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MICROBIAL DIVERSITY IN AN IN VITRO MODEL OF COLONIC FERMENTATION WITH DIFFERENT FOODS

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Microbial fermentation in an in vitro model of colonic fermentation with different foods

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ABSTRACT

The interaction between diet and gut microbiota, and ultimately their link to health, has become the focus of huge research showing that diet and lifestyle have a strong influence on the gut microbiota. That is why there is a growing interest in resolving questions about the relation between the gut microbiome and host metabolism.

The human intestine is densely populated by trillions of microbial symbionts. The symbiotic gut microbiota helps nutrient absorption through the fermentation of dietary fibre, provides protection from invading pathogens and helps to develop and regulate the immune system. However, the mechanisms underlying interactions between diet, gut microbiome and host metabolism are still poorly understood. Here, we discuss how meta-omics datasets can be obtained through an in vitro model model of colonic fermentation in order to study how the food that is consumed can shape the diversity and composition of the gut microbiota. In order to analyze microbial community structure, tools and approaches such as next generation sequencing (NGS) of 16S rRNA amplicons and bioinformatic analysis are used. The study will be performed with bread that will be digested and then submitted to an *in vitro* fermentation process with fecal inocula from healthy Spanish adults from Granada.

In the end, the influence of bread on the structure of the gut microbiota from different types of fecal samples will be evaluated, as well as the variability of the microbioal taxonomy, composition and alpha and beta diversity when fecal samples are analyzed before and after fermentation and before and after a freezing treatment at -80 Cº. Our results suggest that despite a loss of bacterial groups that alters the microbial composition, freezing the samples before fermentation is not a serious problem because the effect of fermentation on the samples remains significant in terms of richness, diversity and abundance of the main bacterial groups. This will be studied in line with the main objectives of a larger project (Stance4Health) of developing a personalised nutrition system that optimizes gut microbiota metabolism.

Keywords: Gut microbiota; Taxonomic richness and diversity; 16S rRNA analysis; Personalised nutrition; Amplicon.

Modelo in vitro de fermentación colónica con diferentes alimentos para estudiar la diversidad microbiana

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RESUMEN

La interacción entre la dieta y la microbiota intestinal, y finalmente su vínculo con la salud, se ha convertido en el foco de una gran investigación que demuestra que la dieta y el estilo de vida tienen una gran influencia en la microbiota intestinal. Es por eso que hay un interés creciente en resolver las preguntas sobre la relación entre el microbioma intestinal y el metabolismo del huésped.

El intestino humano está densamente poblado por billones de simbiontes microbianos. La microbiota intestinal simbiótica ayuda a la absorción de nutrientes a través de la fermentación de la fibra dietética, proporciona protección contra patógenos invasores y ayuda a desarrollar y regular el sistema inmunológico. Sin embargo, los mecanismos subyacentes a las interacciones entre la dieta, el microbioma intestinal y el metabolismo del huésped aún no se conocen bien. Aquí, discutimos cómo los conjuntos de datos meta-ómicos se pueden obtener a través de un modelo in vitro de fermentación colónica para estudiar cómo los alimentos que se consumen pueden dar forma a la diversidad y composición de la microbiota intestinal. Con el fin de analizar la estructura de la comunidad microbiana, se utilizan herramientas y enfoques como la secuenciación de la próxima generación (NGS) de los amplicones del ARNr 16S y el análisis bioinformático. El estudio se realizará con pan que se digerirá y luego se someterá a un proceso de fermentación in vitro con inóculos fecales de adultos sanos españoles (Granada).

Al final, se evalua influencia del pan sobre la estructura de la microbiota intestinal de diferentes tipos de muestras fecales, así como en la variabilidad de la taxonomía microbiana, la composición, y la diversidad alfa y beta cuando las muestras fecales se analicen antes y después de la fermentación y antes y después del tratamiento de congelación a -80ºC. Nuestros resultados sugieren que a pesar de la pérdida de grupos bacterianos que altera la composición microbiana, congelar las muestras antes de la fermentación no es un problema grave debido a que seguimos vienod el efecto de la fermentación en las muestras en términos de riqueza, diversidad y abundancia de los principales grupos bacterianos. Esto se estudiará de acuerdo con los objetivos principales de un proyecto más grande (Stance4Health) de desarrollar un sistema de nutrición personalizado que optimice el metabolismo de la microbiota intestinal.

Palabras clave: Microbiota intestinal; Riqueza y diversidad taxonómica; Análisis de ARN de 16S; Nutrición personalizada; Amplicon.

Dedication

This Bachelor thesis is dedicated to all the women who have made significant contributions to science from the earliest times and have been involved in the progress of new discoveries.
Because being a woman in scientific disciplines has been challenging and lonely at times and my advice to early-career women scientists is to believe in yourself and believe that what you do matters for science and for society.
We should cross sexism barriers, stereotypes and be outspoken about the challenges in order to open new doors for the women of the future.

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Index

TABLE OF CONTENTS

1.Introduction 1
1.1 Functions and composition of the human microbiome 1
1.2 Factors that modulate the gut microbiota 2
1.3 Towards personaliSed nutrition 3
1.4 Different diets that affect THE microbiome 3
1.5 How the way of cooking can affect the gut microbiota 4
1.6 Obesity and cardiovascular diseases 5
1.7 Stance4heatlh project 6

2. Objectives 8

3. Materials and methods 9
3.1 Sampling 9
3.2 Inocula preparation and *in vitro* fermentation 9
3.3 DNA extraction 9
3.4 DNA analysis and amplification 10
3.4.1 PCR Protocol 11
3.4.2 Gel electrophoresis of PCR amplicons 12
3.5 Library preparation 12
3.5.1 Index and PCR test 12
3.5.2 Library quantification and pooling 13
3.6 Library denaturing and Miseq sample loading 13
3.7 Bioinformatic analysis 14
3.8 Bacterial community richness and diversity estimation 15
3.8.1 Alpha diversity indexes 16
3.8.2 Beta diversity indexes 16

4. Results and discussion 17
4.1 Composition 18
4.1.1 Composition of the fresh inoculum 18
4.1.2 Changes in composition of the fresh inoculum after bread fermentation 20
4.1.3 Changes in composition after sample storage at -80 º C. 22
4.1.4 Changes in composition after bread fermentation when using previously frozen samples. 22
4.2 Alpha diversity 23
4.3 Beta diversity 26
4.3.1 Principal components analysis (PCoA) 26
4.3.2 Paired Wilcoxon Tests 27

4. Conclusion 28
Future directions and precision nutrition 28

5. Bibliography 30

FIGURE INDEX

FIGURE 1. DEVELOPMENT OF THE GUT MICROBIOME DURING INFANCY. 2

FIGURE 2. SCHEMATIC OF GUT MICROBIOTA EFFECTS ON RISK FACTORS FOR CVD AND ADVERSE CARDIOVASCULAR EVENTS. 6

FIGURE 3. STANCE4HEALTH OVERALL STRUCTURE (STANCE4HEALTH PROPOSAL). 7

FIGURE 4. 16S V3 AND V4 AMPLICON WORKFLOW. USER-DEFINED FORWARD AND REVERSE PRIMERS THAT ARE COMPLEMENTARY UPSTREAM AND DOWNSTREAM OF THE REGION OF INTEREST. 11

FIGURE 5. 16S LIBRARY PREPARATION WORKFLOW. 12

FIGURE 6. OVERVIEW OF BIOINFORMATICS METHODS FOR GUT MICROBIOTA METAGENOMIC ANALYSIS. ... 15

FIGURE 7. KRONA VIEWER SAMPLE S04_2 19

FIGURE 8. KRONA VIEWER SAMPLE S01_1 21

FIGURE 9. MICROBIAL COMPOSITION OF THE INOCULUM AFTER DIFFERENT TIMES OF FREEZING, 8 DAYS FOR SAMPLE S05_1 (LEFT) AND 16 DAYS FOR SAMPLE S06_1 (RIGHT). 22

FIGURE 10. MICROBIAL COMPOSITION OF SAMPLES S02_1 AND S03_1, WHICH UNDERWENT BREAD FERMENTATION AFTER 8 OR 16 DAYS OF STORAGE AT -80 ° C, RESPECTIVELY. 23

FIGURE 11. CHAO1, SHANNON AND SIMPSON INDEXES AT FAMILY LEVEL FOR INOCULUM AND FERMENTED SAMPLES. 24

FIGURE 12. CHAO1, SHANNON AND SIMPSON INDEXES AT GENUS LEVEL ACCORDING TO DIFFERENT TYPES OF PROCESSES. 26

FIGURE 13. PCOA COMPARING INOCULUM AND FERMENTED SAMPLES. 26

TABLE INDEX

TABLE 1.AMPLICON PRIMERS AND OVERHANGS. 11

TABLE 2. NAME OF THE SAMPLES AND DESCRIPTION 17

TABLE 3. ESTIMATES OF BACTERIAL RICHNESS (CHAO1 INDEX) AND DIVERSITY (SHANNON AND SIMPSON INDEX) COMPARING THE DIFFERENT INOCULUM AND FERMENTED SAMPLES AT DIFFERENT FREEZING TIMES AT FAMILY LEVEL. 24

TABLE 4. ESTIMATES OF BACTERIAL RICHNESS (CHAO1 INDEX) AND DIVERSITY (SHANNON AND SIMPSON INDEX) COMPARING THE DIFFERENT INOCULUM AND FERMENTED SAMPLES AT DIFFERENT FREEZING TIMES AT GENUS LEVEL. 25

TABLE 5. WILCOXON TESTS BETWEEN FERMENTED SAMPLES (MEAN 1) AND INOCULA (MEAN 2) BY TAXA AT GENUS LEVEL. THIS TABLE SHOWS ONLY TAXA WITH SIGNIFICANT CHANGES (P-VALUES <=0.05) AFTER DOING THE WILCOXON. 27

LIST OF ABREVIATIONS

DNA	Deoxyribonucleic acid
SCFAs	Short Chain Fatty Acids
BCFAs	Branched chain fatty acids
BCAAs	Branched chain amino acids
BAS	Bile acids
TLR	Toll-like receptor
APCs	Antigen presenting cells
T1D	Type 1 diabetes
T2D	Type 2 diabetes
NAFLD	Non-alcoholic fatty liver disease
IDB	Inflammatory bowel disease
SPN	Smart Personalised Nutrition
SVD	Strict vegetarian diet
OUT	Operational Taxonomic Unit
TMAO	Trimethylamine N-oxides
WHO	World Health organization
NCD	Non-communicable disease
TBE	Tris/Borate/EDTA
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic acid
ASV	Amplicon Single Variant
PCoA	Principal Components Analysis

1. INTRODUCTION

Over the last two decades, microbiome analysis of faecal samples using culture-independent methods, such as high-throughput DNA sequencing has emerged as a non-invasive tool to study nutrition and health and this has enabled researchers to explore the interaction between diet and gut microbiota (Jain *et al.,* 2018).

1.1 FUNCTIONS AND COMPOSITION OF THE HUMAN MICROBIOME

The gut microbiome acts as an auxiliary metabolic organ and harbours a densely populated microbial ecosystem containing a number of bacterial cells that is larger than the number of eukaryotic cells in the entire human body, and including trillions of microorganisms such as bacteria, archaea, viruses and unicellular eukaryotes. Most gut microbes are facultative obligate anaerobes spanning five different phyla (*Bacteroidetes, Firmicutes, Proteobacteria, Verrumicrobia, and Actinobacteria*), with over 1000 species already identified (Claesson *et al.*, 2009). The composition of the gut microbiota is relatively simple at birth, and it undergoes a series of changes in composition and metabolic functions until it eventually matures between 3–5 years of age (Rodriguez, *et al*., 2015). For any healthy adult individual, the composition of the gut microbiome tends to be stable over time but there are differences in the composition of the gut microbiome within a human population depending, among other factors on the foods they consumed (Faith *et al.,* 2015)

Regarding the function of the colon, it is the major site for the gut microbiota "co-metabolic" activity, which enhances the efficiency of energy harvest from foods and influences the synthesis, bioavailability and function of nutrients. This activity produces different beneficial compounds that regulate host health, such as Short Chain Fatty Acids (SCFAs), polyphenol metabolites, neuroactive chemical species, etc. This leads us to view ourselves as "supra-organisms", composed of our cells and of microbial cells depending on each other for survival (Proposal Stance4health). What's more, the gut microbiota plays a primary function in host health by shaping the development of the immune system, metabolizing dietary nutrients and drugs, and synthesizing vitamins, bioactive molecules, and other beneficial or detrimental metabolites. For example, several complex carbohydrates, not digested by the host intestinal enzymes, are passed to the microbial community, which are then metabolized in the large intestine (Ji *et al.,* 2015). As it has been said, the gut microbiota is involved in metabolism of short-chain fatty acid (SCFAs), but also involved in the processing of branched chain fatty acids (BCFAs), branched chain amino acids (BCAAs), biogenic amines, vitamins, bile acids (BAs), and xenobiotics, as well as the production of gases (e.g., CO2, CH4). (Lamichhane *et al.,* 2018).

Moreover, gut microbes also affect the host immune system, such as by regulating immune homeostasis versus autoimmunity (Napier *et al*., 2019) inducing toll-like receptor (TLR) expression, antigen presenting cells (APCs), and differentiated CD4+ T cells (Valentini *et al*., 2014) and maintaining the stability of the immune system by providing resistance against pathogens. (Sen *et al.,* 2019). This can be linked to the fact that the gut microbiota is directly related to health and disease and it has been proved that imbalance in the gut microbiota has been associated with inflammatory and metabolic disorders including inflammatory bowel disease (Frank *et al*. 2007), irritable bowel syndrome, and obesity (Claesson *et al*. 2012).

Changes in the composition of the gut microbiota flora have also been associated with autoimmune diseases such as type 1 diabetes (T1D) and rheumatoid arthritis, colon cancer (Sears *et al.,* 2014), type 2 diabetes (T2D), obesity (Shoaie *et al*., 2015), cardiovascular disorders (Jonsson *et al.,* 2017), non-alcoholic fatty liver disease (NAFLD) (He *et al*., 2016) or inflammatory bowel disease (IDB) (Wlodarska *et al.,* 2015). Current evidence thus makes Hippocrates' statement "All disease begins in the gut", more convincing today than ever before and shows that the changes in the diet impacted our microbial symbionts, possibly playing a role in the development of several diseases.

1.2 FACTORS THAT MODULATE THE GUT MICROBIOTA

Several genetic and environmental factors such as diet, lifestyle, geography, mode of delivery, infection, infant feeding modality and medication shape the gut microbiota during the early stages of life (Schmidt *et al.,* 2018). However, the gut microbiota can be rapidly affected by diet, one of the main factors that modify its taxonomic diversity and modulate the microorganisms' proportions. So, while genetics, mode of delivery at birth, physical environment, age, stress, and other factors can influence the dynamics of the gut microbiota, diet may be the single most important driver of gut bacterial composition and function. The development of the infant microbiome is dependent on various factors, such as infant feeding method, diet and the environment **(Figure 1)**. Also, the mode of delivery (either vaginal or by cesarean section) affects the early life microbiome. Transfer of bacteria from the mother to the fetus has also been shown, indicating that pregnancy may be important for colonization of the fetal/infant gut

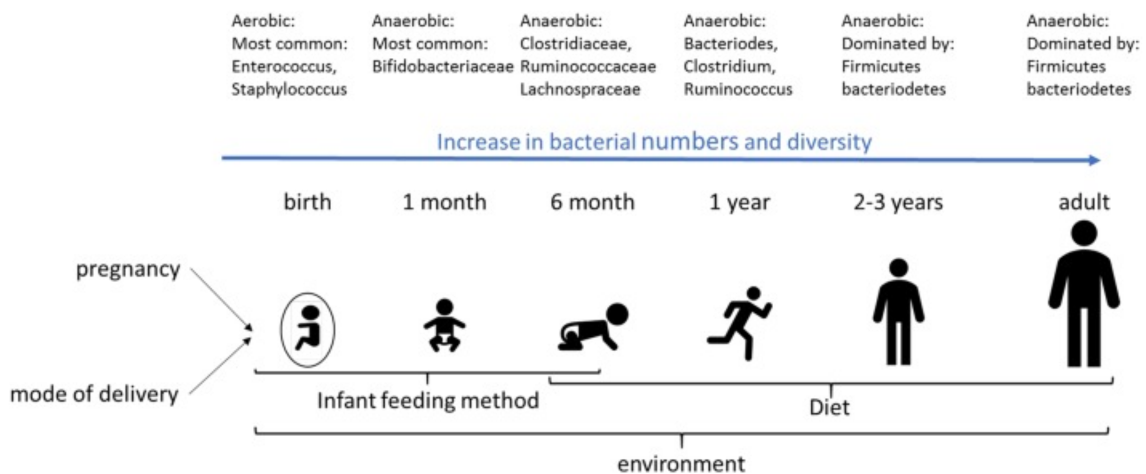

Figure 1. Development of the gut microbiome during infancy.

(Mohajeri *et al.,* 2018)

That is why it is important to unravel the specific effects of foodstuffs from different groups like vegetables, fruits, meat, legumes, or cereals and how different types of food modify the microbial diversity in our gut, taking into consideration that the ability of the gut microbiota to use substrates could also be influenced by the culinary heat treatment undergone by the foodstuffs prior to ingestión or how the fermentation occurred (Pérez-Burillo *et al*., 2018).

1.3 TOWARDS PERSONALISED NUTRITION

Although nutrition research has focused on how direct interactions between dietary components and host systems influence human health, it is becoming increasingly important to consider nutrient effects on the gut microbiome for a more complete picture and it is crucial to understand nutrient-host-microbiome interactions to reveal novel mechanisms of disease etiology and progression, in order to accomplish prevention strategies and to evaluate the safety of food ingredients (Sheflin *et al.,* 2017) and their impact in the human gut. Thus, targeted modulation of the gut microbiota will need to be a key element of a future Smart Personalised Nutrition (SPN) (Johson *et al.,* 2019)

1.4 DIFFERENT DIETS THAT AFFECT THE MICROBIOME

Numerous studies have shown that vegetarians compared to omnivores following a western diet exhibit a lower risk for various chronic diseases including obesity, hypertension, dyslipidaemia, coronary heart disease, stroke, diabetes, and certain cancers.

While vegetarians likely benefit from other health-related behaviours associated with lower disease risk, experimental studies that attempt to control for such confounders still show that changing from a western to a vegetarian dietary pattern reduces a number of disease risk factors (De Filippis *et al.,* 2018). Lower intakes of total fat, saturated fatty acids, dietary cholesterol, and animal proteins among vegetarians are thought to be important features of this dietary pattern linked to lower risk for chronic disease. Regarding the gut microbiota, vegetarians compared to omnivores tend to exhibit greater bacterial diversity and richness and greater ratios of *Prevotella* to *Bacteroides.* Global macronutrient profiles can modulate the intestinal microbiota: saturated fat and animal protein decrease microbial diversity and enrich for *Bacteroidetes* and *Actinobacteria*, whereas plant-based diets with high content of carbohydrates increase microbial diversity, linked with *Firmicutes* and *Proteobacteria* (Bisanz *et a*l., 2019).

Moreover, it has been seen that a strict vegetarian diet leads to a decrease in pathobionts such as the *Enterobacteriace*ae and an increase in commensal microbes such as *Bacteroides fragilis* and *Clostridium* species resulting in reduced intestinal lipocalin-2 and short-chain fatty acids levels. The results indicate that a strict vegetarian diet (SVD) had a significant effect on the composition of the gut microbiota at the operational taxonomic unit (OTU) level. However, there was no correlation between the consumption of an SVD and bacterial diversity. An SVD also reduced body weight and the concentrations of triglycerides, total cholesterol, low-density lipoprotein cholesterol and haemoglobin A1c, and improved fasting glucose and postprandial glucose levels. Kim *et al.* (2013) underscores the benefits of dietary fibre for improving the risk factors of metabolic diseases and shows that increased fibre intake reduces gut inflammation by changing the gut microbiota (Kim *et al*., 2013). Thus, an intake of dietary fibre is thought to reduce the risk for obesity and metabolic diseases by modulating the composition of the gut microbiota.

Both *Bacteroides* and *Prevotella* have already been reported as usually present in the human gut regardless of nationality or continental geography. *Bacteroides* was the most plentiful genus detected in the gut microbiota of those who consumed Western-style-food containing high

protein and animal fat, whereas *Prevotella* was the most abundant genus in the gut microbiota of those who usually consumed a carbohydrate-enriched diet (Claesson *et al.,* 2012). Carbohydrate fermentation results in an increased concentration of short-chain fatty acids, which in turn cause a decrease in pH from 6.5 to 5.5. *Bacteroides* species grow poorly at pH 5.5. This may be the reason why a low abundance of *Bacteroides* was found in vegetarians.

As it has been discussed, diet, microbiota, and the occurrence of disease are linked, therefore dietary modulation studies could provide valuable information to understand diet-microbiota health issues and be useful for medical application, as changing the host's microbiota can lead to better health. It is suggested that the abundance of *Bacteroides* and *Prevotella* may be useful as a prognostic biomarker of disease in combination with other bacterial species from the core gut microbiota, especially *Faecalibacterium prausnitzii* (Ruengsomwong *et al.,* 2016).

As a result, it could be said that the gut microbiota composition changes rapidly in response to dietary adaptations and this is linked to health. For example, foods of animal origin contain higher amounts of choline and L-carnitine, which have been linked to higher risk for cardiovascular disease as a result of their conversion to trimethylamines by gut bacteria, absorption into portal circulation, and conversion to trimethylamine N-oxides (TMAO) in the liver. While the mechanisms by which TMAO increases cardiovascular risk need further clarification, it has been shown that TMAO reduces reverse cholesterol transport and bile acid (Wilson et al., 2013) synthesis, potentially attenuating the normal route of intestinal cholesterol elimination. Vegans and lacto-ovo vegetarians have been shown to have negligible postprandial plasma TMAO concentrations in response to an L-carnitine meal challenge (Koeth *et al*., 2013). This suggest reduction in carnitine and total choline ingestion with attendant reductions in TMAO levels may contribute to the cardiovascular health benefits observed in vegan/vegetarians. Thus it appears that the lower cardiovascular disease risk associated with a plant-based diet could result, in part, from lower circulating TMAO.

To sum up, it could be said that the overall balance in the composition of the gut microbial community and the presence of key species are important in ensuring homeostasis of the intestinal ecosystem to enhance good health.

1.5 HOW THE WAY OF COOKING CAN AFFECT THE GUT MICROBIOTA

Since gut microbiota can be easily affected by diet, it is important to unravel the specific effects of foodstuffs from different groups like vegetables, fruits, meat, legumes, or cereals, among others. However, the ability of gut microbiota to use substrates could also be influenced by the culinary heat treatment undergone by the foodstuffs prior to ingestion.

Upon cooking, many different compounds will be generated, most of them derived from the Maillard reaction. All these neoformed compounds could have some effect over the gut microbiota (Pérez-Burillo *et al*., 2018). This is why cooking methods could play an important role in the modulation of the gut microbiota, due to chemical changes in foodstuffs during the cooking process. There are only few studies on this topic but Shen et al. (2010) for example found that fried beef increased the levels of *Clostridium spp.* and *Bacteroides spp.* more than boiled beef, while decreasing the levels of SCFAs. Marungruang *et al*. (2016) showed that a

heat-treated diet rich in lipids alters the composition and functionality of the gut microbiota in mice and increases adiposity and low-grade inflammation, compared with the same diet not submitted to heat-processing. Heat processing transforms proteins, carbohydrates and lipids due to the Maillard reaction, lipid oxidation and others. These reactions produce new compounds, such as melanoidins, which can exert both antimicrobial and prebiotic activities. Therefore, one of the greatest challenges in nutrition is to interrogate the interactions existing within complex food matrices that integrate a wide range of biologically active compounds. This raises the question of whether there are specific dietary ingredients-nutrients that exert stronger selective forces on the diversity and functional configuration of microbial communities than others, and how different thermal processes affect their microbiota-modulating capacities.

1.6 OBESITY AND CARDIOVASCULAR DISEASES

Nearly two billion people worldwide are overweight or obese (World Health Organization – WHO, 2017), which contributes to an increase in the incidence of Non-communicable disease (NCD) such as obesity, type 2 diabetes and metabolic syndrome kill approximately 38 million people each year (WHO, 2016). The most common NCDs are cardiovascular disease, cancer, chronic respiratory disease, and diabetes. Almost a quarter of people currently employed suffer from some form of chronic disorder, including many that suffer from obesity, which in turn leads to substantial on-the-job productivity losses (UNEP, 2016). The prevalence of NCD in children is also alarming. Prevalence of overweight in children and adolescents has risen dramatically from 4% in 1975 to over 18% in 2016 (WHO, 2017). For example, in Germany 15% of children and adolescents are overweight (Varnaccia *et al*., 2017) while 67% of men and 53% of women are also overweight (Bohn *et al.,* 2017). These children are at increased risk for physical problems, such as cardiovascular disease, diabetes and musculoskeletal disorders, as well as problems related to social and emotional wellbeing and self-esteem. Childhood overweight and obesity have been characterized by the WHO as one of the most serious public health challenges of the 21st century. While excessive energy intake and insufficient physical activity are the main drivers of childhood overweight and obesity, recent research has suggested that other factors such as the gut microbiota may also be involved as shown in **Figure 2**. The gut microbiota contributes to the development of overweight and a lower gut microbial richness and diversity is associated with obesity. Actually, obese individuals appear to harbour a high Firmicutes-to-Bacteroidetes ratio in their gut microbiota.

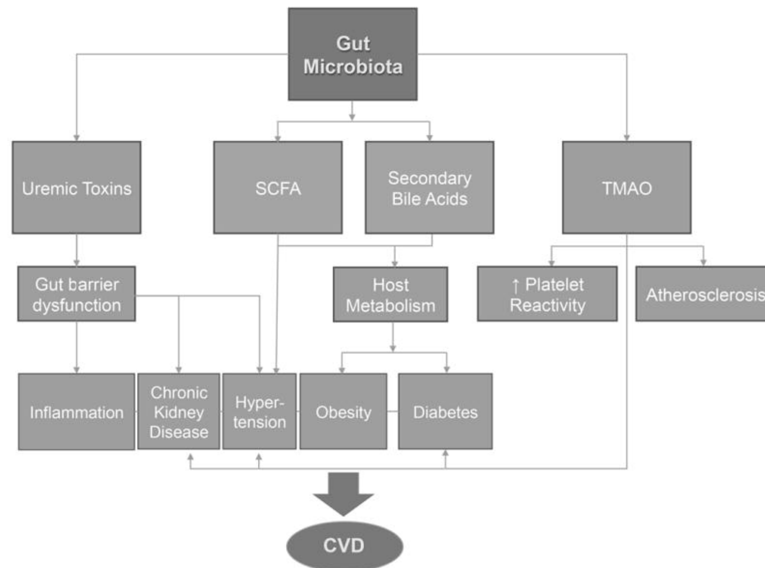

Figure 2. Schematic of gut microbiota effects on risk factors for CVD and adverse cardiovascular events. (Ahmadmehrabi *et al.,* 2017)

However, it has been shown that weight-related parameters (BMI and weight z scores and overweight) are not major drivers of microbial composition in the gut of relatively lean, healthy children. Nonetheless, several specific bacterial taxa appear to be consistently associated with weight-related outcomes and linked to body weight, as well as novel species. Higher abundances of *Prevotella oralis* et rel. and *P. melaninogenica* et rel. were inversely associated with overweight and other bacterial groups, of which *Akkermansia*, *Marvinbryantia formatexigens* et rel., and *Sutterella wadsworthensis* et rel. were consistently associated with all three anthropometric outcomes such as height, weight and percentage body fat (Mbakwa *et al*., 2018).

In addition, other pathologies related with food are also expanding such as oeliac disease that occurs in about 1% of people in most populations. A true rise in incidence rather than an increased awareness and detection of the disease is considered (Lebwohl *et al*., 2017). On the other hand, food allergy has rapidly increased in prevalence over the last 30 years (up to 10% of the European population) and is characterized as a growing and threatening public health concern that can have a significant impact on the quality of life of children and their families. Therefore, an understanding of what constitutes a health-promoting or disease-promoting microbial group and of how this is related to diet has turned into the focus of huge research.

1.7 STANCE4HEATLH PROJECT

The European Commission Recommendation "A healthy diet for a healthy life" (2010/250/EU) (http://www.stance4health.com) states that if common lifestyle risk factors, including diet-related ones, were eliminated, around 80% of cases of heart disease, strokes and type 2 diabetes, and 40% of cancers, could be avoided. Personalised nutrition for the European population is seen as the way forward to tackle this challenge. The Smart Personalised Nutrition approach proposed by Stance4Health will be tailored to different target groups, from healthy children and adults to children with coeliac disease or food allergy, as well as overweight children and adults,

which will have an impact on the development of non-communicable diseases such as obesity or type 2 diabetes.

In a first step, Stance4Health will develop a novel ICT application (the mobile app i-Diet as it is described in **Figure 3**) that will provide individualized counselling about what foods are more recommendable according to the overall needs/preferences of consumers (e.g., health status, gut microbiota composition, lifestyle, food preferences, and socio-economic status). In a second step, Stance4Health will develop customized cereal-derived foods, individualised dietary supplements and nutraceuticals for vulnerable target groups mentioned above (overweight, coeliac disease, food allergy), as well as an in vitro diagnostic test to evaluate gut microbiota activity and a wearable device to help users monitor their body weight over time.

In conclusion, i-Diet will be designed as an innovative and easy tool ecosystem for dietary assessment, allowing measurement of dietary intake in an easy way. All of these technologies will be developed and integrated in Stance4Health by means of preparing an i-Diet prototype, testing new vegetable extracts to produce cereal-derived foods for the target groups and a large-scale validation of all tools developed (mobile app, wearable, new foods and nutraceuticals, in vitro metabolomics diagnostic test). Importantly, i-Diet will have a strong focus on modulating the gut microbiota of an individual through comsumption of a personalised diet in order to improve metabolism and overall health.

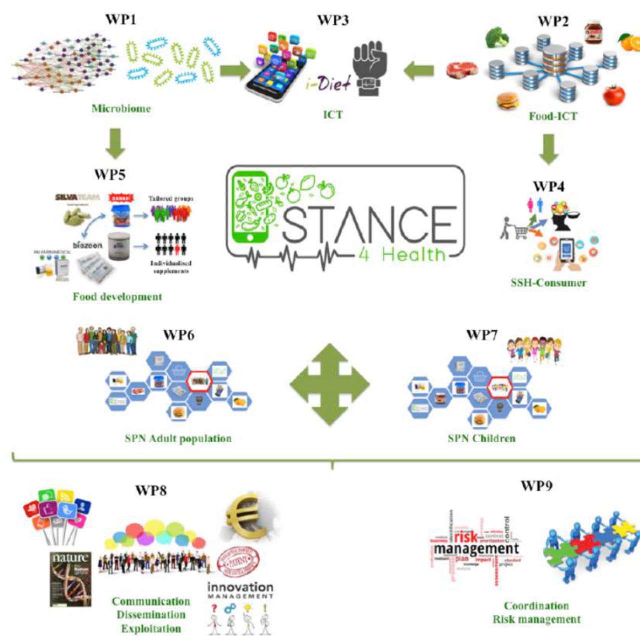

Figure 3. Stance4health overall structure (Stance4health proposal).

2. OBJECTIVES

One of the initial aims of the SPN project Stance4Health is to characterize the bacterial taxa that define the community structure in gut samples from volunteers before and after *in vitro* fermentation assays with different types of foods. The food employed in the assays will first be digested *in vitro* with a protocol developed within the project INFOGEST, and the digested food will then be submitted to an *in vitro* fermentation process with fecal microbiome inocula from Spanish healthy adults, in total there are 11 samples. These experiments will be repeated with a large variety of foods in order to establish a database relating foods to gut microbiota changes; this database will represent an important part of the Stance4Health SPN system by contributing to the design of personalised diets that respond to the specific health needs of each individual.

During the current work, one of the aims is supporting the validity of the experimental protocols that will be employed in the Stance4Health project before scaling up the project experiments.
To achieve the aims of this study, the following specific objectives were stated:

1) To employ 16S rRNA amplicon sequencing to detect gut microbiota alterations produced by the addition of food (bread) to a fecal inoculum in an *in vitro* fermentation assay following *in vitro* food digestion.
2) To elucidate the impact of freezing conditions on the human feces before *in vitro* fermentation assays.

3. MATERIALS AND METHODS

3.1 SAMPLING

Fecal samples were taken in sterile containers from three healthy Spanish adults, residing in Granada, with a normal body mass index and who had not taken antibiotics within the previous three months. At reception, each fecal sample was separated into three different tubes, one for immediate colonic phase in vitro fermentation (fresh feces), one to be stored at -80ºC for 8 days without glycerol, and a last one to be stored at -80ºC for 16 days before fermentation. There are 6 different samples (S01-S06), with 2 copies of each one.

3.2 INOCULA PREPARATION AND *IN VITRO* FERMENTATION

Fermentation assays were performed at the University of Granada in the research group of Prof. José Angel Rufián Henares. Three *in vitro* fermentation experiments were performed, employing fresh feces, 8-days frozen feces and 16-days frozen feces. For each experiment, a pooled inoculum was prepared from the fecal samples of the three donors in order to perform a single experiment controlling for inter-individual variability.

- Inocula preparation**:** each inoculum contains 3 g of feces/10 mL of phosphate buffer 0.1 M pH 7 (1 g of feces from each donor). Fecal material and buffer are mixed by vortexing and centrifuged at 500 rpm for 10 minutes to remove solid particles but not bacteria. Two aliquots of the prepared inoculum are stored for 16S rRNA sequencing, and the rest is used for the *in vitro* fermentation.

- These *in vitro* fermentations are carried out in falcon tubes. Each tube contains 2 mL of inocula, 7.5 mL of fermentation minimum medium (peptone, cysteine, and sodium sulfide), and 0.5 g of food. *In vitro* fermentation proceeds at 37ºC for 20 hours in oscillation.

- After 20 hours of incubation, two 1 mL samples of the fermentation medium were collected and centrifuged to pellet the bacterial cells. The pellets were frozen at -80ºC and subsequently lyophilized for 3 hours before sending to FISABIO (Valencia) for DNA extraction, 16S rDNA gene amplification and sequencing.

Microbiota was characterized before and after fermentation with the fresh feces (day 0), with 8-days frozen feces and 16-days frozen feces. In vitro colonic fermentation was simulated with bread after performing previous luminal digestión of this food according to Menikus *et al*., (2014).

3.3 DNA EXTRACTION

Microbial DNA extraction from lyophilized stool samples (100-200 mg) was performed automatically using the MagNA Pure LC 2.0 robot (Roche) and with the MPLC DNA Isol extraction Kit III for bacteria (Roche), following the manufacturer's instructions. The extraction is carried out by the use of magnetic beads technology. In order to optimize microbial load, 1,5 ml of PBS was added to the samples and these were centrifuged at 2.000 rpm for 3 min. After this, the solid was discarded because the interesting part is the supernatant that is centrifuged again but now at 13.000 rpm in order to recover the bacteria from the new pellet at the bottom of the eppendorf, were the gut microorganisms are settled.

Following the protocol, the extraction is done in the MagNA Pure LC 2.0 robot. 207 µl of lysis buffer and 23 µl of lisozyme (to break down the gram positive bacteria membranes) were added to the pellet. After vortexing the mix, the tube is incubated at 37 ºC for 30 min and in the final step before extraction, 20 µl of proteinase K were added to break down the proteins and free the microbial DNA. The tube was incubated 15 min at 65 ºC and 5 min at 95 ºC with proteinase K before loading the samples in the extraction robot

3.4 DNA ANALYSIS AND AMPLIFICATION

Metagenomic studies are commonly performed by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions. Variable regions of 16S rRNA are frequently used for taxonomic classification of bacteria in diverse microbial populations and this will help us to see the taxonomic differences between samples.

To date, the majority of gut microbiome studies have employed 16S rRNA gene amplification for microbial genotyping. 16S rRNA is a powerful phylogenetic marker and has become a standard in bacterial taxonomic classification because it is easily and rapidly sequenced and contains enough phylogenetical information. Some of the advantages are that it is universally distributed, can be used to measure phylogenetic relationships across different taxa, horizontal gene transfer isn´t a big problem and analysis costs are low. However, the technique incorporates systematic biases, is sensitive to 16S rRNA gene copy-number variations, and overinflates diversity estimates. Moreover, sometimes it is difficult to differentiate between closely related species and the variability in PCR amplification could affect the resultant data. In contrast, shotgun metagenomics sequencing is a non-targeted DNA-based approach that does not incorporate amplification biases.

Which 16S rRNA region to sequence is an area of debate, and our region of interest might vary depending on things such as experimental objectives, design, and sample type. This protocol describes a method for preparing samples for sequencing the variable V3 and V4 regions of the 16S rRNA gene, combined with the Illumina technology MiSeq benchtop sequencing system. MiSeq provides a comprehensive workflow for 16S rRNA amplicon sequencing. 16S rDNA gene amplicons were obtained following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). The gene specific sequences **(Table 1)** used in this protocol target the 16S rDNA gene V3 and V4 regions and are designed with Illumina overhang adapters so the genomic DNA can be amplyfied **(Figure 4)**. The 16S rDNA primers are selected from Klindworth *et al.* (2013). The full length primer sequences, using standard IUPAC nucleotide nomenclature, to follow the protocol:

Table 1.Amplicon primers and overhangs.

16S rDNA gene Amplicon PCR Forward Primer	CCTACGGGNGGCWGCAG
16S rDNA gene Amplicon PCR Reverse Primer	GACTACHVGGGTATCTAATCC
Forward overhang 5'	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Reverse overhang 5'	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

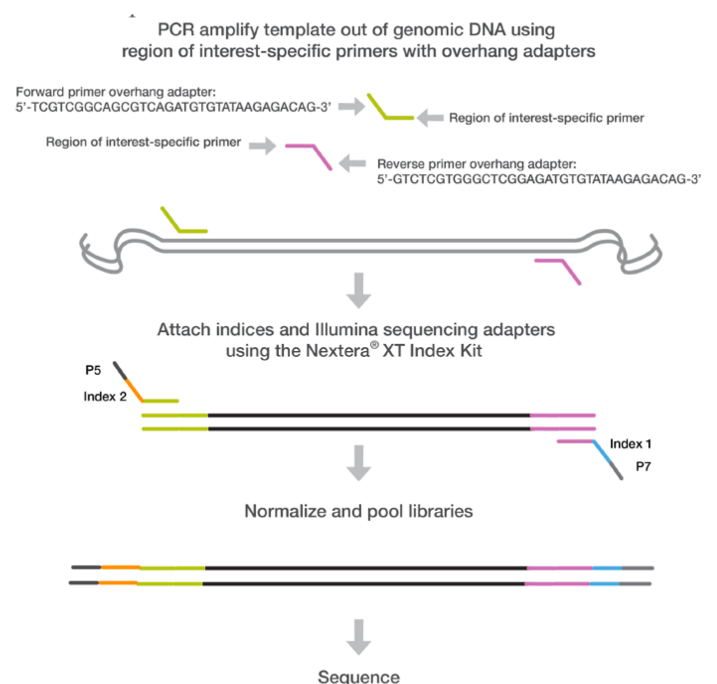

Figure 4. 16S V3 and V4 Amplicon workflow. User-defined forward and reverse primers that are complementary upstream and downstream of the region of interest.

3.4.1 PCR PROTOCOL

The V3-V4 region of the 16S rRNA gene was amplyfied following these steps:

- Mix of a total of 25 µl composed of: 10.5 µl of microbial DNA (1.2ng / µl in 10 mM Tris buffer), 1µl "first forward" primer (5 µM), 1µl "first reverse" primer (5 µM) and 12.5 µl of the Mix 2x KAPA HiFi HotStart polymerase.

- PCR with the following program:
  - 95 ° C for 3 min
  - 25 cycles of:
  - 95 ° C for 30 seconds
  - 55 º C for 30 seconds
  - 72 º C for 30 seconds

- 72 º C for 5 minutes

- Hold at 4 º C

3.4.2 Gel electrophoresis of PCR amplicons

Afterwards, the clean up is done with AMPure XP beads to purify the 16S V3 and V4 amplicon away from free primers and primer dimer species by mixing the dilution of magnetic beads (which are attached to DNA fragments that will capture the 16S rRNA sequences by affinity), with the resulting PCR amplicons **(Figure 5)**.

The amplified products (the PCR fragments) were also confirmed by gel electrophoresis, using a 0.8% agarose gel and TBE 5X (Tris/Borate/EDTA) and 2 µl of red gel stain for labelling the DNA. At the moment of loading the wells in the solid gel, 10 µl of sample + 2 µl of blue loading buffer were mixed in a PCR plate (96 wells). Once the samples were loaded and the DNA molecular weight ladders were added, the is set electrophoresis at 100V for 30 min. The DNA amplicon bands are inspected in order to analyze the quality of the sample, not the quantity.

3.5 LIBRARY PREPARATION

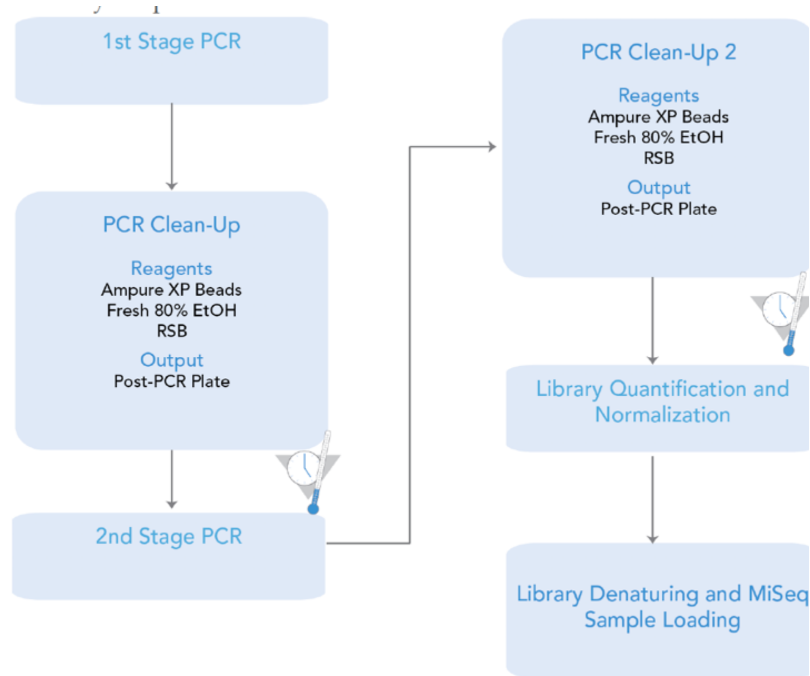

Figure 5. 16S Library Preparation Workflow.

3.5.1 Index and PCR test

After 16S rDNA gene amplification and cleaning, the mutiplexing step was performed using the dual indexing strategy which uses two 8 base indexes, Index 1 (i7) adjacent to the P7 sequence,

and Index 2 (i5) adjacent to the P5 sequence. The P5 and P7 sequences are the Illumina sequencing adapters that attach to the Illumina flowcell where the clustering and sequencing will occur. Dual indexing is enabled by adding a unique combination of Index 1 (i7) and Index 2 (i5) to each sample by means of a short PCR cycle (2nd Stage PCR in **Figure 5**).

In order to perform this 2nd Stage PCR, reactions are set up with 5 µl of 16S rRNA amplicon product, 5 µl of eavh index, 25 µl of Kappa HiFi Hot Start polymerase and 10 µl of H_2O. These primers hybridize to the overhang adapters of the 16S rRNA amplicon and include the index sequences as well as the P5 or P7 sequences.

The 96 sample Nextera XT Index Kit (FC.131– 1002) uses 12 different Index 1 (i7) adapters (N701–N712) and 8 different Index 2 (i5) adapters (S501–S508). In the Index adapter name, the N or S refers to Nextera XT sample preparation, and 7 or 5 refers to Index 1 (i7) or Index 2 (i5), respectively. Dual index barcodes were added to the amplicon target using the full complement of Nextera XT indexes, so that up to 96 libraries could be pooled together for sequencing. The 12 sequencing libraries corresponding to this work were sequenced jointly with 84 other libraries from a different project. After the indexing step AMPure XP beads are used to clean up the final libraries before quantification (PCR Clean-Up 2 in **Figure 5**). After the Clean Up 2 was done, the clean 2nd Stage PCR products is ran on a Bioanalyzer DNA 1000 chip to verify the size; the expected size of the products on a Bioanalyzer trace is ~550 bp.

3.5.2 Library quantification and pooling

The PCR products were quantified using the Invitrogen Qubit 4 Fluorometer for measuring DNA integrity and quality. The kit provides concentrated assay reagent, dilution buffer, and pre-diluted DNA standards. The reagent is diluted using the provided buffer, sample is added (any volume between 1 µl and 20 µl is acceptable), and the DNA concentration is read using the Qubit Fluorometer. Illumina recommends quantifying your libraries using a fluorometric quantification method that uses dsDNA binding dyes. This allows calculating the DNA concentration in nM, based on the size of the DNA amplicons. After quantification, the libraries are diluted because the DNA concentrations were too high for the pool. Each library was diluted to a concentration of 4 nM using 10 mM Tris pH 8.5. Libraries were pooled by mixing aliquots of 5 µl of diluted DNA from each library. Aliquots of the 12 sequencing libraries corresponding to this work were mixed with aliquots of 84 other libraries from a different project to be sequenced jointly in one MiSeq run.

3.6 LIBRARY DENATURING AND MISEQ SAMPLE LOADING

In preparation for cluster generation and sequencing, pooled libraries are denatured with NaOH, diluted with hybridization buffer, and then heat denatured before loading into the MiSeq platform for sequencing. In addition, the denatured amplicon library must be combined with a denatured PhiX control library. Each run must include a minimum of 5% PhiX to serve as an internal control. PhiX is a ready-to-use control library, the sequencing of which informs the MiSeq software about the coordinates in which the fluorescence associated to the sequencing reactions is released.

The pooled libraries were sequenced on a MiSeq Sequencer at the FISABIO Genomics and Health Area, using a 2x300 bp paired-end run with MiSeq Reagent kit v3 (MS-102-3001) according to manufacturer's instructions (Illumina).

During Illumina sequencing, nucleotides are added one by one and only the nucleotide complementary to the base of the template sequence is incorporated, emitting a different fluorescent light according to the type of nucleotide that has been added. It is a sequencing by synthesis. The emitted light signal is detected and with it the sequence. All detected sequences are generated as data in the standard FASTQ format.

3.7 BIOINFORMATIC ANALYSIS

Data have been analyzed using an ad-hoc pipeline written in RStatistics environment (R Core Team, 2012), making use of several R Open Source libraries and of the qiime2 platform (Caporaso *et al.,* 2010). Qiime2 is a sophisticated and tailored platform that combines numerous bioinformatic tools specific for 16S analysis (OTU classification, taxonomic identification, etc).

Quality assessment of sequencing reads was performed with the prinseq-lite program applying the following parameters: a minimal read length of 50 nucleotides and a quality score threshold of 30, evaluated through a mean quality score computed with a sliding window of 20 nucleotides. Sequence denoising, paired-ends joining, and chimera depletion was performed with the DADA2 software (Callahan *et al.,* 2016). Sequence denoising with DADA2 addresses one of the bioinformatics challenges that arise immediately from 16S rRNA sequencing analysis, which is the precise definition of a "unique" sequence. DADA2 models and corrects Illumina-sequenced amplicon errors (https://github.com/benjjneb/dada2) to infer exact, unique sequences, resolving differences of as little as 1 nucleotide. The sequences denoised by DADA2 can then be grouped into clusters of 100% identical sequences, named Amplicon Single Variants (ASV), which can be used as Operational Taxonomic Units (OTU) for further analyses **(Figure 6)**. OTUs defined with this 100% identity criterion are clusters of sequences that are considered to correspond to the same bacterial strain.

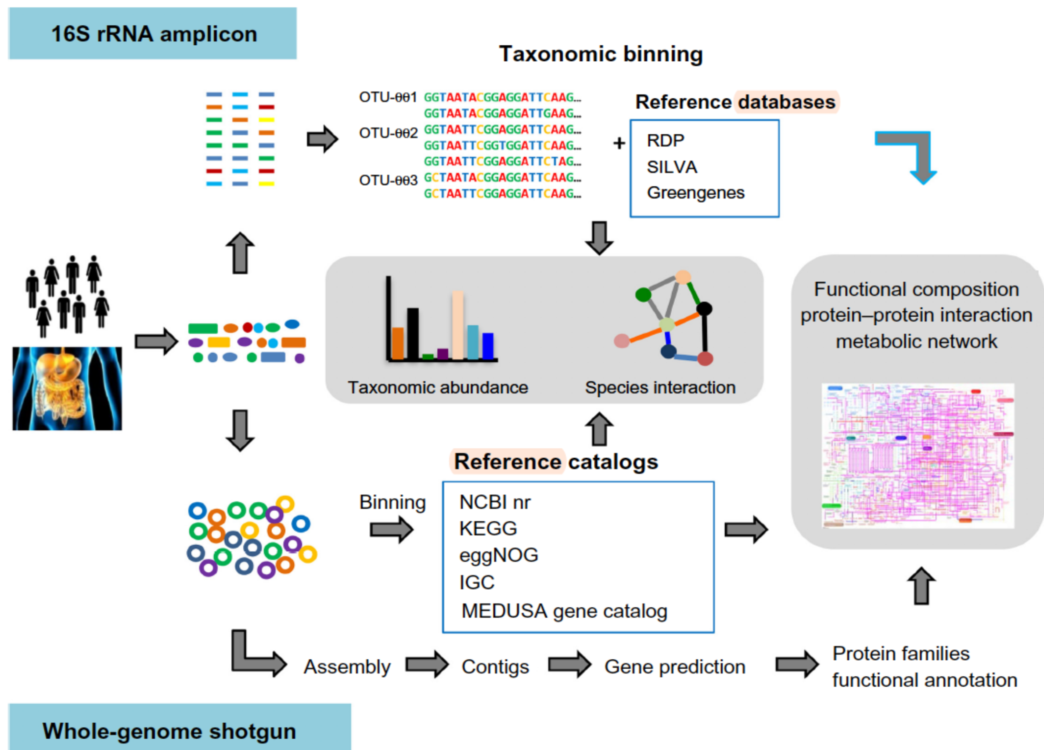

Figure 6. Overview of bioinformatics methods for gut microbiota metagenomic analysis.

(Ji *et al.,* 2015).

The taxonomic assignment of OTUs can then be inferred by similarity-based or composition-based methods. In this work, the taxonomic affiliations of the sequences were assigned by means of the Naive Bayesian classifier integrated in qiime2 using the SILVA_release_132 database (Quast *et al.,* 2013*)*

The Krona representation (Krona hierarchical browser) was used to visualize the microbial composition of the different samples (Ondov, *et al.*, 2011).

3.8 BACTERIAL COMMUNITY RICHNESS AND DIVERSITY ESTIMATION

Diversity within a sample is usually described as alpha-diversity, which captures both the organismal richness of a sample and the evenness of the organism's abundance, whereas the beta-diversity is often referred to as the diversity between multiple microbial samples and the distance between them, information can be extracted about the similarities between several bacterial groups. Diversity analysis is performed using the vegan R library. Vegan is a Community Ecology Package for ordination methods, diversity analysis and other functions for community and vegetation ecologists.

3.8.1 ALPHA DIVERSITY INDEXES

For alpha-diversity, richness estimators such as Chao1 (Chao, 1984) are determined, which gives us information about the number of species and the richness in a sample. The Chao1 estimator was chosen because it is suited for estimating total OTU richness in species-rich samples.

Moreover, Shannon and Simpson indexes were also studied due to the fact that they also consider the microbial distribution. Shannon's Index is more sensitive to species richness while Simpson's Index is more sensitive to species evenness (Johnson *et al.,* 2016). The Shannon index gives us information about the number of species in the sample and the relative abundance of each one. It is normally represented as H and is expressed with a positive number, which in most cases varies between 0.5 and 5, although its normal value is between 2 and 3; values lower than 2 are considered low in diversity and higher than 3 are high in diversity of species. The Simpson index gives us information about the probability that 2 individuals of the same sample belong to the same species. The number 1 indicates presence of 1 species while a number close to 0 indicates more diversity. The information about the heterogeneity inside a bacterial community, the number of species and the relative abundance will be shown.

3.8.2 BETA DIVERSITY INDEXES

In order to analyze beta diversity, two different parameters were employed, the Jaccard and Sorensen indexes. The Jaccard index is a statistic used for gauging the similarity and diversity of sample sets so it is a way to compare populations by determining what percent of organisms identified were present in both populations. The Sorensen index also compares the similarity between samples and is based on presence/absence of data. Both indexes take values from zero to one. In a similarity index, a value of 1 means that the two communities you are comparing share all their species, while a value of 0 means they share none. In a dissimilarity index the interpretation is the opposite: 1 means that the communities are totally different.

In order to visualized the variation in microbiota composition among samples by means of Principal Components Analysis (PCoA) was done using the Jaccard and Sorensen distances. For these analyses, data were grouped according to the metadata file provided that specified whether samples had been taken before or after *in vitro* fermentation and the number of days the sample had been stored at -80 ° C.

4. RESULTS AND DISCUSSION

In this section, the composition of the samples (krona viewer) will be analysed and the calculation of the average abundance of different organisms in the different groups of samples at the level of family and genus will be done. In order to see the specific bacteria that vary in abundance according to the level where you make the comparison. Also, alpha diversity will be described before and after fermentation for samples fermented after 0, 8 and 16 days of storage at -80 ° C, focusing on the Chao1, Shannon and Simpson indexes. At last, beta diversity will be shown with the principal components analysis in order to see differences in taxonomical composition among the different groups of samples and what characteristics divide these samples in groups.

After the bioinformatic analysis, the results in terms of taxonomic composition for all the samples were described in **Table 2**. There are 12 samples in total, i. e. two repetitions for each condition: inoculum and sample after *in vitro* fermentation for a fresh sample (day 0) and for samples having been stored at -80 ° C for 8 or 16 days**.** This allows us to characterize the effect of freezing for different times and how fermentation of bread modifies the intestinal microbiota.

Table 2. Name of the samples and description

Sample ID	Granada_name	Description
S01_1	Pan 1	Fermented bread day 0
S01_2	Pan 1	Fermented bread day 0
S02_2	Pan 2	Fermented bread day 8
S03_1	Pan 3	Fermented bread day 16
S03_2	Pan 3	Fermented bread day 16
S04_1	BI 1	Inoculum day 0
S04_2	BI 1	Inoculum day 0
S05_1	BI 2	Inoculum day 8
S05_2	BI 2	Inoculum day 8
S06_1	BI 3	Inoculum day 16
S06_2	BI 3	Inoculum day 16

4.1 COMPOSITION

4.1.1 COMPOSITION OF THE FRESH INOCULUM

Generally, the phylum *Firmicutes* and *Bacteroidetes* dominate the gut microbial community, while members of *Proteobacteria, Actinobacteria, Verrucomicrobia*, and the candidate phylum are less abundant. Even though this general profile remains constant, gut microbiota exhibits both temporal and spatial differences in distribution at the genus level and beyond. (Jandhyala *et al*., 2015). To describe the differences in the microbiota profile deriving from different food choices, it should be mentioned that *Bacteroides* was linked to diets that were high in protein and animal derived products (mostly omnivorous) and microbiota rich in *Firmicutes* (which includes the enterotype Ruminococcus) was strongly associated with a fat based westernized diet and obesity (Filippo *et al.,* 2010).

In **Figure 7**, the taxonomic composition of the fresh fecal inoculum (S04_1) is ilustrated, so in this case there has been no *in vitro* fermentation with bread or any other food. The phylum *Bacteroidetes* dominates, represented exclusively by the order *Bacteroidales*, followed by *Firmicutes, Proteobacteria, Verrucomicrobia* and *Actinobacteria*. Generally, the phyla *Firmicutes* and *Bacteroidetes* dominate the gut microbial community of healthy adults, while members of *Proteobacteria, Actinobacteria* and *Verrucomicrobia* are less abundant, representing the 17% of the total bacteria. Moreover, *Bacteroidetes* tends to be more abundant than *Firmicutes* in lean individuals (Ley *et al.,* 2006), as it is described in our fresh sample, which is a pool of samples form healthy adults of normal weight.

Bacteroidetes, the major group, is represented by three big genus: *Parabacteroides* (5% of the bacterial community), *Alistipes* (14%) and *Bacteroides* (14%). The children from Europe normally consume a western diet rich in animal protein, sugar, starch and poor in fibers, which is marked by the higher abundance of this phylum.

The second most abundant phylum is that of the *Firmicutes* (representing 24% of the total bacteria) and the main class within this level is *Clostridia,* which represents the 23% of the total composition. This phylum is of interest due to the fact that it shows a large diversity at the family level in comparison to other phyla represented in the fresh inoculum. Some of them could be highlighted, such as *Christensenellaceae, Lachnospiraceae, and Ruminococcaceae,* being this last one the most abundant (16% of all the microbiota). Members of the *Clostridia* confer to the microbiota the metabolic capacity of degrading complex polysaccharides and of fermenting sugars. Thus, they participate in facilitating the absorption and metabolic digestion of the nutritional components in the intestine.

Figure 7. Krona viewer sample S04_2

(Ondov et al., 2011).

The observed abundance of the phylum *Proteobacteria* in the fresh inoculum (20%) is within the range expected for samples taken from healthy adult individuals. The phylum *Proteobacteria* is generally present in healthy mammalian guts as a normal component of the gut microbiota. Under a healthy steady state, the relative abundance of *Proteobacteria* in the human gut can transiently increase to 45% without clinical signs (Caporaso *et al.,* 2011). However, a chronic enrichment of *Proteobacteria* in the gut can represent an imbalanced unstable microbial community structure or a state of disease of the host (Shin *et al*., 2015). Under certain conditions, some *Proteobacteria* can become colitogenic microbes that can trigger inflammatory responses. Moreover, dysbiosis during metabolic disorders often includes an increased prevalence of *Proteobacteria* (Shin *et al.,* 2015) and a correlation has been identified between the abundance of *Proteobacteria* and metabolic diseases such as diabetes. Within the *Proteobacteria*, the main class present in the fresh inoculum is *Gammaproteobacteria* and the prevailing family corresponds to *Enterobacteriaceae. Enterobacteriaceae* is a heterogeneous and extensive family of gram-negative bacilli that reside in the colon without usually causing disease, but they can often be responsible for a considerable number of infections.

Finally, another class to consider is *Verrucomicrobiae (*17% of the total). This class is usually represented specifically by *Akkermansia,* one of the most abundant genera of the microbiota of

the human intestine, generally representing between 1 and 5% of total bacteria. However, in our fresh inoculum it is mainly represented by an unidentified uncultured bacterium. Finally, it is important to consider the phylum *Actinobacteria,* which represents the 2% of all the microbial community.

4.1.2 Changes in composition of the fresh inoculum after bread fermentation

Figure 8 presents the krona viewer image for sample S01_1, which corresponds to the unfrozen sample after *in vitro* fermentation with bread. The *Bacteroidales* and, especially, the *Firmicutes* have become more abundant during the process of bread fermentation, as they now represent 43% and 37% of the total bacteria in the community, respectively, whereas the phylum *Proteobacteria* has strongly decreased (3%). The *Firmicutes* class *Clostridia* is the most abundant overall, as it represents 37% of the total bacterial composition. Within this class, the family *Ruminococcaceae* (order *Clostridiales*) is the most abundant, representing 24% of the total bacteria in the sample. Not to mention, it is important to point out the order *Bacteroidales* with a presence representing 43% of all the microbial diversity. In particular, the genera *Bacteroides* and *Alistipes* represent 23% and 15% of the entire bacterial community, respectively.

Considering all other bacterial groups, there is also a high abundance of the class *Verrucomicrobiae* (13% of all bacteria), although the percentage has decreased in comparison to the fresh sample before fermentation of bread (17%). Not to mention, higher basal abundance of this microorganism is associated with a significant improvement of cardiometabolic parameters in subjects with obesity subjected to caloric restriction (Dao *et al*., 2016). In contrast, the relative abundance of the phylum *Actinobacteria* (4%) has augmented with respect to the unfermented inoculum (2%), although it still represents a small part of the total community.

Figure 8. Krona viewer sample S01_1

(Ondov *et al.,* 2011).

In conclusion, the fermented samples with bread (S01, S02, S03) display a richer composition than the inocula samples without the fermentation process (S04, S05, S06). This could be explained with the fact that the process of fermentation allows ceratin microbial species to develop and grow in the presence of different foods, in this case, bread composition. On one hand, the samples where only the fecal inocula is analysed, the presence of *Proteobacteria* is remarkable and represents half of the microbial composition. Therefore, it is a more heterogeneous samples with lower species richness and unbalanced proportions between taxonomic groups, dominating *Proteobacteria* and *Firmicutes* above all. On the other hand, the samples fermented with bread show a bigger abundance of *Bacteroidales* compared to samples S05 and S06 as well as a greater proportion of *Firmicutes*. In **Figure 8**, a greater richness is visualized, because there is a bigger number of different species and also in similar proportions. It is a more heterogeneous sample. Moreover, it a more diverse sample that the S05 because presents less differences in the number of microbial species.

4.1.3 CHANGES IN COMPOSITION AFTER SAMPLE STORAGE AT -80 ° C.

It is clearly seen that the taxonomical composition of the fecal samples has changed in terms of relative taxon proportions after the freezing treatment. It is a significant difference especially in terms of the proportion of *Proteobacteria*, which increase from a relative abundance of 20% in the fresh sample to 42% and 56% after 8 days and 16 days of storage at -80 ° C, respectively **(Figure 9)**. This increase is accompanied by a substantial decrease in the relative abundances of the *Bacteroidales* and the *Firmicutes.*

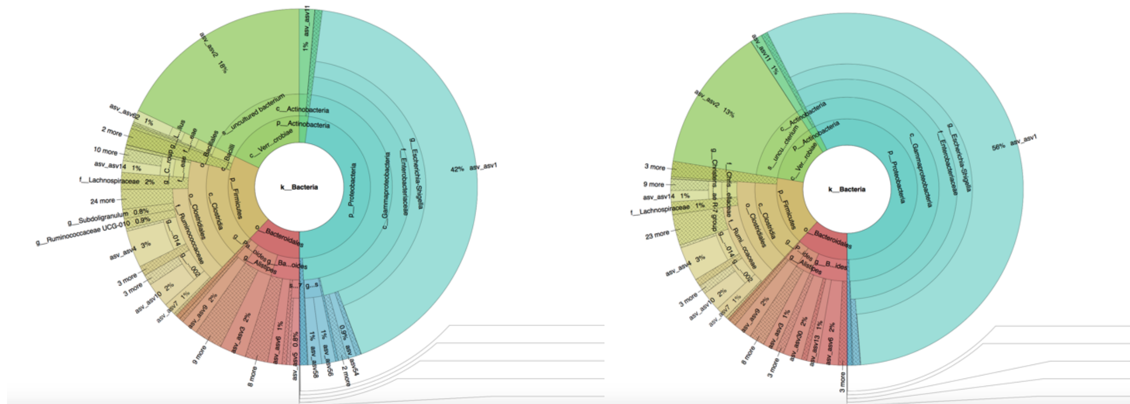

Figure 9. Microbial composition of the inoculum after different times of freezing, 8 days for sample S05_1 (left) and 16 days for sample S06_1 (right).

4.1.4 CHANGES IN COMPOSITION AFTER BREAD FERMENTATION WHEN USING PREVIOUSLY FROZEN SAMPLES

Figure 10 shows that the samples fermented with bread after having been stored at -80 ° C (S02, S03) show an increased abundance of *Bacteroidales* and *Firmicutes* compared to the non-fermented inocula in samples S05 and S06. In contrast, the proportion of *Proteobacteria*, which were greatly over-represented in the inocula prepared from frozen samples, decreases substantially after fermentation. Therefore, the main effects seen after bread fermentation are the same for these frozen samples as those observed with the fresh sample, i. e., in both cases the percentage of *Bacteroidale*s and, especially, that of *Firmicut*es increases with bread fermentation, while that of *Proteobacteria* decreases. When all samples, fresh and frozen, are considered, the differences in the relative abundances of *Fimicutes* and *Proteobacteria* between the inocula and the fermented samples are highly significant (p=0.081 for both comparisons in Wilcoxon tests). However, the difference in the relative abundance of *Bacteroidales* between the inocula and the fermented samples is not significant.

The *Verrucomicrobiae* also tend to increase with bread fermentation, whereas the proportion of *Actinobacteria* remains similar. These trends are different from those observed with the fresh sample for these bacterial groups, since, in that case, the percentage of *Verrucomicrobiae* decreased with bread fermentation, whereas the percentage of *Actinobacteria* augmented.

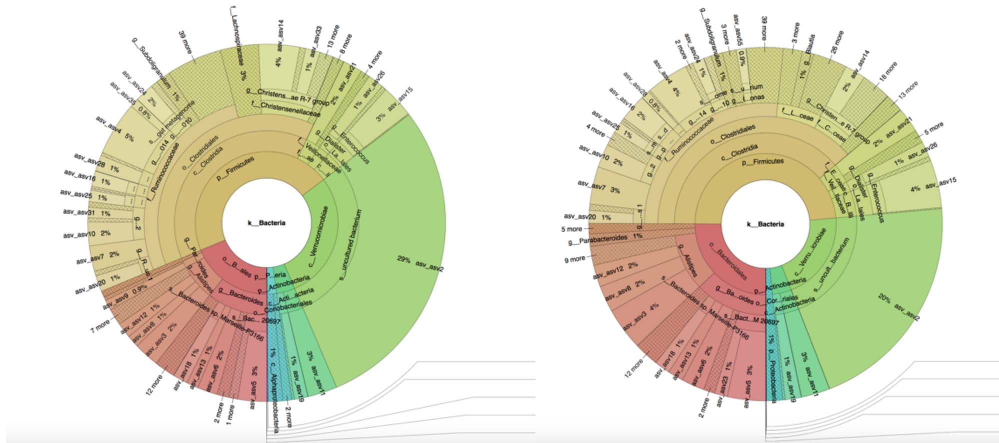

Figure 10. Microbial composition of samples S02_1 and S03_1, which underwent bread fermentation after 8 or 16 days of storage at -80 ° C, respectively.

4.2 ALPHA DIVERSITY

Alpha diversity indexes are generally applied in ecological studies to characterize the number and abundance distribution of species, although they are also occasionally used at higher taxonomical levels, such as genus or family. Given the difficulty of establishing species boundaries in microbiome data, alpha diversity indexes at the level of genera and families are reported.

At family level, the fecal inocula have lower diversity values than the fermented samples, and the diversity of the inocula decreases as the freezing time increases as can be seen in **Table 3**. Thus, the alpha diversity in the sample at day 16 of the freezing treatment (S06_1) is significantly more reduced than the one at day 0 (S04_1), with a Shannon index of 1.55 and 2.29, respectively **(Table 3).**

Furthermore, the range for the Shannon index value is about 0.77 for the inoculum samples (S04-S06), whereas the fermented samples show a range of 0.14 among them. From this, it could be stressed that the alpha diversity takes more stable values in the samples with the fermented bread (S01-S03). This is due to the variation in Shannon index values observed between the fresh and frozen inocula, as the index decreases substantially in the latter **(Figure 11)**. In contrast, diversity is similar for the fermented samples, independently of whether they contained fresh or frozen innocula. Regarding the Chao1 index of richness, there are small variations of values depending on freezing time. The Chao1 values were slightly reduced with the freezing treatment at day 16 (S06_1, Chao1: 35; S06_2, Chao1: 36). The highest Chao1 values were observed for the fermented samples at different freezing times (S01_2 and S03_2, Chao1: 45).

Table 3. Estimates of bacterial richness (Chao1 index) and diversity (Shannon and Simpson index) comparing the different inoculum and fermented samples at different freezing times at family level.

	CHAO1	Shannon	Simpson
S01_1	39	2.24	0.84
S01_2	45	2.28	0.85
S02_2	37	2.17	0.82
S03_1	43	2.31	0.85
S03_2	45	2.27	0.85
S04_1	38	2.29	0.87
S04_2	37	2.32	0.87
S05_1	40	1.91	0.75
S05_2	37	1.82	0.71
S06_1	34	1.55	0.64
S06_2	36	1.72	0.7

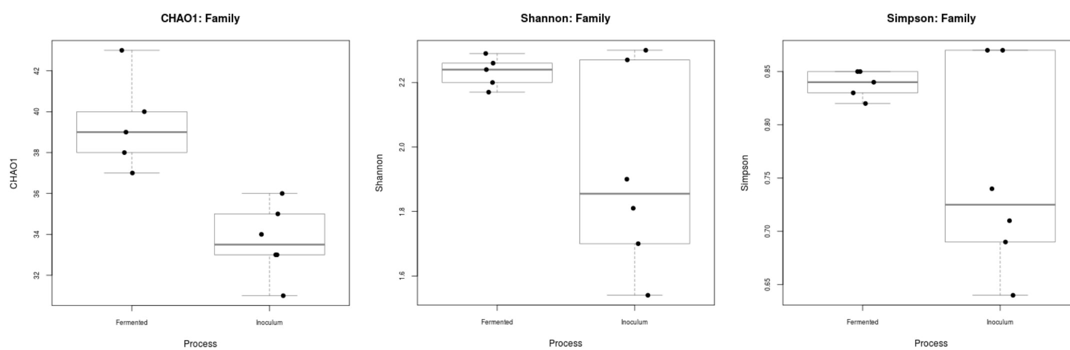

Figure 11. Chao1, Shannon and Simpson indexes at family level for inoculum and fermented samples.

Once into a more specific taxonomic level, such as the genus level, the differences among the samples show different values in terms of richness and diversity**.** At this level, the Chao1 index is much higher in the fermented samples compared to the samples that are only composed of the inoculum itself **(Figure 12)**. As an example of this, the fermented sample S01_2 presents an index of 125**,** to be contrasted with the last inoculum sample with freezing treatment at time 16 (S06_2, Chao1: 79) **(Table 4)**. It is also observed that as the freezing time increases, richness is lost and some bacterial genera are disappearing during this treatment.

Regarding diversity indexes such as Shannon, now in a very specific taxonomic group, a great diversity of microorganisms can be seen. Focusing on the samples in which there has been

fermentation with food, observe an increase in diversity is observed, with values greater than 3 in S01_1, S01_2, S03_1 and S03_2 **(Table 4)**, which means that the *in vitro* fermentation process has enhanced the appearance of more bacterial groups and has generated a more even distribution of organism abundances among these groups. In contrast, for the samples in which there has been no in vitro fermentation with bread, values are still below 3, sometimes even below 2, as in the case of S06_1, which underwent freezing for 16 days. Again, this indicates that the freezing treatment adversely affects both the richness and diversity of the microbiota.

Similarly, the Simpson index shows greater differences between sample values. Before the fermentation process the values are between 0.6 and 0.9, whereas after the fermentation the values are closer to 1 **(Table 4)**. This means that the fermentation treatment generates a more even composition in terms of relative abundance of different microorganisms. In addition, it should be pointed out that the cold treatment also has a great influence on the indexes of the unfermented inocula. This is seen in the fact that in the inoculum sample at day 0 (without freezing treatment) the values of the Simpson index are practically the same as those of the samples after fermentation with bread. However, on days 8 and 16 of the freezing treatment it is shown that the index is decreasing until reaching 0.66 in S06_1, which shows a direct effect of the cold and the loss of microbial diversity.

Table 4. Estimates of bacterial richness (Chao1 index) and diversity (Shannon and Simpson index) comparing the different inoculum and fermented samples at different freezing times at genus level.

	CHAO1	Shannon	Simpson
S01_1	120	3.11	0.9
S01_2	125	3.21	0.91
S02_2	100	2.99	0.89
S03_1	122	3.22	0.92
S03_2	117	3.12	0.91
S04_1	95	2.83	0.89
S04_2	92	2.85	0.9
S05_1	96	2.33	0.78
S05_2	89	2.26	0.75
S06_1	80	1.86	0.66
S06_2	79	2.04	0.71

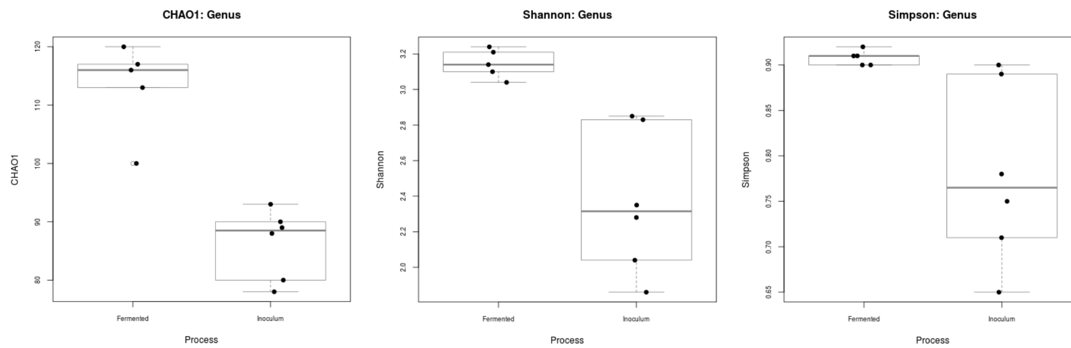

Figure 12. Chao1, Shannon and Simpson indexes at genus level according to different types of processes.

4.3 BETA DIVERSITY

Here, the statistical hypothesis tests in both community composition and microbiome-host interactions are discussed. The utility of various statistical approaches for assessing the diversity of microbiome communities and analyzing and modelling the association between community composition of the microbiome and host are reviewed.

4.3.1 PRINCIPAL COMPONENTS ANALYSIS (PCOA)

To assess beta diversity, principal components analysis was performed with the information obtained from the taxonomy analysis. In PCoA plots, the points that are closer together in the graph represent microbial communities that are more similar in taxonomical composition. **Figure 13** presents a plot of the PCoA analysis performed with taxonomical information at the genus level. The first component (dimension 1) clearly divides the inocula (orange ellipse) from the fermented samples (green ellipse). This dimension explains 41.7% of the total variation among samples. The second component (dimension 2) explains 22.9% of the variation and also clearly separates the unfrozen samples (S01 and S04) from those that have undergone a freezing treatment (S02, S03, S05 and S06).

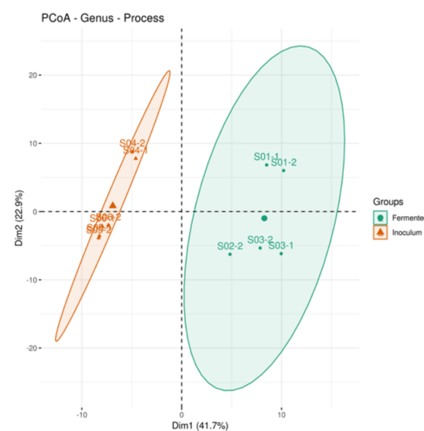

Figure 13. PCoA comparing inoculum and fermented samples.

Moreover, it can be observed that all technical repetitions plot very closely in the PCoA space. It can also be seen that the green ellipse is much wider, indicating that there is more variation among samples after fermentation. This is to be expected, since the changes in the microbiota are expected to be similar but not necessarily identical in independent fermentation experiments, even if the composition of the inocula is very close.

4.3.2 Paired Wilcoxon Tests

In **Table 5** the relative abundance of a microorganism is compared between the inocula and the fermented samples and it is shown whether the difference between the abundance average between the two groups is significant ($p<0.05$). The changes observed for some select genera of the *Clostridiales* families *Lachnospiraceae* and *Ruminococcaceae* are reported. For example, row number 1 compares the abundance of the *Lachnospiraceae* genus *Eisenbergiella*, which is more abundant in group 1 (fermented samples) with a mean of 0.0904%, than in group 2 (inocula) with a mean of 0.022% ($p=0.0055$). Similarly, it can be highlighted that the relative abundances of two other *Lachnospiraceae* (genus *Ventriosum* and *Xylanophilum* group) also increase significantly ($p=0.0081$) in the fermented samples (mean1 = 0.7137 % and 0.1794 %) compared to the inocula (mean2 = 0,1064 % and 0,0493 %). Finally, the last row in **Table 5** compares the relative abundance of the *Ruminococcaceae* genus *Butyricicoccus*, which is more abundant in group 2 (inocula) with a mean of 0.0822 %, than in group 1 (fermented samples) with a mean of 0.005% ($p=0.0055$).

Table 5. Wilcoxon tests between fermented samples (Mean 1) and inocula (Mean 2) by taxa at genus level. This Table shows only taxa with significant changes (p-values <=0.05) after doing the Wilcoxon.

	Phylum	**Class**	**Order**	**Family**	**Genus**	**Mean 1 (%)**	**Mean 2 (%)**	**Wilcoxon p-value**
1	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Eisenbergiella	0.0904	0.0022	0.0055
2	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ventriosum	0.7137	0.1064	0.0081
3	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Xylanophilum group	0.1794	0.0493	0.0081
4	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Butyricicoccus	0.005	0.0822	0.0055

4. CONCLUSION

Working with human feces need stable methods to ensure the viability of the species and conserve them before taking them to the fermentation process. In this project, one of the most relevant aims is to see if freezing the samples before fermentation is worth it in order to work with them or is it better in fresh. Here is discussed if the results are altered after the fermentation once the freezing process is over.

The conclusions that have been reached after this study are:

- It is posible to employ 16S rRNA amplicon sequencing to examine gut microbiota oscillations in terms of taxonomy, composition, diversity and richness produced by the addition of food (bread) to a fecal inoculum in an *in vitro* fermentation assay following *in vitro* food digestion. Moreover, this result demonstrates that *in vitro* fermentation experiments can be used to analyze the effects of diverse foods on fecal microbial communities, in order to provide valuable information to design personalised diets that modulate an individual's gut microbiota.
- The samples fermented with bread display a richer and more diverse composition than the inocula samples without the fermentation and there is an increase in the phylum Firmicutes and a decrease in the phylum Proteobacteria.
- Freezing the fecal sample before *in vitro* fermentation assays has significative effects on the composition and diversity of the inoculum. There is a disturbance in the taxa due to the freezing treatment and the alpha diversity is reduced.
- Nevertheless, in spite of the effects of the freezing treatment, the microbial community in the frozen inocula is modified by the fermentation process in a similar manner to what is observed in fermentation assays with fresh samples.

In the end, despite losing bacterial species and altering the microbial composition, it is not so serious to freeze because the effect of fermentation among the samples remains significant in terms of richness and diversity.

FUTURE DIRECTIONS AND PRECISION NUTRITION

The gastrointestinal microbiome is an essential contributor to mammalian health that participates in vital physiological processes and guides host development. Abnormalities in gut microbial populations have been associated with a variety of gastrointestinal and systemic diseases, making the intestinal microbiome a viable diagnostic and therapeutic target. While recent advances in DNA sequencing and computational technology have revolutionized the field of microbiomics, many fundamental questions remain unanswered. Future directions for research should aim to elucidate the mechanisms underlying interactions between the microbiome and host, describe the process of microbiome maturation during host development

and its impact on early-life and adult health outcomes, clarify its role in the pathogenesis of disease states, and assess the viability of diagnostic tests and therapies designed to assess and treat conditions associated with intestinal alterations such as non-communicable diseases, including cancer and metabolic-related disease.

Not to mention, diet continues to be the most important determinant in shaping the composition, diversity and richness even throughout adulthood. In the last decade, the microbiome field has made tremendous strides in identifying a link between microbiome and diet. However, there are many technological limitations and challenges in meta-omic technologies, bioinformatics analyses and lack of experimental models which have created barriers for microbiome research. What is more, most microbiome clinical studies have been cross-sectional and performed with little or no attention to critical clinical metadata (host physiology, disease subtype stratification, age, race, demography, and other biological variables). Consequently, large amounts of observational data with limited mechanistic insights have been generated. Moreover, given the tremendous inter-individual variation in human gut microbial composition and function, it is unclear to what level these types of studies need to be powered in order to gain better insight.

The analysis of microbial community diversity is rapidly becoming a component of a vast array of different research programs, ranging from neurobiology to nutrition. Personalised nutrition strategies based on individual gut microbiome features are recently emerging and in a next future will allow developing new therapeutic or disease-preventive approaches based on a targeted modulation of gut microbiome through diet (Barko *et al*., 2018). However, the development of innovative technologies and sophisticated bioinformatics tools that integrate the data from both host and microbiome will be necessary to make precision nutrition through the microbiome a reality (Mills *et al.,* 2019).

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APENDIX

BREAD COMSPOSITION

Table 1. Bread composition in nutriets, values for 100g.

Nutrient	Unit	Value per 100 g	Vitamins			Lipids		
Proximates			Vitamin C, total ascorbic acid	mg	0	Fatty acids, total saturated	g	0,529
Water	g	33	Thiamin	mg	0,71	Fatty acids, total monounsaturated	g	0,362
Energy	kcal	272	Riboflavin	mg	0,427	14 1	g	0
Energy	kJ	1139	Niacin	mg	4,817	15 1	g	0
Protein	g	10,75	Pantothenic acid	mg	0,455	16 1 undifferentiated	g	0,011
Total lipid (fat)	g	2,42	Vitamin B-6	mg	0,107	16 1 c	g	0,011
Ash	g	1,96	Folate, total	µg	123	16 1 t	g	0
Carbohydrate, by difference	g	51,88	Folic acid	µg	67	17 1	g	0,001
Fiber, total dietary 1	g	2,2	Folate, food	µg	56	18 1 undifferentiated	g	0,344
Sugars, total	g	4,62	Folate, DFE	µg	170	18 1 c	g	0,34
Sucrose	g	0	Choline, total	mg	8	18 1 t	g	0,003
Glucose (dextrose) 1	g	0,4				20 1	g	0,006
Fructose	g	0,51	Vitamin B-12	µg	0	22 1 undifferentiated	g	0
Lactose	g	0	Vitamin B-12, added	µg	0	22 1 c	g	0
Maltose	g	3,7	Vitamin A, RAE	µg	0	22 1 t	g	0
Galactose	g	0	Retinol	µg	0	24 1 c	g	0
Starch	g	44,23	Carotene, beta	µg	0	Fatty acids, total polyunsaturated	g	0,855
Minerals			Carotene, alpha	µg	0	Fatty acids, total trans	g	0,005
Calcium, Ca	mg	52	Cryptoxanthin, beta	µg	0	Fatty acids, total trans-monoenoic	g	0,003
Iron, Fe	mg	3,91	Vitamin A, IU	IU	1	**Fiber**	mg	0
Magnesium, Mg	mg	32	Lycopene	µg	0	Soluble fiber		
Phosphorus, P	mg	105	Lutein + zeaxanthin	µg	45	Insoluble fiber	%	25
Potassium, K	mg	117	min E (alpha-tocophe	mg	0,21	Total	%	75
Sodium, Na	mg	602	Vitamin E, added	mg	0	Inulin	g	0
Zinc, Zn	mg	1,04	Tocopherol, beta	mg	0,03	Pectin	g	0
Copper, Cu	mg	0,152	Tocopherol, gamma	mg	0,28	Celulose	g	0
Manganese, Mn	mg	0,577	Tocopherol, delta	mg	0,07	Arabinoxilan	g	0,27
Selenium, Se	µg	28,6	Vitamin K (phylloquinone)	µg	0,7	Resistant starch	g	0,98