# Unravelling functional capabilities of the human gut microbiota

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**Declaration**

I declare that this thesis is my own work and the contribution of others has been properly and fully acknowledged.

Choshani Dalukdeniya Arachchilage, 2024

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## General Abstract

The human gut microbiota is crucial for maintaining human health. Largely influenced by diet, its composition and subsequent function impact on a number of host processes such as nutrient absorption, immune function and mental health. As interest in gut microbiota-targeted interventions has grown, understanding the functional capacities of microbial communities has become essential. However, comprehensively understanding these complex interactions remains analytically challenging. The primary goal of this thesis was to unravel these complexities by monitoring selected small communities of bacteria using a combination of microbiology and analytical chemistry approaches.

Through *in vitro* experiments with a nutrient-rich medium mimicking the gut environment, this research explored a simplified nine-gut microbial consortium representing the most abundant genera in the human gut. By dissecting the functional behaviour of these microbial species in various scenarios—pure cultures, co-cultures with a probiotic yoghurt, and mixed culture environments—valuable insights into microbial interactions, metabolic responses, and growth dynamics emerged. Particularly noteworthy was the potential of probiotic yoghurt as a promising dietary intervention strategy for gut microbiota-mediated health benefits.

Metabolic profiling using $^{1}$H-NMR spectroscopy captured the complete metabolic profile of these bacteria, providing insight into microbial metabolic activity. The results showed that all bacteria studied in this thesis produced acetate, lactate, formate, ethanol, and methanol, while specific species like *Bacteroides fragilis*, *Faecalibacterium prausnitzii*, and *Escherichia coli* additionally produced propionate and succinate. *Roseburia intestinalis* synthesised butyrate, and *Bacteroides fragilis* and *Clostridium perfringens* generated gamma amino butyric acid (GABA), with inulin and yoghurt enhancing production of these metabolites. These findings contributed to the creation of an atlas of gut microbial function, offering insights for gut microbiota-targeted interventions.

Furthermore, the thesis compared functional resemblance of the synthetic gut microbial community with human faeces. The novel synthetic gut microbial consortium comprising of the nine bacterial strains, including pathogenic species, was analysed using $^{1}$H-NMR spectroscopy to understand functional behaviour and flow cytometry-fluorescent in situ

hybridisation (FC-FISH) enumeration to monitor the bacterial count. Results showed differences in substrate utilisation and metabolite production between the synthetic mix and human faecal samples, highlighting challenges in replicating the human gut microbiota's complexity.

The study also investigated the effect of a probiotic yoghurt intervention on microbial populations and metabolic responses in a group of school children from South West Uganda, revealing significant increases in total bacterial counts post-intervention and distinct metabolic profiles. The objective was to provide a metabolic perspective on the outcomes observed *in vivo* by leveraging the *in vitro* data collected.

This thesis has contributed to our understanding of gut microbial dynamics, dietary impacts, and therapeutic potentials. Future research directions include exploring diverse dietary substrates, refining synthetic models, and elucidating precise mechanisms underlying probiotic effects, aiming to optimise microbiota targeted interventions and improve human health outcomes.

## List of Abbreviations

| | |
|---|---|
| $^{0}C$ | Degrees celsius |
| 1D | One dimension |
| $^{1}H$-NMR | proton nuclear magnetic resonance |
| AMPs | antimicrobial peptides |
| ANOVA | Analysis of variance |
| APCs | Antigen-presenting cells |
| ATCC | American type culture collection |
| ATP | Adenosinetriphosphate |
| *B. fragilis* | *Bacteroides fragilis* |
| BAZ | Body Mass Index-for-Age Z-score |
| *Bif.* | *Bifidobacterium* |
| BSCFA | Branched short chain fatty acids |
| CA | cholic acid |
| $CaCl_2.6H_2O$ | Calcium chloride hexahydrate |
| CAZymes | Carboydrate Active Enzymes |
| CBM | Carbohydrate Binding Modules |
| CDCA | chenodeoxycholic acid |
| CFU | Colony forming units |
| CoA | coenzyme A |
| *C. perfringens* | *Clostridium perfringens* |
| *Col. aerofaciens* | *Collinsella aerofaciens* |
| CVDs | cardiovascular diseases |
| D20 | Deuterium oxide |
| DF | Dietary Fibres |
| DNA | Deoxyribonucleic acid |
| DSMZ | German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen) |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic acid |
| *F. prausnitzii* | *Faecalibacterium prausnitzii* |
| FC-FISH | Fluorescent in-situ hybridisation coupled with flow cytometry |
| $FeSO_4.7H_2O$ | Iron (II) sulfate heptahydrate |
| FID | Free induction decay |
| FISH | Fluorescent in-situ hybridisation |
| FMT | faecal microbiota transplantation |
| FSC-A | Forward scatter area |
| g | gram |
| GABA | gamma aminobutyric acid |
| GC | Gas chromatography |
| GPCRs | G protein-coupled receptors |
| GRAS | Generally Recognised As Safe |
| h | hour |
| HDAC | histone deacetylase |
| HDL | High density lipoprotein |

| | |
|---|---|
| HMOs | Human milk oligosaccharides |
| IBD | Inflammatory Bowel Disease |
| IBS | Irritable Bowel Syndrome |
| ISAPP | International Scientific Association for Probiotics and Prebiotics |
| IPA | Indolepropionic acid |
| ITF | Inulin Type Fructans |
| JME | Joint Child Malnutrition Estimates |
| K | Kelvin |
| KCl | potassium chloride |
| $K_2HPO_4$ | Dipotassium hydrogen phosphate |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| L | litre |
| LC | Liquid chromatography |
| *L. rhamnosus* | *Lacticaseibacillus rhamnosus* |
| LGG | Lactobacillus rhamnosus yoba |
| $MgSO_4.7H_2O$ | Magnesium sulfate heptahydrate |
| MHz | Mega Hertz |
| min | minute |
| mm | millimetres |
| mM | millimolar |
| mol $l^{-1}$ | Mol per litre |
| MS | mass spectrometry |
| MRS | de Man, Rogosa, and Sharpe |
| mL | millilitre |
| MAMPs | microbial associated molecular patterns |
| Na2HPO4 | disodium phosphate |
| NaCl | sodium chloride |
| NaH2PO4 | monosodium phosphate |
| $NaHCO_3$ | Sodium bi carbonate |
| NaOH | Sodium hydroxide |
| NCTC | National Collection of culture types |
| NMNA | N-methylnicotinamide |
| NMR | nuclear magnetic resonance |
| NRM | Nutrient rich medium |
| $O_2$ | oxygen |
| O-PLS-DA | Orthogonal projection to latent structure discriminative analysis |
| PCA | Principal component analysis |
| PBS | Phosphate buffer solution |
| PFA | Para formaldehyde |
| PHE | Public health England |
| pH | Potential of hydrogen |
| PSA | polysaccharide-A |
| QMP | Quantitative microbial profiling |
| RD | Recycle decay |
| RMP | Relative microbial profiling |
| rRNA | ribosomal ribonucleic acid |
| RS | Resistant Starch |

| | |
|---|---|
| RTIs | Respiratory tract infections |
| *R. intestinalis* | *Roseburia intestinalis* |
| SCFAs | Short Chain Fatty Acids |
| SNV | Netherlands development organisation |
| s | second |
| SSC-A | Side scatter area |
| TCA | Tri carboxylic acid cycle |
| TEM | transmission electron microscopy |
| TIDE | The Inclusive Dairy Enterprise Project |
| TLRs | Toll-like receptors |
| TMA | trimethylamine |
| TMAO | trimethylamine-N-oxide |
| Tm | time |
| Tris/HCL | Tris hydrochloric |
| TSP | 3-(trimethylsilyl) propionic acid-d4 sodium salt |
| WHA | World health assembly |
| WHO | World Health Organisation |
| µl | Micro litre |

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## Conference Abstracts and Awards

**Poster and flash talk '**Developing an atlas of gut microbial function'- Microbiome Interactions in Health and Disease Conference 2022**,** 24-26th October 2022, Wellcome Genome Campus**,** Cambridge.

**Oral presentation** 'A multi-platform analytical approach to characterise the function of the human gut microbiota' Bright Sparks Symposium 2023, 8th September 2023, Victoria Gallery & Museum, Liverpool.

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**Differential Metabolic Responses of Human Gut Bacteria to Dietary Substrates: A Comparative Study of Pure Cultures and Mixed Communities**, In preparation

**Awards**

- Winner - 3 minute thesis competition 2022 University of Reading
- Best Presenter 'Bright Spark Symposium 2023
- 2nd Place – Oral presentation Food and Nutritional Sciences Symposium in years 2019/2021/2022/2023– Department of Food and Nutritional Sciences - University of Reading
- 3rd Place – Poster Presentation – 2nd Year PhD students – 3rd Food and Nutritional Sciences Symposium 2020 - Department of Food and Nutritional Sciences - University of Reading
- 3rd Place – Abstract writing- All PhD students- 3rd Food and Nutritional Sciences Symposium 2020 - Department of Food and Nutritional Sciences - University of Reading

# CHAPTER 1

## Unravelling functional capabilities of the human gut microbiota

### 1.1 Introduction

The human gut microbiota has emerged as a significant player in influencing host health (Forster et al. 2019; Sheflin et al. 2017). Over the past few decades, research has progressively unravelled the evolution, composition and metabolic activities of the gut microbiota (Rinninella et al. 2019; Thursby and Juge 2017; Sheflin et al. 2017). Currently, much research is focussed on the connection between gut microbes and metabolites, and their interactions with numerous disorders such as cardiovascular diseases, inflammatory issues, cancers and cognition related conditions (Morais, Schreiber, and Mazmanian 2021). As a result, manipulating the gut microbiota through dietary interventions is becoming increasingly popular, and diet-based biotics such as probiotics, prebiotics, synbiotics and fermented foods are gaining attention. It is a well-known fact that diet influences microbial composition (David et al. 2014; De Filippis et al. 2016; De Filippo et al. 2010) and that the gut microbiota can directly interact with the host immune system (Rooks and Garrett 2016). Understanding how the microbiota behaves and the response to food can be helpful in developing future gut microbiota targeted interventions. This requires an in depth understanding of the functional capacities of the gut microbiota which would help to better understand their capabilities and contributions to host health. If there exists an atlas that can provide detailed information about individual microbial functional potential and how it behaves in a mixed consortium, then gut microbiota targeted interventional studies could be more optimally designed. Understanding the complex human gut ecosystem is challenging, as it comprises trillions of microbes including bacteria, viruses, fungi and parasites (Rinninella et al. 2019). Metabolism of the gut microbiota is a collective contribution of all these categories. A simplified approach to understanding the functional contribution and dynamics of the gut microbiota would involve monitoring functional capacities of smaller groups of bacterial communities.

This PhD research focuses on unravelling the behaviour of selected gut microbiota, initially studying individual behaviours, progressing to mixed consortia then investigating the influence of different substrates and food items. Additionally, a synthetic gut microbial consortium was developed to mimic the human gut environment. In the latter stages, the

research delves into *in vitro* experiments and offers insights into the impact of a human dietary intervention study on the gut microbiota.

### *1.1.1 Evolution of the human gut microbiota*

Studies such as the Human Microbiome Project and Human Intestinal Tract Project have widely explored the evolution and composition of microbial communities residing in the human gut, shedding light on the diverse array of microbes that collectively form this dynamic ecosystem (Thursby and Juge 2017; Rinninella et al. 2019; Backhed et al. 2012). Exploration of the colonisation process of has gained attention as it can offer valuable insights in understanding human gut microbiota composition.

Bacterial colonisation of the infant gut begins during and after birth (Roswall et al. 2021; Huey et al. 2020), and is influenced by factors such as gestational age, delivery mode and antibiotic use (Wernroth et al. 2022; Bokulich et al. 2016). Preterm babies often show higher levels of Enterobacteriaceae (Arboleya et al. 2017), while the mode of delivery influences the initial microbiota, resembling the maternal vagina for vaginal births and maternal skin or the environment for caesarean delivery (Álvarez et al. 2021; Wernroth et al. 2022). Furthermore, feeding methods, maternal diet, and antibiotic use during pregnancy also play significant roles in infant gut colonisation (Milani et al. 2017). Breastfed infants, for instance, exhibit a higher abundance of bifidobacteria compared to formula fed infants (Stewart et al. 2018). The introduction of breast milk contributes to *Bifidobacterium* growth, as these bacteria can break down the complex sugars found in human milk, known as human milk oligosaccharides (HMOs). HMOs in breast milk act as prebiotics, favouring the growth of beneficial genera like bifidobacteria (Asakuma et al. 2011) and contributing to host health due to its ability to produce beneficial short-chain fatty acids. On the other hand, the gut microbiota of formula fed infants has been demonstrated to have a different microbial profile including high levels of undesirable *Clostridium* and *E. coli* (Tanaka and Nakayama 2017).

The introduction of solid food is another critical milestone impacting gut microbiota composition (Kapourchali and Cresci 2020). A transition to solid foods sees changes in microbial composition, with *Bacteroides* dominating during weaning, and levels of bifidobacteria decreasing. Early-life gut colonisation, dominated initially by Actinobacteria,

Proteobacteria, and Firmicutes, gradually shifts to resemble the adult-like composition dominated by Bacteroidetes and Firmicutes after 2-3 years (Huey et al. 2020; Milani et al. 2017). The abundance of Proteobacteria and Gram-negative anaerobes is very low in the adult gut microbiota (Palmer et al. 2007) even though initial colonisation is dominated by facultative anaerobes of Proteobacteria (Huey et al. 2020). During inadequate nutrition, species in the Proteobacteria phyla (which represents most pathogens) tend to thrive and dominate the infant gut leading to diseases. Research has shown that malnourished infants can have a high proportion of Proteobacteria that can go up to as much as 80% (Million, Diallo, and Raoult 2017).

The infant gut is colonised by bifidobacteria during the first few days and remains the dominant group (Phillips et al. 2021; O'Neill, Schofield, and Hall 2017) in breastfed infants (Stewart et al. 2018). However, the number of bifidobacteria reduces with age and they are present in smaller proportions throughout later life. It is now well established that bifidobacteria are an important group within the human gut microbiota as they are of benefit to health (Fukuda 2011).

Therefore, a healthy infant gut comprised of higher levels of bifidobacteria can be achieved through breastfeeding. This can result in short term and long term health through anti-pathogenic and inflammatory activities, highlighting the pivotal role of early-life gut colonisation in shaping microbial communities. This understanding has prompted much research and development of dietary interventions to augment beneficial bacteria, like bifidobacteria. An identification of beneficial bacteria through understanding of their functional capacities represents an approach to facilitate and target dietary interventions.

### *1.1.2 Age*

Dynamics of the gut microbiota exhibit notable changes throughout the human lifespan. As discussed above, distinct differences in microbial composition between early life and adulthood are evident, with a rapid shift occurring within the first year, eventually stabilising to an adult-like configuration by the age of 2-3 years (Milani et al. 2017; Huey et al. 2020; Roswall et al. 2021). Adolescence introduces further compositional and functional distinctions, as indicated by studies in pre-adolescent children, proposing a more extended

development period toward the adult gut composition (Hollister et al. 2015). While most bacterial groups achieve stability around 3 years of age (Milani et al. 2017), ongoing alterations characterise the adult stage, as influenced by factors such as diet, behaviour, physical activity, disease, and antibiotic use. Beyond 70 years of age, immune activity, digestive changes, disease, inflammation and medication contribute to additional modifications (Odamaki et al. 2016).

### *1.1.3 Diet*

Diet is a major determinant of gut microbiota composition and function (David et al. 2014; Thursby and Juge 2017; Sheflin et al. 2017). Colonic bacteria ferment undigestible dietary residues that escape digestion in the small intestine. The main dietary substrates that reach the colon are carbohydrates, proteins and lipids (Sanders et al. 2019a) and the gut microbiota plays an important role in converting these substrates into metabolites that can influence host health.

Several studies have demonstrated the effect of diet on gut microbiota composition both long term and short term. A study among children consuming an African diet, rich in plant fibres compared to a Western diet, showed that there was a noticeable difference in gut microbial profiles of the two groups. The African diet reported high numbers of Bacteroidetes with the appearance of *Prevotella* and *Xylanibacter* genera which are known to ferment indigestible plant polysaccharides, and low in numbers of Firmicutes phylum. Furthermore, the African diet reported more short chain fatty acids (SCFAs) compared to the Western diet (De Filippo et al. 2010). Similarly, in a study between native Africans and African Americans there was a fundamental difference in microbial composition where native Africans were dominant in species belonging to the *Prevotella* genus and in African Americans, *Bacteroides* were more dominant. Total bacteria and SCFAs were also more abundant in native Africans who followed a plant-based diet (Ou et al. 2013). Similarly, in a study with 153 individuals following omnivore, vegetarian or vegan diets, a significant association between consumption of vegetable-based diets and increased levels of faecal SCFA, *Prevotella* and some fibre-degrading Firmicutes were detected (De Filippis et al. 2016). These studies confirm that long term vegetable consumption increases *Prevotella* spp. in the gut.

In a short term dietary interventional study composed of entirely plant-based vs animal-based diets (David et al. 2014) it was demonstrated that the gut microbiota is altered even within a short time period. The animal-based diet increased the abundance of bile-tolerant microorganisms (*Alistipes, Bilophila* and *Bacteroides*) and decreased levels of Firmicutes such as *Roseburia* spp., *Eubacterium rectale*, and *Ruminococcus bromii* that ferment dietary plant polysaccharides. In another study with a diet rich in inulin-based vegetables (such as artichokes, leeks and garlic), an increase in *Bifidobacterium* spp. was observed (Hiel et al. 2019). These studies provide good evidence that diet leads to modifications in gut microbiota composition. Hence, this approach may be an effective means to favourably alter the gut microbiota in order to improve health.

The aforementioned studies provide evidence for a vital role of diet in shaping the gut microbiota. These investigations strongly indicate that dietary interventions can significantly influence and modulate microbial communities residing in the gastrointestinal tract. Consequently, dietary intervention has gained popularity in influencing gut microbial composition. Gaining a comprehensive understanding of how the gut microbiota responds to varying dietary substrates can help understanding of these areas of research. Exploring functional relationships between diet and the gut microbiota can broaden knowledge of how they interact and open up possibilities to design dietary interventions more conveniently and effectively.

### *1.1.4 Carbohydrates*

Carbohydrate is a major food source which provides energy and fibres (Kumar, Rani, and Datt 2020). The enzymatic digestion of carbohydrates begins in the mouth and continues in the small intestine where digestible carbohydrates are hydrolysed by enzymes and absorbed. Those which cannot be hydrolysed pass down to the colon and can be fermented by the indigenous microbiota (Van der Meulen et al. 2006). Carbohydrate polymers that are not digested or absorbed in the small intestine are defined as dietary fibres (DF). DF can also be considered as soluble and insoluble forms. Insoluble DF such as cellulose and hemicellulose contributes to faecal bulking, whereas the soluble forms are fermented by the gut microbiota to generate metabolites (Makki et al., 2018). Studies have revealed that DF affects both the

composition and function of gut bacteria. For example, *Bifidobacterium* spp. have an ability to ferment a range of DFs such as resistant starch (RS), inulin and oligosaccharides (Falony et al., 2009, Venkataraman et al., 2016), and are commonly elevated following fibre consumption. Inulin type fructans (ITF) are a class of non-digestible carbohydrate widely used as prebiotics. Depending on the degree of polymerisation the ITF are divided into oligofructose and inulin. *Bacteroides* spp. and *Eubacterium rectale* have also been shown to degrade RS, while *Ruminococcus bromii* has been identified as a keystone species in the degradation of RS (Ze et al., 2012).

While some bacteria produce metabolites by degrading DF others can use these metabolites. The phenomenon of exchanging metabolites and nutrients among different species of microbiota creating a complex web of functional interactions is known as microbial cross feeding. This is one important mechanism to be considered during study of functional mechanisms of bacteria. Microbes that are unable to ferment complex carbohydrates feed on breakdown compounds, for example, *Eubacterium* spp. (a butyrate producing bacteria, crossfeeds on mono and oligosaccharides released by primary inulin degraders such as *Bifidobacterium* spp. and *Ruminococcus bromii* (Rios-Covian et al., 2016, Baxter et al., 2019). *In vitro* studies using bacteria isolated from human faeces have shown this mechanism, where acetate or lactate produced by *Bifidobacterium* spp. are utilised by butyrate producing bacteria such as *Eubacterium* spp. (Duncan et al., 2004) and *Faecalibacterium prausnitzii* (Moens et al., 2016). Understanding cross-feeding mechanisms can help in identifying bacterial interactions which can be useful to reveal functional mechanisms among bacteria.

### 1.2 Childhood malnutrition

Childhood malnutrition remains a critical global health issue, contributing to over half of the deaths in children under five years of age (UNICEF 2023). Undernutrition not only increases the vulnerability of children to infections but also exacerbates the frequency and severity of these infections, leading to prolonged recovery times.

Malnutrition significantly alters the composition of the gut microbiota, with studies revealing that malnourished infants can exhibit a high proportion of Proteobacteria, which may constitute up to 80% of their gut microbiota (Million et al. 2017). This dysbiosis is associated

with a shift away from beneficial bacteria, such as Bifidobacterium, which are crucial for maintaining gut health and supporting immune function. The dominance of pathogenic bacteria in the gut can lead to increased inflammation and further compromise the child's health.

Efforts to address childhood malnutrition must focus on identifying convenient and accessible foods that can effectively support undernourished populations. Dietary interventions that promote the growth of beneficial gut bacteria are essential. Therefore, understanding the functional capacities of the gut microbiota is essential for designing effective interventions to address childhood malnutrition. By unravelling the behaviour of individual bacterial species and their interactions within mixed consortia, researchers can gain insights into how the microbiome responds to different substrates and food items. This knowledge can inform the development of targeted dietary interventions and guide the selection of appropriate probiotic strains to restore a healthy gut microbiome in malnourished children.

### 1.3 Gut microbial composition

The concept of a defined healthy gut microbial composition remains elusive, given substantial variabilities observed among individuals. Despite this diversity, approximately 90% of adult gut microbiota primarily falls within two main phyla, Firmicutes and Bacteroidetes, and the remainder comprises Actinobacteria, Proteobacteria, Verrucomicrobia, and Fusobacteria (Rinninella et al. 2019). The enterotype theory categorises these complex microbial compositions into three main clusters: type 1 with an abundance of *Bacteroides*, type 2 dominated by *Prevotella*, and type 3 featuring *Ruminococcus* and bifidobacteria prevalence (Arumugam et al. 2011). These findings suggest that despite complex variations, common structural elements exist within intricate microbial compositions. Understanding functional capacities of these diverse microbial communities holds the key to a clearer picture of the gut microbiota.

This thesis used 9 bacteria representing these main bacteria phyla. The selected bacteria were *Bacteroides fragilis* (Bacteroidetes), *Bifidobacterium longum* (Actinobacteria), *Lacticaseibacillus rhamnosus* (Firmicutes), *Clostridium perfringens* (Firmicutes), *Faecalibacterium prausnitzii* (Firmicutes), *Collinsella aerofaciens* (Actinobacteria), *Roseburia*

*intestinalis* (Firmicutes), *Ruminococcus bromii* (Firmicutes) and *Escherichia coli* (Proteobacteria).

### *1.3.1 Bacteroides fragilis*

*Bacteroides fragilis* (*B. fragilis*) is a key member of the Bacteroides genus in the Bacteroidetes phylum (Phylum Bacteroidetes, Class Bacteroidia, Order Bacteroidales, Family Bacteroidaceae). Bacteroidetes accounts for 1-10% of the total gut microbiota (Rigottier-Gois et al. 2003). *B. fragilis* is a gram negative obligate anaerobe (Eribo, du Plessis, and Chegou 2022; Sun et al. 2019). Under the microscope, *B. fragilis* presents as a rod-shaped cell with rounded ends. When cultured on blood agar, *B. fragilis* appears as smooth, circular, translucent to semi-opaque colonies typically measure 1–3 mm in diameter (Sun et al. 2019). In the colon it is known to degrade carbohydrate and proteins producing short-chain fatty acids (SCFAs) like acetate and propionate as end products (Rigottier-Gois et al. 2003). Studies have indicated that *B. fragilis* has the ability to metabolise glycans derived from both the diet and the host as sources of carbon and energy (Rios-Covian et al. 2015). *B. fragilis* strains play a role in immune system maturation, yet they can also act as opportunistic pathogens (Rios-Covian et al. 2015). It is explored for its ability to produce polysaccharide-A (PSA) involved in immune response-inducing capabilities (Eribo, du Plessis, and Chegou 2022). Factors like diet, health, medication, and lifestyle influence its abundance, with diet playing a significant role (Li et al. 2016).

### *1.3.2 Bifidobacterium longum*

*Bifidobacterium longum*, (*Bif. longum*) a member of the Actinobacteria phylum and the Bifidobacteriaceae family, is a key player in gut health and a predominant species within the human core microbiome (Turroni et al. 2019). Bifidobacteria makes up 3-6 % of the adult gut microbiota (Arboleya et al. 2016). It is a non-motile, non-sporulating, and non-gas-producing gram-positive bacterium (Bottacini et al. 2014). When cultured on de Man, Rogosa, and Sharpe (MRS) media supplemented with 0.05% L-cysteine hydrochloride (Zhao et al. 2021), *Bif. longum* grows, forming creamy or whitish colonies with a smooth texture.

As a pioneer coloniser of the gut, *Bif. longum* plays a vital role in various health-promoting functions. It is particularly abundant in the intestines of breast-fed infants, although its levels decrease but remain relatively stable in adulthood. In infants, *Bif. longum*, *Bif. breve*, and *Bif. bifidum* are typically dominant, whereas *Bif. catenulatum*, *Bif. adolescentis*, and *Bif. longum* are more prevalent in adults (Arboleya et al. 2016). *Bif. longum* is known for its ability to degrade complex polysaccharides using extracellular enzymes, such as glycosyl hydrolases, and internalise resulting mono- and oligosaccharides via specific transport systems. This metabolic versatility allows *Bif. longum* to utilize a wide range of dietary carbohydrates, including plant-derived polysaccharides that escape digestion in the upper intestine (Pokusaeva et al. 2011).

*Bifidobacterium* species, are associated with the production of beneficial metabolites like short-chain fatty acids, conjugated linoleic acid, and bacteriocins (Arboleya et al. 2016). These metabolites contribute to gut homeostasis, immune modulation, and protection against pathogens. Bifidobacteria have been commercially exploited as probiotic agents due to their associated health benefits and GRAS (Generally Recognised As Safe) status (O'Callaghan and van Sinderen 2016). It is therefore commonly used as a probiotic.

### *1.3.3 Lacticaseibacillus rhamnosus*

*Lacticaseibacillus rhamnosus*, previously known as *Lactobacillus rhamnosus* (Zheng et al. 2020), is a widely studied bacterium, particularly due to its probiotic properties (Segers and Lebeer 2014). Lacticaseibacilli accounts for around 1-5% of the gut microbiota. Strains of *L. rhamnosus* are extensively utilised as probiotics in various food formulations and functional foods, owing to their potential health benefits. Notably, the *L. rhamnosus* strain GG, originally isolated from the faecal samples of a healthy human adult, stands out as one of the most well-documented probiotic microorganisms. *L. rhamnosus* is a facultative heterofermentative bacterium capable of fermenting hexoses like lactose and fructose into lactic acid, along with pentoses yielding a mixture of lactic and acetic acids (De Oliveira et al. 2012). In the homofermentative pathway, *L. rhamnosus* primarily converts glucose into lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway, producing lactic acid as the main end product. This process is efficient and typically occurs under conditions where glucose is readily available.

However, under specific conditions such as the presence of pentoses or when glucose is limited *L. rhamnosus* can switch to the heterofermentative pathway. This pathway involves the phosphoketolase pathway, allowing *L. rhamnosus* to produce not only lactic acid but also other metabolites, including acetic acid and ethanol (Tang et al. 2023). This metabolic versatility contributes to its probiotic qualities and the production of beneficial metabolites.

Further, it shows resistance to acidic and bile environments, essential for surviving and persisting within the gastrointestinal tract (Segers and Lebeer 2014). It exhibits robust growth characteristics that enable its survival in challenging conditions. *L. rhamnosus* grows on MRS agar (De Oliveira et al. 2012), and appears as small, round, and creamy-white colonies. In addition to its growth traits, *L. rhamnosus* possesses exceptional adhesion capabilities to the intestinal epithelial layer. This attribute allows it to effectively inhibit the growth and adherence of various pathogens, contributing significantly to gut health (Segers and Lebeer 2014).

The probiotic potential of *L. rhamnosus* extends to various health applications, including the prevention of antibiotic-associated diarrhoea, treatment and prevention of rotavirus diarrhoea (Sindhu et al. 2014) and respiratory tract diseases (Du et al. 2022). Its multifaceted benefits make *L. rhamnosus* a valuable component in probiotic formulations aimed at improving overall gut and immune health.

### *1.3.4 Clostridium perfringens*

Species of clostridia from clusters XIVa and IV are among the predominant gut bacteria, comprising 10-40% of the total bacterial population. In both humans and animals, *Clostridium* species, particularly clusters IV (*C. leptum* group) and XIVa (*C. coccoides* group), play significant roles. *Clostridium* cluster IV includes notable members like *C. leptum*, *C. sporosphaeroides*, *C. cellulosi*, and *Faecalibacterium prausnitzii* (*F. prausnitzii*), while cluster XIVa encompasses 21 species (Guo et al. 2020). Clostridia are early colonisers of the gut and can be detected in faeces within the first week of birth. The composition of *Clostridium* species differs between infants and adults, with infants exhibiting a higher proportion of *Clostridium* cluster I and adults having a higher prevalence of *Clostridium* cluster IV and XIVa (Guo et al. 2020).

*Clostridium perfringens* is an anaerobic, Gram-positive spore-forming bacterium (Ma, Li, and McClane 2012). They can ferment a variety of nutrients, like carbohydrate, protein, organic acid and other organics, to produce acetic acid, propionic acid, butyric acid. When cultured on appropriate media such as blood agar or reinforced clostridial agar, *Clostridium perfringens* forms distinctive large, irregular colonies marked by a characteristic double zone of hemolysis, can be cultured in cooked meat broth (Ma, Li, and McClane 2012).

### *1.3.5 Faecalibacterium prausnitzii*

*Faecalibacterium prausnitzii* (*F. prausnitzii*) is a Gram-negative, strictly anaerobic, rod shaped bacterium. It is notably abundant in healthy adult colons, making around 2-15 % of the total bacteria (Hiippala et al. 2018; Leylabadlo et al. 2020). Taxonomically, *F. prausnitzii* belongs to the *Clostridium* cluster IV (*Clostridium leptum* group) within the Firmicutes phylum, Clostridia class, and Ruminococcaceae family (Duncan 2002).

It serves as a crucial contributor to gut health, due to its role as a major butyrate producer (Duncan 2002). Recent investigations have shown the depletion of *F. prausnitzii* in various gut diseases, highlighting its potential importance in maintaining intestinal homeostasis. The optimal growth conditions for *F. prausnitzii* align with the acidic pH range typically found in the colon, spanning between 5.7 and 6.7 (Lopez-Siles et al. 2017) and extremely sensitive to oxygen (Hu et al. 2022).

### *1.3.6 Collinsella aerofaciens*

*Collinsella aerofaciens* (*Col. aerofaciens*), a rod-shaped nonmotile obligate anaerobe, is highly prevalent in the healthy human gastrointestinal tract (Bag, Ghosh, and Das 2017). It constitutes a significant portion of the Actinobacteria phylum and the Coriobacteriaceae family. *Col. aerofaciens* represent around 1-5 % of bacteria in the human gut. Alterations in *Col. aerofaciens* abundance have been linked to various health conditions, including irritable bowel syndrome (Bag, Ghosh, and Das 2017). This bacterium, formerly classified as *Eubacterium aerofaciens*, stands out for its unique phylogenetic position and characteristics, leading to its reclassification. The genus *Collinsella* (Kageyama, Sakamoto, and Benno 2000),

particularly dominant in the Coriobacteriaceae family, plays a role in metabolism regulation by influencing intestinal cholesterol absorption, liver glycogenesis, and triglyceride synthesis. It has also been associated with modulating gut permeability by impacting tight junction protein expression. Notably, dietary factors significantly influence collinsella abundance, with high-protein diets reducing its levels, while fibre-rich diets promote its growth (Gomez-Arango et al. 2018).

### *1.3.7 Roseburia intestinalis*

*Roseburia intestinalis* (*R. intestinalis*) is an anaerobic gram-positive bacterium, it plays an important role in gut health by producing butyrate and contributing for intestinal well-being (Nie et al. 2021). As part of the Firmicutes phylum and the Lachnospiraceae family, *R. intestinalis* is among the most abundant bacteria in the gut microbiome. *R. intestinalis* cluster usually accounts for 0.9%–5.0% of the total microbiota (Hiippala et al. 2018).

This bacterium is associated with preventing intestinal inflammation and maintaining energy balance through its metabolic activities (La Rosa et al. 2019; Nie et al. 2021). *R. intestinalis* is shown to produce SCFAs like acetate, propionate, and butyrate. Although challenging to culture due to its obligate anaerobic nature, *R. intestinalis* has been successfully isolated using specific culture media and growth conditions, supplemented with sugars (Nie et al. 2021). Its ability to degrade fibres into butyrate highlights its importance in gut health and metabolic modulation. Studies suggest that *R. intestinalis* can contribute to anti-inflammatory effects in the intestine, making it a promising candidate as a potential probiotic for improving metabolic functions (Hiippala et al. 2018; Martinez et al. 2013).

### *1.3.8 Ruminococcus bromii*

*Ruminococcus bromii*, characterised as a non-motile, gram-positive, and anaerobic cocci (La Reau and Suen 2018), belongs to the Ruminococcaceae family of Firmicutes phyla (Crost et al. 2018). This bacteria exhibits a specialisation in utilising complex carbohydrates, mostly resistant starch (RS) (Ze et al. 2012). Its acts as a primary degrader of RS supporting the growth of secondary degraders that produce butyrate, renowned for its anti-inflammatory and anti-

tumourigenic properties (Baxter et al. 2019). This highlights the significance of *Ruminococcus bromii* as a keystone species within the gut microbiome (Ze et al. 2012). *Ruminococcus. bromii* alongside related strains like *Ruminococcus gnavus*, are prevalent in the human gut and constitute a significant portion of the core gut microbiota around 1-5 % (Crost et al. 2018). Furthermore, the abundance of *Ruminococcus bromii* in the large intestine shows a positive response to diets rich in RS (La Reau and Suen 2018).

### *1.3.9 Escherichia coli*

*Escherichia coli* (*E. coli*) is a gram-negative, rod-shaped bacterium commonly found among neonates and is a prevalent member of the intestinal microbiome in over 90% of individuals (Martinson and Walk 2020). As a pioneer of the human gut, *E. coli* is one of the first bacteria to colonise neonates at birth, contributing to the early establishment of the gut microbiota. Being a facultative anaerobe, *E. coli* plays a crucial role in depleting oxygen along the gastrointestinal mucosal surface, thus creating a favourable environment for strict anaerobes to colonise and become dominant (Martinson and Walk 2020). This commensal bacterium is the most common cultivable gram-negative aero-anaerobic bacteria within the gut microbiota (Bonnet et al. 2014).

*E. coli* exhibits versatile metabolic capabilities, including respiration with oxygen, utilization of alternative anaerobic electron acceptors, and fermentation of sugars depending on electron acceptor availability (Fabich et al. 2008). While it thrives on various sugars like mono and disaccharides, it lacks the enzymes needed for complex polysaccharide degradation, relying instead on other gut bacteria like bacteroides for the hydrolysis of complex polysaccharides (Conway and Cohen 2015; Fabich et al. 2008). This cooperative hydrolysis process leads to the production of mono- and disaccharides that *E. coli* can utilise for its metabolic activities (Conway and Cohen 2015). Interestingly, *E. coli* can thrive aerobically but also has the capability to ferment carbon sources anaerobically, producing SCFAs such as acetic acid and related metabolic products like lactic acid (Christofi et al. 2019).

### 1.4 Gut microbial metabolites

The main metabolites produced by fermentation of DF are SCFAs. SCFAs are volatile fatty acids (carboxylic acids with aliphatic chains of C1-C6) which may be present as straight or branched chain fatty acids. Acetate (C2), propionate (C3), and butyrate (C4) are the most abundant SCFA present in the colon (Rios-Covian et al. 2016; Parada Venegas et al. 2019). Branched short chains fatty acids (BSCFA) such as isobutyrate, isovalerate and 2-methyl butyrate are formed from amino acids (Rios-Covian et al. 2016). Bacteria that produce or feed on these SCFA have been studied in detail. *Roseburia* spp., *Eubacterium* spp., *Faecalibacterium prausnitzii* and clostridia are the main butyrate producing bacteria (Walker et al. 2011; Moens, Weckx, and De Vuyst 2016; Baxter et al. 2019). Some bacteria such as *Eubacterium hallii* and *Anaerostipes* spp. are lactate fermenting butyrate producing bacteria (Munoz-Tamayo et al. 2011). Acetate is the most abundant SCFA in the colon and is produced by bacteria such as *Bacteroides* spp., *Bifidobacterium* spp. and *Clostridium* spp. (Parada Venegas et al. 2019). Bacteria that produce propionate belong to mainly to the class Negativicutes, but also *Bacteroides*, *Roseburia* spp., *Ruminococcus* spp. (Reichardt et al. 2014). *Akkermansia muciniphila* is capable of producing both acetate and propionate (Derrien et al. 2004). Therefore, it can be considered that members of the Firmicutes phylum mostly produce butyrate, and Bacteroidetes produce acetate and propionate. Metabolic pathways converting carbohydrates into SCFAs have been well documented (Louis et al. 2004; Munoz-Tamayo et al. 2011). Carbohydrates are hydrolysed into simple sugars and broken down to pyruvate. This is followed by the glycolytic pathway converting pyruvate to acetyl-CoA which reacts further to generate acetate and butyrate (Parada Venegas et al. 2019; Baxter et al. 2019). *Bacteroides* spp. are known to break down higher molecular weight carbohydrates and *Bifidobacterium* spp. are efficient in fermenting low molecular weight polysaccharides (Sanders et al. 2019a). Butyrate is often considered to be the most important SCFA as it is a well-known energy source for colonic cells, promotes epithelial barrier function and has anti-inflammatory as well as anti-carcinogenic effects (Riviere et al. 2016; Parada Venegas et al. 2019). It is formed by butyrate kinase and butyrylcoenzyme A (CoA): acetate CoA-transferase pathways (Louis et al. 2004; Munoz-Tamayo et al. 2011). Acetate, which is the most abundant SCFA in the colon, acts as a cofactor for other bacteria and is used in production of cholesterol and lipogenesis in the liver.

Propionate is also known to have anti-inflammatory, anti-cancer effects and is involved in promoting satiety, lipogenesis and insulin sensitivity (Riviere et al. 2016). Propionate can be produced by three different pathways in the colon, namely: succinate, acrylate and propanediol pathways (Reichardt et al. 2014). These metabolites are capable of regulating signalling mechanisms in different pathways and impacting on immune responses and health (Kayama and Takeda 2016; Kumar, Rani, and Datt 2020). SCFAs bind with G protein receptors in the intestine to regulate energy metabolism, epithelial integrity and immunity (Gentile and Wier 2018; Cani 2018). Mineral absorption and protection against pathogens are also favoured by SCFAs, through reducing pH which facilitates mineral absorption and prevents growth of pathogens (Sanders et al. 2019a). It has also been shown that SCFAs are involved in the production of antimicrobial peptides supressing the growth of pathogens (Zhao et al. 2018). Although most of these mechanisms have been identified using animal studies, there is evidence from human trials where interactions with prebiotics have reduced allergic reactions in infants (Ivakhnenko and Nyankovskyy 2013) and have the potential to produce psychologically relevant aminobutyric and organic acids (Jackson, Wijeyesekera, and Rastall 2023; Jackson et al. 2023). Other than SCFA, less is known about other microbially-derived metabolites and their impact on the host. According to the human metabolome project, there are 112 microbiota derived metabolites that have been detected and quantified in human urine and faecal samples (Wishart et al. 2018). Microbial metabolites are generated as intermediate or end products and include, folate, indoles, cresols, secondary bile acids, trimethylamine-N-oxide (TMAO), neurotransmitters (e.g., serotonin, GABA) and metabolites of amino acids such as indolepropionic (IPA) acid (Cani 2018; Menni et al. 2019). Carnitine and choline found in meat and fish are converted by gut microbes into trimethylamine (TMA), which is then processed by the liver and released into the circulatory system as TMAO. There is evidence that TMAO levels link to the prevalence of microbes associated with diets rich in animal proteins (De Filippis et al. 2016). TMAO has also been shown to be a predictor of cardiovascular diseases (CVDs), high saturated fat diets increase plasma TMAO levels and are associated with high risk of CVDs (Park 2019). Primary bile acids: cholic acid (CA) and chenodeoxycholic (CDCA) are transformed to the secondary bile acids lithocholic acid and deoxycholic acid, respectively (Sheflin et al. 2017). *Clostridium* spp. and *Eubacterium* spp. have been found to be involved in this mechanism. Indolepropionic acid (IPA) is a gut microbiota derived metabolite produced from the deamination of tryptophan. IPA is an antioxidant

predictive of a lower risk of developing type 2 diabetes and is also known to regulate gastrointestinal barrier function. In a study of over 1000 adult women, IPA was shown to be strongly correlated with high microbiome diversity and linked with DF intake (Menni et al. 2019). In a recent interventional study on the effect of chitin-glucan on gut microbiota derived metabolites, bile acids, long- and short-chain fatty acids and an increase in bacterial metabolites including butyric, iso-valeric, caproic and vaccenic acids were detected (Rodriguez et al. 2020).

Recent studies have investigated the role of the gut-brain axis in promoting mental health (Jackson, Wijeyesekera, and Rastall 2023; Huang et al. 2019) by generating both direct and indirect chemical signals that establish communication with the central nervous system. This signalling network involves various compounds, including but not limited to, oxytocin, brain-derived neurotrophic factor, and endothelial factor peptide. Other neurochemicals isolated from the gut bacteria and genera that produce them are gamma aminobutyric acid (GABA) (*Lactobacillus*, *Bifidobacterium*), Serotonin (*Streptococcus*, *Escherichia*, *Enterococcus*, *Lactococcus*, *Lactobacillus*), Norepinephrine (*Escherichia*, *Bacillus*), Dopamine (*Streptococcus*, *Escherichia*, *Bacillus*, *Lactococcus*, *Lactobacillus*), Acetylcholine (*Lactobacillus*, *Bacillus*) and Histamine (*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*) (Wall 2014). Among these, GABA and serotonin have emerged as focal points in discussions surrounding these microbial-generated chemical messengers (Morais, Schreiber, and Mazmanian 2021). Therefore, connections between the gut and central nervous system have sparked interest in how manipulating gut microbiota through dietary interventions could potentially impact mental well-being.

More precise understanding about bacterial interactions in producing these metabolites is important to develop clearer understanding in mapping functional pathways. It is apparent that human colonising microbiota are essential to health. Yet there is no clear understanding on the functional characteristics of a healthy microbiome. Characterisation of the metabolic activity of microbiota can help to fill the gap in understanding the correlation between gut microbiota and related diseases. It can provide a target for dietary interventions and microbial modifications aiming to maintain good health and improve the health status of people exhibiting a disrupted microbiota. Figure 1.1 summarises the discussed information.

### 1.5 Gut-Immune system interactions

A direct interaction of gut microbiota with the host immune system is a well-established fact. Beyond this, the microbiota's indirect contributions through the production of metabolites are acknowledged, influencing immune responses not only within the gut but also in distant organs such as the liver, brain, and central nervous system. This interplay plays a critical role in the gut's contribution to overall health.

The gut microbiota has emerged as a key regulator of health and disease (Gentile and Wier 2018). This is mediated by maintaining gut homeostasis by controlling nutritional metabolism, epithelial barrier integrity and host immunity (Kayama and Takeda 2016). Gut microbiota interactions occur directly through binding by receptors to microbial ligands, or indirectly through metabolites (Cullen et al. 2020). Gut mucosa acts as a barrier composed of different types of epithelial cells, immune cells and the chemical compounds released by these cells. Figure 1.2 shows different types of cells in the gut mucosa. The first line of the barrier is the mucous layer, secreted by goblet cells. Second are the epithelial cells mainly absorptive and paneth cells which secrete antimicrobial peptides (AMPs) to kill pathogens in the gut lumen. The immune system is divided into two types: innate and adaptive. The innate immune system shows quick but less specific responses whereas the adaptive system shows specific responses and creates memory for future attacks. Microbiota co-habit in the gut environment through communication with host immune cells. Antigen-presenting cells (APCs) which protect the body against infections are involved in maintaining immune tolerance to the normal gut microbiota. These cells include dendritic cells and macrophages. They belong to the innate immune system but link with the adaptive immune systems by presenting antigens. Immune cells distinguish commensal and pathogenic bacteria through Toll-like receptors (TLRs) in epithelial cell membranes. This occurs via the recognition of microbial associated molecular patterns (MAMPs) (Lazar et al. 2018). T cells and B cells are adaptive immune cells found in the lamina propria. T cells are categorised into T helper, T cytotoxic and T regulatory cells. T regulatory cells help to prevent immune cells from attacking normal cells of the body.

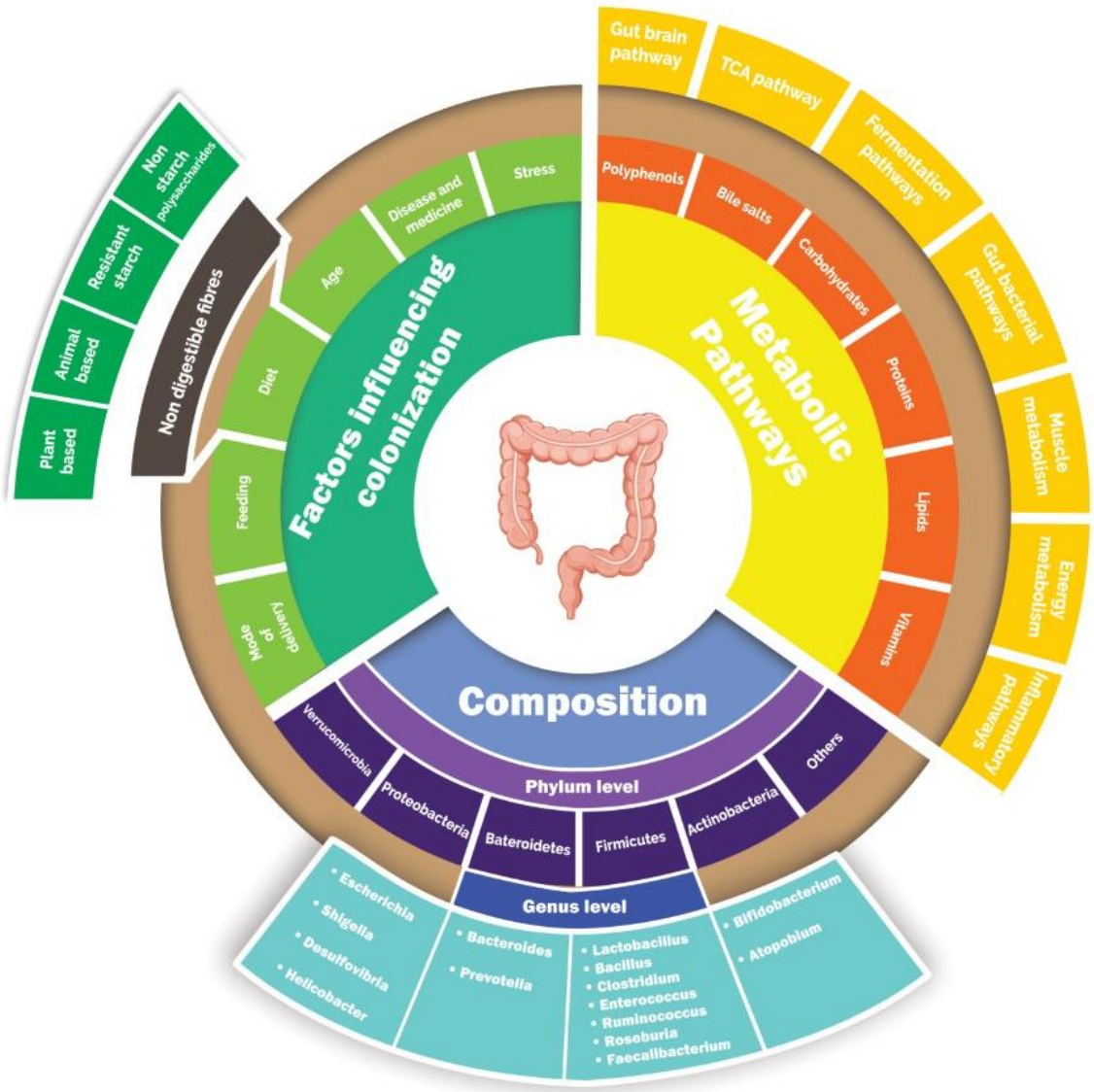


***Figure 1.1:*** *Summary diagram for the composition of gut microbiome, factors affecting gut bacterial composition and contribution of gut bacterial metabolites for metabolic pathways.*

Studies shows that bacterial metabolites such as SCFA can activate different cellular signals and be involved in immune responses (Figure 1.2). SCFAs are capable of communicating with these immune cells and influence inflammatory responses through binding to G protein-coupled receptors (GPCRs) on the epithelial surface, inhibition of histone deacetylase (HDAC) activity and influencing secretions by B cells (Rooks and Garrett 2016; Deehan 2017; Jiao 2020). It has also been demonstrated that SCFAs interact in maintaining barrier defences and gut homeostasis, through enhancing mucous production by goblet cells and maintenance of tight junctions (Kumar, Rani, and Datt 2020). However, these mechanisms have been demonstrated mostly in animal studies. Alterations in microbial community and disruption of

functions (known as dysbiosis), can result in disease development. Extensive research has revealed an association of the gut microbiota and diseases, for example, Inflammatory Bowel Disease (IBD), Irritable Bowel Syndrome (IBS), diabetes, obesity, cardiovascular diseases, neurological diseases and colon cancer (Cani 2018; Sanders et al. 2019a). Thus, there is growing interest in exploring the potential of reducing disease risk, by modulating or altering gut microbiota composition and function.

With comprehensive understanding of interactions between the gut microbiota and host immune system, it becomes more feasible to distinguish which bacteria hold the potential to contribute specific metabolites that enhance immune responses. This knowledge facilitates the ease of targeting particular diseases through interventions based on biotics, as the understanding of microbial contributions becomes more precise.

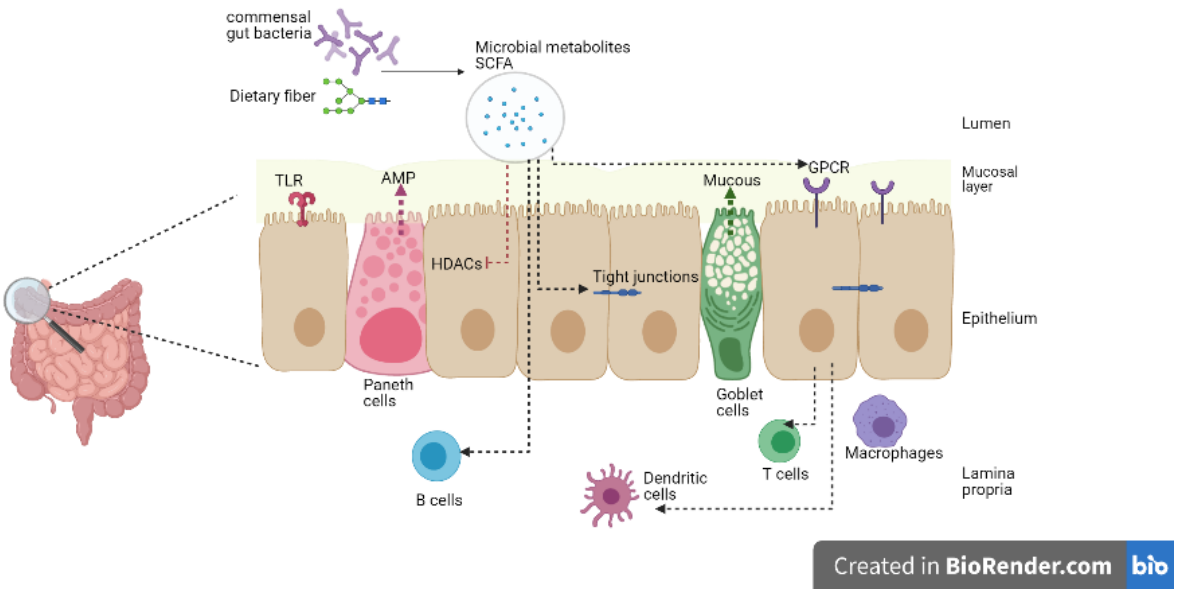


***Figure 1.2:*** *Gut mucosal layer and interaction of SCFAs with immune cells*

*Shows the different types of immune cells in the gut mucosa and the interaction of short chain fatty acids (SCFA) with the immune system through binding to G protein-coupled receptors (GPCRs) on the epithelial surface into T cells and dendritic cells, inhibition of histone deacetylase (HDAC) activity and influencing secretions by B cells and maintenance of tight junctions. Immune cells identify commensal bacteria through Toll like receptors (TLR). Paneth cells produce antimicrobial peptides (AMP), and goblet cells produce mucous as barriers for pathogenic bacteria. Created with BioRender.com*

### 1.6 Biotics based dietary interventions

The use of probiotics, prebiotics and synbiotics has gained popularity in the realm of nutritional interventions. Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host'(Hill et al. 2014). Prebiotics are defined as 'a substrate that is selectively utilised by host microorganisms conferring a health benefit on the host' (Gibson et al. 2017). Synbiotics are 'a mixture, comprising live microorganisms and substrate(s) selectively utilised by host microorganisms, that confers a health benefit on the host' (Swanson et al. 2020). Collectively referred to as biotics, these components, alongside the significant inclusion of fermented foods, constitute a pivotal category in promoting gastrointestinal well-being. Fermented foods are 'foods made through desired microbial growth and enzymatic conversions of food components' (Marco et al. 2021).

Research on the use of probiotics and prebiotics to improve gut health is rapidly increasing. Even though *Lactobacillus* and *Bifidobacterium* spp. are widely used as probiotics, they do not belong to the bacterial genera that produces butyrate which plays a major role in gut homeostasis and health. There is also evidence that gut microbial metabolites contribute to improved human health and maintaining a positive gut environment. However, a more complete description of mechanistic details for their effects remains to be discovered. Therefore, understanding interactions and function of gut microbiota with different food substrates in the complex gut environment is important, in order to target specific species or microbial functions for therapeutic benefits. This knowledge can pave the way to identifying more microorganisms to improve health outcomes.

A recent review by the International Scientific Association for Probiotics and Prebiotics (ISAPP) 2023 indicates a substantial consumer trend, with over 50 percent actively incorporating probiotics into their dietary regimens, primarily with the objective of enhancing gut health. This noteworthy statistic reflects the growing recognition of biotic-based dietary interventions. However, the evolving landscape of biotics necessitates further investigation and scientific exploration to streamline and enhance the efficacy of interventions within this domain. Hence, within the context of this doctoral research, specific substrates were chosen to encompass a spectrum of probiotic, prebiotic, and fermented food components. Notably, *Lacticaseibacillus rhamnosus*, a well-established probiotic species, was selected alongside

Inulin, a recognised prebiotic, and starch, a ubiquitous fermentable compound in the gut environment. Additionally, a probiotic yoghurt, incorporating *Lactobacillus rhamnosus* yoba, was integrated into the experimental design to represent a fermented food source. This substrate selection aimed to capture each category of biotic components.

Subsequently, a human intervention study was conducted utilising the aforementioned probiotic yoghurt, to mirror a real world dietary scenario and the outcomes were subjected to detailed molecular phenotyping analyses. This holistic approach sought to obtain a clear understanding of the interactions and effects of these biotics within the complex milieu of the gut. The integration of *in vitro* studies following the human intervention, provided a comprehensive and multifaceted exploration of the potential impacts of fermented food components on the gut microbiota, contributing to the expanding body of knowledge in this field. Therefore, understanding the functional capacities of gut microbiota through monitoring of metabolites and their metabolic pathways is a promising approach for this area of research.

### 1.7 Functional capabilities: A neglected dimension

Studies of gut bacteria have primarily focused on identifying the types of bacteria present, known as taxonomic composition. However, there is a significant gap in our understanding of the functional capabilities of these bacterial communities. Despite advances in next-generation sequencing, which has enabled us to uncover links between gut microbes and various aspects of human health and diseases, we still lack a comprehensive understanding of their metabolic activities, how they communicate and their combined impact (Li 2018). To bridge this knowledge gap, researchers are increasingly turning to joint analyses of high-throughput multi-omics data. This involves integrating information from metagenomics (study of genetic material in a community of microorganisms) and metabolomics (study of small molecules), along with assessments of host physiology and mechanistic experiments conducted in humans, animals and cells (Fan and Pedersen 2021). These comprehensive approaches represent initial steps toward identifying molecular mechanisms that underlie observed associations between gut microbiota and health.

Recognising the profound impact of gut microbiota activities on host health, gaining a thorough understanding of their functional potential is crucial. This knowledge not only

enhances our understanding of the role of these microbes in health and disease, but also holds promise for predicting how individuals might respond to therapeutic interventions.

This PhD study investigates selected gut microbes in an environment resembling the gut, exploring various substrates using a metabolomics approach in both singular and mixed culture settings. This approach aims to comprehend metabolic potential of these microbiota, facilitating information that could direct therapeutic interventions.

### 1.8 Need for an atlas of functional capabilities

While the functions of gut microbiota have been studied independently and are available in the literature, there is a lack of proper documentation regarding metabolic activities in an environment that resembles the gut. Many studies have explored metabolite production using different substrates, often with just one or two other bacteria or in specific conditions. However, understanding how these bacteria behave in the gut environment with various substrates and mixed culture is crucial for therapeutic studies.

To address this gap, documenting bacteria from major phyla in the human gut can provide a clear understanding of their behaviour. This knowledge is valuable for predicting the outcomes of therapeutic interventions. Having a guide that offers detailed information on the metabolic production of bacteria in a gut-like environment would facilitate the design of studies involving therapeutic biotics. Such a guide would make the planning of therapeutic interventions more straightforward and effective.

This study focuses on monitoring nine selected gut bacteria in a nutrient rich medium in a pure culture system, to generate an atlas of their functional behaviour.

### 1.9 Synthetic microbial communities: Challenges and a simplified approach

It is widely acknowledged that the gut microbiota in the human digestive system exhibits a collective response and intricate interconnections. This complex ecosystem includes bacteria, archaea, bacteriophages, viruses and fungi (Fan and Pedersen 2021) with bacteria being the most functionally predominant. While various studies have attempted to create synthetic bacterial consortia through computational models, a laboratory-based analytical approach is

favoured for providing a more realistic representation of the biological system, accounting for its complexity. However, practical experimental systems for in-depth microbiome study remain limited (Lawson et al. 2019).

Existing microbial consortia resembling faeces are increasingly utilised in various research contexts, particularly in studies focused on gut health, disease mechanisms, and the evaluation of dietary interventions. These consortia provide a more realistic model of the gut environment, allowing researchers to observe microbial interactions and metabolic processes that closely mimic those occurring in the human gut. The primary benefit of using such complex microbial models is their ability to capture the diversity and dynamics of microbial communities, which can lead to more accurate predictions of gut behaviour and better insights into the functional roles of different bacteria. However, there are notable drawbacks associated with these models, including challenges in standardising the consortia, potential variability in microbial composition, and difficulties in maintaining the stability of such complex mixtures over time (Petrof et al. 2013). When a large number of strains are employed the practical feasibility becomes a concern (Petrof et al. 2013) Current model systems often rely on simplified consortia or specific bacterial strains using mathematical models to mitigate these challenges, yet these approaches may not fully replicate the intricate interactions present in the human gut microbiota (Venturelli et al. 2018). This highlights the need for a balanced approach that captures essential microbial interactions while remaining feasible for experimental and commercial applications. By focusing on a smaller, representative group of key bacterial species, my research aims to provide insights into the functional capacities of gut microbiota while addressing the limitations associated with more complex consortia.

Since functions of the main microbiota depend on how bacteria interact in a community, it is important to understand the principles behind these interactions (Weiss et al. 2022). Numerous interactive mechanisms, including cross-feeding and competitive processes like bacteriocin production shape these interactions, as influenced by ecosystem complexity, nutrient availability and reciprocity (Granato, Meiller-Legrand, and Foster 2019; Cornforth and Foster 2013). To unravel such complexities, studying small bacterial groups becomes imperative. Beginning with the observation of individual behaviours and progressing to collective responses, aids in comprehending metabolic cross-feeding and synergistic or antagonistic effects within a consortium.

Complexity of the human gut ecosystem, housing trillions of microorganisms across diverse categories, pose a significant challenge in understanding their functional capabilities. To navigate this complexity, a proposed approach involves monitoring functional capacities of smaller bacterial communities. Initiating with individual species and progressing to pairwise or collective responses in a mixed consortium provides a simplified yet insightful strategy to unravel the dynamics of the gut microbiota, which constitutes a primary focus for this PhD work.

Constructing a synthetic gut microbial consortium capable of replicating functionality of the natural gut microbiota presents a potential solution for replacing faeces in faecal microbiota transplantation (FMT). FMT is an emerging therapeutic approach that involves the transfer of faecal material containing gut microbiota from a healthy donor to a recipient. The primary goal of FMT is to restore the balance of the gut microbiome and treat conditions associated with dysbiosis, such as recurrent *Clostridium difficile* infection. FMT is a clinical approach for recurrent *Clostridium difficile* treatment (Khoruts 2021) and has been extensively studied and successfully used in the treatment of recurrent *Clostridium difficile* (Cammarota et al. 2017), FMT is undergoing exploration for other conditions such as Parkinson's, Alzheimer's and skin diseases (Park et al. 2020). FMT process is rigorous and includes strict donor screening and patient support, it can be administered through various methods, including oral capsules, colonoscopy, retention enemas, and nasogastric tubes (Xiang et al. 2023). A significant challenge in FMT is the donor screening process, which is necessary to ensure the health and suitability of donors. This process can be time-consuming and limit the availability of suitable donors. Additionally, the variability in microbial composition among donors can lead to inconsistent outcomes in recipients, complicating the standardization of FMT procedures. Another limitation is the potential for adverse effects, including the transmission of infections or antibiotic-resistant bacteria, which raises safety concerns. Lastly, patient acceptance of FMT can be a barrier, as some individuals may have reservations about the procedure due to its nature. This suggest that a more stabilised synthetic consortium could enhance acceptance among patients. Hence, the development of a synthetic microbial consortium that closely mirrors functional aspects of the human gut microbiota holds promise for a range of health promoting applications.

### 1.10 Analytical techniques to study gut microbiota

Exploration of gut microbial dynamics has witnessed a transformative shift from conventional culture-based methods to advanced genomic-level analyses. While culture-based techniques provide valuable insights, their limitation in culturing the entirety of microbial organisms *in vitro*, underscores the need for alternative approaches. In this context, *in vitro* fermentation models have emerged as pivotal tools for preliminary examination of gut microbiota behaviour, offering unique advantages at the initial stages of analysis.

In the realm of anaerobic experiments involving pure cultures, the Hungate tube fermentation method is an accessible and efficient choice. This method employs rubber-stoppered vessels filled with boiled medium under anaerobic conditions, providing a straightforward means to inoculate bacteria (Hungate 1944). On the other hand, for investigations involving mixed culture, the reliability of batch culture fermentations comes to the forefront. In these fermentations, vessels operating under anaerobic conditions are inoculated with either fresh human faeces or a defined microbial community. Control over parameters such as temperature, pH, growth medium, and transit time allows for the emulation of specific intestinal regions (McDonald 2017).

This thesis delves into the intricacies of the gut microbiota using Hungate tubes and *in vitro* batch culture for fermentation. Microbial and metabolic profiling approaches were used to unravel the dynamic behaviours and functional capacities of these complex microbial communities. These analytical approaches provide a foundation for the exploration of gut microbiota behaviours. In this study these analytical approaches were applied to unravel dynamic behaviours and functional capacities of the intricate microbial communities dwelling within the gut ecosystem. The amalgamation of molecular techniques sheds light on metabolites, metabolic pathways and cross-feeding mechanisms, ushering in a deeper understanding of gut microbiota dynamics in the context of therapeutic interventions.

Molecular based techniques provide an accurate way to identify microbes within complex ecosystems. Fluorescent in-situ hybridisation techniques (FISH) can be identified as a rapid, reliable and widely used molecular-based technique used to study microbial ecology. The FISH approach uses synthetic 16s rRNA-targeted oligonucleotide probes labelled with fluorescent dye. Fluorescent cells can be counted using FISH coupled with a flow cytometer (FC-FISH).

Profiling of the gut microbiota has generally been undertaken through sequencing of microbial deoxyribonucleic acid (DNA). 16S rRNA gene amplification is a technique used to analyse microbial diversity within a sample. The 16S rRNA gene is a highly conserved region of the bacterial genome. This method is a the most widely used platform for studying gut microbiome (Morgan and Huttenhower 2014).

However, more detailed functional information is possible through capture of metabolic outputs, namely using $^{1}$H-NMR spectroscopy and mass spectrometry (MS) based approaches (Wijeyesekera et al. 2019). $^{1}$H-NMR spectroscopy (proton nuclear magnetic resonance) is an untargeted metabolomic technique enabling extensive and rapid analysis of multiple metabolites present in a sample, with low cost and minimal preparation.

### *1.10.1 Metabolomics*

Metabolomics focuses on the comprehensive study of small molecules, known as metabolites, within a biological system. Metabolites are the end products of cellular processes and include compounds such as amino acids, lipids, sugars, and organic acids. These metabolites exhibit considerable variability with respect to the number of atoms, subgroups, and overall structural diversity. Consequently, consideration of their elemental composition, stereochemistry, and shielding is imperative when monitoring complex systems for metabolic profiling (Fiehn 2002). Therefore, metabolomic analyses involve the use of advanced technologies such as chromatography, MS, nuclear magnetic resonance (NMR) spectroscopy to profile and quantify the abundance of these diverse metabolites within a biological context.

Untargeted metabolomic techniques produces a global overview of the sample. It focuses on the metabolic profiling of the total complement of metabolites to generate a metabolic fingerprint in a sample. The atomic nuclei interact with electromagnetic radiation at specific frequency when placed in a magnetic field. Nuclei in different magnetic fields have characteristic frequencies known as chemical shift which is measured. $^{1}$H-NMR spectroscopy provides a real representation of the distribution of proton nuclei within the molecules and the different concentration levels of the corresponding metabolites in a complex mixture (Emwas et al. 2019). Hence, untargeted metabolic profiling using $^{1}$H-NMR spectroscopy was selected to analyse microbial samples to generate biochemical fingerprints related to

microbial activity. This analytical approach enables elucidation of all potential metabolic pathways and metabolites generated in cross-feeding mechanisms.

### *1.10.2 NMR spectroscopy*

NMR emerges as a non-destructive, unbiased analytical technique that is easily quantifiable, demands minimal to no sample preparation, eschews the necessity for chemical derivatization, and stands as the acknowledged "gold standard" for the identification of novel compounds (Wishart et al. 2022). Moreover, NMR possesses ability of automation and reproducibility, rendering it highly suitable for automated high-throughput metabolomics studies. This capability enhances the feasibility and reliability of such studies in comparison to liquid chromatography-MS (LC-MS) or gas chromatography-MS (GC-MS) methodologies. Beyond these advantages, NMR exhibits particular efficacy in the detection and characterization of compounds that pose challenges for LC-MS analysis, including sugars, organic acids, alcohols, polyols, and other highly polar substances. Notably, unlike NMR, LC-MS is constrained to the detection of compounds that readily ionise, a limitation further compounded by ion suppression phenomena prevalent in complex and heterogenous mixtures (Wishart et al. 2022).

NMR analysis serves as the primary analytical tool in this PhD study. Given the study's central emphasis on elucidating the functional capacities of gut bacteria, an untargeted metabolic profiling approach aligns seamlessly with the research objectives.

## 1.11 Purpose of the PhD

The purpose of this PhD thesis is to further our understanding of the functional capacities of the human gut microbiota, using a combined microbiological and metabolomic approach. Recognising the significance of comprehending the functional intricacies of gut microbial communities, this research delves into the utilisation of NMR spectroscopy as a powerful analytical tool for untargeted metabolic profiling. By employing the NMR technique, this study strives to provide a nuanced understanding of how specific gut bacteria function individually

and in consort with others, thereby contributing insights into the potential impact of targeted nutritional strategies on gut microbial function.

### 1.12 Aims and objectives

The objective is to unravel functional capacities of nine selected bacteria representing the main genera of the human gut microbiota. These bacteria were monitored both in pure culture and within mixed cultures with different substrates. The study specifically aimed to investigate the functional behaviour of these bacteria, with a particular focus on their response to nutritional interventions.

- To investigate the interaction and functional responses of nine representative gut microbiota to various substrates, including starch, inulin, and probiotic yoghurt, within a nutrient-rich medium using metabolic profiling techniques to generate an atlas of gut microbial function.
- To develop a synthetic bacterial consortium comprising the nine selected bacterial strains and monitor their functional behaviour when exposed to the same substrates including a comparison with human faeces.
- To provide an *in vitro* insight into a human intervention that used the same probiotic yoghurt assessing the impact of the probiotic yoghurt on the functional behaviour of the bacteria through *in vitro* batch culture experiments.

### 1.13 Thesis structure

Figure 1.3 shows the layout of the chapters of this thesis. Chapter 2 describes the methods used in the experiments and Chapters 3-5 elaborates the results of a series of investigations conducted for this thesis. Chapter 6 is a general discussion on the overall results of the experiments in achieving the objectives.

Chapter 2: Details the procedures and methods that were used to culture bacteria in Hungate tubes, enumerate using FC-FISH technique, and monitor metabolic function using $^{1}$H-NMR spectroscopy. Data preprocessing, identification of metabolites, multivariate statistical

analysis using Principal component analysis (PCA), Orthogonal-Partial Least Square-Discriminative analysis O-PLS-DA modelling methods are also described.

Chapter 3: Focuses on the behaviour of the nine selected bacteria in nutrient rich medium (similar to that of the human gut environment) in a pure culture system, before and after the addition of different dietary substrates. Bacterial counts and metabolites produced were measured, and contributed to the development of an atlas of human gut microbial function.

Chapter 4: The nine bacteria were combined in a mixed culture system, and analysed using microbial and metabolic profiling. The functional capacity of the synthetic microbial community were compared against human faecal donor samples with a view to assessing whether this mix could be a suitable alternative to FMT.

Chapter 5: *In vitro* and *in vivo* studies to gain metabolic insights in to probiotic yoghurt dietary intervention in Ugandan school children.

Chapter 6 Brings together the above studies in general discussion and discusses the future research directions.

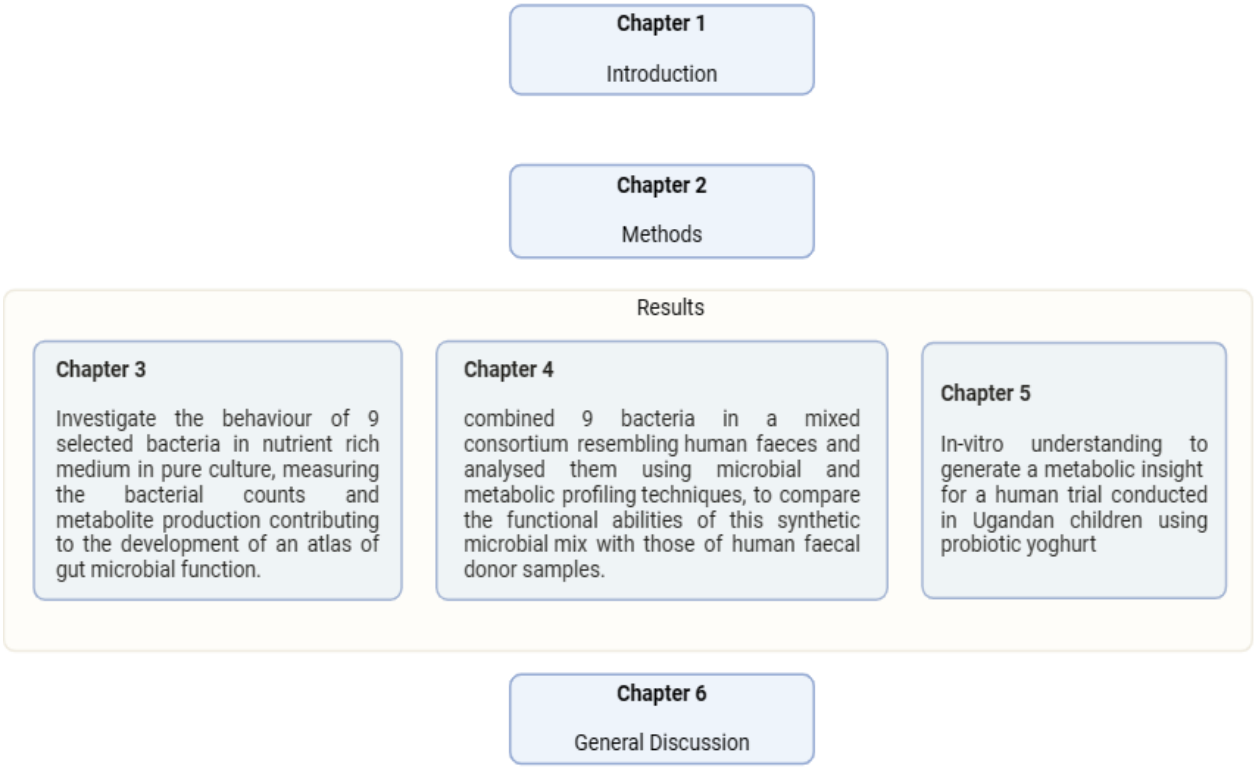


***Figure 1.3:*** *Diagram showing the chapters of the thesis*

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# CHAPTER 2

# METHODS

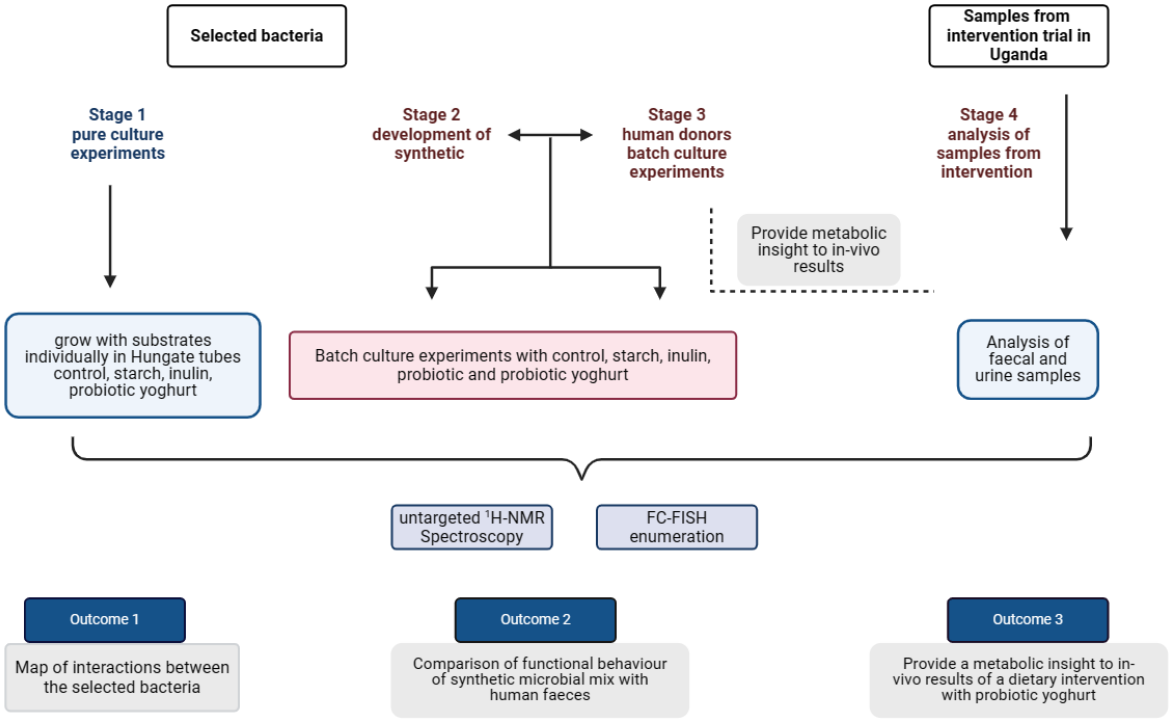


***Figure 2.1:*** *Experimental strategy and outcomes of the research, created in Biorender*

## 2.1 Bacteria

Nine bacterial species representing the core gut microbiota were selected based on human faeces experiments and published literature (Eckburg et al. 2005; Falony et al. 2009; Walker et al. 2011; Ze et al. 2012; Arumugam et al. 2011; Baxter et al. 2019). Pure cultures isolated from human faeces or other human tissue were included in the selection process. Freeze dried pure cultures of these selected bacteria were obtained from culture collections of Public Health England (PHE), DSMZ-German Collection of Microorganisms and Cell Cultures and American Type Culture Collection (ATCC, LGC). Freeze-dried cultures were reconstituted according to the instructions provided by the suppliers and preserved in glycerol stocks to be used in future experiments. Listed below are the nine selected bacteria. *Bacteroides fragilis* NCTC 9343 (Bacteroidetes), *Bifidobacterium longum* NCTC11818 (Actinobacteria), *Clostridium perfringens* NCTC8678 (Firmicutes), *Lactobacillus rhamnosus* NCTC10302 (Firmicutes),

*Collinsella aerofaciens* NCTC11838 (Actinobacteria), *Escherichia coli* NCTC 1093 (Proteobacteria), *Ruminococcus bromii* ATCC 51896 (Firmicutes), *Roseburia intestinalis* DSM 14610 (Firmicutes), *Faecalibacterium prausnitzii* DSM 17677 (Firmicutes).

### 2.2 Nutrient rich medium and substrates

To make 1 L of nutrient rich medium, 5 g starch, 5 g peptone water, 5 g tryptone (Oxoid Hampshire,UK), 4.5 g yeast extract, 4.5 g NaCl (SLS Nottingham UK), 4.5 g KCl, 5 g mucin, 3 g casein, 2 g pectin, 2 g xylan (SERVA Heidelberg, Germany), 2 g arabinogalactan, 0.5 g $K_2HPO_4$, 0.5 g $KH_2PO_4$, 1.25 g $MgSO_4.7H_2O$, 0.15 g $CaCl_2.6H_2O$, 0.005 g $FeSO_4.7H_2O$, 1.5 g $NaHCO_3$, 0.8 g L–cystine HCl, 1 mL Tween 80, 10 µL vitamin K1, 0.05 g haemin, 0.4 g bile salts, 1 g guar gum, 1 g inulin (BENEO-Orafti, Tienen, Belgium), and 4 mL resazurin (pH7) were added into 1 L of deionised water. 10 mL of medium was dispensed into Hungate tubes and autoclaved at 121°C for 15 minutes. To the tubes with substrates, 0.1 g of inulin Orafti® Synergy 1 (BENEO-Orafti, Tienen, Belgium) and starch was added prior to autoclaving. Unless otherwise stated, all reagents were obtained from Sigma Aldrich, Merck (Gillingham UK) (Macfarlane et al. 1998).

### *2.3 In vitro* Hungate tube fermentation

Bacteria were reconstituted from the glycerol stocks in their respective specific media. 100 µl from each bacterium that reached maximum growth was inoculated into Hungate tubes with the nutrient rich medium (as the control), but also tested with inulin and starch added. All conditions were conducted in triplicate. A sample (0.5 mL) was removed from each tube after 0, 12, 24, 48, and 60 h fermentation for metabolite analysis by $^1$H-NMR spectroscopy, and a sample (1.0 mL) was removed at 0, 24, 48, 60 h for bacterial enumeration by FC-FISH.

### 2.4 *In vitro* batch culture fermentation

#### *2.4.1 Synthetic bacterial mix preparation*

The 9 bacteria were reconstituted from glycerol stocks and grown in nutrient rich medium. They were mixed in order to maintain the proportions that were obtained from human

donors: *Faecalibacterium prausnitzii* DSM 17677 -32 %, *Roseburia intestinalis* DSM 14610 - 15%, *Ruminococcus bromii* ATCC 51896 - 16%, *Lactobacillus rhamnosus* NCTC10302 - 3%, *Clostridium perfringens* NCTC8678 - 2% , *Bacteroides fragilis* NCTC 9343 - 12%, *Bifidobacterium longum* NCTC11818 - 15% , *Collinsella aerofaciens* NCTC11838 - 3%, Escherichia coli NCTC 1093 - 2% which contributes to 68 % of Firmucutes, 18% of Actinobacteria, 12 % Bacteroidetes and 2% of Proteobacteria phyla. A volume of 15 mL of the above bacterial mix was immediately used to inoculate each batch culture vessel.

### *2.4.2 Faecal sample preparation*

Freshly voided faecal samples were obtained from 4 healthy adults aged between 30 and 70 years. The donors were those who had not taken antibiotics for at least 4 months before faecal sample donation, had no history of gastrointestinal disorders, were not taking prebiotic or probiotic supplements and who did not follow any restrictive diet. Faecal samples were placed in an anaerobic jar (AnaeroJarTM 2.5 L, Oxoid Ltd) with a gas generating kit (AnaeroGenTM, Oxoid) (<0.1% $O_2$). Once obtained, the faecal samples were diluted 1 in 10 (w/v) using 0.1 mol $l^{-1}$ anaerobically prepared phosphate buffered saline (PBS, Oxoid, Hampshire, UK), pH 7.4. Faecal samples were then homogenised in a stomacher (Seward, stomacher 80, Worthing, UK) for 2 minutes at 260 paddle beats per minute. A volume of 15 mL of faecal slurry was immediately used to inoculate each batch culture vessel.

### *2.4.3 pH controlled, stirred batch culture fermentation*

300 mL glass vessels were set up for the batch culture experiments, with 135 mL of basal nutrient medium aseptically poured in. This system was left overnight with oxygen-free nitrogen pumping through the medium at a rate of 15 mL/min with constant agitation throughout the entire course of fermentation. Before adding the faecal slurry or the bacterial mix, a circulating water bath was used to set the temperature of the basal medium at 37 ◦C, and a pH of between 6.7 and 6.9 (reflecting the distal region of the colon) was maintained automatically using a pH meter (Electrolab pH controller, Tewksbury, UK) via the addition of 0.5 mol $l^{-1}$ HCl or 1.0 mol $l^{-1}$ NaOH as appropriate. Stirring of samples was maintained using

a magnetic stirrer. A sample (6 mL) was removed from each substrate vessel after 0, 12, 24, and 48 h incubation to ensure enough sample was taken for bacterial and metabolite analysis by FC-FISH and $^{1}$H NMR spectroscopy respectively.

### 2.5 Enumeration of bacteria by flow cytometry fluorescence in situ hybridisation (FC-FISH)

Samples (1.0 mL) collected from Hungate tubes were centrifuged at 11 337 × g for 3 minutes. The supernatant was discarded, and the pellet suspended in 375 µl filtered 0.1 mol $l^{-1}$ PBS solution. Then, 1125 µl filtered 4% paraformaldehyde (PFA) at 4°C was added, and samples stored at 4°C for 4 hours. After 4 h, samples were washed three times with PBS to remove PFA and re-suspended in 150 µl PBS and 150 µl 99% ethanol. These fixed samples were then stored at -20°C until FISH analysis by flow cytometry was conducted.

Fixed samples were taken from the freezer and 75 µl mixed with 500 µl filtered 0.1 mol $l^{-1}$ PBS and centrifuged at 11 337 × g for 3 minutes. The supernatant was discarded, and pellets resuspended in 100 µL of TE-FISH (Tris/HCl 1 mol $l^{-1}$ pH 8, EDTA 0.5 mol $l^{-1}$ pH 8, and filtered distilled water with the ratio of 1:1:8) containing lysozyme solution (1 mg/mL of 50 000 U/mg protein). Samples were then incubated in the dark for 10 minutes at room temperature and centrifuged at 11 337 × g for 3 minutes. Supernatants were discarded, and pellets were washed with 500 µl filtered PBS. Next, the samples were centrifuged at 11 337 × g for 3 min and the supernatants were discarded. Then, the pellets were resuspended in 150 µl of hybridisation buffer (30% formamide concentration) and gently vortexed. Samples were centrifuged at 11337 × g for 3 minutes and the supernatants discarded. Thereafter, pellets were resuspended in 1 mL of hybridisation buffer and homogenised. Next, 1.5 mL Eppendorf tubes were labelled and 4 µl of specific probes (50 ng $\mu l^{-1}$) added. A list of the specific probes used is shown in Table 2.1. Then 50 µl of samples suspended in hybridisation buffer were aliquoted into each Eppendorf. Samples were incubated at 35°C overnight in the dark. Following incubation, 125 µl of hybridization buffer was added to each tube, vortexed and centrifuged at 11 337 × g for 3 minutes. The supernatants were discarded and pellets washed with 175 µl of washing buffer solution. These samples were then incubated at 37°C for 20 minutes in the dark and centrifuged at 11 337 × g for 3 minutes. Supernatants were discarded and different volumes of filtered PBS (300 and 600 µl) were added based on flow cytometry

load. Fluorescence measures were performed by a BD Accuri™ C6 Plus (BD, Erembodegem, Brussels) measuring at 488 nm and 640 nm. Thresholds of 9000 in the forward scatter area (FSC-A) and 3000 in the side scatter area (SSC-A) were placed to discard background noise, a gated area was applied in the main density dot to include 90% of the events. Flow rate was 35 uL/min, with limit of collection set for 100,000 events and analysed with Accuri CFlow Sampler software. Bacterial counts were then calculated through consideration of flow cytometry reading and PBS dilution.

***Table 2.1:*** *Name, sequence, and target group of oligonucleotide probes used in this study for FISH of bacterial enumeration*

| Probe name | Sequence (5' to 3') | Targeted groups | Reference |
|---|---|---|---|
| Non Eub | ACTCCTACGGGAGGCAGC | Control probe complementary to EUB338 | (Wallner, Amann, and Beisker 1993) |
| Eub338 | GCTGCCTCCCGTAGGAGT | Most bacteria | (Amann et al. 1990) |
| Eub338II | GCAGCCACCCGTAGGTGT | Planctomycetales | (Daims et al. 1999) |
| Eub338III | GCTGCCACCCGTAGGTGT | Verrucomicrobiales | (Daims et al. 1999) |
| Bif164 | CATCCGGCATTACCACCC | *Bifidobacterium* spp. | (Langendik et al. 1995) |
| Lab158 | GGTATTAGCAYCTGTTTCCA | *Lactobacillus* and *Enterococcus* | (Harmsen et al. 2000) |
| Bac303 | CCAATGTGGGGGACCTT | Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae | (Manz et al. 1996) |
| Rrec584 | TCAGACTTGCCGYACCGC | *Roseburia* genus | (Walker et al. 2005) |

| | | | |
|---|---|---|---|
| Ato291 | GGTCGGTCTCTCAACCC | *Atopobium* cluster | (Harmsen et al. 2000) |
| Fprau655 | CGCCTACCTCTGCACTAC | *Faecalibacterium prausnitzii* and relatives | (Suau et al. 2001) |
| Chis150 | TTATGCGGTATTAATCTYCCTTT | Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II) | (Franks et al. 1998) |
| EC1531 | CACCGTAGTGCCTCGTCA | *E.coli* | (Poulsen et al. 1994) |
| Rbro730 | TAAAGCCCAGYAGGCCGC | *Clostridium sporosphaeroides*, *Ruminococcus bromii*, *Clostridium leptum* | (Harmsen et al. 2002), |
| Erec 482 | GCTTCTTAGTCARGTACCG | Most of the *Clostridium* coccoides-*Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb) | (Franks et al. 1998) |

**2.6 Statistical analysis**

Statistical analysis was carried out using GraphPad Software [version 9.5.1 (733) San Diego, California USA]. A two-way repeated measure ANOVA was used to determine significant differences in microbiota populations and substrates between 0 h and subsequent time points. Post-hoc Tukey test was used to determine differences between treatments at the same time points. Differences are stated as statistically significant at $*(Q < 0.05)$, $**(Q < 0.01)$, and $***(Q < 0.001)$.

## 2.7 $^{1}$H-NMR spectroscopic analysis

### *2.7.1 Sample preparation for $^{1}$H-NMR spectroscopic analysis*

For $^{1}$H NMR spectroscopic analysis, fermentation samples (0.5mL) collected from batch cultures and Hungate tube fermentations that had been stored at -20°C pending analysis, were thawed at 4°C. A phosphate buffer (pH 7·4 sodium phosphate with 0.2M disodium phosphate (Na2HPO4), 0.04M monosodium phosphate (NaH2PO4) in deuterium oxide (99·9 %) was prepared, with 1mM 3-(trimethylsilyl) propionic acid-d4 sodium salt (TSP) and 3mM sodium azide in the solution. 400 µL of each sample were mixed with 200 µL buffer. 550 µL aliquots of supernatant were collected and dispensed into 5 mm NMR tubes. $^{1}$H-NMR spectroscopic analysis was carried out using a Bruker Avance DRX 500 MHz NMR spectrometer (Bruker Biospin, Germany) as described below.

### *2.7.2 Metabolite analysis by $^{1}$H-NMR spectroscopy*

Spectral data were acquired using a Bruker Avance III 500 MHz spectrometer (Bruker, Germany) operating at the $^{1}$H frequency of 500.13 MHz, at a temperature of 300 K. Spectra were acquired using a standard 1D pulse sequence [recycle delay (RD)-90°-t1-90°-Tm-90°-acquire free induction decay (FID)] with water suppression applied during RD of 2 s, a mixing time (Tm) of 100 ms and a 90° pulse set at 7.70 µs. Per spectrum, a total of 128 scans were carried out with a spectral width of 14.0019 ppm. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening.

### *2.7.3 Metabolic data analysis*

Metabolic profiles obtained were subjected to pre-processing (phasing, baseline correction and reference to the TSP (trimethylsilyl-2,2,3,3-tetradeuteropropionic acid) singlet peak (at δ 0.00)) using the Chenomx Processor programme followed by quantification using the Chenomx Profiler programme (Edmonton, Canada). Graphs and statistics were performed using the Graphpad Prism 10 software. SIMCA 13.0 software package (Umetrics AB, Umeå, Sweden) was used to conduct multivariate statistical analysis in Chapter 5.

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# CHAPTER 3

## Developing an atlas of gut microbial function


### Abstract

The gut microbiota plays a significant role in maintaining human health, with dietary choices influencing its composition (and subsequent function). As interest in gut microbiota-targeted dietary interventions grows, there is a need for in-depth understanding of the functional capacity of microbial communities. Given the complexity of the gut environment, characterised by trillions of microorganisms and intricate interactions, comprehensively understanding the microbiota remains a challenge. In this study, we aimed to unravel the functional capabilities of a simplified nine gut microbial consortium, representing the most abundant genera found in the human gut. To achieve this, their behaviour was monitored within a nutrient-rich medium with different substrates including a probiotic yoghurt. $^{1}$H-NMR spectroscopy was used as a strategy to capture the complete metabolic profile generated by these bacteria. Results revealed that metabolites produced were acetate, lactate, formate, ethanol, and methanol. Additionally, B*acteroides fragilis*, *Faecalibacterium prausnitzii*, and *Escherichia coli*, exhibited the production of propionate and succinate. *Roseburia intestinalis* was found to be a producer of butyrate, while *Bacteroides fragilis* and *Clostridium perfringens* synthesised Gamma Amino Butyric Acid. Inulin and yoghurt enhanced the production of these metabolites. The identified metabolites encompass both intermediates and endpoints of biochemical pathways, shedding light on functional behaviour of the selected gut bacteria. This study provides insights to the creation of an atlas of gut microbial function. This atlas holds potential significance in guiding interventions targeting the gut microbiota, uncovering novel mechanisms for microbiota targeted interventions, and advancing the development of next-generation probiotics.

### 3.1 Introduction

The human gut microbiota is a key driver in maintaining human health with diet being a principle determinant (David et al. 2014; Sheflin et al. 2017). Dietary substrates such as some carbohydrates, lipids and proteins reach the colon (Sanders et al. 2019b), where indigenous bacteria ferment them into metabolites that can influence host health. Diet provides the main substrates available to gut microbiota thereby affecting the type and amount of metabolites they produce. As a result, gut microbiota targeted dietary interventions, such as probiotics and prebiotics, are gaining increasing attention. These can influence SCFAs which are the most widely studied and discussed metabolites produced. Butyrate, acetate and propionate are the most abundant SCFA (Rios-Covian et al. 2016; Parada Venegas et al. 2019).

The complex gut environment and ecosystem comprises of many cross feeding, synergistic and inhibitory mechanisms. Therefore, complexity of the gut microbiota are key factors that need to be considered. Microbial cross feeding, whether synergistic or antagonistic, should be considered when studying functional mechanisms of gut microbiota (Li 2018) Obtaining a clear understanding of the metabolites produced by microbiota and how they are modulated with different substrates within the complex nutrient rich gut environment may allow the discovery of probiotics and prebiotics that can influence health. The response of bacteria to food has not been widely studied. Therefore, the behaviour of bacteria with a probiotic yoghurt in terms of growth and metabolite production was studied. Over the past few decades, extensive research has shed light on microbial metabolites and their profound influence on various aspects of human physiology, from metabolism to immune function.

Accumulating evidence shows that other microbial metabolites such as vitamins, amino acids, bile acid transformations, neurotransmitters also contribute to maintaining important host mechanisms (Otaru et al. 2021). These can be precursors to other metabolic pathways or linked to systemic organs such as the brain (Valles-Colomer et al. 2019; Strandwitz et al. 2019; Cryan and Dinan 2012), heart (Nemet et al. 2020), liver (Guo et al. 2022) and skin (Park et al. 2020). Accordingly, it is important to identify metabolites that may contribute towards maintaining host health and to identify potential next generation probiotics that can produce important metabolites.

To investigate this intricate network, there is a developing trend in research to rely on computational models (Muller et al. 2018; Wang et al. 2019; Marcelino et al. 2023). Our study, in contrast, adopts a mechanistic approach, focusing on specific gut bacterial genera within an environment that resembles the gut. We employed an untargeted metabolomics methodology to gain insights into the detailed metabolic interactions taking place in this ecosystem.

Untargeted metabolic profiling is an unbiased approach that allows determination of metabolites produced in a sample, which is a powerful platform that enables identification of metabolic pathways and diseases (Nemet et al. 2020). $^{1}$H-NMR spectroscopy is an untargeted metabolomic technique enabling extensive and rapid analysis of multiple metabolites present in a sample producing a global overview. It focuses on profiling of the total complement of metabolites produced to generate a metabolic fingerprint in a sample. The atomic nuclei interact with electromagnetic radiation at specific frequencies when placed in a magnetic field. Nuclei in different magnetic fields have characteristic frequencies known as chemical shift, which is measured. $^{1}$H-NMR spectroscopy provides a real representation of the distribution of proton nuclei within the molecules and different concentration levels of corresponding metabolites in a complex mixture (Emwas et al. 2019).

FC-FISH enables enumeration of bacterial populations. 16S-rRNA probes can be used to identify changes in the numbers of total bacteria and specifically targeted microbial groups. This information can provide an insight into bacterial counts and how they change during interactions.

This study aims to develop an atlas of gut microbial function using $^{1}$H-NMR spectroscopy using samples collected from a nutrient rich medium mimicking the gut environment, with nine selected gut microbes representing the most abundant genera in the human gut. The change in microbial load was monitored using FC-FISH. As $^{1}$H-NMR spectroscopy allows detection of microbiota produced metabolites, it enables identification of microbial interactions to help elucidate metabolic function of the microbiota.

This study focuses on understanding how these 9 bacteria behave in pure culture in a nutrient rich medium similar to the gut environment, and also with a probiotic yoghurt. By observing metabolic profiles of the bacteria, information can be used to develop an atlas of gut microbial

function. Understanding the individual function of bacteria within a nutrient-rich gut environment and their responses to various substrates and foods is crucial for designing targeted interventions aimed at the gut microbiota. An atlas that comprehensively delineates these functions can streamline complexities within the gut ecosystem. It provides insights into the specific contributions of different bacteria in producing certain metabolites and identifies bacteria responsible for generating intermediate metabolites that facilitate cross-feeding mechanisms. This atlas can be used bidirectionally; first, for the identification of metabolites synthesised by bacteria hence targeting new probiotics and second, for the identification of bacteria with the capacity to produce a targeted metabolite and thereby identifying prebiotics that could be used to enhance the growth of particular bacteria. This dual application holds promise in the recognition and selection of probiotics and prebiotics in future interventional research. Such an atlas serves as a valuable tool for deciphering the intricate interplay of gut microbiota and their metabolites, ultimately paving the way for more effective interventions and therapies targeted towards optimising gut health. Another outcome of this study is that it can lead to designing of a synthetic bacterial mix that can resemble human faeces which is discussed in the next chapter of this thesis.

## 3.2 Materials and methods

### *3.2.1 Bacteria*

9 bacterial species representing the core gut microbiota were selected based on literature sources (Eckburg et al. 2005; Falony et al. 2009; Walker et al. 2011; Ze et al. 2012; Arumugam et al. 2011; Baxter et al. 2019). Listed below are the nine selected bacteria selected: *Bacteroides fragilis* NCTC 9343 (Bacteroidetes), *Bifidobacterium longum* NCTC11818 (Actinobacteria), *Clostridium perfringens* NCTC8678 (Firmicutes), *Lactobacillus rhamnosus* NCTC10302 (Firmicutes), *Collinsella aerofaciens* NCTC11838 (Actinobacteria), *Escherichia coli* NCTC 1093 (Proteobacteria), *Ruminococcus bromii* ATCC 51896 (Firmicutes), *Roseburia intestinalis* DSM 14610 (Firmicutes), *Faecalibacterium prausnitzii* DSM 17677 (Firmicutes).

### *3.2.2 In vitro Hungate tube fermentation*

Bacteria reconstituted from glycerol stocks were grown in specific media (Appendix 3.1) under anaerobic conditions (80% $N_2$, 10% $H_2$ and 10% $CO_2$). 100 µl from each bacterium that reached maximum growth was inoculated into Hungate tubes with the nutrient rich medium (as described in Chapter 2). Initially, duplicate vessels with nutrient rich medium were used to measure growth curves of the bacteria. For the next experiment, individual bacteria were tested with tubes with 1% added substrates (inulin, starch and probiotic yoghurt). All conditions were conducted in triplicate. A sample (0.5 mL) was removed from each tube after 0, 12, 24, 48, and 60 h fermentation for metabolite analysis by $^1$H-NMR spectroscopy, and a sample (1.0 mL) was removed at 0, 24, 48 h for bacterial enumeration by FC-FISH. Figure 3.1 shows the experimental steps for a single bacterial strain. The same procedure was conducted for all 9 selected bacteria.

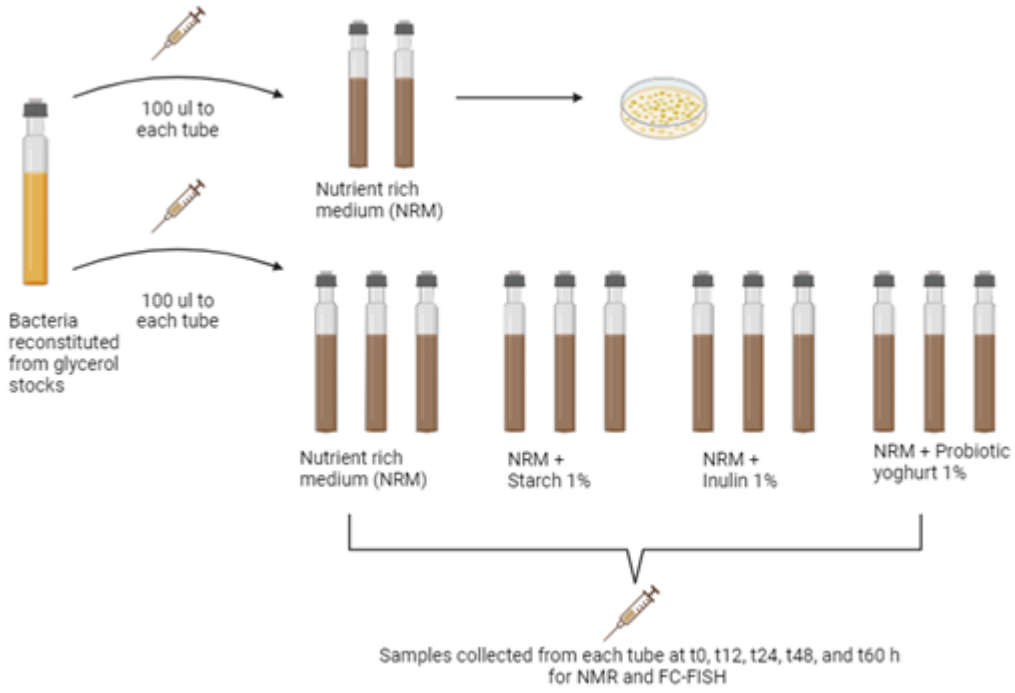


***Figure 3.1**: Experimental method of growing the selected bacteria in Hungate tubes with the different substrates. Image created in Biorender*

### *3.2.3 Metabolite analysis by $^1$H-NMR spectroscopy*

Samples collected were stored and processed as described in Chapter 2. Spectral data were acquired using a Bruker Avance III 500 MHz spectrometer (Bruker, Germany) operating at the

$^{1}$H frequency of 500.13 MHz, at a temperature of 300 K. Acquired spectroscopic data were processed using the TopSpin 3.6.5 software package (Bruker Biospin, Rheinstetten, Germany) and Chenomx NMR Suite 9.0 software package (Edmonton, Canada).

### *3.2.4 Enumeration of bacteria by FC-FISH*

Samples (1.0 mL) collected at t0, t24 and t48 from Hungate tubes were fixed as described in Chapter 2. From the fixed samples, 150 µl was mixed with 500 µl filtered 0.1 mol $l^{-1}$ PBS which was used for the hybridisation process as described in Chapter 2. Bacterial counts for each were analysed and calculated according to the method described in Chapter 2.

Bacterial populations were assessed with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA, as previously described (Costabile 2010). The commercially synthesised probes used in this study are listed in Table 2.1 Chapter 2.

### *3.2.5 Statistical analysis*

Statistical analysis was carried out using GraphPad Software [version 9.5.1 (733) San Diego, California USA]. A two-way repeated measure ANOVA was used to determine significant differences in microbiota populations and substrates between 0 h and subsequent time points (24 h and 60 h). Post-hoc Tukey test was used to determine differences between treatments at the same time points. Differences are stated as statically significant at 0.05 (*), 0.01 (**), and < 0.001 (***). Statistical analyses were conducted on pre-logged values, and data were represented on a log scale to ease visualisation.

## 3.3 Results

### *3.3.1 Pure culture bacterial enumeration and metabolite profiling*

Significant observations were made regarding the behaviour of all 9 bacterial species, as depicted in Figure 3.2. The metabolite production of bacteria were quantified using the spectra shown in Figures 3.3 and 3.4 as shown in Figure 3.5. Notably, the majority of variations were observed within the 24-hour period, with *L. rhamnosus*, *B. fragilis*, *E. coli*, and *C.*

*perfringens* exhibiting the most variations. However, *F. prausnitzii* and *Ruminococcus bromii* showed growth and variations after 48 hours.

*Bif. longum* exhibited a notable response only to inulin, with the highest increase being observed in the inulin vessel compared to all others. This increase was significantly higher compared to control, starch, and yoghurt (*p=0.018). Within the inulin vessel, a significant increase was also observed between T0 and T24 (**p=0.010), indicating rapid changes in response to this substrate. This observation aligns with the highest production of acetate observed in the inulin vessel (Figure 3.5), although starch and yoghurt vessels also exhibited metabolite production compared to the control. Other captured metabolites included formate, lactate, ethanol, and a minimal amount of methanol, with the yoghurt vessel showing utilisation of methanol.

*Lacticaseibacilli rhamnosus* (Figure 3.2) showed a robust response to all substrates, with all three control, starch, and inulin vessels displaying high bacterial counts at 24 hours. Significant differences were observed between control vs starch (*p=0.037), control vs yoghurt (***p<0.001), inulin vs starch (**p=0.006), and both inulin and starch against yoghurt (***p<0.001). Towards the end of fermentation, the inulin, starch, and yoghurt vessels maintained increased counts, while the control vessel exhibited reduced counts, possibly due to substrate depletion. The yoghurt vessel also showed increased counts at 48 hours. *lacticaseibacilli* were also associated with high lactate production in the substrates compared to the control.

*Bacteroides fragilis* exhibited a strong response to all substrates and showed highest growth among the 9 bacterial species. Inulin reported the highest growth, followed by starch, control, and yoghurt. At the end of fermentation, significant differences (***p<0.001) were observed among all vessels, including control vs inulin, control vs starch, control vs yoghurt, inulin vs starch, inulin vs yoghurt, and starch vs yoghurt.

The limitations of this study include the uncontrolled pH of the growth medium. As the bacteria produced SCFAs during fermentation, the pH may have dropped further, potentially affecting the survival of some bacterial species. This uncontrolled pH change could have significantly impacted the co-culture dynamics, as *Lacticaseibacillus rhamnosus* is a heterofermentative bacterium that can tolerate acidic conditions. Therefore, the observed

survival of *L. rhamnosus* may have been partly due to its ability to withstand the acidic environment created by SCFA production, rather than solely due to its competitive interactions with the other bacterial species.

To address this limitation, future studies should monitor and control the pH of the growth medium throughout the co-culture experiments. Hence, the next chapter uses pH controlled anaerobic batch culture experiments for the synthetic consortium experiments.

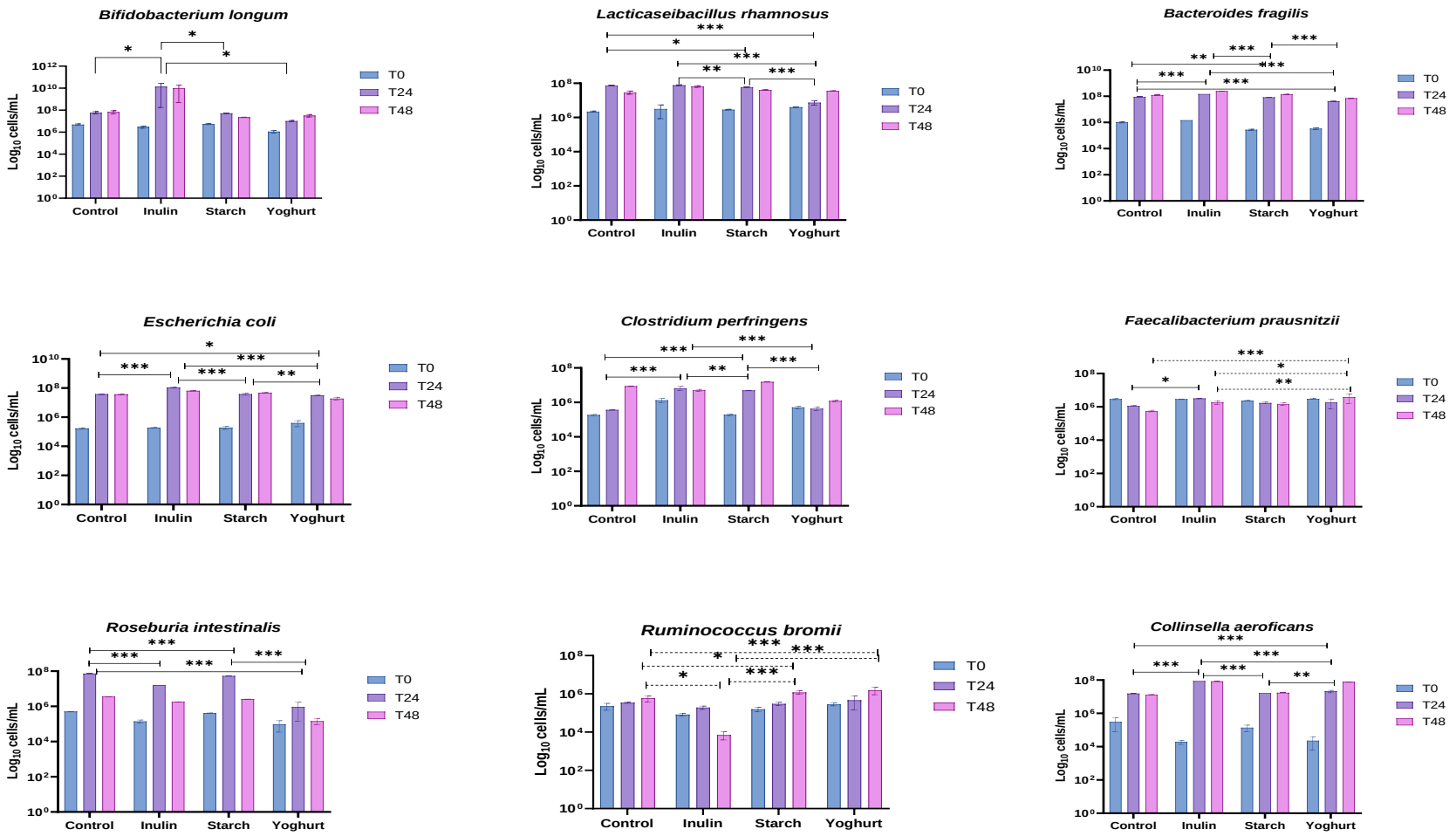


***Figure 3.2:*** *Bacterial population change of each bacteria in the mix consortium over the fermentation time period measured using FC-FISH (Log10 cells/mL) using specific probes for each bacteria. Mean and SE. ∗(Q < 0.05), ∗∗(Q < 0.01), and ∗∗∗ (Q < 0.001) indicate significance compared among substrates at 24h.*

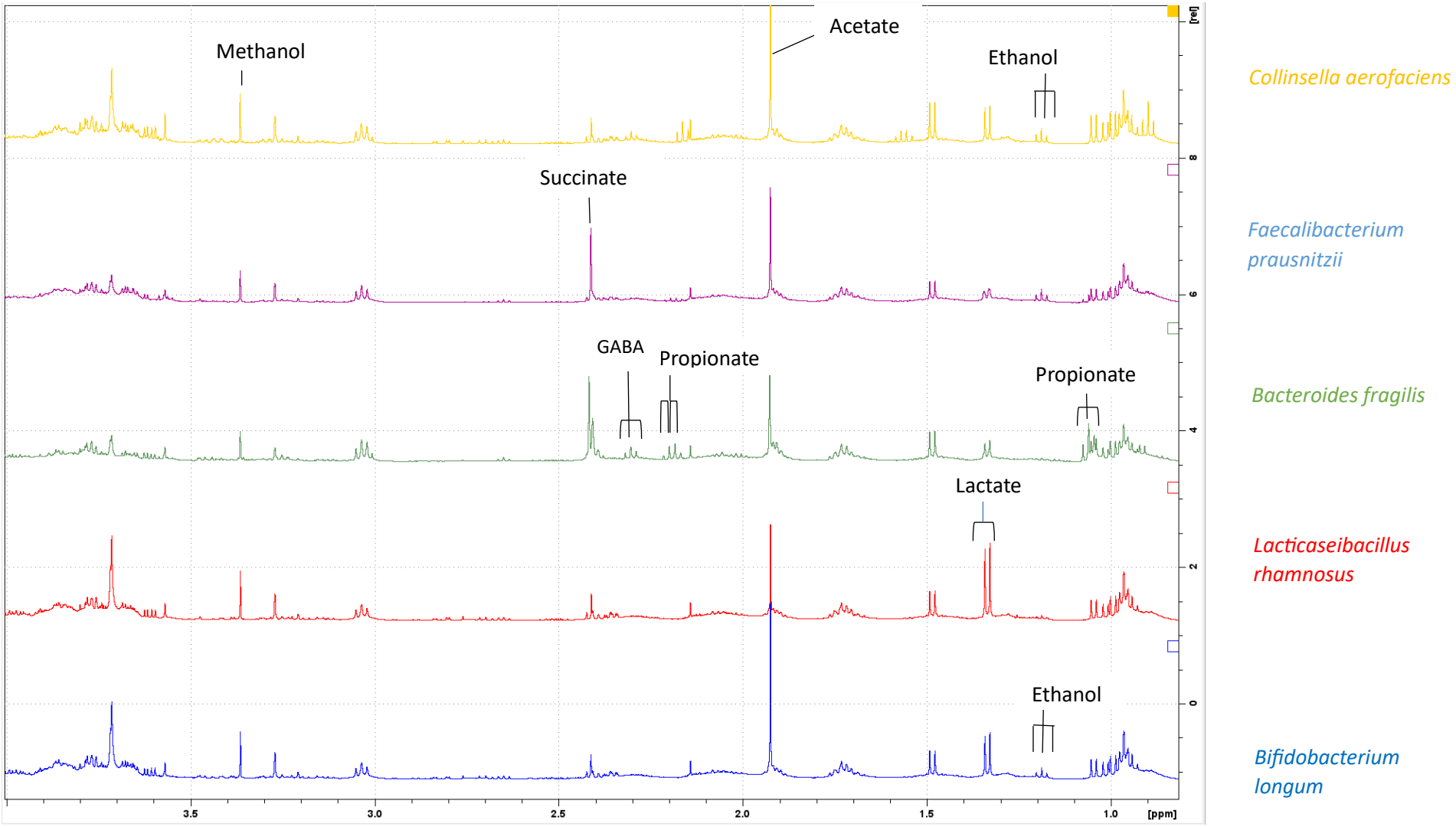


***Figure 3.3:*** *Spectra obtained from Topspin software showing bacteria (Collinsella aerofaciens, Faecalibacterium prausnitzii, Bacteroides fragilis, Lacticaseibacillus rhamnosus and Bifidobacterium longum) and the metabolites produced at their respective chemical shifts*

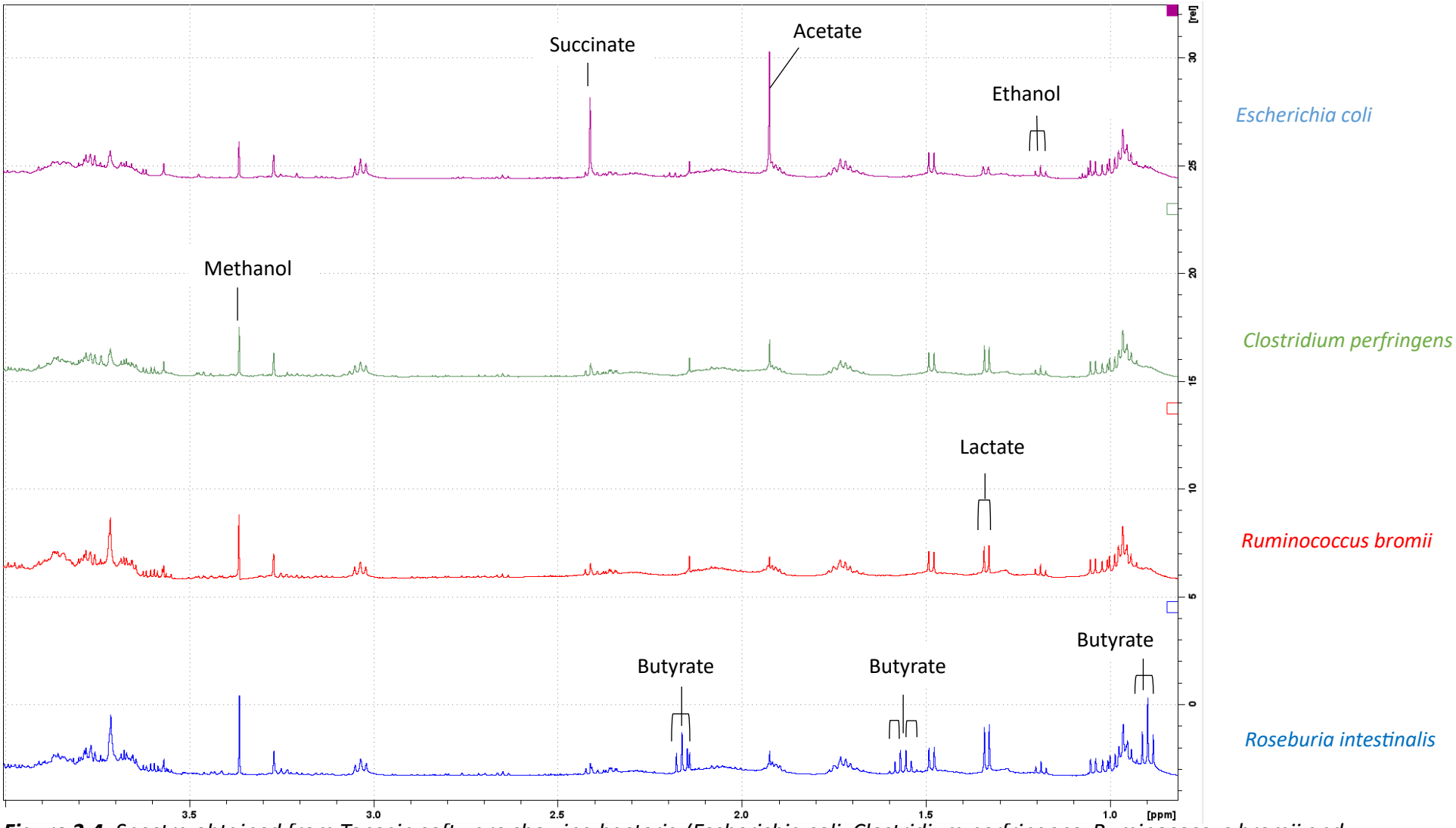


***Figure 3.4:*** *Spectra obtained from Topspin software showing bacteria (Escherichia coli, Clostridium perfringens, Ruminococcus bromii and Roseburia intestinalis) and the metabolites produced at their respective chemical shifts*

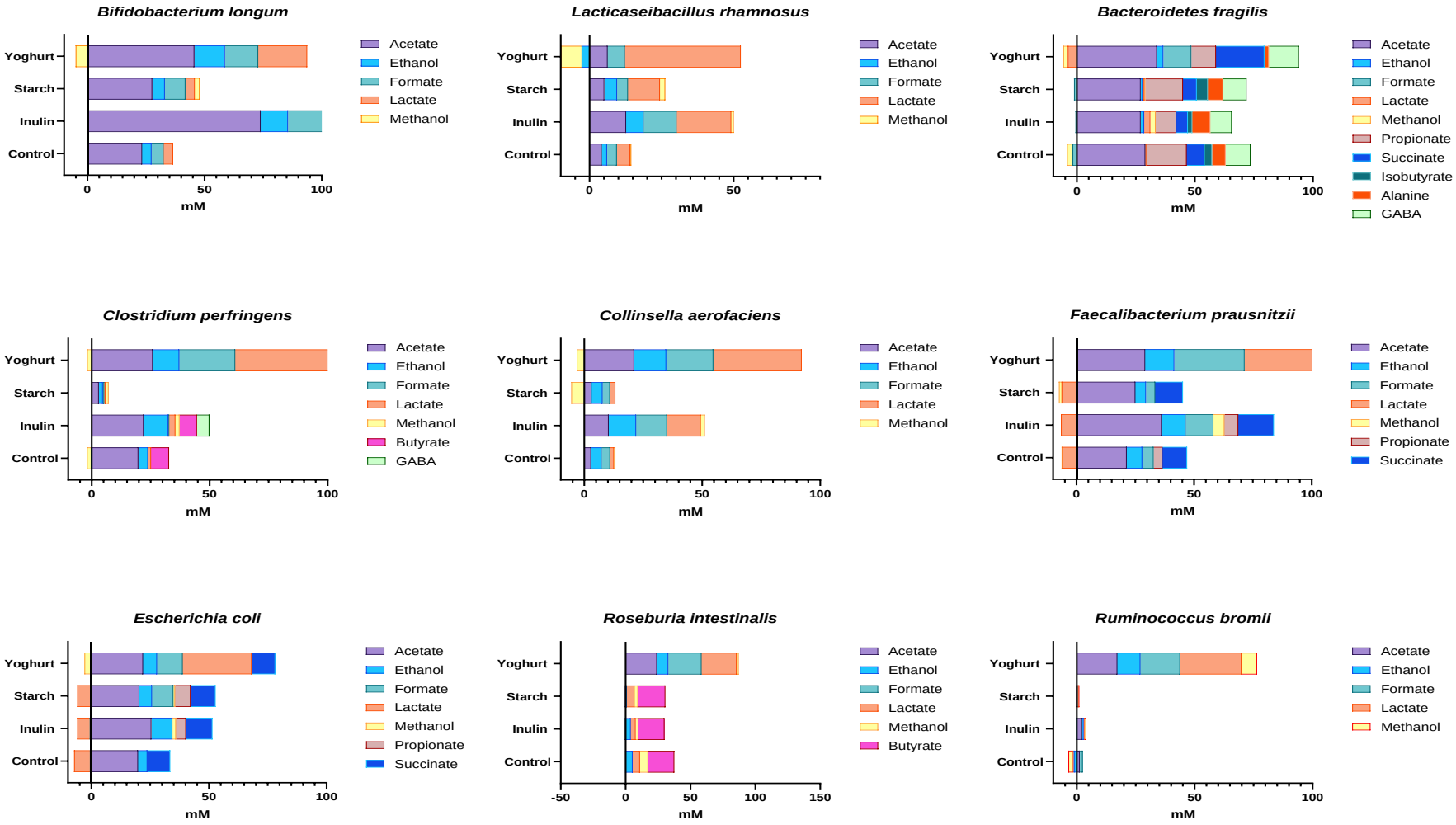


***Figure 3.5:*** *Metabolite production of the bacteria with the different substrates quantified using Chenomx software (mM)*

Appendix 3.2 shows the NMR assignment table and Appendix 3.3 & 3.4 shows the Topspin spectra where the metabolites were quantified for each substrates and media

*Bacteroides* also produced the highest number of metabolites, including GABA and succinate, which are of interest in gut-brain axis studies and cross-feeding mechanisms among bacteria.

*E. coli* displayed a growth pattern similar to that of *Bacteroides* and produced acetate, ethanol, and succinate with all substrates. Additionally, *E. coli* produced lactate with yoghurt, while other substrates showed lactate utilisation. Conversely, growth of clostridia in the yoghurt vessel was significantly lower (***p<0.001) compared to control, inulin, and starch at 24 hours and remained consistent at 48 hours, indicating potential suppression by the probiotic. Clostridia also exhibited different metabolite production across substrates, with GABA production with inulin and high lactate production with yoghurt. Faecalibacteria and ruminococci showed slow initial growth, with *Faecalibacterium* reporting the lowest growth among all bacteria. However, both species exhibited increased counts towards the end of fermentation, particularly in the yoghurt vessel. *Ruminococcus bromii* showed a significant increase (***p<0.001) compared to control and inulin but not starch at 48 hours. *Roseburia* responded well to control, starch, and inulin, but growth in the yoghurt vessel was significantly lower (***p<0.001) compared to other substrates. *Collinsella* responded well to all substrates, with inulin showing a faster response at 24 hours and yoghurt exhibiting a significant increase at 48 hours compared to other substrates (***p<0.001).

### *3.3.2 Co-culture bacterial enumeration with probiotic yoghurt*

A comparative analysis was conducted on the probiotic yoghurt vessel to assess the behaviour of bacteria in co-culture using pre-logged values (Figure 3.6 and 3.7). Results highlighted instances of competition and suppression among the bacterial species. *Bifidobacterium Bacteroides*, and *Collinsella* initially exhibited low percentages (20%, 7%, and 0.5% respectively) but demonstrated rapid growth by 24h of fermentation, reaching percentages of 58%, 84%, and 73% respectively. However, the probiotic lactocaseibacilli experienced a resurgence by 48h, causing the percentages of the other bacteria to decrease to 47%, 66%, and 68% respectively. In contrast, *E.coli* displayed a different pattern, starting at 9%, increasing to 80%, and then dropping to 34%. This fluctuation indicated competition between the probiotic and *E.coli*. The growth of *Clostridium*, *Faecalibacterium*, *Roseburia*, and *Ruminococcus* was suppressed, with the probiotic exerting dominance over these species.

However this cannot be directly concluded as competition as the L. rhamnosus can tolerate acidic environments and hence may have survived better compared to the other strains with the reduction of pH due to the production of SCFAs.

Overall, these results showed the varied responses of 9 bacterial species to substrates, metabolite production and growth dynamics. Metabolites produced by all bacteria included: acetate, ethanol, formate, lactate and methanol. *Bacteroides fragilis*, *Faecalibacterium prausnitzii* and *E. coli* produced propionate and succinate. *Roseburia intestinalis* produced butyrate and *Bacteriodes fragilis* and *Clostridium perfringens* produced GABA. Analysis of metabolic profiles indicated that the substrates did not lead to the generation of new metabolites; rather, they primarily influenced concentrations with inulin and yoghurt being most effective in stimulating. Using this information, an atlas of gut microbial function was generated (Figure 3.8).

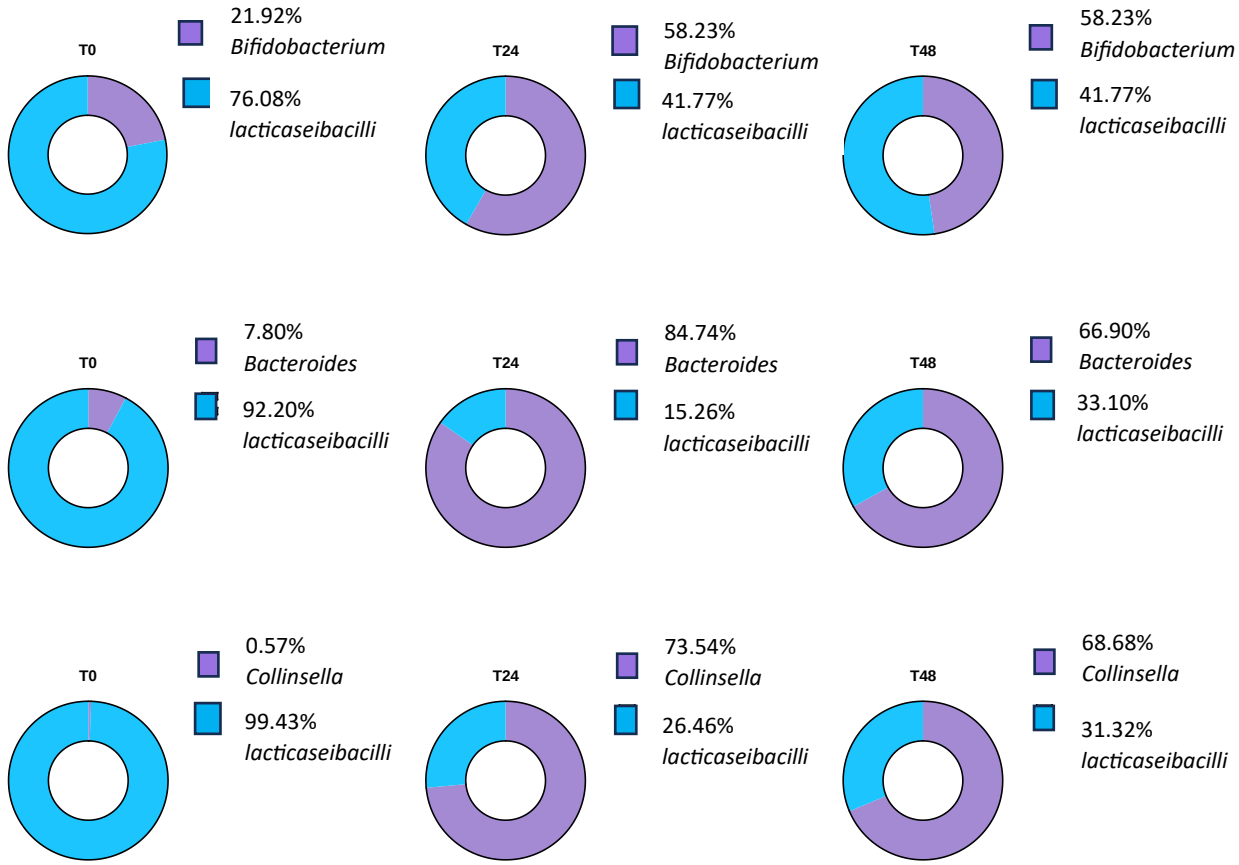


***Figure 3.6:*** *Shift in bacterial percentage in co-culture with probiotic strain Lacticaseibacilli rhamnosus over time (Bifidobacterium, Bacteroides and Collinsella)*

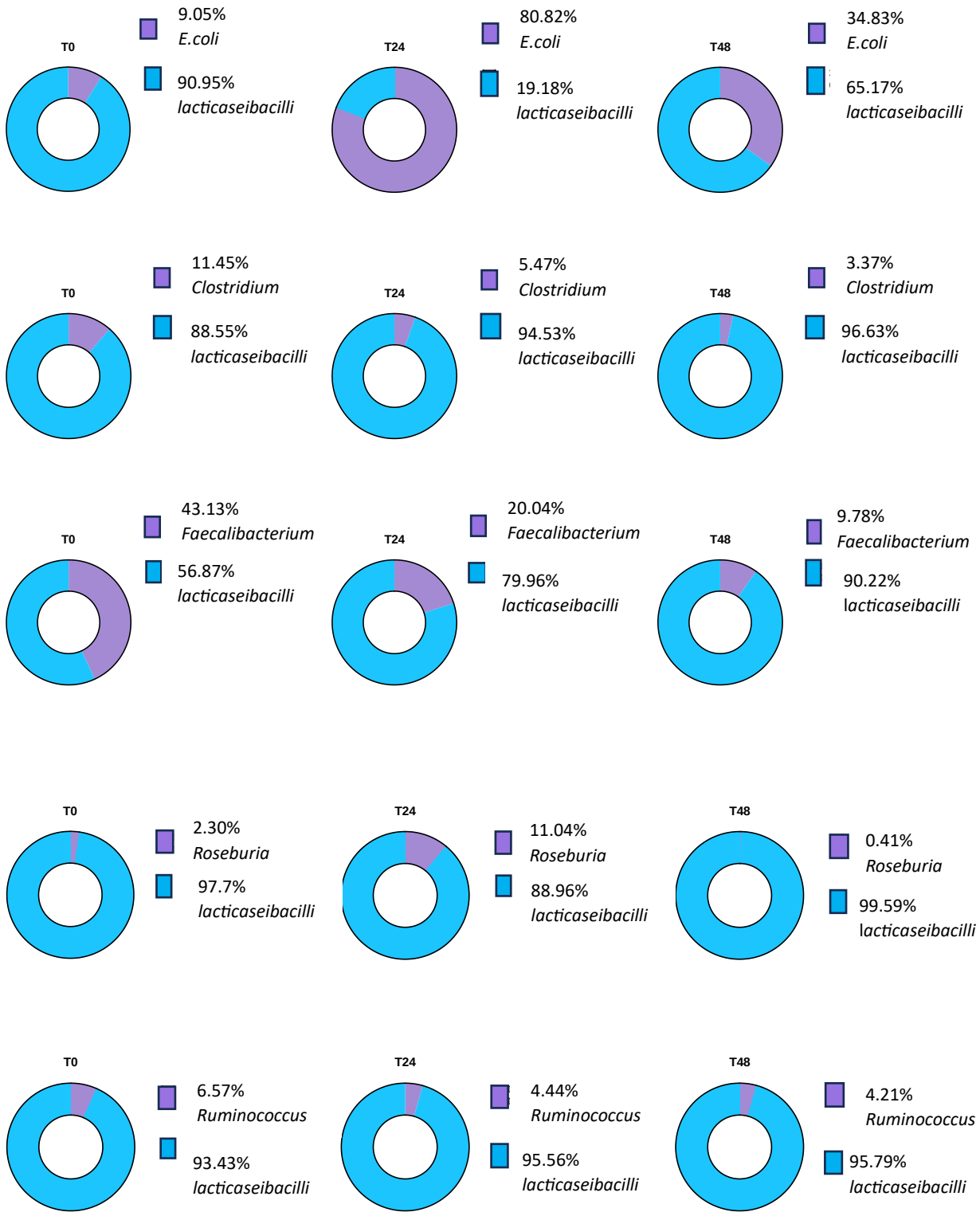


***Figure 3.7:*** *Shift in bacterial percentage in co-culture with probiotic strain Lacticaseibacilli rhamnosus over time (E.coli, Clostridium, Faecalibacterium, Roseburia and Ruminococcus)*

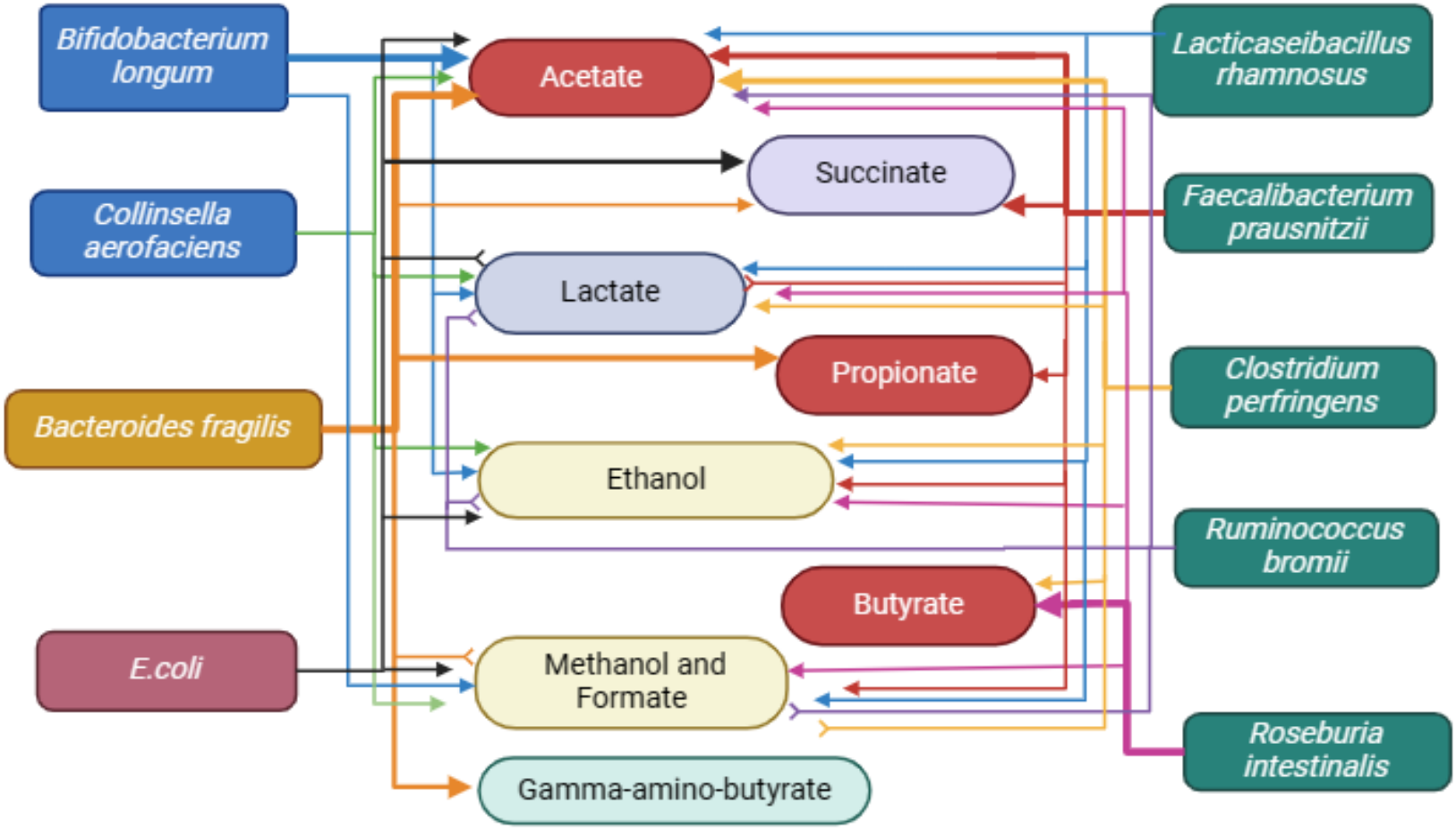


***Figure 3.8:*** *Atlas of gut microbial function for the 9 selected bacteria. Bacteria belonging to the same phyla are shown in same colours, bacteria belonging to phylum Firmicutes shown in green, Bacteroidetes in yellow, Actinobacteria in blue and Proteobacteria in red. Most widely produced metabolites are shown in thick arrows. The inverted arrow (>-) indicates utilisation of the metabolite. Image created in Biorender*

### 3.4 Discussion

In this *in vitro* fermentation study, the experimental design compared the behaviour of 9 different bacteria found in human gut in a nutrient rich growth medium (Macfarlane et al. 1998). Bacteria selected for the study represented the core genera of human gut microbiota (Eckburg et al. 2005; Rinninella et al. 2019). All bacteria showed an increase in growth within the first 24 h of incubation. *Bifidobacterium longum*, *Bacteroides fragilis*, *Lactobacillus rhamnosus*, *Roseburia intestinalis* and *E. coli* showed highest growth of the bacteria tested. *Ruminococcus bromii* and *Faecalibacterium prausnitzii* showed an increased growth at 48h.

Given that the medium contains inulin and other carbohydrates, it is plausible that bifidobacterial counts increased as a result of these nutritional sources. *Bifidobacterium* spp. are known to have necessary intracellular and extracellular mechanisms needed to utilise a wide range of low molecular weight carbohydrates and grow well on inulin (Riviere et al. 2016). *Bacteroides fragilis* showed a good response with the highest number of metabolites, supporting studies that show bacteroides has more affinity towards complex carbohydrates due to the presence of appropriate extracellular enzymes (Cerqueira et al. 2020). *Bacteroides* spp. are shown to have Polysaccharide Utilisation Loci (PULs) and encodes a series of different enzymes (Cheng et al. 2022). Notably, *Bacteroides fragilis* has been reported to harbour around twenty Carbohydrate Binding Modules (CBM) associated with extracellular degenerative enzymes and over two hundred Carboydrate Active Enzymes (CAZymes) (Flint et al. 2012) for glycan degradation. Furthermore, bacteroides are also known for their proteolytic function (Falony et al. 2009; Macfarlane et al. 1988) which was substantiated in this study with the presence of amino acids. Interestingly, bacteroides contributed to the production of GABA which is a neurotransmitter and associated with the gut-brain axis (Cryan et al. 2020) and corresponds with other research (Strandwitz et al. 2019).

Results suggest that *E. coli* and *F. prausnitzii* may be involved in lactate utilisation. These observations align with findings of (Augustiniene and Malys 2022), who demonstrated the presence of lactate metabolism in *E. coli*. However, it is noteworthy that *F. prausnitzii* is primarily recognised as a butyrate producer (Barcenilla et al. 2000; Duncan 2002) whereas this study revealed an absence of butyrate production in *F. prausnitzii*, instead demonstrating synthesis of acetate, propionate, succinate, and utilisation of lactate (Duncan et al. 2004) have reported that *F. prausnitzii* cannot utilise lactate. However, the lactate fermentation of this

strain is accepted by the BRENDA computational pathway annotations (Chang et al. 2021) that the *F. prausnitzii* DSM 17677 (known as *F. dunacaniae* since May 2022) contains enzymes L-lactate dehydrogenase, lactate racemase for lactate fermentation. It is possible that the absence of butyrate in the metabolic profile of this study is due to a relatively low glucose concentration within the medium. A previous study by (Duncan et al. 2004) reported that *F. prausnitzii* utilised acetate while producing butyrate, formate, and D-lactate during glucose fermentation. They also confirmed that acetate was essential for the growth of *F. prausnitzii* (Duncan 2002), implying that further fermentation and breakdown of complex sugars into glucose may influence *F. prausnitzii* towards butyrate production. Lactate utilising bacteria are typically associated with the conversion of lactate into acetate. butyrate and propionate (Louis et al. 2022) while some pathogenic proteobacteria can completely oxidise lactate to carbon dioxide and water (Gillis et al. 2019). Furthermore, it has been documented that the gut environment (Louis et al. 2007) and pH levels (Wang et al. 2020) may influence lactate utilisation. In this context, it is conceivable that uncontrolled pH conditions within the Hungate tube experiment played a pivotal role. Over the course of 24 hours, extensive production of acetate, as evidenced by metabolite production data (Figure 3.5), likely contributed to acidification of the environment. This acidic environment is generally unfavourable for bacterial growth, which raises the possibility that growth rates were adversely affected and as a result most bacteria numbers were reduced by the end of fermentation. Regarding the gut environment, it has been reported that *F. prausnitzii* has limited ability to ferment polysaccharides such as arabinogalactan, xylan, starch and cannot utilise mucin (Lopez-Siles et al. 2017) which are contents in this nutrient rich medium. However, Lopez-siles et al. (2017) reported that pectin was a good medium for *F. prausnitzii* growth. With *Roseburia* spp. the current investigation revealed robust butyrate production corresponding to previous studies (Nie et al. 2021; Duncan et al. 2004). Butyrate is a metabolite of significant importance for maintaining overall health (Parada Venegas et al. 2019; Rios-Covian et al. 2016). Metabolite profile of *Roseburia intestinalis* indicates no acetate production, this observation supports the condition of pH retention that favours butyrate synthesis through the activities of butyryl coenzyme A (CoA):acetate CoA transferase and acetate kinase (Duncan et al. 2002).

This study uncovers key findings regarding bacterial responses to probiotic yoghurt, providing understanding for future interventions aimed at harnessing the potential benefits of real food

with live microbes. Probiotics derived from the *lacticaseibacilli* and *Bifidobacterium* genera have long been celebrated for their positive impact on gut health, as evidenced by acetate production, a result consistent with established literature (Abedi and Hashemi 2020; Louis et al. 2022).

Moreover, our study unveils a diverse metabolite production profile by *Bacteroides fragilis*, encompassing critical compounds like acetate, propionate, and succinate, alongside the neurotransmitter GABA. The involvement of GABA in the gut-brain axis highlights intricate interplay of microbial metabolites with host physiology and neurobiology. Notably, co-culturing *Bacteroides* with probiotic yoghurt resulted in the highest concentration of metabolites, indicating a synergistic response between these microbes and the yoghurt culture.

The distinctive aspect of our research lies in exploring how bacteria respond to a fermented food context, expanding our understanding beyond conventional substrates such as starch and inulin. This investigation sheds light on the nuanced interactions between probiotic-rich foods and the gut microbiota, revealing whether these interactions yield synergistic or antagonistic effects. Such comprehensive insights are important for deciphering complex dynamics within the gut ecosystem and discerning the potential impact of fermented foods containing probiotics on microbial communities.

Findings of this study have provided valuable insights into metabolic capacities of selected bacteria within a nutrient-rich medium, mimicking conditions similar to the gut environment. This information served as a foundational basis for the development of an atlas of gut bacterial function. The utility of this atlas extends to various potential applications, particularly in the context of future interventions targeting the gut microbiota. One immediate application is the selection of beneficial microbes that contribute to the production of specific metabolites. This knowledge empowers researchers to design interventions aimed at promoting the synthesis of metabolites crucial for health and well-being. Furthermore, the atlas provides a foundation for the strategic selection of probiotics. This opens up exciting possibilities for tailoring probiotic approaches to enhance the production of specific metabolites beneficial for health.

The availability of an easily accessible atlas delineating metabolites and their associated bacterial producers stands to greatly simplify the design of interventional studies,

circumventing the need for extensive literature research and data mining. Using this information, an atlas of gut microbial function has been developed, leveraging insights gained from this study. While the substrate only amplified the production of existing metabolites without generating new ones, we utilised these data to construct the atlas with a focus on nutrient-rich medium results as the initial step (Figure 3.8). This atlas offers details into the specific roles of different bacteria in producing specific metabolites and identifies bacteria responsible for producing intermediate metabolites that facilitate cross feeding mechanisms within the gut microbiota.

The dual application of this atlas is particularly promising. Firstly, it aids in identifying metabolites synthesised by bacteria, thereby facilitating the selection of new probiotics. Secondly, it helps in pinpointing bacteria with the capacity to produce targeted metabolites, enabling the identification of prebiotics that can enhance the growth of specific bacteria. This bidirectional approach holds immense potential in the recognition and selection of probiotics and prebiotics for future interventional research aimed at improving gut health.

Such an atlas serves as a tool for unravelling the intricate interplay between gut microbiota and their metabolites. By providing a comprehensive understanding of microbial function and metabolite production, this atlas lays the groundwork for more effective interventions and therapies tailored to optimise gut health and overall well-being.

## 3.5 Conclusion

This study provides information into the complex metabolic responses of human gut bacteria to various dietary substrates including a probiotic yoghurt. Our findings highlight the diverse metabolic capacities of key bacterial genera such as bifidobacteria, lacticaseibacilli, roseburia, and bacteroides, shedding light on their roles in substrate utilisation and metabolite synthesis. Moreover, this study has yielded a comprehensive atlas of gut microbial function, leveraging the insights gained from bacterial responses in the nutrient rich medium. The atlas serves as a repository of functional information, detailing the specific contributions of different bacteria in producing particular metabolites and identifying bacteria responsible for generating intermediate metabolites essential for cross feeding mechanisms within the gut microbiota.

This atlas has dual applications, aiding in the selection of new probiotics with targeted metabolic functions and identifying prebiotics that can enhance the growth of beneficial bacteria.

The atlas simplifies the design of interventional studies by providing accessible information on metabolites and their associated bacterial producers, facilitating the strategic selection of probiotics and prebiotics tailored to optimise gut health. This comprehensive understanding of microbial function and metabolite production lays the groundwork for more effective interventions and therapies aimed at improving gut health and overall well-being.

Future studies can leverage this resource to explore complex microbial dynamics, potentially leading to the development of more representative models and targeted interventions for optimising gut health. Overall, this study contributes significantly to our understanding of gut microbiota function and its potential impact on human health.

This chapter leads to the next chapter which tested the collective response of these bacteria in a synthetic consortium to these substrates and comparing the functional behaviour of the synthetic mix to human faeces.

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# CHAPTER 4

## Developing a synthetic bacterial consortia


### Abstract

Assessment of synthetic gut microbial communities can generate knowledge to assist in advancing interventional studies such as nutritional trials and faecal microbial transplantation (FMT). As an initiation towards this aim, we developed a novel synthetic gut microbial consortium comprising of nine bacterial strains, which represented prominent gut bacterial phyla, including two pathogenic species. $^{1}$H-NMR spectroscopy was used as a strategy to understand functional behaviour of the synthetic mixture. Substrate utilisation was assessed using starch, inulin, a probiotic and probiotic yoghurt, and results were compared against four human donor faecal samples in terms of metabolite production and bacterial counts. In human faeces, end products such as acetate, butyrate and propionate, were detected, whereas the synthetic consortium exhibited intermediate metabolites like succinate and formate, in addition to end products. Furthermore, concentrations of metabolites in the synthetic mix were notably lower than in human faeces. Bacterial counts revealed that *Bifidobacterium* dominated in human faecal inoculated fermenters across all substrates, while bacteroides performed well in the synthetic mix. *E. coli* and clostridia were suppressed with the incorporation of a probiotic. Even though the metabolic profiles at T0 of human vs synthetic mix clustered together, the human donor samples demonstrated clear shifts with time whereas the synthetic mix shifted more slowly in the same direction. This study uncovered novel insights into microbial function in mixed communities however also depicts the challenge of replicating the complexity of the human faecal microbiota. Further investigations, informed by a deeper understanding of the microbial composition of human donors could pave the way for the identification of specific species contributing to cross feeding and pathogen suppression. This knowledge holds the potential to enhance the formulation of mixtures tailored for FMT and related therapeutic applications.

## 4.1 Introduction

Functional capabilities of microbial communities are intricate. Microbial communities contribute to environmental stability and human health by maintaining natural biogeochemical balance. Soil microbiota, waste management and ground water are highlights of environmental significance where scientists and engineers have succeeded in manipulating them for better results such as altering soil microbiota to enhance crop yield (Lawson et al. 2019). When considering human microbial communities, the gut microbiota is under a spotlight. Scientists have understood the composition and importance of the gut microbiota, however, forces and interconnection between this complex gut environment and trillions of microbiota are still not clearly understood (Cullen et al. 2020). Thus, manipulating gut microbiota has limitations such as complex interconnections within microbiota and individual response variations. Dysbiosis in human gut microbiota leads to disease risk (Rooks and Garrett 2016). Use of probiotics, prebiotics and antibiotics have been proven to influence gut microbiota composition (Gibson et al. 2017; Sanders et al. 2019b; Cieplak et al. 2018; Hill et al. 2014). However, manipulating gut microbiota has been a challenge due to the complexity of the microbiota and gut environment along with individual variation. The full functional capacity of human gut microbiota remains elusive.

The human gut microbiota is closely connected to health and disease (Sheflin et al. 2017; Forster et al. 2019). This is influenced by host factors such as immune function (Rooks and Garrett 2016), diet and lifestyle (David et al. 2014; De Filippis et al. 2016) but also a major role is played by microbial cross-feeding (Tramontano et al. 2018). An insight into such interconnections may provide a better understanding of how to manipulate the gut microbial population in a more effective way, for example, through dietary interventions.

The gut microbiota consists of numerous bacterial species composing mainly of Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Rinninella et al. 2019; Thursby and Juge 2017; Graf et al. 2015). While the composition, evolution, dysbiosis and dietary interactions with these bacteria are well documented, there is a lack of information on detailed functional capabilities and interactions in the complex gut environment. As it is challenging to unravel the web of gut microbiome, study on synthetic bacterial consortium can be a fundamental approach to this challenge (Mabwi et al. 2021). Developing a synthetic gut microbial community holds significant potential for various applications. It can help to better understand

the role of gut bacteria in human health, enable personalised healthcare and advance research on metabolic diseases like obesity, diabetes, and gastrointestinal disorders. Additionally, it could aid in creating probiotics and prebiotics to promote a healthy gut and improve well-being. Moreover, a synthetic bacterial consortium has the potential of an alternative to FMT because it excludes viruses and other non-bacterial elements present in faeces leading to a more predictable function as the consortium is precisely defined, allowing for targeted manipulation and enhanced safety in therapeutic applications. It also eliminates the need for using faecal matter, thereby removing any discomfort associated with faeces-related treatments. Understanding functional behaviour of a synthetic bacterial consortium may help to decipher the behaviour of bacteria in a simpler mix than faeces. This thesis chapter explores the potential application of a synthetic bacterial mixture to replace the use of faeces in applications such as in vitro experiments and FMT.

The inclusion of pathogenic bacteria in the synthetic consortium was crucial to monitor whether these potential pathogens would be suppressed in the presence of the other bacterial species. In diseased hosts, there is an increased risk of opportunistic pathogens proliferating and causing further complications. However, if we observe that such pathogens are suppressed within the synthetic microbial mix, it provides hope that these pathogenic strains may not thrive when administered as part of a therapeutic consortium.

By carefully monitoring the interactions between pathogenic and beneficial bacteria in the synthetic consortium, researchers can gain insights into the competitive exclusion mechanisms that may occur in a healthy gut environment. This knowledge can inform the selection of specific bacterial strains and their proportions in the final therapeutic consortium, ensuring that beneficial microbes outcompete potential pathogens and maintain a stable, healthy gut microbiome. The inclusion of pathogenic bacteria serves as a model to study the suppressive effects of a synthetic consortium, but any pathogenic strains must be removed before clinical application to ensure patient safety. The ultimate goal is to develop a defined, non-pathogenic microbial mixture that can effectively restore gut homeostasis and prevent the overgrowth of opportunistic pathogens.

We developed a synthetic bacterial community composing of 9 bacteria representing the major bacterial phyla in human faeces to understand their functional capacity and bacterial count changes in response to the presence of dietary substrates. Functional behaviour was

monitored using an untargeted metabolomic approach, using $^{1}$H-NMR spectroscopy to obtain a "fingerprint" of metabolites produced by the bacteria during the in vitro experiments. Untargeted metabolic profiling is a promising strategy to unravel the functional dynamics of gut microbiota. Monitoring bacterial counts provides understanding of interactive effects of bacteria in relation to suppression of pathogens. This study aimed to monitor the functional behaviour and bacterial enumeration of a synthetic bacterial consortium compared to human faeces in pH controlled in-vitro fermentations with different substrates.

## 4.2 Materials and methods

### *4.2.1 In-vitro batch culture fermentation*

Faecal sample preparation, nutrient rich medium preparation and pH controlled, stirred batch culture fermentation was performed as described in 2.2,2.4.2, 2.4.3 in Chapter 2. For each donor, 4 different substrates were prepared, namely starch, inulin, probiotic and probiotic yoghurt with $10^{9}$ cell/ml probiotic. One vessel was set up as the control with no added substrate. All vessels were inoculated with 15 mL of a 10% (w/v) faecal slurry (diluted with PBS). A sample (6 mL) was removed from each substrate vessel at 0, 12, 24 and 48 hours of incubation. Collected samples were stored at -20$^{o}$C for FC-FISH bacterial enumeration and $^{1}$H-NMR spectroscopy.

### *4.2.2 Developing synthetic bacterial consortium*

FC-FISH analysis was performed on 4 human donors at t0 to determine the composition of the human faeces (Pie chart A Figure 4.1). Based on these results the bacteria and proportions for the synthetic mix was determined. *Bacteroides fragilis* NCTC 9343 (Bacteroidetes), *Bifidobacterium longum* NCTC11818 (Actinobacteria), *Clostridium perfringens* NCTC8678 (Firmicutes), *Lactobacillus rhamnosus* NCTC10302 (Firmicutes), *Collinsella aerofaciens* NCTC11838 (Actinobacteria), *Escherichia coli* NCTC 1093 (Proteobacteria), *Ruminococcus bromii* ATCC 51896 (Firmicutes), *Roseburia intestinalis* DSM 14610 (Firmicutes) and *Faecalibacterium prausnitzii* DSM 17677 (Firmicutes) were the selected bacteria representing the major phyla. Freeze dried pure cultures of the selected bacteria were obtained from

culture collections of Public Health England (PHE), DSMZ-German Collection of Microorganisms and Cell Cultures and American Type Culture Collection ATCC -LGC. The freeze-dried cultures obtained were reconstituted according to instructions provided by the suppliers and preserved in glycerol stocks to be used in future. These 9 bacteria were grown anaerobically in a common nutrient rich medium (described in Chapter 2) in Hungate tubes and their growth phases were monitored by FC-FISH and culture plates (Figure 4.2). According to the growth curves obtained the maximum growth of the bacteria were captured around 24h for all bacteria (Figure 4.3). However, during the time of the enumeration it was revealed that the Erec 482 probe captures bacteria belonging to *Clostridium* XIVa and XIVb and it overlapped with *Ruminococcus bromii* and *Roseburia intestinalis* ATCC33656 (Hold et al. 2003). Therefore, during preparation of the bacterial mix, this was taken into account and the EREC 482 probe was not used. Considering the exclusion of EREC 482 probe, the extra bacteria captured was labelled as 'others' in the Figure 4.1. this proportion was equally distributed among the 9 others. Then, the 9 bacteria (each bacteria at their maximum growth phase) were mixed according to the proportions based on the results from FC-FISH results of batch culture fermentation of human faeces.

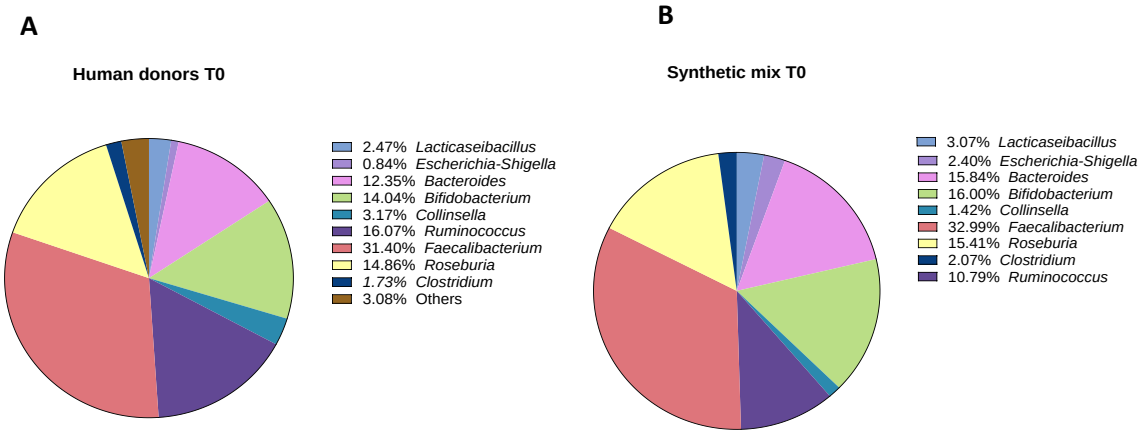



***Figure 4.1:*** *Composition of bacterial groups in human faeces based on FC-FISH analysis (A) and the percentage for the bacteria in the synthetic mix (B)*

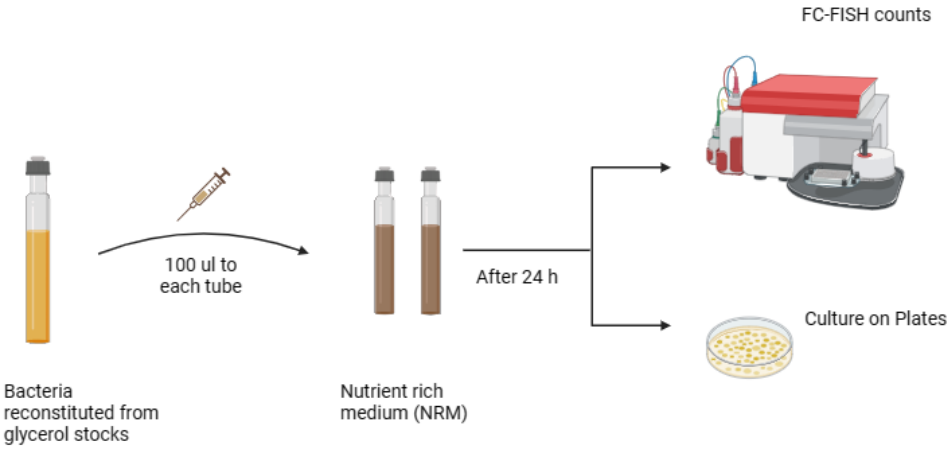



***Figure 4.2:*** *Method of bacterial reconstruction and monitoring growth in a nutrient rich medium*

Images of cultured bacterial colonies shown in Appendix 4.1

Next pH controlled, stirred batch culture fermentation as described in 2.4.3 Chapter 2 was performed in triplicate using the synthetic bacterial mix instead of human faeces Figure 4.4.

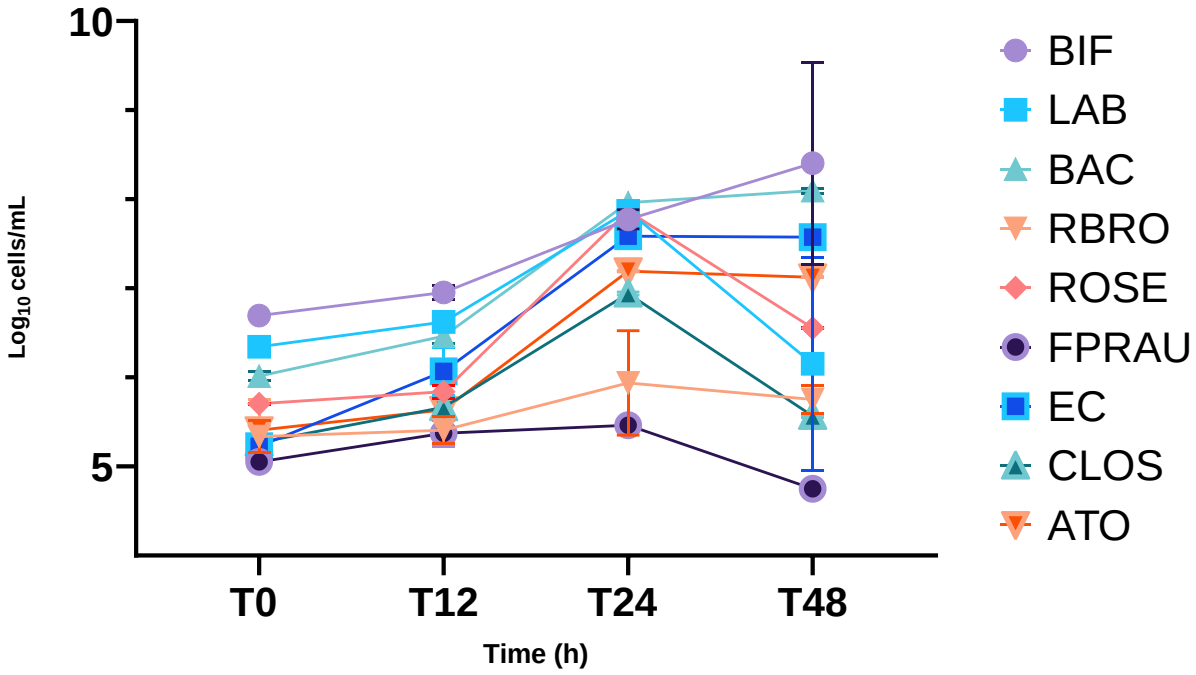



***Figure 4.3:*** *Growth curves of the bacteria (measured individually) in a nutrient rich medium, enumerated using FC-FISH*

### *4.2.3. FC-FISH enumeration*

Bacterial populations were assessed by FC-FISH with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA, as previously described (Costabile 2010). The commercially synthesised probes used in this study are listed in the table 2.1 Chapter 2.

### *4.2.4. 16S rRNA sequencing*

Bacterial DNA was extracted from batch culture sample pellets using the QIAamp PowerFecal Pro DNA Kit (QIAGEN) according to the manufacturer's instructions. DNA samples were sent to Novogene Europe (Cambridge,UK) for 16S rRNA gene sequencing. 16S rRNA genes of 16S V4-V5 regions were amplified using specific primers (GTGCCAGCMGCCGCGGTAA, CCGTCAATTCCTTTGAGTTT). All PCR reactions were carried out with 15 µL of Phusion® High - Fidelity PCR Master Mix (New England Biolabs); 0.2 µM of forward and reverse primers, and

about 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98℃ for 1 min, followed by 30 cycles of denaturation at 98℃ for 10 s, annealing at 50℃ for 30 s, and elongation at 72℃ for 30 s and 72℃ for 5 min. The same volume of 1X loading buffer was mixed with PCR products and electrophoresis was operated on 2% agarose gel for detection. PCR products were mixed in equidensity ratios. Then, the mixture of PCR products were purified. Sequencing libraries were generated and indexes added. The library was checked with Qubit and real-time PCR for quantification and a bioanalyser for size distribution detection. Quantified libraries were pooled and sequenced on an Illumina sequencing platform, according to effective library concentration and data amount required. Paired- end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. The amplicon was sequenced on Illumina paired-end platform to generate 250 bp paired-end raw reads (Raw PE), and then merged using FLASH (V1.2.11) quality filtered using fastp (Version 0.23.1) and pre-treated to obtain Clean Tags (Bokulich et al. 2018). Chimeric sequences in Clean Tags were detected and removed to obtain the Effective Tags which can be used for subsequent analysis. The effective tags were then finally obtained. The Divisive Amplicon Denoising Algorithm 2 (DADA2) method (Callahan et al. 2016) was used for noise reduction. Each de-duplicated sequence generated after noise reduction using DADA2 is called ASVs (Amplicon Sequence Variants). Next, species annotation was performed using QIIME2 software. By applying QIIME2's classify-sklearn algorithm (Bolyen et al. 2019; Bokulich et al. 2018). Annotation database of the project was Silva 138.1. According to the results of ASVs annotations, the species abundance tables at the level of kingdom, phyla, class, order, family, genus, and species were obtained. These results were imported into GraphPad Prism (version 10, USA) for further analysis.

### *4.2.5. Analysis of metabolic profiles using $^{1}$H-NMR spectroscopy*

For $^{1}$H NMR spectroscopic analysis, fermentation samples (0.5mL) collected from batch cultures that had been stored at -20°C pending analysis, were thawed performed as described in Chapter 2.

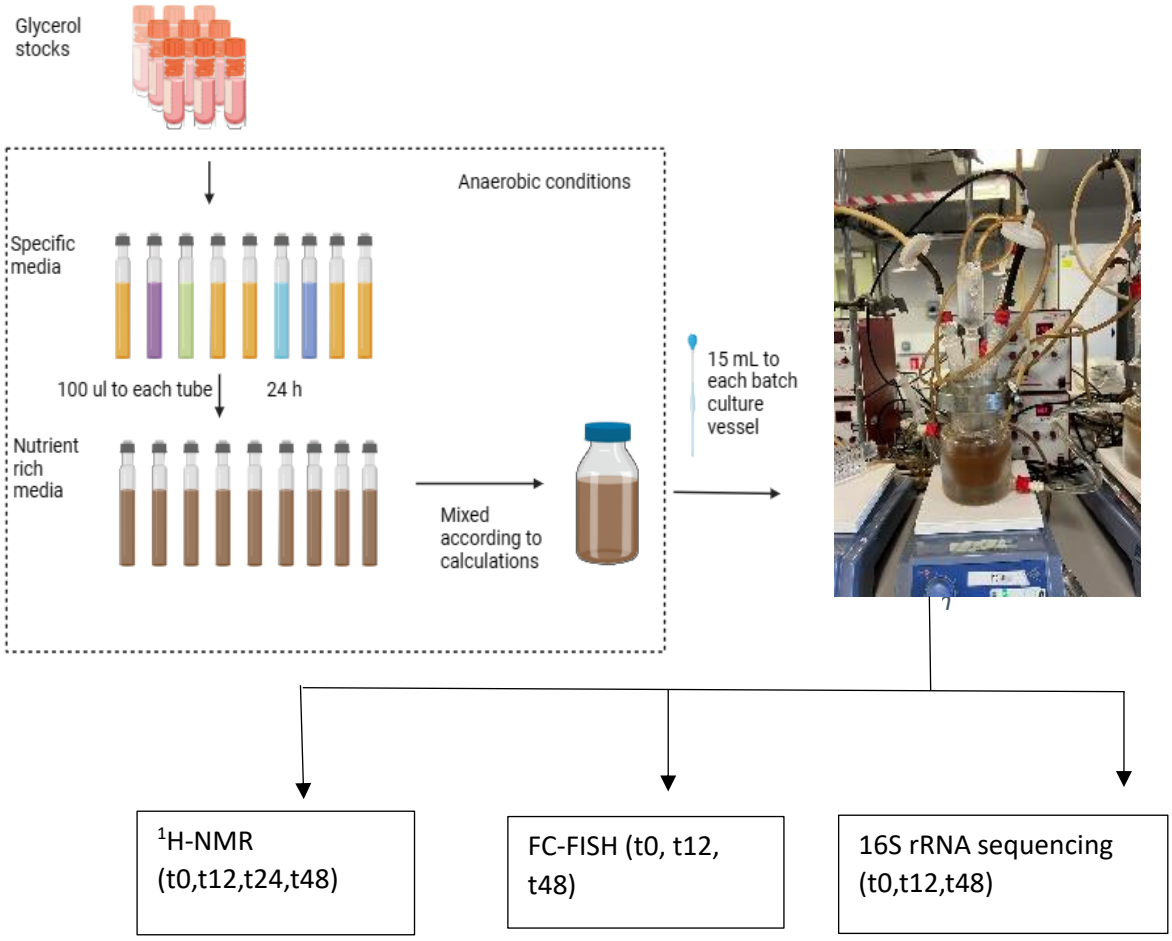



***Figure 4.4:*** *Experimental steps for batch culture fermentations and sample collection*

### *4.2.6. Statistical analysis*

Statistical analysis was carried out using GraphPad Software [version 9.5.1 (733) San Diego, California USA]. A two-way repeated measure ANOVA was used to determine significant differences in microbiota populations and substrates between 0 h and subsequent time points (T0, T24 and T48). Post-hoc Tukey test was used to determine differences between treatments at the same time points. Differences are stated as statically significant at .12 (ns), 0.05 (*), 0.01 (**), and < 0.001 (***).

## 4.3 Results

### *4.3.1. Bacterial enumeration*

To determine changes in bacterial populations, both FC-FISH and 16S rRNA sequencing were used. For FC-FISH, twelve 16S rRNA FISH probes were used to identify changes in the numbers of total bacteria and 10 specifically targeted microbial groups. Results of total bacterial counts during batch fermentation of human faeces and the newly developed synthetic bacterial mix, with different dietary substrates are shown in Figure 4.5. Initial counts of the synthetic mix were low, but had reached similar numbers as the human donors by the end of fermentation. In the human donors, bacterial counts were observed to drop at 48 h while the synthetic mix continued to increase.

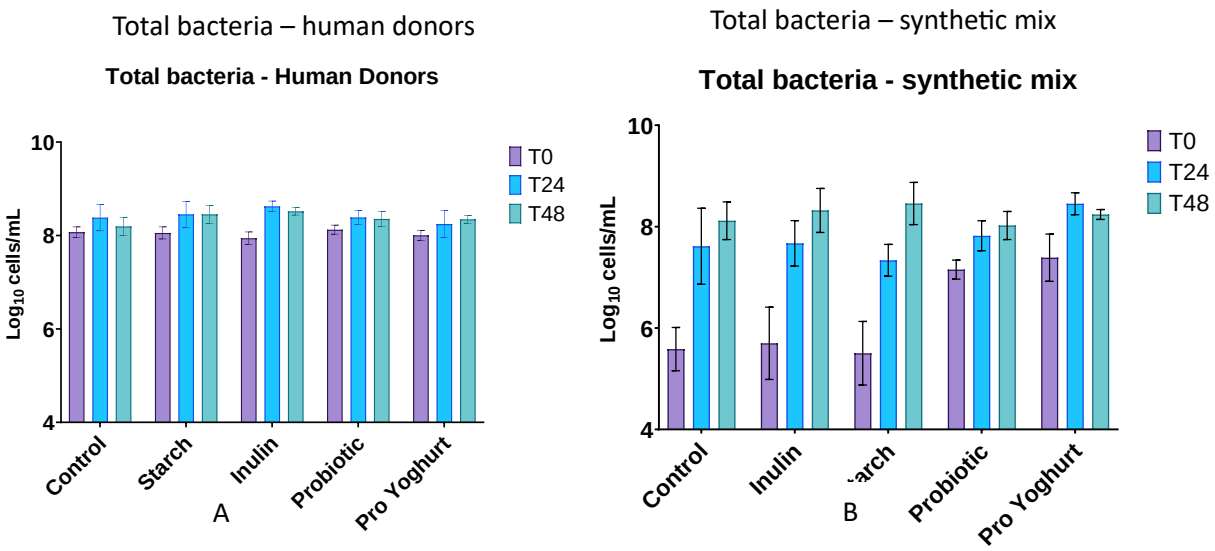



***Figure 4.5:*** *Total bacterial counts Log10 cells/ml with different substrates of human faeces (A) and synthetic bacterial mix (B)*

The counts of specific bacterial functional groups in both human donor and synthetic communities were examined using FC-FISH enumeration, providing detailed insights into population dynamics. Figures 4.6 to 4.14 illustrate changes in these specific bacterial populations. Significant differences at T48 are only shown in the figures.

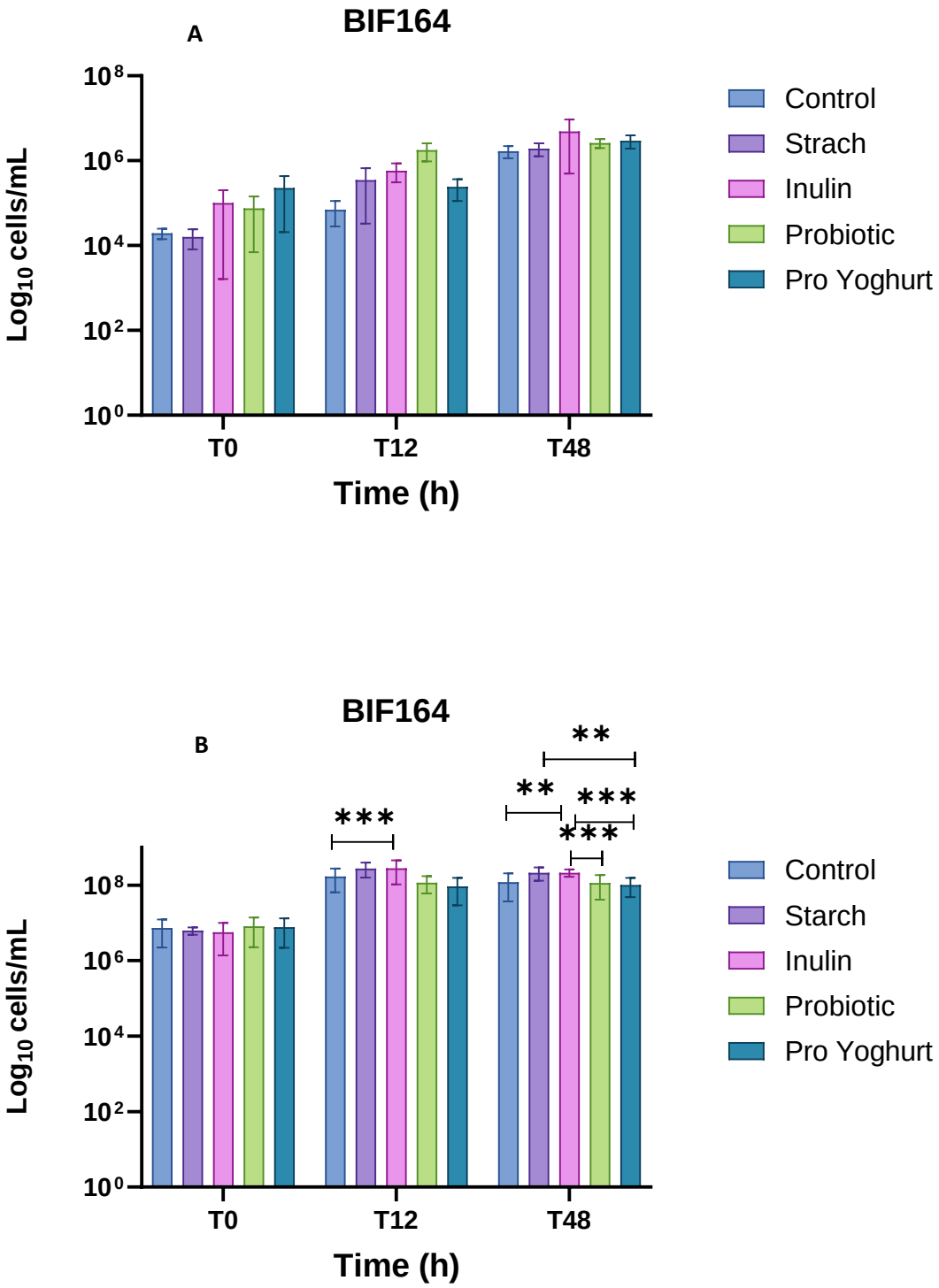



***Figure 4.4:*** Bacteria measured by FC-FISH (Log 10 cells/ml) using (Bif 164) probe Bifidobacterium spp. at 0, 12 and 48 h. Mean and SE (all data points; n = 3) in, **A**- Human faeces and **B**- Synthetic mix

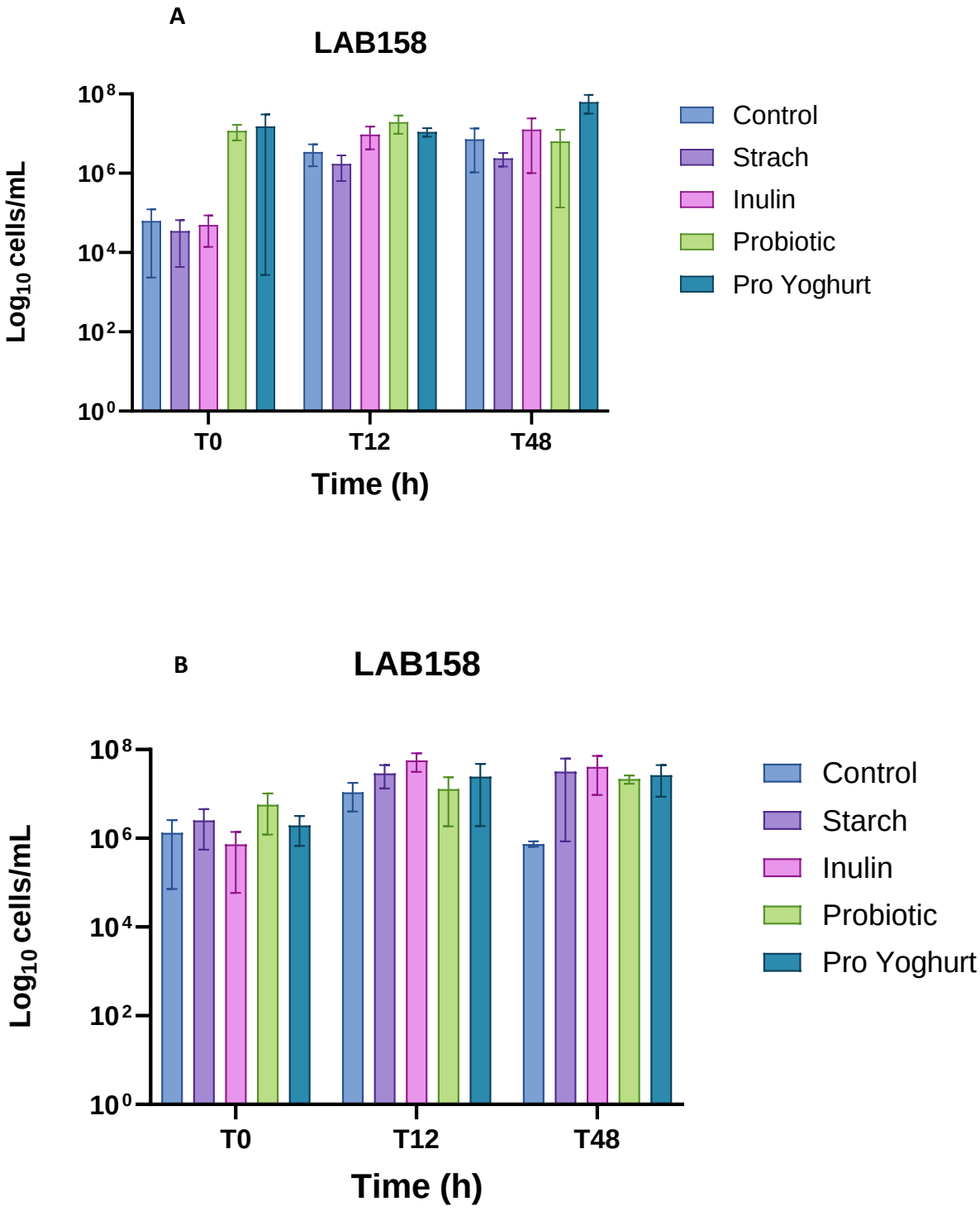



***Figure 4.7:*** *Bacteria measured by FC-FISH (Log 10 cells/ml) using (Lab 158) probe Lactobacillus/Enterococcus spp. at 0, 12 and 48 h. Mean and SE (all data points; n = 3) in* ***A****- Human faeces and* ***B****- Synthetic mix*

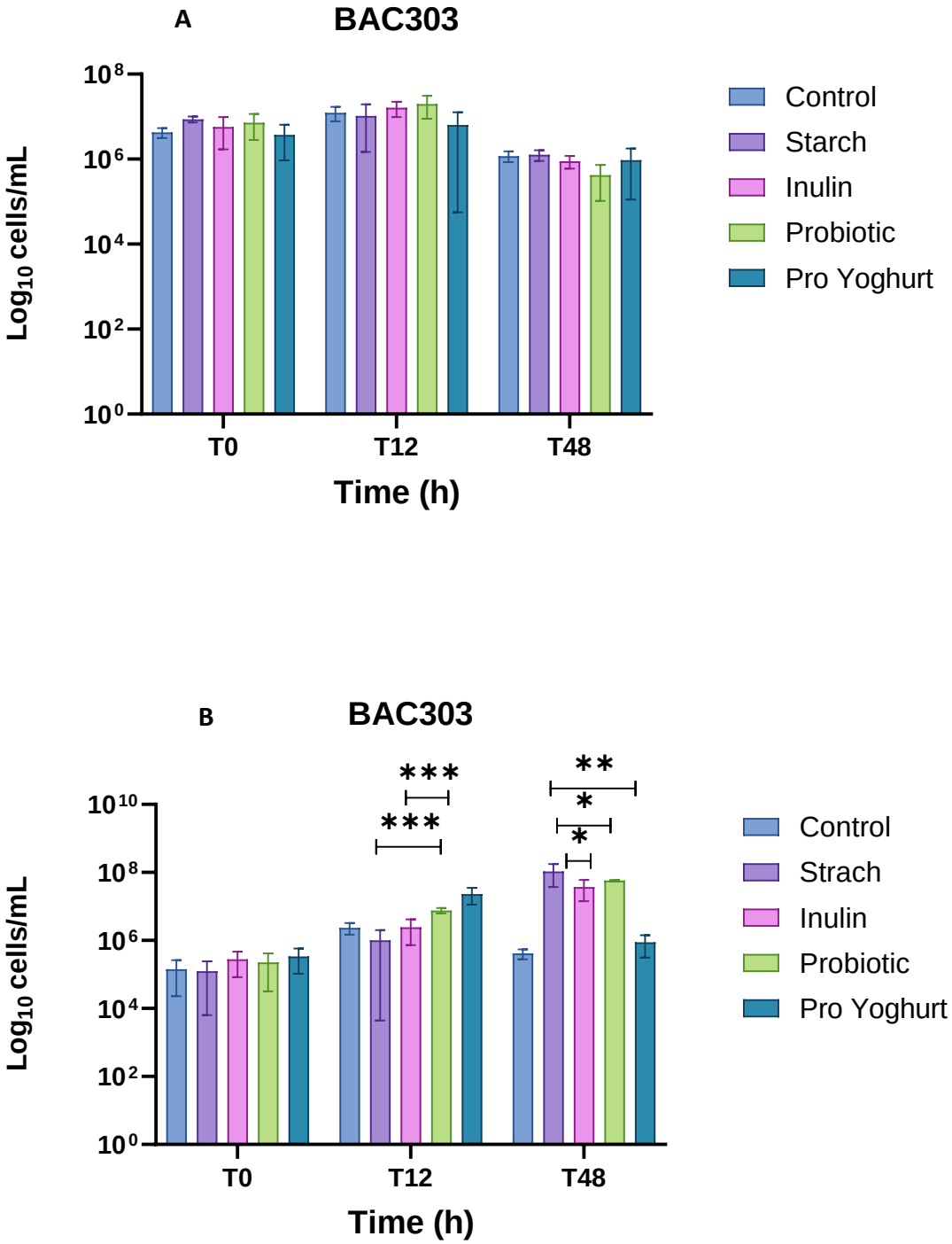



***Figure 4.8:*** *Bacteria measured by FC-FISH (Log 10 cells/ml) using (Bac 303) probe Bacteroidaceae spp. at 0, 12 and 48 h. Mean and SE (all data points; n = 3) in A- Human faeces B-Synthetic mix*

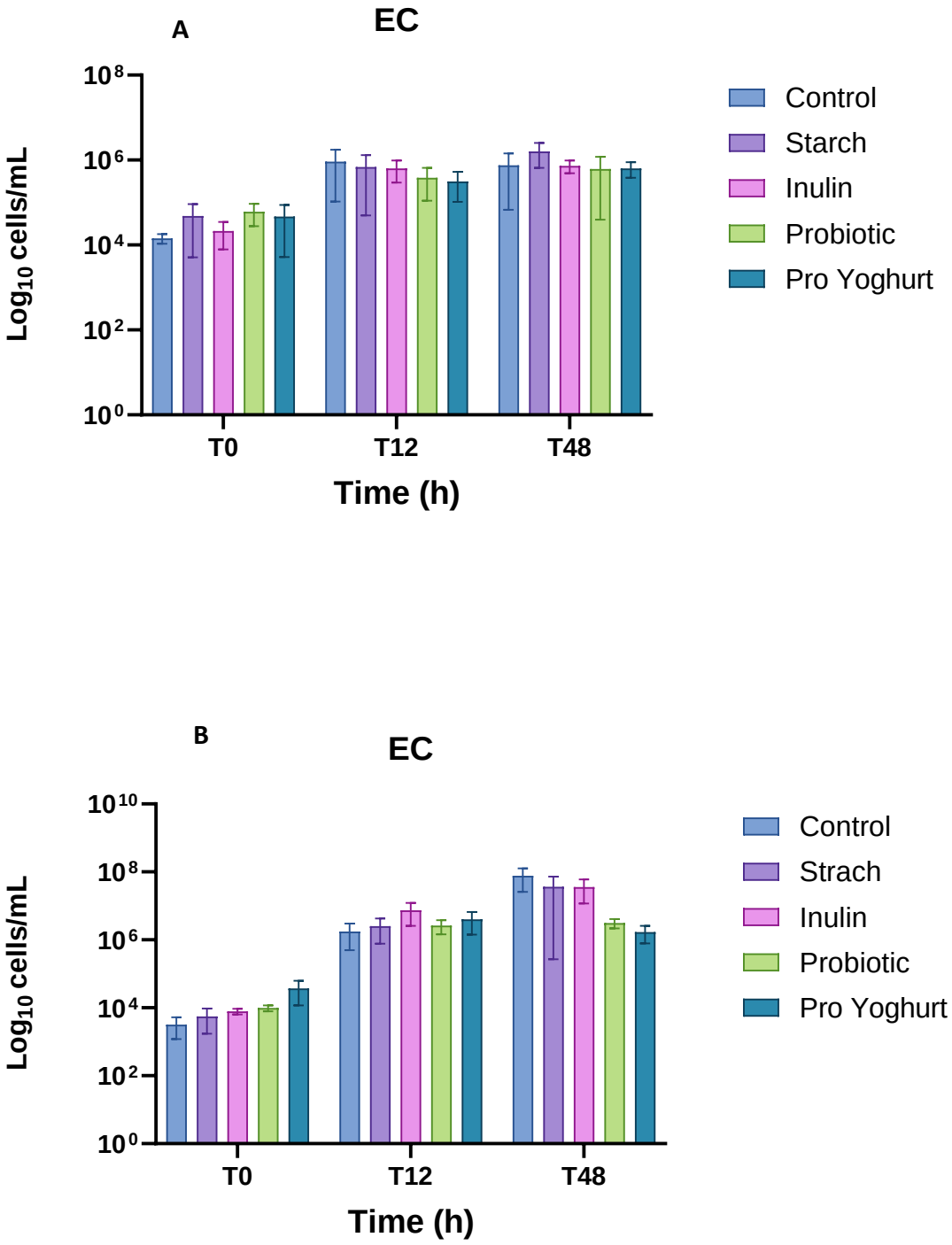



***Figure 4.9:*** *Bacteria measured by FC-FISH (Log 10 cells/ml) using (EC1531) probe Escherichia spp. at 0, 12 and 48 h. Mean and SE (all data points; n = 3) in A- Human faeces, B-Synthetic mix*

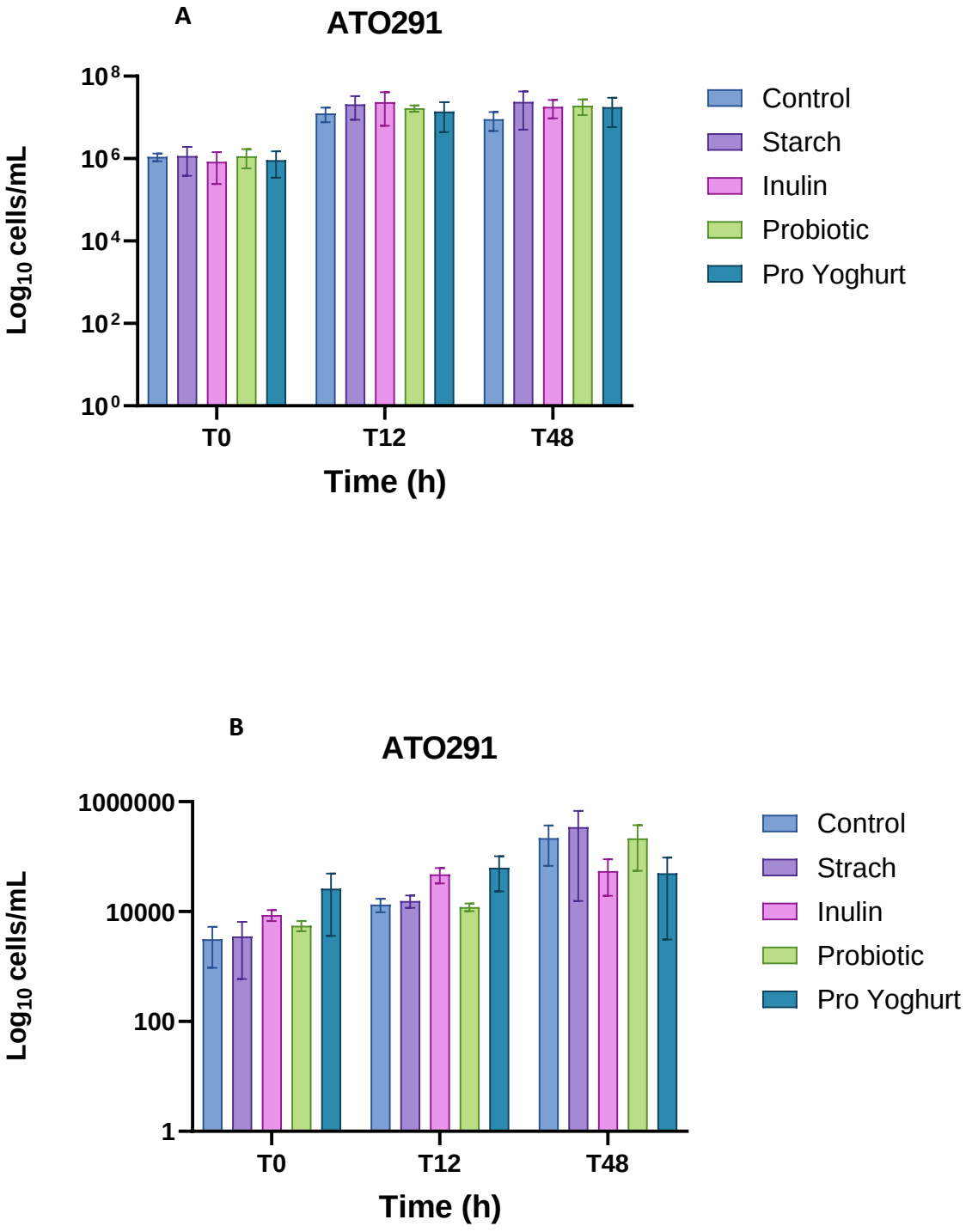



***Figure 4.10:****Bacteria measured by FC-FISH (Log 10 cells/ml) using (ATO291) probe Atopobium cluster at 0, 12 and 48 h. Mean and SE (all data points; n = 3) in A- Human faeces, B-Synthetic mix*

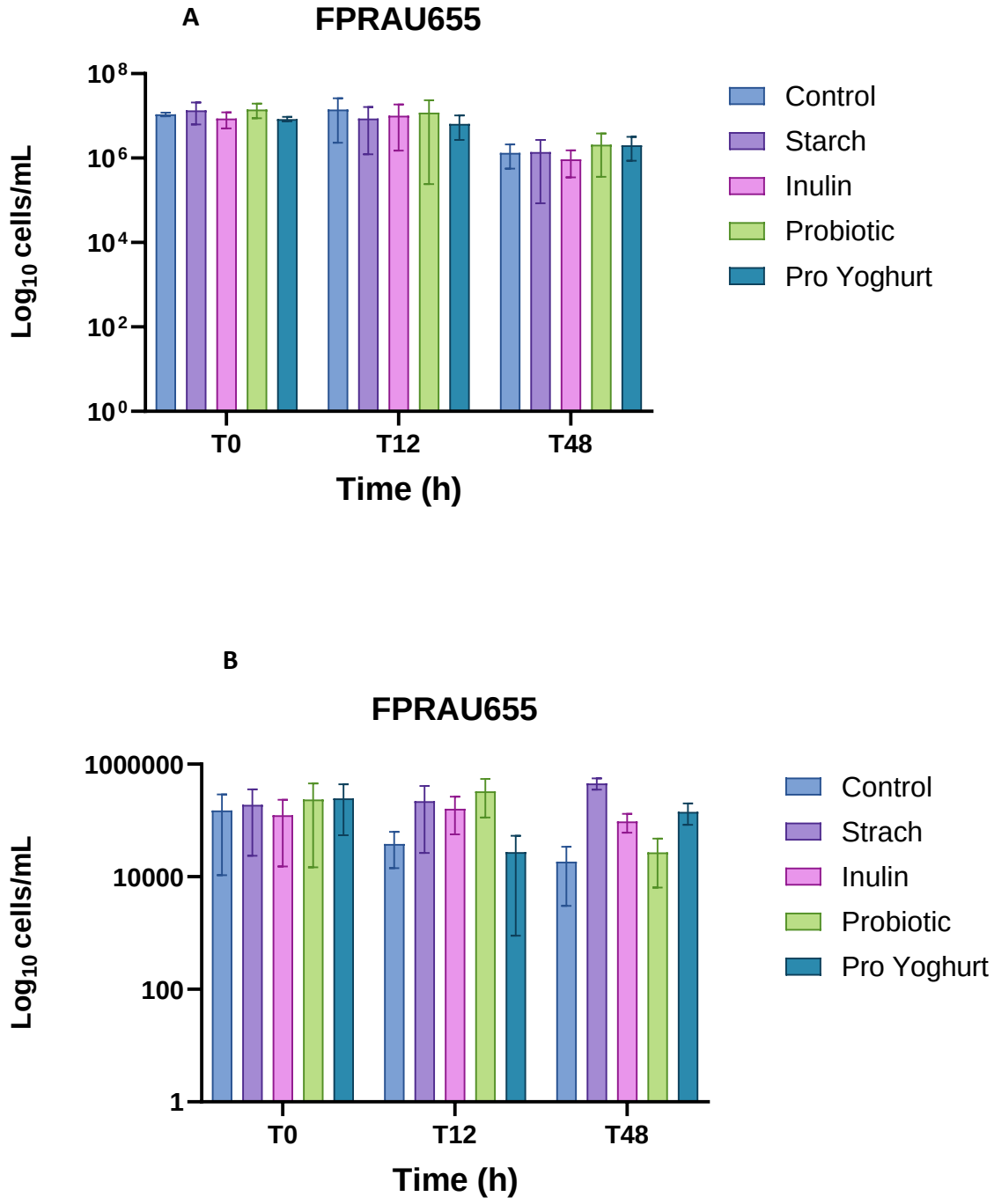



***Figure 4.11:*** *Bacteria measured by FC-FISH (Log 10 cells/ml) using (FPRAU655) probe Faecalibacterium prausnitzii at 0, 12 and 48 h. Mean and SE (all data points; n = 3) in A-Human faeces, B-Synthetic mix*

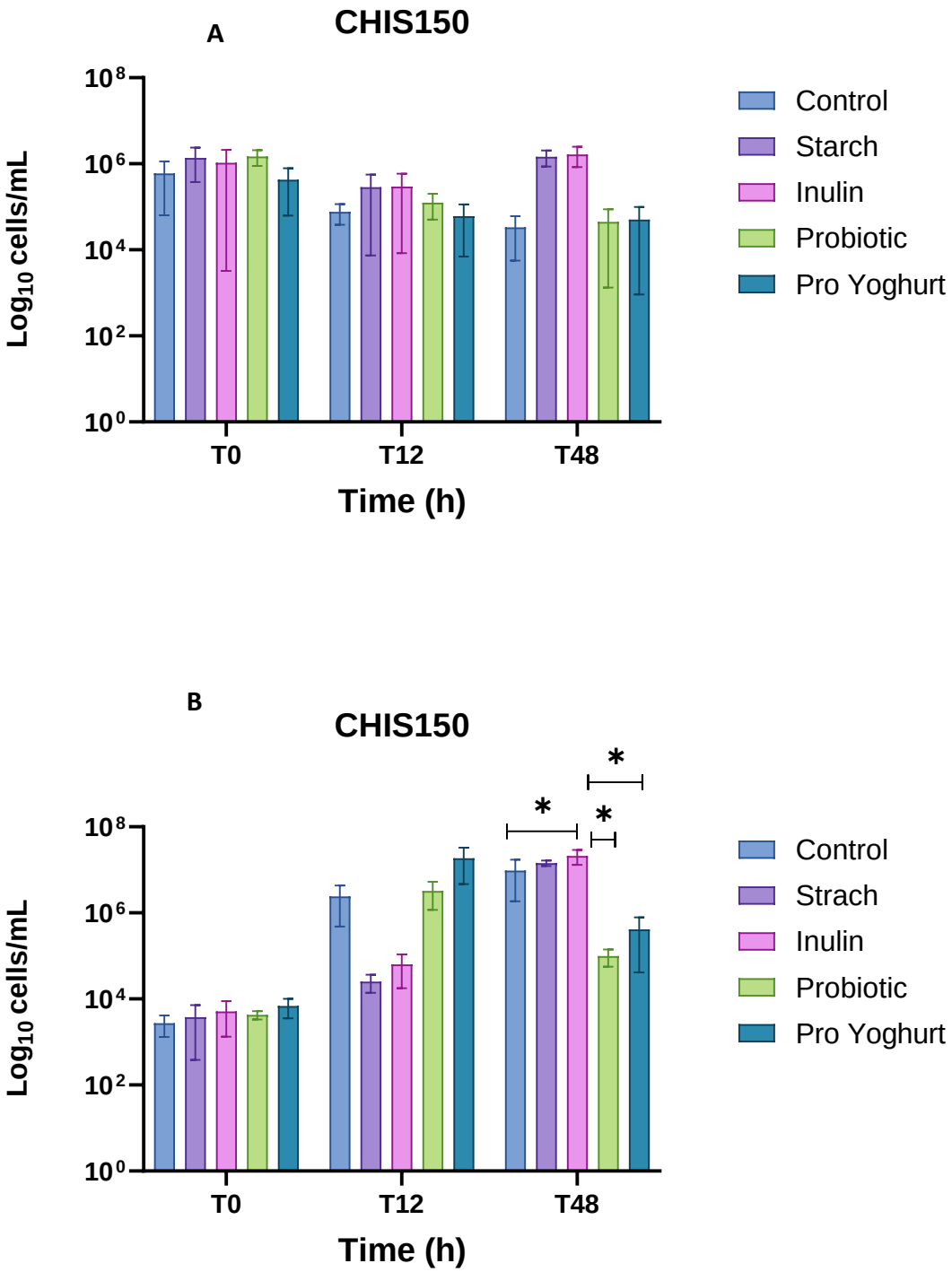



***Figure 4.12:*** *Bacteria measured by FC-FISH (Log 10 cells/ml) using (CHIS150) probe Most of the Clostridium histolyticum group (Clostridium cluster I and II) at 0, 12 and 48 h. Mean and SE (all data points; n = 3) in A-Human faeces, B-Synthetic mix*

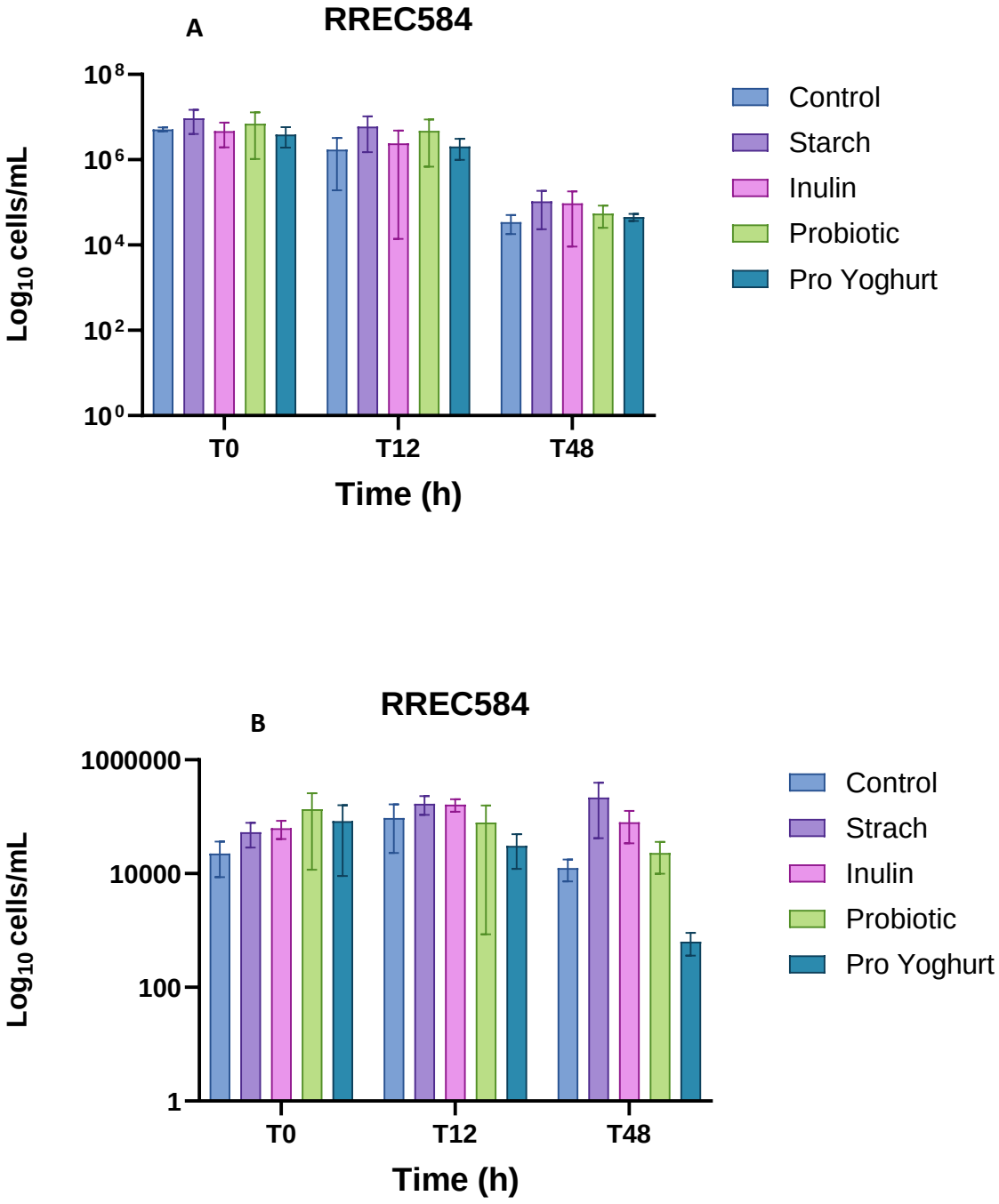



***Figure 4.13:*** *Bacteria measured by FC-FISH (Log 10 cells/ml) using (RREC584) probe Roseburia genus at 0, 12 and 48 h. Mean and SE (all data points; n = 3) in A- Human faeces, B-Synthetic mix*

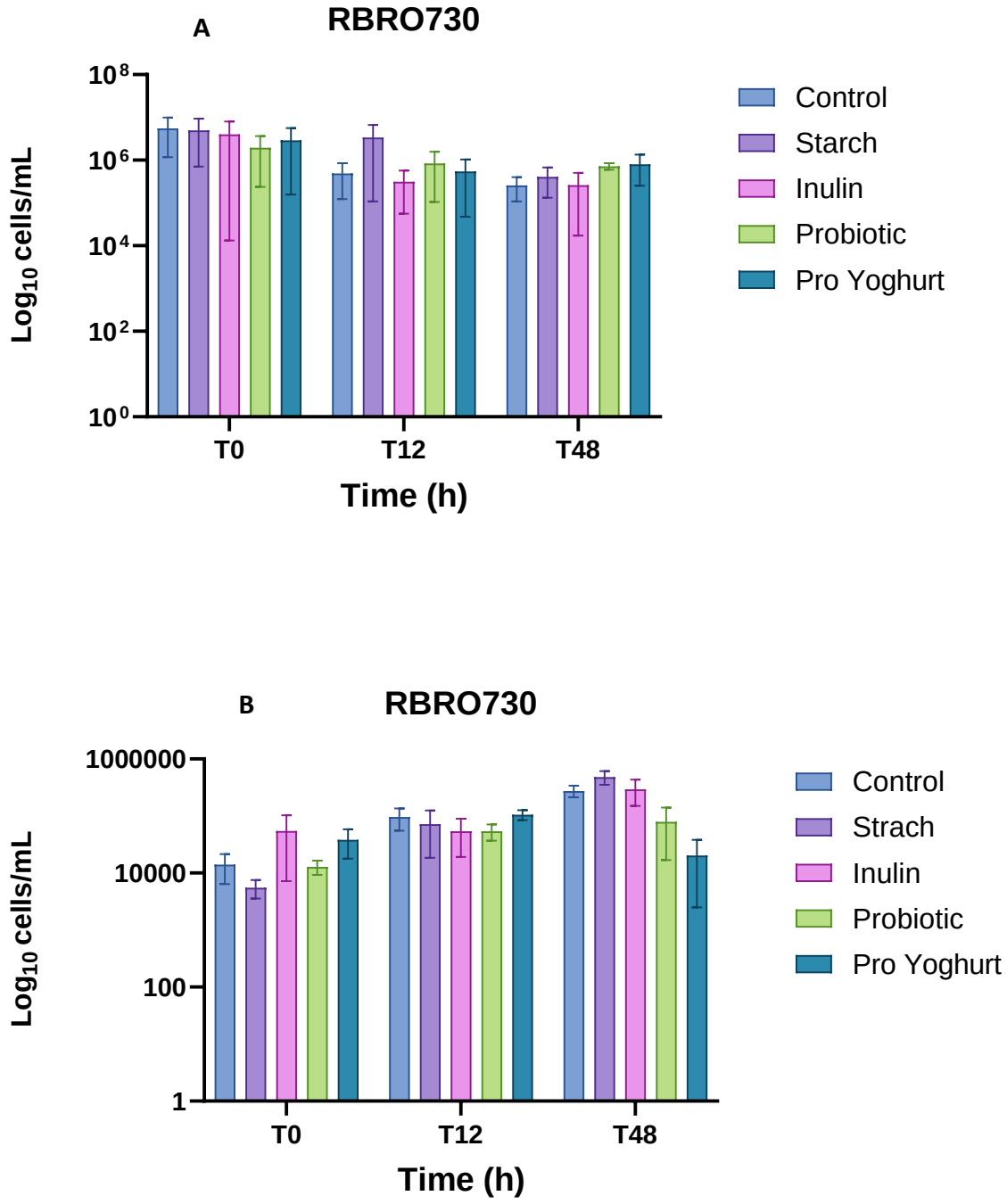



***Figure 4.14:*** *Bacteria measured by FC-FISH (Log 10 cells/ml) using (Rbro730) probe Ruminococcus bromii at 0, 12 and 48 h. Mean and SE (all data points; n = 3) in A-Human faeces, B-Synthetic mix*

Statistical analyses were conducted on pre-logged values, and data were represented on a log scale to ease visualisation. Analysis via two-way ANOVA revealed noteworthy changes primarily within the bifidobacterial genus in human samples (Figure 4.6). Notably, bifidobacteria exhibited a substantial increase by the 12 h of fermentation across all vessels, particularly dominating in the control, starch, and inulin vessels, unlike other tested groups that did not show significant changes. Figures 4.6-4.14 shows changes in bacterial counts categorised by specific bacteria in human faeces vs synthetic mix.

Initially, bifidobacteria (Figure 4.6 A) showed no detectable differences at T0 across substrates. However, by T12, notable changes emerged, particularly between the control and inulin vessels (***$p<0.001$), signifying increased growth in the latter. Further, at T12 significant differences were observed between control and probiotic yoghurt (*$p=0.028$), with the latter exhibiting decreased growth. Significant differences in bifidobacteria continued at the 48 h of fermentation, showing increased growth between control and inulin (**$p=0.002$), control and starch (*$p=0.040$), starch and probiotic (*$p=0.015$), starch and probiotic yoghurt (**$p=0.002$), inulin and probiotic (***$p<0.001$), and inulin and probiotic yoghurt (***$p<0.001$). The probiotic yoghurt vessel demonstrated significant changes between T0 and T12 (**$p=0.009$), as well as T0 and T48 (***$p<0.001$), showing an increase in bifidobacteria populations within this specific environment.

In contrast to human faeces, the synthetic mix (Figure 4.6 B) exhibited notable changes in several tested bacterial genera, including bifidobacteria, bacteroides, and clostridia. Figure 4.8 shows significant changes in bacteroides at both T12 and T48, and for clostridia Figure 4.12. Changes in bifidobacteria were only observed within the starch vessel.

At the end of 12 h fermentation, significant differences were noted between starch and probiotic (***$p<0.001$), as well as inulin and probiotic (***$p<0.001$). However, there was observed growth in bacteroides within the probiotic vessels, although this difference diminished by the end of fermentation. The starch vessel consistently exhibited the highest increase in bacteroides at the end of 48 h. Additional significant changes were observed between starch and inulin at T48 (*$p=0.028$), starch and probiotic (*$p=0.048$), and starch and probiotic yoghurt (**$p=0.002$).

Significant changes in the clostridial genus were observed in the inulin vessel. By the end of fermentation, the inulin vessel reported high clostridia levels, which were significant in inulin vs. control (*p=0.030), inulin vs. probiotic (*p=0.010), and inulin vs. probiotic yoghurt (*p=0.013). Notably, the probiotic vessels appeared to exhibit a suppression of clostridia compared to the inulin vessel, suggesting a potential modulatory effect of the live microbial intervention.

Comparing the FC-FISH data from human and synthetic mix samples revealed significant changes in bacteroides, bifidobacteria, and clostridia. *Bacteroides* spp. exhibited notable changes at T12 between the starch (p=0.008) and probiotic yoghurt (**p=0.002) vessels. Bifidobacteria showed changes at T12 between the control vessel (***p<0.001), starch (***p<0.001), inulin (***p<0.001), probiotic (***p<0.001), and probiotic yoghurt (*p=0.023) vessels. However, by the end of fermentation, bacteroides did not exhibit any significant differences, whereas bifidobacteria and clostridia did vary. The difference in bifidobacteria persisted between the control vessel of human and synthetic mix (***p<0.001), probiotic (*p=0.017), and probiotic yoghurt (*p=0.034) vessels. Clostridia showed significant differences in the inulin vessel (*p=0.011)(Figure 4.15).

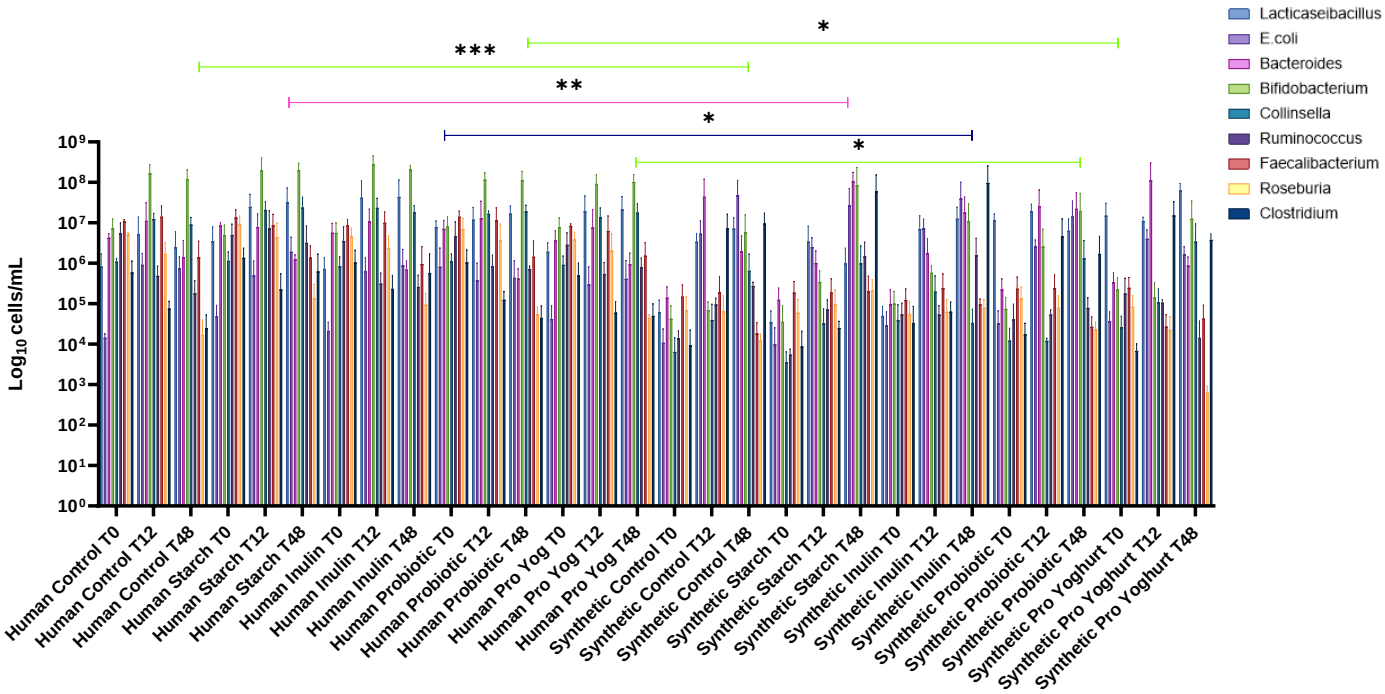



***Figure 4.15:*** *FC-FISH enumeration Log10 cells/mL of both human faeces and synthetic mix*

With the aim of understanding the contributions of different bacterial groups in human faeces, 16S rRNA sequencing was initially conducted. However, given that the data was expressed in abundances (Figures shown in appendix 4.2), quantitative microbial profiling (QMP) was performed to facilitate convenient comparisons with FC-FISH data.

### *4.3.2. Quantitative microbial profiling (QMP)*

Upon statistical analysis of the QMP results from human faecal samples, notable changes were observed in lactocaseibacilli, in addition to bacteroides and bifidobacteria (Figure 4.16-4.24). Mainly, the probiotic vessels exhibited a significant increase in lactocaseibacilli compared to the control vessel, probiotic (*p=0.028), and probiotic yoghurt (*p=0.025) vessels. This finding is particularly significant as 16S data did not detect any lactocaseibacilli in the control vessel of human faeces. Notably, FC-FISH enumeration indicated an increase in lacticaseibacilli numbers over time, although this increase was not statistically significant. However, at T0, the control, starch, and inulin vessels showed a significant difference in probiotic lacticaseibacilli compared to the added lacticaseibacilli, as per the experimental protocol (***p<0.001, **p=0.002, and ***p<0.001, respectively). Interestingly, FC-FISH analysis did not identify this difference as significant. Regarding bifidobacteria, both starch and inulin demonstrated increased growth compared to the probiotic substrates. At the end of fermentation, all control, probiotic, and probiotic yoghurt vessels showed significantly lower bifidobacterial levels compared to inulin (***p<0.001). In the starch vessel at T48, significant changes were observed between probiotic (**p=0.008) and probiotic yoghurt (*p=0.036). This trend of inulin and starch exhibiting a strong response to bifidobacteria compared to probiotic substrates was consistent with the FC-FISH analysis. Bacteroides also exhibited significant changes, notably between inulin and probiotic vessels at T12 (**p=0.007), inulin vs probiotic yoghurt (p<0.001) and within the control and inulin vessels over time (control vessel T12 vs T48, *p=0.011; inulin T12 vs T48, ***p<0.001). Additionally, other bacterial groups exhibited changes within the inulin vessel, notably at T0 vs T12 (*p=0.018) and T0 vs T48 (*p=0.015).

Similarly, QMP was conducted on the synthetic mix (Figure 4.10) and the statistical analysis focused on differences in lactocaseibacilli and bacteroides. Consistent with the FC-FISH results, no significant difference was observed in bifidobacterial levels among the different

vessels. Although FC-FISH indicated an increase within the starch vessel, QMP did not detect any significant changes within any vessel; however, there was an overall increase in bifidobacteria across all vessels. Lacticaseibacilli showed significant differences (***p<0.001) at T12 between control and probiotic, starch and probiotic, and inulin and probiotic vessels, with the probiotic vessel exhibiting highest growth. However, these differences were not observed in the FC-FISH analysis. Similarly, bacteroides displayed significant differences at T48 between control and starch (***p<0.001), starch and inulin (**p=0.006), and starch and probiotic (***p<0.001), in line with the FC-FISH findings. Bacteroides appeared to be the most responsive bacterial group in the starch vessel in the synthetic mix, as indicated by both methods. Analysing the QMP results of human faeces and synthetic mix together revealed noticeable changes in lactobacilli and bifidobacteria (Figure 4.25). Interestingly, only the probiotic yoghurt vessel showed a significant difference (***p<0.001) between human and synthetic mix at T12 in the lacticaseibacilli group, and by the end of fermentation, this difference diminished. Statistically, all other comparisons did not show any significant differences in terms of bacterial changes. Bifidobacteria displayed significant changes between human starch vs synthetic starch at T12 (**p=0.002) and T48 (***p<0.001), as well as human inulin vs synthetic inulin at T48 (***p<0.001). These findings diverged from the FC-FISH analysis, which showed differences in bacteroides, whereas QMP captured differences in lacticaseibacilli; however, both methods detected differences in bifidobacteria. QMP detected changes only in starch and inulin vessels, while FC-FISH detected changes not only in starch and inulin but also in both probiotic containing vessels. The comprehensive analysis of bacterial populations using FC-FISH and QMP of 16S rRNA sequencing provided insights into microbial dynamics within human faeces and a newly developed synthetic mix of bacteria, using different substrates. Significant changes were observed in key bacterial genera such as lacticaseibacilli, bifidobacteria, bacteroides, and clostridia, highlighting complex interconnections in different microbial communities with substrates and microbial responses. While FC-FISH and 16S rRNA sequencing yielded divergent results in some instances, the two methods aligned in capturing changes in lacticaseibacilli and bifidobacteria. However, the comparison of relative abundance given by the 16S data did not offer a clear interpretation of the two populations. Specifically, data from the synthetic mix was confounded by a high abundance of *Escherichia shigella* which was not captured as significant in QMP conversion process nor aligned with the experimental protocol.

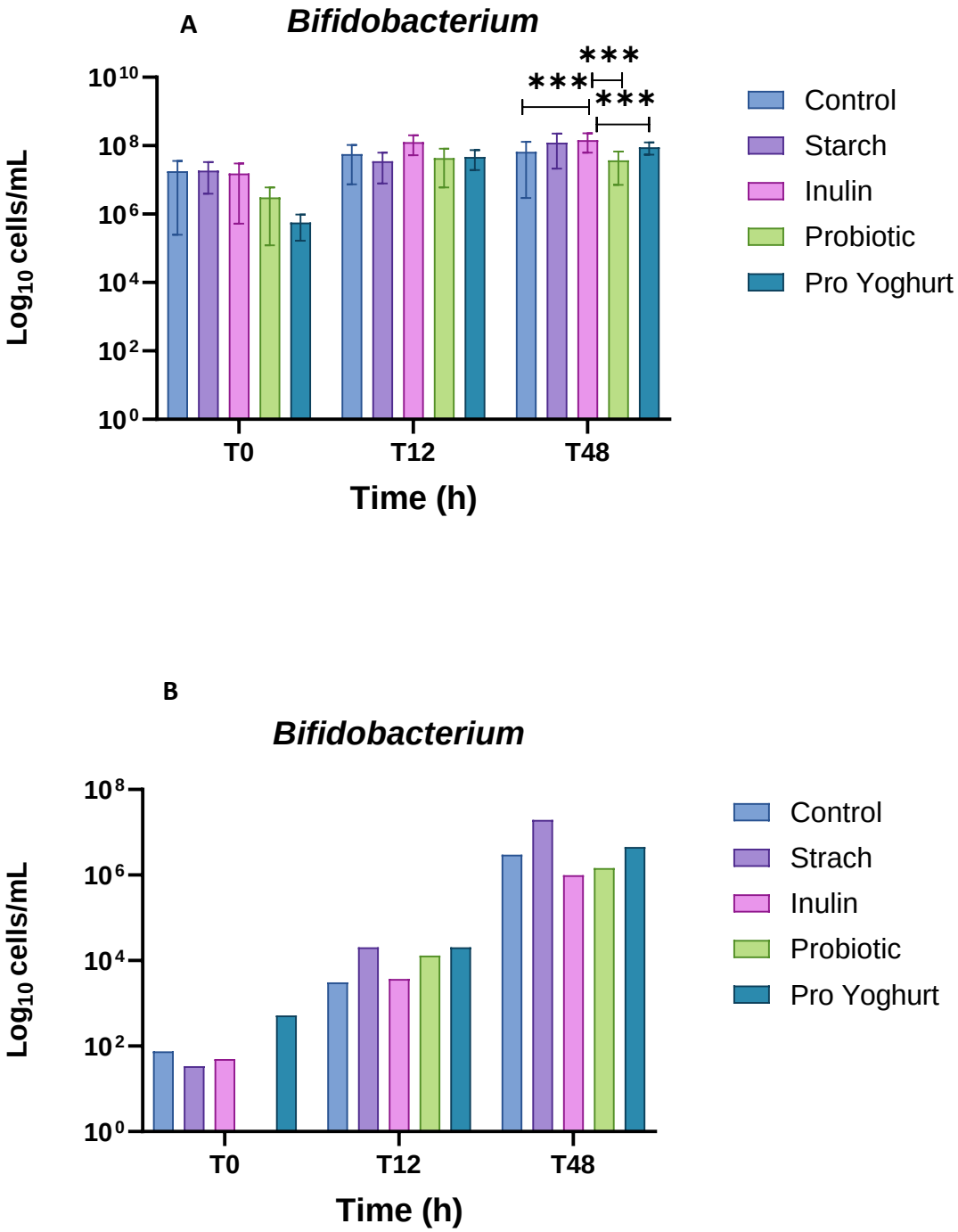
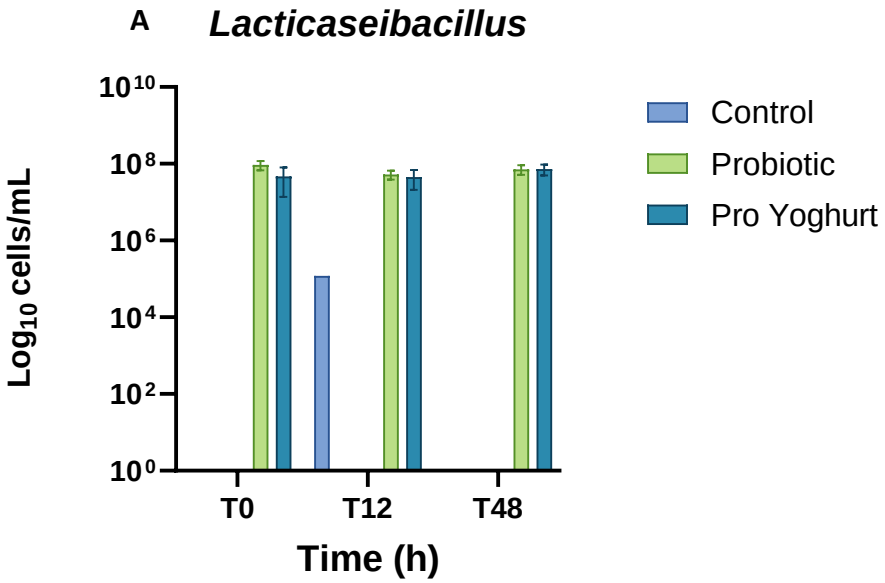



***Figure 4.16:*** *QMP of Bifidobacterium spp. in 16S rRNA Sequencing converted to Log10 cells/mL at 0, 12 and 48 h in A- Human faeces, B- Synthetic mix. Mean and SE (all data points; n = 3)*



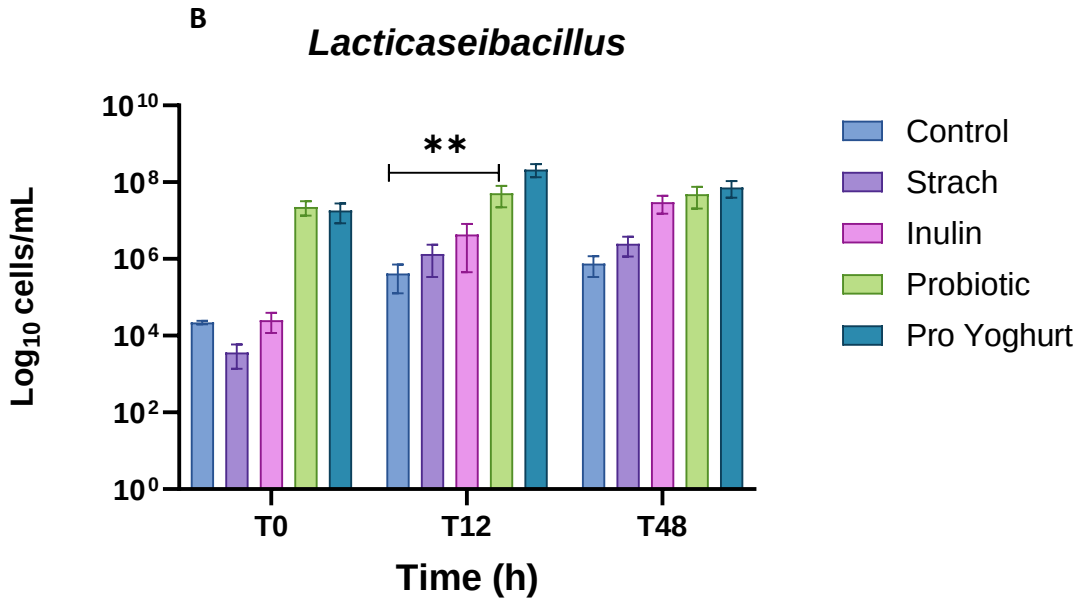




***Figure 4.17:*** *QMP of Lacticaseibacillus spp. in 16S rRNA Sequencing converted to Log10 cells/mL at 0, 12 and 48 h in A- Human faeces, B- Synthetic mix. Mean and SE (all data points; n = 3)*

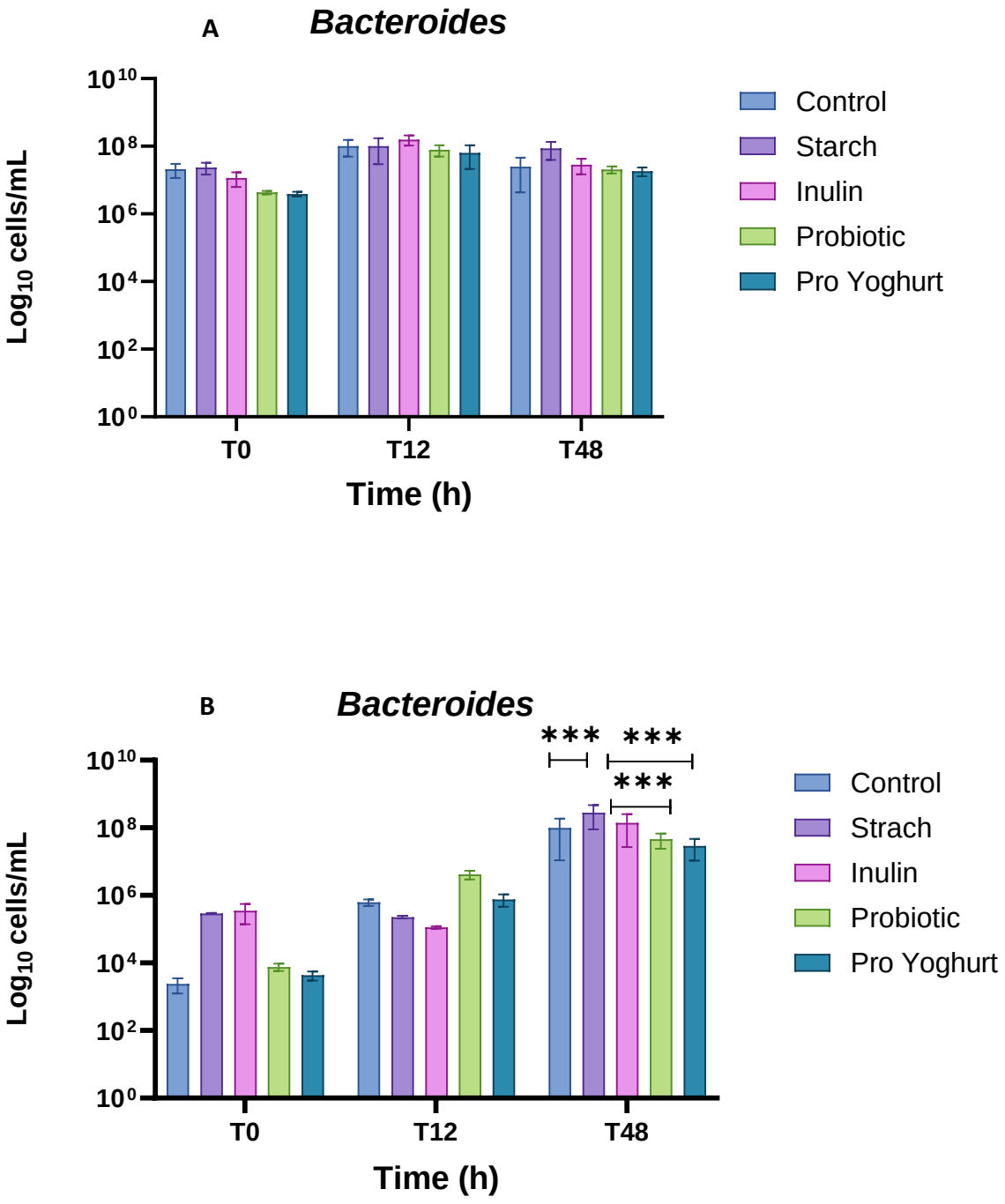
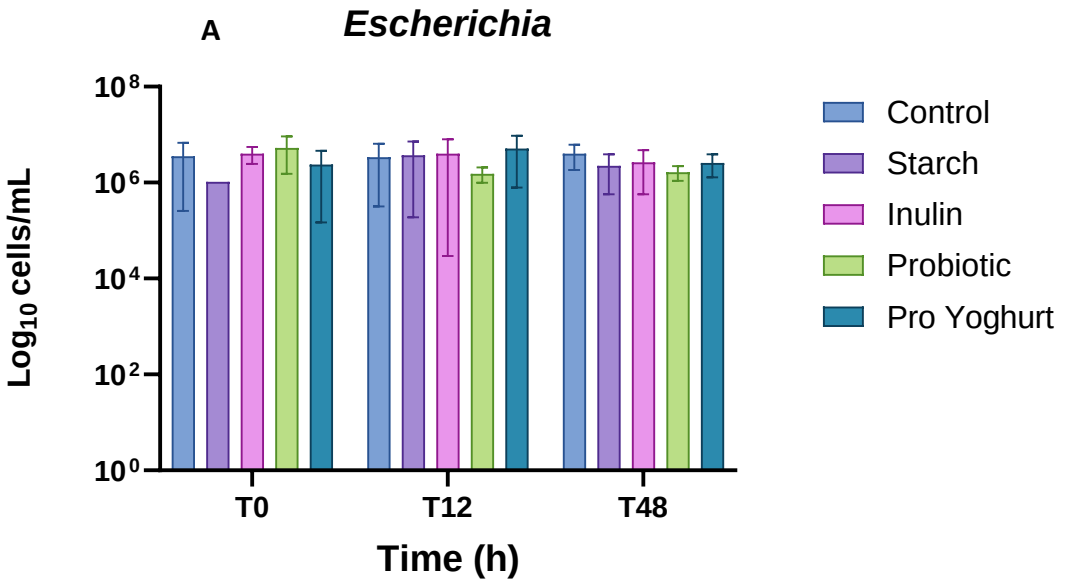



***Figure 4.18**: QMP of Bacteroides spp. in 16S rRNA Sequencing converted to Log10 cells/mL at 0, 12 and 48 h in A- Human faeces, B- Synthetic mix. Mean and SE (all data points; n = 3)*



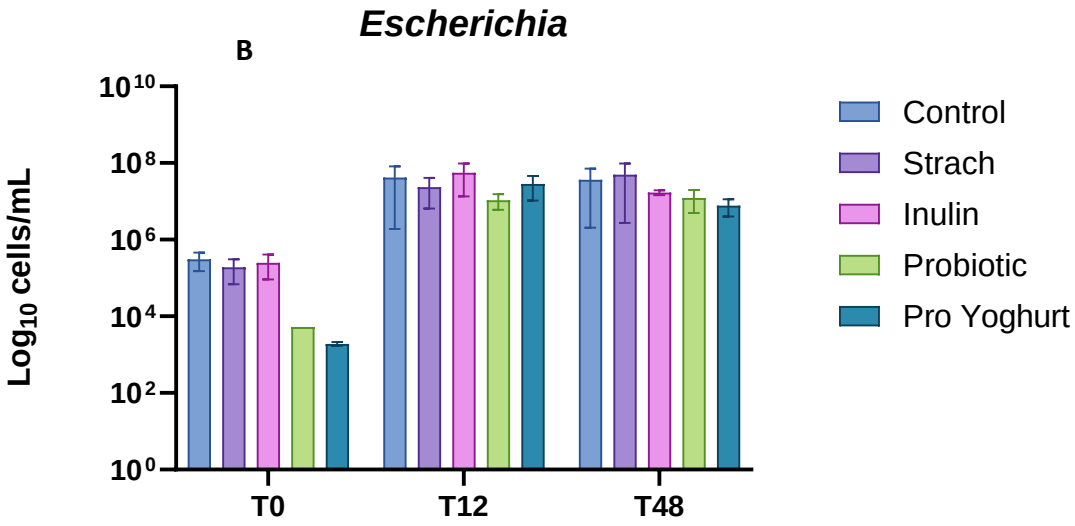




***Figure 4.19**: QMP of Escherichia spp. in 16S rRNA Sequencing converted to Log10 cells/mL at 0, 12 and 48 h in A- Human faeces, B- Synthetic mix. Mean and SE (all data points; n = 3)*

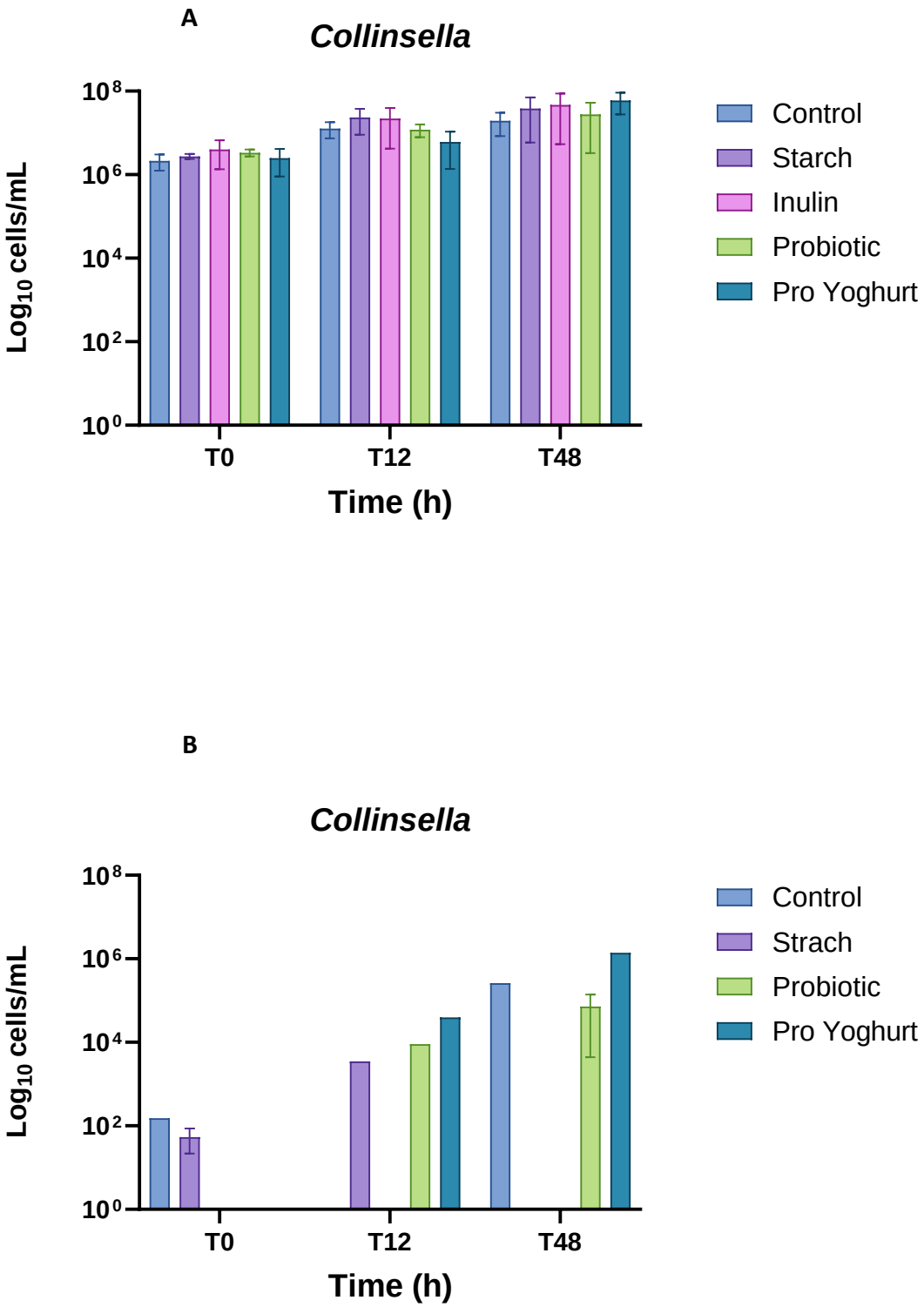



***Figure 4.20:*** *QMP of Collinsella spp. in 16S rRNA Sequencing converted to Log10 cells/mL at 0, 12 and 48 h in A- Human faeces, B- Synthetic mix. Mean and SE (all data points; n = 3)*

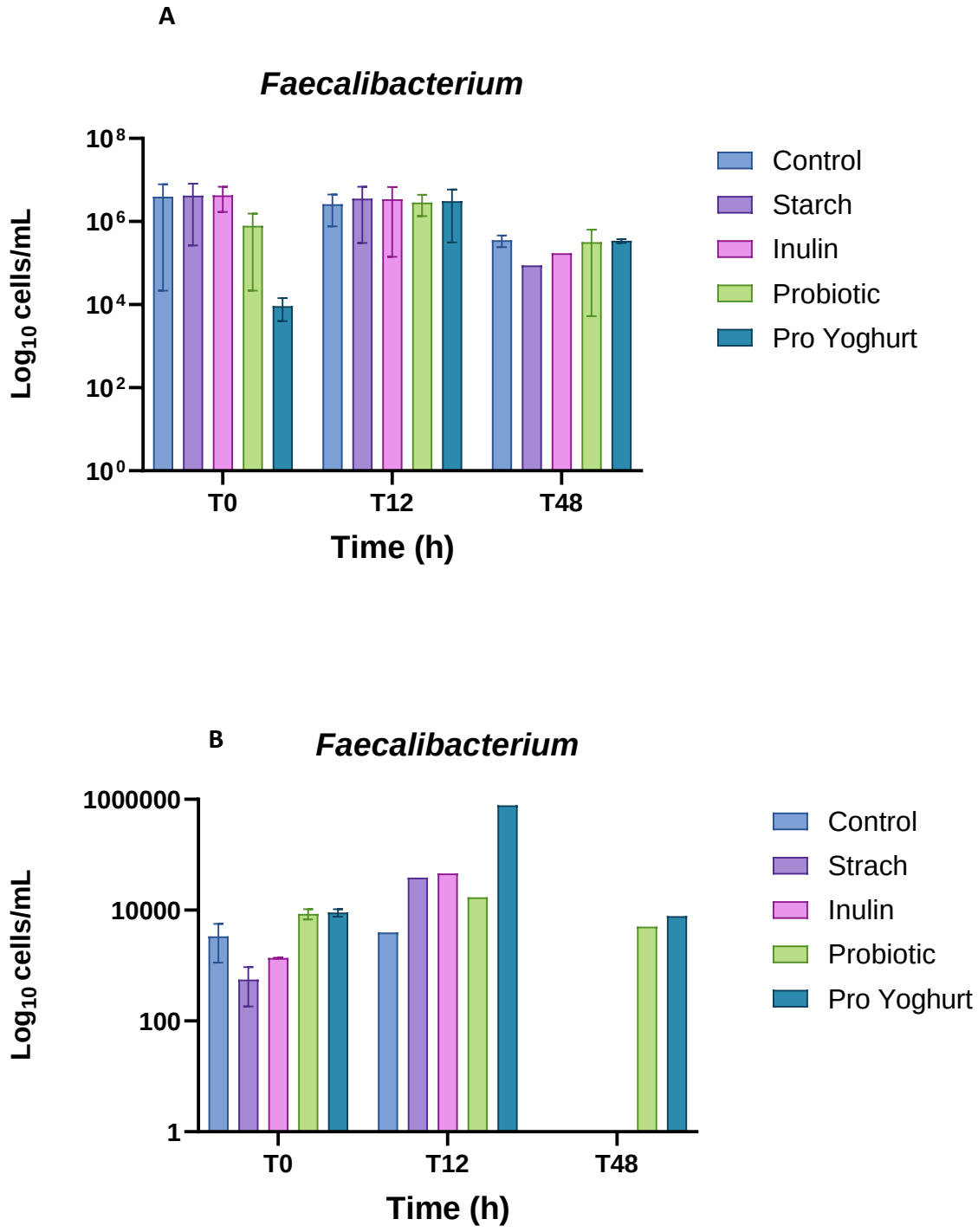



***Figure 4.21:*** *QMP of Faecalibacterium spp. in 16S rRNA Sequencing converted to Log10 cells/mL at 0, 12 and 48 h in A- Human faeces, B-Synthetic mix. Mean and SE (all data points; n = 3)*

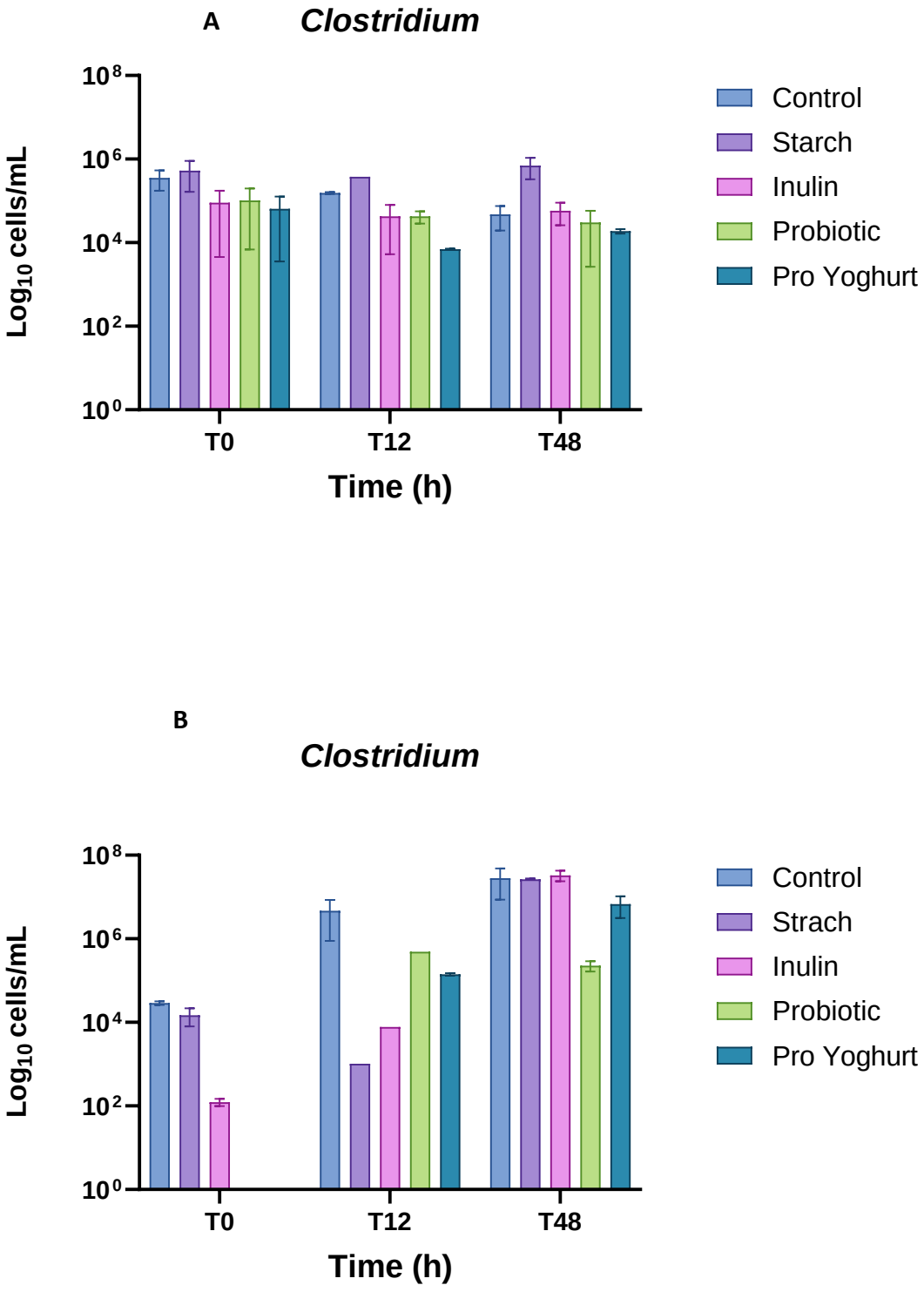



***Figure 4.22:*** *QMP of Clostridium spp. in 16S rRNA Sequencing converted to Log10 cells/mL at 0, 12 and 48 h in A-Human faeces, B-Synthetic mix. Mean and SE (all data points; n = 3)*

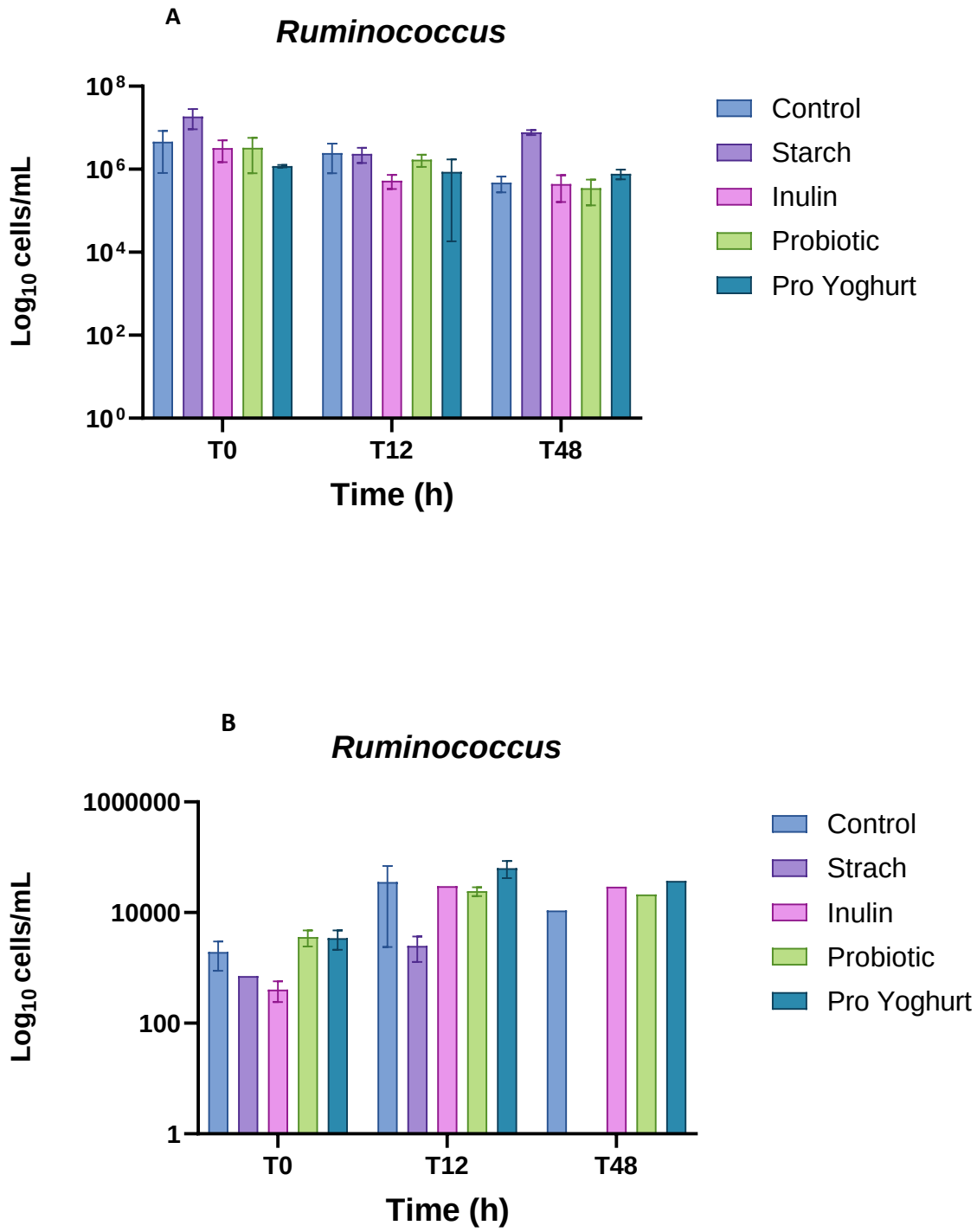



***Figure 4.23:*** *QMP of Ruminococcus bromii. in 16S rRNA Sequencing converted to Log10 cells/mL at 0, 12 and 48 h in A-Human faeces, B-Synthetic mix. Mean and SE (all data points; n = 3)*

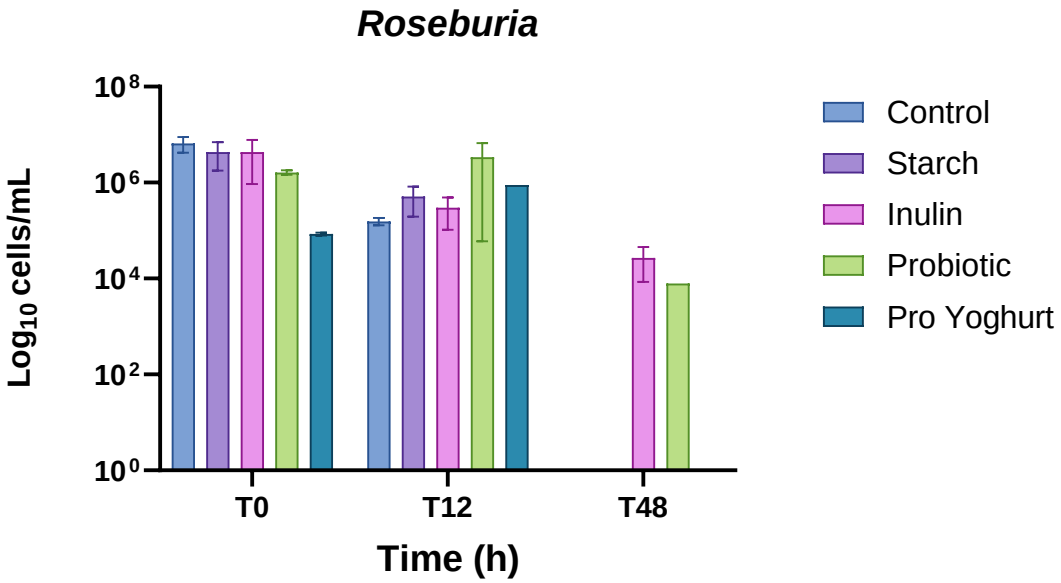



***Figure 4.24:*** *QMP of Roseburia spp. in 16S rRNA Sequencing converted to Log10 cells/mL at 0, 12 and 48 h in Human faeces. Mean and SE (all data points; n = 3)*

*Roseburia spp.* were not detected in the synthetic mix in 16s rRNA sequencing.

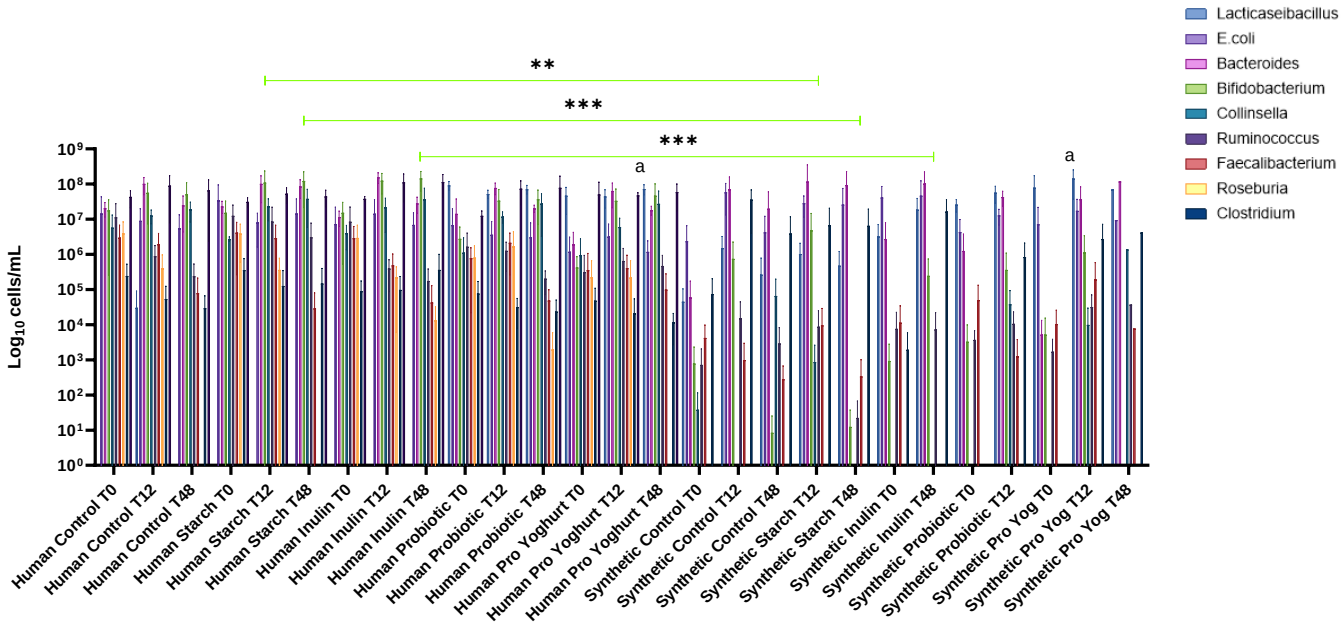



***Figure 4.25:*** *QMP of 16s DNA sequencing of both human and synthetic mix in Log10 cells/mL*

The results from the relative abundance from the 16S rRNA sequencing is shown below on Figure 4.26.

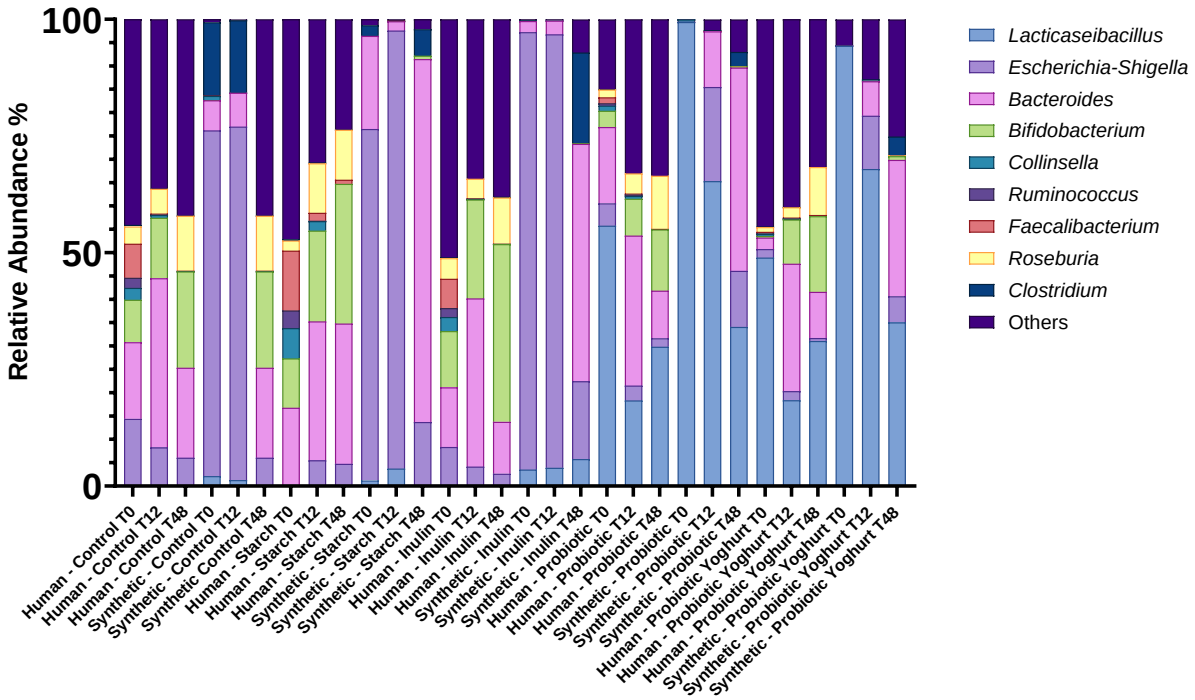



***Figure 4.26:*** *RMP of 16s rRNA sequencing results of human faeces and synthetic mix across different time points*

### *4.3.3. Relative metabolic profiling (RMP)*

Based on the 16S rRNA sequencing data (Figure 4.26), significant variations were observed primarily among lacticaseibacilli, bifidobacteria, bacteroides, and escherichia genera. Notably, lacticaseibacilli levels were significantly higher in the probiotic vessel and probiotic yoghurt vessel at T0 compared to other vessels (***p<0.001). However, a substantial increase (***p<0.001) in lacticaseibacilli abundance was noted in the synthetic mix at T0 compared to the human probiotic vessel, although this difference lost significance by the end of the 48-hour period. Conversely, the *Escherichia shigella* group exhibited a significantly high abundance (***p<0.001) in the synthetic mix's control, starch, and inulin vessels at T0 compared to human donors. Interestingly, this observation conflicted with both QMP and FC-FISH results, where *Escherichia* abundance was not considered significant. The QMP analysis of the synthetic mix did show elevated *Escherichia* levels, but they were not statistically significant, unlike the relative abundance data. However, levels in FC-FISH reported were much lower (in the range of $10^4$) while the QMP was around $10^5$ range. Over time, *Escherichia* abundance decreased without significant differences between synthetic mix and human samples at T48 in these vessels. Notably, there was a significant reduction (***p<0.001) in the *Escherichia* group in the synthetic mix's control, starch, and inulin vessels at the end of fermentation. Regarding bacteroides, no statistical differences were initially reported among any synthetic vessels at T0. However, by T48, substantial growth was observed, with significant changes (***p<0.001) in the synthetic mix's control, starch, and inulin vessels, where bacteroides became the most abundant bacterial group. In contrast, the human donor vessels did not exhibit a significant increase in bacteroides growth, with no statistical differences reported. On the other hand, bifidobacteria displayed a gradual increase in all human donor vessels, reaching highest abundance in the inulin vessel at the end of fermentation (*p=0.033) compared to T0. Human vessels also showed a significant increase compared to the synthetic mix at T48 in starch (*p=0.049) and inulin (***p<0.001) vessels, although no statistical difference was found in bifidobacterial abundance in the control vessel between synthetic mix and human at T48.

Comparing relative abundance results with QMP and FC-FISH, it is evident that the human RMP resembles more closely with both QMP and FC-FISH. However, significant divergence was observed in the initial time points of the synthetic mix when considered in RMP. This

difference suggests that when lower concentrations of bacteria are present, relative abundance may not be the optimal method for interpretation. For comparison with FC-FISH, QMP conversion to a similar platform of cells/mL is preferable, highlighting the reliance of outcomes on the specific techniques used.

### *4.3.4. Metabolite profiles*

In the metabolite profiles of both human faeces and synthetic mix (Figures 4.27, 4.28), prominent metabolites included SCFAs (acetate, butyrate, propionate) and ethanol. Additionally, human faeces yielded trace amounts of lactate, while the synthetic mix produced intermediate metabolites such as formate, lactate, succinate, and methanol alongside the short chain fatty acids. Time point 12h for synthetic mix was analysed and it showed Among human samples, highest acetate production was observed in the starch and inulin vessels, with significant differences compared to the control vessel in both starch (***p<0.001) and inulin (***p<0.001) vessels, as well as the yoghurt vessel (*p=0.036). Conversely, in the synthetic mix, the yoghurt vessel exhibited the highest acetate production, significantly differing from the control vessel (***p<0.001). The concentration of the metabolites produced in the synthetic mix was lower compared to human donors. Average acetate production for the human donors was 272.79 mM while the average acetate production of synthetic mix was 72.81 mM, almost three times higher than the synthetic mix.

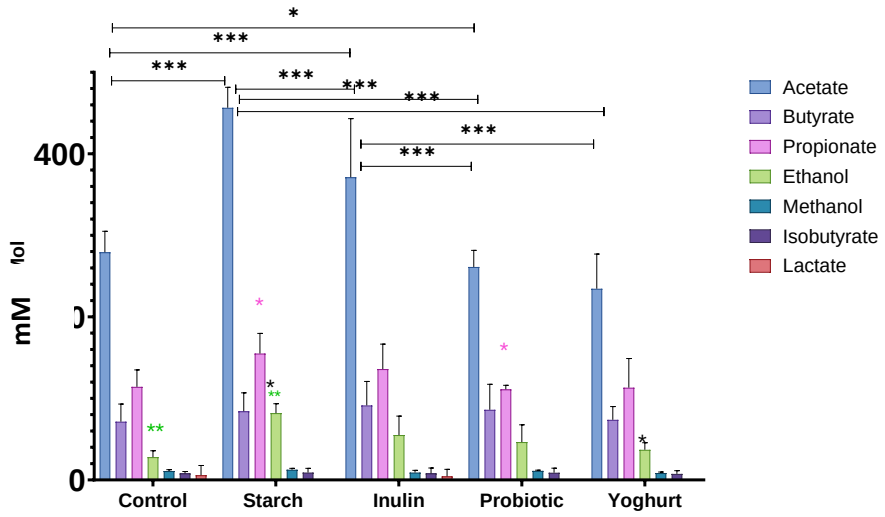



***Figure 4.27:*** *Metabolite profile in mM in human faeces at the end of fermentation 48h*

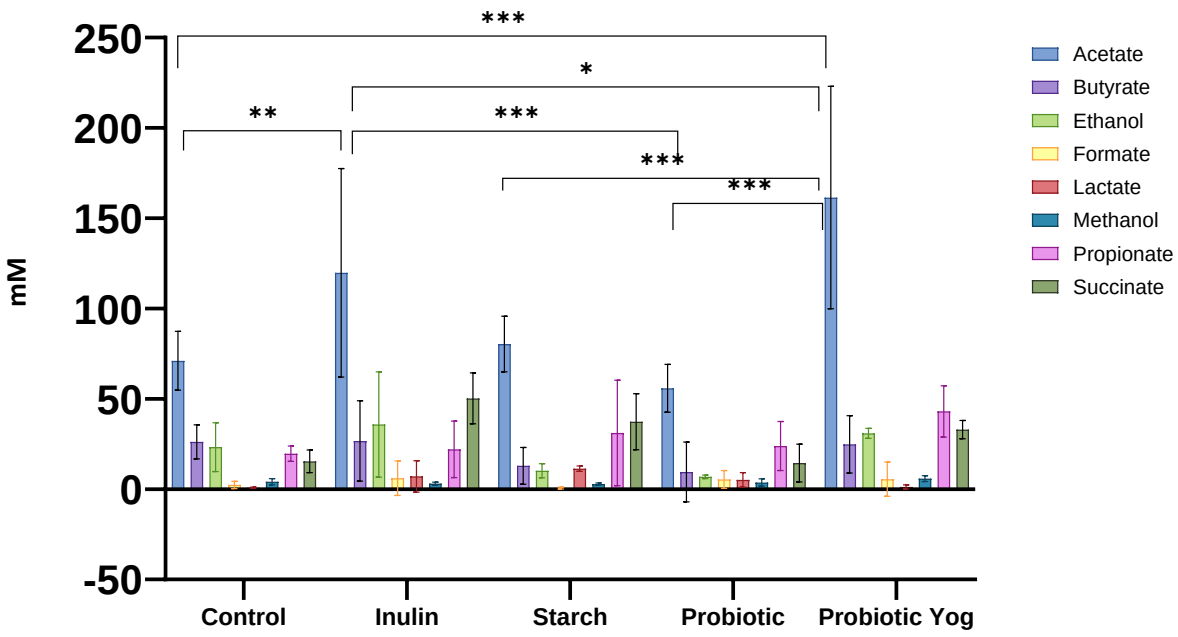



***Figure 4.28:*** *Metabolic profile in mM of synthetic mix at the end of fermentation 48h*

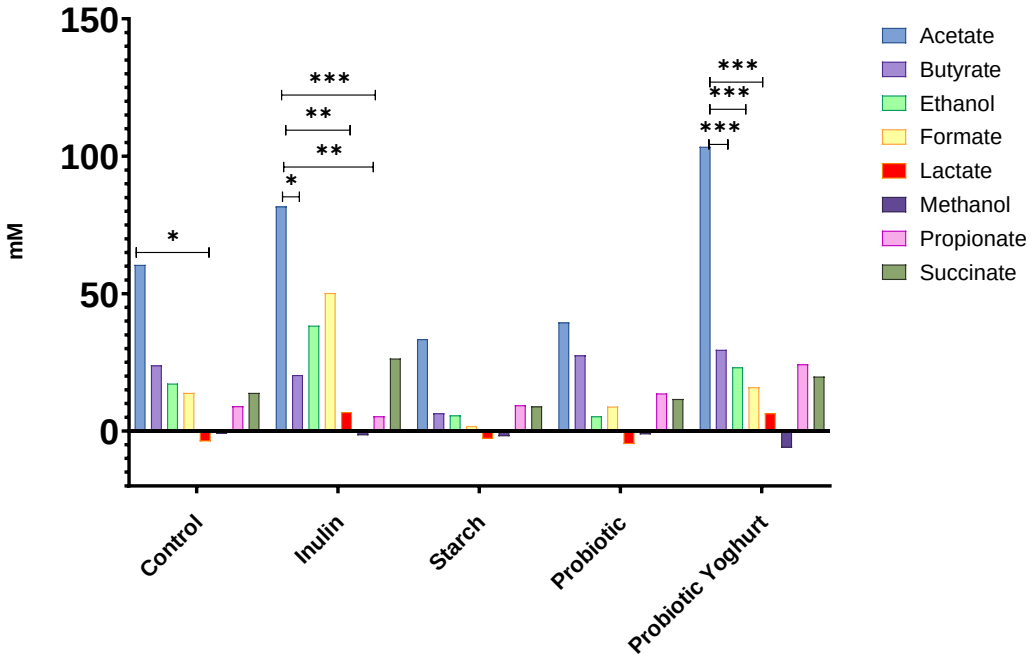



***Figure 4.29:*** *Metabolic profile in mM of synthetic mix at the end of fermentation 12h*

### 4.3.5. Chemometric analysis

Processed spectroscopic data were imported to the SIMCA 17.0 software package (Umetrics AB, Umeå, Sweden) to conduct multivariate statistical analysis. Principal components analysis (PCA) was used initially, to evaluate similarities/differences in the batch culture metabolite composition between human and synthetic mix. The $R^2$ and $Q^2$ variables provided an indication of goodness of fit ($R^2$) as well as goodness of prediction ($Q^2$) of the models.

A scores scatter plot from unsupervised PCA indicated that there was clustering pattern over time (Figure 4.30), as the points showed clustering from T0 to T60 in human donors and synthetic mix individually. At T0, both the human donors and the bacterial mix clustered together showing a similarity. Over time, the human donor clusters shifted away from the samples at T0 (Figure 4.31) but the synthetic mix shifted very slowly. The first two principal components accounted for 43% of the total variation in the dataset. $R^2$Cum=0.436 and $Q^2$Cum=0.416.

Figure 4.31A, shows how both human and synthetic mix cluster tightly at T0 indicating similar metabolic profiles, However at T12 Figure 4.31B, there is a large change in metabolic profile, evidenced by the spread of scores. Then as the fermentation experiment progresses, the human donor samples diverge from the path of synthetic mix samples, indicating growing difference in metabolic profile between the two groups Figure 4.31C and D. However, the synthetic mix seems to follow the direction of the human samples but very slowly. Following unsupervised analysis, the data were then analysed using O-PLS-DA, supervised modelling approach (Figure 4.32 shows the scores plots and Figures 4.33-4.38 shows the S-line loadings for the OPLSDA models with different time combinations). These data showed that there was a clear separation from the functional behaviour of the human donor samples and synthetic mix samples. Therefore, subsequent downstream analysis was not carried out.

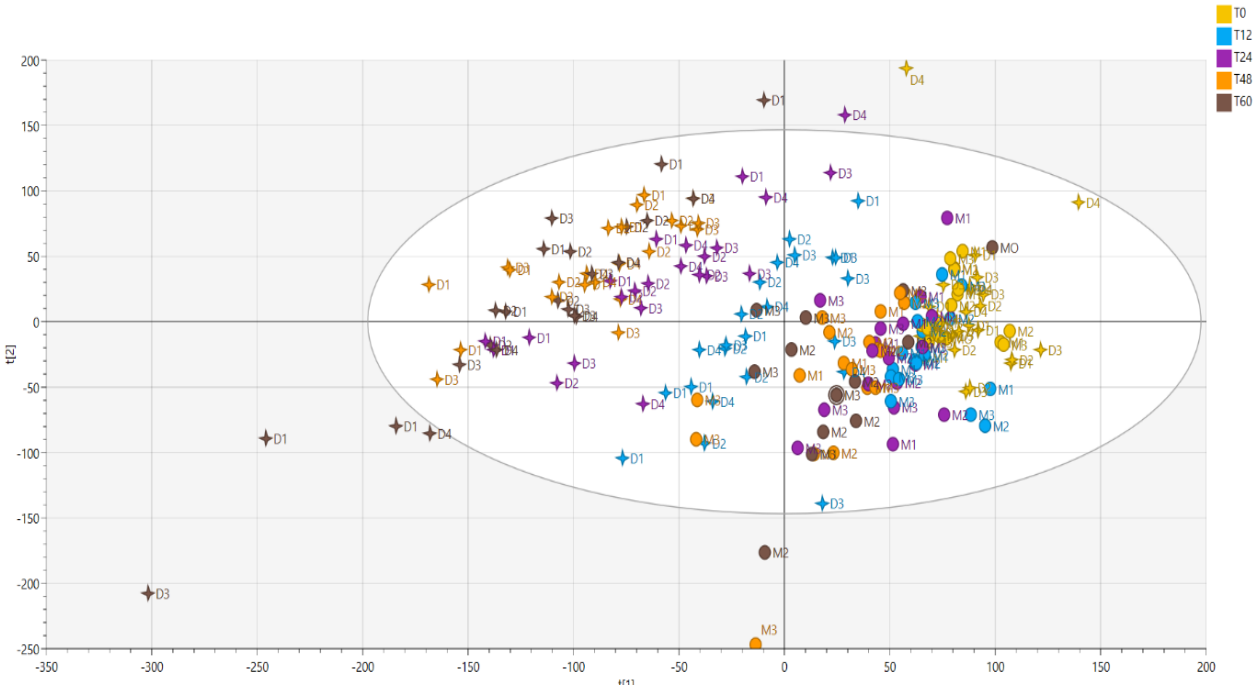



***Figure 4.30:*** *PCA score plot of the metabolites from human and faecal batch culture samples coloured according to time and labelled according to group (D1, D2, D3, D4 = human donor shown by star symbol , M1, M2, M3 = synthetic mix, shown by circles)*

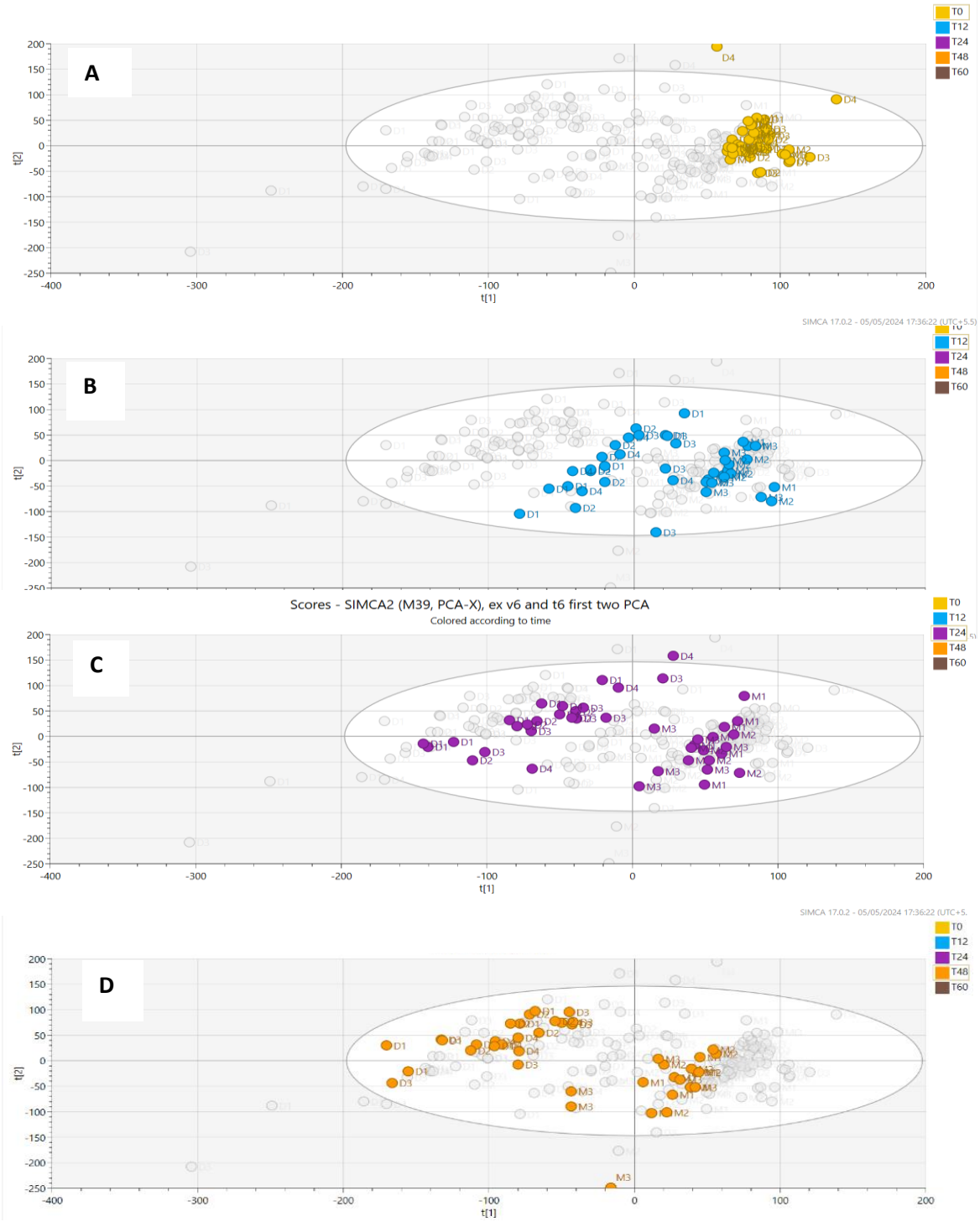



***Figure 4.31**: PCA scores scatter plots shown according to different time points; A t0, B t12, C t24 and D t48*

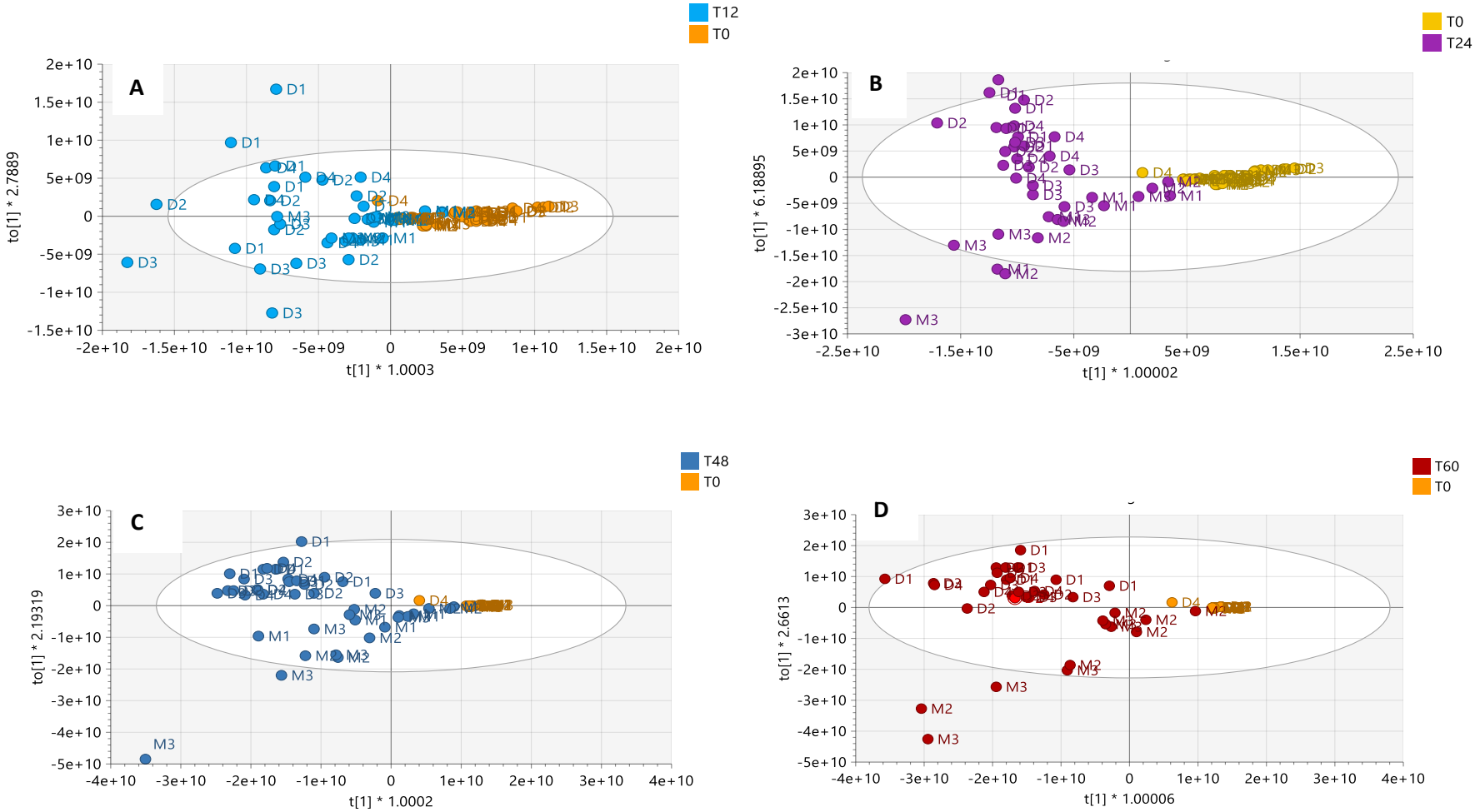



***Figure 4.32:*** *OPLSDA scatter plots showing the clustering of synthetic mix in comparison to human faeces at different time points; A t0 vs t12, B t0 vs t24, C t0 vs t48 and D t0 vs t60*

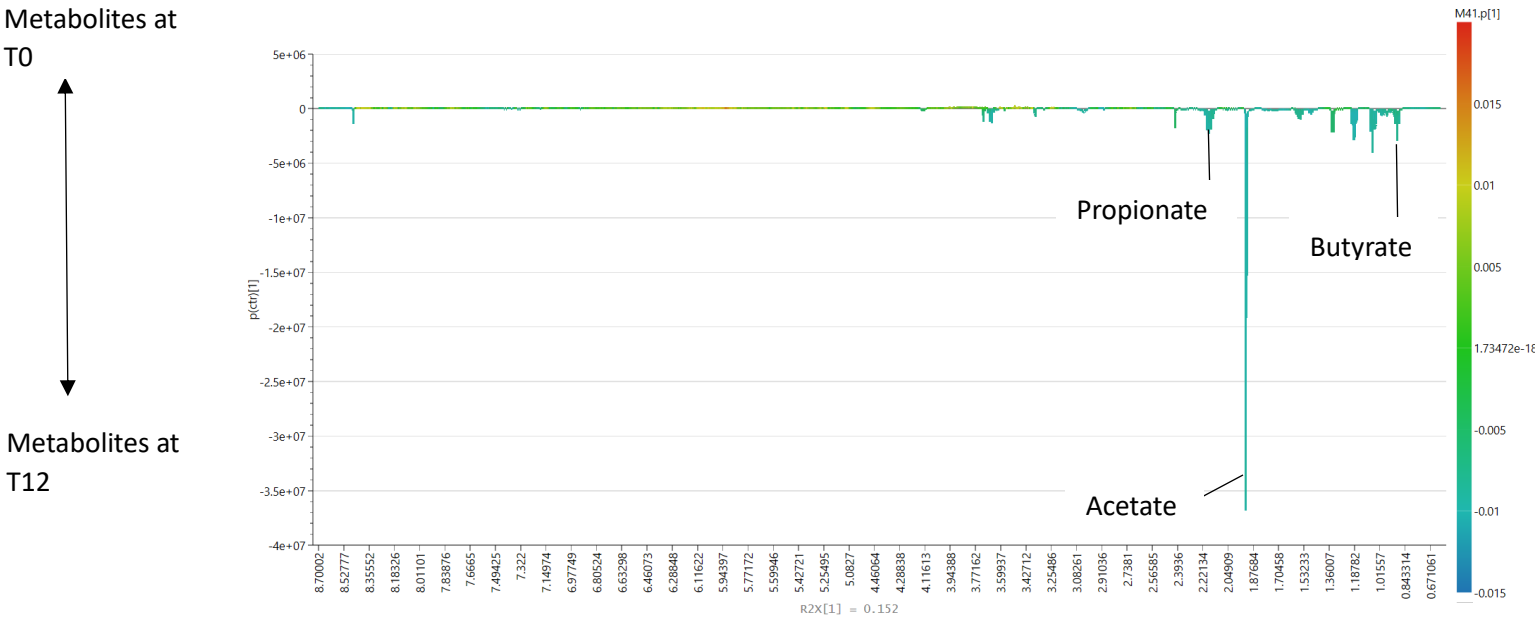



***Figure 4.33:*** *OPLSDA for metabolites at T0 vs T12 (T12 below x axis and T0 above x axis)*

Metabolites at T0



Metabolites at T24

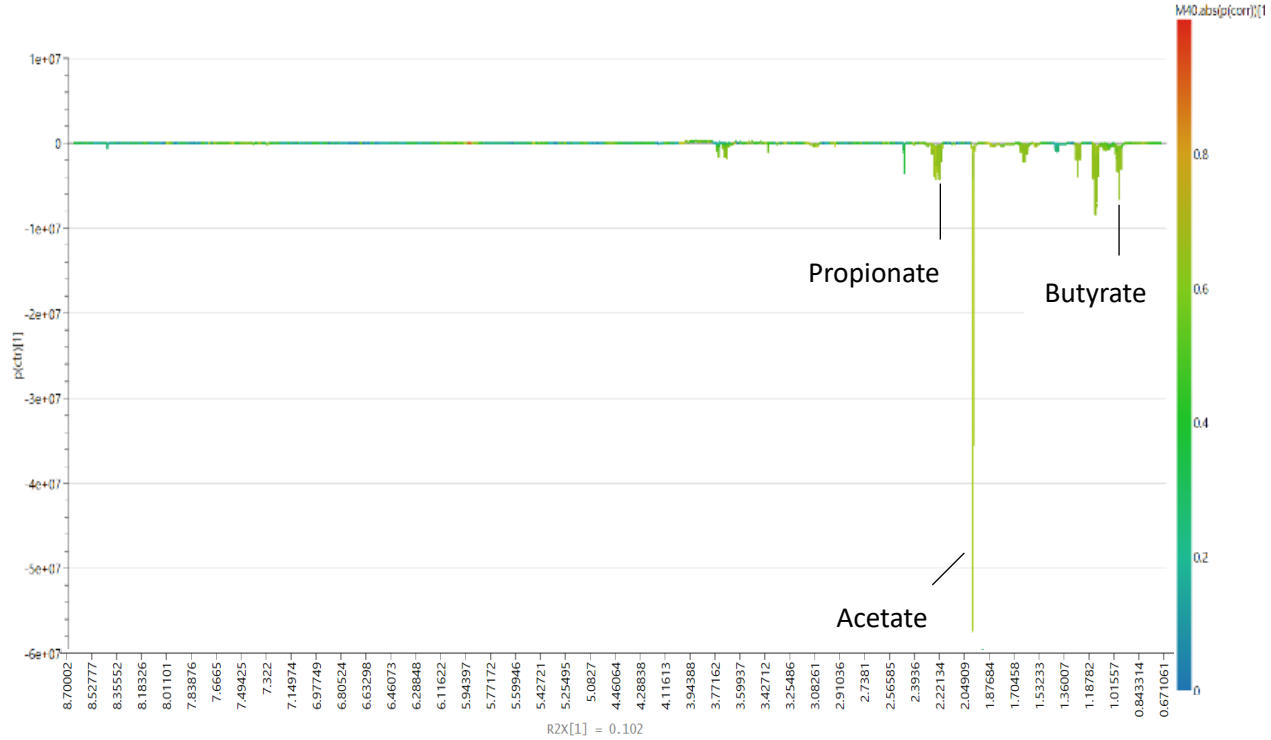



***Figure 4.34:*** *OPLSDA for metabolites at T0 vs T24 (T24 below x axis and T0 above x axis)*

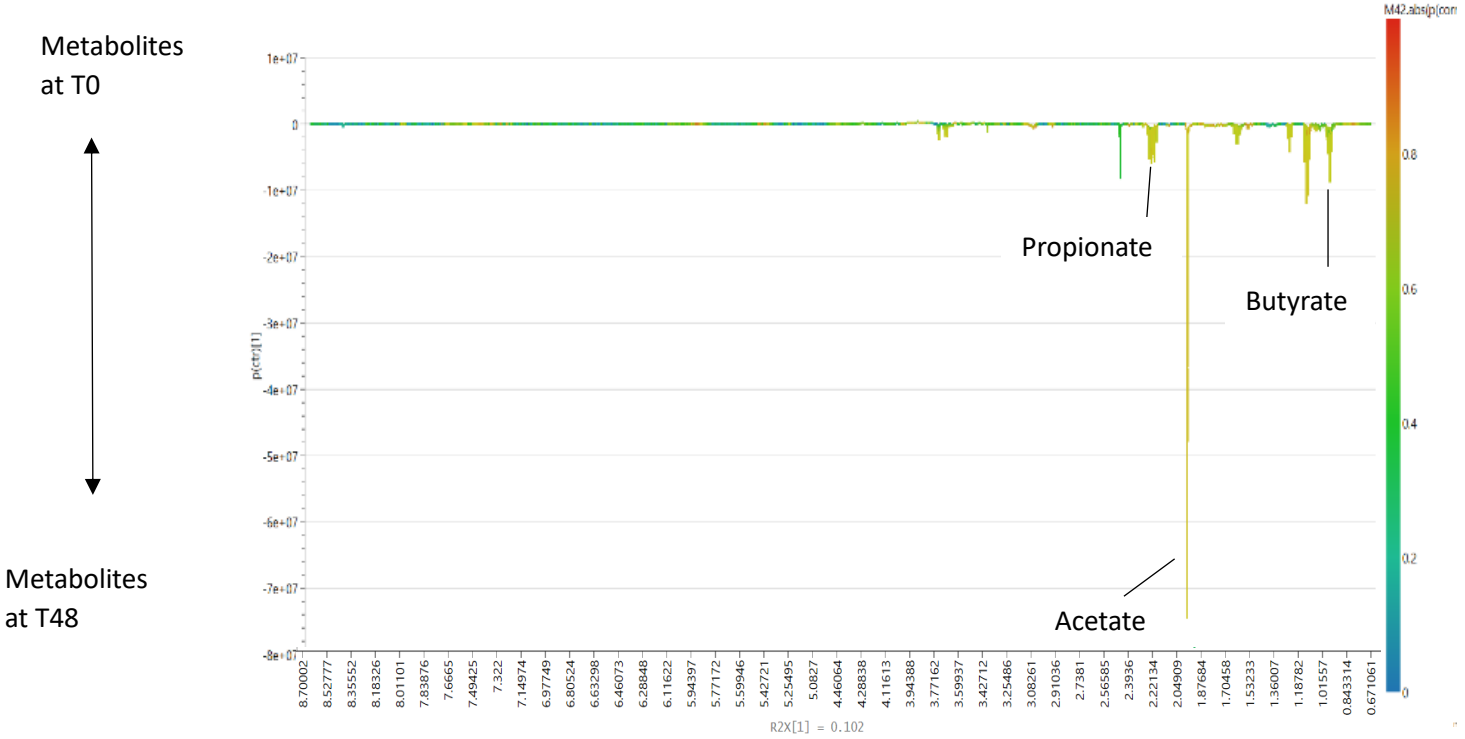



***Figure 4.35:*** *OPLSDA for metabolites at T0 vs T48 (T48 below x axis and T0 above x axis)*

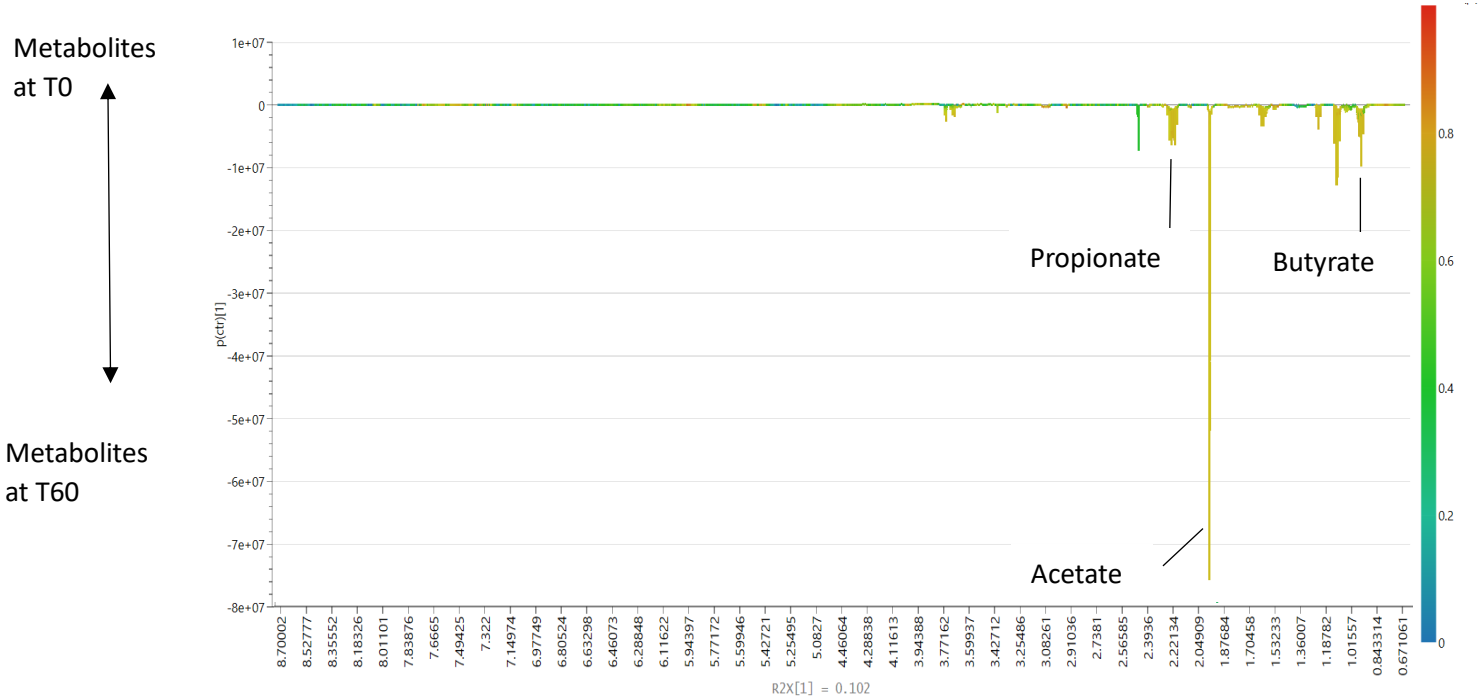



***Figure 4.36:*** *OPLSDA for metabolites at T0 vs T60 (T60 below x axis and T0 above x axis)*

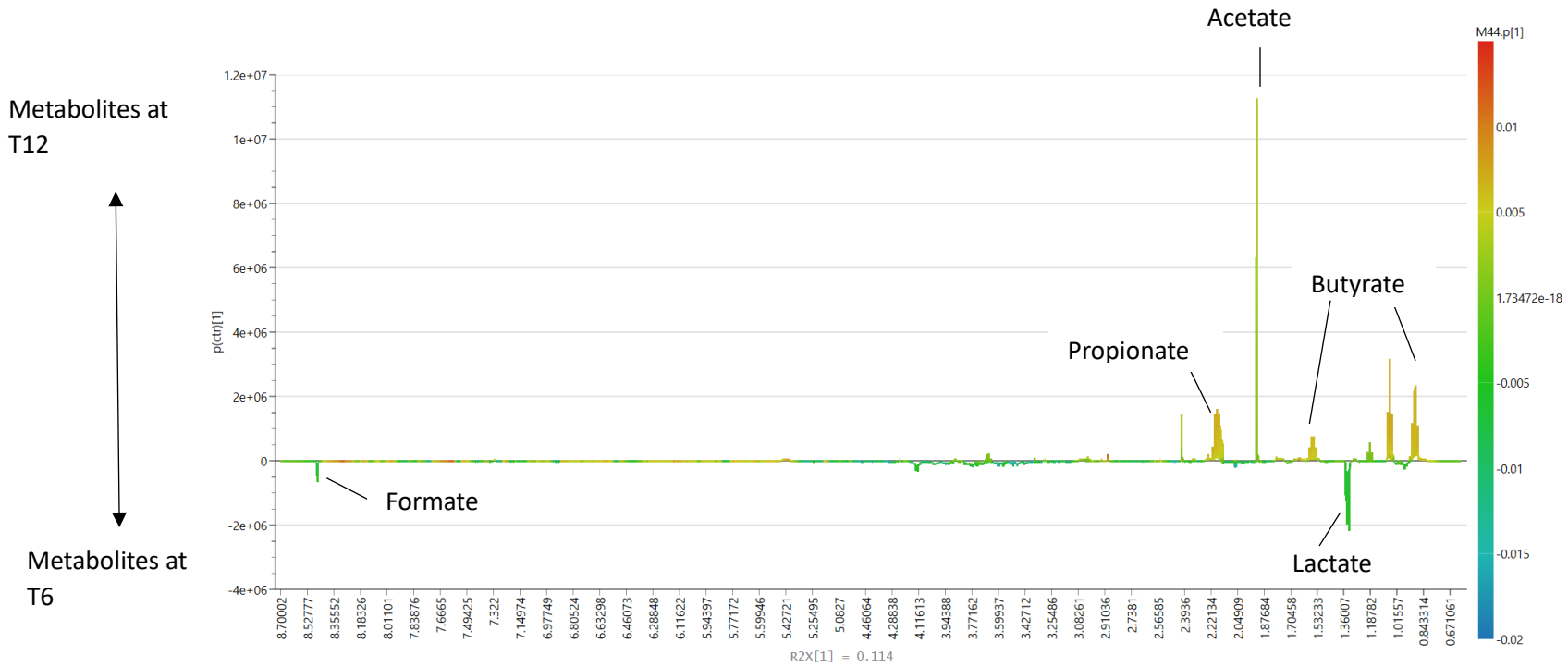



***Figure 4.37:*** *OPLSDA for metabolites at T6 vs T12 (T6 below x axis and T12 above x axis)*

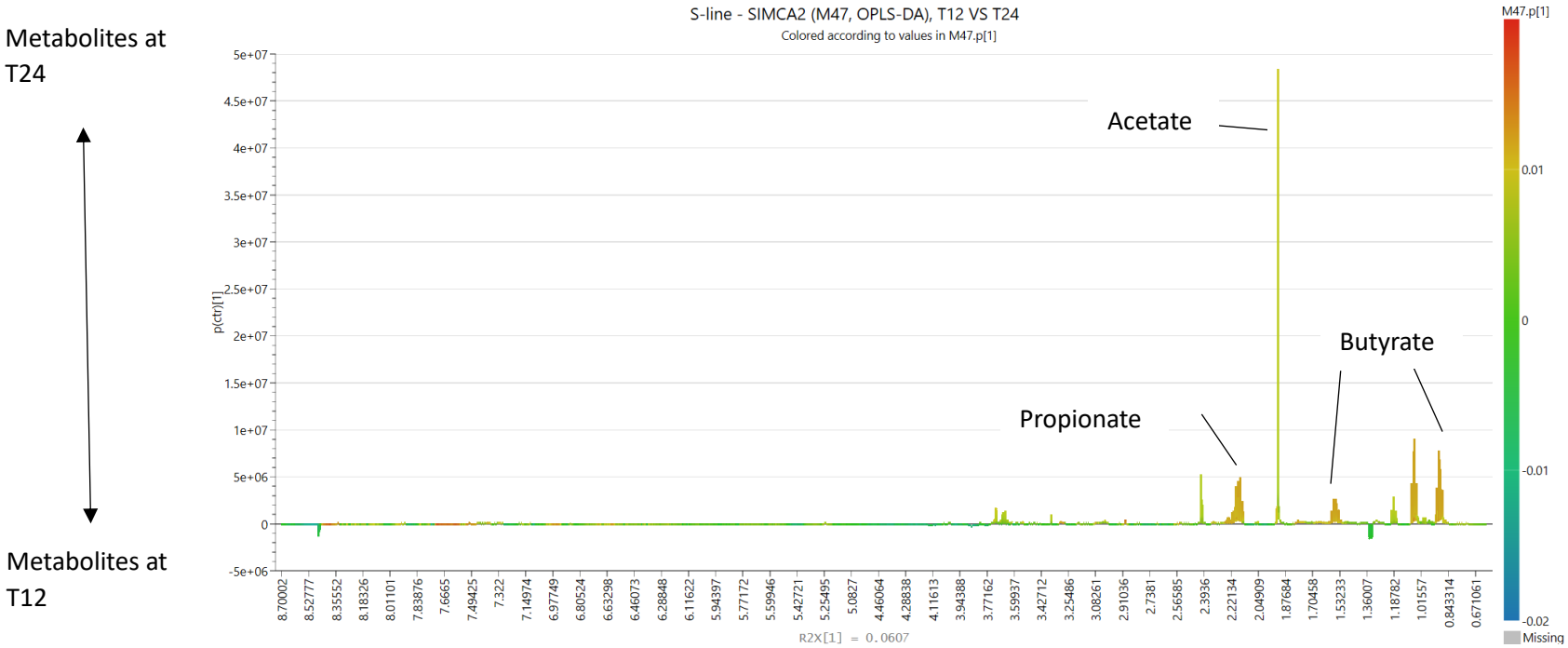



***Figure 4.38:*** *OPLSDA for metabolites at T12 vs T24 (T12 below x axis and T24 above x axis*

## 4.4 Discussion

This study has provided insights into the functional dynamics of synthetic microbial communities in comparison to naturally occurring human faecal microbiota, when exposed to different dietary substrates. Notably, while total bacterial counts remained consistent after 24 hours in both systems, a distinct shift was observed in the composition of dominant bacterial species, favouring the growth of bifidobacteria in human faeces and bacteroides in the synthetic mix. However, at the end of the fermentation experiment, the synthetic mix exhibited the presence of intermediate metabolites such as succinate and formate, suggesting active metabolic pathways, while the human faecal donor samples predominantly recorded end products, including butyrate, acetate, propionate and ethanol. The synthetic mix, despite initially exhibiting lower bacterial counts, ultimately reached levels comparable to those found in human faeces. This suggests the presence of active metabolic pathways within the synthetic mix, facilitating bacterial growth which was evident in the metabolic profiles.

It is notable that total bacterial counts in the synthetic mix increased at the end of fermentation (T48) while a reduction in numbers was observed in human faeces (Figure 4.5). This difference can be attributed to competitive dynamics among bacterial populations. Human faeces, starting with a larger initial bacterial load compared to the synthetic mix, likely encountered an exhaustion of nutrients towards end of fermentation that may have led to a reduction in total bacterial counts. In contrast, the synthetic mix, containing a smaller initial bacterial population, potentially retained sufficient nutrient availability to support bacterial growth, resulting in an increase in total bacterial counts over the fermentation period. This observation shows the complex interplay between bacterial competition and nutrient availability, influencing microbial dynamics (Hibbing et al. 2010) and population sizes in different environments.

In the observed results, shifts in bacterial populations were noted across the fermentations conducted with human donors and synthetic mix. Bifidobacteria exhibited the most notable changes in abundance during the fermentation period with human donors. Conversely, bacteroides emerged as the dominant genus at the end of fermentation in the synthetic mix. The prevalence of bacteroides and the bifidogenic effect varied among different fermentations, indicating substrate-dependent microbial responses. Bacteroides was most abundant in the starch vessel of the synthetic mix, whereas bifidobacteria predominated in the inulin vessel among human donors. The introduction of *Lacticaseibacillus rhamnosus* GG probiotic into the

Probiotic and Probiotic Yoghurt vessels, resulted in stabilisation of lacticaseibacilli populations in both human donors and synthetic mix environments. This stabilisation of lacticaseibacilli was accompanied by the suppression of pathogenic bacteria such as *Clostridium* and *Escherichia*. Previous research has highlighted the ability of lacticaseobacilli to produce an array of inhibitory compounds, including bacteriocins, nisins, organic acids, ethanol and hydrogen peroxide (Vieco-Saiz et al. 2019; Jaiswal 2020). These antimicrobial compounds, specifically bacteriocins, exert antimicrobial activity against bacterial species, while organic acids such as acetate create an acidic environment unfavourable for the growth of pathogenic microorganisms. Furthermore, the production of ethanol and hydrogen peroxide enhances the antimicrobial properties of lacticaseibacilli strains, effectively suppressing the proliferation of pathogenic bacteria. Therefore, the observed suppression of bacteria such as C*lostridium* and *Escherichia* in the presence of *Lacticaseibacillus rhamnosus* GG probiotic in this study, highlights antimicrobial capabilities of lacticaseibacilli species, proving their potential as probiotic agents for promoting gut health.

One of the key concepts of using different substrates in this study was to identify any change in metabolite production and to determine selective changes in microbial composition. In human faecal experiments all substrates demonstrated changes in *Bifidobacterium* counts. Reason for all substrates targeting an increase in bifidobacteria could be due to the presence of intracellular and extracellular mechanisms and transporters needed to utilise a range of low molecular weight carbohydrates (Riviere et al. 2016) which was included in the nutrient rich medium, and also the ability to utilise complex carbohydrates through the 'Bifidus pathway' (Palframan et al. 2002). Even in the probiotic vessels with the presence of the probiotic (which is a lactocaesibacilli strain), bifidobacteria dominated. *Bifidobacterium longum* is reported to have over 50 genes responsible for the uptake of various carbohydrates (Pokusaeva et al. 2011) which may have caused a high affinity to respond to the substrates within a competitive environment. Whereas other bacteria may have been unable to compete with bifidobacteria that are well equipped with enzymes and metabolic pathways for the fermentation of these substrates.

When comparing overall metabolite profiles of human faeces and the synthetic mix, the former displayed a limited spectrum of metabolites, predominantly featuring end products such as acetate, butyrate, propionate, and ethanol. In contrast, the synthetic mix exhibited a more diverse profile, including intermediate metabolites like succinate and formate. This disparity

suggests that complete metabolic reactions may not have occurred within the synthetic mix compared to the human faecal donor samples. This highlights the importance of cross-feeding mechanisms and complex interactions in human faeces that need to be considered in developing synthetic consortia. The term "cross-feeding" refers to the process where different microorganisms in an ecosystem share metabolites with each other. This sharing of metabolites plays a crucial role in establishing stable communities of gut commensals. Essentially, cross-feeding contributes to overall stability and functionality of the gut microbiota, ensuring appropriate functioning and health benefits to the host. (Culp and Goodman 2023). Some authors classify gut microbiota as primary degraders/fermenters which breaks down undigestible food that reached the gut. Certain researchers categorise gut microbiota into trophic levels (Wang et al. 2019; Gralka et al. 2020). At each trophic level, certain microbes utilise nutrients, converting a portion into their biomass and secreting the rest as metabolic byproducts. These byproducts, in turn, serve as nutrients for microbes at the subsequent trophic level (Wang et al. 2019). Primary degraders/fermenters responsible for breaking down undigestible food in the gut. These primary degraders generate metabolites utilised by secondary fermenters to produce SCFAs. Bacteroides are classified as primary fermenters and suppliers of carbon (Escriva, Fuhrer, and UweSauera 2022). In our synthetic mix, bacteroides was observed to be the dominating genus, and also, metabolic profiles indicated a range of intermediate metabolites suggesting a lack of secondary fermenters within the mix that could utilise these metabolites to end products. In human faeces, the metabolic profiles are characterised by end products of metabolism such as acetate, butyrate, and propionate, demonstrating the presence of cross feeding mechanisms among the trillions of bacteria present in the complex gut network. In contrast, the synthetic mix, comprising only nine bacterial strains, displayed a broader range of metabolites including intermediate compounds such as succinate, formate and methanol. This difference in metabolic profiles signifies the influence of bacterial diversity and interaction complexity on the output of metabolites produced. This confirms that more attention should be focussed on cross feeding and complexity of the human gut environment.

Another interesting outcome of this study was the different microbial profiling approaches (FC-FISH enumeration, QMP and RMP). Studies reveal that variations in microbial load across different samples can significantly impact the reliability and accuracy of relative profiling methods (Vandeputte et al. 2017; Morton et al. 2017). This arises because relative profiling does not

account for differences in microbial abundance, potentially leading to biased or misleading interpretations. Therefore, it is essential to consider and address variations in microbial load when utilising relative profiling techniques to ensure robust and reliable correlations with other quantitative data. This could be the reason that the RMP results at T0 of the probiotic incorporated vessel reported 98% of lacticaseibacilli. The same could be said for the high abundance of *Escherichia shigella* in the T0 of the control, starch and inulin vessels of the synthetic mix (as the initial microbial load of the synthetic mix was very low). This discrepancy was not reported in the human faecal vessels as the initial bacterial load was high.

This study suggests that the selection of analytical methods such as RMP and QMP plays a crucial role in interpreting microbial profile data. RMP, based on 16S rRNA sequencing, offers a detailed view of microbial taxa and their relative abundances, making it suitable for samples with high initial bacterial concentrations where taxonomic composition and community structure are of interest. On the other hand, QMP provides absolute quantification of bacterial populations, which is advantageous for samples with lower initial bacterial counts, allowing for precise measurements of population dynamics and treatment effects.

As such, this study's findings highlights the importance of choosing the appropriate method based on sample characteristics, research objectives, and the need for relative versus absolute quantification, highlighting the complementary nature of these analytical approaches in comprehensively understanding microbial dynamics and responses to experimental conditions.

In the context of the chemometric analysis conducted on the metabolic profiles, the intricate complexity of human faeces was further proven. The presence of only 9 bacteria in the mix compared to the diverse microbial communities in the human donors is a crucial factor in microbial function, as observed in the clustering patterns in the PCA scores plot. The mix's limited microbial diversity likely contributes to its tight clustering in the centre of the plot (indicating similar metabolic profiles). In contrast, the human faecal inocula contain a broader range of microbial species, making their microbial activity more dynamic and responsive to changes over time. This diversity can lead to greater variability and movement in the PCA plot, as different individuals within the donor group might respond variably to factors influencing their gut microbiota. The human donors' diversity and complexity of their microbial communities can help explain the observed drift and separation over time. The mix's stability and limited variability can be attributed to its simplified microbial community, whereas the human donors' diversity results

in more pronounced changes in microbiota composition. Despite these differences, the metabolic profile trajectories of the synthetic mix were shown to drift slowly in the direction of the human samples in the PCA plots. This similarity could imply that metabolic activity of the synthetic mix is similar, however it is comparatively slower than that of human faeces, potentially necessitating extended fermentation periods to reach the desired metabolic endpoints. However, this raises questions, particularly in light of the observed increase in pathogenic bacterial counts in control vessel. It is evident that prolonged fermentation could promote the proliferation of pathogenic bacteria, rendering the system unfavourable. This highlights the need for careful consideration when extending fermentation times. However, results of this chapter also show that incorporating starch or a probiotic into the vessel can suppress the growth of the pathogens, which sheds light on the potential to manage the balance of bacterial communities *in vitro*, using dietary substrates. However, the low concentration of metabolite production compared to human faeces should be considered in further investigations.

This study aimed to develop a novel synthetic bacterial community to use as a tool to study the complexity of gut microbial interactions. This approach has potential as an alternative to using faeces in *in vitro* studies and in FMT. The comprehensive monitoring of metabolite production through utilisation of pure culture species offers an effective means of elucidating and tracing their functional behaviours. Within this investigative framework, it becomes evident that both individual and collective responses within the microbial community exhibit notable variances, principally attributed to the production of intermediate metabolites.

Numerous other studies have sought to unravel the complexity of human gut microbiota, each with its unique focus. Certain investigations have centred on specific gut metabolites, such as the production of butyrate (Clark et al. 2021). Additionally, others have used mathematical modelling, striving to predict microbial behaviour (Venturelli et al. 2018). Further, certain studies have explored the potentially deleterious effects of bacterial consortia with an aim to identify keystone species, as demonstrated in the work of (Gutierrez and Garrido 2019). This research collectively highlights the importance of comprehending the complexity of microbial interactions.

This study has contributed to this effort, by elucidating the collaborative role of 9 selected bacterial strains within a synthetic mix. Findings revealed that achieving complete metabolic reactions requires a more intricate bacterial consortium with consideration of primary and secondary fermenters. However, exploration of microbial behaviour within simplified models,

such as this synthetic mix, serves as a stepping stone towards the development of more sophisticated models that can closely approximate functional behaviour of human faecal microbiota.

It is clear that collaboration of many other bacteria and their associated metabolites plays a significant role in the high metabolite production and suppression of pathogens within the human body. Further investigations, informed by a deeper understanding of functional capabilities and metabolic cross feeding could pave the way for the identification of specific species contributing to cross feeding and pathogen suppression more significantly. This knowledge holds the potential to enhance the formulation of mixtures tailored for FMT and related therapeutic applications.

### 4.5 Conclusion

This study depicts the challenge of replicating full complexity of the human faecal microbiota using synthetic bacterial mixes. This complexity arises from the presence of countless trillions of diverse bacteria, alongside bacteriophages, fungi, and a myriad of other microorganisms that collectively constitute this intricate ecosystem. The endeavour to replicate the human faecal microbiota through the inclusion of only nine selected bacterial strains, although representative of the most abundant genera in the human gut, reveals the limitations of such simplified models. The complexity of human faeces, characterised by a diverse microbial community comprising numerous genera, contrasts with the synthetic mix's limited representation of only nine groups. This difference emphasises challenges inherent in replicating the complex microbial composition of human faecal matter in a synthetic model. Human faecal microbiota encompasses a wide range of bacterial taxa, each contributing to the overall ecosystem's functionality and stability. In contrast, the synthetic mix, while designed to simulate certain aspects of this diversity, inherently lacks the richness and complexity observed in natural microbiomes. As a result, the synthetic mix may not fully capture the complex interactions and functionalities present in human faeces, leading to discrepancies in microbial dynamics, metabolic profiles, and other key parameters. However, it is important to recognise the potential for improving synthetic mixes through a continued deep understanding of the human faecal microbiota. These endeavours offer the possibility of creating more sophisticated and precise models of the complex gut ecosystem in

the future. Furthermore, this study highlights the importance of selecting appropriate techniques for bacterial enumeration based on the type of samples.

The limitations of this chapter include the possibility that the time points obtained for the synthetic mix were too late. If the time points from T0 to T12 had been monitored more closely, a clearer understanding of the cross-feeding interactions and the production of intermediate metabolites could have been captured. Additionally, the introduction of pathogenic bacteria raises questions about the safety of administering this mix in a clinical setting, even though it was used to monitor pathogen suppression. Conducting another set of experiments that excludes the pathogenic strains would provide a more comprehensive understanding of the effects of the synthetic consortium.

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# CHAPTER 5

## *In vitro* and *in vivo* studies to investigate the effect of a probiotic yoghurt on microbial and metabolic profiles


### Abstract

This chapter delves into a comprehensive investigation of the impact of a probiotic yoghurt intervention on microbial and metabolic profiles, utilising both *in vitro* and *in vivo* analyses. The study aimed to understand how dietary interventions, particularly probiotic yoghurt, influence gut microbiota and metabolic functions, especially in populations vulnerable to undernutrition-related challenges.

Initial analysis using fluorescent in-situ Hybridization (FC-FISH) revealed a significant increase in total bacterial counts post-intervention in the group of school children receiving probiotic yoghurt compared to the control group. Although the increase in lacticaseibacilli was marginal, the overall rise in total bacteria counts suggests a notable impact on gut microbiota composition.

Further exploration through in vitro batch culture experiments provided insights into shifts in bacterial groups, including bifidobacteria, roseburia, and bacteroides, although these changes were not statistically significant. Nevertheless, observed alterations in bacterial abundance shed light on the collective response of different bacterial groups to probiotic intervention.

Metabolomic analysis of urine samples complemented these findings by highlighting distinct metabolic profiles in both the placebo and intervention groups. The intervention group exhibited increased levels of metabolites such as hippurate and betaine, indicating potential improvements in gut microbial diversity and nutrient utilisation. Parallel studies further supported the potential benefits of probiotic interventions in modulating gut microbial composition and immune function indicating reduced disease recurrence in the group receiving probiotic intervention.

Overall, the results underscore the promising role of probiotics in addressing undernutrition-related challenges and improving overall health outcomes. Further research and clinical studies can build upon these insights to develop targeted interventions and strategies for optimising metabolic and microbial health.

## 5.1 Introduction

Building upon the findings from preceding chapters, particularly regarding the probiotic *Lactobacillus rhamnosus* yoba (LGG) incorporated into yoghurt and its promising outcomes in pathogen suppression and in-vitro establishment, the focus shifted to investigating these outcomes *in vivo*. This led us to explore the dynamics of metabolite production and lacticaseibacilli establishment within a population of pre-primary school children from South West Uganda. Given the well-documented nature of lacticaseiballi as a probiotic, it has been widely utilised in the YOBA for Life project (YOBA4Life) (Kort et al. 2015; Westerik et al. 2020). Through collaboration with Yoba4Life, we had the opportunity to analyse faecal and urine samples collected during an intervention trial involving the use of this probiotic yoghurt.

### *5.1.1 Gut microbiota and health*

The gut microbiota plays a crucial role in various physiological processes, including energy metabolism (Heiss and Olofsson 2018; Duca and Lam 2014), vitamin synthesis such as vitamin K and some vitamin B (LeBlanc et al. 2013; Rowland et al. 2018), immune function (Rooks and Garrett 2016; Lazar et al. 2018; Cullen et al. 2020) and gut-brain communication (Valles-Colomer et al. 2019; Cryan and Dinan 2012). Moreover, the gut microbiota contributes to the production of metabolites, mainly, SCFAs, secondary bile acids, and neurotransmitters, influencing gut barrier function and bidirectional gut-brain signalling (Rios-Covian et al. 2016; Parada Venegas et al. 2019). Consequently, there is growing interest in leveraging dietary interventions, including prebiotics and probiotics, to modulate the gut microbiota and enhance nutritional outcomes and overall health.

There is growing recognition of the potential nutritional and health benefits associated with fermented foods such as yoghurt, kefir, kimchi and kombucha. Fermented foods are defined as “foods made through desired microbial growth and enzymatic conversions of food components” (Marco et al. 2021). Existing data suggest a potential link between fermented food consumption and improved health outcomes such as favourable blood pressure levels, anthropometric measures, triglyceride levels and increased high density lipo-proteins (HDL) levels (Hill et al. 2023; Marco et al. 2017).

Yoghurt is a fermented food derived from milk, which undergoes a fermentation process wherein lactic acid-producing bacteria metabolise the sugar and other nutrients present in milk. As a result of fermentation, organoleptic properties are altered resulting in the formation of yoghurt. Yoghurt is known for its longevity and is a cheap and accessible source of food. Therefore, it is a suitable source for interventional studies based on possible nutritional and health benefits. Cohort studies have indicated correlations between weight maintenance, healthier metabolic profiles and the intake of fermented dairy products (Mozaffarian et al. 2011; Panahi et al. 2017). Additionally, studies have demonstrated improved gastrointestinal conditions including IBS and bowel cancers following yoghurt consumption (Adolfsson, Meydani, and Russell 2004). Individuals who regularly consume yoghurt have also demonstrated reductions in the risk of cardiovascular disease and type 2 diabetes, and overall well-being (Panahi et al. 2017). There is currently no recommended level of live microbial intake, although recent studies have attempted to investigate this (Marco et al. 2020). Recently, the FDA approved the first health claim on yoghurt, stating that at least 3 servings per week may reduce the risk of developing type 2 diabetes (FDA 2024).

Biotic-based approaches, particularly those involving probiotics, are gaining significant popularity in the realm of health and nutrition. Combining probiotics with yoghurt represents a promising strategy within this context, capitalising on the established health benefits of both probiotics and fermented dairy products.

A probiotic is defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill et al. 2014). Among probiotic bacteria, LGG is one of the most extensively studied, and known for its unique characteristics and associated health benefits. Numerous studies have shown no adverse effects of consuming LGG in healthy infants (Petschow et al. 2005; Scalabrin et al. 2017).

Intake of LGG has demonstrated various beneficial effects on health. It enhances feeding tolerance and nutrient absorption (Krajmalnik-Brown et al. 2012; Duca and Lam 2014), potentially leading to increased weight gain in children. Additionally, LGG has been found to bind to Aflatoxin B1, reducing its absorption in the intestine and mitigating aflatoxin-related pathogenicity, including stunting (Wacoo et al. 2020). LGG supplementation has also shown efficacy in reducing the incidence and severity of rotavirus-associated diarrhoea (Sindhu et al.

2014) and various respiratory tract infections (RTI) (Du et al. 2022; Kara, Volkan, and Erten 2019). These findings highlight diverse health-promoting properties of LGG.

Fermented foods, including yoghurt, are gaining recognition for their potential nutritional and health benefits. Yoghurt, derived from milk through fermentation by lactic acid-producing bacteria, is not only affordable and accessible but also associated with improved weight management, metabolic health, and gastrointestinal conditions. Furthermore, probiotics, like LGG, are extensively studied for their ability to confer health benefits, including improved nutrient absorption, reduced gastrointestinal issues, and enhanced immunity. In regions with high child mortality due to diseases like diarrhoea and respiratory tract infections, integrating probiotics like LGG into yoghurt represents a promising approach to address these health challenges and promote better child health outcomes.

### *5.1.2 Childhood undernutrition*

Undernutrition is a significant contributor to more than half the deaths of children under 5 years of age (UNICEF 2023). It increases the vulnerability of children towards infections, the frequency/severity of contracting infections and also delays recovery. The 2023 Joint Child Malnutrition Estimates (JME) highlighted a concerning lack of progress toward meeting 2025 World Health Assembly (WHA) global nutrition goals. Meeting the target of reducing stunting in children (from 178 million to 89 million) by 2030 will require significantly more focused efforts. Based on current trends, it is projected that this goal will be missed by 39.6 million children, with over 80% of these children residing in Africa (UNICEF 2023). As an effort to address this global issue, it is vital to identify convenient and accessible foods that can help undernourished populations .

### 5.1.3 Yoba4Life project

In developing countries such as Uganda, childhood diseases are responsible for high morbidity (Westerik et al. 2020). According to data from the Ugandan National Demographic and Health Survey in 2011, 14% of children under the age of 5 in the Southwestern region have experienced episodes of diarrhoea, while 11% suffered from RTIs (Westerik et al. 2020). As a solution for this

issue the Netherlands Development organisation (SNV) initiated a milk school feeding program, as part of The Inclusive Dairy Enterprise Project (TIDE) in Uganda's southwestern region. By promoting milk consumption in primary schools across seven districts, children receive 100ml of milk five days a week during school terms, integrated into their meals as a hot beverage. Over four years, approximately 300,000 primary and pre-primary school children have benefited from this initiative. Building from the success of the TIDE school milk program, SNV and the Yoba4Life launched a program incorporating locally produced probiotic yoghurt instead. The shift was motivated by the belief that probiotic bacteria, particularly *Lactobacillus rhamnosus* yoba 2012 (a generic form of *L. rhamnosus* GG), could enhance immunity and alleviate common childhood ailments like diarrhoea, colds, allergies, skin issues, and growth delays. As a result, (Kort et al. 2015) introduced the *Lactobacillus rhamnosus* yoba-containing yoghurt drink, which was locally produced and consumed by resource-poor communities in rural Uganda under the YOBA4Life project. With this, development, an observational nutritional trial on effect of probiotic yoghurt containing *Lactobacillus rhamnosus* yoba on RTI and other health outcomes among children aged 3-6 years in Southwest Uganda was conducted which showed a positive effect on common colds and skin infections (Westerik et al. 2020). Followed by the observational study, a randomised double-blind placebo-controlled probiotic yoghurt nutrition intervention study with pre-primary school children in Uganda was conducted where we collaborated with and performed the analysis of urine metabolites and lacticaseibacilli establishment pre and post intervention.

### *5.1.4 Functional assessment of gut microbiota following yoghurt consumption*

Understanding the metabolic processes underlying health benefits of fermented foods and probiotics is crucial, especially given the known effects of gut microbiota and fermented food on overall health. Previous studies have often failed to elucidate underlying mechanisms of probiotic supplementation. By monitoring the behaviour of gut microbiota and analysing urinary metabolites in study participants, and comparing these findings with in vitro experiments, this research aims to understand physiological mechanisms occurring within the human body.

Samples from pre and post intervention from a nutritional trial conducted among children from South West Uganda were analysed using FC-FISH and $^{1}$H-NMR metabolic phenotyping techniques to get an insight into the *in vivo* behaviour on consumption of probiotic yoghurt. Metabolic

phenotyping is a method of analysing metabolites in a biological system, which provides details into how these compounds change in response to various factors. These factors include genetics, environment, diet, lifestyle, and the activities of gut bacteria. By studying metabolic profiles, we can assess the overall metabolic status of a complex system. This analysis helps identify metabolic pathways associated with disease risk and enables the discovery of molecular biomarkers for diagnosis and prediction.

Additionally, to complement findings from $^{1}$H-NMR technique and provide further clarity, results from *in vitro* analyses from batch culture experiments and the atlas of pure culture bacteria were used to provide insights into functional mechanisms of the interactions and contribution of gut microbiota in the human body.

## 5.2 Materials and Methods

### *5.2.1 Intervention study using the probiotic yoghurt*

The interventional study, included two groups - an intervention group consuming probiotic yoghurt with *Lactobacillus rhamnosus* yoba and a control group consuming milk. Each group consisted of approximately 100 children. All children within a school were enrolled in the same group. The study lasted for 11 weeks with 3 weeks baseline period and 8 weeks intervention study, during which children consumed either 100 mL of yoghurt or 100 mL of milk daily, five days a week, while being continuously monitored. Both the milk and yoghurt were sourced locally from the districts where the schools were situated. The study ethics was approved by Ugandan ethics reference MUREC 1/7 (Awarded by Mbarara University Ethics Committee). The probiotic yoghurt was prepared by a local producer according to the protocol described in (Kort et al. 2015). Urine and faecal samples were collected at beginning of the baseline week and end of the intervention week.

### *5.2.2 Collection of urine and faecal samples*

The urine was collected in an 80 mL container, and details such as volume and time of collection were noted. Within 1.5 hours of collection, samples were pipetted in triplicate into 1.5 mL

cryovials and stored at -20°C for up to two weeks. Following this, the samples were transferred to a -80°C deep freezer.

Stool samples were collected from each child once during the baseline period and once during the final week of the study. To ensure purity of the stool sample, children were instructed to urinate before collection. After donation, each stool sample was transferred into 1.5 mL cryovials using small wooden applicator sticks. Within one hour of collection, samples were stored at -20°C then moved to a -80°C deep freezer until microbiological analysis.

### *5.2.3 FC-FISH for faecal samples*

Frozen faecal samples were thawed and diluted 1:10 (w:v) with anaerobic phosphate-buffered saline (PBS, 0.1 M; pH 7.4), then vortexed with 3 mm diameter glass beads for 30 s before being centrifuged at 1,500 x g for 3 min at room temperature. 100 µL of the supernatants were then diluted in 900 µL phosphate buffered saline (PBS mol l-1; pH 7.4) (1:100 dilution), aliquoted into 1.5 mL Eppendorf tubes and stored at -80 °C until cells were fixed. For fixation, samples were centrifuged at 11,337 × g for 5 min and the supernatant decarded. Pellets were then resuspended in 375 µL of 0.1 M PBS and fixed in 4% (w/v) paraformaldehyde (1,125 µL) for 4 h at 4 °C. Fixed cells were centrifuged at 11,337 × g for 5 min at room temperature. Samples were then washed with 1 mL PBS, pellets aspirated and centrifuged at 11,337 × g for 5 min. The washing process was repeated twice more. Samples were re-suspended in 150 µL PBS and stored in ethanol (1:1, v:v) at −20 °C until analysis via FC-FISH.

Bacterial populations were assessed by FC-FISH with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA, as previously described (Grimaldi et al. 2018). Total bacteria and numbers of lactobacilli were quantified. The commercially synthesised probes used to enumerate these bacteria are shown in Table 5.1.

***Table 5.1:*** *Name, sequence, and target group of oligonucleotide probes used in this chapter for bacterial enumeration using FC-FISH*

| Probe name | Sequence (5' to 3') | Targeted groups | Reference |
|---|---|---|---|
| Non Eub | ACTCCTACGGGAGGCAGC | Control probe complementary to EUB338 | (Wallner, Amann, and Beisker 1993) |
| Eub338 | GCTGCCTCCCGTAGGAGT | Most bacteria | (Amann et al. 1990) |
| Eub338II | GCAGCCACCCGTAGGTGT | Planctomycetales | (Daims et al. 1999) |
| Eub338III | GCTGCCACCCGTAGGTGT | Verrucomicrobiales | (Daims et al. 1999) |
| Lab158 | GGTATTAGCAYCTGTTTCCA | *Lactobacillus* and *Enterococcus* | (Harmsen et al. 2000) |

### *5.2.4 $^{1}$H-NMR spectroscopic analysis of urine*

Frozen urine samples were thawed. A phosphate buffer (pH 7·4 sodium phosphate with 0.2M disodium phosphate (Na2HPO4), 0.04M monosodium phosphate (NaH2PO4) in deuterium oxide (99·9 %) was prepared, with 1mM 3-(trimethylsilyl) propionic acid-d4 sodium salt (TSP) and 3mM sodium azide in the solution. 400 µL of each urine sample were mixed with 200 µL buffer. 550 µL aliquots of supernatant were collected and dispensed into 5 mm NMR tubes.$^{1}$H-NMR spectroscopic analysis was carried out using a Bruker Avance DRX 500 MHz NMR spectrometer (Bruker Biospin, Germany) operating at 500.13 MHz. Urine spectra were acquired using a standard 1D pulse sequence [recycle delay (RD)-323 90◦-t1-90◦-Tm-90◦-acquire free induction decay (FID)] with water suppression applied during RD of 2 s, a mixing time Tm of 100ms and a 90o pulse set at 7.70 µs. Per spectrum, a total of 128 scans were carried out with a spectral width of 14.0019 ppm. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz

line broadening. Acquired spectroscopic data were processed using the TopSpin 3.6.5 software package (Bruker Biospin, Rheinstetten, Germany).

### *5.2.5 Chemometric analysis*

Processed spectroscopic data were imported to the SIMCA 13.0 software package (Umetrics AB, Umeå, Sweden) to conduct multivariate statistical analysis. PCA was initially performed to detect any similarities or differences in the urinary spectral profiles and identify outliers based on the distribution of points in the PCA scores scatter plot. Subsequently, supervised modelling using O-PLS-DA was conducted to identify any metabolites that can contribute to changes pre and post intervention. The $R^2$ and $Q^2$ variables provided an indication of goodness of fit ($R^2$) as well as goodness of prediction ($Q^2$) of the models. OPLSDA models were subsequently ran, to maximise separation between the groups in order to determine the metabolites characteristic of pre and post intervention.

### *5.2.6 In-vitro batch culture experiment using probiotic yoghurt*

Batch culture fermentation experiments were conducted following procedures outlined in Chapter 4. Initially, the probiotic strain's colony-forming units (CFU) were determined by plating the yoghurt sample on MRS agar (Appendix 5.1), resulting in a count of $1x10^8$ CFU/mL. Subsequently, a vessel was prepared with probiotic yoghurt containing the same concentration ($10^8$ CFU/mL) to match the initial CFU count. Control vessel contained the nutrient rich medium only.

Bacterial DNA was extracted from batch culture sample pellets using the QIAamp PowerFecal Pro DNA Kit (QIAGEN) according to the manufacturer's instructions. DNA samples were sent to Novogene Europe (Cambridge,UK) for 16S rRNA gene sequencing. The method was as described in Chapter 4.

## 5.3 Results

### *5.3.1 FC-FISH enumeration*

In the interventional study, Group 1 received milk, while Group 2 received the probiotic yoghurt. Pre and post-intervention assessments were conducted using FC-FISH to measure total bacteria and lacticaseibacilli counts. The results indicated a significant increase (*p=0.017) in total bacteria counts in Group 2 between pre and post-intervention (Figure 5.1). Similarly, lacticaseibacilli counts in Group 2 also increased post-intervention, although the increase was marginally significant (*p=0.046) (Figure 5.2). These results suggest that yoghurt intervention led to a substantial increase in total bacterial and an increasing trend in lacticaseibacilli compared to the placebo group. At baseline, the two groups exhibited differences, making it difficult to draw valid conclusions from the post-intervention comparisons.

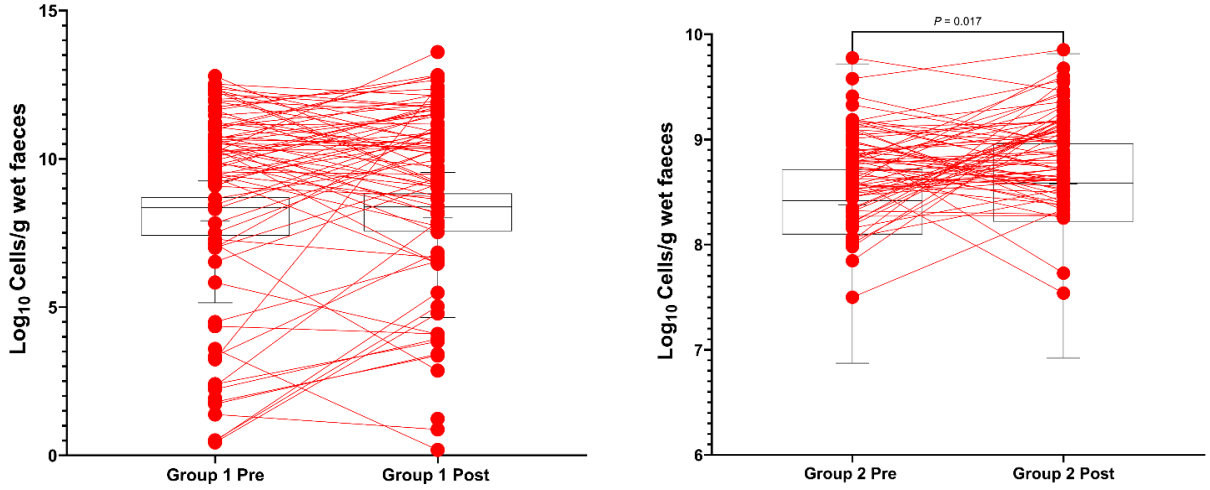



***Figure 5.1**: Total bacteria counts in Log10 cells/g wet faeces in group 1 (A) and group 2 (B) pre and post intervention*

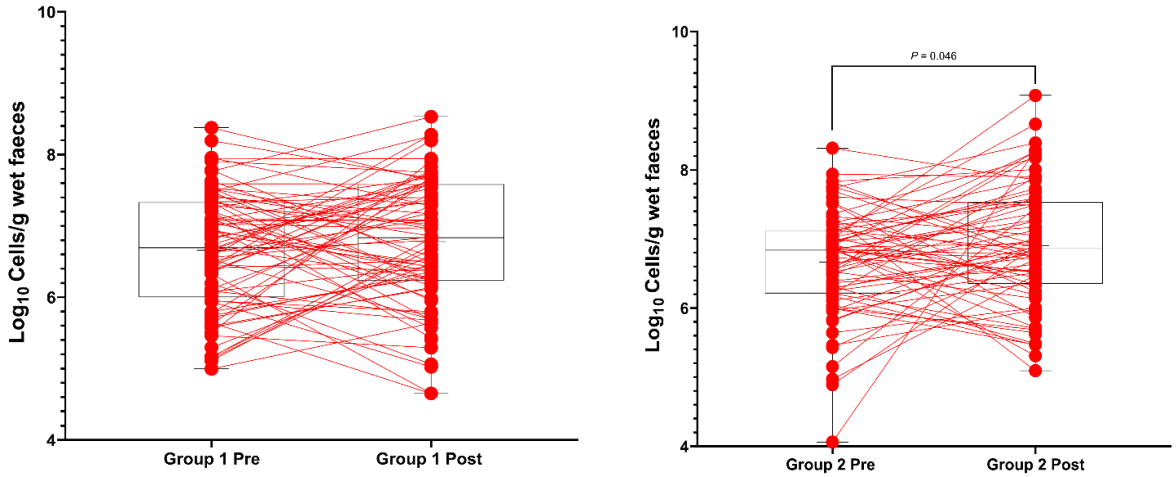
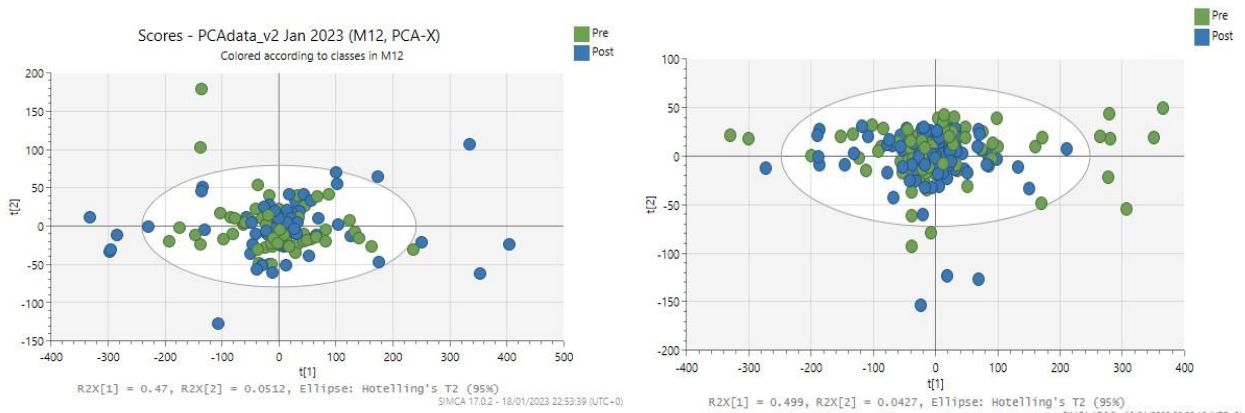



***Figure 5.2:*** *FC-FISH Lactobacilli counts in Log10 cells/ wet faeces in group 1 (A) and group 2 (B) pre and post intervention*

### *5.3.2 Chemometric analysis*

The PCA scores plots for both groups pre and post-intervention (Figure 5.3) did not reveal any intrinsic patterns or trends in the urinary metabolic profile data. A more detailed analysis of the data were then conducted, using O-PLS-DA. The O-PLS-DA loadings line plot showing differences in the metabolic profiles, are shown in Figure 5.4 for group 1, and Figure 5.5 for group 2.




A B

***Figure 5.3:*** *PCA score plots comparing pre and post intervention in group 1 placebo (A) and group 2 intervention (B)*

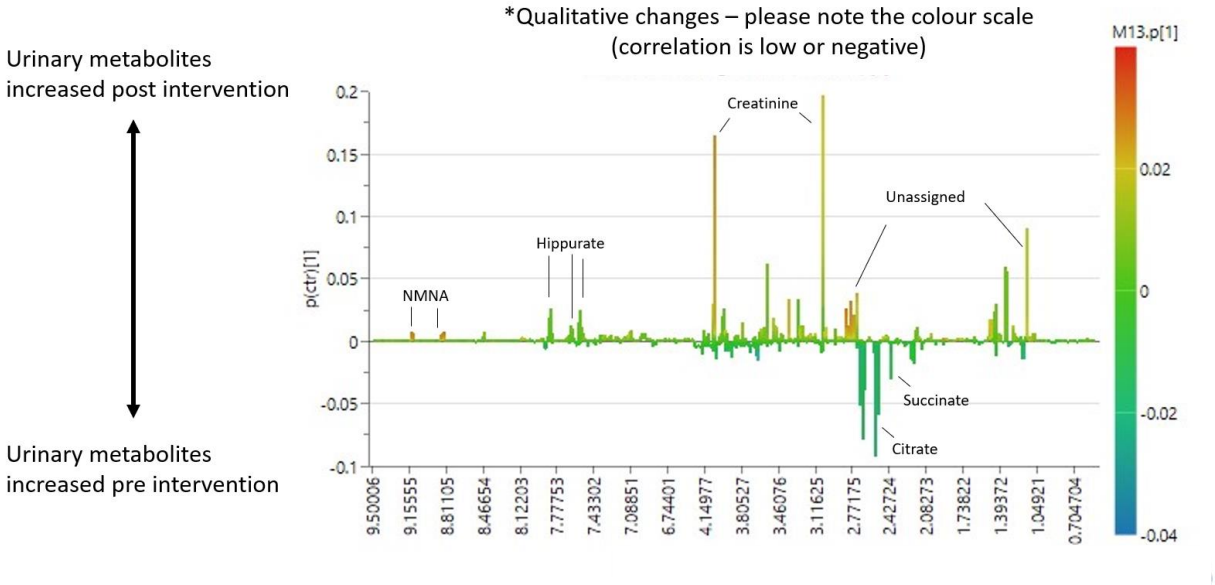



***Figure 5.4:*** *OPLSDA for group 1 placebo to determine the metabolites that had highest influence pre and post intervention*

The OPLSDA analysis revealed distinct metabolic profiles in both groups, with group 1 (the placebo group) showing specific metabolites to be higher pre-intervention compared to post, and group 2 (the yoghurt intervention group) exhibiting different metabolite changes compared to the placebo. In group 1 (Figure 5.4), pre-intervention metabolites such as succinate and citrate suggest ongoing cellular respiration processes, while post-intervention metabolites like NMNA, hippurate and creatinine indicate alterations possibly influenced by the intervention, reflecting changes in energy metabolism and kidney function. On the other hand, group 2 (Figure 5.5) displayed metabolites like creatine and lactate in addition to citrate and succinate pre intervention pointing towards diverse metabolic activities related to energy metabolism and cellular respiration. The appearance of NMNA, hippurate, and creatinine post-intervention in group 2 indicates distinct metabolic changes potentially influenced by the intervention, highlighting shifts in energy metabolism, dietary patterns, and kidney function. These results show that the interventions had a notable effect on metabolic activities and physiological processes in both groups. However, each group had its own unique metabolic characteristics.

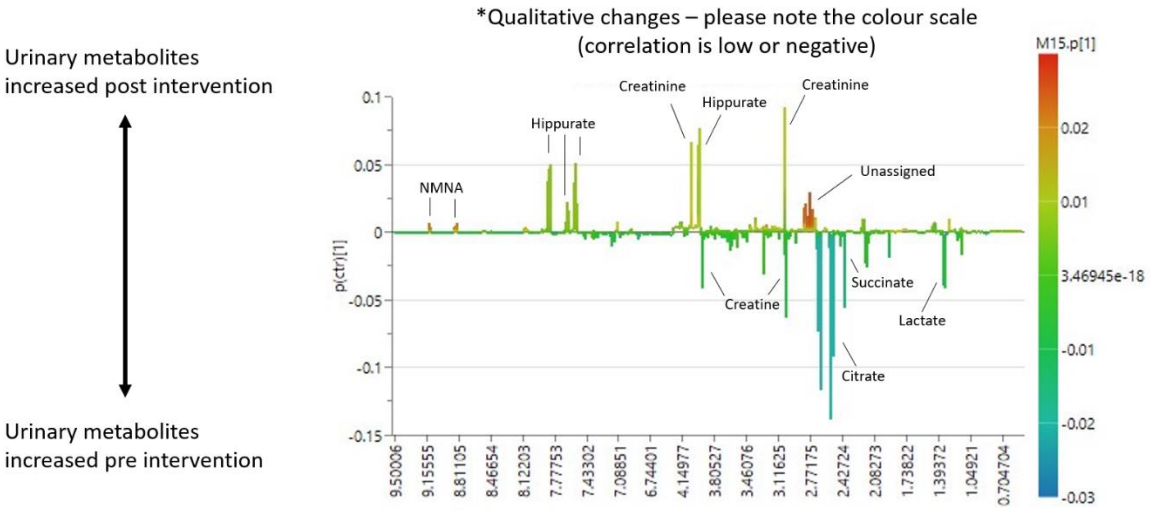



***Figure 5.5:*** *OPLSDA for group 2 intervention group to determine the metabolites that had highest influence pre and post intervention*

Metabolic profiles were further analysed by combining data from both group 1 (placebo) and group 2 (intervention) groups, focussing on differences in both post intervention. No clear separation according to group was observed in the PCA (scores plot shown in Figure 5.6). However, upon conducting O-PLS-DA, (Figure 5.7) it was noted that Group 2 displayed increased levels of hippurate and betaine, while Group 1 showed higher levels of creatine, creatinine, and lactate. These distinct urinary metabolic signatures between the two groups suggest that the interventions may have caused different metabolic responses.

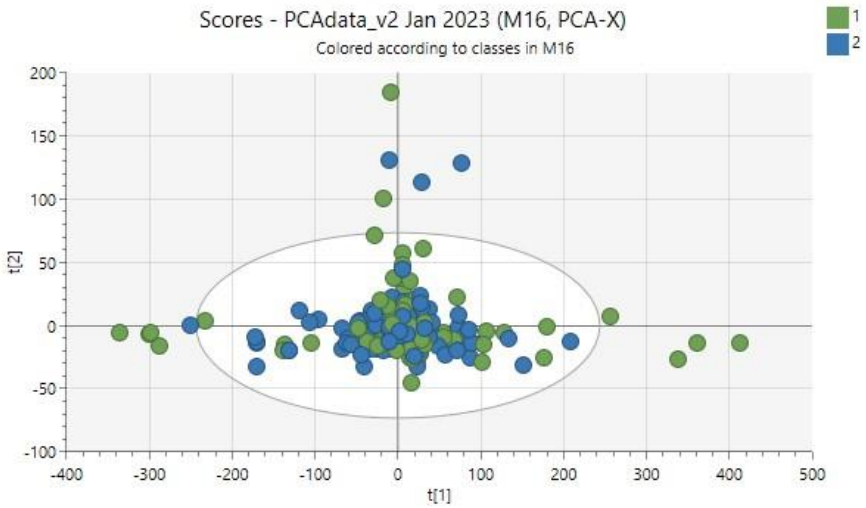



***Figure 5.6:*** *PCA for post intervention between the two groups 1 and 2*

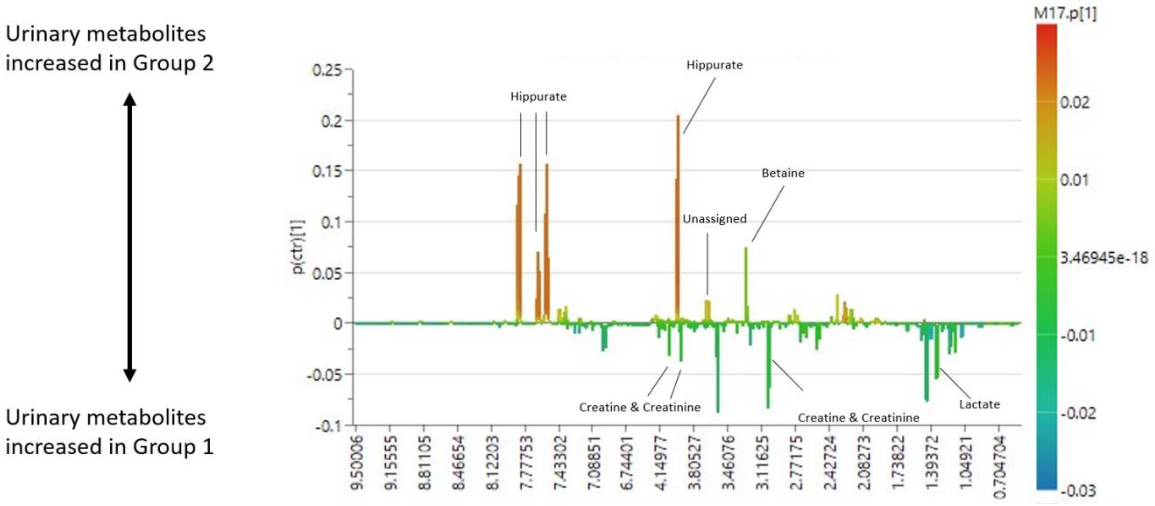



***Figure 5.7:*** *OPLSDA for the two groups post intervention*

The findings indicate heightened microbial activity in group 2, as evidenced by the increased levels of hippurate. Hippurate is a product of microbial fermentation within the gut and serves as a marker for active microbial processes in the intestine. Elevated hippurate levels observed in group 2 suggest a greater extent of microbial fermentation or metabolic activity in group 2 compared to group 1.

## 5.4 Discussion

This chapter sought to investigate the impact of yoghurt intervention on microbial and metabolic profiles, in vitro and in vivo. Initially, FC-FISH enumeration indicated a significant rise in total bacterial counts after intervention in the yoghurt group, along with a minor increase in lacticaseibacilli. However, there was a difference between the two groups at baseline, therefore we cannot compare the two groups post-intervention. From these results it became evident that the intervention had a noticeable impact on the overall bacterial populations rather than specifically on lacticaseibacilli levels. To gain a deeper understanding, we revisited batch culture experiments to gain insights into the impact on the entire microbial profile. Although differences observed were not statistically significant, there was an increase in roseburia and bacteroides,

alongside lacticaseibacilli, based on the relative abundance values obtained from 16S rRNA sequencing. Thus, the elevation in total bacteria can be interpreted as a collective response of different bacterial groups.

This was supported by metabolomic analyses of urine samples from the human study, where the PCA scores plot did not show clear separation between the groups pre and post-study, indicating a lack of intrinsic patterns or trends in the urinary spectral profiles. However, supervised modelling using OPLSDA, revealed distinct metabolic profiles in both groups, with the placebo group exhibiting specific metabolites pre and post-intervention, and the interventional group showing different metabolic changes. Notably, the intervention group displayed increased levels of hippurate and betaine post-intervention, while the placebo exhibited higher levels of creatine, creatinine, and lactate. These distinct metabolic signatures suggest that the interventions may have elicited different metabolic responses in each group.

The findings across FC-FISH enumeration, metabolomics analysis, and human batch culture experiments collectively indicate that dietary interventions induced alterations in total bacterial counts and metabolic profiles. Even though *in vitro* experiments showed a significant establishment of probiotic it was only a marginal increase shown in the *in vivo* trial. Several factors may contribute to these observed results. Firstly, the dosage regimen of the interventions could have influenced their targeted effects. Secondly, adhesion of lacticaseibacilli to the intestinal barrier. Lastly, nutritional status of the studied population, particularly their nutrition condition as the Body Mass Index-for-Age Z-score (BAZ) was only slightly above the WHO standards, could have influenced microbial dynamics and metabolic responses, contributing to the observed outcomes.

In this study, the initial bacterial load in the probiotic yoghurt was $10^8$ CFU/mL, with a daily administration of 100mL resulting in a daily bacterial load of almost $10^{10}$ CFU/mL. Batch culture experiments have demonstrated effective bacterial stabilisation at $10^8$ CFU/mL, suggesting that lower concentrations may not have a significant impact. Considering the ability of lacticaseibacilli to withstand lower pH conditions and bile, as evidenced by previous studies (Doron et al. 2005), it is challenging to deduce that the concentration dropped below $10^8$ CFU/mL. Therefore, it is unlikely that the dose served as a limiting factor for the observed results.

In a parallel study conducted by the YOBA4Life group from the yoghurt intervention in the same population, decrease in disease prevalence among children was observed, it was found that the probiotic yoghurt reduced the incidence rate of common cold and skin infections in the intervention group compared to placebo (Westerik et al. 2020). This observation supports the suggestion that lacticaseibacilli may have played a role in adhering to the intestinal barrier. This is backed by the study group's previous work on developing YOBA yoghurt, which demonstrated that the probiotic function of LGG is linked to its ability to adhere to epithelial cells. Furthermore, transmission electron microscopy (TEM) images have shown the presence of pili structures post-fermentation of yoghurt (Kort et al. 2015). These findings are consistent with (Dunne et al. 2001) indicating that LGG can persist in the host for an extended period. Additionally, studies have shown that *Lacticaseibacillus* species possess significant immunomodulatory capabilities, including enhancing phagocytosis, producing antimicrobial peptides and lysosomal enzymes, improving vaccine effectiveness, regulating interleukins (important in immune responses), stimulating T cells, and reducing intestinal permeability (Maria Remes Troche et al. 2020; Segers and Lebeer 2014).

Pili in LGG are long, thin protrusions found on the surface of certain bacteria and are involved in adhesion to surfaces like mucus and intestinal cells. The discovery of the spaCBA gene cluster responsible for SpaCBA pili production confirmed this adherence mechanism (Lebeer et al. 2012; Segers and Lebeer 2014). Further studies revealed that these pili play a crucial role in sticking to mucus and intestinal cells (Reunanen et al. 2012). Interestingly, LGG retains its pili even under harsh conditions like exposure to bile salts and low pH (Douillard et al. 2013). Therefore, it is plausible that the marginal increase in lacticaseibacilli observed in the intervention group contributed to enhanced immune responses and intestinal permeability, which aligns with evidence of reduced disease recurrence.

Metabolic analyses of the placebo and intervention groups before and after the intervention revealed insights into responses with respective treatments. In the placebo group, metabolites which were higher pre-intervention included citrate and succinate but this was not reported in all individuals. Succinate is an intermediate of the tricarboxylic acid (TCA) cycle, and plays an important role in adenosine triphosphate (ATP) generation in mitochondria. This suggests energy metabolism through the TCA cycle and cellular respiration processes (Arnold and Finley 2023; Choi, Son, and Baek 2021). However, presence of citrate and succinate are identified as

metabolites signalling inflammation (Mills and O'Neill 2014; Verbeke et al. 2015; Tannahill et al. 2013). Interestingly, post-intervention, the intervention group did not detect succinate or citrate instead detected creatine and creatinine indicating ongoing muscle metabolism, renal function, and potential changes in energy production pathways such as glycolysis and creatinine metabolism (Bonilla et al. 2021; Wyss and Kaddurah-Daouk 2000). On the other hand, the intervention group also detected succinate, citrate and additionally displayed creatine and lactate production depicting energy metabolism and possibly anaerobic respiration or inflammation due to lactate production (Verbeke et al. 2015). Following intervention, the detection of N-methylnicotinamide (NMNA), hippurate, and creatinine in the intervention group suggested alterations in gut microbial metabolism and changes in energy utilisation pathways.

NMNA, an end-product of nicotinamide metabolism within the tryptophan-nicotinic acid pathway, signifies active nicotinamide utilisation and potential up-regulation of this pathway, impacting various metabolic processes (Makarov et al. 2019; Zheng et al. 2013). In undernourished children, NMNA detected in urine can serve as an indicator of short-term growth patterns, reflecting metabolic adaptations such as reduced energy usage, as indicated by elevated N-methylnicotinamide and decreased β-aminoisobutyric acid levels (Mayneris-Perxachs et al. 2016). This adaptation is linked to faster catch-up growth, suggesting its presence post-intervention may signify successful metabolic adjustments in both study groups.

The post-intervention detection of hippurate and creatinine further emphasises metabolic shifts post-probiotic yoghurt intervention. Hippurate, a benzoic acid metabolite from dietary sources, reflects changes in gut microbial metabolism and dietary intake, often indicating a more active microbiome (Pallister et al. 2017; Giallourou et al. 2020). Meanwhile, creatinine, a byproduct of muscle metabolism, reveals alterations in muscle mass and overall metabolic activity (Wyss and Kaddurah-Daouk 2000). Notably, betaine detected in the intervention group suggests microbial involvement in betaine metabolism, potentially indicating improved microbial diversity and nutrient utilisation. Betaine levels typically decrease in undernutrition but its presence post-intervention indicates a mitigated impact of undernutrition within this population (Mayneris-Perxachs et al. 2016; Giallourou et al. 2020). This highlights potential enhancements in gut microbial composition, dietary habits, and muscle metabolism due to the probiotic intervention.

Comparing post-intervention outcomes between groups reveals distinct metabolic responses. The placebo group maintained baseline metabolism, while the intervention group exhibited altered gut microbial metabolism and cellular responses, possibly influenced by the probiotic intervention. These findings underscore the multifaceted impact of probiotic interventions on metabolic pathways and microbial interactions, offering valuable insights into potential strategies for addressing undernutrition-related metabolic disruptions.

In light of the modest increase in total bacterial counts and lacticaseibacilli following the intervention, it is worth noting that our in-vitro investigations revealed potential increases in other bacterial groups like bifidobacteria, roseburia, and bacteroides. While these specific bacterial groups were not directly studied in the intervention, considering our previous chapters, we can speculate on the metabolites that might have been influenced if these groups had increased (with the aid of the atlas of gut microbial function Chapter 3). For instance, roseburia is known to produce butyrate, while bifidobacteria and lacticaseibacilli are associated with the production of acetate, lactate, and formate. These metabolites, including succinate, butyrate, lactate, and propionate, play vital roles in energy metabolism and gut barrier function attributed to gut microbiota activities (Martin-Gallausiaux et al. 2021; Riviere et al. 2016; Parada Venegas et al. 2019). Therefore, the presence of urinary metabolites such as hippurate and betaine can be interpreted as indicative of underlying microbial metabolic processes and their functional impacts.

The findings from this study shed light on the intricate metabolic adaptations observed in undernourished children following a probiotic yoghurt intervention. NMNA, reflective of active nicotinamide utilisation, exhibited changes indicative of successful metabolic adjustments linked to short-term growth patterns and enhanced energy utilisation. The post-intervention detection of hippurate and creatinine highlighted notable shifts in gut microbial metabolism and muscle metabolism, suggesting potential improvements in gut microbial composition and nutrient utilisation. Furthermore, the presence of betaine post-intervention suggested microbial involvement and a mitigated impact of undernutrition within the study population. These results underscore the potential of probiotic interventions to positively influence metabolic processes, offering promising avenues for addressing undernutrition-related challenges and improving overall health outcomes in vulnerable populations.

## 5.5 Conclusion

This chapter investigated the effects of a probiotic yogurt intervention on microbial and metabolic profiles in vivo settings, providing insights into the metabolic adaptations in pre-primary school children post-intervention. The results showed that probiotic yogurt has been shown to improve total bacterial counts in the gut, along with increasing metabolites such as NMNA, betaine, and hippurate, which provide metabolic evidence of these changes. These findings suggest that probiotic yogurt can positively impact malnourished populations, highlighting its potential as a beneficial intervention. However, there are limitations to consider. For instance, the study focused solely on lacticaseibacilli, and a more comprehensive analysis using FC-FISH enumeration of all bacteria could have clarified which specific bacteria contributed to the observed increase in total bacterial counts. Furthermore, testing in a healthy adult population would have allowed for better comparisons with in vitro work. Future research with supportive clinical studies can build upon these insights and develop targeted interventions for optimising metabolic and microbial health in undernourished populations.

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# CHAPTER 6

## 6.1 General Discussion

Understanding intricate dynamics of the gut ecosystem presents a formidable challenge. The role of gut microbiota in human health is undeniable, with extensive research aimed at elucidating ways to harness its potential for enhancing well-being. The capabilities of gut microbiota are vast and diverse, encompassing aspects ranging from digestion to influencing brain functions (Rowland et al. 2018; Cryan and Dinan 2012), beginning from birth and extending throughout life (Ronan, Yeasin, and Claud 2021). Decades of research highlight the significance of gut microbiota in aspects such as health, nutrition, immunity, and the modulation of the gut environment. However, maintaining a suitable relationship between the host and microorganisms is vital for proper metabolism, immune function, and disease prevention (Rinninella et al. 2019).

While research has delved into functional behaviour of gut microbiota, there is a need in research regarding molecular mechanisms and interactions between gut microbiota and host of real foods and live bacteria consumption, notably, fermented foods (Hill et al. 2023; Marco et al. 2017).

Thus, this thesis concentrates on a probiotic yoghurt, a well-established fermented food, to investigate the behaviour of nine selected gut microbiota in a nutrient rich medium similar to the gut environment. Other fermentable substrates (starch and inulin) were also tested comparing to nutrient rich medium as the control. Initially studied in pure cultures, they were then examined in co-culture with probiotic yoghurt and eventually as a mixed culture, providing insights into their responses across different scenarios and generating a comprehensive metabolite profile. This approach also sheds light on behaviour of bacteria in both pure and mix culture towards substrates.

Comparing the synthetic mixed culture with human faeces offers insights into its potential applicability in FMT. Additionally, this thesis delves into an intervention involving the same probiotic yoghurt, analysed within a group of children in South West Uganda, revealing positive impacts on gut microbial composition, including total bacteria and lacticaseibacilli. This multi-faceted approach provides a comprehensive view of gut microbial dynamics and responses to dietary interventions, contributing towards enhancing gut health and overall well-being.

Thereby, this study offers significant contributions to our understanding of the functional capacities of the human gut microbiota, particularly through the development of a comprehensive atlas of microbial interactions and metabolic functions. This atlas is a important outcome that serves not only as a research tool but also as a foundational resource for practical applications in gut microbiota-targeted interventions. By providing an understanding of specific microbiota and the metabolites they produce, the atlas enables researchers and healthcare professionals to design precise strategies aimed at improving gut health.

One of the key strengths of this research lies in the practical applicability of the atlas, especially in developing targeted interventions to modulate the gut microbiota. The atlas can guide the enhancement or suppression of specific microbial populations based on their contributions to health or disease. For example, it can help identify beneficial microbes that produce SCFAs and neurotransmitters, which are crucial for maintaining gut integrity and supporting the gut-brain axis. Through dietary modifications, probiotics, or prebiotics, these beneficial microbes can be selectively promoted, leading to improved gut health and overall well-being.

The atlas also provides critical insights for optimising probiotic strains. By understanding the specific conditions under which these probiotics thrive and their interactions with existing gut microbes, more effective probiotic formulations can be developed. These formulations can be designed to integrate seamlessly with the host's microbial ecosystem, enhancing their efficacy in promoting gut health and managing conditions such as dysbiosis. In addition, the atlas offers valuable information for developing prebiotics that selectively promote the growth of beneficial bacteria. This is particularly relevant for creating functional foods that support gut health using local, underutilised resources such as yams, herbs, and coconut, which are abundant in developing countries like Sri Lanka.

One of the most promising aspects of this study is its relevance to addressing malnutrition and food security issues in developing countries. The atlas supports the creation of affordable, accessible functional foods that can be integrated into the diets of vulnerable populations, particularly through the use of local foods as prebiotics or probiotics. This approach not only enhances gut health but also provides a sustainable way to improve overall health and well-being in these communities. For example, utilising underutilised yams and herbs, as well as fruit peels and agricultural by-products, can create functional foods that support gut health while adding value to otherwise wasted resources during glut-production and post-harvest loses.

As a combined outcome of the atlas and the mixed microbial consortium, the practical applications extend well beyond research into clinical and therapeutic settings. The atlas, enriched by insights from the mixed consortium, can be pivotal in guiding interventions, particularly in cases of gut dysbiosis, where the balance of microbial populations is disrupted. By leveraging the detailed microbial mapping provided by the atlas alongside the functional capabilities demonstrated by the mixed consortium, targeted interventions can be developed to restore a healthy microbiota. This approach allows for the identification of specific microbes that are either depleted or overrepresented in disease states, enabling the design of precise strategies to restore microbial balance. Such interventions have the potential not only to prevent but also to mitigate the progression of diseases associated with dysbiosis, offering a framework for both therapeutic and preventative healthcare solutions.

Another promising prospects arising from this study is the potential development of synthetic microbial communities that could serve as viable alternatives to FMT. The use of donor-derived faeces in FMT is associated with several challenges, including variability in microbial composition, the risk of pathogen transmission, and ethical concerns. These limitations underscore the urgent need for safer and more standardized approaches in microbiota interventions (Merrick et al. 2020). The mixed microbial consortium developed in this study, informed by the detailed microbial interactions and functions mapped in the atlas, provides a foundation for creating synthetic microbial communities. These communities could be tailored to mimic the beneficial effects of FMT while minimising the associated risks, offering a more controlled, reproducible, and ethical alternative for restoring gut microbiota in clinical settings.

While the study offers valuable insights, there are certain limitations that should be considered. Although the microbial consortium used provided significant findings, the controlled in vitro nature of the experiments may not fully capture the complexity of the human gut environment. This suggests the importance of future in vivo studies to validate these results in more dynamic, real-world conditions. Additionally, the results could be influenced by specific dietary habits and geographical factors, which might limit the generalizability of the findings. Expanding the research to include diverse populations and diets would enhance the applicability and robustness of the results.

Looking ahead, there is considerable potential for optimising the microbial consortium, particularly by focusing on the exclusion of pathogens and the promotion of commensal

populations. This approach could lead to effective strategies for disease prevention and the restoration of a healthy gut microbiota in cases of dysbiosis. The development of functional foods using local resources, such as underutilised yams and herbs, represents a promising avenue for promoting gut health while simultaneously supporting food security and economic development in developing countries. Furthermore, clinical trials should be conducted to assess the efficacy of the developed probiotic and prebiotic products, particularly in terms of their impact on SCFA production, neurotransmitter synthesis, and overall gut-brain health in populations at risk of malnutrition.

Overall, this research lays a strong foundation for future studies aimed at optimising gut microbiota through diet and probiotic interventions. The atlas generated, combined with the insights gained on probiotics and prebiotics, has significant potential for real-world applications, particularly in improving health and well-being in developing countries. By leveraging local resources and focusing on sustainable practices, this work has the potential to address both health and economic challenges, paving the way for innovative solutions that benefit communities on multiple levels.

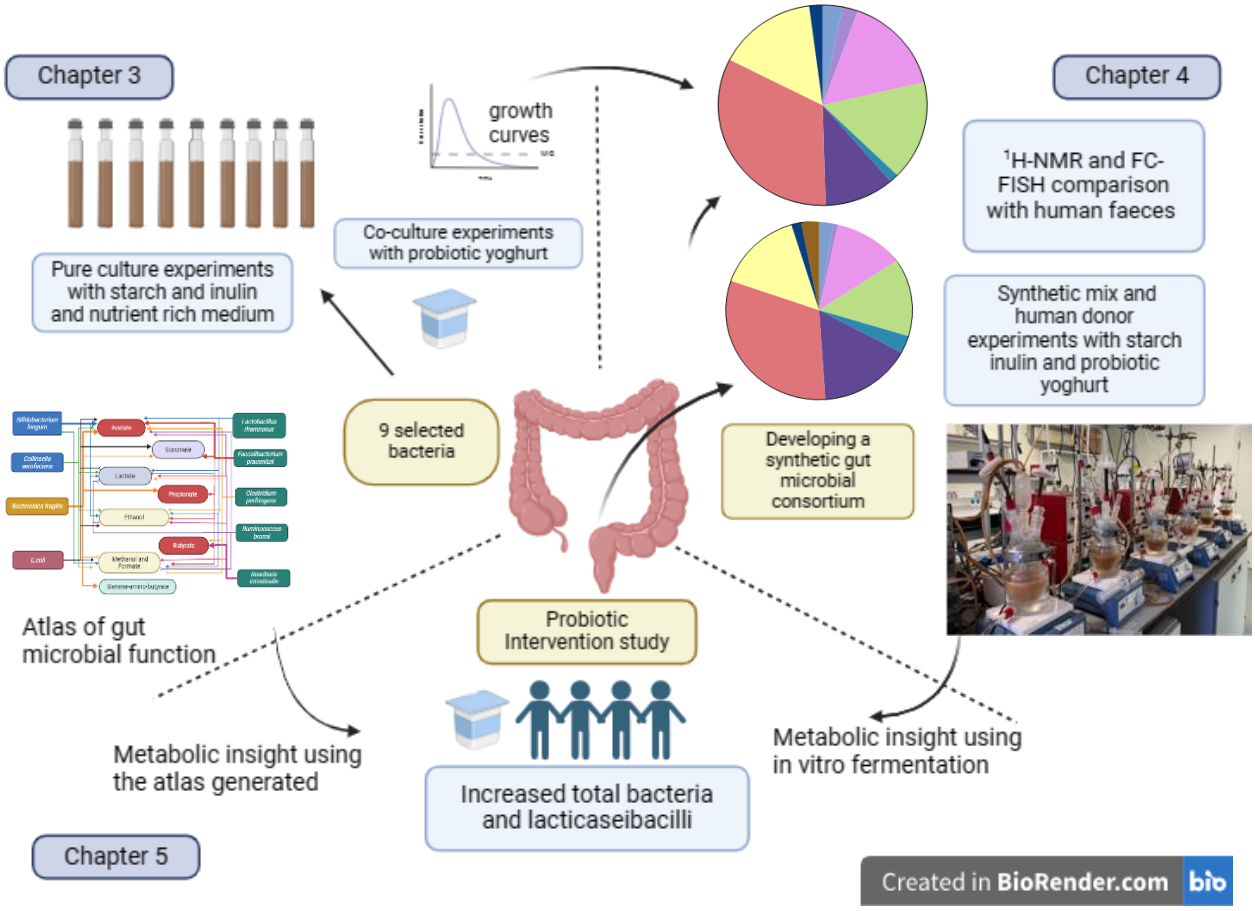


***Figure 6.1:*** *Summary of the results chapters*

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**Appendices – Supplementary Data and Information**

## Appendix 2.1 : Principles of NMR spectroscopy

Figure 1 displays a $^{1}$H-NMR spectrum of *E.coli* in Mueller Hinton medium 1.5h post inoculation. The spectra shows hundreds of peaks and the labelled are the identified peaks corresponding to chemical shifts. The figure is adapted from the study 'Identification of bacterial species by untargeted NMR spectroscopy of the *exo*-metabolomem' (Palama 2016).

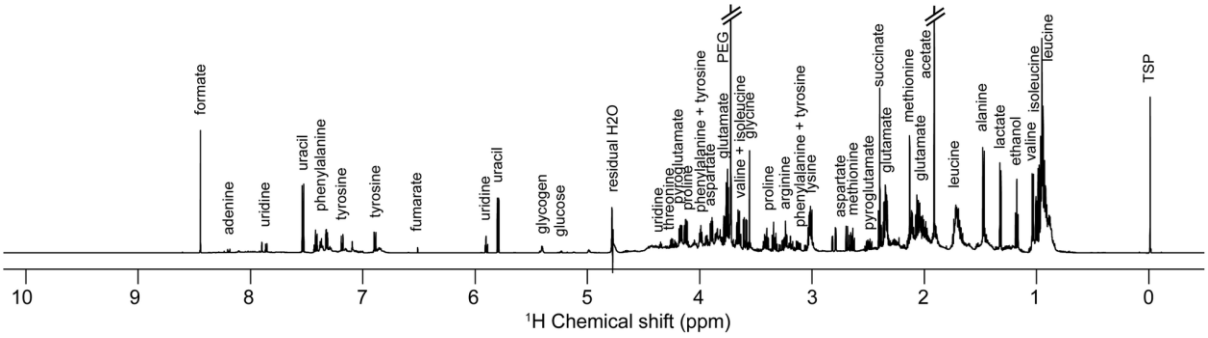



*Figure 1: Representative one-dimensional 1H NMR spectrum of an Escherichia coli sample (culture supernatant) at exponential growth, i.e. after 1.5 hours of culture in a Mueller Hinton medium. (Figure obtained from Palama 2016)*

The figure above illustrates an NMR spectrum from *Escherichia coli*, showcasing its metabolic composition. In this study, similar NMR spectra will be instrumental in understanding the behaviour of the chosen bacteria. By analysing these spectra, the goal is to identify the metabolites produced and uncover the mechanisms involved in cross feeding. This research aims to generate comparable NMR profiles, offering a detailed insight into the metabolic dynamics of the studied bacterial communities.

### Concepts of NMR Spectroscopy

NMR is a spectroscopic technique based on the principles of nuclear magnetic resonance, a phenomenon exhibited by certain atomic nuclei. In NMR, a sample is subjected to a strong magnetic field, and radiofrequency pulses are applied to manipulate the nuclear spins of certain isotopes, such as hydrogen nuclei (protons) or carbon-13. The resulting interactions provide

detailed information about the molecular structure, chemical environment, and dynamics of the sample.

When a chemical compound is placed in a strong magnetic field inside the NMR instrument (designated as B0 along the z-axis), the nuclei start spinning. Different nuclei have different spins. The spin quantum number, I, characterizing the intrinsic spin of a nucleus, is essential for its detectability by NMR. If a molecule has an odd total number of protons and neutrons, resulting in an odd number of nucleons, its spin quantum number is greater than 1, making it observable by NMR. Conversely, molecules with an even total number of nucleons have I=0 and lack spin properties, rendering them non-observable by NMR. Nuclei with odd numbers of protons and neutrons, such as $^{1}H$, $^{13}C$, and $^{31}P$, have I=1/2, making them detectable by NMR.

All nuclei detectable by NMR have a spin angular momentum, represented by the symbol J. This spin comes with a magnetic property called magnetic moment, denoted as μ. The relationship between μ and J is proportional, and this connection is determined by a factor known as the gyromagnetic ratio. The gyromagnetic ratio, denoted by the symbol γ, is a proportionality constant that relates the spin angular momentum to the magnetic moment of a particle.

$$\mu=\gamma J$$

Gyromagnetic ratio is a property of a specific nucleus (is a constant for a given isotope) and is essential in determining the Larmor frequency, a pivotal parameter in NMR spectroscopy. When a nucleus with a 1/2 spin quantum number is exposed to an external magnetic field, the external magnetic field causes the nucleus to precess, or spin, aligning either parallel or antiparallel to the field. Magnetic momentum of the nucleus (μ) interacts with the external magnetic field. In the case of a nucleus with a 1/2 spin quantum number, such as hydrogen nuclei (protons) or carbon-13, the magnetic momentum can exist in two possible orientations, corresponding to the two spin states. These spin states can be characterised by the magnetic quantum number m, at the lower energy level state α, m=1/2 and higher energy level β, m=(-1/2). At equilibrium nuclei align in the direction of B0 (α state) (Figure 2).

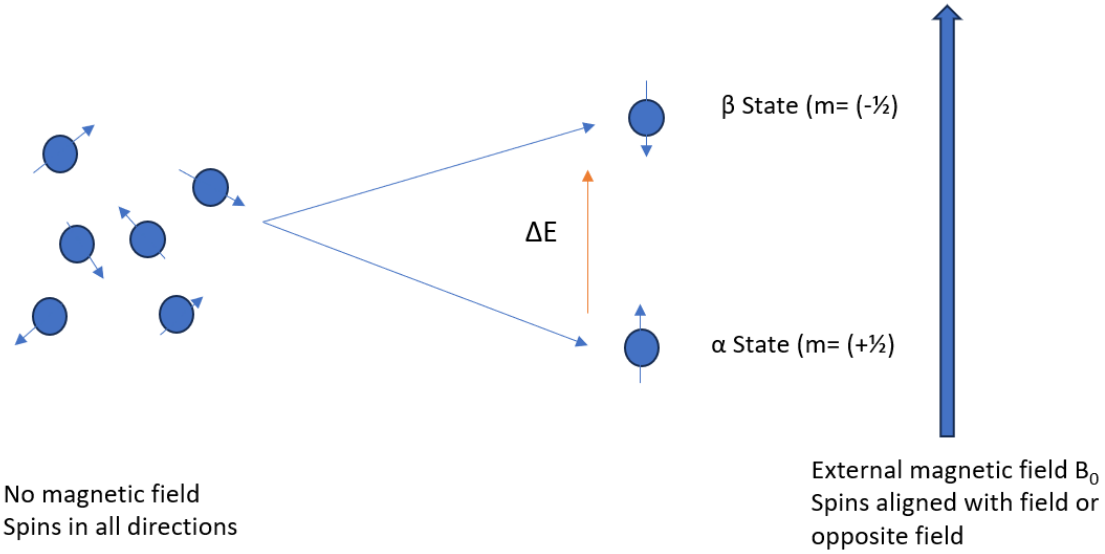



*Figure 2: Effect of an external magnetic field on the magnetic moment of a nucleus showing Zeeman Interactions*

The energy difference between these two states, known as the Zeeman splitting, is directly proportional to the strength of the external magnetic field. The Larmor frequency (ν), measured in hertz (Hz), represents the precession rate of nuclear spins in a magnetic field and is given by the equation, where B0 is the strength of the magnetic field.

$$\nu = \gamma B0/2\pi$$

Nuclides with a larger gyromagnetic ratios have larger magnetic momentums, and therefore more sensitive to NMR instruments. The Larmor frequency, depends on the gyromagnetic ratio and the strength of the magnetic field.

When a short radio frequency is applied at the correct Larmor frequency for a given nuclei, the spins flip the nucleus into the higher β energy state and the nuclear magnetic resonance occurs. This rotation induces a current in the receiver coil, and the resulting signal is detected and amplified. The signal, known as Free Induction Decay (FID), decreases in magnitude as the nucleus realigns with the magnetic field. The FID, representing waves in a time domain, undergoes Fourier transformation, converting it into a frequency domain for visual representation.

**NMR Spectrum**

NMR spectrum is a graphical representation of the radiofrequency signals emitted by atomic nuclei within a sample when subjected to a strong magnetic field. most commonly hydrogen ($^1$H) nuclei are observed. The spectrum is typically plotted as signal intensity (y-axis) against the chemical shift in parts per million (ppm) on the x-axis. Chemical shifts indicate the relative position of different types of nuclei within the molecule. Each distinct peak in the spectrum corresponds to a specific set of nuclei with unique chemical environments. The peak's position (chemical shift), intensity, and shape convey information about the molecular structure, such as the types of atoms present, their connectivity, and the surrounding chemical environment. Integrated NMR spectra reveal the relative abundance of each type of nucleus, aiding in the quantitative analysis of the sample.

**Chemical shift**

Each proton, being in a unique chemical environment, experiences a different magnetic field due to its surrounding electron distribution. The electron generates its own magnetic field, opposing the B0, a phenomenon known as shielding. Protons with higher electron density experience a lower magnetic field and a lower Larmor frequency, and vice versa. NMR data are presented on a scale ranging from 1 to 10, representing chemical shifts in parts per million (ppm). The chemical shift of a nucleus is measured relative to that of a standard compound, which is added to the samples to give a resonance peak position in the frequency domain on a ppm scale. Trimethylsilyl-2,2,2,2-tetradeuteropropionic acid (TSP) is normally used in $^1$H-NMR as the internal reference standard. It is inert, water soluble and gives a single signal at 0 ppm. The area under a resonance signal in the NMR spectrum is directly proportional to the number of protons contributing to that specific signal. Notably, shielded protons yield peaks on the right side of the spectrum, while de-shielded protons result in peaks on the left. The chemical shift values are indicative of the type of hydrogen or carbon within the molecule. Equivalent hydrogen atoms, which produce the same chemical shifts, are those that, when replaced by a test atom such as a halogen, yield identical compounds. Integrated NMR spectra offer valuable insights by revealing the number of hydrogen atoms contributing to each NMR signal. This information corresponds to the count of equivalent

hydrogen atoms responsible for the particular chemical shift, facilitating a detailed understanding of the molecular structure and composition.

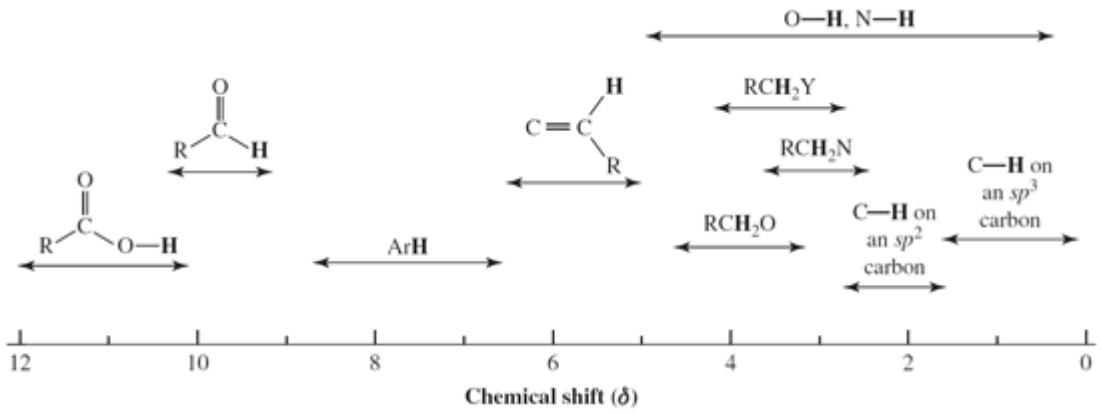



*Figure 3: One-dimensional $^{1}H$ NMR spectrum of an Escherichia coli sample (culture supernatant) at exponential growth, after 1.5 hours of culture in a Mueller Hinton medium.*

Image adapted from Wiley online NMR basics tutorial (WileyPLUS - NMR spectroscopy and nuclear spin (johnwiley.net.au) WileyPLUS - Worked example 4 (johnwiley.net.au)

**Peaks and Splitting**

A molecule with H in three different electronic environments generates three overlapping FIDs, appearing as three distinct peaks after Fourier transformation. Isolated protons create a single peak (singlet) in the NMR spectrum. Protons close enough can interact, leading to spin-spin coupling, causing peaks to split (doublet, triplet, quartet, etc.). Spin-spin coupling provides valuable information about the connectivity of atoms in a molecule. In general signals are split if there are H atoms on adjacent C atoms and the degree of splitting is given by the (n+1) rule. Where n is the number of equivalent H atoms on the adjacent C.

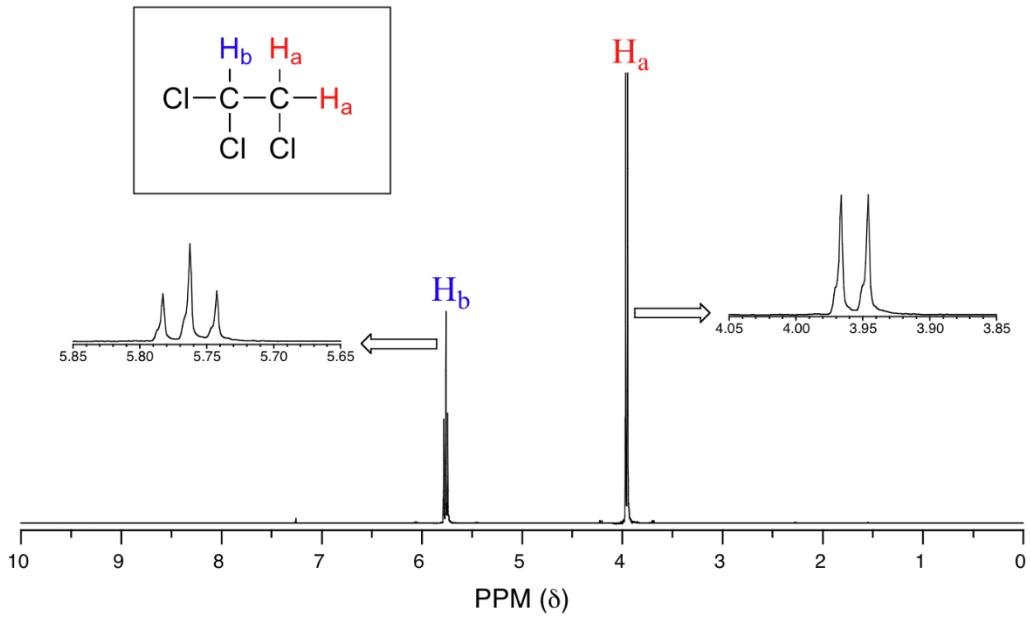



*Figure 4: Signal splitting pattern of 1,1,2-trichloroethane*

Adapted from Spin-spin splitting in proton NMR/ MCC Organic Chemistry (lumenlearning.com)

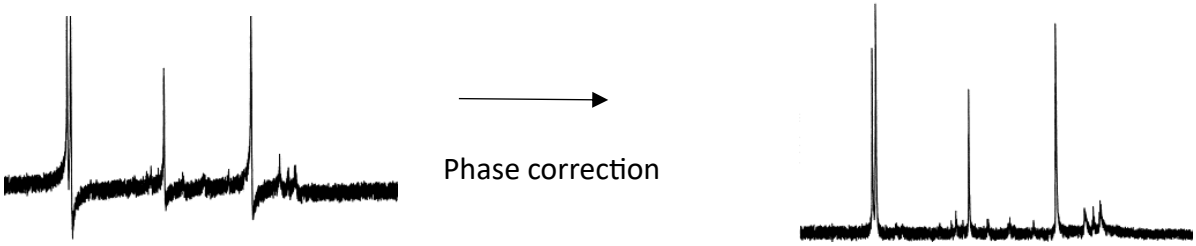
Ha : 1 eqvivalent H atom (Hb ): (n=1) therefore 2 peaks (doublet)

Hb : 2 eqvivalent H atoms (Ha ): 2 equivalent neighbouring H atoms (n=2): 3 peaks (Triplet)

consider the $^{1}$H-NMR spectra of 1,1,2-trichloroethane.

**Preprocessing**

Upon completing a biological experiment and obtaining NMR spectra, essential data processing techniques are employed. This involves correct phasing, baseline correction, and chemical shift referencing to obtain accurate spectra.



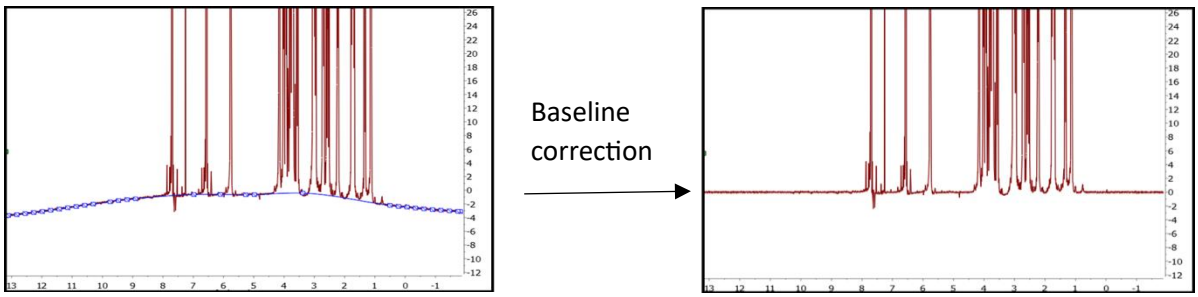



*Figure 5: preprocessing*

**Preprocessing NMR spectra**

The aim of preprocessing is to prepare the data in a manner that allows meaningful analysis through statistical procedures. Preprocessing is transforming raw data to clean data for data processing (Goodacre et al. 2007).

**Multivariate analysis**

After pre-processing NMR data, the next step involves the use of multivariate statistical analysis to understand meaningful patterns within the complex datasets. This analytical approach is proficient at handling information present in NMR spectra, providing a comprehensive exploration of relationships among different metabolites. The analysis falls into two main categories: unsupervised and supervised models. Unsupervised models, like Principal Component Analysis (PCA), unveil inherent structures and trends without external guidance. In contrast, supervised models, such as Orthogonal Partial Least Squares Discriminant Analysis (OPLSDA), incorporate external knowledge to create predictive models and classify samples.

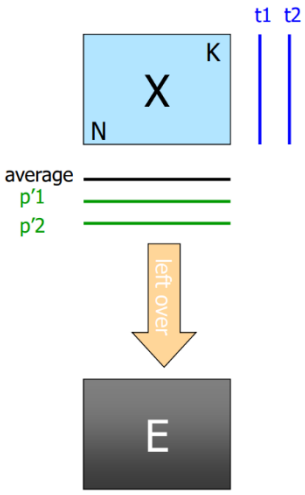
**Unsupervised models PCA**

PCA simplifies complex, high-dimensional data by transforming it into fewer dimensions known as principal components (PCs). As an unsupervised learning method, PCA uncovers patterns without prior knowledge of treatment groups or phenotypic differences. The reduction is achieved by projecting the data geometrically onto lower dimensions, and the first PC is chosen to minimize the total distance between the data and its projection, maximizing the variance.

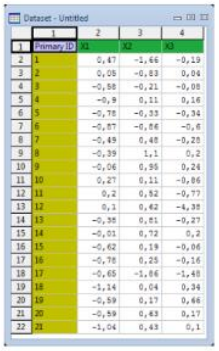
Subsequent PCs are selected to be uncorrelated with previous ones. This no-correlation rule limits the maximum number of PCs to either the number of samples or features, whichever is smaller. The PC selection process maximizes the correlation between data and their projection, resembling multiple linear regression on the original data variables (Lever 2017).

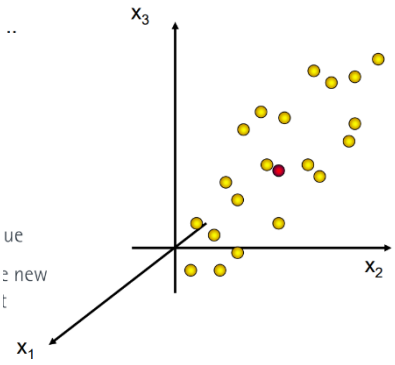
PCA simplifies complex data represented by a matrix, X, with N rows (samples/observations) and K columns (variables or digitised spectral descriptors). It helps uncover patterns by isolating noise, represented by E (Fig X a). In the given example Fig X N=22 and K=3 (X1,X2,X3) (Fig X b). Data is plotted in a multivariate space, and the average is calculated, with values centered around this mean which is coloured in red (Fig X c). In this example K= 3 but in the NMR data matrices K will be equal to the number of digitised spectral descriptors. Principal components (PC) are then selected and fitted to capture the maximum variance. The first PC is a direction in K-dimensional space that explains maximum variance and passes through the origin. The second PC is orthogonal to the first PC and passed through the origin which explains the next highest variance in the dataset (Fig X d). The number of PC to calculate is based on maximising the explained variance ($R^2$ value) and predictive ability ($Q^2$ value), using cross validation to test validity of models against overfitting. The points are then projected to a plane with coordinates t1 and t2 (blue square in Fig X e). The projected components are described through scores and loadings (Fig X f). Scores represent the position of each observation in the new coordinate system, while loadings indicate the contribution of variables to the principal components. This process enables a concise representation of the data's essential features in a lower-dimensional space.

$X = 1^* \bar{x}' + T^*P' + E$

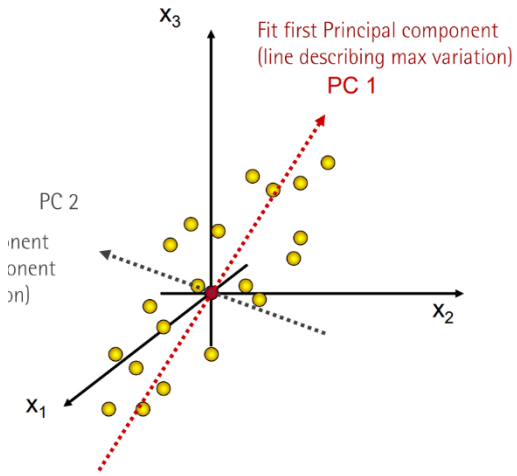



a

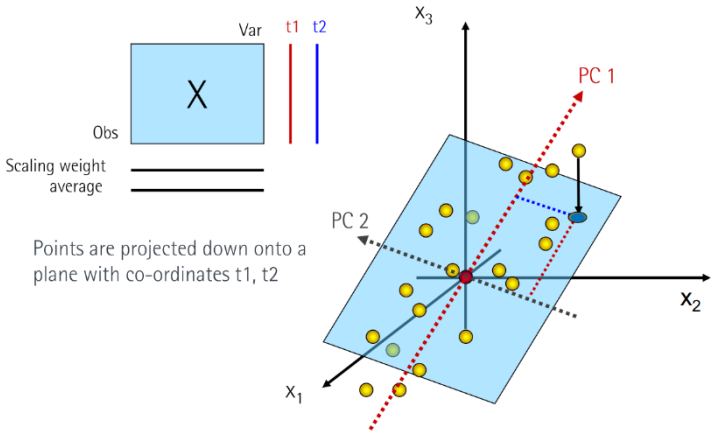




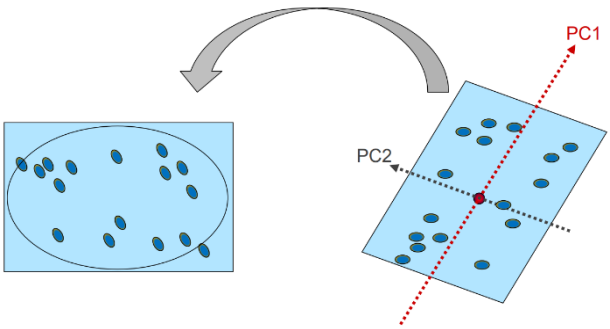

b



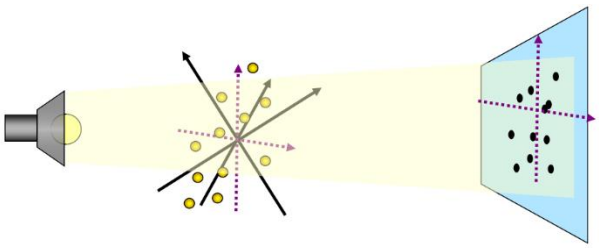

c




d




e



f

Figures adapted from PCA method and observation parameters Sartorius stedim webinar presentation slides.

**Supervised models O-PLS-DA**

PLSDA is a regression extension of PCA which uses prior knowledge of class separation using a dummy matrix Y. The main use of PLSDA is to model the relationship between measured variable X and biological response variable Y (identified as classes). Contribution of certain variables are re-scaled into loadings to achieve maximum separation between the pre-defined classes. PLSDA is important to maximise separation of known classes and also to predict the membership of unknown sample into a particular class.

OPLS-DA, or Orthogonal Partial Least Squares Discriminant Analysis, stands out as a potent statistical tool applied to high-dimensional data matrices, similar to PCA. In a data matrix X with N rows (samples) and K columns (variables), OPLS-DA takes on a distinctive role by specialising in supervised modelling. OPLS-DA efficiently separates systematic variation related to class differences. This supervised modelling approach involves decomposing X into predictive and orthogonal components, effectively capturing and simplifying complex relationships between variables and class information. The resulting model not only provides clear discrimination between classes but also identifies key variables contributing to observed differences. Scores and loadings obtained from OPLS-DA offer insights into the factors driving classification, making it a significant tool for interpreting and understanding intricate data patterns in a supervised context. OPLS-DA is successfully used to identify biomarkers typically using S plots.

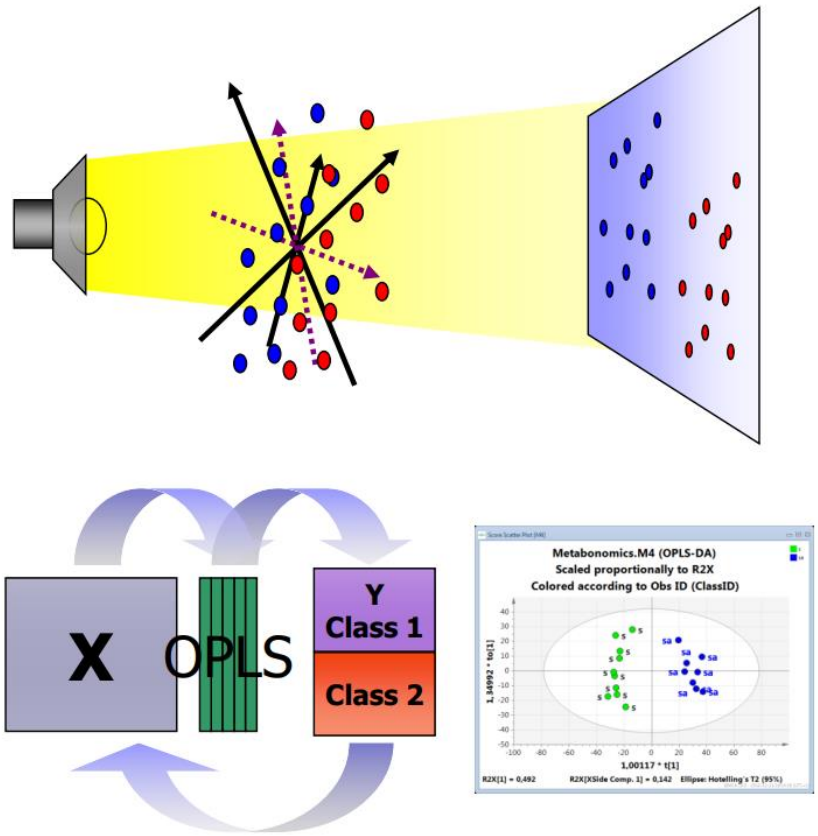



Figure adapted from Sartorius stedim webinar presentation slides.

**References**

Ebbels, Timothy M. D., and Rachel Cavill. 2009. 'Bioinformatic methods in NMR-based metabolic profiling', Progress in nuclear magnetic resonance spectroscopy, 55: 361-74.

Goodacre, Royston, David Broadhurst, Age K. Smilde, Bruce S. Kristal, J. David Baker, Richard Beger, Conrad Bessant, Susan Connor, Giorgio Capuani, Andrew Craig, Tim Ebbels, Douglas B. Kell, Cesare Manetti, Jack Newton, Giovanni Paternostro, Ray Somorjai, Michael Sjöström, Johan Trygg, and Florian Wulfert. 2007. 'Proposed minimum reporting standards for data analysis in metabolomics', Metabolomics, 3: 231-41.

Palama, T.L., Canard, I., Rautureau, G.J.P., Mirande, C., Chatellierb, S., and Elena-Herrmann, B.,. 2016. 'Identification of bacterial species by untargeted NMR spectroscopy of the exo-metabolome', Analyst, 141: 4558-61.

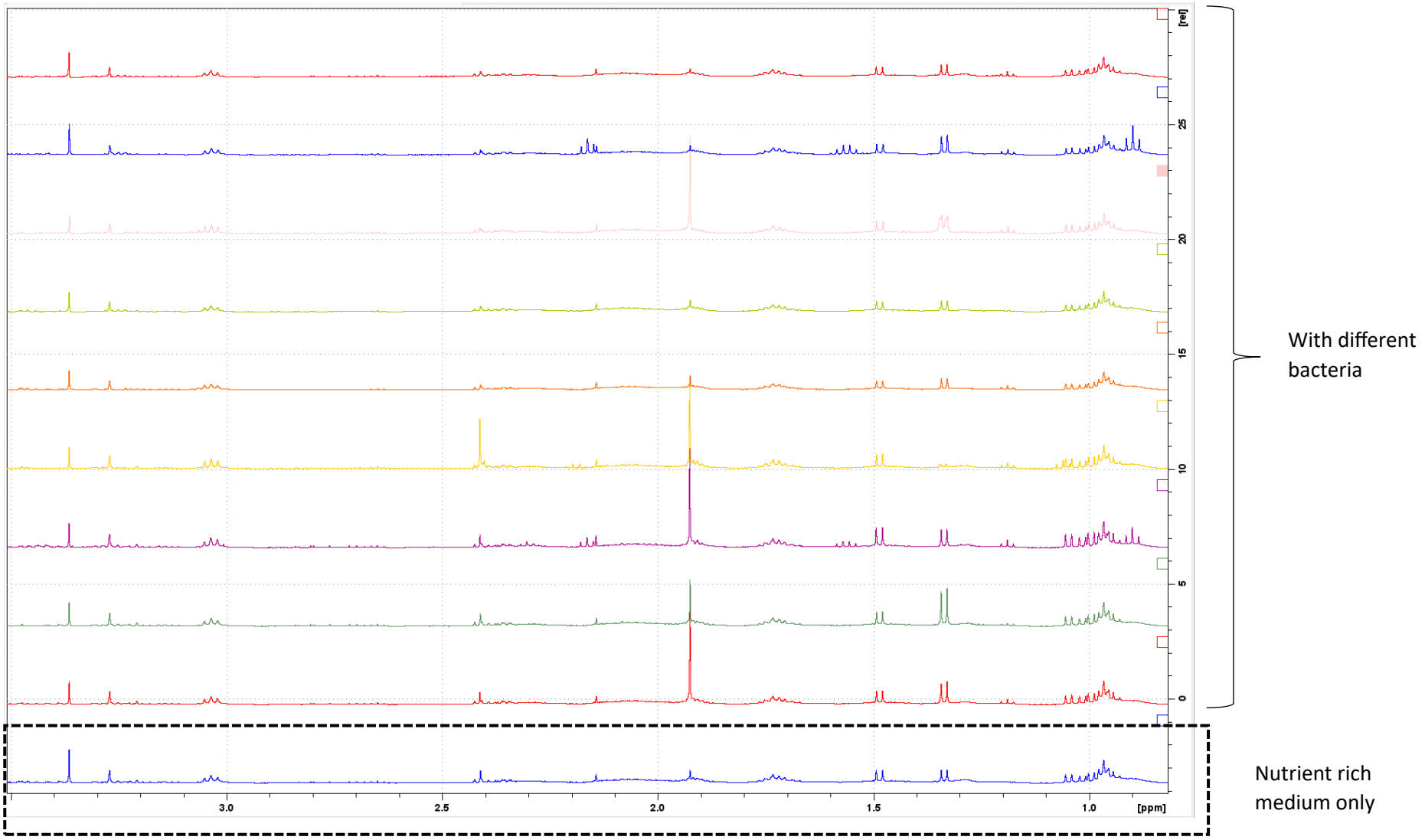
**Appendix 3.1: specific growth media for bacteria**

| Bacteria | Specific medium | No of days |
|---|---|---|
| *Bifidobacterium longum* | MRS + 0.05% L-cystein | 1 |
| *Lacticaseibacillus rhamnosus* | MRS broth | 1 |
| *Bacteroides fragilis* | Nutrient broth | 2 |
| *Clostridium perfringens* | Cooked meat broth | 1 |
| *Faecalibacterium prausnitzii* | YCFAC broth | 2 |
| *Collinsella aerofaciens* | GIFU anaerobic medium | 2 |
| *Ruminococcus bromii* | PYG broth | 2 |
| *Roseburia intestinalis* | YCFAC broth | 1-2 |
| *E. coli* | Nutrient broth | 1 |

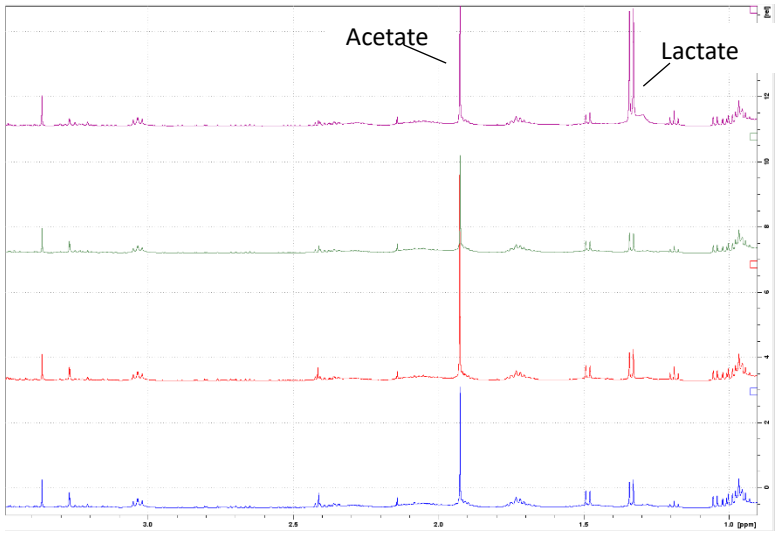
**Appendix 3.2 :NMR Assignment table (Chenomx software data base and in-house databases)**

| Molecule Name | Chemical Shift | Moiety | Multiplicity |
|---|---|---|---|
| Acetate | 1.91 | CH3 | singlet |
| Butyrate | 0.9 | CH3 | triplet |
| Butyrate | 1.56 | betaCH2 | doublet of doublets |
| Butyrate | 2.16 | alphaCH2 | triplet |
| Ethanol | 1.2 | | triplet |
| Gamma-amino-N-butyrate | 1.91 | betaCH2 | quartet |
| Gamma-amino-N-butyrate | 2.3 | alphaCH2 | triplet |
| Gamma-amino-N-butyrate | 3.02 | gammaCH2 | triplet |
| Lactate | 1.33 | CH3 | doublet |
| Lactate | 4.11 | CH | quartet |
| Methanol | 3.4 | | singlet |
| Propionate | 1.06 | CH3 | triplet |
| Propionate | 2.19 | CH2 | quartet |
| Succinate | 2.41 | 2xCH2 | singlet |

**Appendix 3.3 Topspin spectra of nutrient rich medium only vs bacteria at 24 h fermentation**



**Appendix 3.4 Topspin spectra obtained for each bacteria with different substrates**




***Bifidobacterium longum***

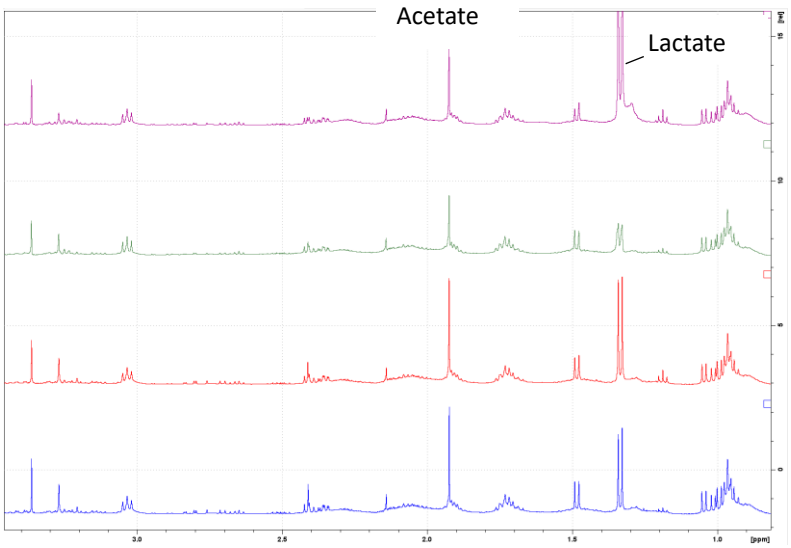
Probiotic yoghurt

Starch

Inulin

Control with nutrient rich medium

***Lacticaseibacillus rhamnosus***



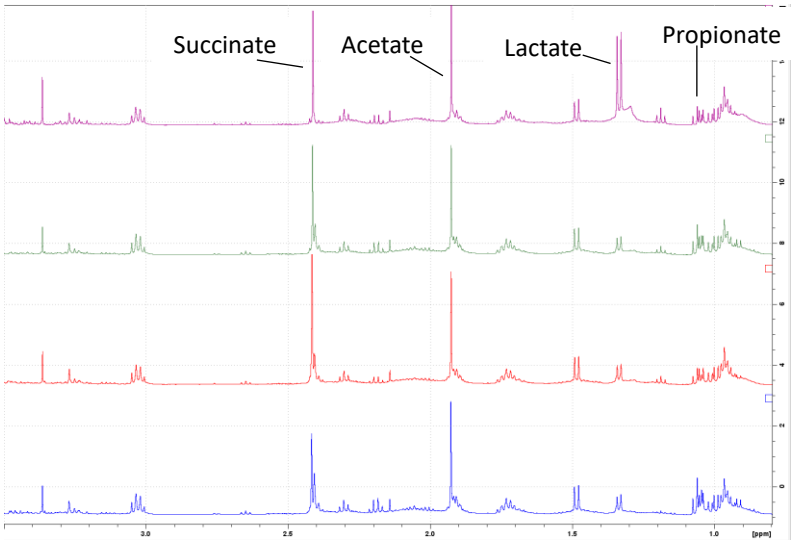

Probiotic yoghurt

Starch

Inulin

Control with nutrient rich medium

***Bacteroides fragilis***



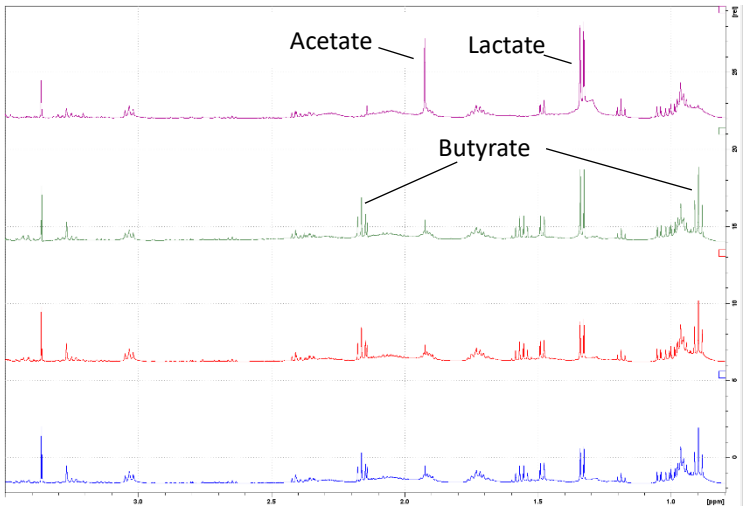

Probiotic yoghurt

Starch

Inulin

Control with nutrient rich medium

***Roseburia intestinalis***



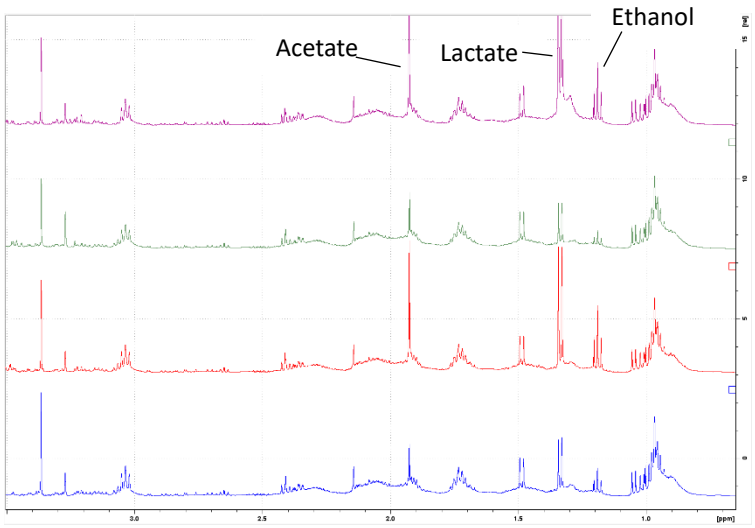

Probiotic yoghurt

Starch

Inulin

Control with nutrient rich medium

***Collinsella aerofaciens***



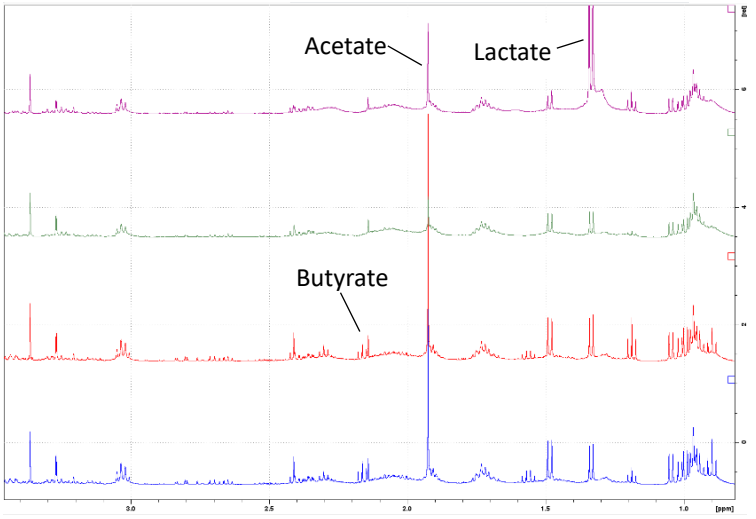

Probiotic yoghurt

Starch

Inulin

Control with nutrient rich medium

***Clostridium perfringens***



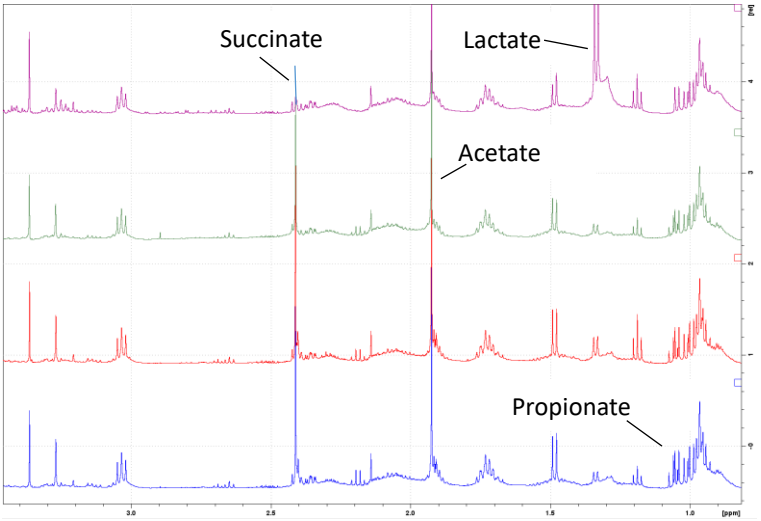

Probiotic yoghurt

Starch

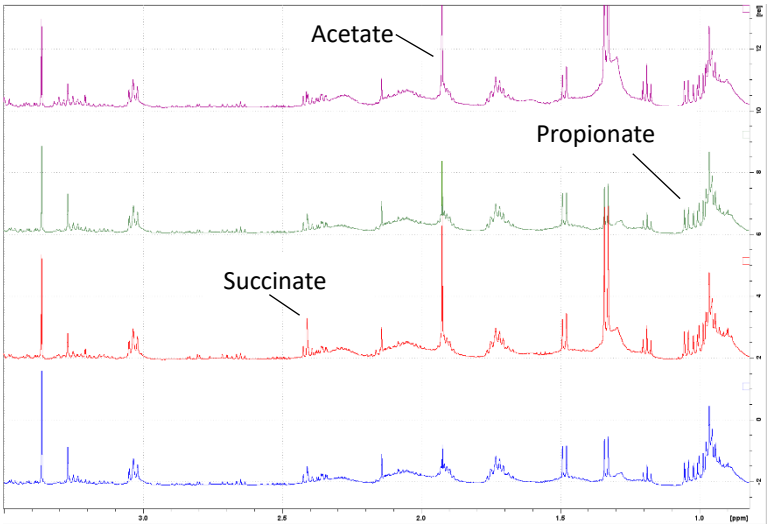
Inulin

Control with nutrient rich medium

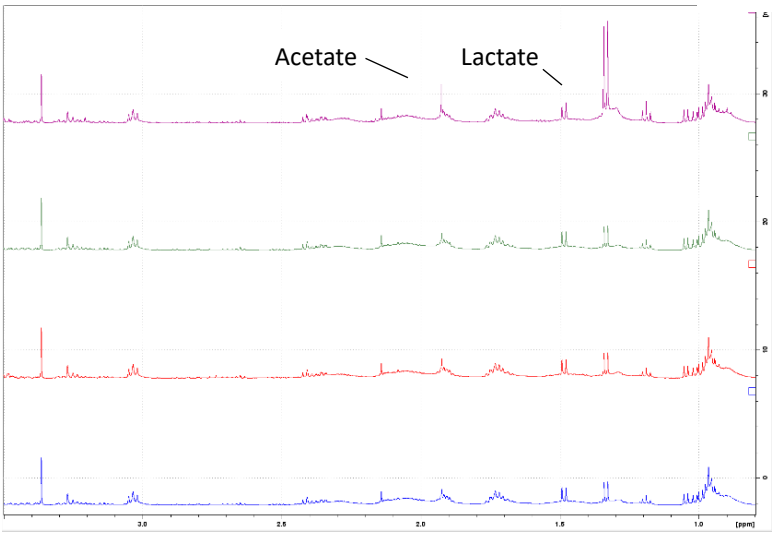
***Escherichia coli***



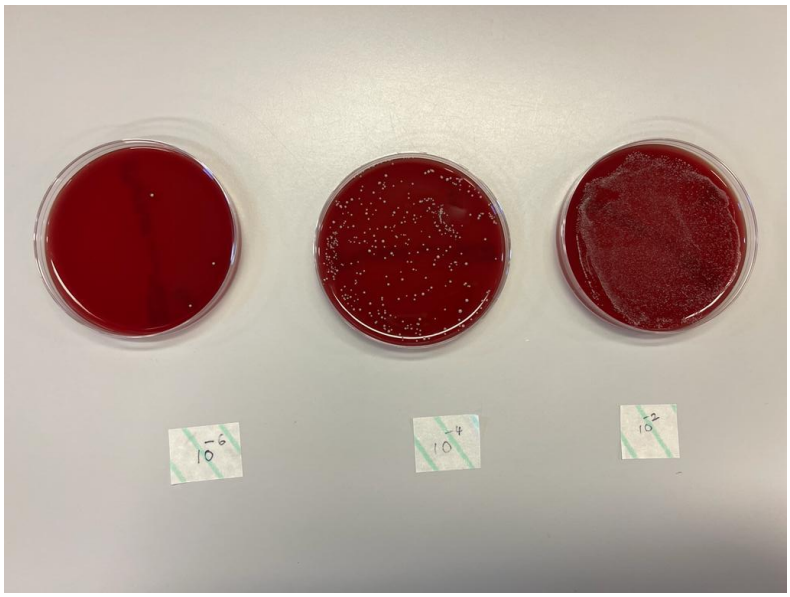

***Faecalibacterium prausnitzii***




***Ruminococcus bromii***

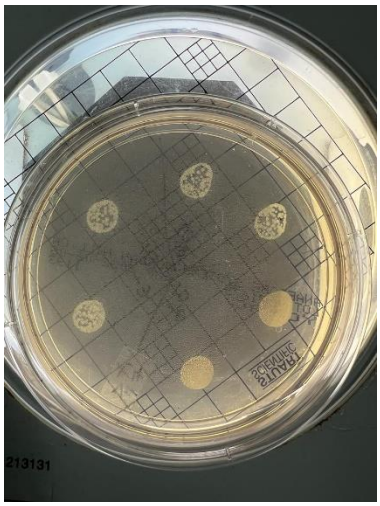


**Appendix 4.1 Images of cultured bacterial colonies**



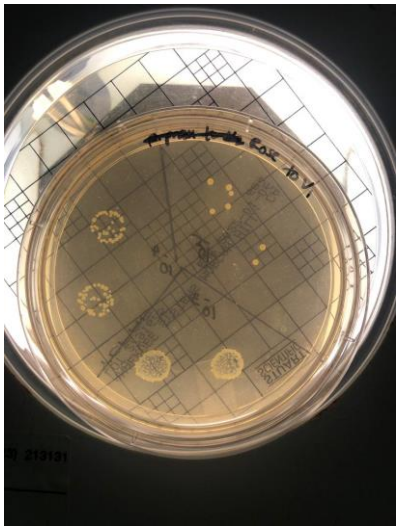
*Ruminococcus bromii* on blood agar plate

*Bacteroides fragilis* on Fastidious anaerobic agar plates

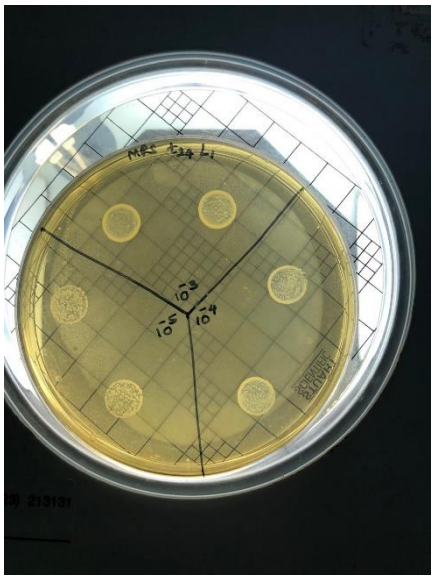


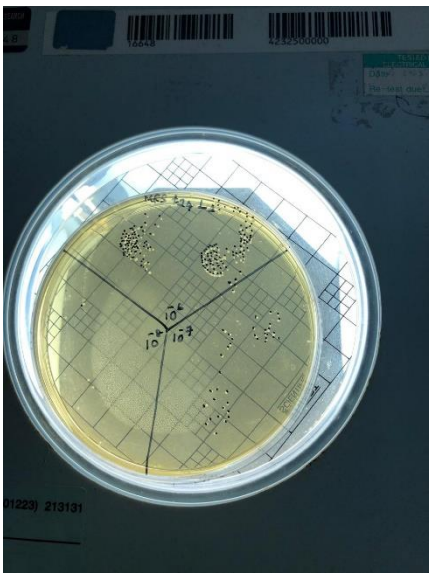
*E.coli* on nutrient agar

*Bifidobacterium longum* on MRS agar

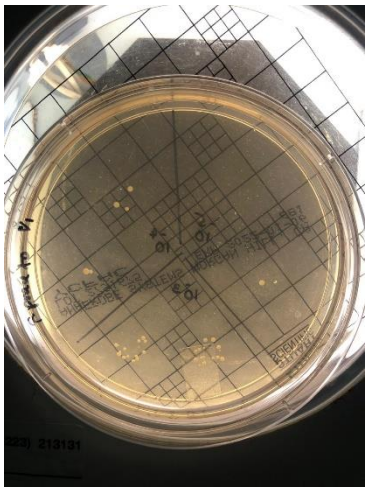


*Roseburia intestinalis* on YCFAC agar

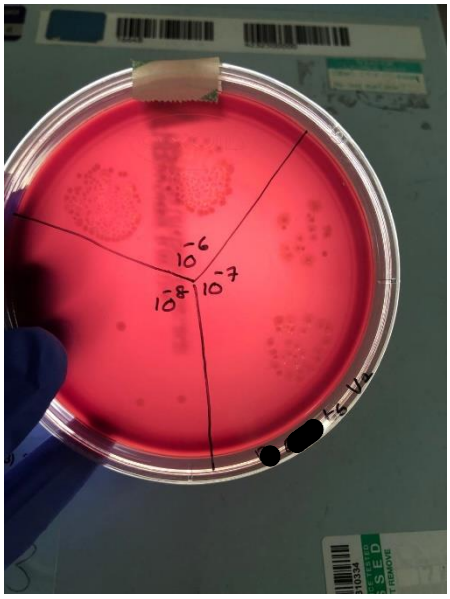




*Lacticaseibacillus rhamnosus* on MRS agar



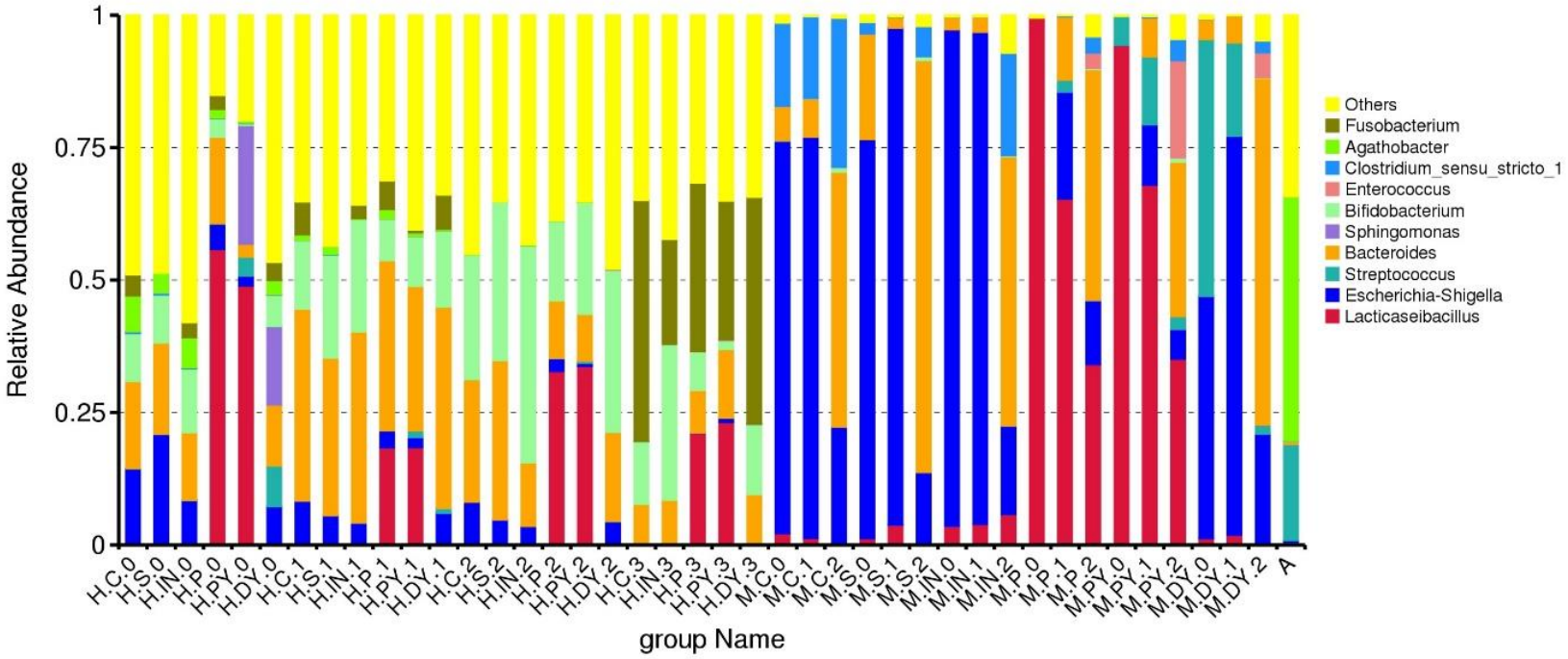

*Faecalibacterium prausnitzii* on YCFAC agar



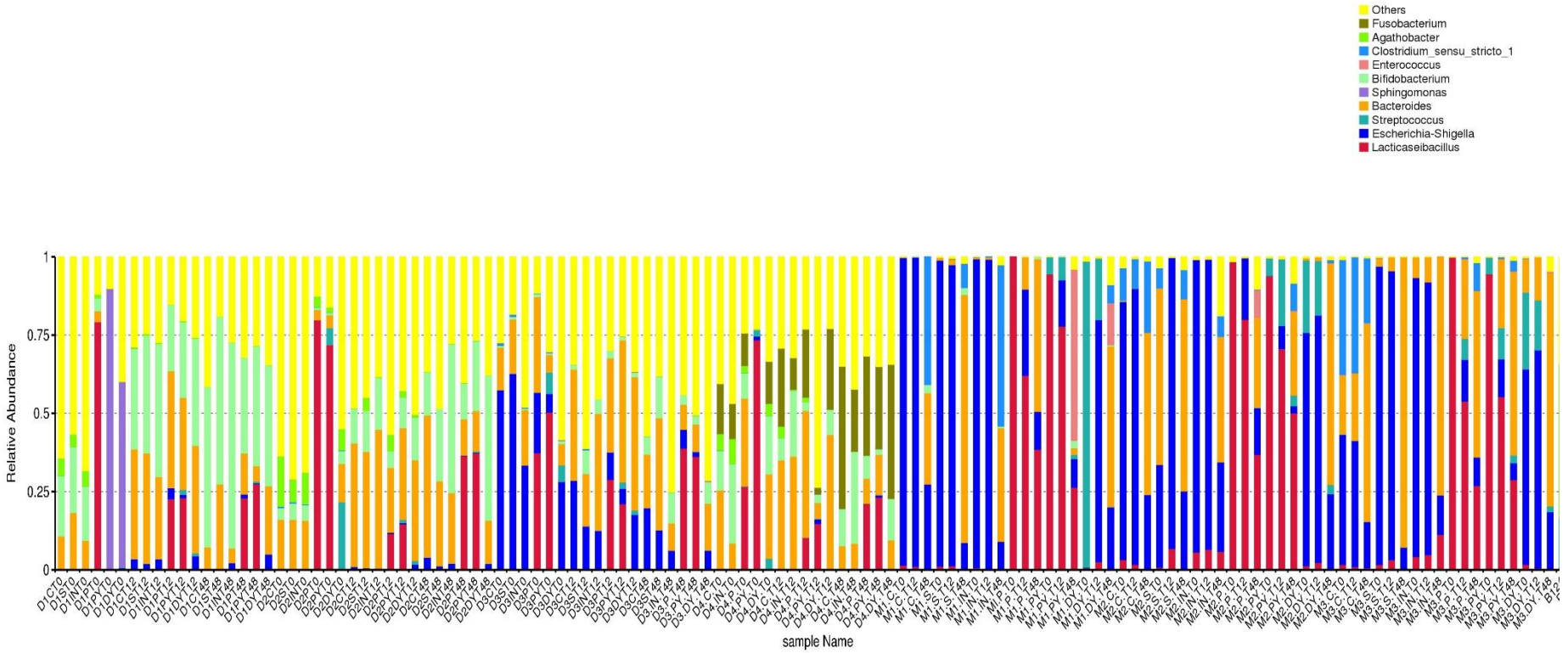

*Clostridium perfringens* on Fastidious anaerobic agar plates

**Appendix 4.2**

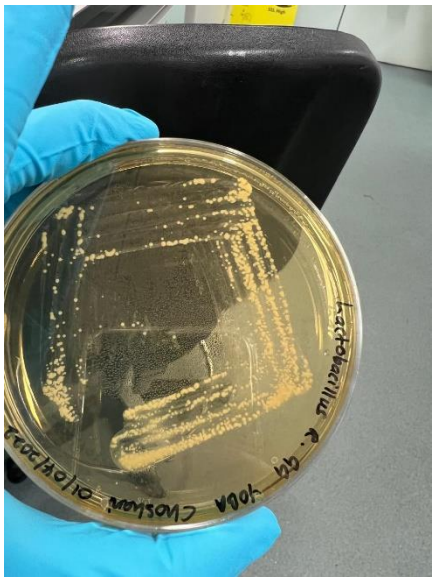
Novogene graphs relative abundance genus level H=Human, M=synthetic mix, C=Control, S=Starch, IN-Inulin, P=Probiotic, PY- Probiotic Yoghurt, 1=T0, 2=T12, 3=T48



Novegene graphs for Relative abundance sample vise



Appendix 5.1



*Lactobacillus rhamnosus* yoba on MRS agar