Takagi R, Sasaki K, Sasaki D, Fukuda I, Tanaka K, Yoshida K, et al. A Single-Batch Fermentation System to Simulate Human Colonic Microbiota for High-Throughput Evaluation of Prebiotics. Wilson BA, editor. PLoS One. 2016 Aug 2;11

3. Kleiveland C, Lea T, Mackie A, Requena T, Swiatecka D, Wichers H. Kitty Verhoeckx · Paul Cotter Iván López-Expósito. The Impact of Food Bioactives on Health In Vitro and Ex Vivo Models. Springer; 2015.

4. Rajilić-Stojanović M, Smidt H, De Vos WM. Diversity of the human gastrointestinal tract microbiota revisited. Vol. 9, Environmental Microbiology. Environ Microbiol; 2007. p. 2125–36.

5. Neri-Numa IA, Pastore GM. Novel insights into prebiotic properties on human health: A review. Food Res Int. 2020 May 1;131.

6. Gouba N, Hien YE, Guissou ML, Fonkou MDM, Traoré Y, Tarnagda Z. Digestive tract mycobiota and microbiota and the effects on the immune system. Vol. 12, Human Microbiome Journal. Elsevier Ltd; 2019. p. 100056.

7. Derrien M, van Hylckama Vlieg JET. Fate, activity, and impact of ingested bacteria within the human gut microbiota. Vol. 23, Trends in Microbiology. Elsevier Ltd; 2015. p. 354–66.

8. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014 Jun 10;11(8):506–14.

9. Markowiak P, Ślizewska K. Effects of probiotics, prebiotics, and synbiotics on human health. Vol. 9, Nutrients. MDPI AG; 2017.

10. Wilkins T, Sequoia J. Probiotics for Gastrointestinal Conditions: A Summary of the Evidence. undefined. 2017.

11. Bindels LB, Delzenne NM, Cani PD, Walter J. Towards a more comprehensive concept for prebiotics. Vol. 12, Nature Reviews Gastroenterology and Hepatology. Nature Publishing Group; 2015. p. 303–10.

12. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics

and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Vol. 14, Nature Reviews Gastroenterology and Hepatology. Nature Publishing Group; 2017. p. 491–502.

13. He M, Shi B. Gut microbiota as a potential target of metabolic syndrome: The role of probiotics and prebiotics. Vol. 7, Cell and Bioscience. BioMed Central Ltd.; 2017.

14. Rastall RA, Gibson GR. Recent developments in prebiotics to selectively impact beneficial microbes and promote intestinal health. Vol. 32, Current Opinion in Biotechnology. Elsevier Ltd; 2015. p. 42–6.

15. Jones JM. CODEX-aligned dietary fiber definitions help to bridge the 'fiber gap'. Nutr J. 2014 Apr 12;13(1):34.

16. Deehan EC, Duar RM, Armet AM, Perez-Muñoz ME, Jin M, Walter J. Modulation of the Gastrointestinal Microbiome with Nondigestible Fermentable Carbohydrates To Improve Human Health. Microbiol Spectr. 2017 Oct 3;5(5).

17. Flint HJ. The impact of nutrition on the human microbiome. Nutr Rev. 2012. p.70.

18. Makki K, Deehan EC, Walter J, Bäckhed F. The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease. Vol. 23, Cell Host and Microbe. Cell Press; 2018. p. 705–15.

19. Li AN, Li S, Zhang YJ, Xu XR, Chen YM, Li H Bin. Resources and biological activities of natural polyphenols. Vol. 6, Nutrients. MDPI AG; 2014. p. 6020–47.

20. Redan BW, Buhman KK, Novotny JA, Ferruzzi MG. Altered Transport and Metabolism of Phenolic Compounds in Obesity and Diabetes: Implications for Functional Food Development and Assessment. Adv Nutr An Int Rev J. 2016 Nov;7(6): p. 1090–104.

21. Cardona F, Andrés-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuño MI. Benefits of polyphenols on gut microbiota and implications in human health. Vol. 24, Journal of Nutritional Biochemistry. J Nutr Biochem; 2013. p. 1415–22.

22. Batch Fermentation - an overview | ScienceDirect Topics [Internet]. [cited 2020 April 28]. Available from: https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/batch-fermentation

23. Wang X, Gibson GR. Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. J Appl Bacteriol. 1993;75(4): p. 373–80.

24. Pérez-Burillo S, Pastoriza S, Jiménez-Hernández N, D'Auria G, Francino MP, Rufián-Henares JA. Effect of Food Thermal Processing on the Composition of the Gut Microbiota. J Agric Food Chem. 2018 Oct 31;66(43): p. 11500–9.

25. Gaisawat MB, Iskandar MM, MacPherson CW, Tompkins TA, Kubow S. Probiotic supplementation is associated with increased antioxidant capacity and copper chelation in C. difficile-infected fecal water. Nutrients. 2019 Sep 1;11(9).

26. Oliphant K, Cochrane K, Schroeter K, Daigneault MC, Yen S, Verdu EF, et al. Effects of Antibiotic Pretreatment of an Ulcerative Colitis-Derived Fecal Microbial Community on the Integration of Therapeutic Bacteria In Vitro . mSystems. 2020 Jan 28;5(1).

27. Tuncil YE, Thakkar RD, Marcia ADR, Hamaker BR, Lindemann SR. Divergent short-chain fatty acid production and succession of colonic microbiota arise in fermentation of variously-sized wheat bran fractions. Sci Rep. 2018 Dec 1;8(1).

28. Wiese M, Khakimov B, Nielsen S, Sørensen H, van den Berg F, Nielsen DS. CoMiniGut-A small volume in vitro colon model for the screening of gut microbial fermentation processes. PeerJ. 2018;2018(1).

29. Gibson GR, Cummings JH, Macfarlane GT. Use of a three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. Appl Environ Microbiol. 1988;54(11). p. 2750–5.

30. Bircher L, Schwab C, Geirnaert A, Lacroix C. Cryopreservation of artificial gut microbiota produced with in vitro fermentation technology. Microb Biotechnol. 2018 Jan 1;11(1): p. 163–75.

31. Silverman JD, Durand HK, Bloom RJ, Mukherjee S, David LA. Dynamic linear models guide design and analysis of microbiota studies within artificial human guts. Microbiome. 2018 Nov 12;6(1).

32. McDonald JAK, Schroeter K, Fuentes S, Heikamp-deJong I, Khursigara CM, de Vos WM, et al. Evaluation of microbial community reproducibility, stability and composition in a human distal gut chemostat model. J Microbiol Methods. 2013 Nov;95(2):167–74.

33. Medina, D.A., Pinto, F., Ortúzar, V., & Garrido, D. (2018). Simulation and modeling of dietary changes in the infant gut microbiome. FEMS microbiology ecology. 2018. P. 94-9.

34. Medina DA, Pinto F, Ovalle A, Thomson P, Garrido D. Prebiotics mediate microbial interactions in a consortium of the infant gut microbiome. Int J Mol Sci. 2017 Oct 1;18(10).

35. García C, Tebbji F, Daigneault M, Liu N-N, Köhler JR, Allen-Vercoe E, et al. The Human Gut Microbial Metabolome Modulates Fungal Growth via the TOR Signaling Pathway. mSphere. 2017 Dec 27;2(6).

36. Santiago-Rodriguez TM, Ly M, Daigneault MC, Brown IHL, McDonald JAK, Bonilla N, et al. Chemostat culture systems support diverse bacteriophage communities from human feces. Microbiome. 2015 Nov 9; p. 3:58.

37. Molly K, Vande Woestyne M, Verstraete W. Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. Appl Microbiol Biotechnol. 1993 May;39(2):p. 254–8.

38. Williams CF, Walton GE, Jiang L, Plummer S, Garaiova I, Gibson GR. Comparative Analysis of Intestinal Tract Models. Annu Rev Food Sci Technol. 2015 Apr 10;6(1):p. 329–50.

39. Giuliani C, Marzorati M, Daghio M, Franzetti A, Innocenti M, de Wiele T Van, et al. Effects of olive and pomegranate by-products on human microbiota: A study using the SHIME® in vitro simulator. Molecules. 2019 Oct 21;24(20).

40. Minekus M, Smeets-Peeters M, Havenaar R, Bernalier A, Fonty G, Marol-Bonnin S, et al. A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. Appl Microbiol Biotechnol. 1999;53(1): p. 108–14.

41. Larsen N, de Souza CB, Krych L, Kot W, Leser TD, Sørensen OB, et al. Effect of potato fiber on survival of Lactobacillus species at simulated gastric conditions and composition of the gut microbiota in vitro. Food Res Int. 2019 Nov 1; p. 125.

42. Zihler Berner A, Fuentes S, Dostal A, Payne AN, Vazquez Gutierrez P, Chassard C, et al. Novel Polyfermentor intestinal model (PolyFermS) for controlled ecological studies: validation and effect of pH. PLoS One. 2013;8(10).

43. Pham VT, Chassard C, Rifa E, Braegger C, Geirnaert A, Rocha Martin VN, et al. Lactate Metabolism Is Strongly Modulated by Fecal Inoculum, pH, and Retention Time in PolyFermS Continuous Colonic Fermentation Models Mimicking Young Infant Proximal Colon. mSystems. 2019 May 28;4(4).

44. Le Blay G, Chassard C, Baltzer S, Lacroix C. Set up of a new in vitro model to study dietary fructans fermentation in formula-fed babies. Br J Nutr. 2010 Feb;103(3):p. 403–11.

45. The Cookbook. Basic Knowledge, reciepes and strategies for microbial bioprocesses. | Infors HT [Internet]. [cited 2020 May 8]. Available from: http://www.infors-ht.com.cn/uploadfile/2018/0515/INFORS-HT_cookbook_en.pdf

46. Rasche U. Bioreactors and Fermentors-Powerful Tools for Resolving Cultivation Bottlenecks. 2019 Jul.

47. Van Nuenen MHMC, Meyer PD, Venema K. The Effect of Various Inulins and Clostridium difficile on the Metabolic Activity of the Human Colonic Microbiota in vitro. MicrobEcol Health Dis. 2003 Nov;15(2–3):p. 137–44.

8. SELF-EVALUATION

At this end of this thesis, it can be concluded that the work done has been satisfactory, although the world's circumstances made some changes in the research.

At first point, the aim of the work was to design an in vitro model of colonic fermentation to study the effects of a polyphenolic compound (Schisandrin B) on gut microbiota. However, due to COVID pandemic, the University of Eastern Finland decided to close all the facilities for 2 months, and the work had to be paused. As a result, there wasn't enough time to take part in all the study, so I decided to include the data from the experiments I had participated: the *Lactobacillus* trials, done during February and half of March, and the first control of the polyphenol study, done during half of May and June.

Nevertheless, to complete this thesis, I took advantage of the two months to do a bibliographic research about previously designed in vitro models, and I included this in the thesis. This gave a better understanding of the in vitro models, the gut environment and allowed to take part more deeply in the design of the in vitro model.

Therefore, I have amplified my knowledge in the gut microbiota population, the different in vitro systems and about the bioactive compounds that modulate the gut environment. Moreover, I have improved my lab skills and becoming more efficient and self-confident. It has been a great chance to improve all the skills acquired during the Biotechnology grade. It was so easy to adapt to all the lab team and they trust on me since the beginning. I was able to solve problems by myself and to propose new ideas during the project. Additionally, I attended to three lessons about Eve program ® so I am able to use and prepare the bioreactor for a batch by my own. Although the current situation made us to stop the experiments, I am satisfied with the result and I would truly recommend to do an internship in the Institute of Public Health and Clinical Nutrition, and to enjoy the beauty of Finland.

9. ANNEXES

9.1 INDEX TO TABLES

Table 15. *Simulated ileal environment media (SIEM) composition, function of the components and amount per litre of medium. Adapted from Gibson et al (1988)[29].*

Component	Function	Weight (g/L)	Vitamin solution	Weight (mg/L)
Pectin	Gelling agent	0.6	Menadione	1.0
Xylan	Carbon source	0.6	Biotin	0.5
Arabinogalactan	Carbon source	0.6	Vitamin B12	0.5
Amylopectin	Carbon source	0.6	Pantothenate	10.0
Litner starch	Carbon source	5.0	Nicotinamide	5.0
Casein	Growth of some lactic acid bacteria	3.0	Para-aminobenzoic acid	5.0
Peptone water	Carbon, nitrogen, vitamins and minerals source	3.0	Thiamine	4.0
K_2HPO_4	Source of phosphorus and potassium and buffering agent	2.0		
$NaHCO_3$	Buffering agent	0.2		
NaCl	Maintenance of ionic strength	4.5		
$MgSO_4$ $7{\cdot}H_2O$	Source of magnesium ions, cofactor of many bacterial enzymes	0.5		
$CaCl_2$ $2{\cdot}H_20$	Source of calcium	0.45		
Cysteine	Source of nitrogen and reducting agent for the growth of lactic acid bacteria	0.4		
$FeSO_4{\cdot}$ $7{\cdot}H_20$	Source of iron	0.005		
Hemin	Nutritious supplement for the growth of some species of anaerobes	0.01		
Bile salt	Simulate the environment in the ileum	0.05		
Tween 80	Emulsifier for the culture media and also needed for the growth of some LAB	2.0		

Table 16. *Composition per litre of Molly et al optimized digested food culture medium. Obtained from Molly et al (1993)[30].*

Component	Weight (g/L)	Vitamin solution	Weight (mg/L)
Polysaccharides (arabinogalactan, pectin, xylan, dextrins, starch)	Variable amount	Menandione	1.0
		Biotin	2.0
		Vitamin B12	0.5
		Pantothenate	10.0
Glucose	0.4	Nicotinamide	5.0
Yeast extract	3.0	Para-aminobenzoic acid	5.0
Proteose peptone	1.0	Thiamine	4.0
Mucin	4.0		
Cysteine	0.5		
K2HPO4	0.04		
NaHCO3	0.2		
NaCl	0.08		
MgSO4	0.008		
CaCl2	0.008		
KH2PO4	0.04		
Hemin	0.005		
Trace elements	1.0		
Tween 80	1.0		

Table 17. *McDonald Gut Media (MDM) composition per litre of media, based on McDonald et al (2013)[32].*

Reagent	Weight (g)
Peptone water	2.0
Yeast extract	2.0
$NaHCO_3$	2.0
$CaCl_2$	0.01
Pectin (from citrus)	2.0
Xylan (from beechwood)	2.0
Arabinogalactan	2.0
Starch (from weheat, unmodified)	5.0
Casein	3.0
Inulin (from Dahlia tubers)	1.0

NaCl	0.1
K_2HPO_4	0.04
KH_2PO_4	0.04
$MgSO_4$	0.01
Hemin	0.005
Menadione	0.001
Bile salts	0.5
l- cysteine HCl	0.5
Porcine gastric mucin (type III)	4.0

Table 18. *Composition per litre of media to simulate chyme entering the colon of 6-month-old infants. Obtained from Le Blay et al (2010)[44].*

Reagent	**Weight (g/L)**	**Vitamin**	**Weight (mg/L)**
Lactose	6.4	Pyridoxine-HCl	100
Casein	0.5	4-aminobenzoic acid (PBA)	50
Whey protein	8.1	Nicotinic acid	50
Peptone	0.5	Biotin	4
Bactotryptone	0.5	Folic acid	4
Mucin	4	Cyanocobalamin	5
Yeast extract	2.5	Thiamine	50
Bile salts	0.05	Riboflavin	50
K_2HPO_4	0.5	Phylloquinone	0.15
$NaHCO_3$	1.5	Menadione	2
NaCl	4.5	d-pantothenic acid	100
KCl	4.5		
$MgSO_4 \cdot 7H_2O$	1.25		
$CaCl2 \cdot 2H_2O$	0.1		
$FeSO_4 \cdot 7H_2O$	0.005		
Hemin	0.01		
Tween 80	1		

Table 19. *Summary of in vitro model's conditions.*

Reference	***Colon***	***Type of model***	***Machine***	***Growing media***	***Medium feed rate***	***Retention time***	***Stabilization time***	***pH***	***Temp.***	***Stirring***	***Anaerobiosis***
Giuliani *et al (*2019)[39]	Distal and proximal	Continuous	**SHIME**	arabinogalactan, pectin,xylan, D-(+)glucose, starch, yeast extract, peptone and pig gastric mucin	x/day	-	2 weeks	5.6-5.9 (asc.), 6.1-6.4 (trans.), 6.6-6.9 (desc.)	37ºC	Continuous stirring	90/10 % N_2/CO_2 gas mixture
Larsen *et al* (2019)[41]	Proximal	Continuous	**TIM-2**	SIEM	40 ml/unit	-	16 h	5.8	37ºC	Continuous stirring	-
Tuncil *et al* (2018)[27]	Distal	Anaerobic chamber	**BACTRONEX Anaerobic Chamber**	carbonate-phosphate buffer with cysteine hydrochloride	-	-	-	Not specified (distal colon pH)	37ºC	150 rpm (shaking water bath)	85% N_2, 5% CO_2, and 10% H_2
Bircher *et al* (2018)[30]	Proximal	Continuous	**Sixfors**	SIEM	25 ml/h	8 h	10 days	5.7	37ºC	Gently agitated	Continuously flushed with CO_2
Silverman *et al* (2018)[31]	Distal	Continuous	**Multifors**	McDonald Gut Media (MDM)	400 ml/day	24 h	-	6.9-7.1	37ºC	100 rpm	1% [O_2], 1 LPM N_2
Pham *et al* (2019)[43]	Infant proximal	Continuous	**PolyFermS**	Le blay *et al* (2010)	40 ml/h	5 and 10 hours	5-7 days	5,0-6,0	37ºC	18 rpm	Constantly sparged with O2-free N_2

Medina *et al* (2018)[33]	Infant proximal	Continuous	**Minibo 250**	mZMB	25 mL/h	-	-	5,5	37ºC	100 rpm	99,99% N_2
Pérez-Burillo *et al* (2018)[24]	Distal	Batch	**Fermentation bottles**	Roowi *et al* (2010)	-	-	-	7	37ºC	Gently agitated	Constantly sparged with O2-free N_2
Gaisawat *et al* (2019)[25]	Distal	Batch	**Fermentation bottles**	Molly et al (1993).	-	-	-	6	37ºC	Continuous stirring	Constantly sparged with O2-free N_2
Oliphant *et al* (2020)[26]	Distal	Batch	**Multifors**	SIEM	400 mL/day	24 h	-	7.0	37ºC	Continuous stirring	Constantly sparged with O2-free N_2
García *et al (*2017)[35]	Distal	Continuous	**Multifors**	McDonald Gut Media	400 ml/ day	24 h	21 days - 10 days	7	37ºC	Gently agitated	Constant bubbling of N_2
Santiago-Rodríguez *et al* (2015)[26]	Distal	Continuous	**Multifors**	SIEM	16.67 mL/h (retention rate)	-	24h	6.9–7.0	37ºC	Gently agitated	Constantly sparged with O2-free N_2
Wiese *et al (*2018)[28]	Distal and transversal	Batch	**CoMiniGut**	SIEM	-	-	-	6.0-6.5 (trans.), 6,5-6,9 (distal)	37ºC	Gently agitated	Constantly sparged with O2-free N_2

9.2 QUESTIONNAIRE FOR FAECAL SAMPLES

Code of the sample:_____

Age: _____

Sex: _____

Height: _____

Weight: _____

Ethnic background: _____

Medical history (any chronic diseases?): _____

Antibiotic consumption in the last 3 weeks? (Y/N)

Laxative consumption in the last 3 weeks? (Y/N)

Prebiotic/probiotic consumption in the last 3 weeks? (Y/N)

How often do you have moderate to vigorous physical activity? (<2/week, 2-5/week, >5/week)

Food consumption:

- Do you have any diet restrictions? (e.g. vegetarian, vegan, etc)
- How many days per week do you consume carbs (bread/pasta/rice)? (0-2, 3-5, >5)
- How many servings of carbs do you consume per day? (0, 1, ≥2)
- Do you consume processed or whole grain carbs more often?
- How many days per week do you consume vegetables? (0-2, 3-5, >5)
- How many servings of vegetables do you consume per day? (0, 1, ≥2)
- How many days per week do you consume fruits? (0-2, 3-5, >5)
- How many servings of fruits do you consume per day? (0, 1, ≥2)
- How many days per week do you consume meat? (0-2, 3-5, >5)
- How many servings of meat do you consume per day? (0, 1, ≥2)
- Do you consume white meat or red meat more often?
- How many servings of yogurt/yogurt drinks do you consume per week? (0, 1-3, ≥4)
- How many servings of fermented food/drinks do you consume per week? (0, 1-3, ≥4)
- How many servings of pastries do you consume per week? (0, 1-3, ≥4)
- How many sugar-sweetened beverages do you consume per week? (0, 1-3, ≥4)
- How many servings of alcohol do you consume per week? (0, 1-3, ≥4)

9.3 SOLOLAB BENCH-TOP ISOLATOR OPERATING PROCEDURES

UNIVERSITY OF EASTERN FINLAND Clinical Nutrition	Valid from: 15.11.19	Page 49/56	METHOD INSTRUCTION

Intended Use

To operate in anaerobic condition

Principle

Sololab can keep nitrogen to maintain an anaerobic condition

Sample Collection and Handling

Reagents

Prepare all materials needed to be placed inside the Sololab before start

IPA 70%

Supplies

Nitrogen gas supply (check the nitrogen container)

Equipment

Sololab bench-top isolator

Nitrogen detector in bioreactor cabinet

Calibration

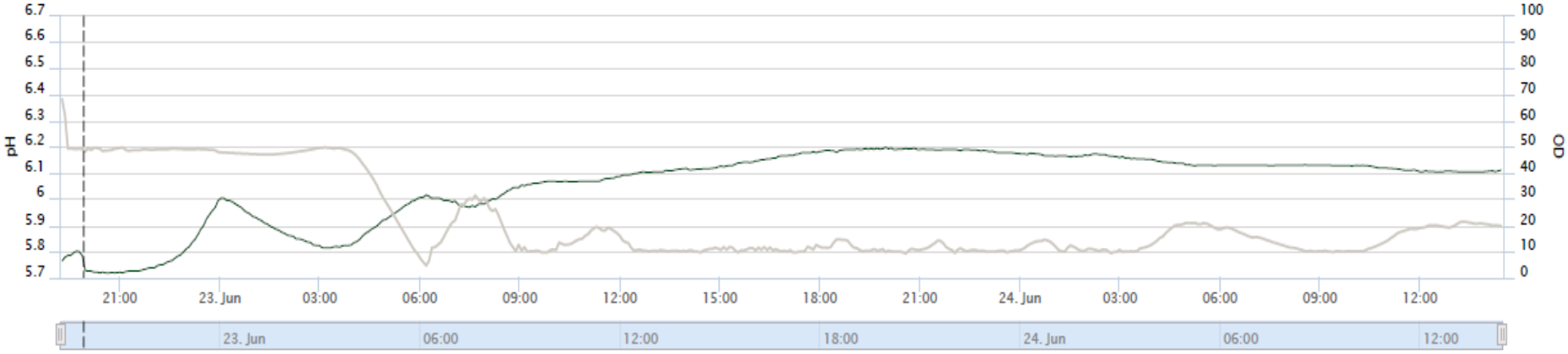
NA

Procedure

1. Turn on the nitrogen container outside (turn anti-clockwise to open, meter closer to us is pressure, inner is amount)
2. Turn on nitrogen detector by pressing OK for 3 seconds
3. Clean the Sololab station with IPA 70%
4. Place the materials into the Sololab (spray them with IPA 70%)
5. The scale has to be open during the nitrogen filling process
6. Zip the Sololab and remove the clip on the outlet
7. Open window (*optional*)
8. Open the nitrogen valve (open by pointing up, horizontal is closed)
9. Around 2 mins is enough to fill the Sololab (for the oxygen level to drop to around 7.4% which use around 100 lb/in^2 nitrogen).
10. Close the nitrogen valve
11. Clip the outlet
12. Operate in the Sololab
13. Refill the Sololab with nitrogen when needed (*optional, before opening each new sample/falcon/container*)
14. Open the Sololab to remove the materials and allow the gas to escape *(Note: it may smell when opening the Sololab*
15. Clean the Sololab with IPA 70%
16. Press both buttons for 3 seconds to turn the nitrogen detector off and place back into the bioreactor cabinet
17. Close the windoy and turn off the nitrogen container outside (Check the remaining nitrogen amount, if it is close to zero, inform Riitta

9.4 EVE GRAPHS

***Graphic 1**. Optical density (OD) vs. time during the stabilization experiment. Obtained from Eve® programm.*



***Graphic 2.** Optical density (OD) and pH vs. time during the control batch experiment. Obtained from Eve® programm.*

9.5 SELECTIVE GROWTH PLATES

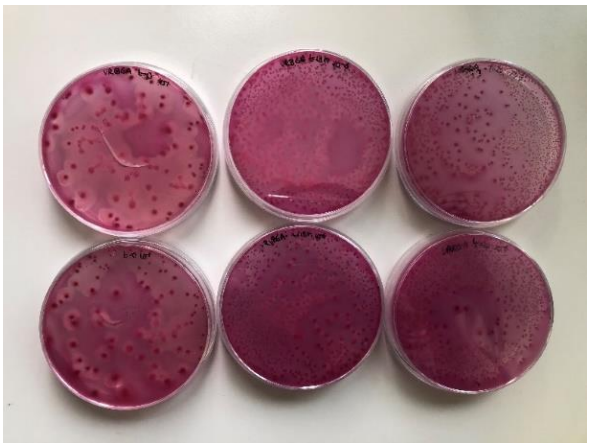


Image 1. *Enterobacteriaceae growth in VRBGA plates at inoculation, time 9 and 2 hours*

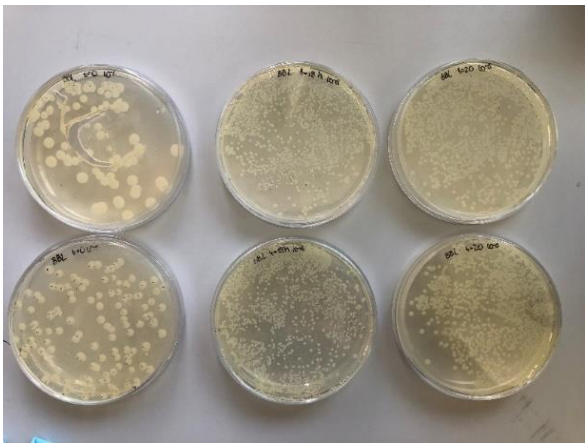


Image 2. *Clostridium growth in BBL plates at inoculation, time 9 and 2 hours*

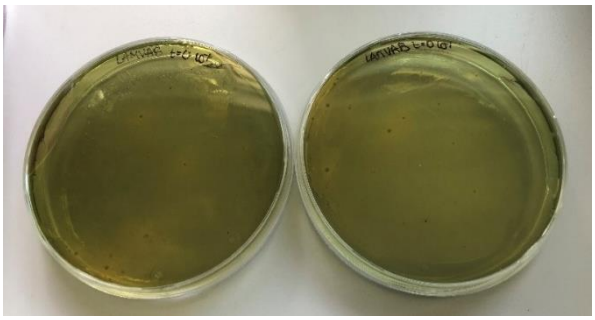


Image 3. *Lactobacilli growth in LAMVAB plates at inoculation, time 9 and 2 hours*