Human Gut Microbiota Modulation through Functional Ingredients and Foods

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Universitat Autònoma de Barcelona

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A ma mare i al meu pare.

LIST OF ABBREVIATONS

AC – Ascendent Colon

Ach – Acetylcholine

ADIPOQ – Adiponectin

AHA – American Heart Association

ALT – Alanine Aminotransferase

ApoAI – Apolipoprotein A-I

AST – Aspartate Aminotransferase

BLAST – Basic Local Alignment Search Tool

BMI – Body Mass Index

CDTI – Centre for Industrial Technological Development

CFU – Colony-Forming Units

CLR – Centered Log-Ratio

CSS – Cumulative Sum Scaling

CYS – Cysteine

DASH – Dietary Approaches to Stop Hypertension

DC – Descendent Colon

DCF – Dichlorofluorescein

DCFDA – Dichlorofluorescine Diacetate

D-CGD – Dynamic-Colonic Gastrointestinal Digester

ESI – Electrospray Ionized Source

ET-1 – Endothelin-1

EVOO – Extra Virgin Olive Oil

FDR – False Discovery Rate

FFAR – Free Fatty Acid Receptor

FFQ – Food Frequency Questionnaire

FID – Flame-Ionization Detector

LPS – Lipopolysaccharide

mAChR – Metabotropic Muscarinic

MCFA – Medium Chain Fatty Acid

MD – Mediterranean Diet

MeJA – Methyl Jasmonate

MetS – Metabolic Syndrome

MGB – 4-Methoxy-Glucobrassicin

MS – Metabolic Syndrome

MUFA – Monounsaturated Fatty Acid

NAC – N,acetylcysteine

NAFLD – Non-Alcoholic Fatty Liver Disease

NCBI – National Centre for Biotechnology Information

NEG – Negative

NGS – Next Generation Sequencing

NHLBI – National Heart, Lung, and Blood Institute

NMDS – Non-Metric Multidimensional Scaling

NGS – Next Generation Sequencing

NHLBI – National Heart, Lung, and Blood Institute

NMDS – Non-Metric Multidimensional Scaling

NO – Nitric Oxide

OTUs – Operational Taxonomic Units

PCA – Principal Component Analysis

PCOS – Polycystic Ovary Syndrome

PERMANOVA – Permutational Multivariate Analysis of Variance

PLS-DA – Partial Last Squares Regression

FOS – Fructooligosacharides

FU – Fluorometric Units

GAPDH – Glyceraldehyde-3-Phosphate Dehydrogenase

GB – Glucobrassicin

GC – Gas Chromatography

GOS – Galactooligosacharides

GSH – Gluthatione

GSLs – Glucosinolates

HBSS – Hank's Balanced Salt Solution

HDAC – Histone Deacetylase

HDL – High-Density Lipoprotein

HGB – 4-Hydroxy-Glucobrassicin

HITdb – Highly Scalable Relational Database

HPLC – High-Resolution Liquid Chromatogram

I3C – Indole-3-Carbinol

IBDs – Inflammatory Bowel Disease

IDF – International Diabetes Federation

IL – Interleukin

IUPAC – International Union of Pure and Applied Chemistry

KEGG – Kyoto Encyclopaedia of Genes and Genomes

PLSDA – Partial Least Squares-Discriminant analysis

POS – Positive

PUFA – Polyunsaturated Fatty Acid

ROS – Reactive Oxygen Species

rRNA – Ribosomal Ribonucleic Acid

RTC – Reverse Cholesterol Transport

SA – Salicylic Acid

SCFAs – Short Chain Fatty Acids

SD – Standard Deviation

SFN – Sulforaphane

SRA – Sequence Read Archive

TC – Total Cholesterol

TC – Transversal Colon

TOF – Time of Flight

UPARSE – Highly Accurate OUT Sequences from microbial amplicon reads

VIP – Value of Variance Importance in Projection

WC – Waist Circumference

WHO – World Health Organization

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ABSTRACT

The gastrointestinal tract processes around 60 tonnes of food in a lifetime. This, together with all the microorganisms that colonise it (gut microbiota), pose a threat on its integrity. Science, studies how to modulate the gut microbiota, through different foods or pharmaceutical products to induce a healthier microbiota profile. These modulatory tools are mainly prebiotics and probiotics, for having the potential to improve its composition and function. Another tool are the postbiotics, defined as metabolic by-products of probiotics, which do not require live bacteria to be administered to the host, being more stable. Among them, SCFAs are the most popular ones, having a wide range of health benefits. The consumption of prebiotics and probiotics can promote the production of SCFAs, highlighting the importance of a healthy and varied diet. The scope of this work lies on specific foods, rich in phytochemicals and fibres, and how, through gut microbiota modulation, may help to fight and prevent the onset of metabolic disorders and obesity. The results show increased levels of probiotics following an increased consumption of plan-based foods. Furthermore, a high intake of fibre promotes fibre-degrading bacteria, increasing the output of SCFAs. However, the effects of these increased bacteria vary depending on the specific species, strains, and individual contexts, therefore it is hard to get final conclusions for everyone.

INTRODUCTION

The BIOTAGUT project

This thesis comes from an industrial project called BIOTAGUT, formed by a consortium of seven Spanish food companies in collaboration with several research centres. The goal of the project was to design ingredients and foods, within the Mediterranean diet (MD), that have the potential to remodel the human gut microbiota towards a healthier status. These foods and ingredients might be used as preventive measures or even therapeutic tools against metabolic pathologies, which have a high prevalence in the Spanish population nowadays. The project starts with the identification of a "healthy microbiome profile" in the Spanish population, and the evaluation of specific MD food groups with modulatory effect on the microbiota, using in vitro and in vivo methods. The project also pursues to study the interactions between certain food components and specific bacterial species from the human gut microbiota, that produce postbiotic metabolites, with a potential healthy effect. This approach was the first step to a further development of new products (ingredients, prebiotics, probiotics and postbiotics) that can be used in the prevention or treatment of obesity and its comorbidities. The project allowed the participating companies to produce new foods, with the capacity to modulate the intestinal microbiome towards a healthier profile. The synergies produced between the companies, each focused on different types of food, allowed a better achievement of this objective. All the data generated during this process, together with the collaboration with the

consortium companies and the specialized research centres, allowed the elaboration of this thesis.

Human gut microbiota

The gastrointestinal tract constitutes the second largest body surface area, between 250 and 400 square meters, similar in size to a tennis court. In average, around 60 tonnes of food are processed through the gut in a lifetime. This, together with all the microorganisms that colonise it, pose a threat on its integrity [1]. Gut microorganisms are a mixture of bacteria, archaea, fungi, and viruses, commonly known as "the gut microbiota" (formerly called "intestinal flora") and has been evolving with the host for over thousands of years to form a symbiotic relationship [2]. However, when this fragile ecosystem is unbalanced, it may tip the balance between mutualism, commensalism, and parasitism [3], compromising the gut integrity, causing various gastrointestinal and extraintestinal diseases, including food allergies and intolerances that are increasingly common among the population nowadays [4]. It is now well established that the composition and diversity of gut microbiota play a crucial role in human health and disease. Factors such as diet, age, genetics, geography, and medication have a great influence [5]. In this thesis we will be focusing on the modulatory effect that diet can have on the microbiota and how this may be conditioning the overall health status. First, it is necessary to deepen in the

microbiota knowledge and in those diet components strong enough to have a modulatory effect that may help to shape it towards a healthier profile.

Among the organisms present in the gut microbiota, bacterial species are the most abundant, and the two dominant phyla are Firmicutes (Bacillota) and Bacteroidetes (Bacteroidota). Other phyla less abundant, including Actinobacteria (Actinomycetota), Proteobacteria (Pseudomonadota), and Verrucomicrobia (Verrucomicrobiota), play essential roles in the gut microbiota's overall function. The composition of the gut microbiota can vary significantly among individuals, but some of the most common bacterial genera in healthy individuals are *Bacteroides, Prevotella*, and *Faecalibacterium* [6]. Dysbiosis is a term used to describe an imbalance in gut microbiota composition and recent studies have shown that alterations in its bacterial composition are associated with various diseases, including metabolic disorders, autoimmune diseases, allergies, and even certain types of cancer [7–9]. For instance, a high Bacillota to Bacteroidota ratio has been previously associated with obesity, while a low ratio has been associated with better glucose metabolism [10]. The gut microbiota interacts with the host through various mechanisms, including nutrient absorption, production of short-chain fatty acids (SCFAs), modulation of the immune system, and regulation of gut motility. SCFAs, such as acetate, propionate, and butyrate, are produced by the fermentation of dietary fibre by the gut microbiota and play a crucial role in maintaining gut integrity,

modulating the immune system, and regulating gut motility. The gut microbiota also helps to maintain gut barrier function, preventing the entry of harmful pathogens into the bloodstream [11]. However, this delicate relation seems to be losing its balance, as intolerances, allergies and gastrointestinal discomfort are increasing among population. Current dietary habits and modern lifestyle seems to be threatening this symbiotic relation for several reasons.

Human genetics has barely changed since the appearance of the *Homo sapiens sapiens*, however industrial revolution together with the intensive agriculture and the development of food-processing techniques have occurred too fast to allow an evolutionary adaptation of the human-microbiota interaction, having important implications for health such as an impaired resistance to disease [12]. In western countries these changes in food habits have been more dramatic, following an increase of refined sugar consumption up to 45 kg per individual per year in the United States [13]; a 10-fold increase in sodium; 4-fold increase in saturated fat, doubling the cholesterol intake, together with a reduced consumption of vegetable fibres, known for having a hypo-cholesterolæmic effect, specially gels from fruit and vegetables, such as pectin and guar gum [14]. There is also a reduction of mineral intake, such as potassium, magnesium, calcium and chromium and a considerable reduction of omega-3 fats, membrane lipids, vitamins, and antioxidants. Therefore, it is not surprising to see an increase in more severe gut affections, such as inflammatory bowel diseases (IBDs) and cancer.

Furthermore, in contrast with ancient methods of food preservation through natural fermentation, enriching the food with a great among of the so-called probiotic bacteria, modern lifestyle has dramatically reduced the number of bacteria present in the food, through extensive hygiene measures and sterile environments for the commercially manufactured food. Nowadays, bacteria are generally regarded as a source of disease, an unwanted organism. This extreme hygiene measures surrounding our lifestyle cause a lame development of the protective gut flora, which could relate to the increased incidence of food intolerances, allergies and infections seen in Western children [15, 16]. For this reason, science is studying how to enrich this impoverished microbiota, through different foods or pharmaceutical products that can provide prebiotics and probiotics to help induce a healthier microbiota profile. From among this microbiota enhancers (prebiotics and probiotics), there is a less known element, which is attracting more and more attention, the postbiotics.

Gut microbiota: prebiotics vs probiotics

Prebiotics and probiotics are often confused, but they are distinct entities with different functions. Prebiotics are non-digestible food ingredients that selectively stimulate the growth and activity of beneficial microorganisms in the gut. Prebiotics include fructo-oligosaccharides (FOS), inulin, galacto-oligosaccharides (GOS), and resistant starch, among others [17]. Probiotics, on the other hand, are live microorganisms that when consumed in adequate amounts, confer a health

benefit on the host [18], including species of the genera *Lactobacillus* and *Bifidobacterium*, among others.

Prebiotics and Probiotics effect

Prebiotics have been shown to modulate the composition and activity of the gut microbiota [19]. A study by Bindels et al. (2015) showed that inulin-type fructans, a type of prebiotic, increased the abundance of Bifidobacteria and butyrate-producing bacteria in the gut, leading to improvements in gut barrier function and reducing inflammation [20]. Similarly, a study by Vandeputte et al. (2017) showed that prebiotic supplementation increased the abundance of beneficial bacteria in the gut, leading to improvements in gut health and metabolic function [21].

Probiotics have also been shown to modulate the gut microbiota, with some species associated with improved gut health. For example, Bifidobacteria and Lactobacilli have been shown to improve gut barrier function and reduce inflammation, among other benefits [22]. A study by Kim et al. (2018) showed that probiotic supplementation with a mixture of Bifidobacteria and Lactobacilli improved gut barrier function and reduced gut inflammation in patients with IBD [23]. However, not all probiotic species confer health benefits, and some may even have negative effects on the gut microbiota [24].

In addition to these two well-known genera, there are other bacterial species that have been studied for their potential probiotic properties. Here are some examples:

- *Streptococcus thermophilus*: this bacterial species is commonly used in the production of fermented dairy products and has been shown to have beneficial effects on gut health. *S. thermophilus* can improve lactose digestion, reduce inflammation, and enhance immune function [25].
- *Bacillus coagulans*: a spore-forming bacterium with beneficial effects on digestive health, including improving symptoms of irritable bowel syndrome and reducing inflammation [26].
- *Escherichia coli Nissle 1917*: this strain of *E. coli* has probiotic properties, including the ability to improve symptoms of ulcerative colitis and reduce inflammation [27].
- *Enterococcus faecium*: it enhances immune function and reduce inflammation [28].
- *Lactococcus lactis*: species commonly used in the production of fermented dairy products. It enhances immune function and reduces inflammation [29].

It is worth noting that not all strains of these bacterial species have probiotic properties, and that the efficacy of probiotics is strain specific. Therefore, it is important to carefully select and evaluate the specific strains of bacteria used in probiotic formulations.

Postbiotic: The Future of Probiotics

Probiotics have been extensively studied for their potential to improve gut microbiota composition and function, however, they are not without limitations, such as poor survival rates during transit through the gastrointestinal tract and limited efficacy in certain populations. For this reason, in the last years the concept of postbiotics has emerged. On one hand, they are officially defined as a preparation of inanimate microorganisms and/or their components that confers a health benefit to the host [30]. One example is *Akkermansia muciniphila*, that has beneficial effects mediated by its outer membrane protein Amuc_1100 [31] or its secreted protein P9 that stimulates GLP-1 secretion [32]. On the other hand, postbiotics, have been defined as metabolic by-products of probiotics. Defined as "bioactive compounds produced by probiotic bacteria during the fermentation of dietary substrates"[33]; could be SCFA, antimicrobial peptides, and vitamins, among others, being the SCFA the most popular ones. Unlike probiotics, postbiotics do not require live bacteria to be administered to the host. This feature allows them to be more stable and have a longer shelf life. Postbiotics are also resistant to gastrointestinal conditions, making them more effective in delivering health benefits to the host [34]. They have a range of health benefits, including:

- **Improved gut health:** improve gut microbiota composition and function, as well as alleviate symptoms of gut disorders such as irritable bowel syndrome and IBD [35]. For example, it is reported that *Bifidobacterium* and *Clostridium*

residing in the gastrointestinal tract convert the nutritional fibers into SCFAs that provide 10% of the body energy [36]. In addition, in patients with Crohn disease the removal of carbohydrates from the nutrition schedule improved the disease outcome, suggesting a role for microbial fermentation in the pathogenesis of the disease [37].

- **Enhanced immune function:** modulate immune function by promoting the growth of beneficial gut bacteria and reducing inflammation [38].
- **Anti-cancer properties:** some postbiotics, such as butyrate, have been shown to have anti-cancer properties, potentially by inhibiting the growth of cancer cells and promoting apoptosis [39].
- **Metabolic benefits:** improve metabolic function, including glucose regulation and lipid metabolism [40]. For example, a study with mice, reported that dietary intake of the three major SCFAs, acetate, propionate, and butyrate, protected against high fat diet-induced obesity, and improved hepatic metabolic conditions via FFAR3 (free fatty acid receptor), demonstrating that SCFAs have anti-obesity effects and could be used to improve metabolic conditions [41].
- **Skin health:** improve skin health by reducing inflammation and enhancing the skin barrier function [42].

Despite their promising potential, research in this field is still in its early stages. Many studies have been conducted in vitro or in animal models, but there is a

lack of human clinical trials to support their efficacy. Furthermore, there is currently no standardized method for the production or identification of postbiotics, which makes it challenging to compare and reproduce study findings [43].

SCFA as postbiotic metabolites

SCFA are organic acids with a carbon chain length of one to six carbons. As mentioned before, the main ones in the human gut are acetic acid (two-carbons), propionic acid (three-carbons), butyric acid (four-carbons) and valeric acid (five-carbons) [44] and they are produced by gut bacteria through the fermentation of non-digestible carbohydrates and fibres such as resistant starch, inulin, and oligosaccharides [45]. The main bacterial species involved in the production of SCFAs are in the Bacillota and Bacteroidota phyla [46]. These organic acids act as signalling molecules in the gut, regulating various physiological functions [47], promote gut motility, stimulate the release of gut hormones, and improve insulin sensitivity [48, 49]. SCFAs have also been shown to play a vital role in the maintenance of gut health by promoting the growth of beneficial gut bacteria and inhibiting the growth of pathogenic bacteria [50]. Their increased production has been associated with a reduction in the risk of various gastrointestinal diseases, including IBDs and colorectal cancer [51, 52]. Furthermore, SCFAs have been shown to have anti-inflammatory effects in the gut, reducing the production of pro-inflammatory cytokines and improving gut barrier function [53]. These anti-

inflammatory properties have also been linked to improvements in other inflammatory conditions such as asthma and allergies [54]. The consumption of dietary fibres and probiotics can promote the production of SCFAs in the gut, highlighting the importance of a healthy and varied diet.

Diet and metabolic health

Diet plays a critical role in maintaining overall health and well-being. Numerous studies have emphasized the significance of dietary patterns in the prevention and management of chronic diseases, such as cardiovascular disease, obesity, and diabetes [55–57]. Probably the most popular and the one that best represents a healthy and well-balanced diet is the Mediterranean diet, characterized by high intake of fruits, vegetables, whole grains, legumes, fish, and olive oil, consistently associated with a reduced risk of cardiovascular disease [58]. In a review from 2020, the MD is associated with a reduction of all-cause mortality. In particular, the microbiota of subjects with a Mediterranean-type diet is significantly different from that of subjects with a Western food model. The Mediterranean microbiota would produce more SCFAs that should be able to contribute on the reduction of the risk of both cardiovascular and some tumour pathologies. The review explored the modulation of the human microbiota, in response to MD adherence, focusing the attention on polyphenols, polyunsaturated fatty acids (PUFA) ω-3 and fibre [59]. Another review from 2021 concluded that MD can modify the gut microbiota in a way that is beneficial to host health, through the

increases in the relative abundance of SCFA-producing bacteria that are considered to possess anti-inflammatory properties [60]. However, this dietary pattern does not consistently alter microbiota composition or metabolism due to the heterogeneity between study populations, analysis methods, duration and characterization of MD and the limited approaches that have been used to characterize the complex ecosystem that constitutes the large bowel microbiome. Further, given the differential effects of individual components of the MD on the gut microbiota, there is a need for a standardization of the composition of experimental MD interventions and of the scoring methods used to assess MD adherence in observational studies [61]. Even thou there are many recognized healthy effects of the MD, this last study evidences the importance a correct contextualization and specification of the interventions, as "good for all" recommendations often do not apply to individuals. However, there are other similar diets, such as the Dietary Approaches to Stop Hypertension (DASH), that also emphasizes fruits, vegetables, whole grains, lean proteins, and low-fat dairy products [62], showing a similar pattern in the beneficial food groups. There are dietary factors that directly relate with specific diseases and conditions. For example, increased consumption of saturated fats, trans fats, and dietary cholesterol has been linked to an elevated risk of coronary heart disease [63]. Conversely, diets rich in omega-3 fatty acids, found in fatty fish, nuts, and seeds, have a protective effect against this condition [64]. A high intake of dietary fibre, particularly from whole grains, fruits, and vegetables, is related with a lower risk

of developing cardiovascular disease [65]. Furthermore, dietary choices have a significantly influence on body weight and the development of obesity. High consumption of energy-dense foods, such as sugary beverages and processed snacks, is positively associated with weight gain and obesity [66]. In contrast, diets rich in lean protein, whole grains, and high-fibre foods promote weight loss and weight maintenance [67].

Despite the concept of diet representing a wide range of different food groups and its proper combination, proportion and distribution, food nutrients have been extensively studied individually for their impact on health outcomes. For instance, calcium and vitamin D intake reduce risk of osteoporosis and fractures [68]. Adequate vitamin C intake is crucial for the prevention of scurvy and maintaining optimal immunity [69], as well as vitamin E and other antioxidants such as polyphenols, found in fruits and vegetables are linked to a lower risk of chronic diseases, including cancer [70].

Modulatory effect of diet on gut microbiota

One important aspect of diet affecting the gut microbiota is the type of macronutrients consumed. For example, the intake of dietary fibre, is strongly associated with increased microbial diversity and the promotion of beneficial bacteria such as Bifidobacteria and Lactobacilli [71, 72]. Conversely, a low-fibre diet, common in Western diets rich in processed foods, prompts a reduction in microbial diversity and an imbalance in the gut microbial composition [73]. In addition to fibre, dietary fat composition also influences the gut microbiota.

Studies have demonstrated that a high-fat diet, particularly one high in saturated fats, can lead to alterations in the gut microbial community, favouring the growth of bacteria associated with inflammation and metabolic disorders [74, 75]. On the other hand, diets rich in unsaturated fats, have been associated with a more diverse and beneficial gut microbiota [76, 77]. Furthermore, the impact of protein intake on the gut microbiota has also been investigated: high-protein diets, especially those rich in animal protein, are associated with a decrease in microbial diversity and an increase in bacteria that produce potentially harmful metabolites [78]. In contrast, plant-based protein sources, such as legumes and soy products, favour the gut microbial profile [79]. There are other bioactive compounds present in foods, such as polyphenols, with the capacity to modulate the gut microbiota, acting as prebiotics or directly influence the growth and activity of specific bacteria in the gut, promoting a more diverse and beneficial microbial community [80].

It is important to keep in mind that individual responses to diet may vary due to other factors (age, gender, genetics, physical activity, geographical location, etc.). Additionally, the gut microbiota is a dynamic ecosystem, and dietary changes may take time to exert significant effects. By understanding the impact of diet on the gut microbiota, we can potentially harness its therapeutic potential for improving health and preventing various diseases. Here are a few more popular bacterial genera and species that have been shown to be influenced by diet:

- *Akkermansia muciniphila*: a mucin-degrading bacterium associated with a healthy gut. Its abundance is linked with dietary factors, including high-fibre diets and polyphenol rich foods, associated with improved metabolic health [81].
- *Blautia:* a genus with probiotic characteristics capable of degrade dietary components such as cellulose and xylan. Some species in the genera *Clostridium* and *Ruminococcus* have been reclassified as *Blautia*, which has gathered recent interest for its ability to regulate host health and alleviate metabolic syndrome [82].
- *Clostridium* clusters IV and XIVa are groups of bacteria that include multiple species involved in the metabolism of dietary fibre and the production of beneficial metabolites, such as butyrate. Although *Clostrodium* are generally perceived as unwanted bacteria, in this case, they have a positive effect. A decrease in these clusters is associated with loss of gut microbiome colonization resistance (reduced diversity and community stability over time) [83].
- *Collinsella:* Its abundance is positively correlated with a proinflammatory effect and a lower gut microbiome diversity. It is more abundant in individuals following a Western-style diet rich in animal products and saturated fats [84].
- *Eubacterium*: a diverse genus of bacteria that includes several species (*Eubacterium rectale* and *Eubacterium hallii*) involved in the fermentation of

dietary fibres and the production of beneficial metabolites, such as butyrate [85]. This genus is also capable to use the subproducts of other bacteria to produce postbiotics (SCFAs) [86].

- *Faecalibacterium prausnitzii:* another butyrate-producing bacterium, also conditioned by fibre intake [87].
- *Prevotella*: a genus associated with a plant-based diet for being a fibre fermenter, producing mainly propionate [88].
- *Roseburia*: a genus including butyrate-producing bacteria, associated with diets rich in fibre and plant-based foods [87].
- *Ruminococcus*: a genus that contributes to starch degradation. Again, associated with fibre-rich diets, producing sub-products used by other beneficial bacteria, like *Eubacterium* [89].

This is a short summary of popular bacteria and the effect they have on human gut microbiota. This thesis is focused on specific foods rich in phytochemicals and fibres, and how may they help to fight obesity and its comorbidities through gut microbiota modulation.

Food compounds: where to put the focus on?

Dietary fibre

Dietary fibre is a crucial component of a healthy diet and is a prebiotic, related with bacterial growth and with SCFAs production. Fibre is defined as the non-

digestible portion of plants and can be classified into two types: soluble and insoluble. Both types play different roles in promoting gut health:

Soluble fibre dissolves in water to form a gel-like substance. It is found in foods such as oats, barley, fruits, and vegetables, regulating blood sugar levels and reducing cholesterol absorption by binding within the gut, preventing it from being absorbed into the bloodstream [90]. This leads to lower cholesterol levels and a reduced risk of heart disease. It also promotes the growth of beneficial gut bacteria, helping digestion and boosting the immune system [91].

Insoluble fibre does not dissolve in water and provides bulk to the stool. It is found in foods such as whole grains, nuts, and seeds and promotes regular bowel movements, preventing constipation [92]. This bulking effect speeds up transit time through the gut and helps to eliminate waste products from the body, reducing the risk of colorectal cancer [93]. It also helps prevent diverticular disease, a condition where small pouches form in the colon [94]. A study with olive pomace, mostly composed by insoluble fibre, showed to confer health benefits to the gastrointestinal tract as promoter of SCFAs production by gut microbiota, in a higher degree than FOS, showing a strong prebiotic effect [95]

Dietary fibre exerts a global anti-inflammatory effect in the gut which is a key factor in preventing the development of several chronic diseases, including IBDs.

Polyphenols

Polyphenols are a class of bioactive compounds widely distributed in plant-based foods, such as fruits, vegetables, cereals, and beverages like tea, coffee, and wine. They are characterized by their complex chemical structure, which includes one or more phenolic rings and may be conjugated to other functional groups like sugars, acids, or lipids. Polyphenols are known for their antioxidant, anti-inflammatory, and antimicrobial properties, which make them potential candidates for preventing or treating various chronic diseases, including cancer, cardiovascular disease, diabetes, and neurodegenerative disorders [96].

Recent research highlights the role of polyphenols in modulating the gut microbiota and improving gut health, through several mechanisms such the promotion of beneficial bacteria, inhibiting the growth of pathogenic ones, and modulating the production of microbial metabolites. For example, some polyphenols like quercetin, resveratrol, and catechins have been shown to stimulate the growth of *Lactobacillus* and *Bifidobacterium*, while inhibiting the growth of harmful bacteria such as *Escherichia coli* and *Clostridium perfringens* [97]. Polyphenols can also act as prebiotics, providing substrates for the growth of beneficial bacteria and promoting the production of SCFAs [98]. The beneficial effects of polyphenols on gut health have been demonstrated in several animal and human studies. A randomized controlled trial involving overweight and obese individuals found that supplementation with a polyphenol-rich extract

from grape and blueberry improved markers of gut health, including faecal microbial diversity and the abundance of beneficial bacteria [99]. Similarly, a study in rats showed that dietary supplementation with green tea polyphenols improved gut barrier function and reduced inflammation in the colon [100]. The mechanisms by which polyphenols exert their effects in the gut and which is the optimal dose, duration, and food sources are not well established yet.

Glucosinolates

Glucosinolates are peculiar of vegetables belonging to Brassicaceae family (present also in few other species) used for human consumption. This includes crops cultivated as vegetables, spices, and sources of oil. These organic compounds play a pivotal role in plant defence mechanisms, acting as key components in deterring herbivores and pathogens. Structurally, glucosinolates are β-thioglucoside N-hydroxysulfates, characterized by a variable side chain derived from an amino acid, most commonly methionine, phenylalanine, or tryptophan [101]. The type and concentration of glucosinolates in food are highly variable depending on several factors, such as genetics, cultivation site, cultivar, growth conditions, developmental stage, plant tissue, post-harvest handling, and food preparation methods. As types and concentration are also the main determinant of their biological activities, estimates of their content in food are essential tool to understand if a certain diet is adequate to deliver qualitatively and quantitatively appropriate glucosinolates and isothiocyanate [102].

Numerous studies have highlighted their chemo-preventive properties, indicating a role in reducing the risk of cancer [103]. Additionally, glucosinolate-derived compounds have demonstrated antioxidant and anti-inflammatory activities [104].

In the gut, glucosinolates encounter the resident gut microbiota, which possesses a repertoire of enzymes capable of metabolizing them through hydrolysis. This enzymatic action leads to the formation of various breakdown products, such as isothiocyanates, nitriles, and epithionitriles, which can be absorbed through the intestinal epithelium and enter the bloodstream [105]. The conversion of glucosinolates by the gut microbiota is a double-edged sword. On the one hand, it can enhance the bioavailability of bioactive glucosinolate breakdown products, which contribute to their beneficial effects. On the other hand, the gut microbiota can also degrade and modify glucosinolates into less bioactive or even harmful compounds [106][107]. Therefore, the interplay between glucosinolates, the gut microbiota, and the resulting metabolites is crucial in determining the overall health effects.

Obesity and comorbidities

Obesity is defined as an abnormal or excessive accumulation of adipose tissue generally attributed to a positive energy balance maintained over the time and accompanied by a sedentary lifestyle [108]. It is a complex and multifactorial condition characterized by excessive accumulation of body fat. It has become a

global epidemic with significant public health implications. This condition is associated with numerous comorbidities, which are additional medical conditions that frequently occur alongside obesity. Understanding these comorbidities is crucial for effective prevention and management of obesity. Furthermore, many of these conditions may occur simultaneously and this is known as metabolic syndrome (MS), which increase the risk of cardiovascular diseases, type 2 diabetes, and stroke. It is characterized by a combination of visceral obesity, dyslipidaemia, hyperglycaemia, and hypertension [109]. This syndrome is considered a significant public health concern due to its increasing prevalence worldwide [110, 111]. The diagnosis of MS is based on a set of criteria established by different organizations, including the World Health Organization (WHO), the International Diabetes Federation (IDF), the American Heart Association (AHA), and the National Heart, Lung, and Blood Institute (NHLBI) [112, 113]. In 2011, a consensus definition was approved by which any patient can be diagnosed with MS when three of the following criteria are present [114, 115]:

1. **Abdominal obesity**: High waist circumference (WC), whose thresholds depend on country-specific populations and definitions (≥102 cm and ≥88 cm for European men and women, respectively) [116].
2. **Blood triglycerides:** blood triglyceride level ≥ 150 mg/dl (1.7 mmol/L).
3. **Elevated blood pressure**: Blood pressure levels equal to or greater than 130/85 mmHg, or receiving treatment for hypertension.

4. **High blood sugar levels**: Fasting blood glucose levels equal to or greater than 100 mg/dL (5.6 mmol/L) or being diagnosed with type 2 diabetes.
5. **Abnormal cholesterol levels**: This includes low levels of high-density lipoprotein (HDL) cholesterol (< than 40 mg/dL (1.03 mmol/L) in men and < than 50 mg/dL (1.29 mmol/L) in women)).

Several factors contribute to the development of MS, including genetics, physical inactivity, unhealthy diet, and obesity [111]. The exact mechanisms underlying MS are complex and not fully understood. However, insulin resistance is considered a key factor in its pathogenesis, leading to a reduced ability of cells to respond to insulin and properly regulate blood sugar levels, resulting in elevated glucose levels and compensatory insulin secretion. This can eventually lead to the development of type 2 diabetes [110].

MS significantly increases the risk of cardiovascular diseases, such as coronary artery disease and stroke [117]. It is also associated with a higher incidence of non-alcoholic fatty liver disease (NAFLD) and polycystic ovary syndrome (PCOS) in women. Furthermore, individuals with MS are more prone to chronic inflammation and oxidative stress, which further contribute to the development and progression of cardiovascular complications. Prevention and management involve lifestyle modifications, including regular physical activity, a healthy diet (such as the Mediterranean or DASH diet), weight loss, and smoking cessation. In some cases, medication may be prescribed to control individual components

of the syndrome, such as antihypertensive drugs, lipid-lowering agents, or medications to manage blood sugar levels [118].

Obesity and microbiota

It seems clear that diet is the corner stone for a healthy microbiota, therefore, those who are over-weight or obese due to an unbalanced diet, will probably have an obesity-related dysbiosis, referring to an imbalance in the composition and function of the gut microbiota. Several studies have investigated the specific bacterial species and genera that are affected by this and how it may impact health. While it is challenging to pinpoint a definitive list of the most affected beneficial bacteria, as research in this field is ongoing, there are some notable findings based on literature. For example, a study where the number of gut microbial genes was compared, showed that individuals with higher overall adiposity, more insulin resistance, dyslipidaemia, and a more marked inflammatory status (overweight and obese subjects) had a lower bacterial richness when compared with lean subjects. The obese individuals belonging to the lower bacterial richness group also gained more weight over time [119]. This correlation between bacterial species and metabolic markers, suggests that a decrease in microbial diversity may be directly associated with metabolic disorders such as obesity. Another research, focused on comparing the gut microbiota composition of obese individuals before and after gastric bypass

surgery, discovered a significant alteration in the gut microbiota following the surgery, confirming again the potential role of the microbiota in obesity [120].

If we focus on specific bacterial genera, a study by Turnbaugh et al. (2009) found that the relative abundance of the bacterial genus *Bacteroides* was reduced in individuals with obesity compared to lean individuals. *Bacteroides* species, such as *Bacteroides thetaiotaomicron* and *Bacteroides fragilis*, are known to play a beneficial role in the gut by promoting the breakdown of complex carbohydrates and producing SCFAs. The reduction of *Bacteroides* species in obesity-related dysbiosis may, therefore, have implications for nutrient metabolism and energy homeostasis [121]. Results from species level must be considered cautiously because this taxonomic level is much more susceptible to external elements, and conclusions can be hard to interpret. For example, Million et al (2012) explored the gut microbiota of obese individuals and identified differences in bacterial populations compared to non-obese controls. Specifically, they found an enrichment of *Lactobacillus (Limosilactobacillus) reuteri* and depletion of *Bifidobacterium animalis* and *Methanobrevibacter smithii* in the obese group. This was the first study to date that links specific species of *Lactobacillus* with obesity in humans [122], which is surprising, as *Lactobacillus* has always been considered a healthy probiotic. However, the *Bifidobacterium animalis* depletion makes much more sense, as the *Bifidobacterium* genus (from the Actinomycetota phylum) has been seen to play a key role in weight regulation (80). The subspecies *Lactis*

Gcl2508 is a probiotic capable of proliferating and producing SCFAs (81). Additionally, the *Lactis* subspecies may have an anti-MS effect. A study with mice has shown that treatment with this probiotic improves energy expenditure by reducing levels of body fat (82). *Methanobrevibacter smithii,* the dominant archaeon in the human gut ecosystem, is known for affecting the specificity and efficiency of bacterial digestion of dietary polysaccharides, thereby influencing host calorie harvest and adiposity. Its depletion is consistent with its role, and supplementation with this species has been approached as a therapeutic tool for reducing energy harvest in obese humans [123]. The number of known species in the human gut is outrageous, so it is impossible to mention all the bacteria implicated in the regulation of a healthy microbiota in obesity, as there are more than one healthy enterotype, with different kind of bacteria confirming it.

As previously explained, an imbalance in the Bacillota-Bacteroidota ratio, towards the Bacillota, may occur in obesity. This phylum-level change in the microbiota reduces bacterial diversity and alters the representation of bacterial genes and metabolic pathways [124]. However, even though an unbalanced Bacillota-Bacteroidota ratio is not wanted, there are many species among the Bacillota known for its healthy effects: *Faecalibacterium* and *Roseburia* are both SCFAs producers and have been associated with beneficial effects on host metabolism and inflammation [125]. Therefore, it is essential to maintain the balance, as too many Bacteroidota will also impair microbiota diversity, with detrimental effects. On the pathological side, certain obesity-related bacterial taxa

may contribute to metabolic dysfunction. For instance, the genus *Clostridium* has been found to be enriched in individuals with obesity [126]. Some species within this genus, such as *Clostridium difficile*, are known to be opportunistic pathogens associated with various diseases, including diarrhoea and colitis. While the role of specific *Clostridium* species in obesity-related dysbiosis is not yet fully understood, their increased abundance may be linked to gut inflammation and metabolic disturbances. It is important to note that the dysbiosis associated with obesity is a complex phenomenon influenced by multiple factors, including diet, genetics, and lifestyle. The specific bacteria affected and their functional implications may vary between individuals. Bacteria from the *Clostridium* genus are part of the normal gut composition but are associated with deleterious effect on human health due to some species involved with intestinal diseases [127]. However, there are other species from this genus, associated with the production of SCFA, known to have a positive impact on gut health [11]. Research in this field is rapidly evolving, and new findings may provide further insights into the specific genera and species affected.

If we widen our scope and move to a broader perspective, even though the microbiota composition of individuals with obesity is not known yet, we can confirm that there is a straight relation. As an example, a study showed that germ-free mice inoculated with microbiota from obese or lean human twins, take on the microbiota characteristics of the donor. Those receiving the obese microbiota (red outline) had an increase in adiposity, whereas those receiving the lean microbiota (blue outline) remained lean [128], figure 1.

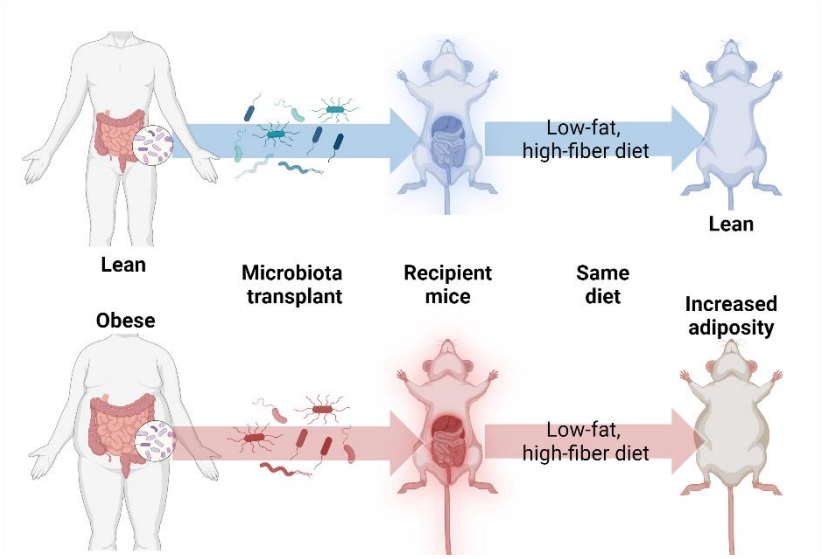


Figure 1. Microbiota transplant from human to mice, and inheritance characteristics.

However, a ternary classification of microbes as pathogens, commensals or beneficial does not exist, as the pathogenicity of a given microorganism is highly dependent on multiple variables, not only related to the bacteria itself but also related to the host. This remark is not restricted to the context of obesity as microbiota ecological principles also apply to other non-communicable diseases. That being said, specific gut microbes have been identified for which causality and effect have been demonstrated often enough, such that they can now be considered beneficial for host health. A search performed on August 2019 in the

databases Pubmed, Scopus, Web of Science, Cochrane library, Lilacs and gray literature using the terms: “microbiota”, “microbiome”, “obesity”, “obesity morbid”, and “humans”, including studies assessing the gut microbiota composition in adults with obesity and lean individuals, concluded that individuals with obesity may show a greater Bacillota/Bacteroidota ratio, Bacillota, Fusobacteria (Fusobacteriota), Pseudomonadota, Mollicutes, *Limosilactobacillus reuteri,* and less Verrucomicrobiota (*Akkermansia muciniphila*), *Faecalibacterium prausnitzii,* Bacteroidota, *Methanobrevibacter smithii*, *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei*. Furthermore, as indicated previously, some bacteria had a clear positive correlation and others have negative correlation with obesity. This study also confirmed that obese individuals have a different gut microbiota profile than lean ones [129]. Further bacterial species have been considered health-promoting by recent literature, such as *Akkermansia muciniphila* [130] and *Faecalibacterium prausnitzii* [131] as promising preventive and therapeutic tools in immune-related diseases and cancer immunotherapy. Others are only considered potential next-generation beneficial bacteria in certain specific conditions such as *Eubacterium hallii* (renamed as *Anaerobutyricum soehngenii*) [132]. There are many other potential beneficial candidates, such as such as *Odoribacter laneus* [133], *Holdemanella biformis* [134], and *Bacteroides uniformis* [135], that have demonstrated beneficial effects on specific conditions like obesity and inflammatory processes like colitis. However, the latest publications still have no proof of a specific microbial culprit

in the onset of obesity or the failure to resolve overweight. However, without diminishing the role of heredity and environmental factors, the gut microbiota clearly makes an important contribution to the development of metabolic disorders and obesity. Maintaining homeostasis and normal metabolism is impossible without restoring the diversity and normal functions of the gut microbiota. Dietary interventions, prebiotics, probiotics, and medication can be useful to achieve this aim [136].

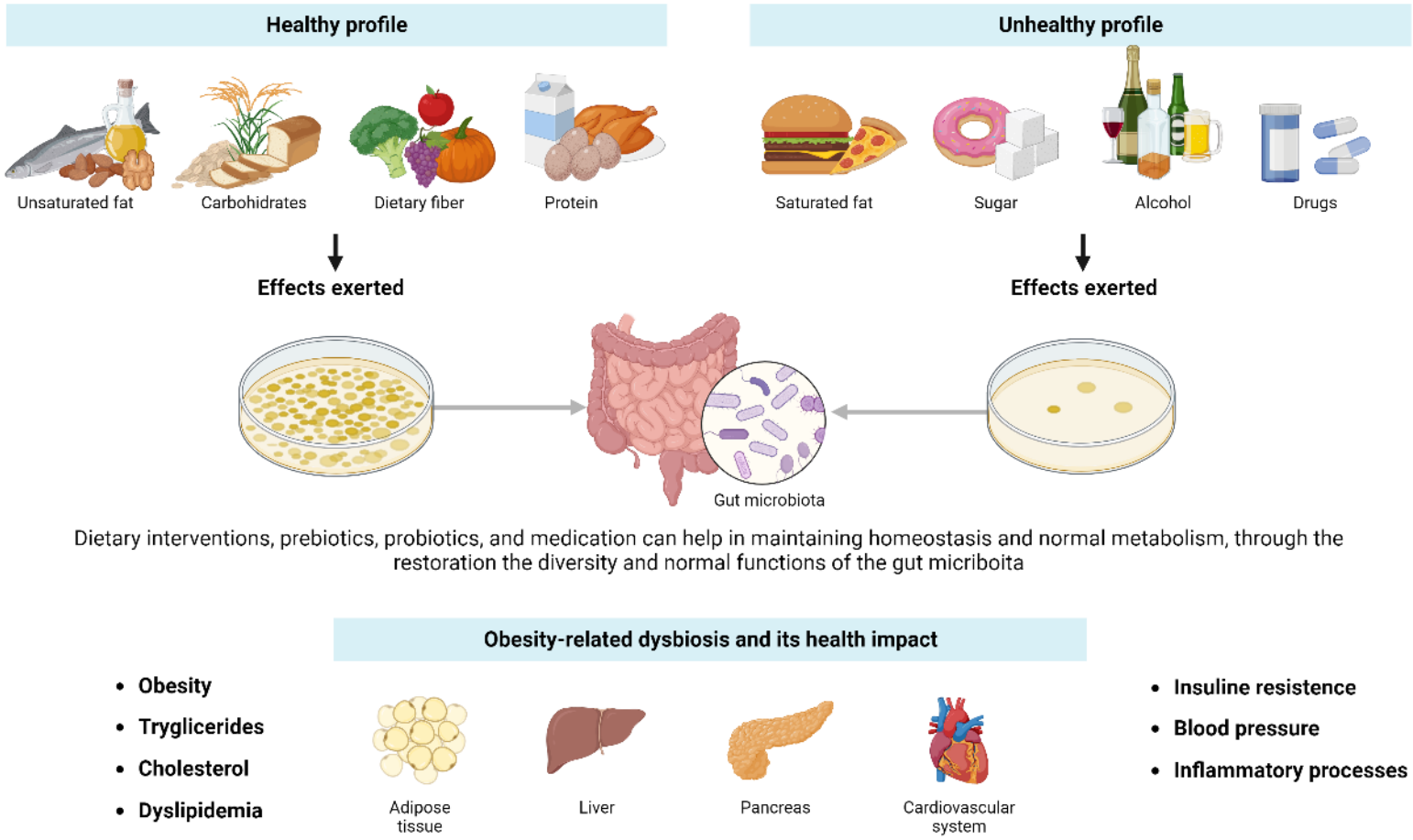


Figure 2. Factors influencing the gut microbiota profile and related health issues.

HYPOTHESIS & AIMS

Hypothesis

Foods and bioactive compounds can modulate gut microbiota composition and function. Some of these foods would be able to revert the obesity-related dysbiosis. This can be studied by different approaches, like in vitro simulators of the gastrointestinal tract or human intervention studies.

Objectives

The present thesis has the three following objectives:

1. To describe the gut microbiota profile associated with Mediterranean diet and specific food groups in Spanish population.
2. To establish an In vitro digester simulator as an approach for the study of the effects of foods on gut microbiota.
3. To study the modulatory effects of different functional foods on human gut microbiota by combining in vitro and in vivo approaches.

METHODS

These is the workplan and the methodology of the BIOTAGUT PROJECT which has allowed the development of this thesis through the following points.

1. Ingredient characterization/definition

    Ingredients and bioactive components bibliography revision

    Food products and ingredient specifications

    Market and regulatory aspects surveillance

2. Microbial identification

    Definition of the microbiota "TYPE": healthy vs metabolic syndrome

3. Food and ingredients design and development

    Ingredients design and development

    Food design and development

4. In-vitro colonic fermentation evaluation

    Determination of the colonic microbiota responses

    Study of the biological effect in-vitro on metabolic syndrome-related features

5. Investigation of possibles probiotic and postbiotics

    Microorganisms culture and isolation

    Probiotic aptitude characterization

    Response quantification to colonic permeability/plasma in relation to the metabolic syndrome

6. Human Trials

    Nutritional intervention with new functional foods, focused on microbiota modulation

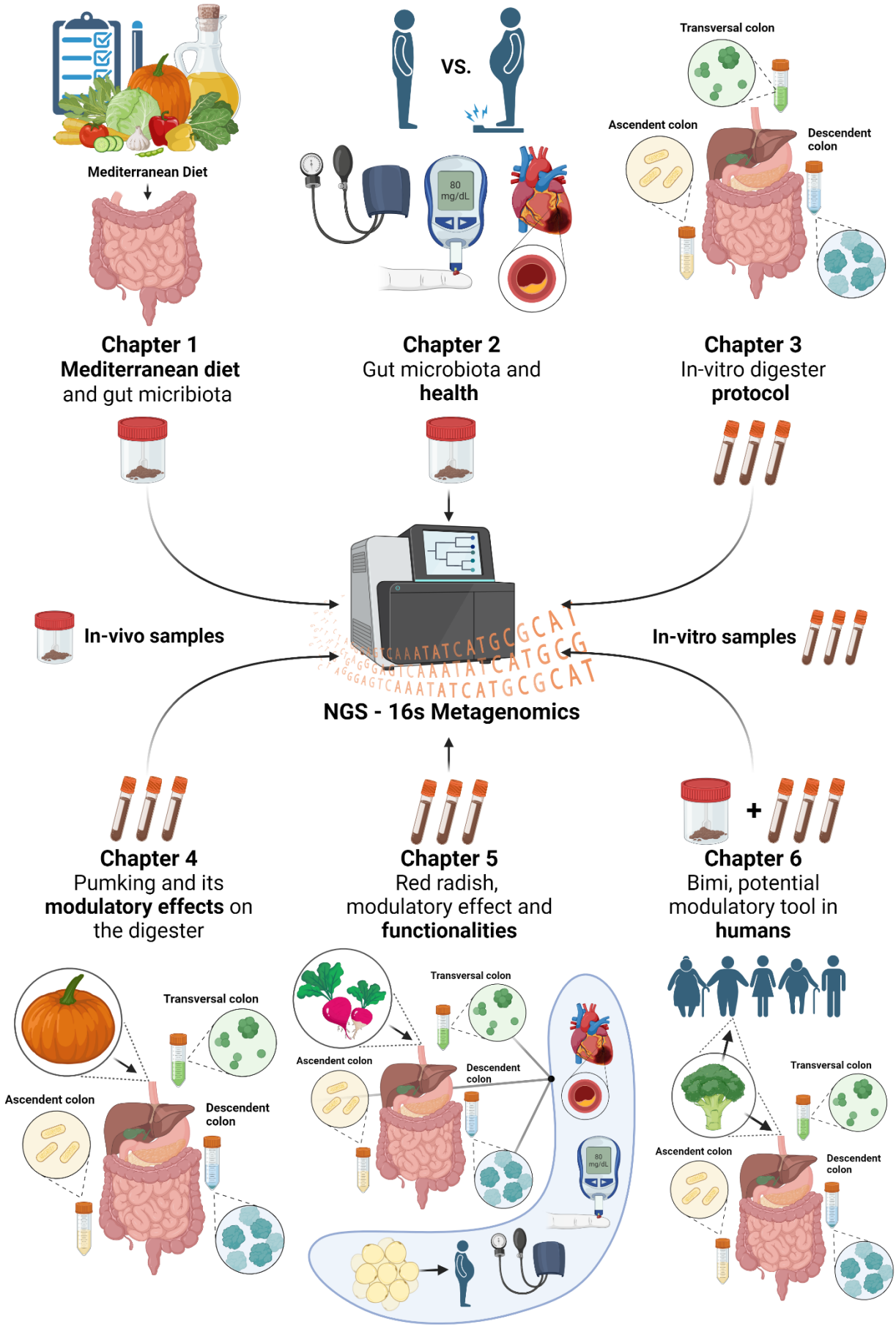


Figure 3. Graphic representation, connecting the workflow of the 6 chapters of this thesis.

In the following paragraphs, there is a short explanation of the main process performed on this thesis. All this methodology is deeply detailed in the corresponding chapters.

Description of the Dynamic gastrointestinal and colonic fermentation model

The equipment simulates in vitro the entire gastrointestinal digestive process. It consists of a computer-assisted model of five interconnected compartments, double jacket vessels, that simulate the physiological conditions of the stomach (R1), small intestine (R2) and the three colonic sections: the ascending colon (R3), transverse colon (R4) and descending colon (R5). R1 and R2 work semi-continuously, while the colon reactors (R3, R4 and R5) work continuously. A peristaltic bomb ensured the flow of the content from one reactor to the next. The system did not simulate water absorption. Further detail in **chapter 3.**

This equipment has been designed by AINIA (Parque Tecnológico de Valencia, Paterna). A frontal view is depicted in the following picture.

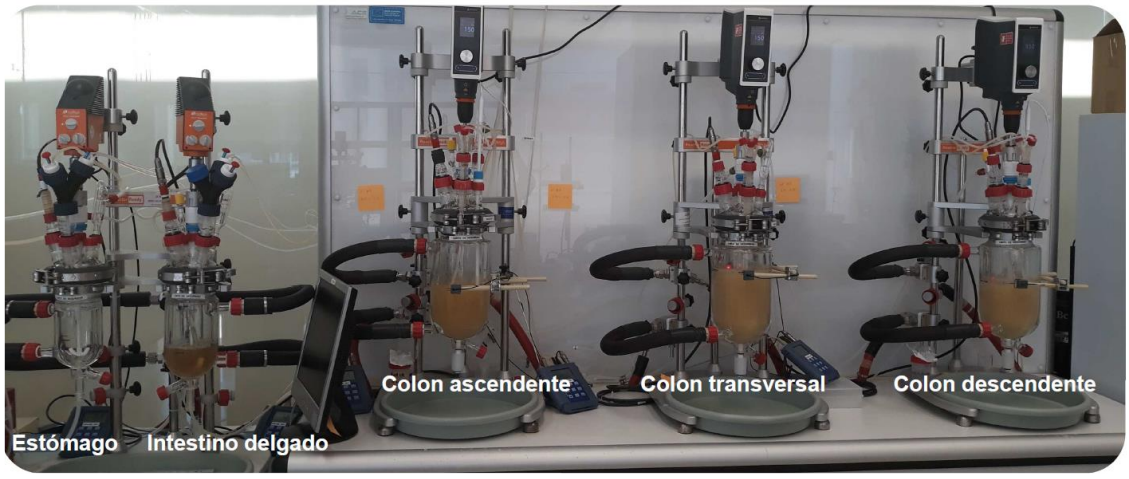


Figure 4. Picture of the *in-vitro* digester designed by AINIA.

Study in humans: Cross-sectional study in the Obekit cohort

Participants, anthropometric and biochemical measurements

This cross-sectional study enrolled 360 Spanish adults of self-reported European ancestry (251 females and 109 males) with ages ranging from 45.0 ± 10.5 years old. Participants were recruited at the Centre for Nutrition Research of the University of Navarra, Spain. Major exclusion criteria included a history of diabetes mellitus, cardiovascular disease, and hypertension, pregnant or lactating women, and current use of lipid-lowering drugs. Patients with a diagnosis of primary hyperlipidaemia were also excluded. Inclusion criteria were body mass index between 25 and 40, a physical examination and assessment of vital signs considered as normal or clinically insignificant by the researcher. Also, in case of individuals with chronic, stable-dose drug treatment during the last three previous months and at baseline, the investigators assessed possible inclusion.

Faecal Sample Collection and DNA extraction

The faecal samples were self-collected by the volunteers, before and after the intervention, using stool preservation kits. The DNA extraction from faecal samples was performed with a DNeasy® PowerSoil® Pro Kit from Qiagen.

Further explanation in **chapter 1 & 2.**

Study in humans: Nutritional intervention with specific foods

The intervention studies in the Obekit project included 8 foods that were given to 4 groups (A, B, C, D) of 15 healthy volunteers. There was a first phase of 3 weeks with one food, then a 3-week washing period to re-establish the basal microbiota, and a second phase of 3 weeks with a new food. Both, at the beginning and the end of the two phases, faecal samples were collected to analyse microbiota, and blood samples were taken to perform untargeted metabolomics in plasma. For example, during the second phase of the intervention period (3 weeks long), participants of group D (n=15) included 90 g of Bimi® (either cooked or row) every two days, into their habitual diet. The details can be found in **chapter 6.** The design of the intervention study is depicted here:

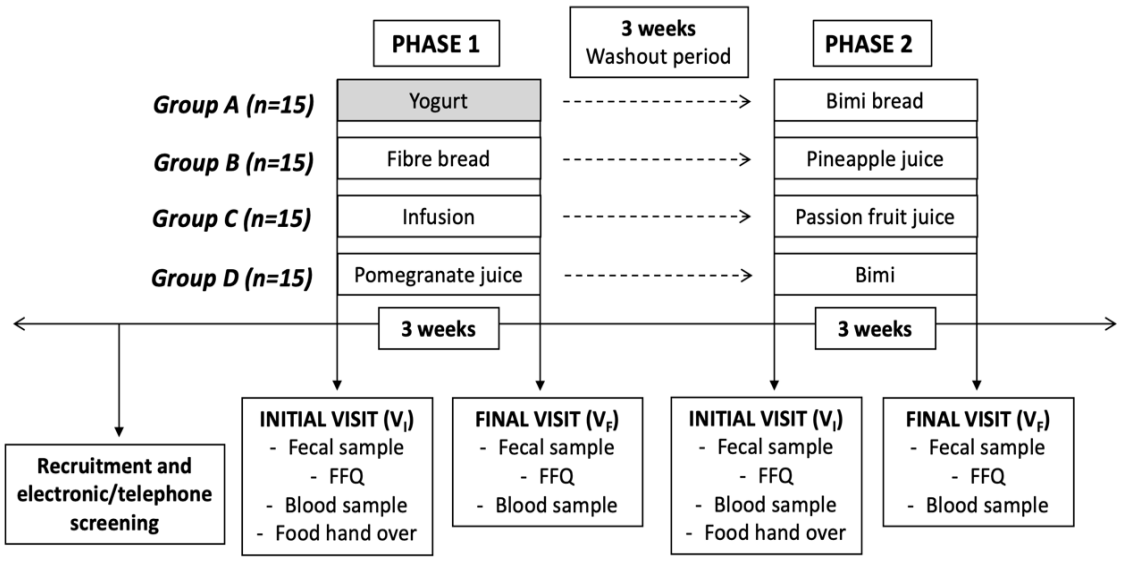


Figure 5. Workflow of the nutritional intervention with specific foods.

The inclusion criteria were the following:

- Men and women between 18 and 70 years old.
- BMI between 19 and 34.9 kg/m^2.
- No weight variations (± 3 kg) during the last 3 months.
- No variations in pharmacological treatment in the last 3 months.
- Without consumption of stomach protectors.
- No gastrointestinal problems.

16s Metagenomics: Library preparation and sequencing

Metagenomics studies were performed by analysing the variable regions V3–V4 of the prokaryotic 16S rRNA (ribosomal Ribonucleic Acid) gene sequences, which gives 460 bp amplicons in a two-round PCR protocol. In a first step, PCR is used to amplify a template out of a DNA sample using specific primers with overhang adapters attached to the flank regions of interest. PCR was performed in a thermal cycler using the following conditions: 95 °C for 3 min, 25 cycles of (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and 72 °C for 5 min. To verify the amplicon, 1 μL of the PCR product was checked in a Bioanalyzer DNA 1000 chip. The expected size on a Bioanalyzer was ~550 bp.

In a second step and using a limited-cycle PCR, sequencing adapters and dual index barcodes were added to the amplicon, which allows up to 96 libraries pooled together for sequencing in NGS. The PCR was performed in a thermal cycler using the following conditions: 95 °C for 3 min, eight cycles of (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and 72 °C for 5 min. Subsequently, the

Index PCR ran a second Bioanalyzer DNA 1000 chip to validate the library. The expected size was ~630 bp. The next step consisted of quantifying the libraries using a Qubit® fluorometer and dilution of the samples before pooling them.

Finally, paired-end sequencing was performed in a MiSeq platform (Illumina) with a 500-cycle Miseq run and with 7 pM sample and a minimum of 25% PhiX. Only samples with more than 40,000 reads were used for further analysis. For more detailed information go to **chapter 3**. Detailed specifications can be found in the specific chapter.

RESULTS

This part is divided in six different chapters, one for each of the articles presented in the thesis. The articles related with the objectives proposed are the following:

Objective 1

Chapter 1: Gut Microbiota Bacterial Species Associated with Mediterranean Diet-Related Food Groups in a Northern Spanish Population.

Chapter 2: Association of the Gut Microbiota with the Host's Health through an Analysis of Biochemical Markers, Dietary Estimation, and Microbial Composition.

Objective 2

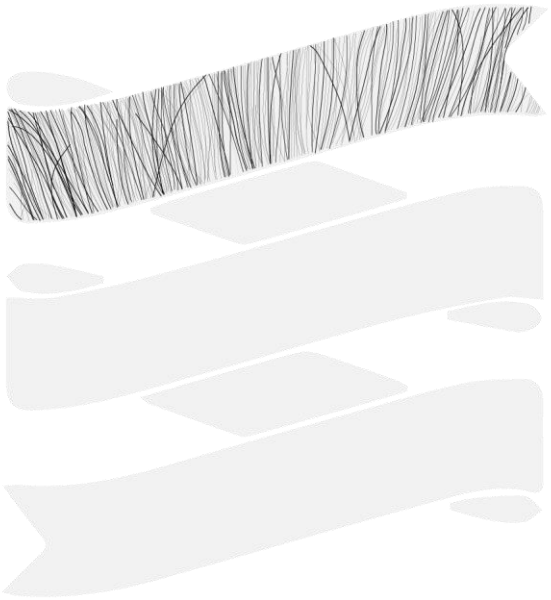
Chapter 3: An In Vitro Protocol to Study the Modulatory Effects of a Food or Biocompound on Human Gut Microbiome and Metabolome.

Objective 3

Chapter 4: Elicited butternut pumpkin (*Cucurbia moschata* D. cv. Ariel) as a natural dietary modulator of the human intestinal microbiota dysbiosis.

Chapter 5: Effects of Glucosinolate-Enriched Red Radish (*Raphanus sativus*) on In Vitro Models of Intestinal Microbiota and Metabolic Syndrome-Related Functionalities.

Chapter 6: Gut microbiota modulatory capacity of *Brassica oleracea* italica x alboglabra (Bimi®).



Chapter 1

Gut Microbiota Bacterial Species Associated with Mediterranean Diet-Related Food Groups in a Northern Spanish Population t

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Article

Gut Microbiota Bacterial Species Associated with Mediterranean Diet-Related Food Groups in a Northern Spanish Population

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Abstract: The MD (Mediterranean diet) is recognized as one of the healthiest diets worldwide and is associated with the prevention of cardiovascular and metabolic diseases. Dietary habits are considered one of the strongest modulators of gut microbiota, which seem to play a significant role in health status of the host. The purpose of the present study was to evaluate interactive associations between gut microbiota composition and habitual dietary intake in 360 Spanish adults from the Obekit cohort (normal weight, overweight, and obese participants). Dietary intake and adherence to the MD tests were administered and fecal samples were collected from each participant. Fecal 16S rRNA (ribosomal Ribonucleic Acid) gene sequencing was performed and checked against the dietary habits. MetagenomeSeq was the statistical tool applied to analyze data at the species taxonomic level. Results from this study identified several beneficial bacteria that were more abundant in the individuals with higher adherence to the MD. *Bifidobacterium animalis* was the species with the strongest association with the MD. Some SCFA (Short Chain Fatty Acids) -producing bacteria were also associated with MD. In conclusion, this study showed that MD, fiber, legumes, vegetable, fruit, and nut intake are associated with an increase in butyrate-producing taxa such as *Roseburia faecis*, *Ruminococcus bromii*, and *Oscillospira (Flavonifractor) plautii*.

Keywords: *Bifidobacterium animalis*; gut microbiota; short-chain fatty acids; obesity; butyrate

Citation: Rosés, C.; Cuevas-Sierra, A.; Quintana, S.; Riezu-Boj, J.I.; Martínez, J.A.; Milagro, F.I.; Barceló, A. Gut Microbiota Bacterial Species Associated with Mediterranean Diet-Related Food Groups in a Northern Spanish Population. *Nutrients* **2021**, *13*, 636. https://doi.org/10.3390/nu13020636

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1. Introduction

Gut microbiota status has an impact on the health and disease of the host [1]. Dietary habits are considered one of the strongest modulators of gut microbiota. However, it is not clear how the two relate to each other or which gut microbiota profile to pursue in order to ensure good health. Currently, the majority of the world's population is exposed to Western diets, characterized by a high intake of saturated and omega-6 fatty acids, reduced omega-3 and fiber intake, an overuse of salt, and too much refined sugar and processed food [2]. Along with a sedentary lifestyle, these factors are increasing the prevalence of obesity worldwide, with half of the world's population now considered to be overweight [3]. As a result of this lifestyle, serious conditions can appear and in a variety of ways. Hypertrophied adipocytes release inflammatory molecules (i.e., interleukins and tumor necrosis factor), which can act as false alarms in the immune system, causing the entire immune system to reduce its sensitivity, such that the response to a real condition may

be delayed [4]. This can aid the development of several inflammation-related disorders such as metabolic syndrome, cardiovascular disease, colorectal cancer, and neurodegenerative diseases [5,6]. Most of these disorders have also been associated with alterations in microbiota composition in humans, especially those with reduced bacterial richness and diversity [7]. These changes have been related to disturbed gut barrier functions, increased gut permeability, and increased plasma concentrations of lipopolysaccharide (LPS) and other bacterial by-products, which cause low-grade inflammation that, again, triggers the development of insulin resistance, obesity, metabolic syndrome, colorectal cancer [8], and autoimmune disorders such as Crohn disease, ulcerative colitis, and allergies [7].

In this context, the MD (Mediterranean diet) is recognized as one of the healthiest diets worldwide as it contains to a high proportion of fiber, mono- and poly-unsaturated fatty acids, antioxidants, and polyphenols, present in vegetables, fruits, pulses, and extra virgin olive oil (EVOO), which are all strongly associated with a reduced risk of developing non-communicable diseases related to Western diet and lifestyle [9,10]. In a study with overweight, obese participants with lifestyle risk factors for metabolic disease, an isocaloric MD intervention reduced their blood cholesterol and caused multiple changes in their microbiome and metabolome, thus improving their metabolomic health [11]. A 12-month-long MD intervention with elderly subjects showed a taxa enrichment that was associated with lower frailty and improved cognitive functions, but negatively correlated with inflammatory markers, thus promoting healthier aging [12]. Carbohydrates and fiber present in the MD are fermented by gut microbiota, through which large quantities of biologically active metabolites such as SCFAs (short-chain fatty acids) are produced [13]. Also, greater concentrations of phenolic metabolites are excreted in feces when there are high concentrations of bioactive compounds coming from polyphenols and fiber [14]. Furthermore, de Filippis et al. reported that a high adherence to a MD rich in plant foods beneficially impacts gut microbiota and the associated metabolome [15]. Therefore, we would expect a modulation of the gut microbiota to be one of the positive health effects of the MD [13]. Mitsou et al. found a positive correlation with gastrointestinal symptoms, fecal moisture, total bacteria, and Bifidobacteria, but a reduced representation of Lactobacilli and butyrate-producing bacteria induced by fast food consumption [16].

The main objective of the present study was to relate the adherence to the MD to specific metagenomic traits, focusing on those bacterial taxa that are more abundant in individuals with a high adherence to the MD. In addition, a specific food group consumption assessment was carried out to increase our knowledge of the impact of diet on gut microbiota composition. The focus was on the bacteria that are most closely associated with a high adherence to the MD and how these bacteria are influenced by specific food groups characteristic of the Mediterranean pattern.

2. Material and Methods

2.1. Participants

This cross-sectional study enrolled 360 Spanish adults of self-reported European ancestry (251 females and 109 males) with ages ranging from 45.0 ± 10.5 years old. Participants were recruited at the Center for Nutrition Research of the University of Navarra, Spain, and took part in the Obekit study. Major exclusion criteria included a history of diabetes mellitus, cardiovascular disease and hypertension, pregnant or lactating women, and current use of lipid-lowering drugs. Patients with a diagnosis of primary hyperlipidemia were also excluded. This investigation followed the ethical principles for medical research in humans from the 2013 Helsinki Declaration [17]. The research protocol (ref. 132/2015) was approved by the Research Ethics Committee of the University of Navarra. Written informed consent from each participant was obtained before the inclusion in the study. The characteristics of the Obekit research project, including study design and registration, have been reported elsewhere [18].

Inclusion criteria were body mass index between 25 and 40, a physical examination and assessment of vital signs considered as normal or clinically insignificant by the researcher.

5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and **Reverse Primer:** 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHV GGGTATCTAATCC. PCR was performed in a thermal cycler using the following conditions: 95 °C for 3 min, 25 cycles of (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and 72 °C for 5 min.

To verify that the specific primers had been correctly attached to the samples, 1 µL of the PCR product was checked on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, USA). The expected size on a Bioanalyzer is ~550 bp.

In a second step and using a limited-cycle PCR, sequencing adapters, and dual indices barcodes, Nextera® XT DNA Index Kit, FC-131-1002 (Illumina, San Diego, CA, USA), were added to the amplicon, which allows up to 96 libraries for sequencing on the MiSeq sequencer with the MiSeq® Reagent Kit v3 (600 cycle) MS-102-3003 to be pooled together.

PCR was performed in a thermal cycler using the following conditions: 95 °C for 3 min, eight cycles of (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and 72 °C for 5 min. Subsequently, the Index PCR ran a second Bioanalyzer DNA 1000 chip to validate the library. The expected size was ~630 bp.

The next step consisted of the quantification of the libraries using a fluorometric quantification and dilution of the samples before pooling all samples.

Finally, paired-end sequencing was performed on a MiSeq platform (Illumina) with a 600 cycles Miseq run [23] and with 20 pM sample and a minimum of 20% PhiX. The mean reads obtained were 164,387. Only samples with more than 40,000 reads were used for further analysis. All the sequencing data were deposited by the authors in SRA (Sequence Read Archive) and the accession key has been included in the text (PRJNA623853).

2.6. Metagenomics Data: Analysis and Processing

The 16S rRNA gene sequences obtained were filtered following the quality criteria of the OTUs (operational taxonomic units) processing pipeline LotuS (release 1.58) [24]. This pipeline includes UPARSE (Highly accurate OTU sequences from microbial amplicon reads) de novo sequence clustering and removal of chimeric sequences and phix contaminants for the identification of OTUs and their abundance matrix generation [25,26]. Taxonomy was assigned using HITdb (Highly scalable Relational Database), achieving up to species sensitivity. BLAST (Basic Local Alignment Search Tool) was used when HITdb failed to reach a homology higher than 97% [27,28]. Thus, OTUs with a similarity of 97% or more were referred to species. However, OTUs that did not reach this percentage of similarity were checked and updated using the Basic Local Alignment Search Tool (BLASTn) to compare with the 16S rRNA gene sequences for bacteria and archaea database of GenBank of National Center for Biotechnology information in order to find an assignment to a species. These sequences in which the BLASTn tool found a new assignment were indicated using the GenBank access number and the percentage of homology following the species name. The abundance matrices were first filtered and then normalized in R/Bioconductor at each classification level: OTU, species, genus, family, order, class, and phylum. This study focused mainly on the species level. Briefly, taxa with a less than 10% frequency in our population were removed from the analysis, and a global normalization was performed using the library size as a correcting factor and log2 data transformation [29].

2.7. Richness and Evenness

Richness was defined as the total of species. Evenness was calculated using Pielou's evenness index according to the following formula: $J' = H/\ln(S)$.

Alpha diversity was assessed using the Shannon index. Beta diversity was calculated using the Bray–Curtis index, PERMANOVA (Permutational Multivariate Analysis of Variance) statistical method, and NMDS (Non-metric Multidimensional Scaling) as ordination methods.

All the sequencing data were deposited by the authors in the Sequence Read Archive (SRA) from NCBI (National Center for Biotechnology Information), with PRJNA623853 as accession key.

2.8. Statistical Analysis

The microbiome Analyst tool [30] was used for statistical differences in microbiota profiles between groups (tertiles) through a Zero-inflated Gaussian approach of MetagenomeSeq and using the cumulative sum scaling (CSS) normalization.

2.9. Prediction of Functional Potential of Gut Microbiota

Computational prediction of the functional capabilities using data from 16S rRNA metagenomics was performed using the Tax4fun tool from MicrobiomeAnalyst [30]. KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthologus (KO) provided by Tax4Fun was comparatively analyzed using the Shotgun Data Profiling section in the MicrobiomeAnalyst. A total of 2094 KO low-abundance features were removed based on prevalence (<20% of prevalence in samples) and 336 low-variance features based on inter-quantile range, with a total of 3018 features remaining after filtering. The EdgeR statistical analysis compared differentially expressed KO between groups (low and high adherence to MD) and allowed to visualization of the results within KEGG metabolic networks, along with pathway analysis between groups.

3. Results

3.1. Participant Characteristics

Baseline characteristics of the population that participated in this study separated by adherence to the MD and BMI (normal weight, overweight, and obese, according to the World Health Organization criteria [31]) are shown in Table 1, including age, anthropometric measures, and biochemical and dietary data. Additional data on the study population (separated by sex and adiposity status) are shown as supplementary material (Supplementary Table S1).

Table 1. Baseline characteristics of the population separated by adherence to the MD (Mediterranean diet).

Variables	High Adherence (3rd Tertile) (n = 94)	Low Adherence (1st Tertile) (n = 128)	p Value
Age (y)	47.3 ± 1.2	41.7 ± 0.9	0.020 *
BMI	27.7 ± 0.6	29.9 ± 0.6	0.002 *
Glucose (mg/dL)	95 ± 2	93 ± 1	0.394
Total cholesterol (mg/dL)	209 ± 4	208 ± 4	0.822
HDL (mg/dL)	59 ± 1	57 ± 1	0.261
LDL (mg/dL)	133 ± 4	132 ± 3	0.904
Triglycerides (mg/dL)	87 ± 5	93 ± 4	0.336
HOMA-IR	1.4 ± 0.15	1.6 ± 0.1	0.011 *
Carbohydrate intake (%)	54.5 ± 0.7	54.7 ± 0.6	0.135
Protein intake (%)	21.8 ± 0.4	22.2 ± 0.4	0.286
Fat intake (%)	23.6 ± 0.5	23.0 ± 0.4	0.085
Fiber intake (g/day)	35.9 ± 1.6	24.5 ± 0.7	0.000 *
Energy intake (kcal/day)	2932 ± 106	2872 ± 84	0.680

The p value was calculated depending on the distribution of the variables by T-Student or Mann–Whitney test. * The score is significant at the 0.05 level.

The high-adherence group was made up of older people, with a significantly lower BMI and less resistance to insulin. Despite the total energy intake being very close, the data in Table 1 indicate that the third tertile values were healthier. Concerning dietary composition, the third tertile was characterized by a higher intake of fiber.

3.2. Microbiota Composition: MD Adherence

MD tertiles 1 and 3 were compared through metagenomeSeq analysis. Significant differences appeared when comparing both tertiles (FDR (False Discovery Rate) < 0.05). Species shown in Table 2 were strongly influenced by the MD score. Participants with a higher adherence to the MD were represented in the third tertile while those who are far from the MD model were in the first tertile.

Table 2. Bacterial species with a significant relation with adherence to the MD (FDR (False discovery rate) < 0.05) by metagenomeSeq test.

High Adherence (3rd Tertile)		Low Adherence (1st Tertile)	
SPECIES	**FDR**	**SPECIES**	**FDR**
Bifidobacterium animalis	1.21×10^{-7}	*OTU100 | NN = Eubacterium saphenum GU427005 | D = 91*	4.44×10^{-5}
Bacteroides cellulosilyticus	4.47×10^{-7}	*OTU375 | NN = Succinivibrio dextrinosolvens Y17600 | D = 97*	0.0001
OTU946 | NN = Paraprevotella clara AB331896 | D = 86.8	1.72×10^{-5}	*OTU759 | NN = Gordonibacter pamelaeae AB566419 | D = 87.6*	0.0005
OTU1682 | NN = Oscillibacter valericigenes AB238598 | D = 91.1	3.42×10^{-5}	*OTU11 | NN = Butyricicoccus pullicaecorum EU410376 | D = 89*	0.0002
OTU1065 | NN = Oscillospira (Flavonifractor) plautii Y18187 | D = 86.6	3.42×10^{-5}	*Christensenella minuta*	0.0020
OTU1173 | NN = Roseburia faecis AY804149 | D = 94.9	0.0008	*Parabacteroides goldsteinii*	0.0073
OTU1517 | NN = Catabacter hongkongensis AB671763 | D = 87	0.0008	*OTU1625 | NN = Anaerotruncus colihominis DQ002932 | D = 89*	0.0120
OTU1296 | NN = Ruminococcus bromii DQ882649 | D = 92.3	0.0120	*Alistipes timonensis*	0.0155
Erysipelatoclostridium ramosum	0.0176	*Prevotella corporis*	0.0192
OTU521 | NN = Papillibacter cinnamivorans AF167711 | D = 89	0.0463		

This work focused on the high-adherence species and their distribution, with box plots (Figure 1). All box plots represent those species with significant differences between high- and low-adherence tertiles to the respective dietary pattern or food group.

According to the species shown in Table 2, the relationship between these bacteria and the intake of certain foods (g of food per day) was analyzed. The food groups chosen for this approach were those most relevant in the MD: legumes, vegetables, fruit, olive oil, nuts, and also total fiber.

3.3. Microbiota Composition: Fibre Intake

It has been proposed that a substantial part of the beneficial effects of the MD could be attributed to a high intake of fiber. Species upregulated in the high MD adherence group were analyzed to see which of them were related to fiber intake (FDR < 0.05) (tertiles 1 and 3). The most abundant species in the tertile of higher fiber intake (third tertile) are shown in Table 3 and Figure 2.

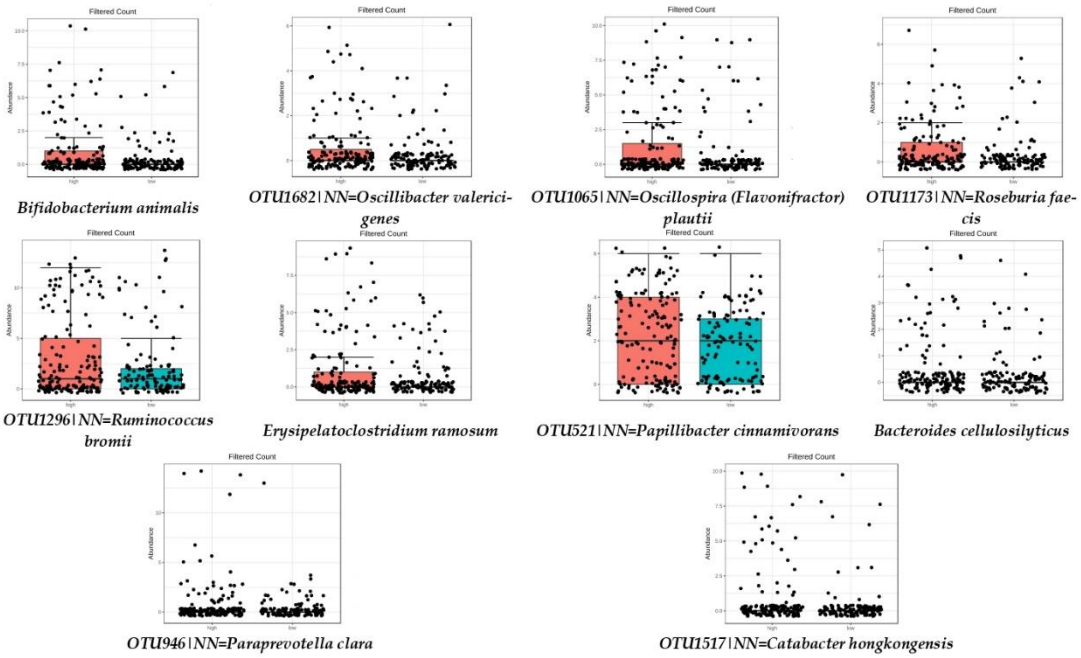


Figure 1. Bacterial species that were significantly more abundant in the group with high adherence to MD (FDR < 0.05) by metagenomeSeq test. Red boxes represent participants with a higher adherence to the MD and blue boxes low adherence.

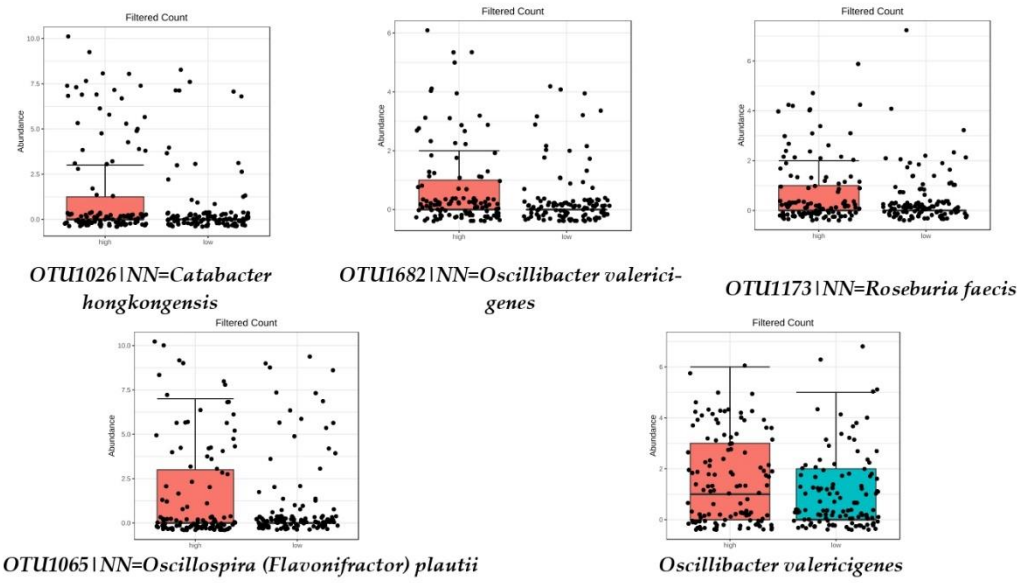


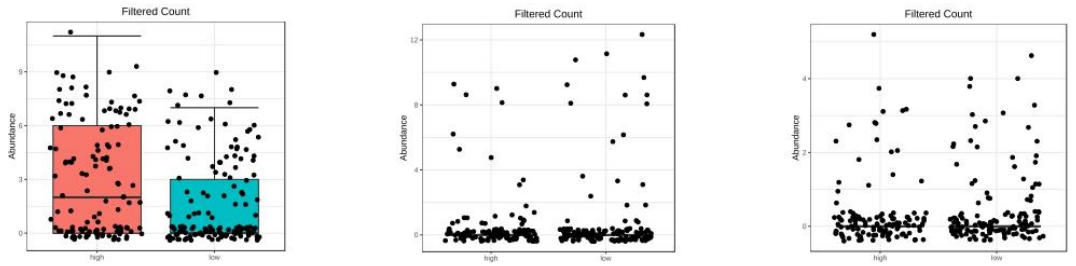
Figure 2. Bacterial species that show significant relation with high FIBRE intake (FDR < 0.05) by metagenomeSeq test. Red boxes represent participants with a higher adherence to the MD and blue boxes low adherence.

Table 3. Bacterial species and OTUs (Operational Taxonomic Units) significantly related (FDR < 0.05) with the main food groups of the MD, by comparing high and low tertiles of the intake of each food group by metagenomeSeq analysis.

SPECIES	FDR
High intake of FIBRE	
OTU1026 | NN = Catabacter hongkongensis AB671763 | D = 82.4	8.48×10^{-11}
OTU1682 | NN = Oscillibacter valericigenes AB238598 | D = 91.1	6.73×10^{-5}
OTU1173 | NN = Roseburia faecis AY804149 | D = 94.9	8.38×10^{-5}
OTU1065 | NN = Oscillospira (Flavonifractor) plautii Y18187 | D = 86.6	0.0018
Oscillibacter valericigenes	0.0045
High intake of LEGUMES	
OTU222 | NN = Ruminococcus bromii DQ882649 | D = 89.9	7.45×10^{-5}
OTU1712 | NN = Catabacter hongkongensis AB671763 | D = 84	0.0011
Bacteroides cellulosilyticus	0.0001
High intake of VEGETABLES	
OTU1517 | NN = Catabacter hongkongensis AB671763 | D = 87	1.19×10^{-11}
OTU1026 | NN = Catabacter hongkongensis AB671763 | D = 82.4	1.43×10^{-6}
OTU521 | NN = Papillibacter cinnamivorans AF167711 | D = 89	0.0019
High intake of FRUIT	
OTU1517 | NN = Catabacter hongkongensis AB671763 | D = 87	0.0008
OTU1173 | NN = Roseburia faecis AY804149 | D = 94.9	0.0012
High intake of NUTS	
OTU1173 | NN = Roseburia faecis AY804149 | D = 94.9	8.11×10^{-5}
OTU855 | NN = Roseburia faecis AY804149 | D = 95.5	0.0001
OTU521 | NN = Papillibacter cinnamivorans AF167711 | D = 89	0.0024

3.4. Microbiota Composition: Food Groups

Focusing on the species influenced by the MD (Table 2), those positively influenced by legumes, vegetables, fruit, and nut intake (FDR < 0.05) are shown in Table 3 and Figures 3–6, respectively. No species from the high-adherence MD group were found in relation to the intake of olive oil.



OTU1712 \| NN=Catabacter hongkongensis ***OTU222 \| NN=Ruminococcus bromii*** ***Bacteroides cellulosilyticus***

Figure 3. Bacterial species that were significantly more abundant in the group with high LEGUMES intake (FDR < 0.05) by metagenomeSeq test. Red boxes represent participants with a higher adherence to the MD and blue boxes low adherence.

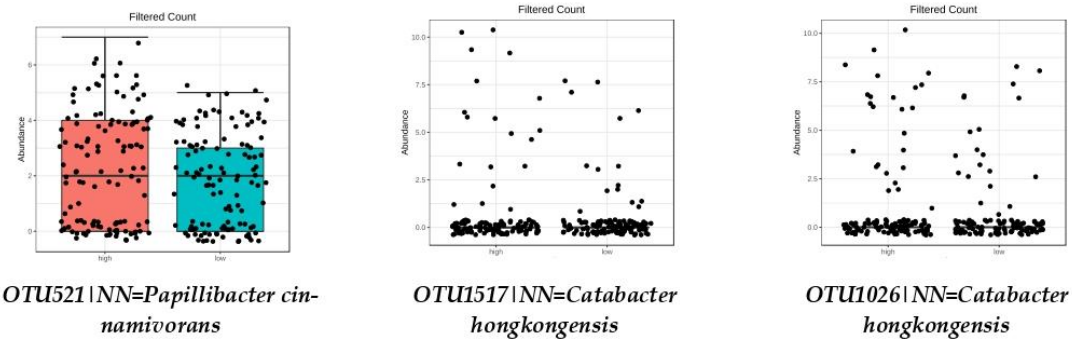


Figure 4. Bacterial species that were significantly more abundant in the group with high VEGETABLES intake (FDR < 0.05) by metagenomeSeq test. Red boxes represent participants with a higher adherence to the MD and blue boxes low adherence.

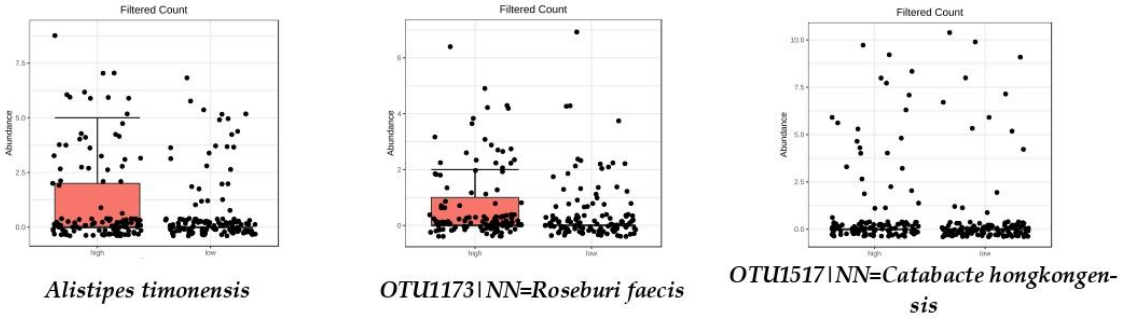


Figure 5. Bacterial species that were significantly more abundant in the group with high FRUIT intake (FDR < 0.05) by metagenomeSeq test. Red boxes represent participants with a higher adherence to the MD and blue boxes low adherence.

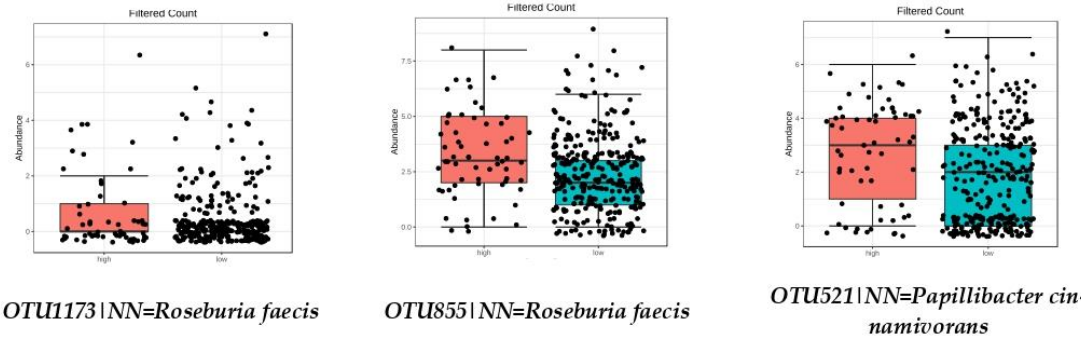


Figure 6. Bacterial species that were significantly more abundant in the group with high NUTS intake (FDR < 0.05) by metagenomeSeq test. Red boxes represent participants with a higher adherence to the MD and blue boxes low adherence.

3.5. Species Richness, Evenness, and Diversity

The total number of species obtained in this study was 4733. Richness, calculated as the number OTUs for each individual, was 535 ± 17.25 for the high and 536 ± 16.84 for the low group ($p = 0.960$). Evenness, as calculated with the Pielou index, was 0.315 ± 0.007 for the high- and 0.303 ± 0.007 for the low-adherence group ($p = 0.220$). Alpha diversity was assessed using the Shannon index, but no significant differences were obtained when comparing low- and high-adherence groups to the MD ($p = 0.220$). As for beta diversity, no

significant differences were observed when comparing low-adherence and high-adherence groups to the MD ($p = 0.554$). The NMDS (Non-metric multidimensional scaling) graph is shown as supplementary material (Supplementary Figure S1).

3.6. Comparison of Functional Potential of the Gut Microbiota

The analysis by Tax4Fun of differential KO abundance between low-adherence and high-adherence groups to the MD revealed a total of five KO (shown in Table 4) that were significantly different (FDR < 0.05). Interestingly, one of these (K02106) was a short-chain fatty acid transporter. The enrichment analysis showed that the sphingolipid metabolism pathway (ko00600), associated with K00720, was significantly upregulated in the high-adherence group ($p = 0.01$).

Table 4. Metabolic activity differences between the tertiles with lower and higher adherence to MD by Tax4Fun test.

K0 Name	Definition	Log2FC	*p* Value	FDR
K00720	Ceramide glucosyltransferase	1.803	2.93×10^{-7}	0.001
K01884	Cysteinyl-tRNA synthetase	1.792	8.67×10^{-7}	0.002
K03653	N-glycosylase/DNA lyase	1.6569	1.57×10^{-6}	0.002
K02106	Short-chain fatty acids transporter	1.0933	2.26×10^{-5}	0.03
K07486	Transposase	0.9605	4.51×10^{-5}	0.04

4. Discussion

It is widely known that the gut microbiota co-develops with the host, and its bacterial proportions are modified by the action of diet and other extrinsic stressors [32], thus determining gut microbiota composition, diversity, and activity [33]. However, despite the fact that diet and health are interrelated, there is little information on the impact of specific components of the diet on microbiota composition. The present study focused on the effect of the MD and its most characteristic food groups (fiber, legumes, vegetables, fruit, and nuts) on gut microbiota composition.

5. MD High-Adherence Species

There is a large amount of written evidence that shows a high adherence to the MD to be beneficial to human health. The MD is a great resource in helping to manage obesity-related comorbidities, such as cardiovascular diseases, type 2 diabetes, and pro-inflammatory conditions [34–36]. Table 2 shows those species that are more strongly associated with the adherence to the MD. The high-adherence group represents those participants that have a diet closer to the MD model. The following species are the most significant.

Bifidobacterium animalis belongs to the phylum Bacteroides. Existing data reveal that this phylum has been associated with obesity-related abnormalities in bacterial gut microbiota and that the genus *Bifidobacterium* might play a critical role in weight regulation [37]. *B. animalis* subsp. *lactis* GCL2508 is a probiotic strain capable of proliferating and producing SCFA in the gut. SCFA are the result of the fermentation of non-digestive polysaccharides in the colon, thanks to bacteria. A study with mice showed increased levels of SCFA were present as a result of treatment with this probiotic, enhancing host energy expenditure and decreasing fat accumulation [38]. These compounds have a regulatory effect on inflammatory conditions [39]. Furthermore, *B. animalis* subsp. *lactis* may have an anti-metabolic syndrome effect [38]. In our study, a K0 (K02106) defined as a SCFA transporter was overrepresented in the group with higher adherence to the MD.

Bacteroides cellulosilyticus received its name because of its ability to degrade cellulose [40]. It is equipped with an unprecedented number of carbohydrate-active enzymes, more than any previously sequenced members of the Bacteroidetes, providing a versatile carbohydrate utilization with a strong emphasis on plant-derived xylans abundant in cereal grains [41]. In our study it was positively correlated with legume intake (Table 3).

Paraprevotella clara, a common member of the human intestinal microbiota [42], is closely related to the carbohydrate-active enzymes of arabinofuranosidase, pectin lyase, and polygalacturonaseand xylanase [43] known to degrade insoluble fiber [44]. Indeed, *P. clara* is known to produce acetic acid [42]. No significant relationship with fiber was found in our results.

Oscillibacter valericigenes produces valeric acid, an SCFA [45]. Valeric acid has been reported to have an inhibitory effect on histone deacetylase (HDAC) isoforms implicated in a variety of disorders such as cancer, colitis, and cardiovascular and neurodegenerative diseases [46]. In this study, *O. valericigenes* showed a positive relationship with fiber consumption (Table 3). Another study observed a significant increase of this species in participants fed resistant starch-rich diets [47].

High levels of *Oscillospira (Flavonifractor) plautii* have been strongly correlated with a high production of SCFA, especially propionate and butyrate [48]. This species is of particular interest since its abundance is found to correlate with a lean host phenotype [49]. Furthermore, the *Oscillospira* genus has been seen to correlate with the production of secondary bile acids known to prevent *Clostridium difficile*-associated infectious diseases in humans [50]. It appears in Table 3 and is positively correlated with fiber intake.

Roseburia faecis is a butyrate producer whose abundance has been related to weight loss and a reduced glucose intolerance in mice [51]. This OTU shows a positive correlation with fiber, fruit, and nut intake (Table 3).

Catabacter hongkongensis, commonly found in the human intestinal microbiota [42,52], is positively influenced by fiber, legumes, vegetables, and fruit intake (Table 3).

Ruminococcus bromii has been linked to diets rich in fiber and resistant starch and greatly contributes to butyrate production in the colon [53]. It is positively correlated with legumes' intake (Table 3), which are rich in resistant starch. It is important to highlight some beneficial effects of butyric acid as it has been reported to improve the intestinal barrier integrity [54], regulate cell apoptosis [55], stimulate production of anaerobic hormones [56], and, by inducing differentiation of colonic regulatory T cells, suppress inflammatory and allergic responses [57]. Furthermore, many conditions have been associated with low levels of butyrate, such as colon cancer or obesity [53]. Thus, increased butyrate production in the colon may be beneficial to human health.

Erysipelatoclostridium ramosum is a member of the Erysipelotrichaceae family known to interfere in various ways with the enterohepatic circulation and excretion of bilirubin, transforming it into urobilin [58]. Although this species has previously been linked to increased fatty acid absorption [59,60] and systemic inflammation [61], this sequence also shares a close homology with other species such as *Erysipelatoclostridium saccharogumia* and *Clostridium cocleatum*. *E. saccharogumia* is a lignin-converting bacterium related to anticancer [62] and osteoprotective effects [63], whereas *C. cocleatum* plays a role in mucin degradation and shows resistance to colonization by *Clostridium difficile* [64].

Papillibacter cinnamivorans is not well known but has been found to be more frequently present, in lower amounts, in centenarians than in any other age groups. In our study, it was shown to have a positive correlation with vegetable and nut intake (Table 3) [65].

A strong adherence to the MD and a high intake of the food groups analyzed in this study clearly modulated the microbiota population profile toward a healthier one. Although several studies have also analyzed the effect of healthy diets, such as the Nordic [66] or the Japanese [67] diets, on gut microbiota composition, it is difficult to compare the results, as the methodologies used were different and the results may not be extrapolated from one study to another due to different classifications: enterotypes, other taxonomic levels, pathogenic bacteria, specific biomarkers, etc. In any case, the present results provide evidence for the notion that a high adherence to the MD leads to increased levels of several fiber- and carbohydrate-degrading bacterial species linked to SCFA metabolism (especially butyrate, which has an anti-inflammatory effect) that may contribute toward a healthier status. Several studies support these results [10–12,16].

One of the reasons for the novelty of the present results may be the specificity of the study population, who were all from a Northern Spanish region and had a specific dietary pattern (close to the MD) that may condition their microbiota profile. In any case, our study revealed that several species that are involved in the production of SCFA, such as *Ruminococcus bromii*, *Roseburia faecis*, *Oscillospira (Flavonifractor) plautii*, *Oscillibacter valericigenes*, *Paraprevotella clara*, and especially *Bifidobacterium animalis*, are more abundant in the Spanish population with a higher adherence to the MD. Furthermore, we were able to associate some of these with the intake of specific food groups. However, we did not observe genera, families, or phyla that significantly differed (FDR < 0.05) between the lowest and highest tertiles of the MD.

However, it remains unclear whether a higher amount of some species is always linked to a healthier status. It seems necessary to perform a more detailed taxonomic classification, as a better classification of OTUs into subspecies would help us to understand why certain species seem to be positively influenced by the MD but might not have positive effects on human health. Also, further research is needed to understand how the growth of specific bacteria can affect microbiota composition and function. Does growth of a beneficial bacterium always mean better health? When is the balance lost toward dysbiosis? These are some of the unknowns for future studies to elucidate.

6. Study Limitations

Following the inclusion criteria of the project, participants included in this study were all overweight or obese, with similar related comorbidities. Other factors such age, socioeconomic conditions, educational, and psychosocial factors were also similar. All participants were from Northern Spain, which may potentially introduce bias in relation to their dietary habits, influenced by the gastronomic culture and habits of that region. Moreover, a larger sample size including participants from other Mediterranean countries could be considered for further studies. The analysis of the V3–V4 regions of the 16S rRNA gene sequence is commonly used in studies aimed at identifying bacterial genera and species, but there are other techniques, such as Nanopore sequencing (Oxford Nanopore®) or SMART sequencing (PACBIO—Pacific Biosciences®), that allow sequencing the whole 16s rRNA gene, which, in some cases, leads to subspecies' identification. Furthermore, a full metagenomic analysis is possible using Shotgun Metagenome Analysis. Finally, we were not able to quantify SCFA in the plasma samples since, when they were extracted and stored, this objective had not been established at that time.

7. Conclusions

Our results indicate that the well-known beneficial factors of a MD may be triggered by changes in intestinal microbiota due to dietary habits. A high adherence to the MD seems to increase the abundance of some species associated with good health. *Bifidobacterium animalis* is the species with the strongest association with the MD. Fiber intake enhances the growth of several SCFA-producing species, such as *Oscillibacter valericigenes*, *Oscillospira (Flavonifractor) plautii,* and *Roseburia faecis. R. faecis* is also enhanced by fruit and nut consumption. Legumes enhance *Ruminococcus bromii* and vegetables increase the *Butyricicoccus pullicaecorum* population. Nut intake benefits *Papillibacter cinnamivorans* growth. This study strongly suggests that a MD and an intake of fiber, legumes, vegetables, and fruit increase butyrate production from *R. faecis, R. bromii,* and *Oscillospira (Flavonifractor) plautii. Erysipelatoclostridium ramosum* is the only bacteria from this study that does not show a clear beneficial effect on health, although this finding should be interpreted with caution. A more detailed taxonomy is required in order to come to definitive conclusions.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6643/13/2/636/s1. Figure S1. NMDS graph comparing the beta diversity of the tertiles with higher (blue) and lower (red) adherence to MD. Table S1: Baseline characteristics of the entire population separated by sex and weight status.

Author Contributions: Conceptualization, A.B. and F.I.M.; methodology, J.I.R.-B., F.I.M., S.Q., and A.B.; formal analysis, C.R. and A.C.-S.; data curation, C.R. and A.C.-S.; writing—original draft preparation, C.R. and A.B.; writing—review and editing, F.I.M., J.I.R.-B., and J.A.M.; supervision, A.B., F.I.M., and J.A.M.; project administration, A.B., F.I.M., and J.A.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of University of Navarra (protocol code 132/2015).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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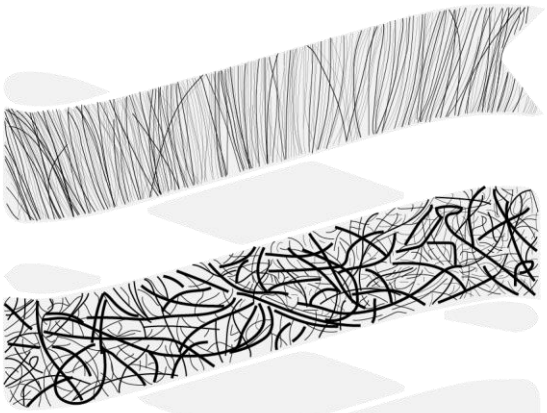

Chapter 2

Association of the Gut Microbiota with the Host's Health through an Analysis of Biochemical Markers, Dietary Estimation, and Microbial Composition.

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Article

Association of the Gut Microbiota with the Host's Health through an Analysis of Biochemical Markers, Dietary Estimation, and Microbial Composition

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Abstract: This study aims to analyze the relationship between gut microbiota composition and health parameters through specific biochemical markers and food consumption patterns in the Spanish population. This research includes 60 Spanish adults aged 47.3 ± 11.2 years old. Biochemical and anthropometric measurements, and a self-referred dietary survey (food frequency questionnaire), were analyzed and compared with the participant´s gut microbiota composition analyzed by 16s rDNA sequencing. Several bacterial strains differed significantly with the biochemical markers analyzed, suggesting an involvement in the participant´s metabolic health. Lower levels of Lactobacillaceae and Oscillospiraceae and an increase in Pasteurellaceae, *Phascolarctobacterium*, and *Haemophilus* were observed in individuals with higher AST levels. Higher levels of the Christensenellaceae and a decrease in Peptococcaceae were associated with higher levels of HDL-c. High levels of *Phascolarctobacterium* and *Peptococcus* and low levels of *Butyricicoccus* were found in individuals with higher insulin levels. This study also identified associations between bacteria and specific food groups, such as an increase in lactic acid bacteria with the consumption of fermented dairy products or an increase in Verrucomicrobiaceae with the consumption of olive oil. In conclusion, this study reinforces the idea that specific food groups can favorably modulate gut microbiota composition and have an impact on host´s health.

Keywords: gut microbiota; dysbiosis; metabolic health; insulin resistance; diet

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1. Introduction

The microbiome is defined as the collective genomes of the microorganisms inhabiting a specific environment [1]. The intestinal microbiota comprises trillions of microbes and is being studied over the past years due to their physiological functions and role in the host's health [2]. There is evidence of a bidirectional relationship between the gut microbiota and many organs in the human body, including the brain [3].

A microbial alteration is crucial to the development of pathogenesis affecting the host's health. An alteration in the composition or function of the gut microbiota is known as dysbiosis [2], which is a word that refers to a decrease in microbial diversity, a lower amount of beneficial microorganisms, or an increase in potentially harmful microbes [4].

Several chronic disorders such as cardiovascular diseases, type 2 diabetes, inflammatory bowel disease, and non-alcoholic fatty liver disease (NAFLD) have been identified

to have specific dysbiotic patterns [5]. Many of the pathologies related to gut microbiota dysbiosis are associated with metabolic complications related to insulin resistance, chronic inflammation, and oxidative stress.

The implication of gut microbiota in the host's health is attributed to the extraction of calories from the diet, absorption of nutrients, fat deposition in adipose tissue, hepatic inflammation, and the ability to provide energy and nutrients for microbial growth and proliferation [6]. The relationship between the microbial community and the host is modulated by environmental factors such as diet and lifestyle, which may trigger changes in gut microbiota composition. The manipulation of the microbiota through nutritional strategies is considered a potential alternative when treating or preventing the development of diverse metabolic disorders.

Several patterns in the microbiota have been associated with specific dietary habits, food groups, and specific nutrients. For example, a high-fat diet with low dietary fiber and high sugars could disrupt the intestinal eubiosis and impair gut permeability. It could also contribute to the reduction in microbial diversity [7]. A diet rich in simple sugars has been associated with pro-inflammatory effects, which may mediate alterations in the epithelial integrity and impair gut microbiota composition, which can lead to the promotion of metabolic endotoxemia, systemic inflammation and metabolic dysregulation [8].

The consumption of specific foods and macronutrients, the modulation of the microbiome, and the association with health and disease are closely correlated. For example, adherence to a Mediterranean dietary pattern (MD) rich in fruit, vegetables, antioxidants, and monounsaturated and polyunsaturated fatty acids has been demonstrated to favorably modulate the gut microbiome, which probably contributes to the positive health effects of the MD [9]. These effects seemed to be caused by specific food groups characteristic of this diet.

In the present work we hypothesize that the diet, though specific compounds such as fibers, pre and probiotics, and phytonutrients, can modulate the gut microbiota. This will consequently affect the host's health, which is evaluated by anthropometric and biochemical markers. The general aim of this study is to analyze the relationship between the participants' gut microbiota composition and their health and nutrition status by analyzing serum biochemical markers and food intake.

2. Materials and Methods

2.1. Participants

The individuals participating in the BIOTAGUT project were recruited at the Center for Nutrition Research of the University of Navarra, Spain. The present study included 60 Spanish adults (41 females and 19 males) aged 47.3 $\pm$ 11.2 years old. The study included participants with a body mass index (BMI) between 19.0 and 34.9 kg/m^2, without weight variations ($\pm$3 kg), changes in pharmacological treatment or consumption of stomach protectors, and gastrointestinal problems during the last three months. Subjects had to understand and be willing to sign the informed consent form and comply with all the procedures and requirements of the study. Primary exclusion criteria included a history of hypertension, cardiovascular disease, and diabetes mellitus, patients diagnosed with primary hyperlipidemia or current use of lipid-lowering drugs, and lactation or pregnancy. The study followed the ethical principles of the 2013 Helsinki Declaration [10]. The Ethical Committee in Research of the University of Navarra approved the study protocol (reference 2021.074).

2.2. Anthropometric and Biochemical Measurements

Anthropometric measurements, including body weight (kg) and height (cm), were collected by trained nutritionists using conventional validated methods. The SECA 216 stadiometer (SECA gmnh &co. kg, Hamburg, Germany) and the TANITA SC-330 body composition monitor (Tanita Corp, Tokyo, Japan) were used. BMI was calculated as the ratio between body weight and squared height (kg/m^2) and was classified following the World Health Organization (WHO) standards [11].

Blood samples were drawn by venipuncture after an overnight fasting period in a clinical setting. Two tubes with EDTA and two tubes without anticoagulants were collected from each volunteer. Tubes were centrifuged for 15 min at 4500 rpm, aliquoted, and stored at −80 °C. Serum samples were used for analyzing glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and insulin. These markers were analyzed with a Pentra C200 clinical chemistry analyzer (HORIBA Medical, Madrid, Spain) and suitable kits provided by the company. Serum insulin was quantified using a specific enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden) and read with an automated analyzer system (Triturus, Grifols, Barcelona, Spain).

2.3. Dietary Estimation

A validated food frequency questionnaire (FFQ) that included 137 food items with corresponding portion sizes was used to estimate the habitual dietary intake [12]. Participants indicated the number of times they had consumed each food group or specific food during the previous month according to frequency categories, including daily, weekly, or monthly. Macronutrient (%) and total energy intake (kcal) were estimated with ad hoc software and the information available from valid Spanish food composition tables. Specific food groups include vegetables, fruit, legumes, cereals, whole grains, dairy intake, yogurt intake, fermented dairy products, meat, cold meat (*embutidos*), olive oil, soda, and light soda. A specific analysis of the fat consumption included total cholesterol, trans fat, monounsaturated (MUFA), polyunsaturated (PUFA), and saturated fat intake.

2.4. Fecal Sample Collection, DNA Extraction, and Metagenomic Data

The fecal samples were self-collected by the volunteers using OMNIgene.GUT kits from DNA Genotek (Ottawa, ON, Canada), according to the instructions provided by the company. The DNA extraction from fecal samples was performed with a QIAamp® DNA kit (Qiagen, Hilden, Germany), following the manufacturer's protocol.

Bacterial DNA sequencing was performed by the Servei de Genòmica from the Universitat Autònoma de Barcelona (Bellaterra, Cerdanyola del Vallés, Spain). We analyzed the variable regions V3–V4 of the prokaryotic 16S rRNA (ribosomal Ribonucleic Acid) gene sequences, which gives 460 bp amplicons in a two-round PCR protocol. Initially, PCR is used to amplify a template out of a DNA sample using specific primers with overhang adapters attached that flank regions of interest. The full-length primer sequences were: Forward Primer: 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and Reverse Primer: 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHV GGGTATCTAATCC.

PCR was performed in a thermal cycler using the following conditions: 95 °C for 3 min, 25 cycles of (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and 72 °C for 5 min. To verify that the specific primers had been correctly attached to the samples, 1 µL of the PCR product was checked on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, USA). The expected size on a Bioanalyzer was ≈550 bp.

Following this procedure, using a limited-cycle PCR, sequencing adapters, and dual indices barcodes, Nextera® XT DNA Index Kit, FC-131-1002 (Illumina, San Diego, CA, USA), were added to the amplicon, which allows up to 96 libraries for sequencing on the MiSeq sequencer with the MiSeq® Reagent Kit v3 (600 cycles) MS-102-3003 to be pooled together.

The libraries were quantified using a fluorometric method and dilution of the samples before pooling all samples. Finally, paired-end sequencing was performed on a MiSeq platform (Illumina) with a 600 cycles Miseq run, a 20 pM sample, and a minimum of 20% PhiX. The mean reads obtained was 164,387. Samples with more than 40,000 reads were used for further analysis. The authors deposited all the sequencing data in SRA (Sequence Read Archive), and the accession key has been included in the text (PRJNA623853).

The 16S rRNA gene sequences obtained were filtered following the quality criteria of the OTUs (operational taxonomic units) processing pipeline LotuS (release 1.58). This

pipeline includes UPARSE (Highly accurate OTU sequences from microbial amplicon reads), de novo sequence clustering, and removal of chimeric sequences and phix contaminants for identifying OTUs and their abundance matrix generation. Taxonomy was assigned using HITdb (Highly scalable Relational Database). OTUs with a similarity of 97% or more were referred to as species. The abundance matrix of species, genera, families, class, order and phyla was normalized using the centered log-ratio (CLR) transformation using the R packages "compositions" and "zCompositions" [13].

2.5. Statistical Analysis

Microbiome Analyst [14,15] was used to analyze the participants' microbiome composition comparing the different anthropometric measures, biochemical markers, and dietary estimation. For each variable, the population was divided into two groups (high and low levels) according to the median. To analyze the statistical differences in microbiota profiles between groups, a Zero-inflated Gaussian approach of Metagenome-Seq using the cumulative sum scaling (CSS) normalization and Student's t-test through a CLR normalization, followed by FDR correction, were performed.

3. Results

3.1. Characteristics of the Study Population

Characteristics of the population that participated in this study, including age, anthropometric measures, and biochemical data, are shown in Table 1. Data for the whole population, and for the high and low groups according to the median, are shown.

Table 1. Characteristics of the participants.

Variables	All Participants (n = 60)	High Group (n = 30)	Low Group (n = 30)
Age (y)	47.3 ± 11.2	55.8 ± 5.8	38.3 ± 9.3
Weight (kg)	69.9 ± 14.1	79.8 ± 11.6	59.0 ± 5.0
BMI (kg/cm^2)	24.6 ± 3.9	27.5 ± 2.8	21.4 ± 1.6
Glucose (mg/dL)	94.5 ± 11.3	101.3 ± 11.1	86.3 ± 3.9
Total cholesterol (mg/dL)	219.4 ± 35.4	247.5 ± 21.7	192.8 ± 21.0
HDL (mg/dL)	64.3 ± 15.6	78.0 ± 11.9	52.7 ± 6.8
Triglycerides (mg/dL)	79.0 ± 36.6	102.2 ± 36.1	53.1 ± 9.0
Insulin (µIU/mL)	8.4 ± 4.3	11.4 ± 3.7	5.2 ± 1.6
AST (µ/L)	22.0 ± 14.3	27.3 ± 17.7	16.2 ± 2.4
ALT (µ/L)	22.0 ± 20.6	30.2 ± 25.1	12.8 ± 2.4

Data are expressed as mean ± standard deviation.

3.2. Dietary Intake Characteristics

A FFQ evaluated the dietary intake characteristics of the population participating in this study. Table 2 shows the main characteristics of the dietary intake from the self-reflected questionnaire. Data for the whole population, and for the high and low groups according to the median, are shown.

Table 2. Nutritional characteristics of the population.

Variables	All (n = 60)	High Group (n = 30)	Low Group (n = 30)	Variables	All (n = 60)	High Group (n = 30)	Low Group (n = 30)
Energy intake (kcal/day)	2381 ± 796	3118 ± 801	1819 ± 299	Yogurt (g/day)	75.4 ± 78.2	129.9 ± 72.4	18.0 ± 21.8
Carbohydrate intake (%)	38.2 ± 7.7	43.8 ± 4.2	31.9 ± 5.3	Fermented dairy (g/day)	93.0 ± 79.8	149.6 ± 72.2	34.3 ± 22.7
Protein intake (%)	18.3 ± 3.4	21.1 ± 3.0	15.9 ± 2.0	Meat (g/day)	169.4 ± 75.6	263.8 ± 166.0	115.1 ± 34.6
Fat intake (%)	41.3 ± 6.8	46.8 ± 5.1	36.3 ± 3.2	Cold meat (g/day)	7.5 ± 9.2	12.8 ± 10.1	1.7 ± 1.7
Fiber intake (g/day)	28.9 ± 11.8	39.9 ± 9.6	20.1 ± 4.7	Olive oil (g/day)	27.6 ± 27.2	45.8 ± 30.5	12.6 ± 5.6
Vegetables (g/day)	432.6 ± 206.8	632.5 ± 194.1	281.3 ± 90.6	Soda (g/day)	13.2 ± 29.5	26.5 ± 36.5	0.0 ± 0.0

Table 2. *Cont.*

Variables	All (n = 60)	High Group (n = 30)	Low Group (n = 30)	Variables	All (n = 60)	High Group (n = 30)	Low Group (n = 30)
Fruit (g/day)	301.8 ± 204.7	527.6 ± 343.2	156.6 ± 65.3	Soda light (g/day)	20.3 ± 42.2	45.9 ± 59.2	0.0 ± 0.0
Legumes (g/day)	23.8 ± 12.6	32.2 ± 12.0	14.9 ± 4.7	Total cholesterol (mg/day)	526.5 ± 210.7	727.4 ± 237.0	379.5 ± 88.0
Cereals (g/day)	166.3 ± 98.5	241.0 ± 83.4	97.1 ± 36.9	Trans fat (g/day)	0.8 ± 0.5	1.1 ± 0.4	0.4 ± 0.1
Whole grains (g/day)	35.8 ± 39.9	64.8 ± 36.8	5.8 ± 8.5	Monounsaturated fat (g/day)	48.8 ± 22.0	69.2 ± 22.7	33.4 ± 7.6
Dairy intake (g/day)	301.3 ± 187.5	442.3 ± 142.3	154.8 ± 80.1	Polyunsaturated fat (g/day)	16.8 ± 6.5	24.3 ± 7.6	11.6 ± 2.7
				Saturated fat (g/day)	30.4 ± 11.9	41.3 ± 8.7	21.4 ± 5.8

Data are expressed as mean ± standard deviation.

3.3. Microbiota Composition: Biochemical Markers

The population was divided into two groups (low and high levels) according to the median of the circulating levels of the biochemical parameter, as shown in Table 1. Significant relations (FDR < 0.05) were observed between some of the biochemical markers analyzed and specific families or genera (Figure 1). No statistical differences were found at the levels of phylum, class, order and species. The biochemical markers that were significantly related to changes in the gut microbiota composition were AST, HDL cholesterol, and insulin levels. The following data are expressed as box plots in Figure 1.

Several beneficial bacteria, such as Oscillospiraceae, Lactobacillaceae, Rikenellaceae, and Porphyromonadaceae, were less abundant in the participants with higher AST levels. However, Pasteurellaceae, *Phascolarctobacterium*, and *Haemophilus* were more abundant in the same group.

Higher levels of the Christensenellaceae and lower levels of Peptococcaceae were found in the individuals with higher HDL-cholesterol. In addition, higher levels of *Phascolarctobacterium* and *Peptococcus* and a lower abundance of *Butyricicoccus* was detected in individuals with higher insulinemia. The whole summary of these results at Family and Genus levels are presented as Supplementary Material in Figure S1.

The different diversity and richness indexes (i.e., Shannon, Chao-1 or Simpson) were not associated with any of the biochemical markers in the studied population.

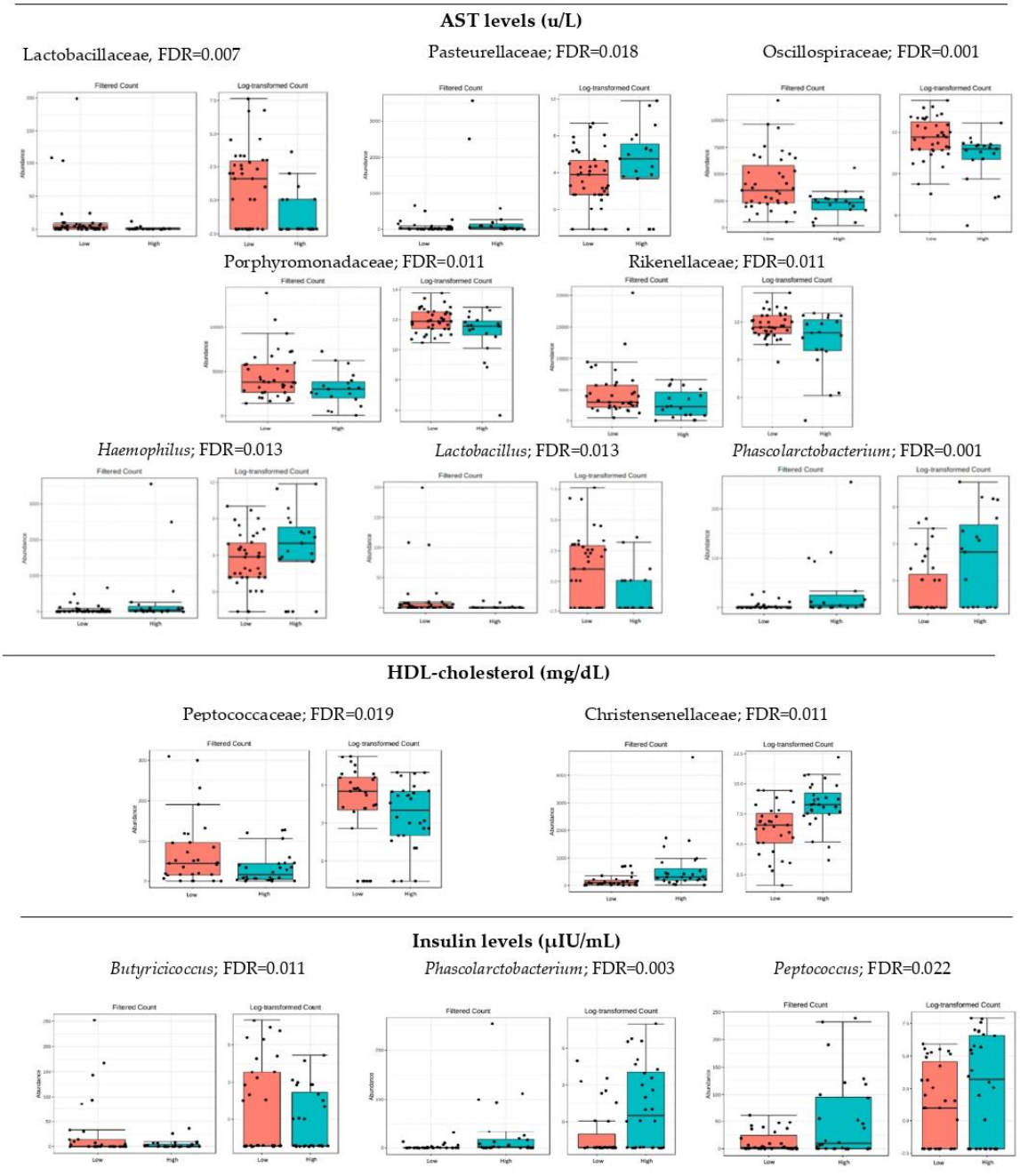

Figure 1. Bacterial taxa differing significantly in abundance when comparing the groups of individuals with high and low levels of biochemical biomarkers (FDR < 0.05): AST, HDL-cholesterol and insulin. Red boxes represent participants with lower levels, and blue boxes represent participants with higher levels of the specified biochemical marker.

3.4. Microbiota Composition: Nutritional Markers

As shown in Table 2, the population was divided into two groups (low and high intake levels) according to the median of the intake of the different nutritional data. The bacteria whose levels were significantly different (FDR < 0.05) when comparing both groups, high and low intake of each parameter, are shown in Figures 2–4. These figures represent the interface between the dietary factors presented in Table 2 and the gut bacterial composition at the different taxonomic levels. No statistical differences were found at the levels of phylum, class, order and species.

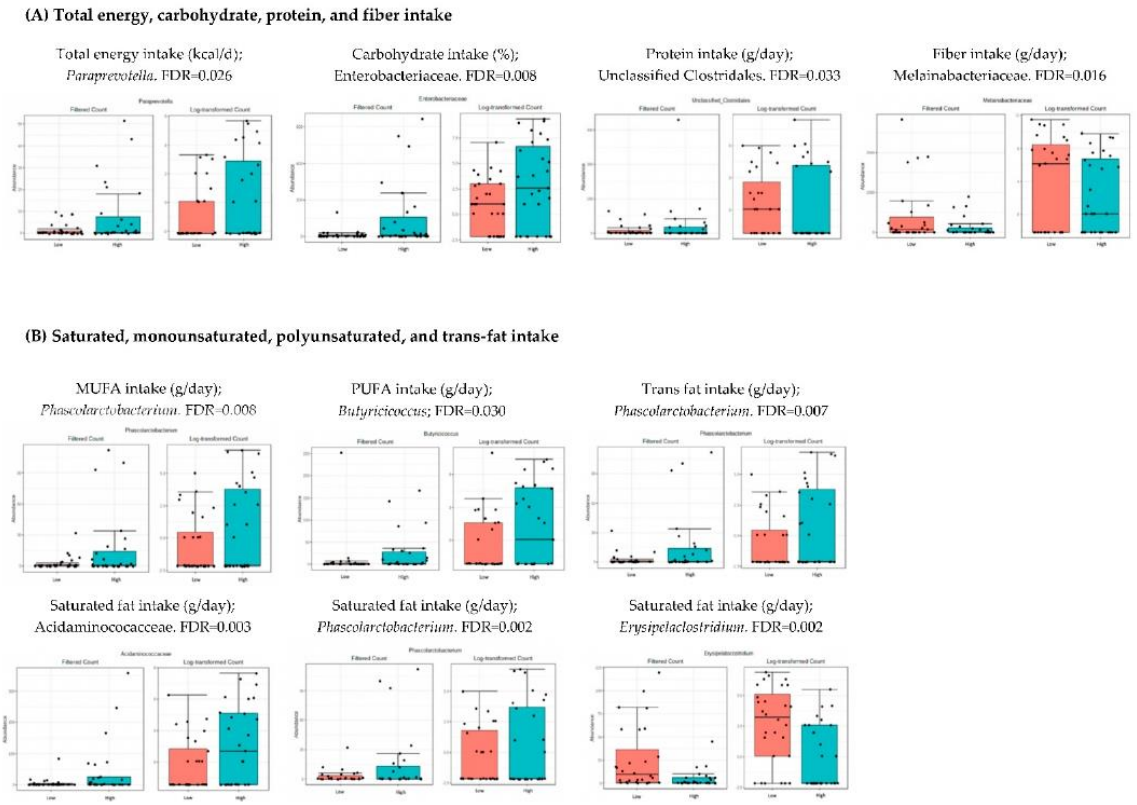

Figure 2. Bacterial taxa differing significantly in relation to total energy, carbohydrate, protein, and fiber intake (**A**), as well as different types of fat: monounsaturated, polyunsaturated, saturated, and trans (**B**) (FDR < 0.05). Red boxes represent participants with lower consumption, and blue boxes represent participants with higher consumption of the specified food group compared to the median.

Total energy, carbohydrate, and protein intake were associated with higher abundance of *Paraprevotella*, Enterobacteriaceae, and Unclassified *Clostridium*, respectively. Lower levels of Melainabacteriaceae were found in individuals with higher fiber intake. There were significant differences when comparing the bacterial abundance of the individuals with higher and lower consumptions of different types of fats, including monounsaturated, polyunsaturated, saturated, and trans. For example, higher levels of *Phascolarctobacterium* and *Butyricicoccus* were found in the groups with higher intake of monounsaturated and polyunsaturated fats, respectively. Higher consumption of saturated fat was associated with an increase in Acidaminococcaceae and *Phascolarctobacterium*, and a decrease in *Erysipelaclostridium*. A higher intake of trans fat was related with higher levels of *Phascolarctobacterium*.

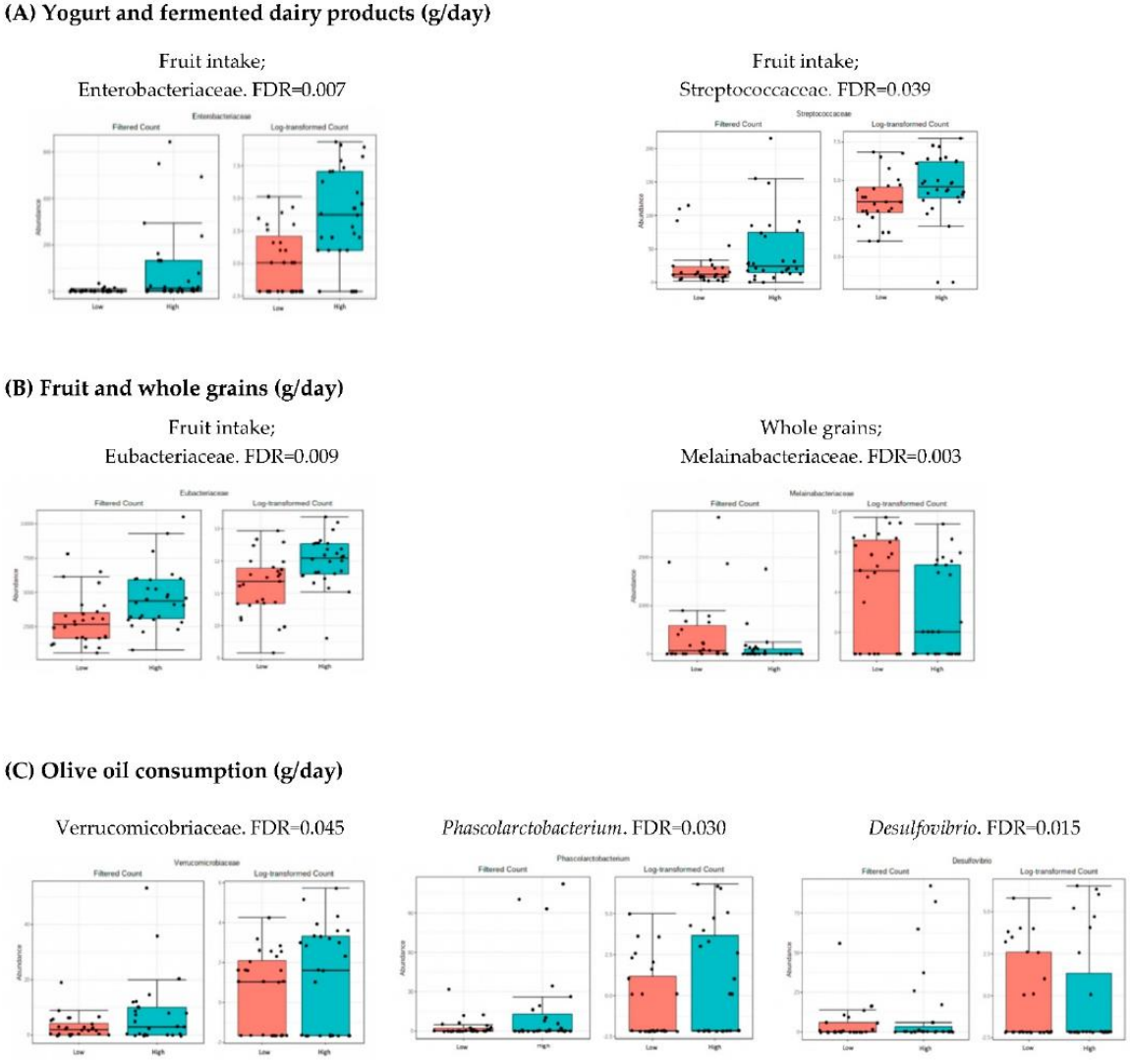

Figure 3. Bacterial taxa differing significantly when comparing individuals with higher and lower consumption of yogurt and fermented dairy (**A**), fruit and whole grains (**B**), and olive oil (**C**) (FDR < 0.05). Red boxes represent participants with lower consumption, and blue boxes represent participants with higher consumption of the specified food group.

The results in Figure 3 show higher levels of Enterobacteriaceae, Eubacteriaceae, and Streptoccaceae were observed in individuals with higher fruit consumption. Lower levels of Melainabacteriaceae were found in the group of individuals with higher consumption of whole grains. The results also show higher levels of the Lactobacillaceae family in individuals with higher consumption of yogurt and fermented dairy products. A high consumption of olive oil was related to lower amounts of *Desulfovibrio* but higher levels of *Phascolarctobacterium* and Verrucomicrobiaceae (very close taxonomically to the genus *Akkermansia*).

Individuals with higher consumption of light soda had lower abundance of the families Clostridiaceae, Methanobacteriaceae, and Dehalobacteriaceae and lower levels of the genera *Ruminococcus* and *Eggerthella*. A higher consumption of meat was related with higher levels

of *Phascolarctobacterium* but a decrease in *Oscillospira*. *Butyricicoccus*, a butyrate-producing genus, was less abundant in individuals with lower consumption of cold meat (*embutidos*).

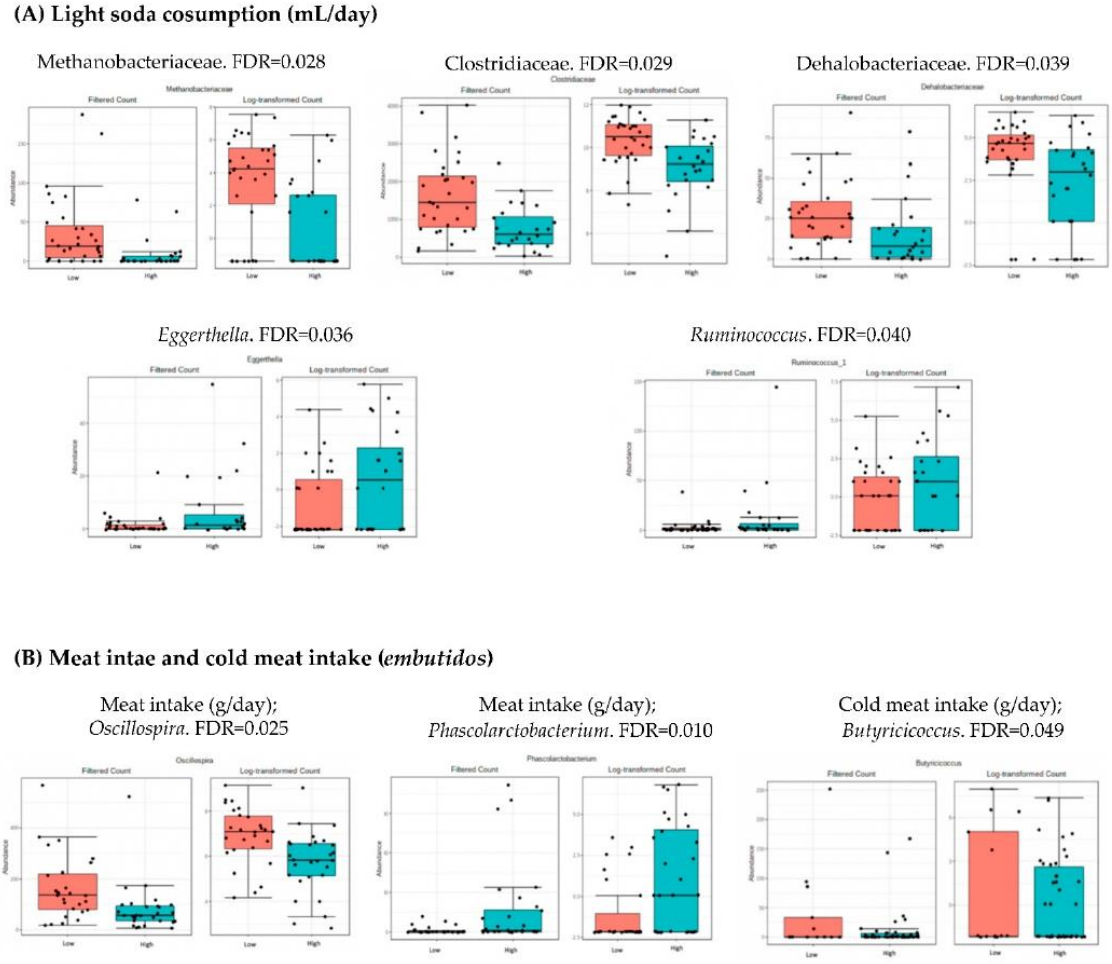

Figure 4. Bacterial taxa differing significantly in relation to the consumption of light soda (**A**) and meat and cold meat intake *(embutidos)* (**B**) (FDR < 0.05). Red boxes represent participants with lower consumption, and blue boxes represent participants with higher consumption of the specified food group compared to the median.

The whole summary of the relationship between nutritional markers and microbiota composition at Family and Genus levels are presented as Supplementary Material in Figures S2 and S3, respectively. In the studied population, the different alpha-diversity indexes were not associated with any of the nutritional markers evaluated.

4. Discussion

4.1. Analysis of Microbiota with Metabolic and Hepatic Health

In the analysis between the gut microbiota and health status, we identified biochemical markers of metabolic and hepatic health that presented strong associations with specific bacteria in the microbiome. Metabolic pathologies related to obesity, insulin resistance, inflammatory conditions and metabolic endotoxemia are highly associated with dietary and lifestyle factors, which have a crucial role in the modulation of the microbiome [16]. Some

specific bacteria have been related to metabolic benefits, and others have been related to a worse metabolic state [17]. Some metabolic diseases, particularly those related to insulin resistance and low-grade inflammation, have been associated with lower microbial diversity and dysbiosis. On the contrary, metabolic health and leanness have been associated with higher gut microbial diversity and richness [17].

Gut microbiota dysbiosis has been also implicated in the pathogenesis of liver diseases such as alcoholic and non-alcoholic fatty liver disease [18]. Concerning hepatic health, a decrease in Oscillospiraceae and Lactobacillaceae and an increase in Pasteurellaceae, *Phascolarctobacterium* and *Haemophilus* has been described in individuals with altered liver function through the analysis of AST and ALT [16–22]. Elevated levels of AST have been associated with hepatic dysfunction and higher levels of inflammation [19]. In our results, higher AST levels were related to lower abundance of *Oscillospira* and *Lactobacillus*, which are two genera that have been previously associated with health benefits in humans.

Different studies have described that metabolic pathologies are usually accompanied by gut barrier dysfunction, which may cause increased gut permeability, translocation of bacteria, and a pro-inflammatory state in the body. This state of gut dysbiosis can contribute to an increased absorption of lipopolysaccharides and metabolic endotoxemia, which has also been related to an increased risk of insulin resistance [6,17].

Our results show that some SCFA-producing bacteria, such as *Butyricicoccus,* are more abundant in participants with lower insulin resistance. This suggests that butyrate might benefit insulin metabolism. In our results, there was also an increase in the *Phascolarctobacterium* genus in individuals with higher levels of AST, as well as a significant correlation between *Phascolarctobacterium*, insulin levels and trans-fat intake. Previous evidence stated that an increase in the *Phascolarctobacterium* was associated with a decrease in bacterial diversity in patients with NAFLD, type 2 diabetes, and hepatitis B infection [23].

Critical cardiometabolic parameters and dyslipidemia (i.e., low HDL-c) have been negatively associated with higher BMI and abdominal obesity. These parameters can be modulated by dietary and lifestyle factors that contribute to changes in gut microbiota composition [7,24]. In our results, individuals with higher levels of HDL-c presented a higher abundance of Christensenellaceae, suggesting a possible relation between Christensenellaceae and lower cardiometabolic risk [25].

On the other hand, some bacteria may contribute to impair the cardiometabolic state. For example, low levels of Peptococcaceae have been associated with higher levels of HDL-c, whereas higher levels of Peptococcaceae have been positively correlated with insulin resistance [26]. Insulin resistance is associated with worse metabolic health and several cardiometabolic risk factors, such as low HDL-c [27]. Therefore, lower levels of Peptococcaceae and higher HDL-c seem to be associated with better metabolic health [28]. In our study, we have observed that Peptococcaceae was negatively associated with HDL-cholesterol.

4.2. Analysis of Microbiota and Dietary Intake

Diet and lifestyle are the most important modulators of gut microbiota composition. There are complex interactions between gut microbiota, dietary factors and the genetic background that are crucial for the development of metabolic syndrome features [29]. In this research, the consumption of specific food groups, such as fermented dairy products, meat, fat, and olive oil, was significantly associated with specific bacteria in the gut microbiome. For example, we found that a high caloric intake was associated with an increase in *Paraprevotella* genus and Enterobacteria. These results contribute to previous evidence stating that an elevated consumption of simple sugars and carbohydrates is associated with a worse metabolic state due to the impairment in epithelial integrity and increased inflammation [30].

The association between the consumption of fermented dairy products and an increase in *Lactobacilli* has been previously reported. Different strains from this bacterial group are used as probiotics and contribute to the prevention of obesity and other metabolic patholo-

gies [26,31]. In this context, we have also observed that there were higher *Lactobacillus* levels in the individuals with higher consumption of fermented dairy products and yogurt.

High consumption of meat has been associated with an increase in inflammatory markers and adiposity [32]. Our results show a decrease in the levels of *Oscillospira*, a putative beneficial genus, in the individuals with higher meat consumption, suggesting a negative correlation between *Oscillospira* and meat intake.

Extra virgin olive oil has been extensively associated with anti-inflammatory effects [33]. Our results show that olive oil consumption is associated with an increase in the abundance of Verrucomicrobiaceae, which is very close taxonomically to the genus *Akkermansia*. *Akkermansia muciniphila* has been negatively associated with overweight, obesity, hypertension, and type 2 diabetes [34].

On the contrary, a high-fat diet rich in saturated and trans fats has been associated with a pro-inflammatory state characterized by a reduction in microbial diversity, increased intestinal permeability, and lipopolysaccharide translocation [35]. In our study, individuals with higher consumption of saturated and trans fat had higher amounts of *Phascolarctobacterium*, which is a lipolytic genus that secretes extracellular esterase to break down triglycerides and its hydrolyzates [36].

5. Conclusions

This study suggests associations between gut microbiota composition, hepatic health, and insulin resistance status, where the consumption of specific foods related with inflammation features plays an important modulatory role.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu14234966/s1, Figure S1: Biochemical markers and microbiota composition: Family and Genus, Figure S2: Nutritional markers and microbiota composition: Family, Figure S3: Nutritional markers and microbiota composition: Genus.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Navarra.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Conflicts of Interest: The authors declare no conflict of interest.

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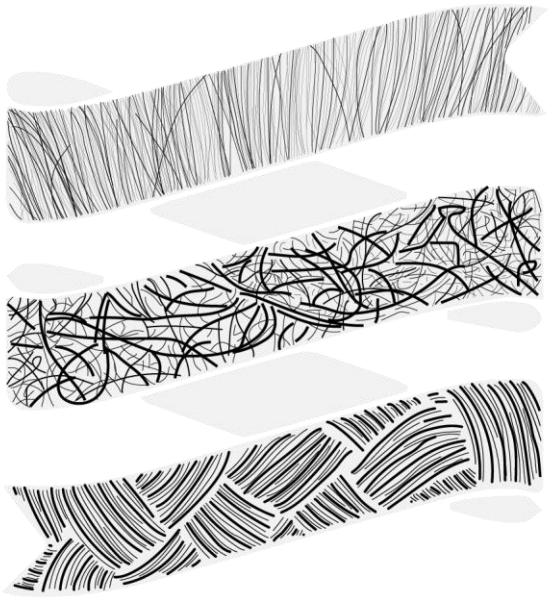

Chapter 3

An In Vitro Protocol to Study the Modulatory Effects of a Food or Biocompound on Human Gut Microbiome and Metabolome, published in the journal Foods from the MDPI editorial.

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An In Vitro Protocol to Study the Modulatory Effects of a Food or Biocompound on Human Gut Microbiome and Metabolome

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Abstract: The gut microbiota plays a key role in gastrointestinal immune and metabolic functions and is influenced by dietary composition. An in vitro protocol simulating the physiological conditions of the digestive system helps to study the effects of foods/biocompounds on gut microbiome and metabolome. The Dynamic-Colonic Gastrointestinal Digester consists of five interconnected compartments, double jacket vessels that simulate the physiological conditions of the stomach, the small intestine and the three colonic sections, which are the ascending colon, transverse colon and descending colon. Human faeces are required to reproduce the conditions and culture medium of the human colon, allowing the growth of the intestinal microbiota. After a stabilization period of 12 days, a food/biocompound can be introduced to study its modulatory effects during the next 14 days (treatment period). At the end of the stabilization and treatment period, samples taken from the colon compartments are analysed. The 16S rRNA gene analysis reveals the microbiota composition. The untargeted metabolomics analysis gives more than 10,000 features (metabolites/compounds). The present protocol allows in vitro testing of the modulatory effects of foods or biocompounds on gut microbiota composition and metabolic activity.

Keywords: microbiota; digestor; food compounds; colonic fermentation; metabolome

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1. Introduction

The outcome of the ingested food components in the human digestive system is an area attracting interest among researchers because of its relation to nutrition and health. Digestion of food is a complex combination of various physiochemical processes that disintegrate the food into more suitable forms for its absorption and transportation to related organs, and discarding the remaining waste [1]. In this context, the gut microbiota plays a key role in the host's health by metabolizing dietary compounds such as fibres and polyphenols that have a potential prebiotic capacity and is a determinant factor in the development of obesity and other diseases [2]. The diet composition determines the gut microbiota profile, and therefore the diet–microbiota interaction is crucial for synthesizing vitamins and other beneficial bioactive molecules, such as postbiotics [3]. The microbiota also plays a key role in the maintenance of the intestinal functions, modulating the immunological response and working as a barrier against certain pathogens [4]. The composition of our microbiota is influenced by host genotype, environment, and diet. A gut microbiota in a eubiotic status is characterized by a preponderance of potentially beneficial species,

belonging mainly to the two bacterial phylum, Firmicutes and Bacteroidetes [3], while potentially pathogenic species, such as those belonging to the phylum Proteobacteria (i.e., Enterobacteriaceae) are present, but in a very low percentage. In case of dysbiosis "good bacteria" no longer control the "bad bacteria" which take over [5]. The composition of the intestinal microbiota and the metabolic activity can be modulated through diet, which opens an opportunity for intervention with dietary strategies to balance the intestinal microbiota and reverse the state of dysbiosis. Additionally, the characterization of new beneficial molecules that are generated by the microorganisms during the colonic fermentation of foods, which are called postbiotics [6]. Increased interest in modifying the matrix and structural characteristics of foods to optimize their digestion, absorption and particularly the microbiota profile (related to health benefits), requires implementation of many food digestion studies in the digestive tract; therefore, it is essential to design an in vitro protocol for analysing the effect of foods and dietary bioactive compounds on the microbiota profile and metabolite production.

The main objective of the present study was to standardize a protocol that allows testing, in vitro, of the modulatory effects of foods or biocompounds on gut microbiota composition and metabolic activity.

2. Material and Methods

2.1. In Vitro Digestion Models

In vitro models have been developed since the 1990s to be used in food digestion studies. The models can help to design novel food products by estimating the in vivo behaviour after meals [7]. Many of these models are designed to work in static conditions, and therefore cannot reproduce the dynamic conditions that occur in the digestive system. Furthermore, these models have limitations for predicting food behaviour and nutrient bioavailability [8]. The interest that has arisen in the last years about the gut microbiota physiology has obliged to design new in vitro models more centred on colonic fermentation. In this context, very few dynamic in vitro models mimic the mechanic, dynamic and chemical conditions of the human digestive system. Additionally, multi-compartmental systems usually have gastric and small intestinal compartments, but very few include the colon, which is essential to study of the gut microbiome and metabolome. Dynamic models can simulate the change in pH, enzyme secretion, peristaltic forces and microbial fermentation continuously [9].

2.2. Description of the Dynamic Gastrointestinal and Colonic Fermentation Model

The equipment simulates in vitro the entire gastrointestinal digestive process. It consists of a computer-assisted model of five interconnected compartments, double jacket vessels, which simulate the physiological conditions of the stomach (R1), the small intestine (R2) and the three colonic sections: the ascending colon (R3), transverse colon (R4) and descending colon (R5). This model was developed by AINIA (Valencia, Spain) [10] based on the work of Van de Wiele et al. [11] and Marzorati et al. [12]. R1 and R2 work semi-continuously, while the colon reactors (R3, R4 and R5) work continuously. A peristaltic bomb ensured the flow of the content from one reactor to the next. The system did not simulate water absorption.

The volumes and transit times for each region of the gastrointestinal tract were: 260 mL for 2 h in R1, 410 mL for 6 h in R2 and the colon, 1000 mL for 20 h in R3, 1600 mL for 32 h in R4, and 1200 mL for 24 h in R5. [13–15]. The temperature was kept at 37 °C during the entire process. An anaerobic environment was maintained, and gaseous N_2 was flushed for 15 min twice a day.

Gastric digestion was simulated by continuous addition of a 0.03% (*w*/*v*) pepsin solution (2100 units/mg) during 2 h (total volume of 60 mL). A typical gastric digestion pH curve (based on in vivo data) was simulated by adding a 1 M HCl solution. The gradual decrease in the pH of the stomach to pH 2 was carried out. The pH control was carried out by adding HCl. HCl secretion to control pH is based on the pH curve reported by Conway

et al. (1987), which represents the pH profile in the stomach after human volunteers consumed yogurt [16]. Digestion of the small intestine was mimicked by the continuous addition of a pancreatin solution (0.9 g/L), NaHCO3 (12 g/L) and oxgall dehydrated fresh bile (6 g/L) in distilled water (total volume of 440 mL), maintaining the intestinal content at pH 6.5.

2.3. Faecal Inoculum

Fresh faeces from human subjects were used to reproduce the gut conditions. A list of characteristics can be added in the recruitment procedure depending on the aim of the experiment. As the faeces profile determines the characteristics of the microbiota reproduced in the digestor, especially when a dysbiosis or normobiosis environment is the study aim, this parameter should be considered. In this case, the inoculum was prepared using faeces from four adult volunteers with pathologies associated with obesity and/or metabolic syndrome (BMI 35–40; age 30–50), non-smokers, no history of antibiotic treatment in the last three months, and no intestinal disease background [17–19].

Faecal samples were collected and maintained in special anaerobic plastic bags (BD GasPak™ systems). Faeces were diluted with thioglycolate 20% (*w*/*v*) and homogenized with a stomacher to obtain a faecal slurry. The faecal suspension was centrifuged at 3000 g for 3 min and the collected supernatant was immediately inoculated in the colon vessels (50, 80 and 60 mL for R3, R4 and R5, respectively). The reactors were filled with culture medium up to a total volume of 1000, 1600 and 1200 mL, respectively. The composition of the culture medium followed Molly et al. (1993, 1994) [20,21], providing the necessary nutritional components to simulate the conditions of the human colon and allowing the intestinal microbiota to grow. Each reactor was maintained at different optimal pH levels. The bacteria present in each region of the colon have an optimal pH of action: pH 5.5–6 in the ascending colon (R3); pH 6–6.4 in the transverse colon (R4) and pH 6.4–6.8 in the descending colon (R5). In order to regulate the pH changes that occur during fermentation and maintain them in the optimal intervals for each region, acid or base were added.

2.4. Process Description and Duration

After faecal inoculation, a stabilization period of 12 days was required to allow bacteria to grow and reach stable levels [22,23]. During this period, 200 mL of cultured medium was added to the stomach (R1) three times a day. At this point, the system was ready to start the sample treatment period to study its modulation effects on the gut microbiota [10,11]. The treatment period was conducted by feeding the equipment with sample once a day (in culture medium up to 200 mL) and with 200 mL of cultured medium twice a day. The treatment period was 14 days. The maintenance of the microbial population during the stabilization (time 12) and treatment period (time 14) was checked by bacteria plate counts. The following bacterial groups were quantified by growth on specific medium, expressing the result as CFU/mL of colonic medium: *Lactobacillus* (MRS agar; the MALDI-TOF technique was employed to verify lactobacilli colonies), *Bifodobacterium* (TOS-propionate agar enriched with MUP), Enterobacteriaceae (VRBD agar), *Clostridium* (TSC Agar enriched with cycloserin) and total anaerobic bacteria (Agar Schaedler). Then, 10 mL of the samples was taken from each reactor (R3, R4 and R5) and serially diluted in saline solution. Plates were inoculated with 1-mL sample of four serial dilutions by duplicate and incubated at 37 °C under aerobic or anaerobic conditions. In addition, samples were taken from each reactor at the end of the stabilization and treatment period and stored at −20 °C to determine the short chain fatty acid content in the colonic media and to conduct the metabolomic analysis, 10 mL and 3 mL, respectively. For the metabolomic analysis, these samples were centrifuged (15,000× *g*, 15 min) and filtered through a 0.22-µm-Ø Millipore filter (Billerica, MA, USA) into vials for UHPLC-ESI-QqQ-MS/MS analysis. In addition, 1.5 mL of the inoculum, as well as samples from each reactor at the end of the stabilization and in the treatment period were collected at baseline using OMNIgene.GUT kits from

DNA Genotek (Ottawa, ONT, Canada), according to the standard instructions provided by the company.

2.5. Description and Volume of the Product to Be Tested

A vegetable drink based on oats, fruit, vitamins B2, B5, B12 and D2, iodine, calcium, beta-glucans and postbiotics, with a 6% sugar content, was pasteurized to ensure a viability of, at least, 15 days. It was stored at −20 °C. The dosage administrated during the treatment was about 100 mL/day, once a day. The product was provided by the company AMC Natural Drinks, a partner of the BIOTAGUT Project (Modulation of the microbiome and postbiome by the intelligent design of food promoters of a healthy microbiota in relation to metabolic syndrome), which is supported by the Ministry of Science, Innovation and Universities of Spain, through CDTI (Centre for Industrial Technological Development, Madrid, Spain).

2.6. Short Fatty Acid Analyses

Short and medium chain fatty acids were extracted from the sample using a liquid–liquid extraction with diethyl ether. The resulting extract was filtered and subsequently analysed using a AS 800 C.U. gas chromatograph (CE Instruments, Wigan, United Kingdom) equipped with a HP-FFAP 25 m × 0.2 mm × 0.33 mm column (Agilent Technologies) and a flame-ionization detector (FID). The samples were quantified by interpolation in the calibration curve using capric acid as an internal standard. The concentration of the fatty acids was provided directly by the software using a 1/× linear regression. The results were expressed as mg of compound per Kg of colonic medium.

2.7. DNA Extraction

The DNA was extracted with the QIAamp® DNA kit (Qiagen. Hilden, Germany) following the manufacturer's protocol [24].

Microbiota composition according to 16S rRNA analysis with Next Generation Sequencing (NGS).

2.8. Metagenomic Data: Library Preparation

Metagenomics studies were performed by analysing the variable regions V3–V4 of the prokaryotic 16S rRNA (ribosomal Ribonucleic Acid) gene sequences, which gives 460 bp amplicons in a two-round PCR protocol.

In a first step, PCR is used to amplify a template out of a DNA sample using specific primers with overhang adapters attached to the flank regions of interest. The full-length primer sequences, using standard IUPAC (International Union of Pure and Ap-plied Chemistry) nucleotide codes, were: Forward Primer: 5′TCGTCGGCAGCGTCAGATGTGTATAAG AGACAGCCT ACGGGNGGCWGCAG; and Reverse Primer: 5′GTCTCGTGGGCTCGGAG ATGTG TATAAGAGACAGGACTACHVGGGTATCTAATCC. PCR was performed in a thermal cycler using the following conditions: 95 °C for 3 min, 25 cycles of (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and 72 °C for 5 min.

To verify the amplicon, 1 µL of the PCR product was checked in a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, USA). The expected size on a Bioanalyzer was ~550 bp.

In a second step and using a limited-cycle PCR, sequencing adapters and dual index barcodes, Nextera® XT DNA Index Kit, FC-131-1002 (Illumina, San Diego, CA, USA), were added to the amplicon, which allows up to 96 libraries pooled together for sequencing in the MiSeq sequencer with the MiSeq® Reagent Kit v2 (500 cycle) MS-102-2003 to be pooled together.

The PCR was performed in a SimpliAmp thermal cycler (Applied Biosystems®, San Francisco, CA, USA) using the following conditions: 95 °C for 3 min, eight cycles of (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and 72 °C for 5 min. Subsequently, the Index

PCR ran a second Bioanalyzer DNA 1000 chip to validate the library. The expected size was ~630 bp.

The next step consisted of quantifying the libraries using a Qubit® 2.0 fluorometer (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA), and dilution of the samples before pooling them.

Finally, paired-end sequencing was performed in a MiSeq platform (Illumina) with a 500-cycle Miseq run and with 7 pM sample and a minimum of 25% PhiX. The mean reads obtained were 164,387. Only samples with more than 40,000 reads were used for further analysis [25].

2.9. Data Analysis

For the Short-chain fatty acid (SCFA) production and the bacterial growth analysis, a Mann–Whitney U Test was applied for differences between two groups on a single, ordinal variable with no specific distribution [26]. These analyses were carried out using GraphPad Prism version 6.0c for MAC OS X, GraphPad Software, San Diego, California USA, www.graphpad.com (accessed on 13 September 2021).

2.10. Metagenomic Data: Analysis and Processing

The 16S rRNA gene sequences obtained were filtered following the quality criteria of the OTU (operational taxonomic units) processing pipeline LotuS (release 1.58) [23]. This pipeline includes UPARSE (Highly accurate OTU sequences from microbial amplicon reads) de novo sequence clustering and removal of chimeric sequences and phix contaminants for identifying OTUs and their abundance matrix generation [27,28]. The taxonomy was assigned using HITdb (Highly scalable Relational Database), achieving up to species sensitivity. BLAST (Basic Local Alignment Search Tool) was used when HITdb failed to reach a homology higher than 97% [29,30]. Thus, OTUs with a similarity of 97% or more were considered as species by themselves. However, OTUs that did not reach this percentage of similarity were checked and updated using the Basic Local Alignment Search Tool (BLASTn), comparing them with the 16S rRNA gene sequences of the bacteria and archaea database of GenBank of the National Center for Biotechnology Information to obtain an assignment to a species. The sequences in which the BLASTn tool found a new assignment were indicated using the GenBank access number and the percentage of homology following the species name. The abundance matrices were first filtered and then normalized in R/Bioconductor at each classification level: OTU, species, genus, family, order, class, and phylum. This study focused mainly on the species level. Briefly, taxa with less than 10% frequency in our population were removed from the analysis, and a global normalization was performed using the library size as a correcting factor and log2 data transformation [31].

2.11. Richness and Evenness

Richness was defined as the total of species. Evenness, defined as the alpha diversity index, was calculated using the Shannon index [32] according to the following formula:

$$H = -\text{sum}(Pi \ln[Pi]) \quad (1)$$

2.12. Untargeted Metabolomics

The chromatographic analysis was performed with a high-resolution liquid chromatogram (HPLC) from Agilent (model 1100). The detector used was a TOF (Time of Flight) Mass Accuracy from Agilent, model 6220. The stationary phase was a chromatographic column, Zorbax SB-C18 (Agilent Technologies) of 150 × 46 mm and 5 µm pore size. The column temperature was kept at 40 °C. The mobile phase was made of milliQ with 0.1% formic acid (canal A) and methanol with 0.1% formic acid (canal B). The system worked with a gradient, screening the entire polarity range from 100% of A until 100% of B. The injected volume was 15 µL.

The TOF detector is made of an electrospray ionized source (ESI). The flow of gas drying was 10 L/min, at a 40 psig pressure and a temperature of 350 °C. The capillary voltage was 4000 V, the fragmentor voltage was 175 V, and the skimmer voltage 65 V. The relation m/z range, for the detection, was between 100 and 2000 and the acquisition ratio was 1.03 spectre/sec.

All samples coming from the digestor were analysed following the same sequence. The samples were analysed in positive polarity (POS group) and negative polarity (NEG group). To apply this process, sample duplicates were needed to perform the two analyses.

To analyse the results, an alignment with XCMS Online (The Scripps Research Institute, La Jolla, CA 92037, United States) software was carried out according to the relation m/z (mass/charge) and the retention time. Raw data were normalized by logarithmic transformation followed by Pareto scaling. Compounds with the same m/z relation and the same retention (5 mDa tolerance for the m/z relation; 0.5 min for the retention time) were considered the same.

Later, comparisons of two vs. two were carried out, for example: R3 T12 POS vs. R3 T14 POS and R3 T12 NEG vs. R3 T14 NEG. The same with the three reactors. These comparisons show the impact of the chosen treatment on the microbiota of the in vitro digestor. The XCMS Online software gave features (metabolites/compounds) detected from each chromatogram, showing more than 10,000 features. Finally, the MetaboAnalyst software (Xia Lab of McGill University, Quebec, Canada) processed the list file using statistical multivariable tools, such as Partial last squares regression (PLS-DA), *Random Forest* and Volcano Plot. Outliers were excluded through the *Random Forest* model, followed by the other statistical tools mentioned above. With these tools, features with more discriminating outputs were chosen between the two groups of the polarity group:

- Volcano Plot: p-value < 0.001 and Fold change >10.0 (100.0 for negative polarity).
- PLSDA: Value of Variable Importance in Projection (VIP) >3.0

Subsequently, the features that passed the selection criteria were identified through the database Metlin (Scripps Research Institute).

Certain conditions must be established to set the tolerance limit: 5 mDa in relation to the m/z ratio (specifically the mean m/z relation of the csv file, generated from the XCMS Online). For the positive group, the marked ions were $[M+H]^+$, $[M+Na]^+$ and $[M+H-H2O]^+$. For the negative group, the ions were $[M-H]^-$ and $[M-H2O-H]^-$. In both cases, those metabolites classified as toxic were taken out of the database [33].

3. Results and Discussion

In the colonic fermentation studies, two different phases exist: the stabilization part, which aims to create a stable microbiome from the faecal inoculum, and is considered as the control, and the treatment one, where the purpose is to analyse the behaviour of the stabilized microbiota under the effects of the chosen food or biocompound, added after the stabilization period is over. During both periods, samples are taken from the colonic digestors, allowing the comparation between the metabolites production by the microbiota itself (stabilization period), and due to the chosen food or biocomppound added (treatment phase).

The test results showed a stabilization of the faecal microbiota during the first period (12 days) in R3, R4 and R5. The recovered levels (for each ml of cultured medium) of total anaerobic microorganisms were above 10^7 cfu in all three sections. During the treatment period (14 days after reaching the stabilization point), 100 mL of the vegetable drink were added every day. In this period, the total anaerobic microorganisms remained stable in the three sections. According to the total anaerobic level, it was concluded that the digestor conditions were favourable for the development of the intestinal microbiota during the two weeks while the product was being tested. R3 shows a stabilization of the anaerobic bacteria while R4 and R5 show a very significant increase ($p < 0.025$). Furthermore, bacteria from the Enterobacteriaceae family, including potentially pathogenic species, show a very significant ($p < 0.025$) reduction in all reactors. These results are shown in Table 1 and Figure 1.

Table 1. Microbiological count of the three colonic reactors during the fermentation of the vegetable drink. R3T12 represents the end of the stabilization period and R3T14 the end of the treatment period. Results are shown as log (CFU/mL).

	Days	Bifidobacterium	Lactobacillus	Enterobacteria	Clostridium	Total Anaerobic
R3T12	5	7.48 ± 0.05	6.19 ± 0.04	5.4 ± 0.01	1.00 ± 0.02	7.53 ± 0.11
	8	5.59 ± 0.03	5.5 ± 0.57	5.48 ± 0.01	1.00 ± 0.02	8.2 ± 0.07
	12	6.16 ± 0.01	4.31 ± 0.01	7.49 ± 0.12	1.00 ± 0.01	8.05 ± 0.09
R3T14	2	4.00 ± 0.02	3.2 ± 0.02	5.43 ± 0.07	1.00 ± 0.01	5.88 ± 0.11
	7	4.59 ± 0.11	6.55 ± 0.01	5.42 ± 0.04	1.00 ± 0.02	6.66 ± 0.19
	10	4.25 ± 0.02	6.66 ± 0.08	4.98 ± 0.04	1.00 ± 0.01	6.62 ± 0.23
	14	5.45 ± 0.01	6.68 ± 0.21	6.48 ± 0.01	1.00 ± 0.01	7.97 ± 0.06
R4T12	5	7.97 ± 0.08	7.79 ± 0.07	6.04 ± 0.02	4.37 ± 0.07	8.34 ± 0.12
	8	6.60 ± 0.06	6.37 ± 0.78	6.42 ± 0.08	3.25 ± 0.08	8.4 ± 0.09
	12	5.93 ± 0.05	4.87 ± 0.24	6.74 ± 0.19	2.25 ± 0.05	7.37 ± 0.08
R4T14	2	4.30 ± 0.02	3.89 ± 0.05	5.16 ± 0.12	1.93 ± 0.09	7.65 ± 0.09
	7	4.60 ± 0.13	5.85 ± 0.07	4.34 ± 0.26	1.00 ± 0.02	7.9 ± 0.07
	10	5.20 ± 0.02	6.02 ± 0.04	5.16 ± 0.05	1.00 ± 0.01	7.73 ± 0.05
	14	5.91 ± 0.03	5.48 ± 0.02	4.35 ± 0.07	1.00 ± 0.01	8.20 ± 0.05
R5T12	5	7.95 ± 0.02	7.48 ± 0.02	6.85 ± 0.02	6.46 ± 0.01	8.28 ± 0.04
	8	7.06 ± 0.02	7.14 ± 0.25	6.34 ± 0.01	5.33 ± 0.04	7.97 ± 0.02
	12	5.66 ± 0.07	5.19 ± 0.07	6.16 ± 0.02	3.88 ± 0.05	7.63 ± 0.07
R514	2	4.00 ± 0.03	4.61 ± 0.20	5.09 ± 0.03	3.46 ± 0.03	7.66 ± 0.09
	7	4.72 ± 0.09	6.22 ± 0.01	4.27 ± 0.05	2.41 ± 0.01	7.8 ± 0.07
	10	4.62 ± 0.07	5.99 ± 0.04	5.30 ± 0.66	3.06 ± 0.02	7.91 ± 0.03
	14	5.46 ± 0.02	6.16 ± 0.02	4.93 ± 0.16	2.46 ± 0.08	8.11 ± 0.05

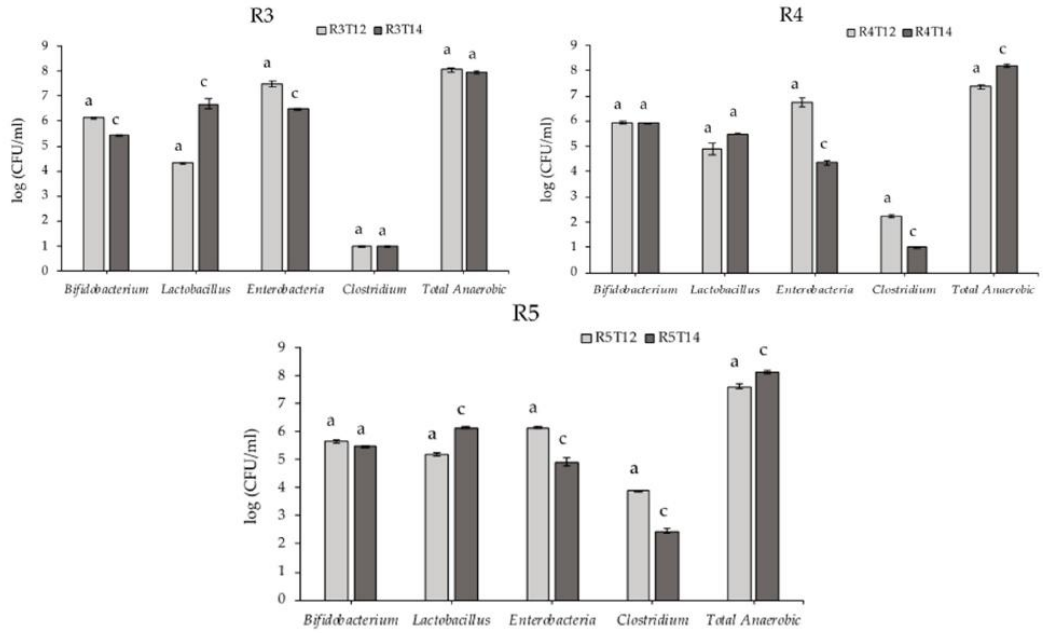

Figure 1. Microbiological count of the three colonic reactors: (**R3**, **R4** and **R5**) before (T12) and after the treatment (T14) ($n = 2 \pm$ SD). A Mann–Whitney U Test was applied for differences between two groups on a single, ordinal variable with no specific distribution. Different letters mean statistically significant differences: a, no significant difference ($p > 0.05$) and c, for a very significant difference ($p < 0.025$). Results are shown as log (CFU/mL).

Short-chain fatty acid (SCFA) production showed a clear tendency towards increased levels of acetic and butyric acids in the three sections after the treatment (Table 2). Propionic acid level showed a very significant ($p < 0.025$) increase after the treatment period in the three reactors (Figure 2).

Table 2. SCFA production in the three reactors after the stabilization period (T12) and the treatment period (T14) (mg/Kg of colonic medium).

	Acetic Acid	Butyric Acid	Propionic Acid
R3T12	999 ± 98	441 ± 37	258 ± 17
R3T14	1281 ± 127	597 ± 54	1543 ± 116
R4T12	2157 ± 226	561 ± 52	509 ± 40
R4T14	2267 ± 222	766 ± 69	2531 ± 117
R5T12	2155 ± 209	646 ± 59	509 ± 42
R5T14	2645 ± 238	835 ± 76	2684 ± 196

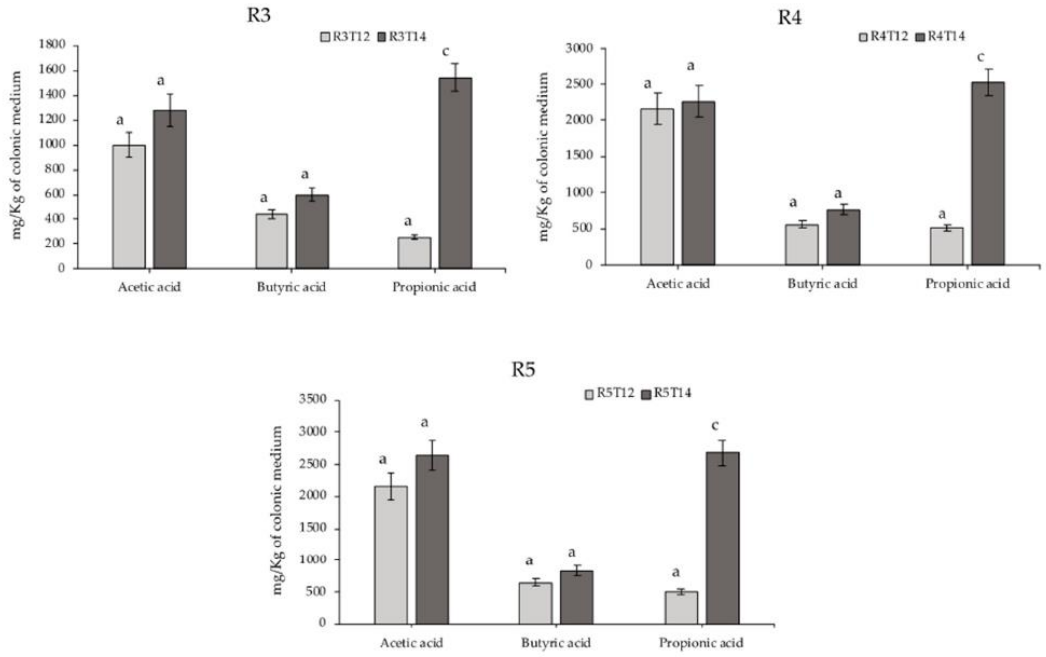

Figure 2. Representation of the SCFA production on the different reactors: (**R3**, **R4** and **R5**) before (T12) and after the treatment (T14) ($n = 2 \pm$ SD). A Mann–Whitney U Test was applied for differences between two groups on a single, ordinal variable with no specific distribution. Different letters mean statistically significant differences: a, no significant difference ($p > 0.05$) and c, a very significant difference ($p < 0.025$).

The metagenomic analysis showed an increase in some bacterial species, (Figure 3), especially butyrate-producer species such as *Alistipes putredinis* and *Eubacterium desmolans*. Other SCFA-related species (*Clostridium lactatifermantans* and *Phascolarctobacterium succinatutens* (propionic)) also increased their levels; these results are shown in Table 3 and Figure 3. Results on the genus rank show a growth of some genera related to SCFA production, such as *Anaerotruncus* [34], *Cloacibacillus* [35], and *Parasutterella* [36]. The genus *Acidaminococcus* [37] has attracted scientific interest due to its high resistance to antibiotics. These results are shown in Table 4.

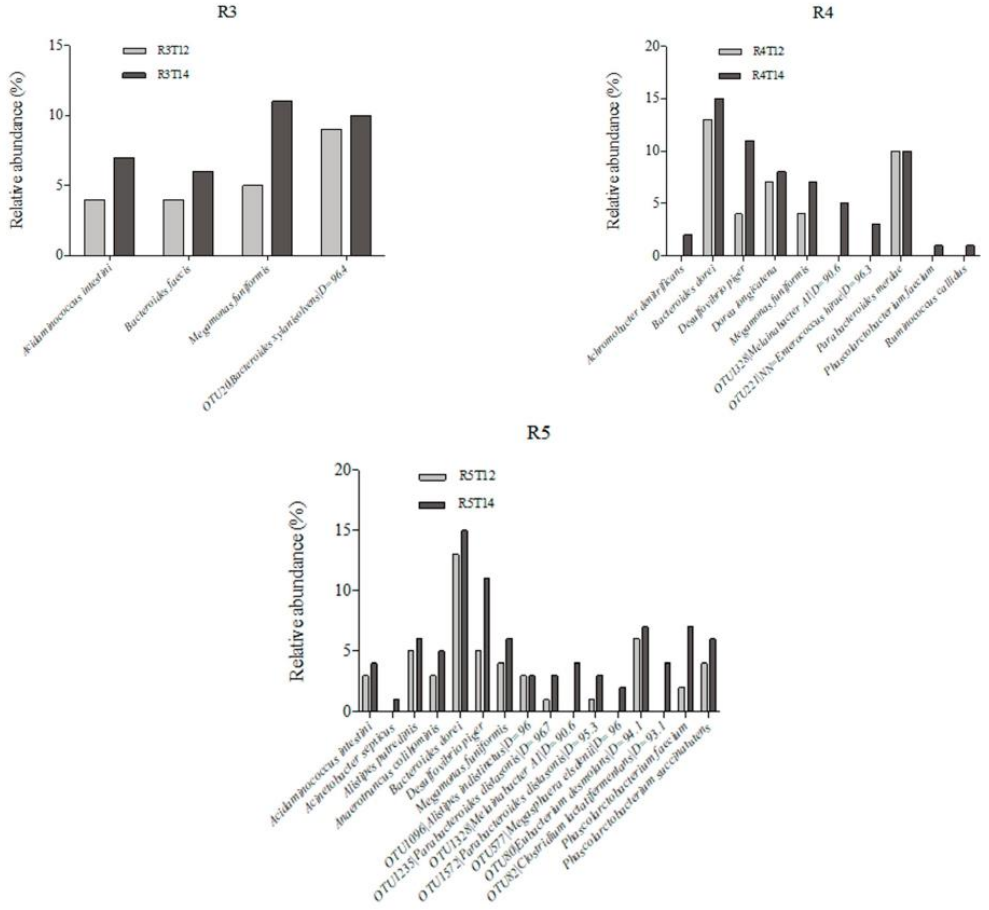

Figure 3. Representation of the bacteria species relative abundance on the different reactors: (**R3**) ascending colon, (**R4**) transversal colon, (**R5**) descending colon, before (T12) and after the treatment period (T14) with the biocompound.

Table 3. Bacterial species abundance in the three colonic sections. R3T12 represents the end of the stabilization period and R3T14 the end of the treatment period. Values represent relative abundance %.

R3	R3T12	R3T14	R5	R5T12	R5T14
Acidaminococcus intestini	4.45	6.96	*Acidaminococcus intestini*	2.79	3.88
Bacteroides faecis	3.63	5.56	*Acinetobacter septicus*	0.00	0.94
Megamonas funiformis	4.68	11.17	*Alistipes putredinis*	5.32	6.29
OTU20 | *Bacteroides xylanisolvens* | D = 96.4	9.38	10.14	*Anaerotruncus colihominis*	3.36	4.74
R4	R4T12	R4T14	*Bacteroides dorei*	13.09	14.85
Achromobacter denitrificans	0.00	1.87	*Desulfovibrio piger*	4.85	10.99
Bacteroides dorei	12.98	14.78	*Megamonas funiformis*	3.86	6.03
Desulfovibrio piger	3.56	11.21	OTU1096 | *Alistipes indistinctus* | D = 96	2.60	3.06
Dorea longicatena	7.32	7.60	OTU1235 | *Parabacteroides distasonis* | D = 96.7	0.88	2.70
Megamonas funiformis	4.15	6.83	OTU1328 | *Melainabacter* A1 | D = 90.6	0.00	4.27
OTU1328 | *Melainabacter* A1 | D = 90.6	0.00	4.78	OTU1572 | *Parabacteroides distasonis* | D = 95.3	0.88	3.47
OTU221 | *Enterococcus hirae* | D = 96.3	0.00	2.65	OTU577 | *Megasphaera elsdenii* | D = 96	0.00	1.91
Parabacteroides merdae	9.95	10.03	OTU80 | *Eubacterium desmolans* | D = 94.1	5.76	6.82
Phascolarctobacterium faecium	0.00	0.91	OTU82 | *Clostridium lactatifermentans* | D = 93.1	0.00	4.47
Ruminococcus callidus	0.00	0.91	*Phascolarctobacterium faecium*	2.39	7.08
			Phascolarctobacterium succinatutens	4.29	5.85

Table 4. Bacterial genus abundance in the three colonic sections. R3T12 represents the end of the stabilization period and R3T14 the end of the treatment period. Values show relative abundance %.

Tax	Inoculation	R3T12	R3T14	R4T12	R4T14	R5T12	R5T14
Acidaminococcus	0.00	4.45	6.96 ^	3.03	2.65 *	3.24	3.88 ^
Acinetobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.94 ^
Anaerotruncus	2.22	0.00	0.00	3.03	0.00 *	3.36	4.74 ^
Bacteroides	12.83	15.16	10.62 *	14.90	14.98 ^	14.79	14.94 ^
Chryseobacterium	0.00	0.00	6.77 ^	0.00	0.00	0.00	0.00
Cloacibacillus	0.00	0.00	0.00	0.00	0.91 ^	0.00	3.47 ^
Desulfovibrio	9.04	0.00	0.00	3.56	11.21 ^	4.85	10.99 ^
Dorea	8.05	0.00	0.00	7.32	7.60 ^	8.63	6.64 *
Enterobacter	0.00	7.07	10.03 ^	4.64	4.95 ^	4.41	5.30 ^
Enterococcus	0.00	3.10	0.00 *	0.00	3.16 ^	0.00	0.94 ^
Lachnoclostridium	11.62	1.54	0.00 *	10.65	10.72 ^	11.07	11.69 ^
Lachnospira	10.64	0.00	4.99 ^	7.65	0.91 *	8.38	3.59 *
Lysinibacillus	0.00	11.20	11.20 ^	6.63	5.78 *	2.79	7.06 ^
Megamonas	0.00	4.68	11.23 ^	4.15	6.85 ^	3.86	6.07 ^
Megasphaera	0.00	5.86	8.20 ^	4.05	3.74 *	3.48	4.52 ^
[Melainabacter]	7.09	0.00	0.00	1.14	10.04 ^	0.00	9.24 ^
Olsenella	2.22	0.00	0.00	0.00	0.00	0.00	1.91 ^
Parabacteroides	9.59	0.00	0.00	9.95	10.03 ^	10.42	8.67 *
Parasutterella	4.99	0.00	0.00	1.14	3.74 ^	2.60	4.40 ^
Phascolarctobacterium	8.22	0.00	0.00	0.00	0.91 ^	4.57	7.59 ^
Propionibacterium	0.00	0.00	0.00	0.00	0.00	0.00	1.50 ^
Tyzzerella	6.89	0.00	0.00	5.84	0.00 *	5.19	6.32 ^

^: Bacterial genus increase; *: Bacterial genus decrease.

A total of 219 bacterial species were obtained in this experiment. The result of alpha diversity assessed using the Shannon index showed an increase at R3 with the treatment, however not at R4 and R5, where it decreases (Table 5)

Table 5. Bacterial diversity numeric classification of the inoculum and the three reactors. T = 12 represents the end of the stabilization period and T = 14 the end of the treatment period.

SW Div	Inoculation	R3	R4	R5
T = 12	3.87	2.03	3.51	3.55
T = 14		2.10	2.61	3.02

The metabolomic analysis showed a dominance of peptides consisting of up to four amino acids, phenolic compounds, phosphatidylcholines, fatty acids and terpenoids (Table 6). For example, in negative polarity, several interesting metabolites increased their levels. In R3, there are some catechin derivatives and an isoflavone; in R4, a metabolite related to vitamin D3; in R5, beta-carotenes or lycopene; and in R3 and R5, there is a terpenoid. In positive polarity, there is an increase in choline in R4. Figure 4 shows the PLS-DA Score Plots obtained in the comparisons among all groups in positive (A) and in negative (B) polarity.

Table 6. Some metabolites whose levels changed in the three reactors.

m/z	Tr: (min)	Reactor (Polarity)	Intensity T = 12 (Days)	Intensity T = 14 (Days)	Putative Metabolites
415.1389	5.64	R3 (neg)	4.19	6.14	Heptamethoxyflavanone/Eleganin
367.1145	5.75	R3 (neg)	4.19	5.93	Barpisoflavone/Glisoflavone
401.3422	5.81	R4 (neg)	3.27	5.68	Hydroxy-dihydrovitamin D3
517.4152	7.22	R5 (neg)	3.54	5.62	Carotene/Lycopene
104.1036	2.82	R4 (pos)	5.60	7.13	Choline

Tr: retention time; intensity expressed on a logarithmic scale.

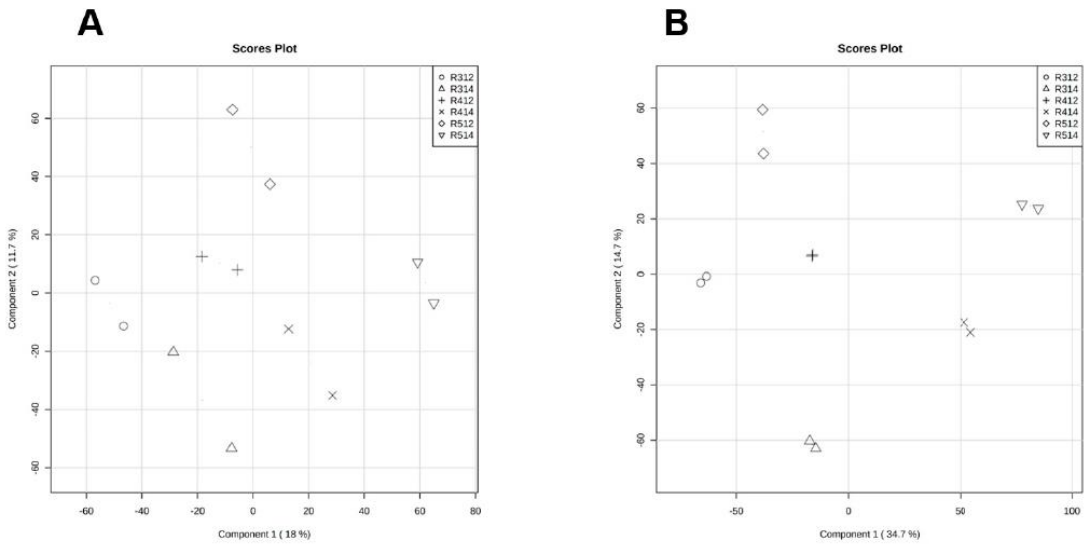

Figure 4. Representation the PLS-DA Score Plots obtained in the comparisons among all groups in positive (**A**) and in negative (**B**) polarity.

With this model, mechanic, dynamic and chemical conditions of the human digestive system can be outlined, including transit times of ingested meals, pH profiles, temperature, contractions, and peristaltic movements, digestive secretion rates, and absorption of water and nutrients. Moreover, it also allows analysis of the microbiota fluctuation and the metabolites produced along the whole process. However, as it is mainly designed for studying the effects of foods on microbiota composition and gut metabolites, it does not take into account nutrient absorption and gut leak. The main differences of the intestinal fermentation protocol established in this article compared to other similar protocols (SHIME, TIM-2, SIMGI, . . .) previously published [1,38] are:

The system can adapt the gastrointestinal transits and the addition of digestive secretions to the state of fasting or fed to study the behaviour of the dosage form of bioactive compounds and food.

It presents great versatility since it allows reproduction of the physiological conditions of human digestion (digestive secretions, pH and gastric and intestinal transits, etc.) for different population groups (children, adults, elderly,...) and certain pathological situations (obesity, etc.), as well as reproducing the gastrointestinal and colonic conditions of other monogastric animals (pig,...).

The intestinal absorption of the digested food can be carried out by using intestinal cell lines in combination with the colonic fermentation system.

It allows maintenance of the anaerobic conditions required in the fermentation process, to protect the oxygen-sensitive compounds involved in the colonic fermentation.

The design allows collection of samples of the luminal content of the three sections of the colon at any time during the fermentation process of the food for analysis (metagenomic, metabolomic, SCFA,...) and to obtain information on the transformation of the food during digestion.

It incorporates a computer program for the automated control of different physiological conditions applied in colonic fermentation assays.

The characteristics of the system and the computer-control of physiological parameters open possibilities for variation of conditions that would allow the simulation of microbial dysbiosis associated to pathological conditions or due to unbalanced diets.

4. Study Limitations

All the data in this article come from an industrial project formed by a consortium of food sector companies. Each in vitro digestor test takes a month to complete and every metagenomic analysis represents a large part of the budget, so there are not enough resources to replicate the process more than one time for each product (food or biocompound). That is why there are not enough data to perform statistical analysis regarding the bacteria species relative abundance on the different reactors, in Figure 3.

5. Conclusions

Combining the in vitro colonic fermentation with 16S rRNA-based metagenomic analysis with Next Generation Sequencing (NGS) and the UHPLC-ESI-QqQ-MS/MS metabolomic analysis provides an appropriate methodology for reproducing the microbiota environment under diverse conditions (eubiosis or dysbiosis) to study the potential modulatory effects of bioactive compounds, ingredients or foods. The in vitro colonic fermentation makes it possible to reproduce the typical microbiota under dysbiosis conditions, whereas the 16S rRNA protocol allows characterization of the change in microbiota composition up to species level. In addition, the deep chemical characterization of the colonic medium allowed ascertainment of the main metabolites generated among a wide range of determined compounds. The capacity of the proposed protocol was corroborated with the studied sample. The treatment with the vegetable drink produced changes in the microbiota. Although there was no clear improvement in the diversity, the population of some bacteria increased, such as some SCFA-producing bacteria that produce propionate, acetic and butyrate. The metabolomic analysis showed an increased production of some compounds that may ameliorate health status. In conclusion, we present an interesting protocol for testing of the modulatory effects of foods or biocompounds on gut microbiota composition and metabolic activity

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of University of Navarra (protocol code 132/2015).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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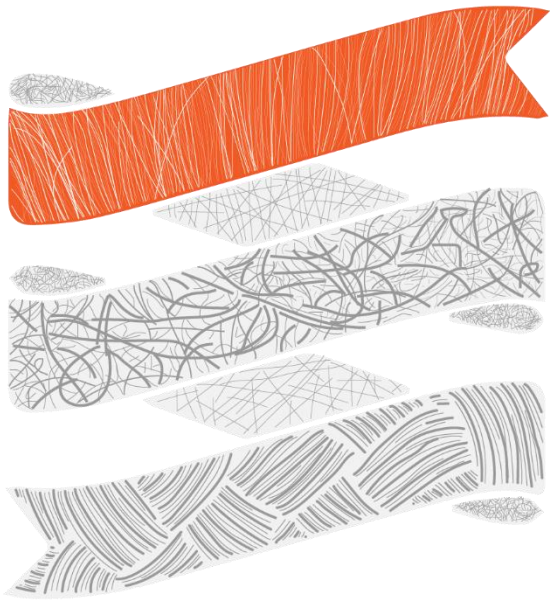

Chapter 4

Elicited butternut pumpkin (Cucurbia moschata D. cv. Ariel) as a natural dietary modulator of the human intestinal microbiota dysbiosis.

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Elicited butternut pumpkin (*Cucurbita moschata* D. cv. *Ariel*) as a natural dietary modulator of the human intestinal microbiota dysbiosis

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Abstract

Elicited pumpkin was evaluated as a potential daily consumption product able to modulate the microbiota profile. An *in vitro* dynamic colonic fermentation performance with microbiota from obese volunteers was used. Prebiotic effects were observed after the pumpkin treatment. *Bifidobacterium* abundance was maintained during the treatment period whereas *Lactobacillus* increased in the transversal and descending colon. Conversely, Enterobacteriaceae and *Clostridium* groups were more stable, although scarce decreasing trends were observed for same species. Increments of *Lactobacillus acidophilus* and *Limosilactobacillus fermentum* (old *Lactobacillus fermentum*) were observed in the whole colonic tract after the treatment period. However, modulatory effects were mainly observed in the transversal and descending colon. Diverse bacteria species were increased, such as *Akkermansia muciniphila*, *Bacteroides dorei*, *Cloacibacillus porcorum*, *Clostridium lactatifermentans*, *Ruminococcus albus*, *Ruminococcus lactaris*, *Coprococcus catus*, *Alistipes shahii* or *Bacteroides vulgatus*. The prebiotic effect of the elicited pumpkin was provided by the fiber of the pumpkin, allowing the release of pectin molecules in the transversal and distal colonic tract through low cellulosic fiber degradation, explaining the increases in the total propionic and butyric acid in these colonic sections. Also, a possible modulatory role of carotenoids from the product was suggested since carotenes were found in the descending colon. The results of this research highlighted pumpkin as a natural product able to modulate the microbiota towards a healthier profile.

Keywords: *pumpkin, microbiota, dysbiosis, colon, prebiotic, short chain fatty acids*

1. Introduction

An imbalance in the microbiota, known as dysbiosis, increases the bacterial population associated with the generation of lipopolysaccharides generation, causing endotoxemia and therefore, a low-grade systemic inflammation. The consequences of which can include diverse chronic pathologies, such as obesity, degenerative diseases, or metabolic syndrome [1]. In this regard, diet pattern plays a significant role in the development of dysbiosis, principally with high protein, saturated lipid, and/or free sugars consumption, as in the Western Diet [2]. Therefore, the intake of products with a natural prebiotic effect because of their dietary fiber content or bioactive compounds can be used as a strategy to promote the reversal of the dysbiosis state to a eubiosis profile [3-5].

Pumpkin (*Cucurbtia moschata*) belongs to the *Cucurbitaceae* family. The flesh section represents approximately 80% of the fresh weight, with the seeds and peel close to 20% of the total fresh weight [6]. Pumpkin flesh, seeds and leaves contain diverse bioactive compounds, such as carotenoids, polysaccharides, pectins, proteins and peptides, para-aminobenzoic acid, sterols, polyphenols and other phytochemicals such as phenolic glycosides [7]. It is well-known that pumpkin has noticeable amounts of carotenoids, ranging from 234.21 to 404.98 μg/g or even up to 600 μg/g, being violaxanthin, lutein, α-carotene, and β-carotene the main compounds [8]. In terms of dry matter, carbohydrates are the main compounds (42-60%) with significant polysaccharides content, comprising mainly of starch or pectins [9-11], but also lower amounts of free sugars principally represented by glucose, fructose, galactose and sucrose [8]. A wide range of polysaccharides with different degrees and compositions of molecular polymerization have been observed in pumpkin flesh. Pumpkin polysaccharides are mainly composed of arabinose, mannose, glucose, galactose [12], rhamnose [13], fructose, and xylose [11]. High molecular weight polysaccharides with a

85 molecular mass ranging between 10-23 kDa have been found, such as acidic
86 polysaccharide of 22.6 kDa composed of rhamnose, galacturonic acid, galactose, and
87 arabinose in a molar ratio of 7.4: 25: 28: 2.6 [14] or a 10.1 kDa heteropolysaccharide
88 (LGPP2-1) mainly containing 1,6-disubstitued-α-galactopyranosyl [15]. In addition, low
89 molecular mass pumpkin polysaccharides (< 10 kDa) have also been identified.
90 Recently, a new low molecular branched polysaccharide from pumpkin with a
91 molecular mass of 3.5 kDa (SLWPP-3) has been identified. Its composition is based on
92 rhamnose, glucose, arabinose, galactose and uronic acid with a weight ratio of 1: 1: 4: 6:
93 15 [16].
94 Few research has investigated the effect of pumpkin consumption on the human colonic
95 microbiota, suggesting a prebiotic effect [17,18]. During gastrointestinal digestion,
96 pumpkin starch is digested releasing glucose molecules [11]. Some polysaccharides are
97 acid degraded into lower molecular weight compounds during gastric digestion and low
98 degraded during intestinal digestion. Therefore, no release of reducing sugars occurs
99 during intestinal digestion [19], allowing the pumpkin polysaccharides fibre to reach the
100 colonic tract. Once the fiber reaches the colonic tract, it can be fermented by the colonic
101 microbiota [20].
102 In this study, elicited pumpkin was evaluated as a potential natural product able to
103 modulate dysbiotic microbiota collected from volunteers with metabolic syndrome.
104 Volunteers did not intake probiotic or fiber supplements. An *in vitro* dynamic colonic
105 fermentation was conducted to simulate the human gastrointestinal digestion and
106 colonic fermentation. The effect of this consumption was evaluated by determining the
107 microbiota changes which occurred after simulating a chronic consumption through
108 metagenomic analyses (*16S* rRNA analysis) with Next Generation Sequencing (NGS),

as well as the microbiota metabolic activity by determining the generated metabolites during colonic fermentation through HPLC ESI-TOF/MS analysis of the colonic media.

2. Materials and Methods

2.1. Samples

Butternut pumpkin (*Cucurbita moschata* D. cv. *Ariel*) was cultivated in the Experimental Station of Sakata Seed Ibérica, located in the Spanish region of Murcia (southwest of the country). Random collection of pumpkin samples was carried out, which allowed considering for the inter-individual variability existing in the samples belonging to the same crop area. After sample collection, pumpkins were stored at room temperature and transported to the laboratory in a few days in order to avoid the degradation processes. Samples were treated within 3 days after reception of the samples. The skin and seeds were carefully removed by hand with a knife, and the fleshy section was cut in circular cross section slides and subsequently in cubes of approximately 1-2 cm in width. The resulting cubes were immediately frozen at – 20 ºC for 24 h. Then, the samples were chopped in their frozen state and the resulting frozen powder was immediately stored at -80 ºC in separated plastic vessels until use.

2.2. Pumpkin elicitation and carotenoids quantification

Different treatments with methyl jasmonate (MeJA) were assessed in the field when the colour of the pumpkins started to change from green to orange. Three different concentrations were sprayed twice, with six-days in-between sprayings. The selected treatments were: i) 100 µM MeJA, ii) 150 µM MeJA, and iii) 200 µM MeJA. Concentrations and methodology were based on the patent PCT/ES2019/07045, in order to adjust the dosage for the pumpkin field elicitation. In this way, all treatments were

dissolved in 0.2 % ethanol and with the surfactant concentration specified in the patent (PCT/ES2019/070457). Also, 100 mL of each elicitor were applied to each plant.

For the total carotenoid quantification, an extraction with 5 mL of methanol for each 1g of fresh pumpkin flesh was conducted. After 24 h of incubation in darkness, the supernatant was measured by spectrophotometry, performing three measurements per treatment of three different randomly selected pumpkins from each treatment [21]. The following absorbance wavelengths were measured: 450, 645 and 663 nm. The formulas employed were:

$$C_a = (12.7\ x\ A_{663}) - (2.69\ x\ A_{645})$$

$$C_b = (22.9\ x\ A_{645}) - (4.68\ x\ A_{663})$$

$$Carotenoids = (4.07\ x\ A_{450}) - [(0.0435\ x\ C_a) - (0.367\ x\ C_b)]$$

2.3. Microbiota modulation

2.31. Simulated digestion in the dynamic colonic model

The potential modulatory activity of the colonic microbiota was evaluated using the Dynamic-Colonic Gastrointestinal Digester (D-CGD), developed by AINIA Technology Center (Valencia, Spain), and previously reported in Garcia-Ibañez et al., [22] and Rosés et al., [23,24]. It is a computer controlled simulator developed according to the validated apparatus carried out by Molly et al. [25,26]. D-CGD is comprised of 5 double jacket vessels interconnected through peristaltic pumps, representing the stomach, small intestine, ascending colon, transverse colon and descending colon. Thee system set up, that is, the volumetric capacity, pH, anaerobiosis (O_2 and CO_2 levels), and temperature (37 ◦C) were controlled. Anaerobiosis of the system was achieved by the addition of nitrogen and the pH value corrected in each vessel using hydrochloric acid or sodium hydroxide accordingly, to be in the range from 5.6 to 6.4 (ascending, pH

159 = 5.6–5.9; transverse, pH = 6.15–6.4, and descending pH = 6.6–6.9) according to
160 according to Molly et al. [25].
161 The compartments that corresponded to the colonic section (vessels 3, 4 and 5), were
162 colonized with microbiota bacteria obtained from 4 adults volunteers with dysfunctions
163 or pathologies associated with obesity and/or metabolic syndrome. The study was
164 approved by the Ethics Committee of the University of Navarra (protocol code
165 2019.169, date 12 December 2019) and conducted according to the guidelines of the
166 Declaration of Helsinki. Informed consent was obtained from all subjects involved in
167 the study. Feces were collected in special anaerobic plastic bags (GasPak, BD, Canada)
168 from non-smoking donors with these characteristics who had not ingested antibiotics for
169 at least 6 months before the study, had not consumed diets rich in fiber, had not
170 followed any weight loss diets and had not suffered acute inflammation or diseases of
171 the gastrointestinal tract, were collected in special anaerobic plastic bags (GasPak, BD).
172 Samples were diluted and regenerated in a physiological phosphate buffer (Merk, Spain)
173 with thioglycolate 20% (w/v) (Merk, Spain), according to [27]. This mixture was then
174 homogenized in a stomacher and centrifuged at 3000g for 15 min (Heraeus Multifugue
175 x3R Centrifuga, Thermo Scientific, Spain). The supernatant was collected and
176 inoculated in the colon vessels according to van den Abbeele et al. [28] with
177 modifications [23]. Hence, 50, 80 and 60 mL of the collected supernatant were placed in
178 the AC, TC and DC, respectively, and filled with culture medium up to a total volume
179 of 1000, 1600 and 1200 mL, allowing simulation of the conditions of the human colon
180 media. The colonic culture media was composed according to Molly et al. [26].
181 The whole *in vitro* simulation consisted in two step, a gastric digestion (2 h) followed
182 by an intestinal digestion (3 h), simulating the upper gastrointestinal digestion; and a
183 second step corresponding to the subsequent colonic fermentation simulation. The upper

184 gastrointestinal digestion that comprises the gastric and intestinal digestion was
185 conducted according to Nieto et al. [29] with modifications [22,27]. Gastric digestion
186 was simulated for 2 hours in vessel 1 by adding a continuous flow of 0.03% (w/v)
187 pepsin (pepsin from porcine mucosa, ≥ 2500 unit/g; P7012–56, Sigma-Aldrich, Spain)
188 in a gastric electrolytic solution (2100 U/mg) (NaCl, KCl, CaCl2, Merck, Germany).
189 Gastric pH medium was set up according to a pH curve observed under *in vivo* data by
190 adding a HCl solution (1 M) (HCl, 37 % purity, VWR Chemicals, Spain). After 2 h, the
191 generated gastric digested material was immediately transferred to the intestinal vessel
192 (vessel 2), where small intestinal digestion was conducted via the continuous addition of
193 a simulated intestinal solution consisting of pancreatin (1.9 g/L) (pancreatin P1750–100
194 G, Sigma-Aldrich, Spain), $NaHCO_3$ (12 g/L) (Merck, Germany), and oxgall dehydrated
195 fresh bile (6 g/L) in distilled water (240 mL for the whole intestinal digestion step)
196 (Oxgal, bile bovine, dried, unfractionated, B3883). After that, the generated intestinal
197 digested was immediately transferred to the third vessel (comprising of the AC) for 30
198 min, simulating the digestion transfer through the iliocecal valve to the AC. The
199 transferred digested material is maintained in the whole colonic segment for 76 h under
200 a continuous dynamic flow from the AC entrance to the DC exit, according with Rosés
201 et al. [23,24].
202
203 *2.3.2. Experimental protocol: in vitro colonic fermentation*
204 The experimental period was carried out according with Garcia-Ibañez et al. [22] and
205 Rosés et al. [23,24], lasting 4 weeks. The experiment was composed by two different
206 steps, first a stabilization period for 12, where a stable colonic microbiota was reached
207 (microbiota was not treated with the sample), followed by a treatment period for 14
208 days, where the microbiota was fed with the sample once per day. For the microbiota

209 stabilization period, the system was fed three times per day with 200 mL of culture
210 medium (without sample) for 12 days [30]. After this period, the system was fed twice
211 per day with 200 mL of culture medium (without sample) and once per day with 55 g of
212 chopped pumpkin sample mixed with culture medium until a final volume of 200 mL
213 was reached, for 14 days [22-24].
214 Samples of the colonic medium from the AC, TC and DC vessels were collected at time
215 0 and 14 of both stabilization period and at time 14 of the treatment period to conduct
216 the metagenomics and metabolomics analyses in order to observe the differences
217 between the end of the stabilization period (control) and the end of the treatment period
218 (sample). Collected samples were immediately stored at -20 ºC for further analyses of
219 the short chain fatty acids, metabolic analyses and microbial metagenomic analyses. In
220 this context, time 0 of the treatment period correspond with time 14 of the stabilization
221 period [24,27,31]. Additionally, samples were collected at the treatment period in days
222 0, 2, 7, 10 and 14 to conduct the bacteria group analyses through plate counting [23,24].
223 Samples were conducted in duplicate.
224
225 *2.3.3. Microbial analyses: plate counting method.*
226 Plate counting analyses of the colonic medium (AC, TC and DC) were conducted for
227 the stabilization and treatment period according to Rosés et al. [23,24]. Five bacteria
228 groups were analysed, being *Bifidobacterium ssp., Lactobacillus spp.*, *Clostridium spp.*,
229 Enterobacteriaceae ssp., and total anaerobes. Each bacterial group was cultured in its
230 specific culture medium, prepared in disposable petri dishes. The material used for these
231 analyses are indicated below: Saline solution (NaCl)-peptone (NaCl)-peptone (SSP
232 medium, Scharlau, Sapin), TOS-Propionate Agar (Base, 1000430500), mupirocin, and
233 MRS Agar (VM856760) were purchased from Merk (Sigma-Aldrich, Spain), VRBD

(Violet Red Bile Dextrose) Agar (1.10275.0500) was purchased from VWR, Schaedler agar for total anaerobes (Schaedler Anaerobe Agar, CM0437B), and TSC agar and cycloserin for Clostridium spp. (TSC Agar) was obtained from Thermo Fisher Scientific (Thermo, Spain).

10 mL of samples were collected from AC, TC and DC reactors at different times during the stabilization and treatment periods. Ten-fold serial dilutions of the collected fermentation medium were carried out in a physiological solution composed of a saline solution (NaCl)-peptone. 1 mL of generated serial dilutions samples were incubated through pour plate method (excepting for MRS Agar medium, that was incubated in surface incubation) in specific culture medium for each bacterial group. Cultivated petri dishes were maintained under anaerobiosis conditions at 37 ºC for 24 h (*Enterobacteriaceae ssp.*, *Clostridium spp.*) or 48 h (*Bifidobacterium ssp., Lactobacillus spp.*, and total anaerobes). Duplicates of two independent samples collected at any time from AC, TC and DC, were done. Colony-forming units (CFU/mL) were counted, and the results were expressed as log CFU/mL of slurry of any mentioned bacterial groups.

MALDI-TOF technique was used to verify lactobacilli colonies. Briefly, bacteria colonies with different phonotypical characteristics were collected from incubated MRS Agar petri dishes. Collected colonies were dispersed in 1.2 mL of water: ethanol, homogenised and centrifuged for 2 min at 10000 rpm. The resulting precipitate was collected and recuperated in 100 µL of a solvent mix composed by formic acid (70%) and acetonitrile (1:1 v/v). The mix was homogenised and centrifuged again. The resulted sample was mixed with 1 µL of HCCA (matrix for MALDI-TOF-MS, Bruker), and collected drops were placed in the MALDI-TOF plate to conduct the protein profile analysis in a MALDI Biotyper Microflex (Bruker) based on matrix-assisted laser desorption.

259 *2.4. Microbiota composition: 16S rRNA analysis with Next Generation Sequencing*
260 *(NGS)*
261 The microbiota profile of each colonic reactor (AC, TC and DC) at the end of
262 stabilization and treatment periods were analysed through metagenomic analyses of the
263 extracted 16S rRNA from the microbiota bacteria, as previously reported by our group
264 [22-24].
265
266 *2.4.1 DNA Extraction*
267 1.5 mL of samples were collected from the inoculum and from each reactor at the end of
268 the stabilization and at the treatment period at baseline using OMNIgene.GUT kits from
269 DNA Genotek (Ottawa, ONT, Canada), according to the standard instructions provided
270 by the company. DNA was extracted with the DNeasy® PowerSoil® Pro Kit (250)
271 (Qiagen. Hilden, Germany) following the manufacturer´s protocol [32].
272
273 *2.4.2 Metagenomic Data: Library Preparation*
274 Metagenomic studies were conducted according by analysing the variable regions V3–
275 V4 of the prokaryotic 16S rRNA (ribosomal Ribonucleic Acid) gene sequences, which
276 gives 460 bp amplicons in a two-round PCR protocol [22-24].
277 PCR was performed in a thermal cycler using the following conditions: 95 °C for 3 min,
278 25 cycles of (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and 72 °C for 5 min.
279 Specific primers with overhang adapters attached that flank regions of interest were
280 used. The full-length primer sequences, using standard IUPAC (International Union of
281 Pure and Applied Chemistry) nucleotide codes, were as indicated below:
282
283

Forward Primer:

[5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG]

Reverse Primer:

[5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC].

To verify the amplicon, 1 µl of the PCR product was checked in a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, USA), where the expected size is ~550 bp. After that, a limited-cycle PCR was used. Sequencing adapters and dual indices barcodes, Nextera® XT DNA Index Kit, FC-131-1002 (Illumina, San Diego, CA, USA), were added to the amplicon, allowing up to 96 simultaneous libraries for sequencing in the MiSeq sequencer with the MiSeq® Reagent Kit v2 (500 cycle) MS-102-2003 to be pooled together. The PCR was performed in a SimpliAmp thermal cycler (Applied Biosystems®, San Francisco, CA, USA) under the following conditions: 95 °C for 3 min, eight cycles of (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), and 72 °C for 5 min. Subsequently, the Index PCR ran a second Bioanalyzer DNA 1000 chip to validate the library (expected size was ~630 bp).

The next step consisted of the quantification of the libraries using a Qubit® 2.0 fluorometer (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA), diluting the samples before pooling them. Finally, paired-end sequencing was performed in a MiSeq platform (Illumina) with a 500 cycles Miseq run and with 7 pM sample and a minimum of 25% PhiX. The mean reads obtained were 164,387 and only samples with over 40,000 reads were used for further analysis.

2.4.3. Metagenomics Data: bioinformatic analysis

The *16S rRNA* gene sequences obtained were filtered according to the quality criteria of the OTUs (operational taxonomic units) processing pipeline LotuS (release 1.58)

309 [22-24]. This pipeline includes UPARSE (Highly accurate OTU sequences from
310 microbial amplicon reads) de novo sequence clustering and removal of chimeric
311 sequences and phix contaminants for the identification of OTUs and their abundance
312 matrix generation. Taxonomy was assigned using HITdb (Highly scalable Relational
313 Database), achieving up to species sensitivity. BLAST (Basic Local Alignment Search
314 Tool) was used when HITdb failed to reach a homology higher than 97% [33-34]. Thus,
315 OTUs with a similarity of 97% or more were referred to species. However, OTUs that
316 did not reach this percentage of similarity were checked and updated using the BLAST
317 to compare with the 16S rRNA gene sequences for bacteria and archaea database of
318 GenBank of National Center for Biotechnology Information in order to find an
319 assignment to a species. These sequences in which the BLASTn tool found a new
320 assignment were indicated using the GenBank access number and the percentage of
321 homology following the species name. The abundance matrices were first filtered and
322 then normalized in R/Bioconductor at each classification level: OTU, species, genus,
323 family, order, class, and phylum. This study focused mainly on the species level.
324 Briefly, taxa with a less than 10% frequency in our samples were removed from the
325 analysis, as a cut off, as they are not representative of a global effect. Reducing the
326 number of species allowed us to focus on the most important ones and to obtain more
327 conclusive results. A global normalization was performed using the library size as a
328 correcting factor and log2 data transformation [22-24].
329
330 *2.5. Nontargeted metabolomics: HPLC ESI-TOF/MS analysis*
331 Nontargeted metabolomics analysis of the microbiota fermentation was conducted
332 through a HPLC ESI-TOF/MS [22,23].

333 Aliquots of the colonic media (3 mL) from each reactor were collected at the end of the
334 stabilization and treatment periods. These samples were centrifuged (10,000 x g, 10
335 min) and filtered through a 0.22 µm-Ø Millipore filter (Billerica, MA, USA) into vials.
336 The chromatographic analysis was carried out through a high-resolution liquid
337 chromatogram (HPLC) from Agilent (model 1200). The detector used was a TOF (Time
338 of Flight) Mass Accuracy from Agilent, model 6220.
339 The stationary phase was a chromatographic column, Zorbax SB-C18 (Agilent
340 Technologies) of 150 x 46 mm and 5 µm pore size, maintained at 40 °C. Mobile phase
341 consisted of milliQ with 0.1% formic acid (solvent A) and methanol with 0.1% formic
342 acid (solvent B), applying a gradient able to screen the whole polarity range, from the
343 100% of A until the 100% of B. The injected volume was 15 µL.
344 The TOF detector is made of an electrospray ionized source (ESI). The flow of gas
345 drying was of 10 L/min, at a 40 psig pressure and a temperature of 350 °C. The
346 capillary voltage was of 4000 V, the fragmenter voltage 175 V, and the skimmer
347 voltage 65V. The relation m/z range, for the detection, was between 100 and 2000 and
348 the acquisition ratio was 1.03 spectre/sec. All samples analyses were conducted in
349 duplicate to be analysed in positive mode (ESI+) and negative mode (ESI-).
350 To analyse the results, an alignment with XCMS Online 3.7.1 software (The Scripps
351 Research Institute, La Jolla, USA) was done [35], according to the relation m/z
352 (mass/charge) and the retention time. Raw data was normalized by logarithmic
353 transformation followed by Pareto scaling. The alignment applied a 5 mDa mass
354 tolerance and 0.5 min retention time windows.
355 Later, comparisons of two vs two were done (data obtained at the end of the
356 stabilization period against data of the end of treatment period in each reactor, that is,

357 AC, TC and DC). These comparisons show the impact of the chosen treatment over the
358 microbiota of the *in vitro* digestion.
359 The alignment results were processed by MetaboAnalyst software 5.0 (Xia Research
360 Group, University of Alberta, Quebec, Canada) [36] using statistical multivariable tools,
361 such as Principal Component Analysis (PCA), Partial last squares regression (PLS-DA),
362 Random Forest and Volcano Plot. Outliers are excluded through the RandomForest
363 model, followed by the other statistical tools mentioned before. Discriminating features
364 among groups with more discriminating outputs were chosen with the following
365 conditions: between both groups of the polarity group:
366 - Volcano Plot: p-value < 0.001 and Fold change > 100
367 - PLSDA: Value of Variable Importance in Projection (VIP) > 3.0
368 Subsequently, the discriminating features that pass the selection criteria were identified
369 through the database Metlin (The Scripps Research Institute, La Jolla, USA) within a
370 mass precision below.
371 Certain conditions must be established to set the tolerance limit: 5 mDa in relation to the
372 m/z ratio (specifically the mean m/z relation if the csv file, generated from the XCMS
373 Online) [37]. For the ESI+ mode the characteristic ions of positive mode were used in
374 the identification positive group, the marked ions were $[M+H]^+$, $[M+Na]^+$ and $[M+H-H_2O]^+$
375 while ESI- mode, the ions were $[M-H]^-$ and $[M-H_2O-H]^-$. In both modes, those
376 metabolites classified by Metlin as toxic were deleted in the identification process.
377

378 *2.6. Short fatty acids (SCFA) analyses*

379 SCFAs analyses were conducted following the method of Sivieri et al. [38] with some
380 modifications [22]. 10 mL of colonic media from each reactor were collected at the end
381 of the stabilization and treatment periods and stored at -20 °C to determine the short

chain fatty acids (SCFA) content in the colonic media. Short and medium chain fatty acid composition of the fermentation medium was determined according to the relative percentage of the chromatographic areas of their corresponding methyl esters. First, the fat was extracted according to the Folch method (cold extraction) using diethyl ether and the esterification of the free fatty acids was carried out using a methanol solution of potassium hydroxide. The methyl esters of fatty acids were analysed by gas chromatography (GC) coupled to a FID detector. To this purpose, equipment Agilent GC-FID, model 8860 GC, coupled with a column Agilent GC HP-FFAP (25m X 0.2 mm X 0.33 µm) (Agilent, Spain) was used. N2 gas was used as carrier gas (flow rate of 20 mL/min). The column temperature was set at 130 °C and the temperature of the injector was 195 °C. This methodology also allowed the analysis of the content of medium chain fatty acids (MCFA) in the colonic medium. The results were expressed as mg of compound per Kg of fecal medium.

2.7. Statistical Analysis

Carotenoids and SCFA results were expressed as mean ± standard deviation (SD). Analysis of variance (one-way ANOVA) followed by HSD Tukey as a post hoc test was used to discriminate among means at $p < 0.05$ for treated pumpkin samples with MeJA, as well as for log CFU/ml colonic media, and SCFAs levels between initial and final sample during *in vitro* colonic fermentation. The statistical analyses were conducted using the statistical program Statgraphics Centurion XVI (Statistical Graphics Corp., Warrenton, VA, USA).

3. Results

3.1 Pumpkin elicitation

Elicitation results are represented in Table 1. The total carotenoid content (mg/g D.W.) revealed that the three treatments increased the concentration of carotenoids when compared to control samples ($p < 0.05$). Specifically, elicitation treatment with 150 µM MeJA increased the carotenoid content of the pumpkin samples in an approximately a 140 % when compared to the control plants ($p < 0.05$). Hence, pumpkin samples for the microbiota modulation studies were obtained applying a plant treatment with 150 µM MeJA, which maximize the carotenoids amount.

3.2 In vitro colonic fermentation. Plate counting method

The *in vitro* colonic fermentation comprised a stabilization period (12 days) followed by a 14 days treatment period where a chronic intake simulation of fresh pumpkin flesh was conducted. The colonic bacteria profile in each reactor (AC, TC and DC) was monitored through the whole study by evaluating 5 bacteria groups: *Bifidobacterium ssp.*, *Lactobacillus spp*., Enterobacteriaceae *ssp*., *Clostridium spp*. and total anaerobes. Results were expressed as CFU/mL of colonic media in each reactor (Figure 1). To identify specific lactobacilli colonies, MALDI-TOF technique was applied.

Total anaerobic bacteria reached stables levels close to 8 log units of CFU/mL on the digested media in the three colonic sections. These results confirm the stabilization of the microbiota after the stabilization period in each colonic section (day 0 of the treatment period).

During the treatment period, different trends could be determined between the diverse monitored bacteria groups, being specific of each studied group. The *Bifidobacterium* group showed a fluctuant growth but generally stable at 7 log units of CFU/mL in the

whole colonic system during the treatment period, with a slight increasing trend in the TC and DC. Conversely, *Lactobacillus* showed a clear noticeable increase during the treatment period. A similar behaviour was observed in the three colonic sections for this bacteria group, raising up progressively from 6 log units of CFU/mL to close to 8 log units of CFU/mL (7-8 log units of CFU/mL), especially in AC and TC. On the other hand, the Enterobacteriaceae and *Clostridium* bacteria group were stable during the treatment period, showing a fluctuant growth for the Enterobacteriaceae, with a marginal reduction trend in the DC, as well as for *Clostridium* bacteria group in AC and DC.

These results suggest that the studied elicited pumpkin enriched in carotenoids has a potential modulatory effect in the evaluated bacterial groups. These results indicate a clear prebiotic capacity by increasing *Lactobacillus* bacteria in the whole colonic tract and maintaining the *Bifidobacterium* population, while marginal potential bacteriostatic or reducing capacity was suggested against Enterobacteriaceae and *Clostridium* group.

3.3 Effects of pumpkin on the microbiome: metagenomics

The alpha index, that indicates the variability degree of the species existing in a bacterial community, was evaluated through the Shannon Wiener test, comparing the stabilization and treatment values of each colonic tract (AC, TC and DC).

When species level was considered, important changes were observed in AC, TC and DC. However, these changes were more remarkable for TC and DC. Diverse *Lactobacillus* species increased after the treatment period in the whole colonic system (Figure 2), being in concordance with the results obtained by plate counting (section 3.2). Regarding AC, a significant increase in *Lactobacillus acidophilus* and lower increases of *Limosilactobacillus fermentum* occurred. Both bacteria were not detected in

457 the AC at the end of the stabilization period, indicating a clear growth promoted by the
458 pumpkin treatment. Additionally, a slight increase of *Stenotrophomonas maltophilia*
459 was observed. TC and DC showed a more complex modulation effect mediated derived
460 from the pumpkin treatment. In this context, a similar trend was observed in both
461 colonic segments, with an increase in of many bacteria species. *Akkermansia*
462 *muciniphila*, *Bacteroides dorei*, *Cloacibacillus porcorum*, and *Clostridium*
463 *lactatifermentans*, as well as *Lactobacillus acidophilus* and *Limosilactobacillus*
464 *fermentum*, significantly increased I both colonic segments. Coinciding with AC, both
465 *Lactobacillus* bacteria were not previously quantified at the end of the stabilization
466 period, indicating a clear growth promoted by the pumpkin treatment. A similar trend
467 was observed for *Ruminococcus albus* and *Ruminococcus lactaris*. Other bacteria with
468 slight increases in TC and DC were *Stenotrophomonas maltophilia, Coprococcus catus*,
469 *Alistipes shahii* or *Bacteroides vulgatus*.
470
471 *3.4 Metabolites generated during colonic fermentation.*
472 HPLC ESI-TOF/MS method allowed the detection of 79,936 features in the ESI+ mode
473 and 71,118 features in the ESI- mode (data not shown). The PCA analysis (Figure 3)
474 showed a clear tendency of separation among the data set of samples from six groups,
475 thus reflecting differential metabolic characteristics in these groups. Nontargeted
476 metabolomics analyses of the colonic media of the three colonic sections showed the
477 increment or appearance of diverse metabolites (Table 2), mainly as metabolized
478 phenolic compounds, vitamins, oligopeptides and diverse lipids. It is important to point
479 out that after the treatment period, increments of a compound previously not detected in
480 the stabilization period was observed, identified as a carotenoid compound (probably
481 lutein or zeaxanthin), a characteristic compound of pumpkin [39].

482 *3.5 SCFAs production during colonic fermentation*

483 The metabolic activity of the microbiota was measured by analysing the amount of
484 SCFAs and MCFAs after the stabilization and treatment periods in the whole colonic
485 tract. As a consequence, the potential prebiotic effect was determined for the pumpkin
486 as a natural product able to increase the SCFAs levels. Results are shown in Figure 4.
487 Significant changes in total SCFAs production were determined for the TC and DC,
488 whereas marginal changes could be observed for the MCFAs. Conversely, no
489 significant changes were observed in the AC. TC and DC showed a similar trend in the
490 SCFAs and MCFAs generation. A clear increase of total SCFAs occurred as a
491 consequence of a noticeable generation of butyric acid and, to a lesser extent, of
492 propionic acid, whereas acetic acid levels were maintained. Also, a minimal increase in
493 caproic acid was observed.

494

495 **4. Discussion**

496 Pumpkin is a natural product rich in bioactive compounds, such as carotenoids,
497 polysaccharides, pectin, and phytochemicals. Pumpkin polysaccharides mainly consist
498 of starch or pectin [7,11], which are principally comprised of arabinose, mannose,
499 glucose, galactose [12], rhamnose [13], fructose, and xylose. Pumpkin starch is
500 hydrolysed during gastrointestinal digestion, releasing glucose molecules [11].
501 However, although pectin is commonly considered as dietetic fiber compounds, it has
502 been suggested that pumpkin polysaccharides seems to be acid degraded into lower
503 molecular weight compounds during gastric digestion and low degraded during
504 intestinal digestion. As consequence, a portion of the pumpkin dietetic fiber would
505 reach the colonic tract, integral or pre-degraded [19].

506 It is well known that polysaccharides fermentation by microbiota bacteria reduces
507 polysaccharides to constitutive monosaccharides. After, these compounds are
508 metabolized generating pyruvate, CO_2, H_2 or other metabolic intermediates. Then,
509 generated pyruvate can be converted into diverse compounds, mainly into acetate,
510 propionate or butyrate but also lower amounts of MCFAs such as valerate or hexanoate
511 [40]. However, the ratio of the SCFAs production is a consequence of two combined
512 factors; the microbiota profile (specific fermentative bacteria) and the kind of fiber
513 intake. In general, acetate and butyrate are released from aldehydes fermentation
514 (glucose, galactose, mannose, xylose) whereas propionate is generally generated during
515 ketonic glucosides fermentation (fructose, arabinose, tagatose). In this context,
516 *Bifidobacterim, Lactobacillus* and *Prevotella* species have been associated with high
517 butyrate increases [20].
518 Few studies have been previously conducted to investigate the impact of the
519 consumption of pumpkin flesh in the microbiota. In this study diverse bacteria species
520 able to ferment pectin, generating butyric acid, increased at the end of the experiment,
521 such as *Lactobacillus acidophilus*, *Limosilactobacillus fermentum, Akkermansia*
522 *muciniphila*, *Bacteroides dorei*, *Cloacibacillus porcorum*, *Clostridium*
523 *lactatifermentans, Ruminococcus albus* and *Ruminococcus lactaris, Coprococcus catus*,
524 *Alistipes shahii, Bacteroides vulgatus,* or *Stenotrophomonas maltophilia*. A slow
525 degradation process of the cellulose material at TC and mainly in DC [20], may release
526 into these colonic segments pectins, rhamnogalacturonans and xylogalacturonans
527 molecules, originally enclosed by cellulose and hemicellulose [41]. These results are in
528 concordance with Parkar et al. [42], which did not observed gas production during the
529 first 10 hours of pumpkin fermentation, which could indicate a delayed fermentation for
530 this vegetable. Hence, these released compounds in the lasted colonic segments,

531 especially pectins, are degraded by microbiota bacteria into constitutive
532 monosaccharides, such as arabinose, mannose, glucose, galactose [12], rhamnose [13],
533 fructose and xylose [11]. The fermentation of these released monosacharides explains
534 the increases of SCFAs in TC and DC but not in AC. These results are also in
535 agreement with the observed propionic and butyric acid increases but not acetic acid. In
536 this regard, *Ruminococcus albus*, which increased in this study, has been previously as a
537 bacteria capable of fermenting vegetable fibers such as cellulose, xylanes or pectins
538 [43]. On the other hand, pectin fermentation promotes specific propionic and butyric
539 acid generation [44], being in concordance with the results observed in this study.
540 Therefore, the release of butyric acid is explained because of the increase in identified
541 butyrogenic bacteria, such as *Blautia faecis, Clostridium lactatifermentans,*
542 *Coprococcus catus, Eubacterium desmolans, Gemmiger formicilis* and *Ruminococcus*
543 *lactaris* and other bacteria strains such as *Lactobacillus*. Besides, *Parasutterella*
544 *excrementihominis* has been previously associated with propionic increases (Wang et
545 al., 2019). These results are in concordance with previously research that observed
546 increases in *Ruminococcus* [41]. Additionally, Agarkova et al. [17] observed that a
547 mousse containing pumpkin fibers (pectin) significantly increased *Lactobacillus* in lab
548 rats, whereas Wu et al. [18], observed high amounts of *Akkermansia* after pumpkin fiber
549 intake.
550 Previous studies has pointed out that diets rich in free sugars and low fibers and
551 complex carbohydrates reduces the presence of *Bifidobacterium* and some *Clostridium*
552 groups such as *Roseburia* and *Eubacterium rectale*, as well as butyric levels in feces
553 [45]. Conversely, high intake of fiber and vegetables promote the bacteria able to
554 degrade fiber, producing beneficial metabolites such as SCFAs [46]. In this context, the

555 increase of butyric acid is associated with diverse health benefits, among them,
556 protective effects against colon cancer and lipid metabolism regulation [47]
557 On the other hand, other typical bioactive compounds present in pumpkin have been
558 detected in fecal samples, indicating that these compounds reach the colonic tract, such
559 as carotenoids [48]. During fermentation process, carotenoids could be released from
560 the fiber matrix, providing these bioactive compounds to the microbiota [49]. Previous
561 studies indicate that carotenoids such as astaxantina can contribute to modulation of the
562 intestinal microbiota [50,51]. It has been reported that microbiota α-diversity is
563 positively correlated with α-carotene and β-carotene [52]. Also, carotenoids seem to
564 contribute to an increase in typical butyrogenic bacterial species, such as
565 *Lachnospiraceae*, *Ruminococcaceae, Faecalibacterium* [50,51,53]. While in this study
566 changes in microbiota α-diversity were not observed in TC and DC, significant changes
567 occurred in the microbiota profile after the treatment with pumpkin. Therefore, since
568 carotenoids were detected in the DC (but not in previous colonic sections), these results
569 suggested a carotenoid is released from the pumpkin fiber matrix, contributing to
570 modulate the microbiota and increasing the butyrogenic species such as *Ruminococcus*
571 *albus* and *Ruminococcus lactaris*, and thereby, increasing the SCFA amount in the
572 distal colonic segments. In this context, Liu et al. [54] found that pumpkin intake in lab
573 rats increased propionic and butyric acids but not acetic acid, being in concordance with
574 this study.
575

576 **5. Conclusions**

577 In this work, an optimum elicitation protocol for pumpkin plants was achieved with
578 150 µM Methyl Jasmonate, based on the previous determined concentrations of the
579 patent PCT/ES2019/070457, thus, increasing the total carotenoid concentration. Elicited

pumpkin shows a prebiotic effect. As a consequence, increases in the *Lactobacillus* species occurs while *Bifidobacterium* species remained stable. In addition, bacteria species associated with beneficial health benefits, such as *Akkermansia muciniphila* and *Bacteroides dorei*, were also increased. The prebiotic effect can be associated with increments on the generation ratios of butyric and propionic acid in the TC and DC. The general increments of SCFAs in TC and DC but not in the AC suggest a low fermentability fiber fermentation (cellulosic fiber) that uses to occur in the TC and DC, releasing high fermentability fiber (pectins) in this colonic section, responsible of the SCFAs increases in this colonic section.

As a conclusion, daily consumption of fresh products rich in fibers and carotenoids, such as pumpkin, or their use as prebiotic ingredients, may contribute to the modulation of the microbiota dysbiosis to promote a healthy microbiota profile, becoming an interesting nutritional intervention strategy for obesity and dysbiosis states. More studies have to be conducted to elucidate the whole potential of fresh pumpkin as a microbiota modulator.

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Figure 1

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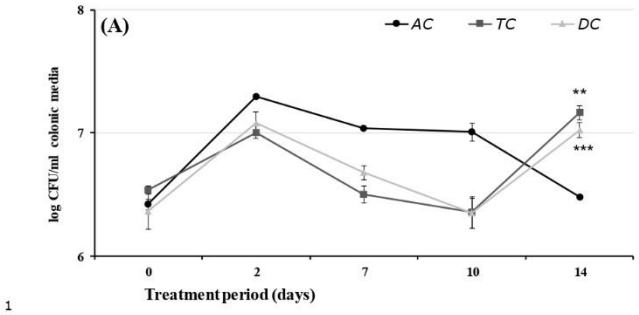

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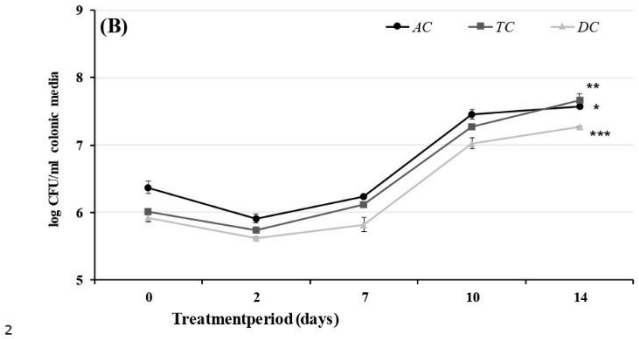

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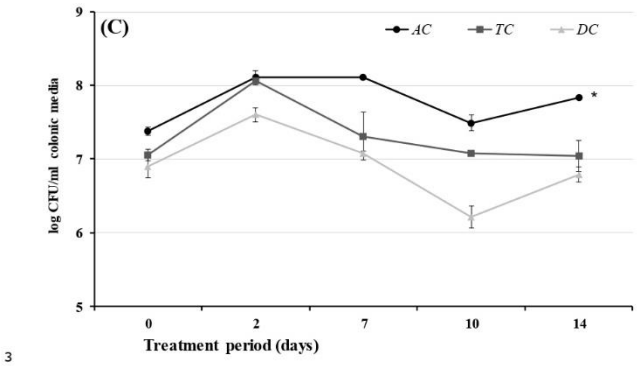

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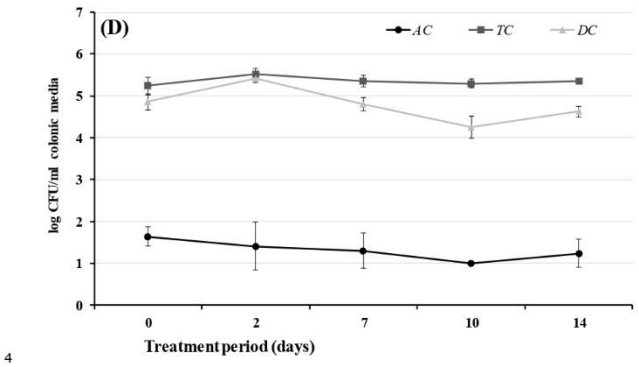

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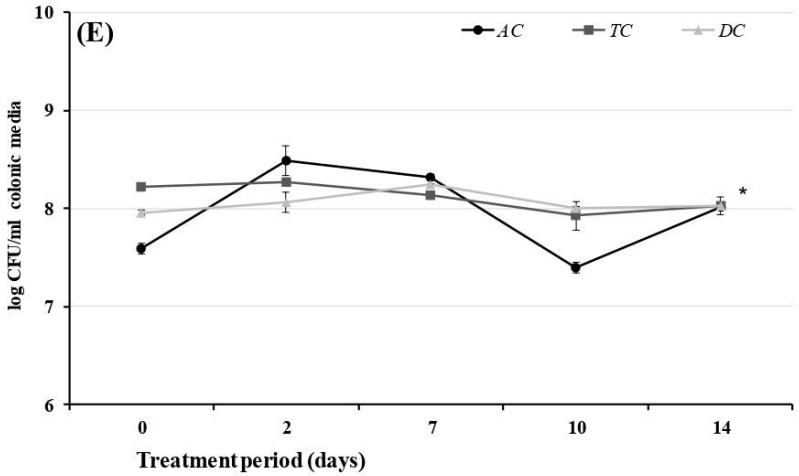

Figure 1. Bacteria populations of diverse bacteria groups from the colonic media on the three colonic sections (ascending colon, AC; transversal colon, TC; descending colon, TC) during stabilization (day 0-12) and treatment periods (day 12-14), expressed as log UFC/mL of fecal sludge. (A) *Bifidobacteria*, (B) *Lactobacillus*, (C) *Enterobacteria*, (D) *Clostridium*, (E) Total anaerobic bacteria. Results (n = 2) are expressed as log CFU/mL of colonic medium (mean ± S.D.).

Statistical differences in mean values by *post hoc* Tukey test between initial (time 0 days, end of stabilization period) and final (time 14, end of treatment period) are indicated as * for AC, ** for TC and *** for DC. Lack of symbol indicates no differences between initial and final data ($p < 0.05$).

Figure 2

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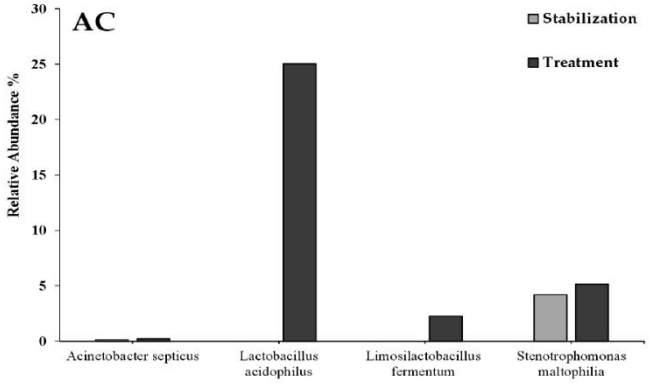

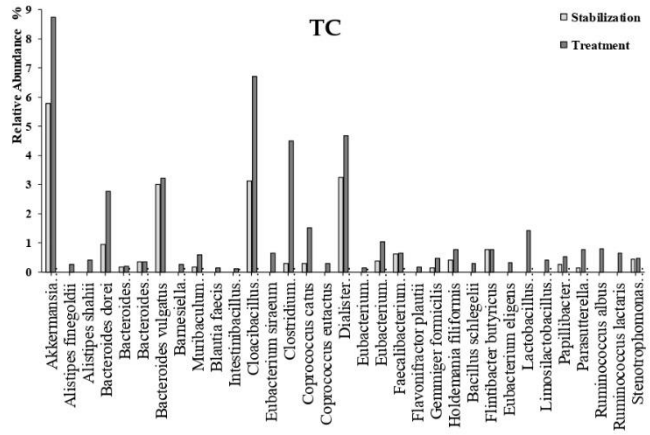

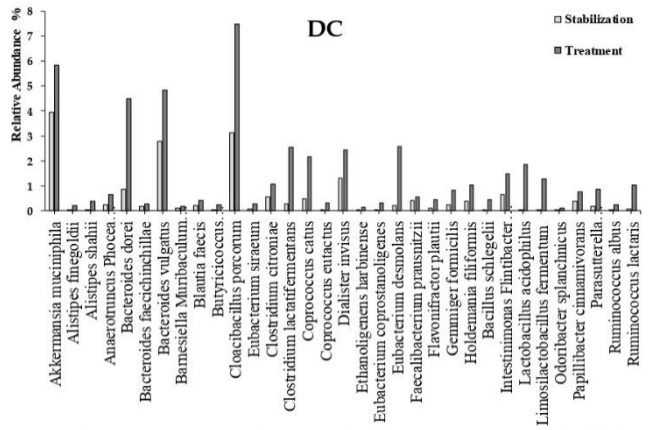

Figure 2. Representation of the relative abundance of bacterial species in the different reactors: (AC) ascending colon, (TC) transverse colon, (DC) descending colon, before (Stabilization) and after the treatment period (Treatment) with fresh pumpkin.

Figure 3

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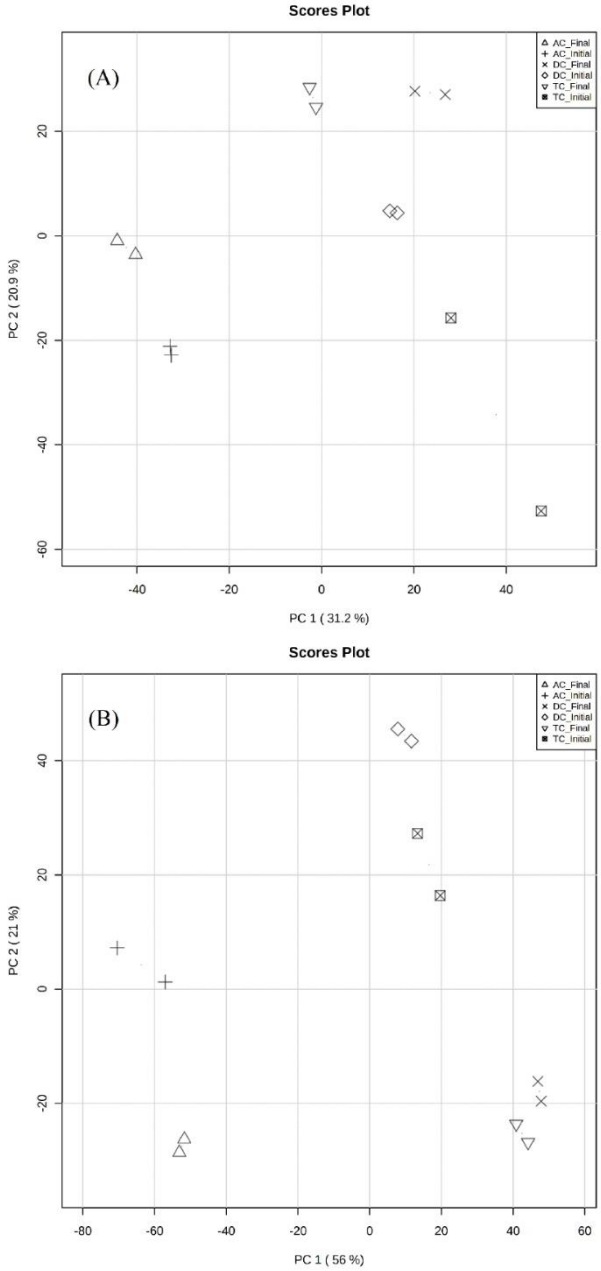

Figure 3. PCA Score Plots obtained in the comparisons among ascending colon (AC), transversal colon (TC) and descending colon (TC) at the end of the stabilization period (Initial) and at the end of the treatment period (Final). (A) Positive polarity (B) Negative polarity.

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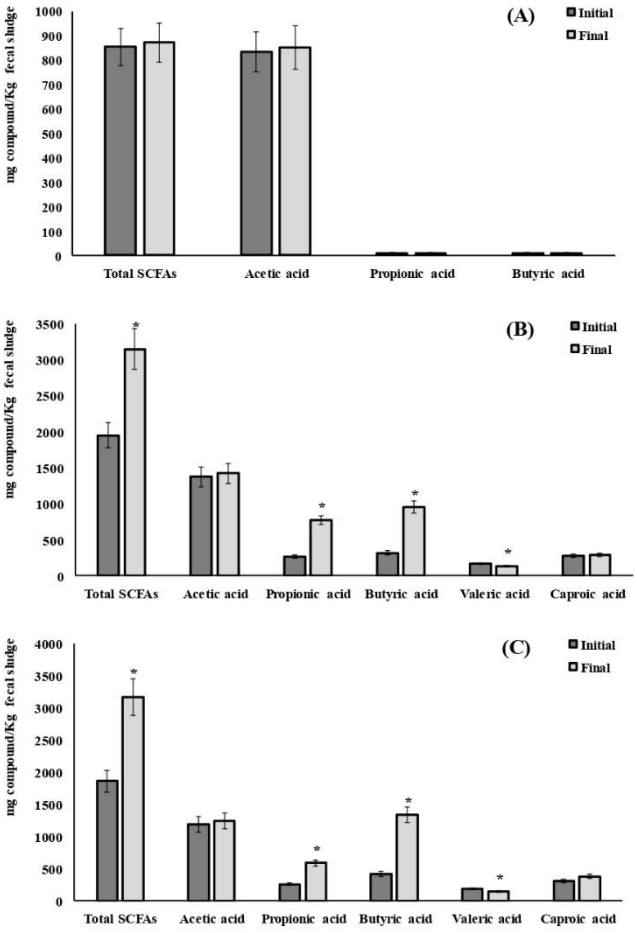

Figure 4. Short chain fatty acids production, expressed as mg of compound/ Kg of fecal sludge, at the end of the stabilization period (Initial) and at the end of the treatment period (Final) on each colonic segment. (A) ascending colon, (B) transversal colon, (C) descending colon (n = 2). Data represent means ± S.D.

* Indicate significant differences between compounds level (initial compared to final) by Tukey test at $p < 0.05$ (ANOVA).

Table 1

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Table 1. Carotenoid concentration in pumpkin flesh with different MeJA treatments (mg/g D.W.). Data are represented as mean ± standard deviation (S.D., n=3). Different letters show statistically significant differences in the HSD Tukey *post hoc* analysis performed after a one-way ANOVA statistic test ($p < 0.05$).

	Treatment with Methyl Jasmonate			
	Control	**100 µM**	**150 µM**	**200 µM**
Total carotenoids	23.81 ± 3.97^{c}	39.07 ± 1.76^{b}	56.78 ± 3.65^{a}	43.27 ± 0.75^{b}

[a,b,c] Different letters indicate statistically significant differences between applied treatments.

Table 2

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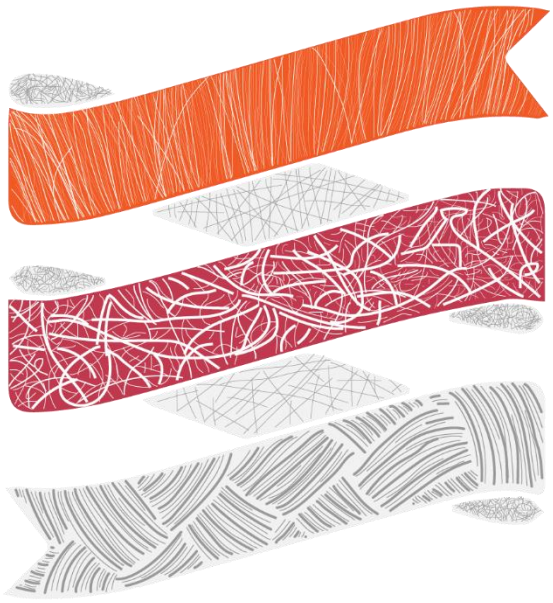
Table 2. Main discriminating putative metabolites.

	Polarity	Colonic section	UP / DOWN* 12 days vs 14 days	VIP score	RT (min)	Detected mass (m/z)	Putative metabolite	Assignation	Mass difference (mDa)
1	NEG	DC	UP	4.68	5.08	429.2330	Tetrapeptide Gly, Glu, Leu, Leu	$[M-H]^-$	-2.50
2	NEG	TC	DOWN	4.39	6.39	607.4750	DG(17:2/20:4/0:0)	$[M-H-H_2O]^-$	2.40
3	NEG	AC	DOWN	4.20	4.13	617.4640	Isomers glucopyranosyl-octacosanetriol	$[M-H]^-$	0.60
4	NEG	AC	DOWN	4.04	3.67	955.8676	TG(19:0/20:1(11Z)/20:1(11Z))[iso3]	$[M-H]^-$	-2.30
5	POS	TC	UP	4.01	4.56	475.2313	Tetrapeptide Phe, Val, Asn, Asn	$[M+H-H_2O]^+$	-2.87
6	POS	AC	UP	3.94	8.35	499.3754	Isomers dihidroxy-dimethyl-tetrahydro-vitamin D3	$[M+H]^+$	-2.80
7	NEG	TC	UP	3.82	3.98	725.5625	SM(d18:2/18:1)	$[M-H]^-$	2.20
8	POS	AC	DOWN	3.64	1.34	575.1775	5,4'-Dimethoxyflavone 7-xylosyl-(1->6)-glucoside	$[M+H-H_2O]^+$	1.00
9	POS	DC	UP	3.64	2.33	765.5107	PG(15:1/22:4)	$[M+H-H_2O]^+$	3.69
10	POS	TC	DOWN	3.28	2.76	287.2559	Hydroxy heptadecanoic acid	$[M+H]^+$	-2.20
11	POS	TC	UP	3.03	2.28	551.4257	Zeaxanthin/Lutein	$[M+H-H_2O]^+$	0.40

* Stabilization period (12 days) *versus* treatment period (14 days)
POS = positive; NEG = negative; AC = ascending colon; TC = transversal colon; DC = descending colon; RT = retention time; VIP = variable importance in projection.



Chapter 5

Effects of Glucosinolate-Enriched Red Radish (*Raphanus sativus*) on In Vitro Models of Intestinal Microbiota and Metabolic Syndrome-Related Functionalities.

Carles Rosés[1], Paula Garcia-Ibañez[2,3], Agatha Agudelo[4,5], Blanca Viadel[9], Lidia Thomas[9], Elisa Gallego[9], Micaela Carvajal[2,3], Fermin I. Milagro[6,7,8] and Anna M Barceló[1]

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Article

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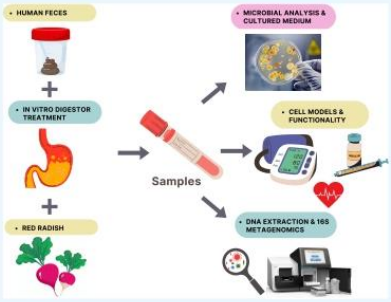
ABSTRACT: The gut microbiota profile is determined by diet composition, and therefore this interaction is crucial for promoting specific bacterial growth and enhancing the health status. Red radish (*Raphanus sativus L.*) contains several secondary plant metabolites that can exert a protective effect on human health. Recent studies have shown that radish leaves have a higher content of major nutrients, minerals, and fiber than roots, and they have garnered attention as a healthy food or supplement. Therefore, the consumption of the whole plant should be considered, as its nutritional value may be of greater interest. The aim of this work is to evaluate the effects of glucosinolate (GSL)-enriched radish with elicitors on the intestinal microbiota and metabolic syndrome-related functionalities by using an *in vitro* dynamic gastrointestinal system and several cellular models developed to study the GSL impact on different health indicators such as blood pressure, cholesterol metabolism, insulin resistance, adipogenesis, and reactive oxygen species (ROS). The treatment with red radish had an influence on short-chain fatty acids (SCFA) production, especially on acetic and propionic acid and many butyrate-producing bacteria, suggesting that consumption of the entire red radish plant (leaves and roots) could modify the human gut microbiota profile toward a healthier one. The evaluation of the metabolic syndrome-related functionalities showed a significant decrease in the gene expression of endothelin, interleukin IL-6, and cholesterol transporter-associated biomarkers (ABCA1 and ABCG5), suggesting an improvement of three risk factors associated with metabolic syndrome. The results support the idea that the use of elicitors on red radish crops and its further consumption (the entire plant) may contribute to improving the general health status and gut microbiota profile.



1. INTRODUCTION

The gut microbiota is closely linked with the host's health and has a key role in the development of several diseases, such as obesity. Its profile is determined by the diet and therefore this interaction is essential for synthesizing vitamins and beneficial bioactive molecules derived from polyphenols and fibers and postbiotic molecules released by bacteria following their death.[1] These compounds are capable of promoting specific bacterial growth and enhancing the health status because gut microbiota is also involved in the maintenance of intestinal functions, modulating the immune system response, and working as a barrier against certain pathogens. Many studies suggest a link between gut microbiota and obesity[2] and other interrelated metabolic diseases, including hyperglycemia, hyperlipidemia, insulin resistance, and hepatic steatosis,[3] that are defined as metabolic syndrome (MS). In humans, the development of obesity correlates with shifts in the relative abundance and diversity of bacterial phyla and species in the gut.[4] Current diet health standards include more vegetables and fruits daily.[5]

Radish (*Raphanus sativus L.*), belonging to the Brassicaceae family, is a major root vegetable crop that is widely cultivated and consumed. Radish plants consist of two main organs: a large, extended root, which acts as a storage organ, and the aboveground leaves. Radish roots are commonly harvested and consumed as vegetables.[6] They contain various minerals, nutrients, and bioactive compounds, including polysaccharides, organic acids, phenolic compounds, alkaloids, nitrogen compounds, and glucosinolates (GSLs).[7,8] Several studies have shown that radish contains several secondary plant metabolites that can exert protective effects on chemically induced carcinogenesis in animals and tumor growth and

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of the small intestine were mimicked by the continuous addition of a pancreatin solution (0.9 g/L), NaHCO3 (12 g/L), and oxgall dehydrated fresh bile (6 g/L) in distilled water (total volume of 440 mL), maintaining the intestinal content at pH 6.5.

2.2.1. Fecal Inoculum. Fresh feces were used to reproduce the gut conditions from four adult participants with pathologies associated with obesity and/or metabolic syndrome (BMI 30−40 kg/m^2; age 30−50 y.o.), nonsmokers, no history of antibiotic treatment in the last three months, and no intestinal disease background.[44] Fecal samples were collected and maintained in special anaerobic plastic bags (BD GasPak systems). Feces were pooled and diluted with thioglycolate 20% (w/v) and homogenized with a stomacher to obtain a fecal slurry. The fecal suspension was centrifuged at 3000*g* for 3 min, and the collected supernatant was immediately inoculated in the colon vessels (50, 80, and 60 mL for R3, R4, and R5, respectively), followed by culture medium up to a total volume of 1000, 1600, and 1200 mL, respectively. The composition of the culture medium followed by Molly et al.[45,46] provides the necessary nutritional components to simulate the conditions of the human colon and allows the intestinal microbiota to grow. Each reactor was maintained at different optimal pH levels. The bacteria present in each region of the colon have an optimal pH of action: pH 5.5−6 in the colon ascending (R3), pH 6−6.4 in the transverse colon (R4), and pH 6.4−6.8 in the descending colon (R5). To regulate the pH changes that occur during fermentation and maintain them in the optimal intervals for each region, an acid or a base was added.

2.2.2. Process Description and Duration. After fecal inoculation, a stabilization period of 12 days (T12) was required to allow bacteria to grow and reach stable levels.[47,48] During this period, 200 mL of cultured medium was added to the stomach (R1) three times a day. At this point, the system was ready to start the sample treatment period to study its modulation effects on the gut microbiota.[38,39] The treatment period was conducted for another 14 days (T26) (a total of 26 days of the experiment) by feeding the equipment with 25 g of crushed raw red radish with a regular hand blender once a day (in culture medium up to 200 mL) and with 200 mL of cultured medium twice a day. The maintenance of the microbial population during the stabilization and treatment period was checked by bacteria plate counts. The following bacterial groups were quantified by growth on specific media, expressing the result as CUF/mL of colonic media: acid−lactic bacteria (MRS agar, the matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) technique was employed to verify lactobacilli colonies), bifidobacteria (TOS-propionate agar enriched with MUP), enterobacteria (VRBD agar), clostridia (TSC agar enriched with cycloserin), and total anaerobic bacteria (agar Schaedler). Then, 10 mL of the samples was taken from each reactor (R3, R4, and R5) and serially diluted in a saline solution. Plates were inoculated with 1 mL of the sample of four serial dilutions by duplicate and incubated at 37 °C under aerobic or anaerobic conditions. In addition, samples were taken from each reactor at T12 and T26, 10 mL and 3 mL, respectively, and stored at −20 °C to determine the short-chain fatty acid (SCFA) content in the colonic media and to conduct the metabolomic analysis. In addition, for the metagenomic analysis, 1.5 mL of the inoculum, as well as samples from each reactor at the end of T12 and T26, were collected using OMNIgene GUT kits from DNA Genotek (Ottawa, ONT, Canada), according to the standard instructions provided by the company. Finally, for the functionality analysis, two samples were taken from the *in vitro* digester at two different stages:

(a) the sample taken at the end of T12 when the microbiota has adapted to the *in vitro* conditions (**Mbasal**). This sample is obtained from the mixture of each of the three reactors ("R3" ascending, "R4" transversal, and "R5" descending).

(b) the sample taken at the end of T26, after the addition of the radish whole plant treated with MeJA on the *in vitro* digester, and the reaction of the microbiota (**Mtt**). This sample is obtained from the mixture of each of the three reactors ("R3" ascending, "R4" transversal, and "R5" descending).

The samples were diluted 1/2 with Hank's balanced salt solution (HBSS), selected dilution 1/2.

2.2.3. SCFA Analyses. For the metabolomic analysis, these samples were centrifuged (15,000*g*, 15 min) and filtered through a 0.22-μm-Ø Millipore filter (Billerica, Massachusetts) into vials for UHPLC-ESI-QqQ-MS/MS analysis. Short- and medium-chain fatty acids were extracted from the sample using liquid−liquid extraction with diethyl ether. The resulting extract was analyzed using an AS 800 C.U. gas chromatograph (CE Instruments) equipped with an HP-FFAP 25 m × 0.2 mm × 0.33 mm column (Agilent Technologies) and a flame-ionization detector (FID). The samples were quantified by interpolation in the calibration curve with an internal standard. The concentration of the fatty acids was provided directly by the software using a 1 × linear regression. The results were expressed as mg of the compound per kg of colonic medium.

2.2.4. Microbiota Composition and 16S rRNA Analysis. DNA was extracted with the QIAamp DNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.[49] The microbiota composition was analyzed according to the 16S rRNA protocol with next-generation sequencing (NGS) using a MiSeq (Illumina, San Diego, California). A first PCR was performed using 12.5 ng of genomic DNA obtained from the samples and 16S-Fw and 16S-Rv primers. After that, a second PCR reaction was performed using 5 μL of DNA and the Nextera XT DNA Index kit (FC-131-1002, Illumina). Then, the process quality was verified using a Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). When all of the samples were obtained, they were multiplexed by mixing equimolar concentrations from each sample and the internal standard Phix. The mix was diluted until obtaining a concentration of 8 pM. The sequencing was performed in the MiSeq using a MiSeq Reagent v2 (MS-102-2003) kit (Illumina).

2.2.5. Bioinformatic Analysis. The 16S rRNA sequences obtained were curated following the quality criteria from the OTU processing protocol using the LotuS pipeline.[50] This protocol includes the clustering of de novo sequences by UPARSE and the deleting of chimeric and contaminant sequences for OTU identification. Also, this software generates the corresponding abundance matrix. An OTU is defined as organisms that are clustered according to the similarity of their DNA sequence. The taxonomy was assigned by using BLAST and HITdb, reaching a species sensitivity level. The abundance matrix was curated and normalized in R and Bioconductor. Global normalization was performed using the library size as a correction factor. Data were transformed to Log2.

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respectively. The higher the absorbance, the more the ROS generated by the cells.

2.3.7. Gene Expression Analysis: Quantitative PCR. Gene expression analyses were performed after total RNA extraction from cells. The extraction was performed automatically with MAXWELL equipment (Promega, Madison, Wisconsin). cDNA was obtained from RNA using the high-capacity cDNA reverse transcription kit transcriptase enzyme (Applied Biosystems, Foster City, California). From cDNA, the real-time PCR was performed using the previously acquired primers of the selected biomarkers specified in Table 1.

Table 1. Specification of the Gene Primers Used in the Quantitative PCR for Each Functional Study

models	gene primers
2.3.2. Endothelial model to study changes in blood pressure	Endothelin-1 (**END-1**)
2.3.3. Liver model to study modulation of cholesterol metabolism	Cholesterol transporters (**ABCA1** and **ABCG5**)
2.3.5. Model with adipocytes to study adipogenesis or fat formation	Adiponectin (**ADIPOQ**) Interleukin 6 (**IL-6**)
Gene Primer References	
END-1: Hs00174961_m1 → Thermo-Fisher Scientific (Waltham, Massachusetts)	
ABCA1 and ABCG5: Hs01059137_m1 and Hs00223686_m1 → Thermo-Fisher Scientific (Waltham, Massachusetts)	
ADIPOQ and IL-6: Mm00456425_m1 and Mm00446190_m1 → Thermo-Fisher Scientific (Waltham, Massachusetts)	
Control Gene References	
Beta-actin (B-Act): 4326315E → Thermo-Fisher Scientific (Waltham, Massachusetts)	
GAPDH: Mm99999915_g1 → Thermo-Fisher Scientific (Waltham, Massachusetts)	

Beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as control genes, whose expression is constitutive in these cells. The amplification conditions in the thermocycler (7300 AppliedBiosystem) were the universal ones, and the quantification of gene expression was performed in a relative way so that the magnitude of the physiological changes in the biomarker gene is obtained in comparison with a reference gene. For the calculations, the formula $2^{-\blacksquare\Delta\Delta Ct}$[50] was used.[57]

2.4. Data Analysis. Results are expressed as the mean and standard deviation of the mean of at least two to six replicates per study. Statistical analysis was usually carried out with the Student *t*-test to compare the results obtained between Mbaseline and Mtt or between the included controls (* $p < 0.05$; ** $p < 0.025$). For the field elicitation experiment, one-way analysis of variance (ANOVA) was performed using Tukey's HSD as a post-hoc test. For the SCFA production and the bacterial growth analysis, a Mann−Whitney *U* Test was applied for differences between two groups on a single, ordinal variable with no specific distribution.[58] These analyses were carried out using GraphPad Prism version 6.0c for MAC OS X, GraphPad Software, San Diego, California, www.graphpad.com (13/09/2021).

3. RESULTS AND DISCUSSION

3.1. Plant Material and Treatments. The influence of the elicitors on the fresh weight is a parameter used to determine its efficiency. Since these compounds simulate a defense reaction in the plant, at some dosage, they could cause a growth arrest, which would result in a lower field yield.[59] Table 2 (Figure 7 in complementary materials) shows the effect of these treatments on the whole plant, the aerial part, and the edible part of fresh red radish (g). When comparing the weight of the treated red radishes with the control plants, a decrease in biomass by 16% is shown when applying 100 μM MeJA and its combination with SA (SA + MeJA, $p < 0.05$). Nevertheless, no changes were observed with 200 μM SA alone, suggesting that the main effect could come from the MeJA application ($p < 0.05$). Furthermore, when analyzing the edible part and the aerial part separately, the main effect in biomass was observed in the bulb (edible part) after applying the treatments, which include MeJA ($p < 0.05$). However, since the total biomass only decreases by 16%, the treatment with these elicitors could achieve a good balance between plant yield and this enrichment in health-promoting metabolites.

3.1.1. Glucosinolates Concentration. The effect of the elicitors on the GSL content (μg mL^{-1}) is represented in Table 3, which varies between the plant parts (Figure 8 in complementary materials).

- Glucoraphanin levels decreased after the three treatments in the edible part but increased in the aerial part by 69% with MeJA ($p < 0.05$ for each of the comparatives).
- Dehydroerucin raised up to 130% in the edible part with SA and up to 140 and 180% in the aerial part with MeJa and SA + MeJA, respectively ($p < 0.05$).
- 4-Hydroxy-glucobrassicin (HGB) concentrations increased up to 130% in the edible part and up to 67% in the aerial part with MeJA ($p < 0.05$). With SA, concentrations increased by 35% in the edible part and 20% in the aerial part ($p < 005$ for both parts).
- Glucobrassicin (GB) showed the highest increase in both plant parts with MeJA—5 times higher in the edible part and 2 times in the aerial part.
- 4-Methoxy-glucobrassicin (MGB) levels increased in the aerial part with all of the treatments ($p < 0.05$), but none of them caused any increase in the edible part ($p > 0.05$).

In previous studies with brassica plants, elicitors like MeJA or combined with SA have already been seen to increase the total content of glucosinolates.[34,36] For example, with Bimi

Table 2. Study of the Elicitors' Effect on the Plant Biomass (g) of Red Radish. Data Show the Means ($n = 3$) Along with the Standard Deviation[a]

plant biomass (g)	control	200 μM SA	100 μM MeJA	SA + MeJA
whole plant	12.12 ± 0.83 a	10.40 ± 0.66 a	9.58 ± 0.65 b	8.13 ± 1.03 b
edible part	6.15 ± 0.29 a	5.38 ± 0.25 a	5.56 ± 0.22 a	5.23 ± 0.35 a
aerial part	5.75 ± 0.58 a	4.73 ± 0.49 ab	3.77 ± 0.47 bc	3.25 ± 0.67 c

[a]Different letters show statistically significant differences between samples ($p < 0.05$), using an HSD Tukey as a post-hoc test. The statistics were performed within each plant part. MeJA: methyl jasmonate and SA: salicilic acid.

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Lactobacillus also had a decrease in R3 but had an opposite response in R4 and R5 (Table 4). Total anaerobic bacteria did not have a clear response, as some genera increased and others decreased.

3.2.1. Bacterial Metabolome: SCFA. After treatment with fresh radish, there was a significant increase in acetic and propionic acid production in the three sections of the colon. Butyric acid only showed a significant increase in R3 but decreased in R4 and R5. These results are shown in Table 5 (Figure 10 in complementary materials). SCFA-producing bacteria have a positive impact on gut health, improving the integrity of the intestinal barrier.[65] Butyrate regulates cell apoptosis,[66] stimulates the production of anaerobic hormones, and through cell differentiation regulation,[67] suppresses allergic and inflammatory responses.[68] Reduced levels of butyrate have been related to diseases such as colon cancer and obesity,[69] so increased levels could be beneficial for health. Propionate is also considered to have health benefits, including the promotion of satiety, reduction of cholesterol, and adipogenesis inhibition. High levels of propionic acid could be related to a hypocholesterolaemic effect.[70] Acetate is a net fermentation product for most gut anaerobes that is also produced by reductive acetogenesis and, almost invariably, achieves the highest concentrations among the SCFA in the gut lumen.[71] Previous studies showed that the butyryl-CoA: acetate-CoA-transferase pathway is the main process for the biosynthesis of butyrate,[72,73] so acetate is utilized by butyrate producers to produce butyrate.[74] Thus, it seems logical to expect a butyric increase following the acetic increase; however, the results do not show that behavior. In the

Table 5. SCFA Production in the Three Reactors (R3, R4, and R5) after the Stabilization Period (T12) and the Treatment Period (T26)[a]

reactor/phase	acetic acid	butyric acid	propionic acid
R3T12	1667 ± 168	161 ± 15	178 ± 11
R3T26	2041 ± 202	440 ± 41	836 ± 64
R4T12	1841 ± 185	1165 ± 106	360 ± 28
R4T26	2657 ± 266	722 ± 66	784 ± 59
R5T12	1756 ± 171	1407 ± 130	382 ± 31
R5T26	3084 ± 309	915 ± 82	856 ± 64

[a]Data are expressed as mg/Kg of colonic medium.

Table 6. Bacterial Genus Abundance in the Three Colonic Sections (R3, R4, and R5)[a]

tax	inoculation	R3T12	R3T26	R4T12	R4T26	R5T12	R5T26
Acidaminococcus	0.00	3.58	5.02^	0.00	0.00	2.48	2.55^
Acinetobacter	0.00	0.00	0.00	1.46	8.82^	0.00	9.65^
Akkermansia	0.00	0.00	0.00	0.00	0.00	1.10	2.77^
Alistipes	1.40	0.00	0.00	0.00	0.00	8.29	8.43^
Anaerofustis	0.00	0.00	0.00	0.00	0.00	2.62	4.54^
Anaerotruncus	0.86	0.00	0.00	0.00	0.00	3.17	4.48^
Bacillus	0.00	3.17	8.29^	2.18	2.24^	0.00	0.00
Bifidobacterium	3.98	4.08	7.02^	0.00	0.00	0.00	0.00
Barnesiella	7.90	0.00	0.00	0.00	0.00	5.70	5.84^
Blautia	10.56	0.00	0.00	4.47	4.50^	5.67	5.82^
Catabacter	7.11	0.00	0.00	0.00	6.71^	3.08	6.48^
Christensenella	4.63	0.00	0.00	4.22	5.11^	5.58	5.69^
Cloacibacillus	4.97	0.00	0.00	0.00	1.69^	0.00	0.00
Clostridium	11.97	0.00	0.00	0.00	0.00	5.82	9.66^
Desulfovibrio	7.28	0.00	0.00	0.00	0.00	0.00	1.97^
Dialister	0.00	0.00	0.00	0.91	1.99^	0.00	1.56^
Dorea	8.22	0.00	0.00	7.39	7.75^	7.40	7.76^
Enterobacter	0.00	5.21	6.32^	0.00	0.00	3.88	5.00^
Enterococcus	1.40	0.00	0.00	0.00	11.76^	1.44	11.68^
Eubacterium	11.79	0.00	0.00	7.02	10.14^	6.89	8.91^
Faecalibacterium	5.50	0.00	0.00	0.00	0.00	6.78	7.43^
Holdemania	4.58	0.00	0.00	0.00	0.00	7.68	8.09^
Lachnoclostridium	11.66	0.00	0.00	0.00	0.00	9.50	9.99^
Lachnospira	10.56	0.00	0.00	8.77	10.43^	8.95	10.36^
Lactobacillus	4.93	5.90	12.11^	0.00	0.00	0.00	0.00
Lysinibacillus	0.00	2.00	2.05^	0.00	0.00	0.00	0.00
Melainabacter	7.45	0.00	0.00	0.00	11.86^	0.00	11.77^
Odoribacter	8.00	0.00	0.00	0.00	0.00	4.71	5.28^
Oscillibacter	11.37	0.00	2.87^	11.15	11.91^	11.08	11.21^
Parabacteroides	7.79	0.00	0.00	5.77	5.83^	5.65	6.02^
Propionibacterium	0.00	1.00	2.05^	0.00	0.00	1.95	1.97^
Ralstonia	2.35	2.32	4.53^	1.46	1.69^	0.00	0.00
Roseburia	11.27	0.00	3.76^	0.00	0.00	5.05	5.98^
Ruminococcus	11.00	0.00	2.87^	6.68	10.97^	7.22	10.50^
Victivallis	5.58	0.00	0.00	0.00	0.00	7.29	8.69^

[a]T12 represents the end of the stabilization period, and T26 is the end of the treatment period. Values show the relative abundance %. ^: Bacterial genus increase.

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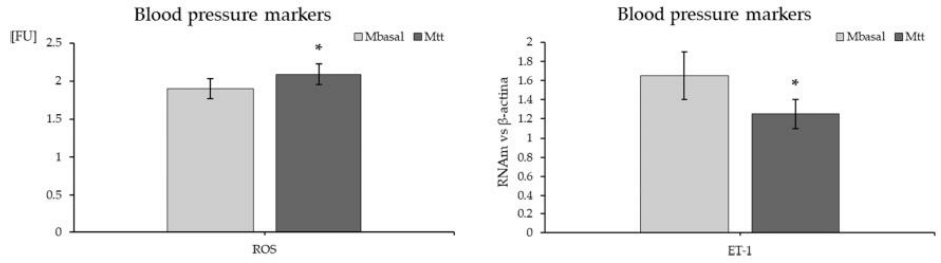

Figure 3. Blood pressure biomarker changes: reactive oxygen species (ROS) in fluorometric units [FU] on the left and endothelin-1 (ET-1) expressed in genic biomarker units on the right (n = 6). A Mann−Whitney U Test was applied for differences between two groups on a single, ordinal variable with no specific distribution. "*" indicates a very statistically significant difference (p < 0.025).

various diseases. It is located mainly in the mucosa of the colon, involved in maintaining intestinal integrity.[93] *Bacteroides clarus*[94] and *B. uniformis* can interact with the host modifying its immune cells and regulating metabolites. They can improve various intestinal and behavioral disorders, cardiovascular diseases, and cancer. The use of *Bacteroides* in food processing has been authorized by the European Commission after evaluating its safety. They are considered a source of next-generation probiotics.[95] *Desulfovibrio piger* uses lactate, pyruvate, ethanol, and hydrogen as electron donors for sulfate reduction. It also oxidizes lactate and pyruvate incompletely to acetate.[96] *Holdemania filiformis* is a carbohydrate-fermenting bacterium that produces acetic, lactic, and succinic acids.[97] *Turicibacter sanguinis* appears to have robust host effects impacting metabolism and overall gastrointestinal health.[98] *Barnesiella intestinihominis* is considered an "oncomicrobe" of great value since it improves the efficacy of alkaline immunomodulatory compounds for cancer treatment.[99] These results seem to indicate that the use of elicitors on red radish crops increases their glucosinolate content, enhancing the beneficial effect of this root (when consuming the entire plant) on the colon integrity and on the microbiota health status through an improved bacterial profile and SCFA production. This translates into a favored growth of several bacterial genera and species that are known to have a positive impact on the overall health status, working synergistically and improving the metabolome and therefore the intestinal functionality.

3.3. In Vitro Study of Functionalities Related to Metabolic Syndrome. *3.3.1. Endothelial Model to Analyze Blood Pressure Changes.* Changes in the blood pressure markers ROS and END-1, after inducing a "hypertension" phenotype to the cell models, are represented in Figure 3. The treatment did increase ROS levels (Mbasal: 1.90 ± 0.13 vs Mtt: 2.09 ± 0.133 fluorometric units [FU]), but it decreased the gene expression of endothelin (END-1) (Mbasal: 1.65 ± 0.25 vs Mtt: 1.25 ± 0.15). Therefore, fresh radish may have a potential antihypertensive effect. These results are consistent with a previous study where an aqueous extract of radish seed showed a hypotensive effect in rats[100] and another where an ethyl acetate extract of radish leaves improved hypertension by increasing serum nitric oxide (NO) levels.[101] It seems to be a link between the increase of ROS post-treatment and the decrease of ET-1, as NO, in conjunction with other ROS, contributes to oxidative stress[102,103] and is also a potent vasodilating substance implicated in vasodilatation.[104]

3.3.2. Modulation of Cholesterol Metabolism in Hepatocytes. In the evaluation of biomarkers associated with cholesterol metabolism, the treatment induced a significant increase in the gene expression of the transporters ABCA1 (Mbasal = 1.00 ± 0.1 vs Mtt = 1.44 ± 0.07: relative units of biomarker mRNA vs beta-actin mRNA) and ABCG5 (Mbasal = 1.00 ± 0.09 vs Mtt = 2.08 ± 0.23: relative units of biomarker mRNA vs beta-actin mRNA), as shown in Figure 4. ABCA1 plays a key role in regulating cholesterol efflux. Reverse

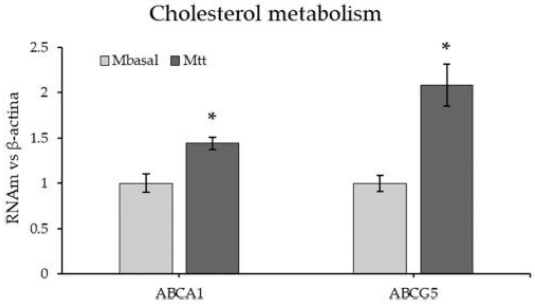

Figure 4. Changes in the gene expression of ABCA1 and ABCG5, associated with cholesterol metabolism, after being in contact with the red radish samples (n = 4). A Mann−Whitney U Test was applied for differences between two groups on a single, ordinal variable with no specific distribution. "*" indicates a very statistically significant difference (p < 0.025).

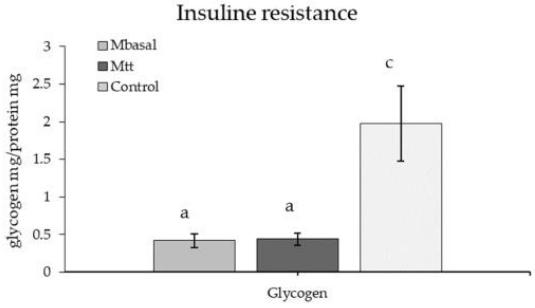

Figure 5. Changes in the intracellular glycogen associated with insulin resistance. The + control is an internal control that increases intracellular glycogen uptake (n = 4). Relative units: mg glycogen/mg protein. A Mann−Whitney U Test was applied for differences between two groups on a single, ordinal variable with no specific distribution. Different letters mean statistically significant differences: a, no significant difference (p > 0.05), and c, a very significant difference (p < 0.025).

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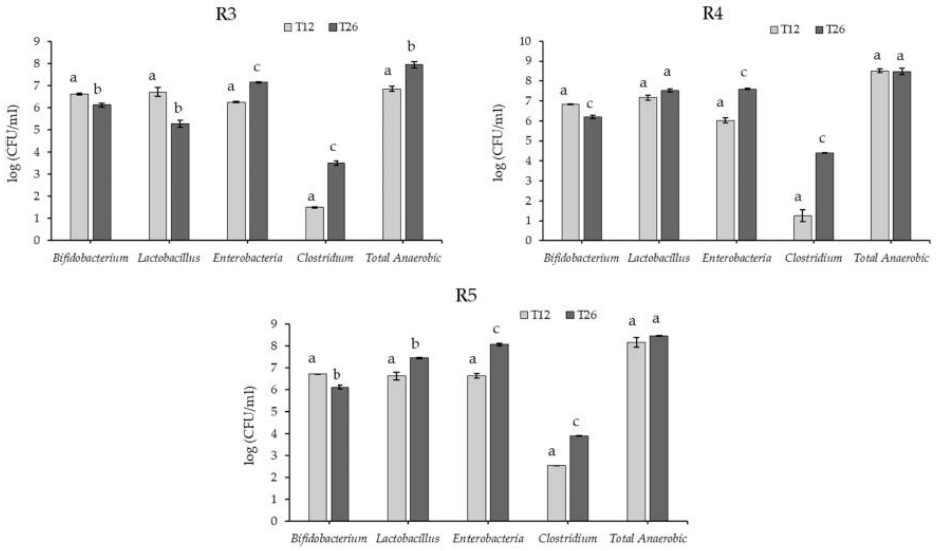

Figure 9. Microbiological count of **R3**, **R4**, and **R5** at T12 and T26 ($n = 2 \pm$ SD). The Mann−Whitney *U* test was applied for differences between two groups on a single, ordinal variable with no specific distribution. Different letters mean statistically significant differences: a, no significant difference ($p > 0.05$), and c, a very significant difference ($p < 0.025$). Results are shown as log (CFU/mL).

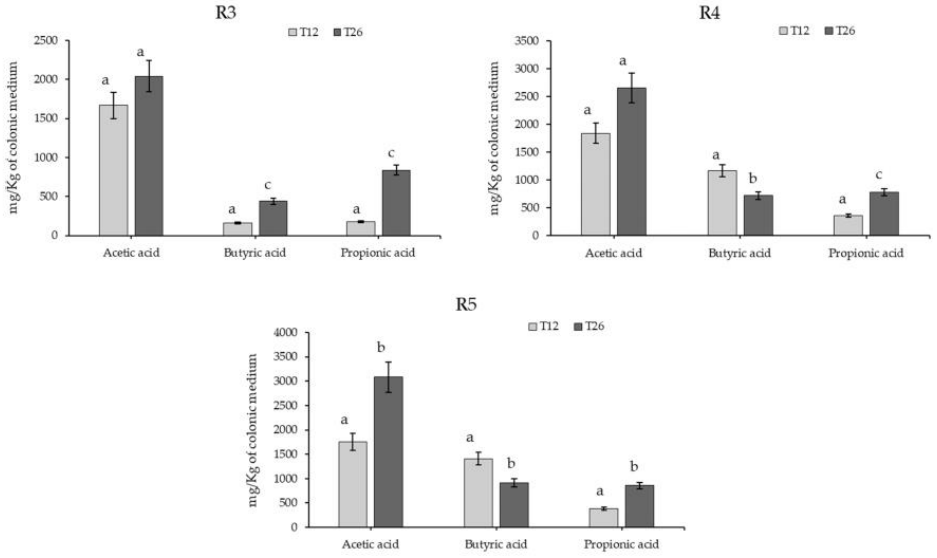

Figure 10. Representation of the SCFA production on **R3**, **R4**, and **R5** at T12 and T26 ($n = 2 \pm$ SD). The Mann−Whitney *U* test was applied for differences between two groups on a single, ordinal variable with no specific distribution. Different letters mean statistically significant differences: a, no significant difference ($p > 0.05$), and c, a very significant difference ($p < 0.025$).

and the gene expression of adiponectin (ADIPOQ) (an adipocyte-secreted factor, which is an insulin-sensitizing and anti-inflammatory adipokine, concentrations of which are decreased in obesity[109] and interleukin (IL)-6 cytokine (an inflammatory mediator) have been measured. The intracellular level of accumulated lipids was not modified with the treatment (Mbasal: 1.00 ± 0.11 vs Mtt: 1.02 ± 0.13); however, ADIPOQ showed a tendency toward reduced levels (Mbasal: 1.00 ± 0.15 vs Mtt: 0.77 ± 0.21), and IL-6 showed a significant reduction (Mbasal: 1.00 ± 0.28 vs Mtt: 0.031 ± 0.004) (Figure 6). *In vitro* and *in vivo* studies carried out with a radish extract alone or mixed with other vegetables have observed an improvement of some obesity-related parameters, such as the accumulation of fat or the modulation of adipokines such as adiponectin.[106,110] Thus, the results obtained regarding the decrease in interleukin IL-6 are consistent with the literature.

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Notes

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The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Navarra (protocol code 132/2015).

Informed consent was obtained from all subjects involved in the study.

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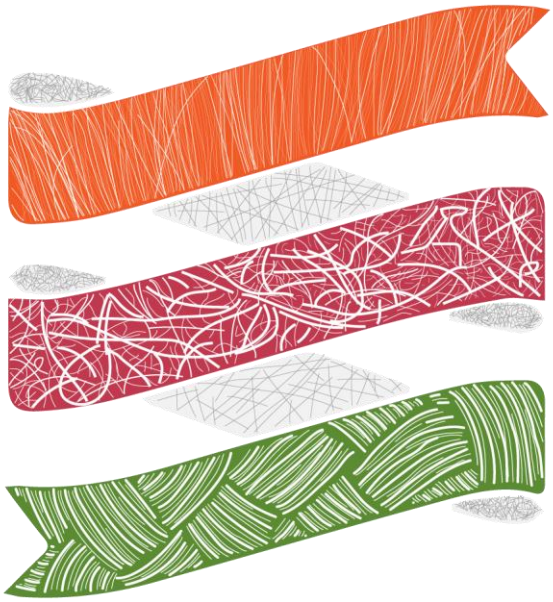

Chapter 6

Gut microbiota modulatory capacity of *Brassica oleracea* italica x alboglabra (Bimi®).

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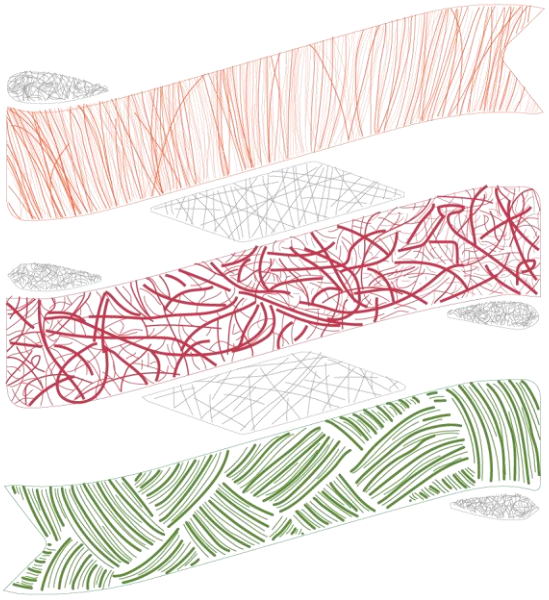
GENERAL DISCUSSION



The relationship between diet and the human gut microbiota is highly significant and has been the subject of extensive research. The gut microbiota consists of bacteria, fungi, viruses, and other microbes, that reside in the gastrointestinal tract. Diet plays a crucial role in shaping its composition, and different dietary components, such as carbohydrates, proteins, fats, fibres, and various bioactive compounds, can have a direct or indirect influence. For example, a high-fat diet has been associated with a decrease in microbial diversity, while a high-fibre diet promotes a more diverse and beneficial gut microbiota [137]. It is important to note that the gut microbiota is highly individualized and can vary among individuals based on factors like genetics, lifestyle, and overall health. The gut microbiota's role in health is still being actively studied, however, maintaining a balanced and diverse gut microbiota through a healthy diet is generally associated with improved overall health and a reduced risk of various diseases. The present investigation, through different methodology such as bacteria plate counts, metagenomics, SCFA quantification and the analysis of specific biochemical markers, aims to investigate the interaction between gut microbiota and food consumption patterns in a health context. More specifically, this research work sought:

- Firstly, to **understand the complex interaction between gut microbiota and diet-related food groups, particularly those more characteristic of the MD,** to further relate it with the overall health status of the host; figure 2.

- Secondly, to **determine which microbiota profiles, specifically bacterial genera and species, are related to better host health**.
- Thirdly, to **elucidate the possibility to use specific food products as a promising tool for the stimulation of beneficial bacteria** in order to achieve a successful treatment for a better gut and general health.

Interaction between gut microbiota and diet in the context of health

The analysis of the gut microbiota and its association with health reveals intriguing insights into the complex interplay between our microbiome, dietary factors, and various health conditions.

Bifidobacteria and Lactobacilli

To begin with, we will have a quick overview on the behaviour that Bifidobacteria and Lactobacilli have shown through the 6 chapters of this thesis. These two extended genera exert multifaceted effects on gut health, largely attributed to their ability to modulate the gut microbiota composition and function. These beneficial bacteria have been shown to enhance the diversity and stability of the gut microbial community, through their fermentative capacity, immunomodulatory effects, and metabolic activities, that contribute to the prevention of dysbiosis [138, 139]. By competitively excluding potential pathogens, they reinforce the gut barrier and help to protect against intestinal infections, improving mucosal immune responses.

The following table summarizes the results related to the Bifidobacteria and Lactobacilli bacteria along the 5 chapters that analyse the impact of certain products. Chapter 3 is not included because is mainly a methods article, and it is not focused on the potential effect of the tested products.

Table 1. Results of the genus *Bifidobacterium* and *Lactobacillus* along the different chapters. The underlined titles indicate the techniques used to analyse bacterial behaviour.

	Bifidobacterium	***Lactobacillus***
Chapter 1	**16s-metagenomics: in-vivo** *Bifidobacterium animalis* shows a very strong positive association with adherence to the MD.	**16s-metagenomics: in-vivo** NO RESULTS
Chapter 2	**16s-metagenomics: in-vivo** NO RESULTS	**16s-metagenomics: in-vivo** High levels of the Lactobacillaceae family in individuals with higher consumption of yogurt and fermented dairy products. Higher AST levels, related to lower abundance of this family (hepatic function).
Chapter 4	**Plate counts** *Bifidobacterium* generally stable at 7 log units of CFU/mL in the whole colonic system during the treatment period, with a slight increasing trend in the TC and DC. **16s-metagenomics: in-vitro** *Bifidobacterium* had an increase in the TC.	**Plate counts** *Lactobacillus* increased during the treatment period, rising progressively from 6 log units to close to 8 log units (CFU/mL), especially in AC and TC. **16s-metagenomics: in-vitro** *Lactobacillus* increased in all reactors. *Lactobacillus acidophilus* had a strong increase after the treatment in AC.
Chapter 5	**Plate counts** *Bifidobacterium* had a slight decrease in all reactors. **16s-metagenomics: in-vitro** *Bifidobacterium* increased in AC. The specie *Bifidobacterium bifidum* increased also in AC. The specie *Bifidobacterium adolescentis* increased in DC.	**Plate counts** *Lactobacillus* had a decrease in AC but had an opposite response in TC and DC. **16s-metagenomics: in-vitro** *Lactobacillus* showed a marked increase in AC The specie La*ctobacillus paracasei* also increased in the first reactor.
Chapter 6	**Plate counts** *Bifidobacterium* had a slight decrease in all reactors. **16s-metagenomics: in-vitro** *Bifidobacterium adolescentis* increased. **16s-metagenomics: in-vivo** *Bifodibacterium* decreased	**Plate counts** *Lactobacillus* had a slight decrease in all reactors. **16s-metagenomics: in-vitro** NO RESULTS **16s-metagenomics: in-vivo** *Lactobacillus* increased

Before analysing the results is important to keep in mind that an in vitro digester is not able to fully reproduce the natural conditions, as it is a static environment, and therefore there are no other stimuli such as natural body movement, hormones, trophic activity, interaction with other humans, the outside world, etc. In this kind of in vitro digestors, the natural tendency is to observe a reduction in bacterial diversity and population. Moreover, because the gut microbiota is a delicate and fragile environment, a consistent growth of a certain population could have a detrimental effect on their neighbours, so it is tricky to analyse an isolated group of bacteria without considering the general behaviour of the whole ecosystem.

Bifidobacteria and Lactobacilli are known for being two type of beneficial bacteria that are commonly found in the human gut. While they share some similarities in their roles and interactions with diet, there are also notable differences between them. Both bacteria can ferment dietary fibre, breaking down complex carbohydrates and fibres into beneficial by-products such as SCFAs, contributing to the fermentation process, although they have different preferences for specific types of fibre. In general, *Bifidobacterium* growth has been related to pectin content [140] increasing its levels with dietary pectin presence while *Lactobacillus* is more related with resistant starch and xylo-oligosaccharides, as they produce lactic acid from fermented sugars [141]. Moreover, it is important to note that the effects of Bifidobacteria and Lactobacilli

can vary depending on the specific species and strains within each genus. Different strains may have different metabolic capabilities, preferences for dietary substrates, and interactions with the host. It is crucial to consider strain specificity when evaluating the effects of these bacteria on diet and health. However, it is important to know that the methodology used in this thesis (Illumina 16S Metagenomic Sequencing) is not able to differentiate bacterial strains. Overall, both genera play important roles in gut health and can be influenced by dietary factors. Including a variety of plant-based foods and dietary fibres in the diet can help promote the growth of these beneficial bacteria, favouring a healthy gut microbiota.

If we focus in the Table 1, the presence of vegetables enhances the growth of the *Bifidobacterium,* as it is the genus most significantly related with MD, results coming from the human trial in chapter 1, a very reliable result. However, not all techniques used in the different chapters find the same tendencies. The plate counts show no positive impact of the treatments on this genus. As mentioned before, this could be attributed to the natural competition between different bacteria and moreover, plate counts only focuses on 4 different genera, giving a limited view of the global microbiota modulation. The 16s-metagenomiuc analysis clearly reveals that the species *Bifidobacterium adolescentis* significantly increases with red radish and Bimi consumption, in the in vitro digester.

Moving onto *Lactobacillus*, we observed no clear association with MD from chapter 1, however on this occasion, plate counts generated a favourable output: a general increase with the pumpkin treatment in all reactors and increased counts with the red radish, especially on the TC and DC, which are the most relevant ones, as they represent the last segments of the colon, the richest and most diverse parts. Pumpkin and red radish, from chapters 4 and 5 showed an increase of two well-known species, *Lactobacillus acidophilus* and *Lacticaseibacillus paracasei* in the digester, showing a similar effect from these two foods. Chapter 2 revealed lower presence of the Lactobacillaceae family in those subjects with worse hepatic health, figure 6. The human trials revealed that higher consumption of yogurt and fermented products, as well as the consumption of Bimi positively correlates with a growth of this genus.

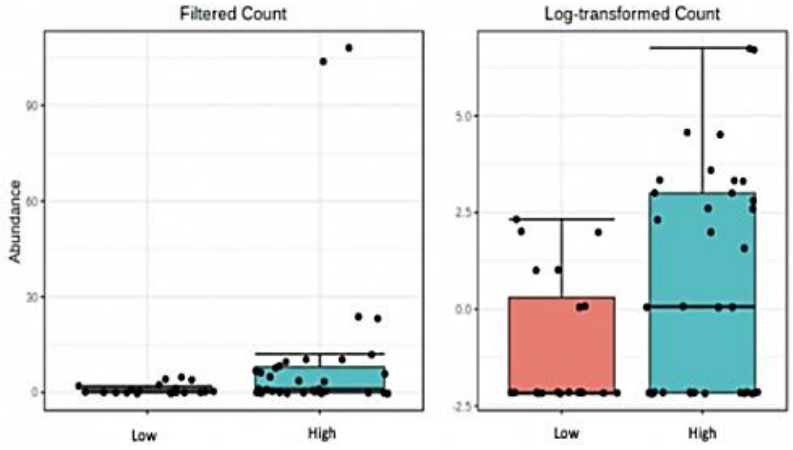


Figure 6. Lactobacillaceae differing significantly when comparing individuals with higher and lower consumption of fermented dairy (FDR < 0.05). Red boxes represent participants with lower consumption, and blue boxes represent participants with higher consumption compared to the median.

In summary, including a variety of fermented and plant-based foods, as well as dietary fibres in the diet, can promote the growth of *Bifidobacterium* and

Lactobacillus, contributing to a healthy gut microbiota. However, the effects of these bacteria can vary depending on the specific species, strains, and individual contexts. A deeper and more specific analysis is required to elucidate specific roles and interactions with diet, food, and the health outcome.

Gut microbiota, diet, and foods

A healthy and balanced diet with abundance of fibre-rich plant foods promotes a diverse and beneficial gut microbiota, while a poor diet can disrupt its balance and lead to health problems. In this section the scope will be held on the modulatory effect resemblances among plant foods from chapters 4, 5 and 6. The three foods tested are from the family Brassicaceae (genera *Raphanus* and *Brassica*) and Cucurbitaceae (genus *Cucurbita*). We will also consider the bacterial species from chapter 1. There should be some similarities as the MD is characteristic for its abundance of food plants. Table 2 shows those genera and species that correlate positively with the MD and that have been enhanced by the treatment with pumpkin, red radish or Bimi. Those listed in table 2 are the ones that appear in more than one chapter simultaneously or concur in another article.

Table 2. Genera and species in common across chapters 1, 4, 5 and 6, that appear to have a positive influence on gut health and show an increased population after the treatment.

	Genera	Species
Chapter 1 MD	NO RESULTS	**<u>16s-metagenomics: in vivo</u>** *Bacteroides cellulosilyticus* (legumes) *Catabacter hongkongensis* *Oscillibacter valericigenes* *Oscillospira (Flavonifractor)Plautii* *Paraprevotella clara* *Roseburia faecis* *Ruminococcus bromii*

Pumpkin Chapter 4	**16s-metagenomics: in vitro** *Akkermansia* *Alistipes* *Catabacter* *Christensenella* *Cloacibacillus* *Clostridium* *Coprococcus* *Eggerthella* *Eubacterium* *Faecalibacterium* *Fusicatenibacter* *Gemmiger* *Holdemania* *Intestinimonas* *Methanobrevibacter* *Oscillibacter* *Oscillospira* *Parasutterella* *Phascolarctobacterium* *Prevotella* *Roseburia* *Ruminococcus*	**16s-metagenomics: in vitro** *Akkermansia muciphila* *Alistipes shahii* *Bacteroides dorei* *Bacteroides vulgatus* *Cloacibacillus porcorum* *Clostridium lactatifermentans* *Gemmiger formicilis* *Lactobacillus acidophilus* *Lactobacillus fermentum* *Ruminococcus albus* *Ruminococcus lactaris*

Red radish Chapter 5	**16s-metagenomics: in vitro** *Akkermansia* *Alistipes* *Anaerotruncus* *Catabacter* *Christensenella* *Cloacibacillus* *Clostridium* *Eubacterium* *Faecalibacterium* *Holdemania* *Lachnospira* *Oscillibacter* *Parabacteroides* *Roseburia* *Ruminococcus*	**16s-metagenomics: in vitro** *Akkermansia muciniphila* *Bacteroides clarus* *Christensenella minuta* *Gemmiger formicilis* *Holdemania filiformis* *Lactobacillus paracasei* *Oscillibacter valericigenes* *Oscillospira plauti* *Victivallis vadensis*

Bimi Chapter 6	**16s-metagenomics: in vitro** *Anaerotruncus* *Eubacterium* *Eggerthella* *Holdemania* *Lachnospira* *Parabacteroides* *Paraprevotella* *Parasutterella* *Phascolarctobacterium* *Ruminococcus* *Victivallis* **16s-metagenomics: in vivo** *Alistipes* *Eubacterium* *Fusicatenibacter* *Lachnospira*	**16s-metagenomics: in vitro** *Akkermansia muciniphila* *Alistipes obesi* *Alistipes putredinis* *Anaerotruncus colihominis* *Bacteroides cellulosilyticus* *Eggerthella lenta* *Eubacterium eligens* *Holdemania massiliensis* *Intestinimonas butyriciproducens* *Oscillibacter valericigenes* *Oscillospira capillosus* *Parabacteroides goldsteinii* *Paraprevotella clara* *Parasutterella excrementihominis* **16s-metagenomics: in vivo** *Alistipes putredinis* *Bacterioides coprocola* *Christensenella minuta* *Clostridium clariflavum* *Oscillibacter valericigenes* *Ruminococcus bromii*

Fibre degrading bacteria.

The first name worth mentioning from Table 2 is the genus *Akkermansia* and the species *Akkermansia muciniphila.* As explained in the introduction, they are mucin-degrading bacteria associated with a healthy gut, linked with high-fibre diets and polyphenol-rich foods [81]. With these results we could assume that the three tested products have a positive effect on this bacterium, especially the pumpkin and the red radish, as they cause a growth in both, genus and species levels. Red radish and pumpkin are plant-based sources of dietary fibre. The proportions and types of fibre can vary between different crops and plant species, however, both plants contain a combination of soluble and insoluble fibre and different fibre molecules such as cellulose, hemicellulose, pectin, and potentially lignin. With the Bimi, we could expect to find a similar effect, as it is also an excellent source of dietary fibre, rich in vitamins, minerals and glucosinolates, but, during the test in the digester of chapter 6, only the R5 (transversal colon) showed a mild increase of this bacterium. However, it seems clear, from what we have seen in the introduction and in chapter 1, 4, 5 and 6, that fibre is one of the strongest gut microbiota modulators, and that is because its fermentation by the microbiota bacteria reduces it to constitutive monosaccharides, which, when metabolized, generate pyruvate, CO_2, H_2 or other metabolic intermediates. Generated pyruvate can be converted into diverse compounds, mainly acetate, propionate, or butyrate, all three SCFAs with great potential as microbiota modulators and contributors to good health.

The ratio of this SCFAs production is consequence of two combined factors: the microbiota profile (specific fermentative bacteria) and the kind of fibre intake. In general, acetate and butyrate are released from aldehydes fermentation (glucose, galactose, mannose, xylose) [142] whereas propionate is generally generated during ketonic glucosides fermentation (fructose, arabinose, tagatose) [143]. The genera *Bacteroides* and *Bifidobacterium* are a good example of cross-feeding interactions between members of different type of genera. *Bacteroides* is considered an important primary degrader of complex non-digestible carbohydrates (dietary fibre), thereby generating oligosaccharides, which in turn can be fermented by secondary degraders, for example the *Bifidobacterium* as an important and purported probiotic representative of secondary degraders [144]. In the first chapter we found positive correlation between *Bacteroides cellulosilyticus* and fibre intake. This species is equipped with an unprecedent number of carbohydrate active enzymes, providing a versatile carbohydrate utilization with a strong emphasis on plant-derived xylans [145]. It appears again in chapter 6, with the Bimi, obviously enhanced by its fibre content. This species would be considered a primary degrader, as it is able to degrade cellulose. However, within this genus, there are other species that would be considered secondary degraders that also appear in our chapters: *Bacteroides dorei* and *vulgatus* [146] in chapter 4 (pumpkin) both known for being butyrate producers. The ratio of fibre types in each of the tested foods is going to stimulate a different

bacterial response. Let's have a look now on which are the fibre fermenters that appear in more than one chapter, and which their effects are.

Butyrate producers

Bacteria from *Alistipes* [147], *Eubacterium* [148] and *Ruminococcus* [149] genera are butyrate producers through fibre degradation. Within the *Alistepes* genus, the pumpkin enhances its growth and of the species *Alistipes shahii*. The red radish also stimulates it; however, the most remarkable effect is the one of the Bimi, which increases *Alistipes obesi* and *Alistipes putredinis* in the digestor in addition to positive results of both groups (genus and species) in the human trial, extrapolating the results from the digestor into a real scenario. With *Ruminococcus* we find a very similar scenario, as all three products also stimulate their growth. The genus increases in the three in vitro tests, with specific species appearing with the pumpkin (*Ruminococcus albus* and *lactaris*) and again, with the *Ruminococcus bromii* extrapolating the positive effect in the human trial. *Eubacterium* follows the same pattern, increasing in the 3 chapters in the in vitro part (the species *Eubacterium eligens* exclusively with the Bimi), and showing significant results of the genus in the in vivo part. Another butyrate-producing genus that seems to be stimulated by the treatment of the tested foods is *Anaerotruncus* [150], increased with red radish and Bimi but not with pumpkin, *Cloacibacillus* and *Cloacibacillus porcorum* [151], stimulated by pumpkin and red radish but not Bimi, and *Parabacteroides* [152], increased with red radish and Bimi but not pumpkin.

Among the butyrate producers, a genus that gathers scientific interest is *Roseburia* [153], that although only appears with the pumpkin and the red radish, evidence shows a potential therapeutic role of this bacteria in human disease, especially in regulating barrier homeostasis, immune cells, and cytokines release through its metabolite butyrate in scenarios such as IBDs, type 2 diabetes, antiphospholipid syndrome, and atherosclerosis [154]. To finish with the increased butyrate producers, we must mention *Intestinimonas butyriciproducens* with the Bimi and *Gemmiger formicilis* with pumpkin and red radish. In this context, *Bifidobacterium*, *Lactobacillus* and *Prevotella* species have been associated with high butyrate increases.

Other SCFA producers

There are many other SCFA producers known to have a positive impact on health, that also appear in Table 2: *Oscillibacter valericigenes*, a valeric acid producer, has been reported for having an inhibitory effect on a variety of pathologies such as cancer, colitis, and cardiovascular and neurodegenerative diseases [155]. The decrease of its genus, *Oscillibacter*, may promote inflammation [156]. This species has a positive correlation with fibre intake (chapter 1) and again is positively influenced by red radish and Bimi treatment. It is also significantly increased in the human trial with the Bimi consumption, reinforcing the idea that fibre consumption has strong modulatory effect on gut microbiota, towards a healthier status, through a huge variety of mechanisms.

Oscillospira plauti, strongly related to elevated production of propionate, butyrate, and total SCFA, associated with thin phenotypes, and *Victivallis vadensis*, an acetate producer, are increased in Chapter 5 (red radish) and Chapter 6 (Bimi).

These species demonstrate a consistent increase in various chapters and indicate their potential role in SCFA production and fibre degradation. Their presence suggests a similar influence on SCFA production across different fibre-rich treatments (pumpkin, red radish and Bimi), highlighting the importance of fibre in promoting gut health. The specific bacterial taxa and their metabolic capabilities play a crucial role in determining the types and proportions of SCFA generated, which will be specifically analysed later. Understanding the interplay between dietary fibre, gut bacteria, and SCFA production can provide valuable insights into the potential health benefits associated with fiber consumption and inform about strategies for promoting gut health and overall well-being.

No fibre-degrading bacteria

Worth to mention is the increase in *Christensenella minuta*, which has been described to be enriched in subjects with low BMI (Goodrich et al. 2014). *C. minuta* administration in mice has been reported to prevent the onset of obesity through unknown biological mechanisms [157]. Many studies have shown human health associations with Christensenellaceae, going from serum lipid levels to longevity to metabolic disorders [158, 159]. Table 2 shows an increase of

the genus *Christensenella* with pumpkin and red radish, and a significant increase of the species *C. minuta* in the in vivo part of Bimi. This group, even thou do not seem to be a fibre fermenter, seems to be enhanced, rather by these foods' components or by the metabolic by-products generated by other bacteria. Even thou the gut microbiota is a very fragile environment, the fact that the three plant foods reproduce similar results, favouring the growth of this bacterium, indicates that they all have a similar impact on this ecosystem. Although we cannot know for sure which is the exact mechanism that stimulates these responses, our results confirm that the intervention with the chosen products has a marked modulatory effect on the microbiome, reinforcing many bacteria with a known positive role in the human gut microbiota. In the following part we will focus on other components of the tested foods that may have a modulatory effect on the microbiota.

Bioactive compounds: glucosinolates (GLS)

Bioactive compounds are natural compounds found in foods that are not essential nutrients like proteins, carbohydrates, and fats, but their consumption has been linked to positive health outcomes. It is worth noting that the bioavailability and health effects of these compounds can vary depending on factors such as food processing, cooking methods, and individual differences in metabolism [160]. Incorporating varied fruits, vegetables, whole grains, and plant-based proteins in the diet can help ensure an intake of diverse bioactive compounds that contribute to overall health and well-being.

Chapter 5 and 6 present two food products rich in GLS. In the next paragraphs we will try to elucidate whether these compounds have a positive impact on the overall health status through an amelioration of the microbiota profile, and if it is so, through which mechanism. In chapter 5, the use of elicitors in red radish crops stimulates the GLS content of the plant, especially methyl jasmonate (MeJA), widely found in cruciferous plants, such as red cabbage, reporting an increase in GSL concentration [161]. In chapter 6, no elicitors were used, however Bimi is a hybrid plant from Chinese kale (*Brassica oleracea* var. alboglabra) and Broccoli (*Brassica oleracea* var. italica), both plants standing out for their high content in GLS and phenolic compounds [162, 163], therefore a similar gut modulation could be expected from their GLS content. In table 2 we can see that there are three bacterial species with increased population in both chapters: *Akkermansia muciniphila, Christensenella minuta* and *Oscillibacter valericigenes.* The three of them are known for having a positive effect on the microbiota, however, through different mechanisms. For example, *A. muciniphila* and *O. valericigenes* are both fibre fermenters, but not *C. minuta*. Furthermore, only *O. valericigenes* is a SCFA producer, specifically of valeric acid. Maybe the combination of fibre and GLS is what is specifically stimulating the same three species in both food products. Concerning genera, there are three names that match in the in vitro part, *Eubacterium, Holdemania* and *Lachnospira*, meanwhile *Alistipes* and *Eubacterium* match between the in vitro from the red radish and the human trial from the Bimi.

Several studies showed that the human gut microbiome can provide myrosinase activity that potentially can raise the beneficial effects of consumption of vegetables rich in GLS [164]. Myrosinase is an enzyme that coexists in the same plants but is normally kept apart from GLS in different apparatus. When GLS coexist with myrosinases, this enzyme hydrolyses the GLS into the bioactive form, such as active isothiocyanates [165]. A key point is that myrosinase is temperature sensitive and can be inactivated upon exposure to temperatures over 60 °, as typically occurs during cooking. However, studies using animal models and population trials have suggested that human gut bacteria might act like an 'organ' in that they can secrete their own myrosinase [160], as rats and humans are short on this enzyme.

In a randomised crossover nutritional study where the effects of a high-cruciferous vegetable diet on gut bacterial community profile was evaluated, it was shown that the gut bacterial composition differed significantly [166]. *Eubacterium hallii, Phascolarctobacterium faecium, Alistipes putredinis*, and *Eggerthella* spp. were found as the microbial taxa closely associated with cruciferous vegetable intake [164]. Results from table 2 show a very similar profile of bacteria, indicating a similar microbiota modulation: *Eubacterium, Alistipes* and *Alistipes putredinis* increase in both chapters, and *Egerthella* and *Phascolrctobacterium* increase with the Bimi treatment.

The main difference between chapters 5 and 6 is that red radish is normally consumed raw, while Bimi is more usually cooked, therefore myrosinase activity from the Bimi would be inactive. The fact that both foods stimulate similar profiles could be explained by the hypothesis that gut microbiota is implicated in GLS metabolism. However, when comparing our bacteria with the known strains with myrosinase activity [167], there are no microorganism matching the bibliography, therefore it is possible that the main microbiota modulator is the fibre content in these two products and not the GLS bioactive forms. However, even thou we have no results showing a gut microbiota modulation from the GLS bioactive forms, there are many studies that outline the role of the polyphenols, glucosinolates and fibres against cancer progression in the gastrointestinal tract [168] and for their protective properties against IBD [169]. For further research, it would be interesting to replicate a similar intervention with raw versus cooked Bimi, to see if any difference in the microbiota modulation is due to the preserved myrosinase in the raw Bimi.

Gut microbiota and disease: metabolic disorders

Chapters 2 and 5 study several health markers and gut microbiota composition in relation to metabolic disorders, hepatic function, and cardiometabolic risk factors. In chapter 2, blood samples were drawn from the participants involved in the study, as well as faecal samples collected and analysed through 16s-metagenomics. Chapter 5 however, did not perform any human trial, it was all done in cell models, as explained in chapter 5, to evaluate the effect that red radish could have on several health markers and through an in vitro digestor to study its modulatory capacity. Therefore, as the results are more limited, it is trickier to relate the cell models with the microbiota. However, we will try to elucidate if there is any relation regarding metabolic disorders and gut microbiota through the results we have, as both chapters put the focus on the health biomarkers. Table 3 presents the association between bacteria and the different heath disorders from chapter 2. We will try to relate those bacteria groups with the ones from the in-vitro digester in chapter 5 and see if there are any coincidences regarding health benefits.

Table 3. Relation between the gut microbiota and the health markers presented in chapter 2 Bacterial taxa differing significantly in abundance when comparing the groups of individuals with high and low levels of biochemical biomarkers (FDR < 0.05)

Biochemical markers	Increase ↑	Decrease ↓
AST (μ/L)	**Family** Pasteurellaceae	**Family** Lactobacillaceae Oscillospiraceae Porphyromonadaceae Rikenellaceae

	Genera *Haemophilus* *Phascolarctobacterium*	**Genera** *Lactobacillus*
HDL-cholesterol (mg/dl)	**Family** Christensenellaceae	**Family** Peptococcaceae
Insulin levels (μIU/mL)	**Genera** *Phascolarctobacterium* *Peptococcus*	**Genera** *Butyricicoccus*

Hepatic affection

AST or aspartate aminotransferase is a hepatic biomarker that indicates an altered liver functions and higher levels of inflammation, when elevated [170]. Therefore, those bacteria that show a lower abundance with increased AST levels could be associated with healthier status in humans. In chapter 5, cholesterol metabolism is evaluated through changes in the gene expression of the membrane transporters, ABCA1 and ABCG5. These two are implicated in reverse cholesterol transport (RCT) and prevention of atherosclerosis. In the liver, ABCG5 has been proposed to efflux sterols into the bile for excretions. It also limits absorption of dietary cholesterol and plants sterols in the intestine [171]. ABCA1 can be found in macrophages, mediating cholesterol removal from these cells to HDL [172]. These ABC transporters are regulated by the liver X receptors (LXR), important regulators of cholesterol, fatty acids, and glucose homeostasis [173]. Elevated levels of these transporters could help in liver metabolism, enhancing liver function, and that's what we see in chapter 5 after the treatment

with red radish in the cellular models. In the in vitro digester, the first thing that gets our attention is the increase of *Lacticaseibacillus paracasei.* In chapter 2 there is lower abundance of the genus *Lactobacillus* when AST levels are high. Could this be suggesting that the bacteria from the *Lactobacillus* are in some way, related to hepatic health? Let's have a look on the other families from chapter 2, related with hepatic health and see if we can find any relation with chapter 5.

- Bacteria from the family Oscillospiraceae (see Table 3), that show an increase after the treatment with red radish:

Genera	**Species**
Oscillibacter	*Oscillibacter valericigenes*
Ruminococcus	*Oscillospira plautii*
	Ruminococcus callidus
	Ruminococcus bromii

- Bacteria from the family Rikenellaceae (see Table 3), that show an increase after the treatment with red radish.

Genera	**Species**
Alistipes	*Alistipes indistinctus*
	Alistipes putredinis

All the bacteria that appear here are related to SCFA production and as already explained, known for promoting beneficial gut bacteria and for their anti-inflammatory properties. The only species not mentioned previously in this thesis is *Oscillospira plautii,* which not surprisingly is another SCFA producer, and is associated with thin phenotypes [174]. Could these results link the consumption of red radish with hepatic health, suggesting that the consumption of foods rich in fibre and glucosinolates has the capacity to modulate the gut

microbiota towards a more protective one, with anti-inflammatory effect? Let's check the other markers and see if we can find a global relation.

Cardiometabolic health

Low levels of HDL cholesterol are considered a critical cardiometabolic parameter; however, this parameter can be modulated by dietary and lifestyle factors that contribute to changes in gut microbiota composition [175]. In chapter 2, individuals with higher levels of HDL-c presented a higher abundance of Christensenellaceae, suggesting a possible relation between this bacterial family and lower cardiometabolic risk. In chapter 5 we observe that red radish consumption stimulates the growth of the genus *Chrisensenella* and the species *Christensenella minuta,* which has been considered as a putative therapeutic candidate for the management of obesity and associated metabolic disorders [176]. Furthermore, blood pressure markers from chapter 5 seem to indicate a potential antihypertensive effect of fresh red radish.

Metabolic health

The adipose tissue is a dynamic organ, well known for its function in energy storage and mobilization according to nutrient availability and body needs, in charge of keeping the energetic balance of the organism [177]. During the last decades, adipose tissue has emerged as the largest endocrine organ in the human body, being able to secrete hormones as well as inflammatory molecules and having an important impact in multiple processes such as adipogenesis,

metabolism and chronic inflammation [178]. In chapter 5 there are two biomarkers (ADIPOQ, an adipocyte-secreted factor known as adiponectin, and the cytokine interleukin (IL)-6, an inflammatory mediator) that indicate an improvement of some obesity-related parameters after red radish consumption, specifically a reduction in adipogenesis and inflammation markers. Another crucial parameter in relation to metabolic health is the insulin resistance. It is considered the prelude to diabetes and tightly related to MS [179]. In chapter 2, we observe that high levels of insulin are related to low levels of the genus *Butyricicoccus,* again a butyrate-producing bacteria, considered a good probiotic candidate for its beneficial effects [180]. Even thou chapter 5 does not show an improvement in insulin resistance after the treatment, there is some bibliography that shows improved blood glucose levels after the consumption of radish [181]. In any case, we must emphasize that it is difficult to observe an amelioration of insulin resistance in our population since this parameter was not elevated at baseline.

The correlation of certain bacteria with health biomarkers from chapter 2 can be linked with the modulatory effect of the red radish in the digester microbiota. The cell models from chapter 5 help to translate the bacterial modulation in the digester, on health impact. All seems to indicate that health and gut microbiota are tightly related, and certain food composition may be enough stimulus for bacterial populations to affect the overall health status through their metabolites,

reducing inflammatory levels and improving markers related to cholesterol and fat metabolism, indicators of diseases such as obesity, diabetes and MS.

Gut microbiota modulation: a holistic view, through different methods and their pros and cons.

Along the chapters presented in this thesis, different methods have been used to study the gut microbiota modulation, with results sometimes hard to understand, as some of them seem contradictory. In this part, we will try to summarize them and compare the pros and cons of the different methodology and try to reach a final consensus about the impact of specific foods and if there is any pattern that could be reproduced, on the pursuit of a healthier status.

Plate counts

Table 4. Microbiological plate counts through chapters 3, 4, 5 and 6. Green arrows indicate increased populations and red arrows decreased.

Taxa	Chapter 3 Bio compound	Chapter 4 Pumpkin	Chapter 5 Red radish	Chapter 6 Bimi
Bifidobacterium	=	↑	↓	↓
Lactobacillus	↑	↑	↑	↓
Enterobacteria	↓	↓	↑	↑
Clostridium	↓	↓	↑	↑
Total Anaerobic	↑	=	=	=

Table 4 summarizes the results obtained by this technique along 4 different chapters, where a food modulatory capacity was tested. It seems a good indicator of the general microbiota fluctuation during the treatment period, but the problem is that only focuses on 4 different genera, giving a limited view of the global microbiota modulation. For example, *Clostridium* may be considered a not desirable bacteria cluster in general, but as previously explained, many

Clostridium species are capable of fibre degradation even to produce SCFA, considered healthy metabolites. The same problem may appear with the beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus*. It may seem that the global population is decreasing but the result may be that a specific bacterial known for its great probiotic proprieties had a great increase, therefore this analysis is not conclusive at all.

PROS: quick and affordable techniques that gives a first glimpse into the treatment result.

CONS: very low specificity and short range. Further analysis is required to know the behaviour of the microbiota in general.

In-vitro digester

The modulatory capacity of the chosen foods was tested in the in vitro digester. This technique gives more detailed results, very specific and a great range. It could be a first step before studying the product in humans. The only problem with this tool is the amount of time required to test every product. Each of them needs about a month to be tested. Because of that, there are no duplicates of the in vitro assays. Another problem is that this kind of digesters is not dynamic, as it is a human digestive system, and therefore it lacks other stimuli such as natural body movement, hormones, trophic activity, etc. This fact causes that the microbiota population loses richness and diversity during the experiment and

sometimes this may mask the modulatory effect of the tested product, as the general population diminishes.

PROS: generates very specific and detailed results, without the need to test in humans.

CONS: takes a lot of time and is not able to fully reproduce the natural conditions, as it is a static environment.

SCFA measurement

SCFA measurement is another affordable and easy technique to do, that gives a better idea about the treatment influence. Higher levels of SCFA are usually related to a better gut health [182]. However, we have found a couple of situations where the SFCA levels did not match with what we would have expected according to the modulated bacteria profile. For example, in chapter 5, there was a significant increase in acetic and propionic acid in the three sections of the colon after red radish treatment. Butyric acid only showed a significant increase in AC but decreased in TC and DC, although there were many butyrate-producing bacteria in those reactors according to the metagenomic results. However, in chapter 4 and chapter 6, where similar results regarding the butyrate producers were found, butyric acid levels showed a marked increase. A possible explanation is that the butyryl-CoA: acetate-CoA-transferase pathway is the main process for the biosynthesis of butyrate, so acetate is utilized by butyrate

producers to produce butyrate. Thus, a butyrate increase following the acetate increase would be expected. In Chapter 6, acetate levels decrease in TC and DC after the treatment. As acetic and butyric acids work synergically, this reduction of acetic acid could be explained by this huge global increase of butyric acid in all reactors, as acetate may be consumed by the butyrate producers. A hypothesis for this not happening in chapter 5 could be that, with more time, butyrate levels would have increased, as expected by the acetic levels and the bacterial profile. Red radish should stimulate the butyrate synthesis at some point.

PROS: an affordable and quick technique that allows to reach a preliminary conclusion about the treatment effect on the gut microbiota

CONS: it needs to be contrasted with bacterial populations as it can be misleading.

Biomarkers

The colon is the primary site for the biotransformation of bioactive compounds from food that are not absorbed in the small intestine, but the use of static in vitro methodologies may be overly simplistic due to the continuous influx of complex endogenous compounds throughout the digestive tract, such as intestinal secretions, absorption of fermentation products, and interactions between the host and bacterial population. In chapter 5, cellular models of the target organ or biological function were integrated to the dynamic in vitro model, allowing the reproduction of physiological states at a laboratory scale, where manipulation

and assay conditions are controlled. Intestinal cells were seeded onto bicameral polycarbonate supports and treated with samples T12 and T26 from the digestor. After the incubation time, the content of the basal chamber was collected for use in other cellular models. The use of this medium on the other cellular models permitted elucidation of the compound's mechanism of action. Chapter 5 cell models (cellular lines from human enterocytes CaCo-2, endothelial human cell line EA.hy926, and human epithelial liver cells HepG2) studied functionalities related to MS: blood pressure, cholesterol metabolism, insulin resistance, fat accumulation and adipogenesis. This helped to understand how the red radish modulatory effect may translate into systemic changes. Furthermore, the results obtained were very promising.

PROS: it is a relative affordable technique that allows a better understanding of the compound mechanism of action, being a perfect complement of the microbiota analysis.

CONS: in an in vivo situation, some of these cells are not going to be in direct contact with the intestinal content, so the results of this model might not represent real physiological effects.

Human intervention studies:

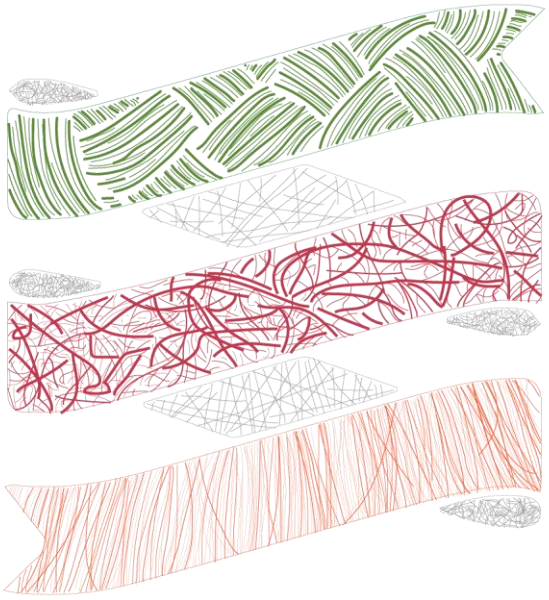
Human trials are the last step of any study before its application or commercialization. Chapters 1, 2 and 6 give a great overview of the relation between diet, health, and microbiota in vivo. Some results can be linked between

chapter 2 and 5 about metabolic disorders and its associated microbiota profile. Chapters 1 and 6, both including human trials, allow associating diet characteristics with specific bacterial species. In both chapters *Oscillibacter valericigenes* and *Ruminococcus bromii* appears, both known for having beneficial effects through their SCFA production. No results exhibit any potential danger for human health of the tested products. No dysbiosis was observed in any of the participants.

PROS: it is the best way to evaluate how a certain intervention is going to work and its safety or hazardousness.

CONS: it is a complex intervention that requires a huge implication from the participants and the scientific personal. Resource and time demanding, needs a good teamwork. Many personal working on, which implies a great chance for human error. Also, the huge variability in gut microbiota composition between the different subjects implies that the sample size must overcome this fact.

COROLLARY



The discussion over the 6 different chapters shed some light into the relation between dietary habits and microbiota. Including a variety of plant-based foods in the diet, seems to promote the growth of *Bifidobacterium.* The human data reveals that higher consumption of yogurt and fermented products, as well as the consumption of Bimi, positively correlates with a growth of *Lactobacillus*. Moreover, a high intake of fibre from vegetables promotes fibre-degrading bacteria, increasing the output of beneficial metabolites such as SCFA. It is noteworthy to mention the increase of the genus *Alistipes*, butyrate producers enhanced by the three foods tested. Another SCFA producer worth mentioning is *Oscillibacter valericigenes*, standing out by its anti-inflammatory effect, strongly correlated with MD, and increasing again within chapters 4, 5 and 6. *Akkermansia muciniphila* and *Christensenella minuta* are healthy bacteria that appear to be related to both, fibre, and bioactive compounds: Bimi and red radish may have synergistic effect on their modulation due to their specific composition. However, we have observed that the effects of these bacteria vary depending on the specific species, strains, and individual contexts, therefore it is hard to adopt a general recommendation for everyone. Regarding the glucosinolates, results are not conclusive whether the gut bacteria may have enzymatic capacity to transform these compounds into their bioactive forms, highlighting the necessity to consume raw foods rich in GLS as the only way to ensure the consumption of the enzymes able to transform these compounds into their bioactive forms.

For closure, we must mention that the variety of methods used to evaluate the tested food effects on gut microbiota allowed a wide perspective. The in vitro digester is a good approach for the development of new food products, as it allows a first evaluation of its modulatory capacity. The wide variety of techniques allowed a better understanding of what was happening and fulfilled some gaps that otherwise, would have been hard to read. However, the fact that this thesis had come from an industrial project, has limited the possibility to modify the plan during the process, which could have offered a chance to repeat or modify some techniques. Further research is still needed to discern if the presentation of foods (raw or cooked) has a detrimental effect on food properties.

Conclusions

This thesis associates the gut microbiota profile with MD and specific food groups and confirms the potential of new designed functional foods on gut microbiota modulation. Altogether, the results of this work can be summarized in the following conclusive statements:

Chapter 1 & 2

1. A high adherence to the MD seems to increase the abundance of some species associated with good health. *Bifidobacterium animalis* is the one with the strongest association with MD.
2. Fiber intake enhances the growth of several SCFA-producing species, such as *Oscillibacter valericigenes*, *Oscillospira (Flavonifractor) plautii*, and *Roseburia faecis*. *R. faecis* is also enhanced by fruit and nut consumption.
3. Legumes enhance *Ruminococcus bromii*, vegetables increase the *Butyricicoccus pullicaecorum* population and nut intake benefits *Papillibacter cinnamivorans* growth.
4. Higher meat consumption shows a negative correlation with *Oscillospira* and higher consumption of fermented dairy a positive relation with *Lactobacillus* levels. A decrease in Oscillospiraceae and Lactobacillaceae has been described in individuals with altered liver function.

5. Olive oil consumption increases the abundance of Verrucomicrobiaceae, the family of *Akkermansia muciniphila*, negatively associated with overweight obesity, hypertension, and type 2 diabetes.

Chapter 3

6. The in vitro colonic fermentation is a useful tool to reproduce the gut microbiota under diverse conditions and treatments. The 16S rRNA analysis, allows characterization of the changes in microbiota composition up to species level.

Chapter 4, 5 & 6

7. Elicited pumpkin shows a prebiotic effect and increases ratios of butyric and propionic acid in the TC and DC.
8. The treatment of the whole red radish plant has a greater and more varied content of isothiocyanate, suggesting a healthier impact. Its consumption increases SCFA production, especially acetic and propionic acid, as well as many butyrate-producing bacteria.
9. There is an increase in acetic, butyric, and propionic levels accompanied by the growth of many SCFA-producing bacteria with Bimi® treatment in the *in vitro* model. Its consumption stimulates probiotics presence in humans.

Take Home Message

To pursue a healthy gut microbiota, be sure that your daily diet includes abundance of plant food, of a wide variety of colours and origins.

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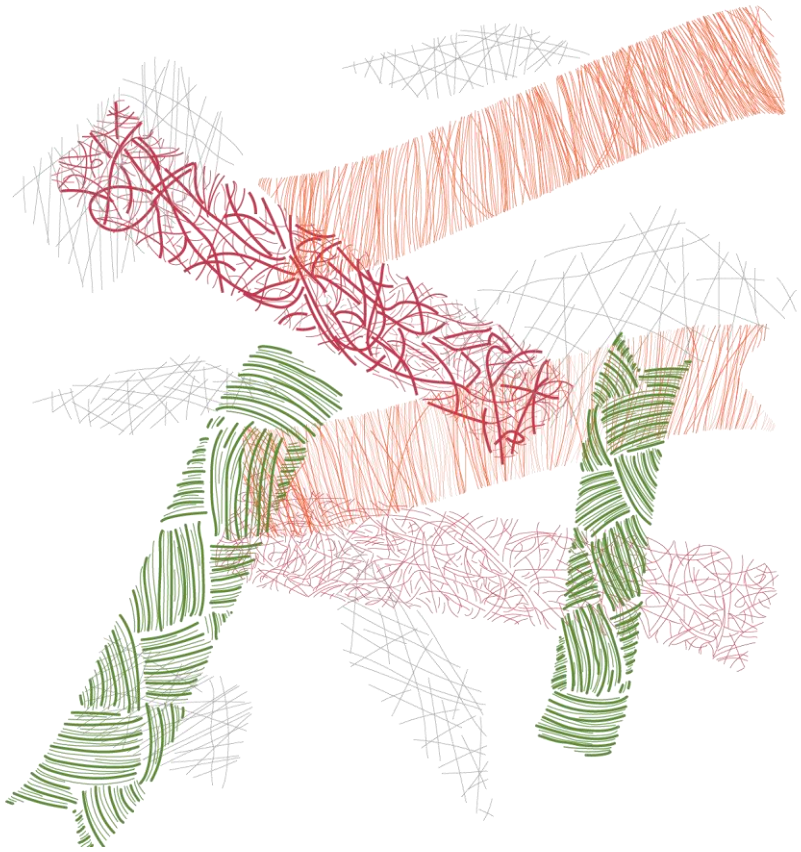

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