Ph.D. SCHOOL IN FOOD SYSTEMS

Department of Food, Environmental and Nutritional Sciences

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Evaluation of the impact of dietary interventions on the human intestinal microbial ecosystem through improved bioinformatics and statistics

[AGR 16]

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Preface

"SOCRATES: Well, then, let us see in what way the self-existent can be discovered by us; that will give us a chance of discovering our own existence, which otherwise we can never know.

ALCIBIADES: You say truly.

SOCRATES: Come, now, I beseech you, tell me with whom you are conversing? — with whom but with me?

ALCIBIADES: Yes.

SOCRATES: As I am, with you?

ALCIBIADES: Yes.

SOCRATES: That is to say, I, Socrates, am talking?

ALCIBIADES: Yes.

SOCRATES: And Alcibiades is my hearer?

ALCIBIADES: Yes.

SOCRATES: And I in talking use words?

ALCIBIADES: Certainly.

SOCRATES: And talking and using words have, I suppose, the same meaning?

ALCIBIADES: To be sure.

SOCRATES: And the user is not the same as the thing which he uses?

ALCIBIADES: What do you mean?

SOCRATES: I will explain; the shoemaker, for example, uses a square tool, and a circular tool, and other tools for cutting?

ALCIBIADES: Yes.

SOCRATES: But the tool is not the same as the cutter and user of the tool?

ALCIBIADES: Of course not.

SOCRATES: And in the same way the instrument of the harper is to be distinguished from the harper himself?

ALCIBIADES: It is.

SOCRATES: Now the question which I asked was whether you conceive the user to be always different from that which he uses?

ALCIBIADES: I do.

SOCRATES: Then what shall we say of the shoemaker? Does he cut with his tools only or with his hands?

ALCIBIADES: With his hands as well.

SOCRATES: He uses his hands too?

ALCIBIADES: Yes.

SOCRATES: And does he use his eyes in cutting leather?

ALCIBIADES: He does.

SOCRATES: And we admit that the user is not the same with the things which he uses?

ALCIBIADES: Yes.

SOCRATES: Then the shoemaker and the harper are to be distinguished from the hands and feet which they use?

ALCIBIADES: Clearly.

SOCRATES: And does not a man use the whole body?

ALCIBIADES: Certainly.

SOCRATES: And that which uses is different from that which is used?

ALCIBIADES: True.

SOCRATES: Then a man is not the same as his own body?

ALCIBIADES: That is the inference.

SOCRATES: What is he, then?

ALCIBIADES: I cannot say."

(from PLATONE, Alcibiade first, F. Adorno, in *Opere cit., vol. V, p. 81-83)*

What is a man?

Socrates with the word "man" includes/speaks of all he is concerned with and puts great attention to the thought and the concept of soul. Although it is not the purpose of this thesis to deal with this topic, more relevant to the studies of metaphysics, this question should be kept in mind and used as a background guideline along this work.

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1. State of the art

1.1 Microbiota

1.1.1 The super-organism

In the past decades, the scientific community has made progress in studying not only the physical object "human" in terms of its innate complexity and nature, but also the close and fundamental symbiosis with complex, dynamic communities of other organisms.

This discovery defined a new realm of individuality called a 'super-organism' or 'holobiont' (Greek, from holos, whole; bios, life; -ont, to be; whole unit of life), (Dheilly 2014; Gilbert 2014) composed of host cells (or human body) and persistent populations of symbionts that inhabit it (de Moreno et al. 2014). These complex, ecological symbiotic communities are composed of commensal, symbiotic and pathogenic microorganisms, such as bacteria, fungi (mainly yeasts), protozoa, archaea, and viruses (mainly bacteriophages) (Ferranti et al. 2014). The whole system is entitled "microbiota". The microbiota is located at different sites of human body, mainly the ones exposed to the outer world, (e.g. gastrointestinal tract, skin, oral and nasal cavity, vagina and urogenital apparatus, with more than 70% of all microbes in the human body situated in the colon). It is estimated that the number of the microorganisms residing in the gastrointestinal tract is likely 10 times higher than the number of somatic cells composing the whole body (approximately 100 trillion (Sandrini et al. 2015). They add 1.5-2 kg to the total body weight and their gene content, called microbiome, is about 150-200 times larger than that of the human genome (Gilbert 2014).

In conclusion, the "super-organism" is the new subject to be investigated in order to answer the question "what is a man?" and the topic has to be approached by using the "complex systems theory".

A “complex system” is defined as the structure in which individual parts are affected by short-range interactions which lead to changes in the overall system. The inability to study as a whole system, leaves us with one preferential option: studying the local changes and short-range interactions in order to establish the general rules that govern the whole system, always keeping in mind the quote by French philosopher Edgar Morin "*in complex systems, unpredictability and paradox are always present and some things will remain unknown.*" (La connaissance de la connaissance, 1989).

1.1.2 The intestinal microbial ecosystem

The discovery of the numerous microorganisms strictly interacting with the human body led to numerous questions pertaining to the influence that gut microbiota (composed of mostly anaerobic bacteria) might have on the physiology of the human organism. The gut microbiota is potentially expressing around 4 million distinct genes. Most of these genes encode for proteins (i.e. enzymes) involved in the digestion of food and metabolic reactions essential for host homeostasis. This complex system could be compared to a bioreactor that generates molecules interacting, both directly and indirectly, with immune-system, epigenome and metabolism of the host (Li et al. 2008). Extensive research in this field together with the advancement of high-throughput technologies, helped elucidating significantly the influence of intestinal microbial ecology (IME) on human physiology. This influence is evident in many different processes, from the modulation of the effects that ingested food has on host’s health (Bourassa et al. 2016) to the direct influence on host’s gene expression (Forsythe and Kunze 2013).

1.1.3 Inter-personal microbiota variability

IME influences human processes that determines also our behaviour and the fundamental aspects of our personality (Cryan and Dinan 2012), leading to an obvious but important question: if the microbiota is so fundamental for the human physiology, how conserved is its composition over time and between different people?

There is an extraordinary interpersonal microbiota variability within the human gut. Each individual has a unique microbiota composed of distinct combination of microorganisms. This observation led to the hypothesis that each individual possesses a unique microbiota in composition, like a microbial 'fingerprint'. This diversity arises from all the diverse factors that can affect the development and stability of the microbiota, such as host genetics (Goodrich et al. 2014), physiological status, pathologies, environment, diet and lifestyle (Schippa and Conte 2016). In fact, while serial gut microbiota samples coming from the same organism typically show similar microbiota composition over time, the similarity in microbiota composition among gut samples from different organisms is significantly lower. According to the study of Tojo et al., more than 80% of individuals maintains the same microbiota composition during life, which can be used as a unique identifying marker (Tojo et al. 2014). The observation that less than 50% of bacterial taxa at the species level are shared between monozygotic twins is another proof of the microbiota originality (Tojo et al. 2014). Despite the considerable variability in the composition of the gut microbiota between individuals, the core set of redundant genes, among different bacterial species, remains stable over years. This 'functional core' is indispensable for basic, house-keeping metabolic activities and remains quite similar between individuals (Schippa and Conte 2016).

1.1.4 Personalized responses based on microbiota

The variability of microbiota observed between individuals is a consequence of different host responses to the external stimuli like therapeutic treatment, diet and lifestyle. Intestinal microbiota can be considered an optimal target for therapeutic manipulation due to its accessibility. The therapeutic manipulation could be performed by simple procedures such as addition of probiotic microorganisms in the diet, use of specific antibiotic molecules, or the modulation of nutritional sources.

It has been observed that the gut commensals are able to modify drugs through a variety of bio-transformation processes, such as hydrolysis and reduction, significantly impacting also on the efficacy of therapeutic treatments (Iida et al. 2013). The dietary habits have the dominant role in shaping the IME. There is evidence indicating that different glycaemic

responses given by different individuals to the identical meals are associated with differences in microbiota composition (Suez et al. 2014). Specifically, the metabolic functions of the intestinal microbiota of a single subject regulate food processing and therefore nutrient availability. Significant changes in microbiota composition affect the reactions that transform ingested food into nutrients available for the absorption. Based on these observations, nutritional science should take into account also the connection between dietary intervention and microbiota. This correlation has been confirmed by several cases. For instance, the study performed by Hazen and colleagues (Koeth et al. 2013) demonstrated a cause-effect link between the gut microbiome, red meat consumption and atherosclerosis. The authors showed that specific gut bacteria transform L-carnitine, a nutrient abundant in red meat, into trimethylamine (TMA), which is a rapidly absorbed volatile molecule, subsequently converted to the atherogenic molecule TMAO by liver flavin monooxygenases. The abundance of TMA-producing bacteria is variable among people and largely depends on the dietary pattern. In fact, the same study showed a reduced ability to convert carnitine into TMA and then TMAO by vegetarians compared to omnivore people (Hu et al. 2000). Then, the consumption of red meat may promote cardiovascular diseases particularly in people with increased susceptibility due to a specific microbiome profile.

Regarding the needs to personalize universal dietary recommendations, a recent study showed the microbiome-dependent induction of glucose intolerance caused by consumption of non-caloric artificial sweeteners (Suez et al. 2014). In a pilot prospective study, it was shown that even short-term consumption of non-caloric artificial sweeteners may increase glucose intolerance in a subpopulation of human individuals that possess a peculiar intestinal microbiota structure. These data suggest that general recommendations for the reduction of sugar consumption via the widespread use of non-caloric artificial sweeteners may be harmful for some people.

1.1.5 The concept of ‘enterotype’

Nowadays, the importance of the role of the microbiota for human health has become evident. The wealth of knowledge acquired during the last 15 years by the research on the

intestinal microbiota and microbiome has been used to define proper biomarkers of health and disease through the definition of microbial co-occurrence patterns. In this context, the concept of "enterotypes" was proposed (Arumugam et al. 2011). The enterotypes are classes of gut microbiotas distinguished according to the abundance of specific bacterial groups. It has been suggested that the human gut microbiota fall into three distinct enterotypes defined by differential representation of genera. Enterotype I is rich in *Bacteroides* and other bacteria belonging to Bacteroidete*s* phyla that take energy above all from protein and carbohydrates fermentation. Enterotype II is enriched in *Prevotella* and *Desulfovibrio*, which are bacteria specialized in the degradation of complex dietary fibers. Enterotype III is rich in *Ruminococcus* and *Akkermansia* that are mucin degradation bacteria (Arumugam et al. 2011). The enterotypes described so far appear to be continent, ethnicity, age and gender independent. Furthermore, notably, only a habitual long term diet can influence the foundation of a specific enterotype (Wang et al. 2015).

However, the actual existence of the enterotypes is controversial; for instance, almost two-thirds of the Russian faecal samples analysed in a recent study were not dominated by either *Prevotella* or *Bacteroides* genera, but contained novel community structures that were not observed in non-Russian metagenomes (Tyakht et al. 2013). This notion challenges current microbiota subdivision, suggesting probably the existence of other enterotypes. Nonetheless, there are also studies that contest these findings, supporting a continuous gradient of dominant taxa rather than distinct clusters (Schippa and Conte 2014).

1.1.6 Intra-subject evolution of the gut microbiota

Human beings constantly evolve during their life span. Since the bacterial communities live in close interaction with the host, a question arises about how host variation impacts the development of bacterial communities. The differences observed in the microbiota structures among individuals and during life in a single subject cannot be determined by different diet habits only and, therefore, remain at least partly unexplained. Microorganisms first colonize the host during the transition from the in utero environment to the mother's vagina, which changes its IME just before birth (Biasucci et al. 2008). The study by Dominguez-Bello et al.

(2010) showed that the microbiota of newborns differs drastically depending on the type of childbirth (natural or caesarean). It has been proposed that the new-born microbiota resembles the mother's vaginal or skin microbiota depending on the first bacteria communities that the new-born encounters during the delivery (Dominguez-Bello et al. 2010). Therefore, there exists a similarity between mother's and newborn's microbiota promoted by the vertical transmission of bacteria (faecal-oral, oral–oral and skin-oral routes) from the mother to the newborn (Adlerberth and Wold 2009). The study by Palmer and collaborators showed that the earliest gut colonisers are generally facultative anaerobes, followed by strict anaerobes. Palmer advanced the hypothesis that the intestinal microbiota is strongly influenced by maternal communities (Palmer et al. 2007).

1.1.7 Macronutrients and microbiota

Nutritional components (macro/micronutrients) are not only important for the host nutrition, but also for shaping the microbiota (Wu et al. 2011).

The major macronutrients implicated in the gut microbiota modulation are the carbohydrates. The complex polysaccharidic fibers that are not absorbed by the proximal intestine represent the principal source of energy for the intestinal microbes (Conlon and Bird 2014). The gut bacterial communities produce carbohydrate-active enzymes (CAZymes) that facilitate the degradation/fermentation of a vast range of (otherwise non-degradable) carbohydrates, with production of organic acids and other nutrients used by the host and the surrounding bacteria (Gill et al. 2006). The most important catabolites of this process for host's physiology are the short chain fatty acids (SCFAs), especially: acetate, butyrate and propionate (Tojo et al. 2014).

Proteins are another macronutrients that influence microbial growth. Dietary proteins serve as the major source of nitrogen (including ammonia) for colonic microbial growth (Schippa and Conte 2014). Notably, diets that promote microbial protein synthesis (and the use of ammonia as N source), efficiently redirect systemic N excretion from kidneys to faecal stream, resulting beneficial for renal health. (Conlon and Bird 2014). Conversely, protein fermentation increases the formation of putrefactive products and, consequently, the generation of gases (Backus et al. 2002). Also, many end-products of protein fermentation (e.g. ammonia,

hydrogen sulphides, amines, phenols, thiols and indoles) have been shown to have cytotoxic, genotoxic and carcinogenic effects in vitro and in animal models (Amstberg et al. 1980, Backus et al. 2002)

Dietary fats may influence the gut microbiota indirectly through bile acids, although the exact mechanism of this interaction is not completely understood. For instance, it was shown that members of the intestinal microbiota produce the enzyme 7 α-dehydroxylase transforming the primary into secondary bile acids such as deoxycholic acid, which exerts hepatotoxic effects (Yoshimoto et al. 2013). It is also known that the excess of lipids in the diet increases intestinal permeability, leading to enhanced translocation of bacterial lipopolysaccharide in the bloodstream (Conlon and Bird 2014, Moreira et al. 2012). Further investigations are required to clarify the interaction between fats introduced with the diet, bile acids and microbiota modifications.

1.1.8 Short chain fatty acids (SCFAs)

Acetate, propionate and butyrate are the major SCFAs present in the colon, accounting for 90% of the total SCFAs produced, with molar ratios approximately of 60:25:15. Other minor organic acids produced include lactate, succinate, valerate, pyruvate, and formate. Branched-chain SCFAs (e.g., isobutyrate and isovalerate) are formed during the fermentation of branched chain amino acids (Wong et al. 2006).

In the caecum and colon the fermentation is very intense, leading to a high production of SCFAs, pH reduction (range 5-6) and rapid bacterial growth. In the distal colon the fermentation process almost stops due to the lack of substrates, leading to a higher pH and putrefactive processes.

The SCFAs have many important functions in the host physiology. i) Butyric acid for consistency serves as the principal source of energy for colonocyte differentiation and apoptosis, and therefore it is essential for maintaining mucosal integrity and protection against colon cancer (Brinkworth et al. 2009). Furthermore, butyrate modulates intestinal inflammation responses and regulates glucose metabolism through the hormone glucagon-like peptide-1. ii) Propionic acid positively influences the growth of hepatocytes and it is

considered an additional source of energy for the host (used mainly for the synthesis of glucose and lipids). iii) Acetic acid has a positive effect on the development of peripheral tissues, in particular in muscles (Conlon and Bird 2014). Moreover, SCFAs modulate glucose and cholesterol metabolisms and stimulate the absorption of calcium, magnesium and iron (Wong et al. 2006).

1.1.9 Probiotics and prebiotics

It has been proposed that the observed differences in the intestinal microbiota composition of Western country people compared to people in developing countries and rural areas of Africa and South America, are plausibly due to the industrialization processes in food manufacturing, as all foodstuff nowadays is processed in order to increase shelf-life, decreasing at the same time the presence of microorganisms. In comparison, the diet of our forefather consisted of fermented foods (fermentation was a widespread method for food conservation), that was also a way to introduce considerable quantities of different bacteria, increasing the overall microbial diversity in the intestine. Of note, nowadays the overall microbiome richness is also low in people living in the urban areas of the Western world, as a possible consequence of a general distancing from natural environments with a consequential little exposure to soil, animals, and associated microbes (Ferranti et al. 2014). These environmental and lifestyle changes in the Western world have created a need to reintegrate live bacteria that are no longer introduced with the diet. In this context, strong industrial interests arose to implement the knowledge on and production of probiotics.

The term probiotic, meaning “for life,” is derived from the Latin word (*pro*, “in favour of”) and the Greek word (*bios*, “life”). The word “probiotic” was first used in 1965 to describe “substances secreted by one microorganism which stimulates the growth of another” in antithesis with the term antibiotic (Lilly and Stillwell 1965). Almost 10 years later, Parker (Parker 1974) proposed a different definition of probiotic (“organisms and substances which contribute to intestinal microbial balance”) which is not far from the currently accepted definition: “*Live microorganisms which when administered in adequate amounts confer a health benefit on the host*” (FAO May 2002, FAO/WHO).

Probiotic microorganisms have been demonstrated to possess several health promoting properties. Probiotics may exert a regulatory effect on the intestinal epithelial barrier in different ways, for instance through a direct action on the intestinal epithelium, by stimulating the synthesis and secretion of mucin by the goblet cells. Probiotic microorganisms may also antagonize pathogens by competing for the adhesion to epithelial surfaces, or through the production of antimicrobial molecules Probiotic microbes can moreover improve the stability of tight-junctions, reducing the epithelial permeability and possible translocation of pathogenic agents and their products. (Patel RM and Lin 2010). In addition, several probiotic strains have been demonstrated to modulate the host immune system (Ashraf and Shah 2014).

Besides the administration of live bacteria, gut homeostasis also benefits from diet integration with prebiotics molecules. A prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit." (Gibson et al. 2017). The concept of prebiotics has attracted increasing attention, stimulating both scientific and industrial interest. However, several food components, especially oligosaccharides and polysaccharides, have been claimed to possess prebiotic activity even though a scientific demonstration is missing (Roberfroid 2007). Not all dietary carbohydrates are prebiotics, and there are specific criteria for classifying a food ingredient as a prebiotic. According to Gibson et al. (2014), these criteria are:

a) Non-digestibility: testing of prebiotic resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption.

b) Fermentation by the colonic microbiota.

c) Selective stimulation of growth and/or activity of intestinal bacteria.

1.2 Microbiome data analysis

The step that allowed the progression in the scientific research on microbial ecosystems was the introduction of next-generation sequencing technologies (NGS). NGS allowed for fast and low-cost DNA sequencing, permitting the generation of millions of sequences. Deep

sequencing analysis enables characterization of microbial communities in different environments without the need of culture-based methods increasing enormously the information obtained from complex microbial ecosystems

A critical issue of the microbiota study is the correct analysis and interpretation of the data. The data produced in the microbiota studies has several biases due to the complexity of this system and due to the technology used. These aspects of the data analysis in microbiota research are discussed in the next paragraphs.

1.2.1 Next generation sequencing technologies

NGS technologies come from the Sanger method based on the selective incorporation of chain-terminating dideoxynucleotides in the first automatic sequencing machine was produced by Applied Biosystems in 1987; (Liu et al. 2012). Currently, there are several platforms available for sequencing. Each one has advantages and disadvantages regarding generation of largest total throughput per run, read length, accuracy and time. In the studies included in the present PhD thesis two different sequencing platforms have been used: Ion PGM from Ion Torrent and Illumina's MiSeq.

The output data originating from NGS consist of hundreds of thousands of reads, which can be counted as a discrete number of counts. The analysed samples will express their diversity qualitatively and quantitatively in terms of diversity in the sequence of bases and library size. In this context, statistics has a pivotal importance to properly manage and interpret the data generated by the different NGS approaches.

1.2.2 Amplicon sequencing

NGS has been used to characterize the human intestinal microbiota. PCR amplification of the 16S rRNA gene is considered the most suitable way to define the taxonomic composition of the bacteria in a metagenomic sample. The 16S rRNA gene is a region of the bacterial genomes that includes both very conserved and variable sequences. Conventionally, only specific regions of 16S rRNA gene are sequenced in microbiomics. In fact, due to the length of the

whole gene (about 1.5 kb), sequencing the full gene would lead to decreased depth of sequence coverage, making it more difficult to detect the rare taxa present in the sample.

Specifically, the 16S rRNA gene contains nine hypervariable regions (Vn), which possess a sequence divergence that permit the potential discrimination of bacterial taxa to the level of species. Not all the Vn have different sequences among different species. In fact, some species can be distinguished only by one of the nine Vn. Schloss (2010) reported that the use of different variable regions influences the richness and evenness of communities (Schloss 2010). Since the usage of any specific Vn of the 16S rRNA gene has advantages and disadvantages, some researchers opt to sequence more than one variable region simultaneously to get a clearer view of the composition of the microbiome. According to the study of Shah et al., the V1–V3 or V1–V4 regions should be targeted for microbiome sequencing, although the choice should be made by taking into account the aim of the experiment and at the nature of samples (Shah et al. 2011).

Another issue is given by the primers used for the amplification of the 16S rRNA gene region. Even though these primers are considered “universal” (or, more correctly, pan-bacterial), they preferentially anneal on the 16S rRNA gene of certain taxa over others, resulting in the over-representation of specific taxa. Another bias is introduced by the fact that the 16S rRNA gene is present in multiple copies in the bacterial genome (Acinas et al. 2004) leading to the over-representation of the abundance of certain taxa. Furthermore, in certain bacterial species (e.g., extremophiles; [Acinas et al. 2004]), the sequences of the multiple 16S rRNA genes in the same genome diverge, resulting in the overestimation of the taxonomic diversity.

1.2.3 Taxonomic association

The sequencing process produces an output file containing all DNA sequences obtained, which are conventionally called “reads”. The reads are associated to an identification code (called barcode) used to pair each sequence to a unique sample. The pairing phase leads to the reads count, in which each sample has a precise pool of sequences that expresses the numerical data of 16S rRNA gene copies present in the metagenomic DNA; next, the taxonomic association is performed. Each read is compared by a Bayesian taxonomic classifier with the

16S rRNA gene sequences present in a database in order to be associated to a specific bacterial species. Important considerations have to be taken in account to perform this bioinformatics step. Sequencing errors can lead to minor different amplicon sequences within a species creating an overestimation of bacterial diversity. To limit this bias, the reads are aligned based on their similarity and grouped in clusters. Typically, 97% similarity is used as a cut-off for the approximate clustering with species-level resolution, that corresponds to the operational taxonomic units (OTUs) (Goebel and Stackebandt 1994). Based on the number of clustered sequences present in the sample, it is possible to extrapolate the richness of the biological sample, even if the clusters are not associated to a taxonomy at this point. This phase is called "OTU picking". Two approaches can be adopted to determinate the OTUs. (i)The most common one is "*de novo* OTU picking". In this approach, all reads are compared to each other and grouped into OTUs based on their similarity. (ii) The second approach is named "reference picking". Here, the sequences are compared to a database and clustered into OTUs based on their similarity to a known sequence in the database.

Each approach had pros and cons. For example, the de novo picking includes more erroneous sequences than the reference picking. At the same time de novo OTU picking does not discard any sequence and it is not limited by the quality of the database used for the annotation. Even though reference picking is quicker and creates less errors, this approach has the disadvantages of discarding many potentially valid sequences due to factors such as mismatches in the database.

The annotation of the OTUs is the last step in the bioinformatics pipeline. For this step, one sequence is selected per each cluster and compared with the sequences in a 16S rRNA database in order to assign the taxonomy to each specific OTU cluster.

1.2.4 Biodiversity determination

The biodiversity of the microbiota can be analysed through several different methods, the most commonly used being the alpha and beta diversity indexes.

The alpha diversity indexes are defined by two different parameters: the richness index and the diversity index. The richness index indicates the number of different bacteria present in a

sample and it is determined by the number of OTUs present in the sample (e.g., Chao1 index) (Chao, 1984). The diversity index combines richness with the uniformity of distribution of the different bacteria in a sample (evenness). Diversity index is commonly determined by using the Simpson diversity index and the Shannon diversity index (Li et al. 2008). In general, the alpha diversity indexes measure the intra-sample diversity.

The beta diversity indexes measure the biodiversity by evaluating the similarities between different group of samples. The beta diversity accounts for the presence or absence of OTUs, their abundance and their phylogeny; therefore, in this analysis, an addition or removal of a samples will change the final outcome. More specifically, the beta diversity indexes are based on a multivariate analysis (Ramette 2007). The bacterial profiling defines each sample in analysis. Each sample, which is characterized by a large range of variables derived from the bacterial profiling, is compared to the other samples, creating a distance matrix based on the grade of dissimilarities. The distance matrix considers the phylogeny as well as the abundance of the bacteria.

The output of the beta diversity analysis is a square matrix that determines the distance between samples. This matrix is called distance matrix. The UniFrac distance analysis, which also consider the phylogenetic information of the ecosystem under study, is the most frequently used algorithm to build the distance matrix. The UniFrac distance analysis can be performed in two ways: (i) by generating distance matrix only based on similarity between the bacteria present in the samples without considering their abundance (Unweighted UniFrac); (ii) by including both similarity and abundance of the bacteria (Weighted UniFrac) (Lozupone et al. 2011).

The final results of the beta diversity are represented in a graph where, conventionally, the samples are plotted in multidimensional Euclidean space. The abscissa separates the points in the space and refers to the first principal coordinate (PC); the ordinate is the second PC. Each PC is associated with a percentage value which describes the variation explained by the axes. A maximum of three PCs should be used per analysis. The principal coordinates analysis (PCoA) and the principal composition analysis (PCA) are the most common multivariate analyses used to obtain the coordinates used to represent samples in the graph. PCoA derives from a

dissimilarity matrix that emphasizes the most abundant OTUs present in the samples. On the other hand, PCA gives more importance to the OTUs that have a greater variance between samples. It is still controversial which is the best approach between PCoA and PCA to evidence biologically relevant differences between groups.

In addition, the analysis of similarity based on distance metric like ANOSIM can be also used to establish significant differences in the microbiota composition between groups of samples (Clarke 1993).

1.2.5 Feature-comparison methods

The output of the sequencer is a list of sequences (reads) that could be analysed as counts data or compositional data. It is, however, uncertain if it is more appropriate considering reads as counts or compositional data. If reads are considered compositional data, they cannot be analysed by the use of the statistical analyses that assume independence of the data because the reads are not actually independent. DNA fragments prior to sequencing are immobilized on the sequencing chip. The area available for sequence immobilization is limited allowing only for a small fraction of the fragments present in the sample to actually be bound and sequenced. On the other hand, if considered as counts data, reads correspond to gene expression data. In this work, the reads are taken in consideration like counts data, therefore the sequences of a samples are independent from each other.

Another feature of the sequencer's output is the different library size for each sample. The library size is the quantity of good quality reads that are generated for a single sample.

The methods to analyse the differences between samples take into account the independence of the data. They are mainly three:

a) Counts table transformation in relative amount of OTU. Where the sum of all the OTUs for each sample will equal to 1;

b) The rarefying method; (Hughes et al. 2005)

c) The Differential gene expression analysis (DESeq2) normalization; (Anders and Huber 2010).

The first method reports the relative abundance of the reads number per sample without regard to the "compositional" feature of the data. The second method is based on sample rarefying; in specific, first, the minimum library size is established (rarefying level) and, then, the reads are randomly selected for each sample until the number of read per each sequence equals to the rarefying level. At this point, the data are normalized by a linear normalization method. This principle does not apply to DESeq2 normalization, which is a method evolved from the gene expression statistical theory.

Poisson distribution is often used to model the gene expression data. The Poisson distribution is based on the assumption that the mean and variance of the data are the same. If this assumption is not met, an extension of the Poisson model needs to be applied. This extension of the model provides for the addition of the dispersion factor Θ. The Gamma-Poisson model, also called the negative binomial (NB) model, includes this dispersion factor. NB is used to test if the difference in expression of different OTUs is significant. To control the rate of false positive genes, it is necessary to recognize the factor $\Theta > 0$ and to estimate its value. Many false positive OTUs appear significantly differentially expressed between experimental conditions under the assumption of a Poisson distribution ($\Theta = 0$) but, nevertheless, they are not significant in tests that account for the larger variance as in the 16S rRNA sequencing. With respect to the previously described methods, the use of NB model solves both the deep differences of the library size and the lack of dispersion factor that causes the overestimation of the OTU variations.

1.2.6 Correlation and regression models

The analyses described above allow only for identification of OTUs differentially abundant between different groups of samples (representing different conditions), but they do not provide any information about the causing factors leading to this differential abundance. In order to speculate on the potential causes at the origin of observed microbiota variations (differential OTU abundances), the correlation analysis may be performed between differentially abundant OTUs and different biological parameters potentially involved in this variation. Due to the compositional nature of NGS data, the correlation analysis requires the

fulfilment of different assumptions. First, data need to be normalized and false positives need to be eliminated or reduced as much as possible. Kendall rank based correlation analysis is the most appropriate for this purpose because this statistical test uses a system of data perturbation that improves identifying and removal of false positive correlations. This specific correlation analysis allows for the establishment of an interdependence link between a single OTU and a biological (e.g. physiological or immunological) parameter linked to a sample group. Next, the regression analysis is used to further establish if the variation of a certain biological parameter leads to differential OTU abundance in a direct or indirect manner. To this end, a linear model is computed to estimate the influence of a specific biological parameter over differential OTU abundance. If the obtained R^2 value is close to 1, this indicates that the parameter is a good predictor for the differential OTU abundance.

1.3 References

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2. Aim of the PhD work

The aim of this PhD work was the characterization of the intestinal microbial ecosystem through bioinformatic and statistical analyses of the microbiomics data originated from three studies carried out on different human populations: healthy (non-diseased) adults, hyperlipidemic and normo-lipidemic children and adolescents, and subjects with diagnosed irritable bowel syndrome (IBS).

Specifically, the three studies presented in this PhD theses were as follows:

- **Probiotic crossover intervention study:** A randomized, double-blind, crossover, placebo-controlled intervention study was carried out to determine the impact of a *Bifidobacterium bifidum* strain on the IME of adult healthy volunteers.
- **Children's dyslipidemia single arm intervention study:** In this trial, the IME of children and adolescents with primary hyperlipidemia (a risk factor for cardiovascular diseases) was compared with the IME of control (normo-lipidemic) group. In addition, the modulatory effect of the regular intake of hazelnuts on hyperlipidemic subjects's IME was examines in order to evaluate if the modulation of IME by hazelnuts ameliorates the lipid profile.
- **Irritable bowel syndrome (IBS) observational study:** The aim of this trial was the characterization of the IME in human subjects affected by IBS. The characterization was performed through the analysis of the bacterial communities present in the faecal samples collected during a multicentre intervention trial (Cremon et al., 2017). Irritable bowel syndrome is the most common gastrointestinal disorder in western countries. IBS is conventionally classified in four different subtypes based on bowel clinical symptoms: constipation, diarrhoea, alternating constipation and diarrhoea, and unsubtyped IBS. Clinical and immunological data collected during the trial were used to investigate the potential correlations between the IME characteristic for a specific IBS subtypes and the physiological and clinical parameters of the host, including bowel symptoms, faecal levels of IgA and cytokines, and depression/anxiety scores.

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3. Results

3.1 Consumption of a *Bifidobacterium bifidum* Strain for 4 Weeks Modulates Dominant Intestinal Bacterial Taxa and Faecal Butyrate in Healthy Adults

3.1.1 Introduction

The prevailing notion that the deliberate intake of viable cells of certain microorganisms through food and supplements may be beneficial for health underlies the worldwide commercial success of probiotic products. Probiotics have been defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill C. et al. 2014). Therefore, a positive consequence on consumer health is an intrinsic feature of any formulation that is considered a probiotic. Accordingly, the European Union considers the term probiotic a health claim *per se* ("reference to probiotic/prebiotic implies a health benefit" [Food Safety Authority of Ireland]). However, although numerous studies have demonstrated the efficacy of different probiotic preparations in a number of pathological conditions (Sanders ME. et al. 2013), the potential benefits associated with probiotic consumption by the general (healthy) population remain unclear (Kristensen NB. et al. 2016). The lack of a clear cause-and-effect relationship between probiotic intake and health benefits for the general consumer is the most frequent reason stated by the European Food Safety Authority (EFSA) for the rejection of all health claims requested for probiotics.

The intestinal microbiota has been proposed as an additional organ of the human body that performs numerous functions, ranging from vitamin production and immunomodulation to improvement of nutrient bioavailability and competitive exclusion against potential

detrimental microorganisms (Goulet O. 2015). Therefore, modification of the intestinal microbial ecosystem (IME) may potentially induce functional changes that affect host physiology (Goulet O. 2015) and is generally recognized by certain health agencies (e.g., Italian Ministry of Health and Health Canada Federal Department) as the primary element supporting probiotic efficacy. Studies that describe and demonstrate the ability of specific probiotics to impact the IME of healthy consumers, however, remain limited (Ferrario C. et al. 2014, Veiga P. et al. 2014). Contradictory results have also been obtained (Derrien M. et al. 2015), possibly owing to differences in the microbial strain used, the number of viable cells administered, and the product formulation. Furthermore, the deep complexity and profound variability of microbiota composition among subjects can hinder the recognition of actual modifications. As a consequence, the impact of a probiotic, dietary, or pharmacologic intervention on the IME can be studied only by adopting sensitive analytical tools, such as 16S rRNA gene profiling, and an appropriate trial design.

We therefore performed an intervention trial with a crossover design and used 16S rRNA gene profiling together with short-chain fatty acid (SCFA) quantification in faecal samples from healthy adults to investigate the impact on the IME of a product containing a single *Bifidobacterium bifidum* strain. The strain was selected as a representative of a species that has been reported to possess numerous host interaction properties (Turroni F. et al. 2014), including marked adhesion to enterocytes (Guglielmetti S. et al. 2013, Turroni F. et al. 2013), immunomodulation (Turroni F. et al. 2013, Guglielmetti S. et al. 2014), and metabolism of mucin and human milk oligosaccharides (Wada J. et al. 2008, Duranti S. et al. 2015).

3.1.2 Materials and methods

3.1.2.1 Participants

Thirty-eight healthy human volunteers (21 women and 17 men; age, 24 to 54 years; mean, 31 years) participated in the study, named PROBIOTA-Bb: “Effect of the probiotic strain *Bifidobacterium bifidum* Bb on the faecal microbiota of healthy adults.” All patients provided written informed consent, and the study protocol was approved by the Ethics Committee for Research of the Università degli Studi di Milano (opinion 37/12, 19 December 2012). The

procedures were carried out in accordance with the approved trial synopsis. The following inclusion criteria were adopted for the enrollment of participants: age between 18 and 55 years, good general health, and a signed consent form. The following exclusion criteria were adopted: antibiotic therapy during the 1 month prior to the first visit, intentional intake of probiotic or prebiotic products 1 month before the first visit, viral or bacterial enteritis during the 2 months before the first visit, presence of gastrointestinal disorders (e.g., diarrhoea, inflammatory bowel disease, or irritable bowel syndrome), pregnancy or breastfeeding, and recent or presumed episodes of alcoholism or drug addiction. Participants in the study were prohibited from eating probiotic foods and supplements and any foods or supplements enriched in prebiotic compounds. Traditional yogurt was allowed.

3.1.2.2 Experimental design

The PROBIOTA-Bb study was a randomized, double-blind, and placebo-controlled crossover trial with two parallel groups (**Figure 1**). The study consisted of a 4-week prerecruitment (run-in) phase, followed by random assignment of participants to group A (n = 16) or group B (n = 19). The group A protocol included a 4-week probiotic treatment (one capsule every day for 4 weeks in addition to habitual diet), followed by a 4-week washout period and a 4-week placebo phase. Group B followed the opposite sequence, with placebo, washout, and then probiotic treatment. Participants received written and oral instructions to store the capsules at room temperature, to avoid exposure of the capsules to heat sources, and to consume one capsule every day in the morning at least 15 min before breakfast with natural (not sparkling) water (alternatively, to consume the capsule in the evening at least 3 h after the last meal of the day). No research has tested which mode of administration is better, ingesting probiotics on an empty stomach or with meals. In this study, we decided to invite volunteers to consume capsules on an empty stomach because food intake may vary enormously from meal to meal and from subject to subject, differently affecting cell transit through the stomach and therefore diversely influencing probiotic activity.

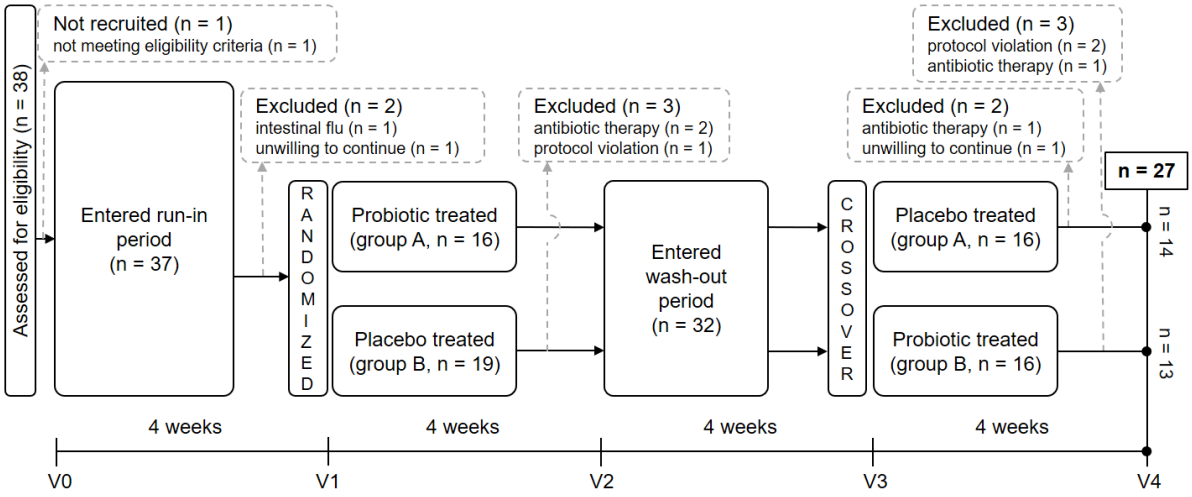

Figure 1. Schematic of study design and flow.

The study consisted of five visits: before the run-in period (visit V0), before and after the first treatment (V1 and V2, respectively), and before and after the second treatment (V3 and V4, respectively) (**Figure 1**). During each consultation, participants completed a short food frequency questionnaire that was specifically prepared to include a section for items considered potential sources of prebiotic fibers. In addition, participants compiled a weekly Bristol stool chart to report their bowel habits.

3.1.2.3 Products used in the trial

The probiotic preparation consisted of gelatin uncoated capsules filled with *Bifidobacterium bifidum* Bb isolated from the stool from a healthy adult woman and available in the culture collection of the section of Food Microbiology and Bioprocesses at the Department of Food, Environmental and Nutritional Sciences (University of Milan). Viable and total bacterial cell counts were performed approximately 1 week before the beginning of the trial on several randomly selected capsules. Viable counts were performed by serial dilutions and plating on de Man-Rogosa-Sharpe (MRS) agar plates with the addition of 0.05% cysteine-HCl. After 72 h of anaerobic incubation at 37°C, we calculated that each capsule contained $1.3 \times 10^9 \pm 0.1 \times 10^9$ CFU. Total bacterial cell counts, which were performed by cytofluorimetry (BD Accuri C6;

Becton Dickinson Italia, Milan, Italy) upon SYBR green cell labeling, revealed that each capsule contained 3.8×10^9 bacterial cells per capsule. The capsules also contained maltodextrin, cellulose powder, dextrose, a separating agent (magnesium salts of edible fatty acid), and silica. Placebo capsules were identical and were filled with maltodextrin instead of dry powder probiotic bacteria. This trial was registered at **www.isrctn.com/search?q=ISRCTN56945491** under trial no. ISRCTN56945491.

3.1.2.4 Collection of faecal samples and extraction of metagenomic DNA

A faecal sample was collected from each participant in a sterile plastic pot no more than 24 h before visits V1, V2, V3, and V4. According to the recommendations for "storage conditions of intestinal microbiota matter in metagenomic analysis" (Cardona S. et al. 2012), participants were asked to preserve the sample at room temperature until delivery to the laboratory. At delivery, stool specimens were immediately stored at −80°C until metagenomic DNA extraction, which was performed within 14 days by means of a QIAamp DNA stool minikit (Qiagen, Valencia, CA), according to the manufacturer's specifications, adopting a temperature of 95°C in step three to maximize bacterial cell lysis.

3.1.2.5 Profiling of faecal microbiota composition

The bacterial community structure of faecal samples was determined by 16S rRNA gene profiling, as previously described (Milani C. et al. 2013). In brief, a DNA fragment encompassing the variable region V3 of the 16S rRNA gene was amplified from metagenomic DNA with the primers Probio_Uni (5'-CCTACGGGRSGCAGCAG-3') and Probio_Rev (5'-ATTACCGCGGCTGCT-3') and was sequenced by means of Ion Torrent PGM sequencing technology (Life Technologies, Carlsbad, CA). Specifically, emulsion PCR was performed using the Ion OneTouch 200 template kit version 2 DL (Life Technologies, Guilford, CT), according to the manufacturer's instructions. Amplicon library sequencing was performed on 316 Chips using the Ion sequencing 200 kit (Life Technologies). The sequencing runs were multiplexed, and barcode sequences were used to discriminate the samples. Sequence reads were then analyzed using the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME)

version 1.7.0 (Caporaso JG. et al. 2010) with the GreenGenes database updated to version 13.5. Bacterial relative abundances in each faecal sample were reported at the taxonomic levels of phylum, class, order, family, and genus.

3.1.2.6 Quantification of faecal SCFAs

SCFAs were quantified in the faecal samples from 25 out of 27 subjects who completed the intervention trial. The remaining two subjects were excluded from the analysis due to insufficient faecal material availability. Faecal samples were extracted according to Huda-Faujan et al. (Huda-Faujan N. et al. 2010), with some modifications. In detail, stool specimens (100 mg) were suspended in 2 ml of 0.001% HCOOH and vortexed for 1 min. The suspension was centrifuged at 1,000 × *g* for 2 min at 4°C, and the supernatant was recovered. The residue was extracted again, as described above. The supernatants were combined, and the volume was adjusted to 5 ml with a solution of 0.001% HCOOH in water. All extracts were stored at −20°C. Before ultraperformance liquid chromatography–high-resolution-mass spectrometry (UPLC-HR-MS) analysis, samples were diluted 1:100 in 0.001% HCOOH and centrifuged at 3,000 × *g* for 1 min.

UPLC-HR-MS analysis was carried out on an Acquity UPLC separation module (Waters, Milford, MA, USA) coupled with an Exactive Orbitrap MS with an HESI-II probe for electrospray ionization (Thermo Scientific, San Jose, CA, USA). The ion source and interface conditions were as follows: spray voltage, −3.0 kV, sheath gas flow rate, 35 arbitrary units; auxiliary gas flow rate, 10 arbitrary units; temperature, 120°C; and capillary temperature, 320°C. A 1.8-µm HSS T3 column (150 by 2.1 mm; Waters) was used for separation at a flow rate of 0.2 ml/min. The eluents were 0.001% HCOOH in MilliQ-treated water (solvent A) and CH_3OH:CH_3CN (1:1 [vol/vol], solvent B). A 5-µl aliquot of the sample was separated by the UPLC using the following elution gradient: 0% B for 4 min, 0 to 15% B in 6 min, 15 to 20% B in 5 min, 20% for 13 min, and then return to initial conditions in 1 min. The column and samples were maintained at 30 and 15°C, respectively. The UPLC eluate was analyzed in full-scan MS in the range *m/z* 50 to 130. The resolution was set at 50 K, the automatic gain control (AGC) target was 1E6, and the maximum ion injection time was 100 ms. The ion with *m/z* 91.0038,

corresponding to the formic acid dimer $[2M-H]^-$, was used as the lock mass. The mass tolerance was 2 ppm. The MS data were processed using Xcalibur software (Thermo Scientific). Analytical-grade SCFAs were used as standards (Sigma-Aldrich, Milan, Italy). Five-point external calibration curves were adopted to quantify pyruvic, lactic, succinic, acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids in faecal samples. SCFA concentrations were expressed in milligrams per kilogram of wet feces.

3.1.2.7 Statistics

Statistical analyses were performed using the R statistical software (version 3.1.2). To measure valid outcomes, only participants with 100% compliance with the treatments and the experiment protocol were included in the analysis (per-protocol analysis). The numerical value of 0 was given to any taxon that was undetected in a specific sample to allow a comparison. Because of the necessary crossover design for significant results, intention-to-treat analysis was not performed. Differences between the effects on microbiota composition of probiotic and placebo treatments were evaluated by analyzing the data with nonparametric Wilcoxon-Mann-Whitney test with Benjamini-Hochberg correction using paired data. In particular, we performed two separated statistical analyses: in the first analysis, we compared data for each taxon before versus after probiotic intake; in the second, data from the same subjects were compared before versus after placebo. This analysis allowed us to decide whether the population distributions were identical without assuming them to display a normal distribution. A nonparametric test derived from the Shapiro-Francia test was performed for the composite hypothesis of normality. The *P* value was computed from the formula given by Royston (Royston P. 1993). Repeated-measures analysis of variance (ANOVA) was used when the data were consistent with the assumption of a parametric test for normal distribution. Statistical significance was set at a *P* value of ≤ 0.05, and mean differences with $0.05 < P \leq 0.10$ were accepted as trends. To group subjects into enterotypes, the tutorial by the European Molecular Biology Laboratory (EMBL) was used (**http://enterotype.embl.de/**). With respect to differences in the absolute quantity of SCFAs, the subjects were clustered using a Jensen-Shannon divergence (JSD) distance and the Partitioning Around Medoids (PAM) algorithm based on the SCFA concentration. Moreover, the treatment response was evaluated by

performing the Wilcoxon-Mann-Whitney test with Benjamini-Hochberg correction using the paired-data test.

Accession number(s). Sequence reads have been deposited in NCBI's Sequence Read Archive (ENA) under accession no. **PRJEB11694**.

3.1.3 Results

3.1.3.1 Study compliance and questionnaire analyses

All participants tolerated the capsules well, and no adverse events were reported. Participants maintained their usual dietary habits during the study, and no significant differences in the intake of potentially prebiotic foods were observed. The only registered modifications were related to slight seasonal differences in the availability of fruits and vegetables (the study began in June and ended in October). Participants' adherence to the study protocol was assessed based on capsule counts and faecal sample collection, and compliance was higher than 95%. In total, 38 participants were assessed for eligibility, and 35 participants were randomly assigned; 27 participants (77%) concluded the study, with 14 participants in the randomization group A (6 females and 8 males) and 13 participants in group B (7 women and 6 men). The drop-out rate of volunteers who began the first treatment was 20% (n = 7), consistent with the literature (Ferrario et al. 2014), and apparently justified by the strict exclusion criteria and quite long duration of the study (four months). No significant changes in stool consistency and evacuation frequency were noted according to the analysis of data reported in stool diaries (data not shown).

3.1.3.2 16S rRNA gene profiling revealed that the faecal microbiota composition was markedly varied among the participants in the PROBIOTA-Bb study

A total of 15,845,061 filtered high-quality sequence reads were generated (average, 135,428 reads per sample), with a mean ± standard deviation (SD) length of 179 ± 4 bp. Rarefaction curves indicated that most faecal microbiota diversity had been covered (see **Figure S1A** in the supplemental material). We identified a total of 93 bacterial families and 190 bacterial

genera, with a minimum number of 23 families and 38 genera and a maximum of 65 families and 125 genera per faecal sample. Only 17 genera were detected in all subjects at the 4 time points (8.9% of all detected genera), and 47 genera were present in at least one sample for all subjects (approximately 25% of all detected genera).

Subsequently, microbiota profiling data were stratified by enterotyping based on the relative abundances of the bacterial genera (Arumugam et al. 2011). The microbiota compositions of all samples in this study were clustered in two groups (Silhouette index [SI] = 0.25; see **Figure S2** in the supplemental material) resembling the Bacteroides-dominant (Ba) and the Prevotella-dominant (Pr) enterotypes (Arumugam et al. 2011). During the study, 8 of 27 subjects changed enterotypes; specifically, we observed 11 shifts from one enterotype to the other (accounting for the 14.7% of all possible shifts), with 3 during the probiotic intervention, 5 in the placebo treatment, and 3 in the washout phase (**Figure S2B**). Therefore, the treatments did not affect the affiliation of subjects to enterotypes. Interestingly, bacterial richness differed significantly between the two enterotype clusters: the α-diversity estimated by the Chao1 index was significantly higher in the Ba group than in the Pr group of samples (see **Figure S3** in the supplemental material).

The analyses described below were conducted to identify the effects induced by the probiotic intake on faecal microbial ecology and were restricted to the participants who received the intended interventions in accordance with the protocol (per-protocol analysis).

3.1.3.3 *B. bifidum* intake did not modify the α- and β-diversities of the faecal microbiota

The effect of the probiotic intervention was first investigated with respect to the modification induced by *B. bifidum* intake on the richness and evenness of the operational taxonomic units (OTUs) in each sample (α-diversity) and the intersample relationship of the bacterial compositions (β-diversity). According to both parametric (repeated-measures ANOVA) and nonparametric (Wilcoxon text) statistics, the intake of *B. bifidum* did not significantly affect the intrasample biodiversity as measured by the Chao1 and Shannon coefficients (predictors of taxonomic richness and evenness; **Figure S1A** and **B** in the supplemental material) or the

intersample diversity determined by principal-coordinate analysis (PCoA) based on weighted and unweighted UniFrac distances (measure of β-diversity; **Figure S1C**).

3.1.3.4 Probiotic intervention with *B. bifidum* modified the relative abundance of dominant taxa in the faecal microbiota

The Wilcoxon test with Benjamini-Hochberg correction was used to identify the bacterial taxa (from phylum to genus) that were significantly affected by the probiotic or placebo treatments. This nonparametric statistical analysis revealed that *B. bifidum* Bb intake had a greater impact than placebo on the relative abundance of OTUs (see **Table S1** in the supplemental material). In detail, the probiotic intervention induced the significant modification of 25 taxa, whereas only 13 taxa were different after placebo. More importantly, only 1 OTU that changed during the placebo treatment had a relative abundance above 1% (undefined members of the *Bacteroidales* order, from 1.10 to 0.46%; *P* = 0.036; **Table S1**), whereas probiotic intake significantly modulated the abundance of several dominant taxa of the faecal microbiota, including the families *Prevotellaceae* (mean value of the relative abundances from 14.18 to 11.97%; *P* = 0.041), *Rikenellaceae* (from 3.99 to 5.92%; *P* = 0.010), and *Ruminococcaceae* (from 12.21 to 15.27%; *P* = 0.039), and the genus *Prevotella* (from 14.16 to 11.96%; *P* = 0.034) (**Figure 2**; see also **Table S1**).

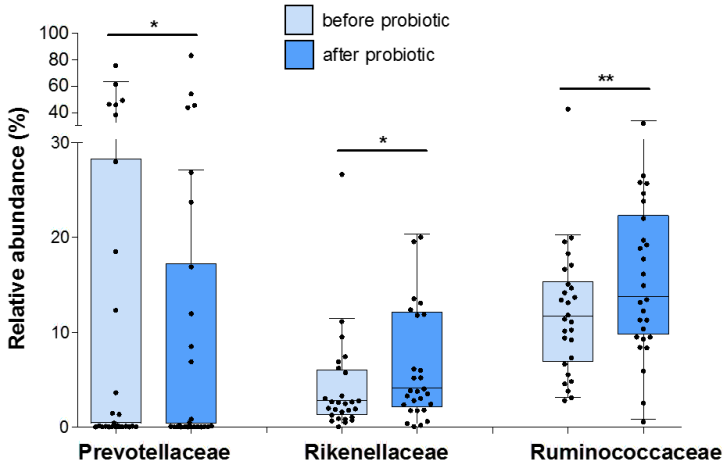

Figure 2. Relative abundance of dominant bacterial families in faecal samples significantly modified by the probiotic treatment. Data are shown as Tukey box plots. Statistically significant differences are according to the Wilcoxon-Mann-Whitney test with Benjamini-

Hochberg correction; *, $P < 0.05$.

The statistical strength of the observed differences between the effects induced by the two treatments is corroborated by the comparison of OTU relative abundances before treatments (i.e., for samples collected at V1 and V3; **Figure 1**). The Wilcoxon test revealed that only three OTUs with relative abundances of less than 1% were significantly different between the placebo and probiotic pretreatment phases (see **Table S1** in the supplemental material).

We did not find a significant modulation of the genus *Bifidobacterium* by probiotic treatment, suggesting that the intake of a billion *B. bifidum* cells was not enough to affect the relative abundance of the entire genus. On the contrary, we found that the relative abundance of the reads associated with the *B. bifidum* species increased importantly upon probiotic consumption, changing from a median below the detection limit to 0.005%. In contrast, during placebo treatment, the relative abundance of *B. bifidum* reads decreased from 0.002% to below the detection limit.

Finally, we also observed that the impact of the probiotic intervention on enterotypes was greater on the *Pr* group of samples (see **Table S2** in the supplemental material); this result, however, may have been merely a reflection of the change in *Prevotella* levels.

3.1.3.5 Probiotic intervention modulated the faecal levels of butyrate

We quantified the concentration of SCFAs in faecal samples by UPLC-HR-MS (see **Table S3** in the supplemental material). Statistical analyses (repeated-measures ANOVA and Wilcoxon test) revealed that neither the probiotic nor placebo intervention significantly affected the levels of SCFAs quantified in all faecal samples (see **Table S4** in the supplemental material).

Subsequently, we clustered subjects by PCoA based on the concentrations of the most abundant faecal SCFAs, i.e., acetate, butyrate, and propionate. According to the highest Silhouette coefficient of clustering prediction (SI = 0.43), we separated the samples in two groups (**Figure 3A**), which do not correspond to enterotype clusters. SCFA group H was characterized by higher concentrations of acetate, butyrate, isovalerate, propionate, succinate, and valerate than the second group (SCFA group L; **Figure 3B**). Furthermore, SCFA group H exhibited significantly lower acetate/butyrate and acetate/propionate ratios than

SCFA group L (**Figure 3C**). Subsequently, we investigated whether the intervention affected faecal levels of taxa and SCFAs in the two groups. We did not find significant modifications of taxon relative abundance upon probiotic or placebo treatment in the SCFA groups (see **Table S5** in the supplemental material). On the contrary, we determined that the probiotic (but, notably, not the placebo) treatment induced a significant change in the faecal level of butyrate in both SCFA groups (**Figure 3D**; see also **Table S3** in the supplemental material). Specifically, the median of butyrate levels increased from 5.2 to 8.2 mg kg^{-1} of wet feces in group L and dropped from 17.9 to 12.0 mg kg^{-1} of wet feces in group H, erasing the significant difference initially existing in the levels of this SCFA between the groups (**Figure 3D**). None of the other SCFAs were significantly affected by the treatments (**Table S3**).

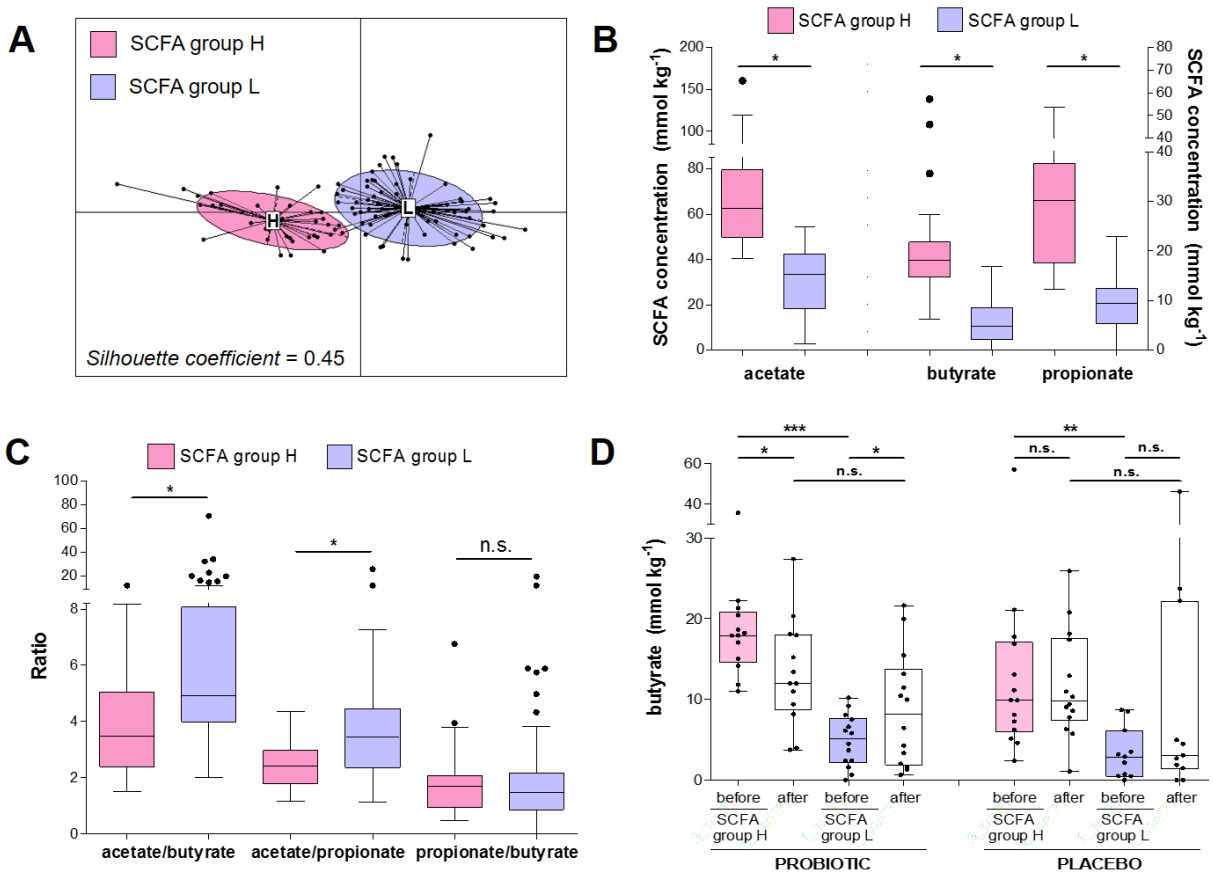

Figure 3. Short-chain fatty acid (SCFA) cluster analysis. (A) Principal-coordinate analysis (PCoA; the first two principal components are shown); clustering was based on the faecal concentrations of acetate, butyrate, and propionate using JSD distance and the Partitioning around Medoids (PAM) algorithm. (B) Tukey box plots representing the proportion of main faecal SCFAs in groups H (displaying higher concentrations of SCFAs) and L (lower SCFA concentrations). (C) Tukey box plots of the ratios between the three main faecal SCFAs in groups H and L. (D) Tukey box plots representing the effect of the probiotic and placebo intervention on butyrate levels in SCFA groups H and L. Asterisks are according to the Wilcoxon-Mann-Whitney test (with paired data, when possible) with Benjamini-Hochberg correction to determine statistically significant differences between groups (B to D) and before and after the probiotic treatment (D). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., no significant difference.

3.1.4 Discussion

The PROBIOTA-Bb trial was undertaken to contribute to the elucidation of the probiotic potential of strain of the species *Bifidobacterium bifidum*, which is a specialized human commensal possessing a large arsenal of host interaction properties (Turroni et al. 2014). Specifically, we studied the impact of *B. bifidum* Bb on the intestinal microbial ecology of healthy adults. Strain Bb was isolated from the feces from a healthy adult woman and, according to *in silico* analysis of its draft genome (our unpublished data), possesses the genetic determinants known to support the *B. bifidum*-host interaction, including genes encoding sortase-dependent pili (Turroni et al. 2013), mucin-metabolizing enzymes (Turroni et al. 2011), human-milk oligosaccharide hydrolases (Ashida H. et al. 2009), BopA outer surface lipoprotein (Guglielmetti et al. 2008), and Tal transaldolase (González-Rodríguez et al. 2015). In this study, we used capsules containing approximately one billion viable Bb cells, which corresponds to the minimal daily dosage recommended for probiotics by the Italian Ministry of Health (Ministero della Salute 2013).

16S rRNA gene profiling revealed considerable variation of the faecal microbiota composition of the volunteers enrolled in the PROBIOTA-Bb trial, consistent with previous studies (Yatsunenko et al. 2012, Davenport et al. 2014). These interindividual differences support the choice of the crossover design, in which individual participants serve as their own controls, resulting in the reduction of interindividual variation.

To characterize the wide interindividual variability of the faecal microbiota, we clustered samples according to common features in the taxonomic composition. In light of the theory suggesting that intestinal microbiota variation is generally stratified and not continuous (Arumugam et al. 2011), we adopted the enterotype classification to cluster the data collected during the PROBIOTA-Bb study, according to the relative abundance of bacterial genera. The proposed approach for enterotyping is subject to limitations. In particular, enterotyping reflects overconfidence in the assumption of discrete enterotypes without consistent evidence to refute the simpler hypothesis of continuous variation of the microbiota (Knights et al. 2014). The existence of discrete structures for gut microbiota has not been convincingly

demonstrated (Gorvitovskaia et al. 2016) and represents a crucial assumption in applying an appropriate prediction model. Nonetheless, we used the original tutorial to define enterotypes (**http://enterotype.embl.de/enterotypes.html**) in the present study, because it has been demonstrated to be useful to correlate the gut microbial community structure with host biomarkers and diet (Wang et al. 2014, Vandeputte et al. 2016). In our study, enterotyping clustered the microbiota taxonomic structures of the faecal samples in two groups corresponding to the most common enterotypes of healthy adult populations, namely, the *Bacteroides*-dominant (*Ba*) and the *Prevotella*-dominant (*Pr*) enterotypes (Arumugam et al. 2011, Vandeputte et al. 2016).

In our trial, the α- and β-diversities were not significantly affected by probiotic intervention. In a recent intervention study that was performed adopting the same trial design (Ferrario et al. 2014), the administration of capsules containing the probiotic strain *Lactobacillus paracasei* DG did not modify the α-diversity of participants' faecal samples, but, in contrast to the results of this study, induced a significant change in the β-diversity in terms of weighted UniFrac distances. Kim et al. (Kim et al. 2013) did not observe significant alterations in α- and β-diversities following the consumption of various probiotic products; however, a very limited number of subjects per group (only three) were used in that study. Furthermore, no alterations in α- and β-diversities were observed in the faecal microbiota of 1- to 2-year-old children following the consumption of probiotic milk containing *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus acidophilus* La-5, and *Bifidobacterium animalis* subsp. *lactis* Bb-12 (Dotterud et al 2015). Other intervention trials investigating the effect of probiotics on the intra- and interindividual biodiversities of the gut microbiota in healthy subjects have not been reported. Taken together, these data confirm the literature (although limited) suggesting that probiotic intake may be an insufficiently weak perturbation to modify the α- and β-diversities of the intestinal microbiota of healthy adults. This hypothesis is plausible in light of the recognized stability throughout adulthood and the reported resilience of the human intestinal microbiota to short-term dietary changes (Lozupone et al. 2012).

This study revealed that the intervention with strain Bb affected the relative abundances of several dominant taxa of the intestinal microbiota; specifically, the families *Ruminococcaceae*

and *Rikenellaceae* increased, whereas *Prevotellaceae* decreased. Remarkably, *Ruminococcaceae*, *Prevotellaceae*, and *Rikenellaceae* (together with *Lachnospiraceae* and *Bacteroidaceae*) have been identified by metatranscriptomics as the predominant families of the active microbiota (Gosalbes et al. 2011).

Ruminococcaceae is a family of obligate anaerobes that include bacteria (e.g., *Faecalibacterium*, *Ruminiclostridium*, and *Ruminococcus* spp.) that may degrade numerous polysaccharides in the lower gastrointestinal tract, such as starch, cellulose, and xylan, and produce SCFAs (Flint et al. 2008). The expansion of *Ruminococcaceae* in centenarians has been reported, with a positive correlation with high-fiber diets (Wang et al. 2015) and after intervention with resistant starch (Salonen et al. 2014). Furthermore, in the study by Martínez et al., *Ruminococcaceae* were more dominant in normoweight than obese individuals and negatively correlated with markers of inflammation (Martínez et al. 2012). A lower abundance of this taxon was associated with exaggerated Toll-like receptor 2 (TLR-2) responses and an increased risk of developing IgE-associated eczema in infants (West et al. 2015). The relative abundance of *Ruminococcaceae* was also lower in acute-chronic liver failure patients (Chen et al. 2015); in the same study, a negative correlation of *Ruminococcaceae* with tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) and endotoxemia was also observed. Finally, two recent studies reported that *Ruminococcaceae* are diminished in the guts of inflammatory bowel disease (IBD) patients, particularly those with ileal Crohn's disease (Willing et al. 2010, Morgan et al. 2012). Therefore, a number of observations suggest that *Ruminococcaceae* are commonly associated with a healthy gut microbiota and may exert a protective role on host health.

A couple of studies have also suggested a potential positive role for *Rikenellaceae*, a *Bacteroidales* family significantly enhanced by probiotic treatment with strain *B. bifidum* Bb. Specifically, *Rikenellaceae* family members were depleted in patients who had chronic HIV infection relative to HIV-uninfected controls (Dinh et al. 2015) and suppressed in IBD patients relative to healthy controls (Morgan et al. 2012, Scaldaferri et al. 2015). Conversely, one study reported a higher abundance of *Ruminococcaceae* and *Rikenellaceae* and a decrease in the

abundance of *Prevotellaceae* in the terminal ileum microbiota of subjects with ankylosing spondylitis (AS) compared with healthy controls (Costello et al. 2015).

In our study, the probiotic intervention reduced the relative abundance of the family *Prevotellaceae* and, particularly, the genus *Prevotella*. The relative richness of *Prevotella* spp. and *Prevotellaceae* is frequently modulated following dietary interventions (Clemente-Postigo et al. 2013, Karl et al. 2015) and, in general, by lifestyle modifications, suggesting that these microorganisms represent active and rapidly reacting components of the human intestinal microbiota (Gosalbes et al. 2011). Notably, *Prevotella* spp. are the dominant colonizers of agrarian societies and are associated with long-term diets rich in plant carbohydrates and fibers, whereas the abundance of *Bacteroides* spp. is increased in individuals from urbanized societies and is associated with diets high in animal fat and proteins (Wu et al. 2011, De Filippo et al. 2010). Although all subjects enrolled in the present study lived in an urbanized European area (Lombardia region), *Prevotella* was the dominant genus in approximately 30% of study participants (8 of 27 subjects at V1). This high prevalence of *Prevotella* in the population under study may potentially be explained by the higher-than-average fruit and vegetable (and thus fiber and starch) intake of Italian subjects (De Filippis et al. 2015).

A few studies have suggested that the abundance of *Prevotellaceae*/*Prevotella* (*Bacteroidetes* phylum) is inversely associated with the relative richness of *Ruminococcaceae* (*Firmicutes* phylum). For instance, increased *Ruminococcaceae* have been proposed to compensate for lower levels of *Prevotellaceae* in Parkinson's disease patients (Scheperjans et al. 2015). Conversely to *Ruminococcaceae*, *Prevotellaceae* have been observed to be overrepresented in obese people (Zhang et al. 2009). Furthermore, although typically associated with plant carbohydrate consumption, enriched abundance of *Prevotella* has also been linked to the high consumption of L-carnitine-containing foods, such as red meat (Koeth et al. 2014).

Notably, several studies also suggested a potential role of *Prevotellaceae* as intestinal pathobionts. The family *Prevotellaceae* was, in fact, demonstrated to elicit a strong inflammatory response in the guts of mice (Elinav et al. 2011) and is overrepresented in patients with ulcerative colitis (Lucke et al. 2006). An increase in *Prevotella* spp. was also reported in the intestinal lumen microbiota of colorectal cancer patients (Chen et al. 2012)

and in children diagnosed with irritable bowel syndrome (Rigsbee et al. 2012). Finally, the species *Prevotella copri* was identified as strongly correlated with disease in new-onset untreated rheumatoid arthritis patients (Scher et al. 2013). However, increased abundance of *P. copri* has also been associated with dietary fiber-induced improvement in glucose and insulin responses (Kovatcheva-Datchary et al. 2015). In addition, low *Prevotellaceae* levels have been reported in patients with type 1 diabetes (Brown et al. 2011) and children with autism (Kang et al. 2013). However, a subsequent study involving a larger population of autistic children reported the opposite result (i.e., a significant increase in *Prevotella* spp. [Son et al. 2015]). In conclusion, the scientific literature is far from a final and unambiguous understanding of the role of a specific taxon in host health; plausibly, the same bacterial taxa of the gut microbiota may exert opposite effects on the host (health preserving versus health threatening), depending on physiological background. This variability is particularly true for *Prevotella* spp., which appear to be critical bacteria for healthy microbiota that have been linked to plant-rich diets but also to chronic inflammatory conditions (Ley et al. 2016). In addition, genera such as *Prevotella* include numerous species that possess wide genetic diversity and strain differences within species, which may at least partially explain the observed differences in interactions between *Prevotella* and its host (Ley et al. 2016).

Modification of the intestinal microbiota structure may potentially lead to alterations of the gut levels of SCFAs, which are microbial metabolic products exerting a number of effects on host physiology (den Besten et al. 2013). Diet (probiotics included) may modify the level of SCFAs in the intestinal lumen by affecting their uptake/utilization by host and intestinal microbes or by changing the relative abundance of specific butyrate-producing bacteria. In our study, we observed the modification of *Clostridiales* bacteria of the family *Ruminococcaceae*, which are among the primary producers of SCFAs and, in particular, butyrate in the human large intestine. We therefore quantified the concentrations of SCFAs in faecal samples by UPLC-HR-MS. We observed that subjects could be clustered into groups, H and L, according to the levels of the three most abundant SCFAs. Group H was characterized by higher concentrations of several SCFAs, including butyrate, than those in group L. Notably, we observed that the probiotic treatment induced a significant change in butyrate levels,

which decreased in group H and increased in L. Notably, this modification was not observed with placebo treatment. A similar effect on faecal butyrate levels was also observed during an intervention study with the probiotic strain *Lactobacillus paracasei* DG (Ferrario et al. 2014). In light of literature suggesting that excessive intestinal butyrate may be detrimental in certain physiological conditions, such as irritable bowel syndrome (IBS) or metabolic syndrome (Bourdu et al. 2005, Payne et al. 2011), although considered improbable by some researchers (Conlon et al. 2015), we recently postulated the potential existence of an optimal butyrate concentration range in the human intestine (Ferrario et al. 2014, Guglielmetti and Riso 2015). In this context, the hypothesis can be made that probiotics might be used to decrease high butyrate concentrations or increase low butyrate concentrations to maintain butyrate homeostasis in healthy people, potentially preventing disorders associated with altered butyrate levels (Guglielmetti and Riso 2015).

3.1.5 Supplementary material

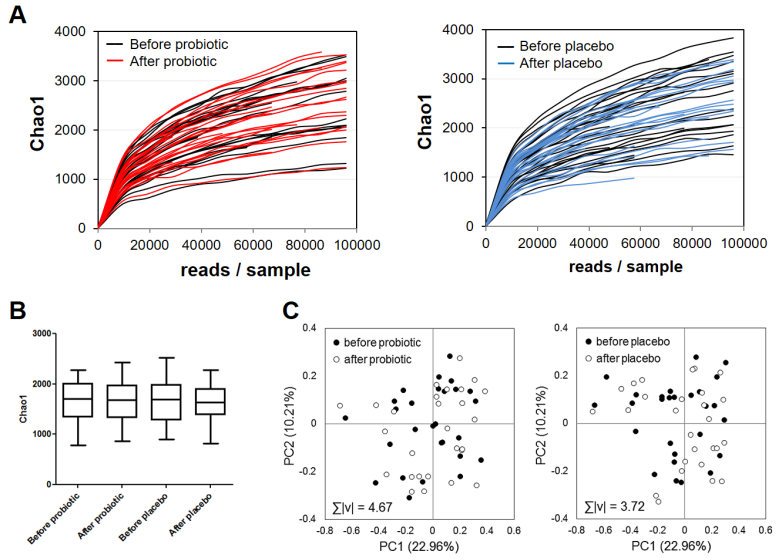

Figure S1. Analyses of the overall faecal microbiota diversity in (panels A and C) and among (panel B) the Probiota-Bb faecal samples. **A**, bacterial richness estimated through rarefaction curves using Chao1 coefficient as α-diversity predictors. **B**, biodiversity according to Chao1 coefficient of α-diversity, before and after the treatments. **C**, Principal Coordinate Analysis (PCoA) based on Weighted Unifrac expressing the β-diversity of samples. Axes of the two panels are the two most informative components explaining the differences among samples (percent of explained variation is shown between brackets on each axis). Each sample is represented by the overall microbiota composition of a single faecal specimen. Samples are separated into four categories: before and after probiotic treatment (panels A), and before and after probiotic treatment. ∑|v|, the sum of absolute Euclidean distances of paired points calculated as the sum of square variances of the coordinates of each point before and after a treatment ($|v| = \sqrt{[(x_i-x_j)^2+ (y_i-y_j)^2]}$, where «i» indicates before treatment and «j» after treatment). Paired points are the sample before and the sample after a treatment for a specific subject. For coordinates PC1 vs PC2, absolute distances were not significantly different between the probiotic and the placebo treatments.

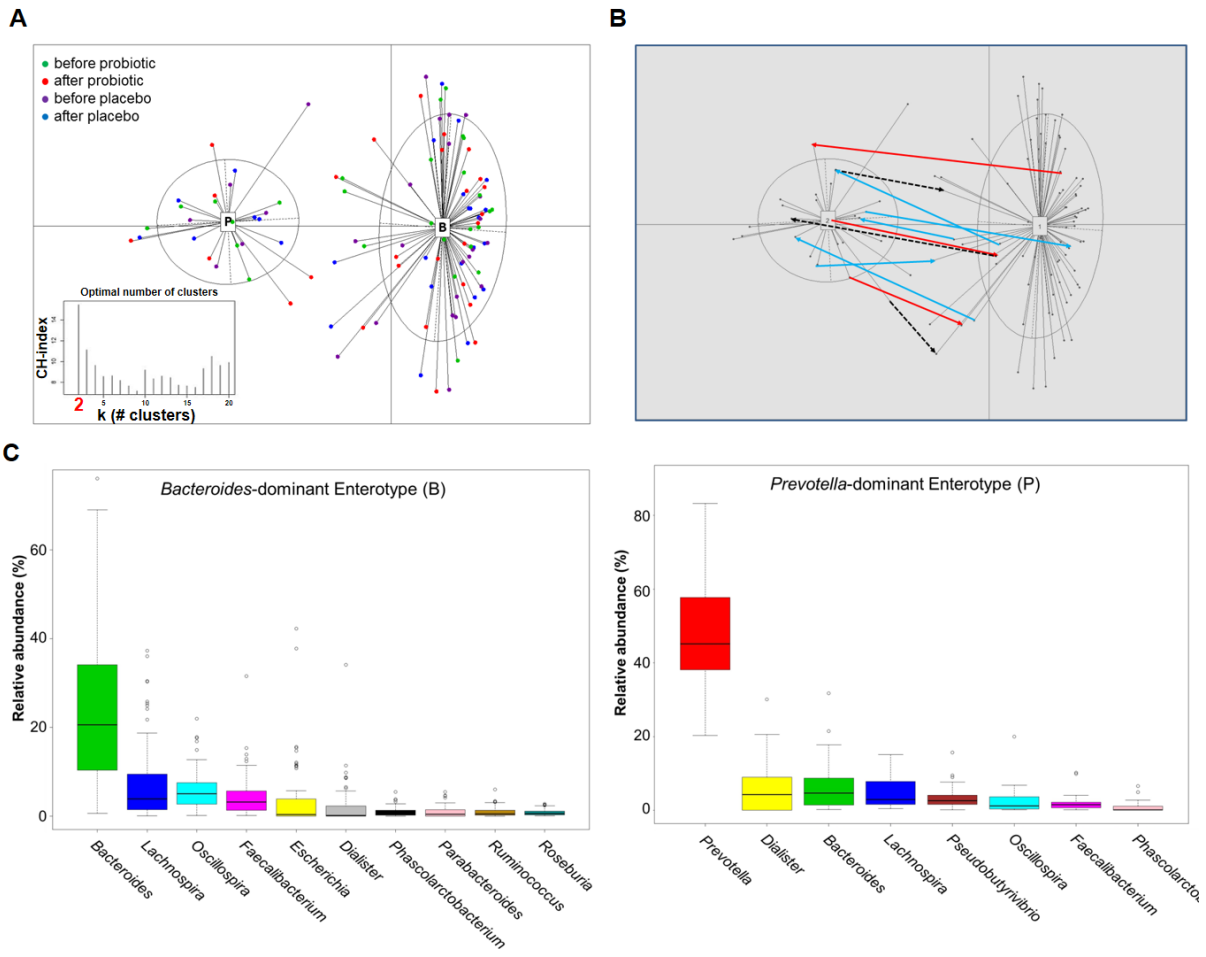

Figure S2. Analyses of enterotypes in samples of the PROBIOTA-Bb trial. **A**. Principal Coordinated Analysis (PCA; the first two principal components are shown); clustering was based on genus relative abundance using JSD distance and the Partitioning Around Medoids (PAM) algorithm. The optimal number of clusters was determined through Calinski-Harabasz (CH) index (59) and the Silhouette coefficient. **B**, shifts between enterotypes of faecal microbiota compositions in a single subject; red, blue, and dotted-black arrows indicate shifts that occurred across the probiotic, placebo, and wash-out phases respectively. **C**, Tukey boxplots of the dominant bacterial genera distribution in the two identified enterotypes.

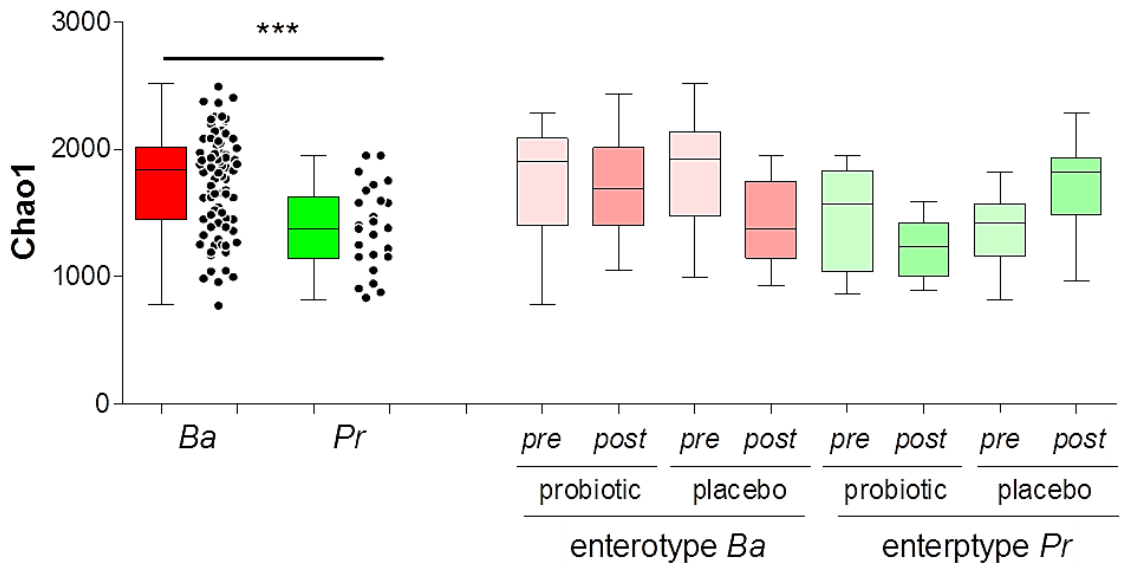

Figure S3. Tukey boxplots representing bacterial taxonomic richness of enterotypes assessed with a Chao1 estimator of α-diversity. Ba, Bacteroides-dominated enterotypes; Pr, Prevotella-dominated enterotype. Statistically significant difference is according to Mann Whitney test; ***, $P < 0.001$.

ALL SUBJECTS	p values	baseline	post
PROBIOTIC			
k_Bacteria;Other	**0.030**	**1.536%**	**2.812%**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_;g_	0.018	0.162%	0.364%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae	**0.041**	**14.179%**	**11.973%**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella	**0.034**	**14.158%**	**11.963%**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae	**0.010**	**3.987%**	**5.919%**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_undefined	**0.013**	**3.823%**	**5.640%**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;Other	0.015	0.120%	0.158%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_Alistipes	0.013	0.044%	0.122%
p_Firmicutes;c_Bacilli;o_Bacillales;f_Staphylococcaceae;g_Staphylococcus	0.023	0.000%	0.005%
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_;g_undefined	0.044	0.000%	0.000%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_;Tissierellaceae;g_Peptoniphilus	0.029	0.001%	0.002%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae	0.005	0.405%	0.838%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_undefined	0.006	0.389%	0.810%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;Other	0.006	0.016%	0.027%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_Christensenella	0.042	0.000%	0.001%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Dehalobacteriaceae	0.042	0.002%	0.005%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Dehalobacteriaceae;g_Dehalobacterium	0.046	0.001%	0.004%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae	**0.039**	**12.212%**	**15.271%**
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Anaerotruncus	0.029	0.026%	0.031%
p_Firmicutes;c_Clostridia;o_Clostridiales;Other	**0.008**	**1.289%**	**1.794%**
p_Firmicutes;c_Clostridia;Other	0.030	0.107%	0.215%
p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_;Eubacterium;	0.010	0.023%	0.056%
p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;Other	0.038	0.004%	0.017%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfovibrionales;f_Desulfovibrionaceae	0.014	0.133%	0.245%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfovibrionales;f_Desulfovibrionaceae;g_Bilophila	0.010	0.051%	0.110%

PLACEBO			
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;Other	**0.036**	**1.095%**	**0.457%**
p_Firmicutes;c_Bacilli	0.012	0.030%	0.104%
p_Firmicutes;c_Bacilli;o_Gemellales;f_Gemellaceae;g_undefined	0.038	0.000%	0.001%
p_Firmicutes;c_Bacilli;o_Lactobacillales	0.030	0.027%	0.094%
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Carnobacteriaceae;g_Granulicatella	0.008	0.000%	0.002%
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus	0.019	0.001%	0.002%
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus	0.019	0.022%	0.087%
p_Firmicutes;c_Bacilli;o_Turicibacterales;f_Turicibacteraceae;g_Turicibacter	0.006	0.001%	0.008%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Peptococcaceae	0.025	0.001%	0.006%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_undefined	0.021	0.024%	0.010%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Veillonella	0.017	0.018%	0.038%
p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Holdemania	0.032	0.003%	0.015%
p_Proteobacteria;c_Gammaproteobacteria;o_Pasteurellales;f_Pasteurellaceae;g_Haemophilus	0.012	0.002%	0.019%
BEFORE TREATMENTS	p values	V1	V3
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24;7;g_undefined	0.010	0.025%	0.033%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia	0.004	0.936%	0.413%
p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Bulleidia	0.012	<0.001%	<0.001%

Table S1. Bacterial taxa that were significantly modified by probiotic or placebo treatments. Mean bacterial relative abundances are shown at baseline (before treatment) and post (after treatment), and at visit 1 (V1) and 3 (V3). Visits are according to Figure 1. p values are according to Wilcoxon-Mann-Whitney test with Benjamini-Hochberg correction to determine statistically significant differences before and after the treatment. Bacterial taxa with a relative abundance higher than 1% are shown in bold. The taxonomic lineage of each taxon is shown; k, kingdom; p, phylum; c, class; o, order; f, family; g, genus.

Bacteroides-dominated enterotype	p values	baseline	post
PROBIOTIC			
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_;g_	0.011	0.026%	0.072%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_;Paraprevotellaceae;g_;Prevotella;	0.021	0.001%	0.367%
p_Firmicutes;c_Clostridia;o_Clostridiales;Other;Other	**0.039**	**1.169%**	**1.686%**
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;Other	0.005	0.012%	0.032%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_undefined	0.034	0.549%	0.655%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_Christensenella	0.022	0.001%	0.001%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfovibrionales;f_Desulfovibrionaceae;g_Bilophila	0.044	0.061%	0.146%
PLACEBO			
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;Other;Other	**0.036**	**1.657%**	**0.457%**
p_Firmicutes;c_Bacilli;o_Gemellales;f_Gemellaceae;g_undefined	0.038	0.001%	0.001%
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Carnobacteriaceae;g_Granulicatella	0.008	0.001%	0.002%
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus	0.019	0.001%	0.002%
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus	0.019	0.025%	0.087%
p_Firmicutes;c_Bacilli;o_Turicibacterales;f_Turicibacteraceae;g_Turicibacter	0.006	0.001%	0.008%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_undefined	0.021	0.032%	0.010%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Veillonella	0.017	0.017%	0.038%
p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Holdemania	0.032	0.003%	0.015%
p_Proteobacteria;c_Gammaproteobacteria;o_Pasteurellales;f_Pasteurellaceae;g_Haemophilus	0.012	0.002%	0.019%

Prevotella-dominated enterotype	p values	baseline	post
PROBIOTIC			
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;Other	0.024	0.051%	0.023%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella	**0.014**	**34.416%**	**23.547%**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;Other	0.002	0.057%	0.208%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_undefined	**0.005**	**3.118%**	**6.478%**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_Alistipes	0.024	0.063%	0.172%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_;Barnesiellaceae;g_undefined	**0.003**	**0.388%**	**1.092%**
PLACEBO			
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Veillonella	0.031	0.010%	0.084%

Table S2. Bacterial taxa that were significantly modified by probiotic or placebo treatments in enterotypes. Mean bacterial relative abundances are shown before treatment (baseline) and after treatment (post). p values are according to Wilcoxon test with Benjamini-Hochberg correction to determine statistically significant differences before and after the treatment. Bacterial taxa with a relative abundance higher than 1% are shown in bold. The taxonomic lineage of each taxon is shown; k, kingdom; p, phylum; c, class; o, order; f, family; g, genus.

Subject	Phase	Pyruvate	Lactate	Acetate	Succinate	Propionate	Butyrate	Isobutyrate	Isovalerate	Valerate
S02	Before placebo	-	0.14	20.48	0.77	5.37	2.19	1.25	1.59	1.51
S03		0.40	0.78	39.45	1.72	9.91	5.16	-	2.77	2.73
S04		0.26	0.47	18.12	1.71	14.82	8.51	-	2.29	2.62
S05		-	0.47	160.15	19.39	39.77	57.22	32.31	0.59	2.49
S07		0.44	0.95	47.04	1.73	14.18	2.41	-	3.19	3.25
S08		0.25	0.55	6.59	1.97	2.72	3.22	-	1.51	1.85
S13		0.25	0.35	21.65	1.47	16.16	-	-	5.11	2.35
S14		0.27	0.28	35.84	3.57	9.57	16.89	-	0.72	2.37
S15		0.29	0.31	34.95	1.71	9.55	0.49	-	4.47	3.89
S16		0.26	0.30	50.38	1.21	17.72	17.77	-	2.06	2.91
S17		-	0.25	64.07	0.42	42.39	6.27	3.25	0.77	0.65
S19		0.26	0.34	19.33	1.75	15.32	8.71	-	2.64	2.53
S20		-	0.22	119.78	0.74	43.94	21.10	11.86	8.34	8.38
S23		-	0.15	4.98	0.59	4.28	0.73	0.33	1.27	1.04
S25		-	0.26	49.38	1.36	17.00	8.11	5.29	1.76	2.43
S26		-	0.08	15.19	0.44	3.07	2.90	1.34	1.06	0.92
S28		-	0.41	51.50	0.78	14.17	7.25	5.28	3.57	2.99
S32		-	0.19	25.33	0.49	10.60	6.17	3.42	0.69	0.98
S33		-	0.25	43.62	0.59	11.55	9.91	5.23	1.35	3.39
S34		-	0.14	18.16	0.63	5.21	0.53	0.95	1.80	1.26
S35		-	0.18	45.81	0.42	19.61	13.07	6.52	0.68	1.46
S36		-	0.06	43.04	0.52	12.40	11.14	7.18	2.26	2.40
S37		-	0.24	13.37	0.65	2.98	3.52	2.29	1.45	1.08
S38		0.34	0.66	26.48	2.03	22.92	4.61	-	1.19	2.30
S39		-	0.19	39.75	9.83	21.80	9.93	5.79	0.59	1.03
S02	After placebo	-	0.14	12.89	0.72	2.37	-	-	0.72	0.80
S03		0.32	1.22	44.58	1.90	7.99	8.59	-	1.84	2.83
S04		0.33	0.45	50.94	1.78	32.35	23.72	-	2.05	7.03
S05		-	0.13	54.62	0.84	10.84	10.32	6.11	0.58	1.22
S07		0.37	0.72	85.83	2.29	53.83	25.93	-	12.23	10.80
S08		0.27	0.44	100.66	1.55	25.59	46.09	-	11.89	3.69
S13		0.39	3.73	39.93	1.63	1.55	-	-	1.00	1.98
S14		0.23	0.25	26.11	2.74	13.60	12.94	-	1.37	3.02
S15		0.26	0.43	13.30	1.34	7.44	3.08	-	2.29	3.13
S16		0.27	0.25	29.15	1.47	6.75	6.30	-	1.18	1.87

S17		-	0.23	40.35	0.44	34.27	9.05	4.77	0.21	1.27
S19		0.28	0.26	48.10	1.66	30.41	22.20	-	3.00	6.60
S20		-	0.22	96.93	0.81	39.93	20.78	13.14	2.40	5.98
S23		-	0.51	10.79	0.64	7.33	1.89	1.47	1.55	1.10
S25		-	0.31	40.49	0.60	11.36	5.75	3.92	2.30	2.64
S26		-	0.84	34.78	0.76	7.99	4.99	2.96	1.95	1.56
S28		-	0.11	34.66	0.40	10.20	7.77	4.13	0.19	1.26
S32		-	0.15	19.44	1.28	6.87	4.49	2.30	0.68	0.83
S33		-	0.16	24.86	0.59	4.17	1.10	0.44	0.93	1.72
S34		-	0.10	10.02	0.57	4.28	1.49	1.92	3.31	2.15
S35		-	0.53	63.90	0.60	33.36	17.38	8.52	1.13	2.50
S36		-	0.12	40.79	0.66	9.57	9.41	6.61	3.30	2.22
S37		-	0.27	15.25	0.48	2.69	2.71	1.39	1.06	0.92
S38		0.32	1.01	66.25	1.81	43.33	10.98	-	2.45	3.83
S3		-	0.14	61.15	1.44	36.84	18.10	10.55	1.93	3.07

Subject	Phase	Pyruvate	Lactate	Acetate	Succinate	Propionate	Butyrate	Isobutyrate	Isovalerate	Valerate
S02	Before Probiotic	-	0.77	60.76	6.86	13.97	11.82	6.55	0.46	1.15
S03		0.32	0.55	48.09	1.97	26.18	15.02	-	1.90	5.21
S04		0.38	-	48.16	1.91	6.62	2.43	-	1.11	1.79
S05		0.29	0.41	45.62	1.86	24.70	14.18	-	1.81	4.69
S07		0.26	-	53.96	1.42	17.56	35.63	-	1.44	3.87
S08		0.33	0.93	68.78	1.95	27.35	17.92	-	3.54	4.65
S13		0.19	0.24	50.64	1.35	12.21	21.31	-	0.96	2.26
S14		0.39	0.10	55.40	1.56	23.03	10.99	-	5.62	6.51
S15		0.33	1.81	11.26	1.54	2.32	-	-	1.55	2.00
S16		0.25	0.74	34.82	1.29	13.21	7.53	-	5.21	4.18
S17		-	0.23	23.95	0.85	9.09	3.71	2.60	2.19	2.62
S19		0.27	0.22	19.41	1.62	9.52	5.82	-	1.78	2.64
S20		-	0.23	33.17	0.86	18.85	8.09	4.46	1.06	1.63
S23		-	0.28	103.38	0.72	38.66	18.21	11.16	2.37	6.07
S25		-	0.44	2.77	0.39	1.00	0.67	-	0.43	0.60
S26		-	0.56	53.49	1.25	25.25	22.22	13.35	2.05	2.66
S28		-	0.19	40.23	0.39	16.29	6.60	3.70	0.42	0.97
S32		-	0.95	38.44	0.55	11.18	9.18	4.93	0.96	2.29
S33		-	0.18	17.55	0.45	6.14	1.61	1.86	2.74	1.46
S34		-	0.15	44.40	0.90	9.68	4.51	2.73	1.67	1.56
S35		-	0.24	44.48	0.46	5.07	10.21	5.44	0.94	2.61

S36		-	0.16	37.61	0.59	11.39	6.16	3.28	1.38	2.41
S37		-	0.30	93.50	0.84	37.23	20.43	12.63	3.02	4.40
S38		-	0.14	48.60	0.44	15.82	17.06	11.29	4.34	3.25
S39		-	0.17	37.38	0.70	9.41	2.45	2.31	2.75	2.00
S02	After Probiotic	-	0.16	51.95	0.87	11.69	11.98	7.54	0.82	1.38
S03		0.37	0.46	72.93	1.46	33.96	20.33	-	10.18	6.95
S04		0.38	1.02	22.04	1.61	8.78	2.03	-	2.40	2.10
S05		0.30	0.27	64.70	13.36	29.99	17.95	-	8.98	5.71
S07		0.22	-	45.78	1.83	15.27	27.40	-	1.77	2.70
S08		0.36	1.51	47.32	2.32	6.77	3.99	-	2.48	2.72
S13		0.27	0.25	8.31	1.44	6.37	3.76	-	2.46	2.57
S14		0.35	1.92	76.83	1.70	26.52	9.40	-	5.26	5.71
S15		0.36	1.96	42.40	1.56	7.59	1.32	-	3.91	2.53
S16		0.22	0.36	41.66	1.56	14.03	19.97	-	3.18	3.34
S17		-	0.22	10.39	0.46	2.40	0.65	-	0.65	0.84
S19		0.34	0.29	33.83	1.82	13.34	9.99	-	2.93	3.08
S20		-	0.24	110.13	1.07	49.00	15.48	9.41	2.68	4.23
S23		-	0.74	32.00	2.46	10.27	8.17	4.31	0.43	1.06
S25		-	0.16	40.94	0.52	12.79	11.48	7.77	3.92	3.47
S26		-	0.42	41.81	1.17	17.25	18.09	10.81	3.77	2.73
S28		-	0.28	52.20	0.41	35.10	13.17	7.41	0.49	2.09
S32		-	0.26	12.12	0.94	7.84	4.28	2.76	1.96	1.67
S33		-	0.32	18.60	0.53	3.58	1.66	1.11	1.63	1.06
S34		-	0.23	30.22	0.75	7.91	6.47	4.05	2.14	1.55
S35		-	0.19	63.94	0.42	16.85	21.64	11.98	1.03	4.10
S36		-	0.17	42.63	0.74	20.16	10.47	5.92	1.71	2.15
S37		-	0.09	77.39	0.98	30.90	15.21	9.51	2.59	3.94
S38		-	0.08	43.43	0.49	-	11.00	8.21	4.32	2.54
S39		-	0.09	47.98	0.62	9.89	3.36	2.57	2.53	1.57

Table S3. Levels of short chain fatty acids in the faecal samples. Data are reported as mg per kg of feces. -, not detectable.

	All subjects			SCFA group L			SCFA group H		
	p values	baseline	post	p values	baseline	post	p values	baseline	post
PROBIOTIC									
Acetate	0,833	101,44	106,58	0.268	30.97	40.65	0.365	62.02	51.13
Butyrate	0,271	22,78	27,51	**0.025 ***	**4.93**	**8.71**	**0.042 ***	**18.62**	**13.39**
Isovalerate	0,853	5,37	6,15	0.217	1.73	2.23	0.465	2.50	3.92
Lactate	0,653	0,82	1,27	0.808	0.41	0.41	0.919	0.39	0.54
Propionate	0,711	38,70	44,49	0.068	9.27	14.95	0.102	23.81	17.18
Succinate	0,508	5,65	2,90	0.502	0.89	0.93	0.278	1.84	2.55
Valerate	0,426	5,88	7,40	0.502	2.06	2.41	0.365	4.06	3.45
PLACEBO									
Acetate	0,937	111,58	113,16	0.179	29.53	40.23	0.125	98.60	55.27
Butyrate	0,916	27,38	26,92	0.083	5.97	10.89	0.375	25.59	11.61
Isovalerate	0,120	5,1708	7,42	0.393	2.00	2.72	0.125	2.94	1.09
Lactate	0,989	1,00	1,170	0.338	0.33	0.57	0.250	0.31	0.21
Propionate	0,874	39,17	39,82	0.203	11.58	16.82	0.125	35.95	22.95
Succinate	0,164	3,27	4,108	0.473	1.65	1.21	0.875	5.44	0.89
Valerate	0,853	7,35	7,179	0.128	2.11	3.03	0.250	3.61	2.58

Table S4. Effects of the probiotic and placebo treatments on faecal short chain fatty acid (SCFA) concentration. Mean SCFA concentration at baseline (before treatment) and post (after treatment) are reported as mg per kg of feces. p values are according to Wilcoxon-Mann-Whitney test with Benjamini-Hochberg correction to determine statistically significant differences before and after the treatments. The asterisk (*) indicates statistically significant differences ($p < 0.05$).

3.1.6 References

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3.2 Evidence of dysbiosis in the intestinal microbial ecosystem of children and adolescents with primary hyperlipidemia and potential role of regular hazelnut intake

3.2.1 Introduction

Hyperlipidemia is comprehensively defined as a lipoprotein metabolism disorder mainly manifested by an increase of total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C) and triglyceride concentrations. Hyperlipidemia may be either the result of a genetic defect in the lipid metabolism pathways (primary) or secondary to underlying diseases. It may occur from pediatric age and represents an unquestioned risk factor for cardiovascular diseases (D'Adamo et al. 2015).

Dietary interventions appear a promising strategy to manage premature hyperlipidemia. For instance, the intake of a food supplement containing fiber was shown to reduce total cholesterol, LDL-C levels, and apolipoprotein B in hypercholesterolemic children (Guardamagna et al. 2013). In addition, the daily consumption of yoghurt supplemented with phytosterols modulated lipid profile by reducing LDL-C (Guardamagna et al. 2011a). In another trial, a probiotic formulation resulted in the decrease of LDL-C and in the improvement of trygliceridaemia and HDL-cholesterol (HDL-C) levels (Guardamagna et al. 2014). Mechanisms supporting the potential efficacy of the above mentioned interventions include the suppression of liver cholesterol synthesis (Guardamagna et al. 2011b), the reduction of the intestinal cholesterol absorption (Guardamagna et al. 2011a), and the production of conjugated linoleic acid in the gut (Guardamagna et al. 2014).

In principle, the modulation of the gut microbiota could represent an additional strategy for the amelioration of the lipid profile. Expanding scientific evidence, in fact, indicate that the gut microbiota mediates physiopathological mechanisms that alter lipid metabolism and other related metabolic traits (Ghazalpour et al. 2016). Particularly, the intestinal microbiota has been recognized as a metabolically active endocrine organ of the human superorganism that can be a therapeutic

target for hyperlipidemia and associated cardiometabolic diseases (Brahe et al. 2016, Ghazalpour et al. 2016).

Although altered microbiota composition (generally called “dysbiosis”) has been associated to diseases that are characterized by hyperlipidemia such as obesity (Kobyliak et al. 2016), diabetes (Yamaguchi et al. 2016), metabolic diseases (Woting and Blaut 2016) and non-alcoholic fatty liver disease (Wang et al. 2016), the intestinal microbial ecosystem (IME) has never been thoroughly investigated in young people with inherited hyperlipidemia.

Hyperlipidemic subjects could benefit from dietary patterns/food products able to affect lipid metabolism through the modulation of intestinal microbiota. In this context, nuts have been suggested as lipid-lowering products due to their richness in unsaturated fats and other bioactive compounds (such as L-arginine, fiber, minerals, vitamin E, phytosterols and polyphenols), which may synergistically contribute improving plasma lipid profile and providing overall cardiovascular benefit (Ros 2015). In light of the above considerations, in this study, we characterized the IME of children and adolescents with primary hyperlipidemia by means of 16S rRNA gene profiling and short chain fatty acids (SCFAs) quantification in faecal samples. Furthermore, the potential modulatory effects of the regular intake of hazelnuts on microbiota composition and SCFAs was investigated. Finally, we studied the potential correlations existing between the IME and hyperlipidemia-related clinical parameters.

3.2.2 Materials and methods

3.2.2.1 Ethics approval and consent to participate

The study protocol conformed with the principles outlined of the Declaration of Helsinki and was approved by the Ethics Committee of the City of Health and Science University Hospital of Turin, Italy (EC:CS377). The protocol and the aim of the study were explained in detail to all participants and their legal guardians, who signed an informed consent before the enrollment into the study.

3.2.2.2 Participants

Volunteers considered in the present study derive from a subgroup of children and adolescents with primary hyperlipidemia, aged between 6 and 17 years old, participating to a project aimed to characterize this pediatric population (Deon et al. 2017b) and to investigate the effect of the regular

intake of hazelnuts with skin for eight weeks on several markers related to lipid metabolism and oxidative stress (Deon et al. 2017a, Deon et al. 2017b). The trial was registered under ISRCTN.com (identifier no. ISRCTN12261900). Subjects participating to the intervention study were recruited among patients cared at the Department of Health Science and Pediatrics of the University of Turin (Italy) after a screening for eligibility. In addition, 15 age-matched normolipidemic volunteers (mean age 11, median 10; min 7, max 17 years old) were recruited as controls for the microbial ecology of the faecal samples.

The recruited hyperlipidemic subjects were asked to collect a stool sample before and after the intervention. Thirty-four stool samples were collected for analysis at baseline, but only 15 were available after the eight weeks of hazelnut with skin consumption (mean and median age: 11; min 5, max 17 years old). The effect of the dietary intervention on the faecal microbiota composition was analyzed in 15 subjects, whereas the levels of SCFAs were evaluated in the whole group of children and adolescents with primary hyperlipidemia who collected stools at baseline.

To be eligible, screened children and adolescents were required to be normal-weight with diagnosis of primary hyperlipemia including familial hypercholesterolemia (FH), familial combined hyperlipidemia (FCHL) or polygenic hypercholesterolemia (PHC), with total serum cholesterol (TC) and/or triglycerides (TG) levels higher than age- and sex-specific 90^{th} percentile. Diagnostic criteria of primary hyperlipidemia were based on accepted international standards as previously reported (Guardamagna et al. 2009). FH was diagnosed in presence of LDL-C $\geq 95^{th}$ percentile, parental LDL-C $\geq$ 190 mg dl^{-1}, tendon xanthomas and/or cardiovascular disease (phenotype IIA). FCHL was diagnosed in children showing TC and/or TG $>90^{th}$ age- and sex-specific percentile, with at least one parent affected by hypercholesterolemia, hypertriglyceridemia, or both (IIA, IV, or IIB phenotype, respectively), with concomitant individual and familial lipid phenotype variability. Children with LDL-C levels $>90^{th}$ percentile and a family history of dominant inherited hypercholesterolemia, but not fulfilling the biochemical international diagnostic criteria of FH or FCHL were diagnosed with PHC. Subjects were excluded if they had food allergies or specific aversion for nut consumption, secondary hyperlipidemias, obesity (body mass index, BMI, $\geq$ 90th percentile, age and sex matched); renal, endocrine, liver or gastrointestinal disorders (e.g., diarrhoea, inflammatory bowel disease, or irritable bowel syndrome) or chronic diseases requiring drug treatment (i.e., immunologic, neurologic, or oncohematologic disorders). Subjects were also excluded from the study if they were

taking lipid-lowering treatments, antibiotics, probiotic or prebiotic products one month prior to the first visit, or if they had viral or bacterial enteritis two months before the first visit.

3.2.2.3 Experimental design

All patients enrolled were under nutritional recommendations suggested for pediatric hyperlipidemia based on the *cardiovascular health integrated lifestyle diet* (CHILD-1) (Expert Panel on Integrated Guidelines for Cardiovascular et al. 2011). Dietary intervention consisted of 8-week intake of hazelnut with skin (HZN+S). Subjects were provided with pre-weighed vacuum packed portions of Italian roasted *Corylus avellana* L. (cultivar 'Tonda Gentile delle Langhe' from Piedmont, Italy). The quantity of hazelnuts per packet was calculated by considering doses generally advised to adults and adjusted to children body weight (about 0.43 g kg^{-1} body weight until a maximum of 30 g, which is the recommended daily dose for an adult). All participants were encouraged to maintain the same dietary pattern and lifestyle habits throughout the 8-week intervention study. Subjects had to exclude the intake of other nuts, dried fruits, probiotic or prebiotic foods or supplements from one month before the beginning of the study until the end of the experimentation. Traditional yogurt was allowed. To check the compliance to the dietary recommendations, subjects and their families were asked to fill in weekly food diaries.

At baseline and at the end of the HZN+S intervention (0 and 8 weeks), each study participant underwent a medical examination after an overnight fast, during which biological samples and physical parameters (including height, weight and blood pressure measurements) were obtained. The serum levels of TC, HDL-C and TG were directly determined by an automatic biochemical analyzer (Olympus AU2700, Japan), while the LDL-C concentration was estimated by using the Friedewald formula (LDL=TC-(HDL+TG/5) and non-high density lipoprotein cholesterol (non–HDL) was calculated subtracting HDL-C from TC.

The faecal samples were collected from each participant in a sterile plastic pot within 24 h before visits at baseline and following the HZN+S intervention. A single faecal sample was also collected from the 15 age-matched controls. According to the recommendations for "storage conditions of intestinal microbiota matter in metagenomic analysis" (Cardona et al. 2012), participants were asked to deliver the faecal sample to the laboratory within 24 h. Since delivery, stools were stored

at -80°C until DNA extraction. Subjects were asked to give back any uneaten HZN+S package at the visit. Compliance was assessed by weighing returned packages and by checking weekly food diaries.

3.2.2.4 Bacterial profiling of faecal microbiota

The bacterial community structure of faecal samples was determined by 16S ribosomal RNA gene profiling with Illumina MiSeq System at the Center for life – Nanoscience, Istituto Italiano di Tecnologia (Roma, Italy). Briefly, metagenomic DNA was extracted from 200 ± 10 mg of stool within 30 days from delivery by means of a PowerFaecal DNA Isolation Kit (Mo Bio Laboratories, Cabru s.a.s., Biassono, Italy) according to the manufacturer's specifications. A DNA fragment encompassing the V3 and V4 regions of the 16S rRNA gene was amplified from metagenomic DNA with the primer pair selected by Klindworth et al. (Klindworth et al. 2013). The sequencing runs were multiplexed and barcode sequences were used to discriminate the samples. 16S rRNA gene sequence reads were analyzed through the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0 using the last version of GreenGene (gg_13_5) as reference taxonomic database. Bacterial abundances in each faecal sample were determined at the operational taxonomic unit (OTU) level. Sequence reads from 16S rRNA gene profiling have been deposited in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under accession code PRJEB10296.

3.2.2.5 Quantification of faecal short chain fatty acids (SCFAs)

SCFAs were quantified in the faecal samples collected from 34 subjects, including the 15 who completed the intervention trial. SCFA quantifications were performed as previously described (Gargari et al. 2016). In brief, stools (200 ± 10 mg) were extracted in 10 ml of 0.001% HCOOH by vortexing for 1 min. The supernatant was then recovered through centrifugation at 1000 x *g* for 2 min at 4 °C. All extracts were stored at -20 °C until UPLC-HR-MS analysis, which was carried out on an Acquity UPLC separation module (Waters, Milford, MA, USA) coupled with an Exactive Orbitrap MS with an HESI-II probe for electrospray ionization (Thermo Scientific, San Jose, CA, USA). The ion source and interface conditions and other detailed information were as specified in (Gargari et al. 2016). The eluents were 0.001% HCOOH in MilliQ-treated water (solvent A) and CH_3OH:CH_3CN (1:1, v/v, solvent B); UPLC elution gradient: 0% B for 4 min, 0-15% B in 6 min, 15–20% B in 5 min, 20% for 13 min, and then return to initial conditions in 1 min. The UPLC eluate was analyzed in full-scan MS

in the range 50–130 *m/z*. The resolution was set at 50 K, the AGC target was 1E6, and the maximum ion injection time was 100 ms. The MS data were processed using Xcalibur software (Thermo Scientific). Five-point external calibration curves prepared with analytical grade SCFAs (Sigma-Aldrich, Milan, Italy) were adopted to quantify acetate, butyrate, isobutyrate, isovalerate, lactate, propionate, pyruvate, succinate, and valerate in faecal samples. SCFA concentrations were expressed in millimoles per kilogram of wet feces.

3.2.2.6 Statistical analyses.

Statistical analyses were performed using R statistical software (version 3.1.2). The numerical value of 0 (zero) was given to any taxon that was not detected in a specific sample to allow comparison. All the statistical tests were performed considering three study populations: (i) hyperlipidemic subjects before HZN+S intake, (ii) hyperlipidemic subjects after HZN+S intake and (iii) normolipidemic subjects as controls. Differences in microbiota composition between these populations were determined using Wald test following DESeq2 (paired between the comparison of hyperlipidemic subject populations) read counts normalization. After DESeq2 normalization, only OTUs with a number of reads higher than 5 in at least one quarter of the samples were considered for the statistical analysis.

Differences in SCFA concentrations were evaluated by analyzing the data with non-parametric Wilcoxon-Mann-Whitney test using paired data, when allowed.

The correlation analyses were carried out using the Kendall and Spearman formulas with bacterial taxa abundances (DESeq2-normalized data), lipidemic profile data and faecal SCFA concentrations in hyperlipidemic subjects.

Statistical significance was set at $P \leq 0.05$; the mean differences with $0.05 < P \leq 0.10$ were accepted as trends. When p-values correction was applied, the false discovery rate (FDR) adjustment was used.

3.2.3 Results

16S rRNA gene profiling and SCFAs quantification revealed significant differences in the faecal microbial ecosystem between hyperlipidemic and control subjects. A total of 15’069’105 filtered high-quality sequence reads was generated (an average of 334’869 reads per sample). We identified

a total of 109 bacterial families (100 in hyperlipidemic samples and 89 in controls) and 269 bacterial genera (244 in hyperlipidemic subjects and 193 in controls). We found a minimum number of 39 families and 69 genera, and a maximum of 63 families and 110 genera per faecal sample in hyperlipidemic subjects; a minimum of 44 families and 80 genera, and a maximum of 64 families and 113 genera were found in controls. Only about 15% of families (n=16) and 10% of genera (n=26) were detected in all 45 analyzed faecal samples; the same 16 families and 26 genera were the only detected in all 30 hyperlipidemic faecal samples, whereas 30 families and 46 genera were found in all 15 control samples. These data suggest that the bacterial composition varied more among hyperlipidemic than control faecal samples, as also evidenced by the analysis of β-diversity (**Figure 1**).

Fig. 1

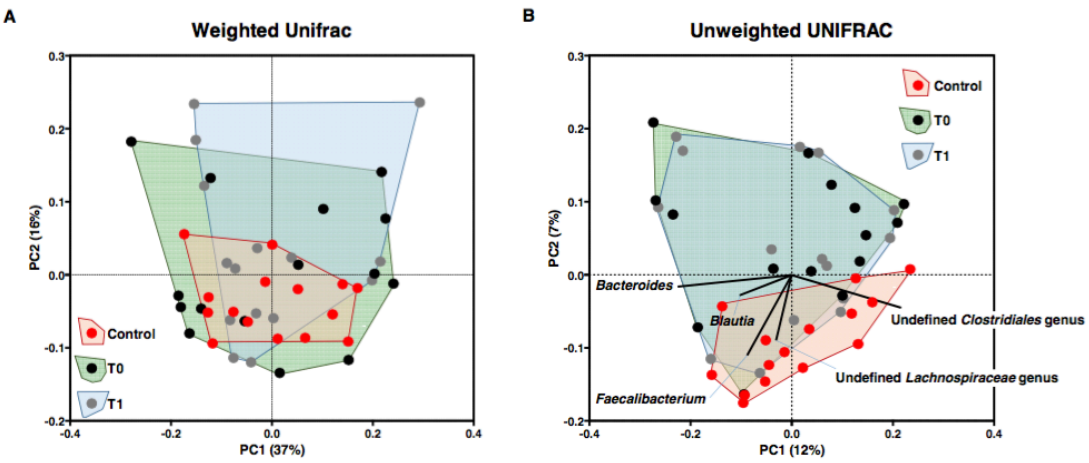


Figure 1. Inter-sample ecological diversity based on 16S rRNA gene profiling data of faecal samples from hyperlipidemic subjects participating to the hazelnut intervention trial and normolipidemic controls. Principal coordinates analysis of weighted (A) and unweighted (B) UniFrac distances based on the medians of OTU abundances. The first two coordinates (PC1 and PC2) are displayed with the percentage of variance explained in brackets. In panel B, a bi-plot is represented showing five of the genera that better describe the diversity among samples.

The analysis of the β-diversity performed with the unweighted UniFrac algorithm evidenced a good segregation of controls from hyperlipidemic samples, which was principally led by the abundance of *Faecalibacterium* spp. and two unidentified *Clostridiales* genera (**Figure 1B**). Also, the intrasample (α)-diversity analyzed through Chao1 index evidenced a difference between hyperlipidemic and

control samples (**Figure 2**). In specific, Chao1, which estimates the abundance of operational taxonomic units (OTUs) in a single sample, was significantly higher in controls; nonetheless, we did not find any difference between groups when we analyzed the α-diversity with the inverse Simpson index, which estimates biodiversity also considering OTUs' evenness (**Figure 2B**).

Fig. 2

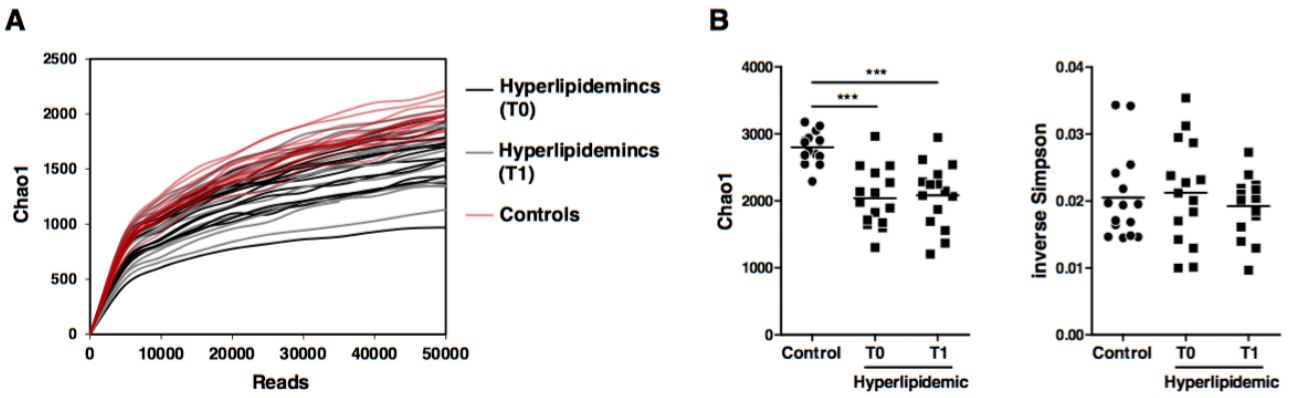


Figure 2. Intra-sample ecological diversity based on 16S rRNA gene profiling data of faecal samples from hyperlipidemic subjects participating to the hazelnut intervention trial and normolipidemic controls. A, rarefaction curves obtained using Chao1 index fixing a maximum of 50.000 reads per sample. B, α-diversity analysis based on Chao1 and inverse Simpson indexes. T0, samples before the hazelnut intervention; T1, samples after the hazelnut intervention. ***, P<0.001 according to Mann-Whitney U (unpaired) test.

To infer taxonomic signatures distinguishing the faecal microbiota structure of the 15 hyperlipidemic participants and the 15 aged-matched normolipidemic controls, we performed a comparative analysis at OTU level through the DESeq negative binomial distribution method. We found 229 OTUs whose abundance significantly differed between the two groups of subjects at baseline (**Figure S1** and **Figure 3**): 193 OTUs were increased in the controls, whereas only 36 OTUs in the hyperlipidemic samples (**Figure S1**). Most of the OTUs belonged to the phylum *Firmicutes* and, particularly, to the families *Ruminococcaceae* and *Lachnospiraceae*; notably, controls were enriched in OTUs belonging to well-recognized butyrate producing bacteria such as the genus *Roseburia* and the species *Faecalibacterium prausnitzii*. We also observed a significant reduction of an OTU

ascribed to the species *Akkermansia muciniphila* in hyperlipidemics. In addition, 23 OTUs belonging to the phylum *Bacteroidetes* were significantly enriched in controls compared to hyperlipidemic samples, whereas only 3 *Bacteroides* OTUs were reduced (**Figure S1**).

Fig. 3

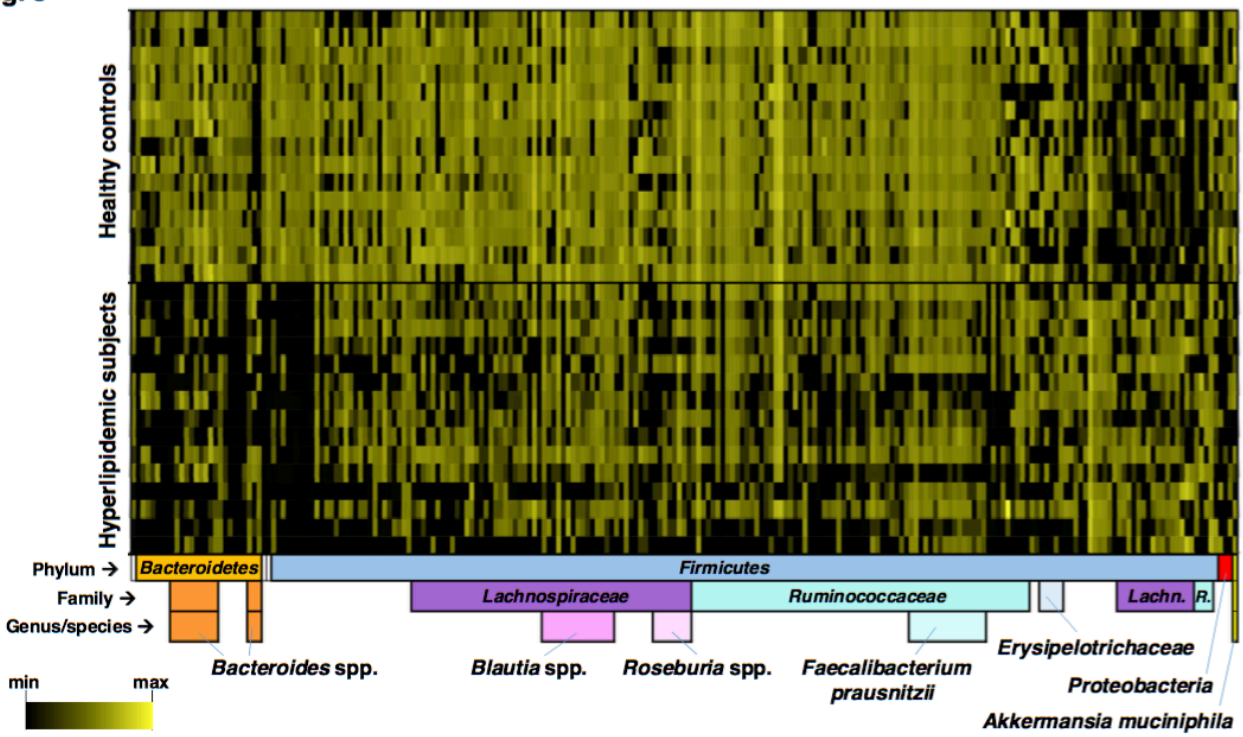


Figure 3. Taxonomic units in faecal samples distinguishing hyperlipidemic from control subjects. Heat map based on the normalized abundance of OTUs (horizontal axis) for individual faecal sample (vertical axis). The figure includes only the OTUs that were significantly different between hyperlipidemic and control samples through the DESeq2 negative binomial distribution method. Lachn., *Lachnospiraceae*; R., *Ruminococcaceae*.

Finally, the IME of enrolled children and adolescents was characterized by quantifying 8 SCFAs and lactate in faecal samples by UPLC-HR-MS (**Table S1**). Specifically, we analyzed the data from the faecal samples of 15 hyperlipidemic subjects undertaking the hazelnut intervention together with 19 additional faecal samples from hyperlipidemic subjects at baseline. We found that the faecal levels of several SCFAs were significantly different between hyperlipidemic and control subjects; specifically, hyperlipidemia was associated with significantly lower concentrations of acetate, butyrate and propionate, whereas lactate, isobutyrate and pyruvate were significantly increased

compared to control subjects (**Figure 4**). Notably, the faecal levels of acetate, propionate and (as a trend) butyrate were significantly lower in hyperlipidemic samples also when we included in the analysis the data collected in a previous study of 25 healthy adult volunteers (Gargari et al. 2016) (**Figure S2**). The levels of valerate, isovalerate and succinate were not significantly different between groups.

Fig. 4

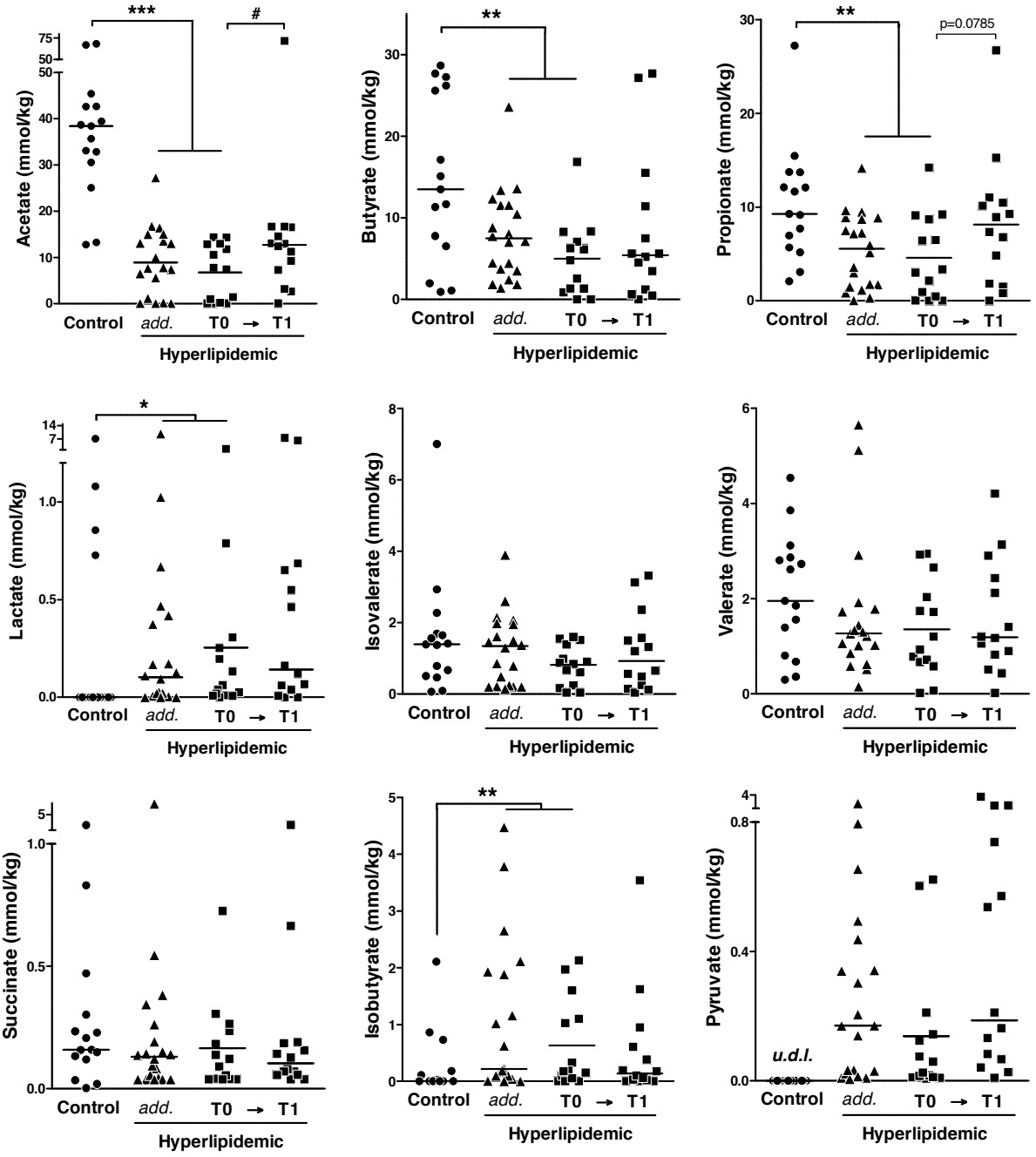


Figure 4. Faecal levels of short chain fatty acids and lactate in hyperlipidemic and control subjects. *add*, hyperlipidemic subjects not included in the intervention trial; T0, samples before the hazelnut intervention; T1, samples after the hazelnut intervention. *u.d.l.*, under detection limit. *, $p < 0.05$; **, $p<0.01$, ***, $p<0.001$ according to Mann-Whitney U (unpaired) text. #, $p<0.05$ according to Wilcoxon (paired) text.

Overall, these data suggest that hyperlipidemia is potentially associated with gut microbiota dysbiosis, which can be characterized by the alteration of numerous OTUs associated to SCFAs-producing bacteria, and the reduction of the faecal levels of acetate, butyrate and propionate.

3.2.3.1 Hazelnut intake induced limited changes in the bacterial abundances but modulated SCFA levels in feces

The α- and β-diversity of faecal samples were not significantly affected by the eight-week hazelnut intervention (**Figure 1** and **Figure 2**). Furthermore, we did not find any OTU that was modified with a FDR adjusted P value lower than the significance limit of 0.05 (**Figure S3**). Adopting the p value without adjustment, the 0.05 threshold was reached by 30 OTUs, 29 of which ascribed to the order *Clostridiales*. Specifically, 13 OTUs decreased after the dietary intervention, whereas 17 OTUs increased, including one OTU ascribed to *Faecalibacterium prausnitzii* and one belonging to an undefined species of the genus *Roseburia* (**Figure S3**).

Subsequently, we quantified the faecal level of 8 SCFAs and lactate after hazelnut consumption in 14 hyperlipidemic subjects that concluded the intervention (**Figure 4**). We found that acetate increased significantly over the intervention. A trend of increase (P=0.079) was also observed for propionate.

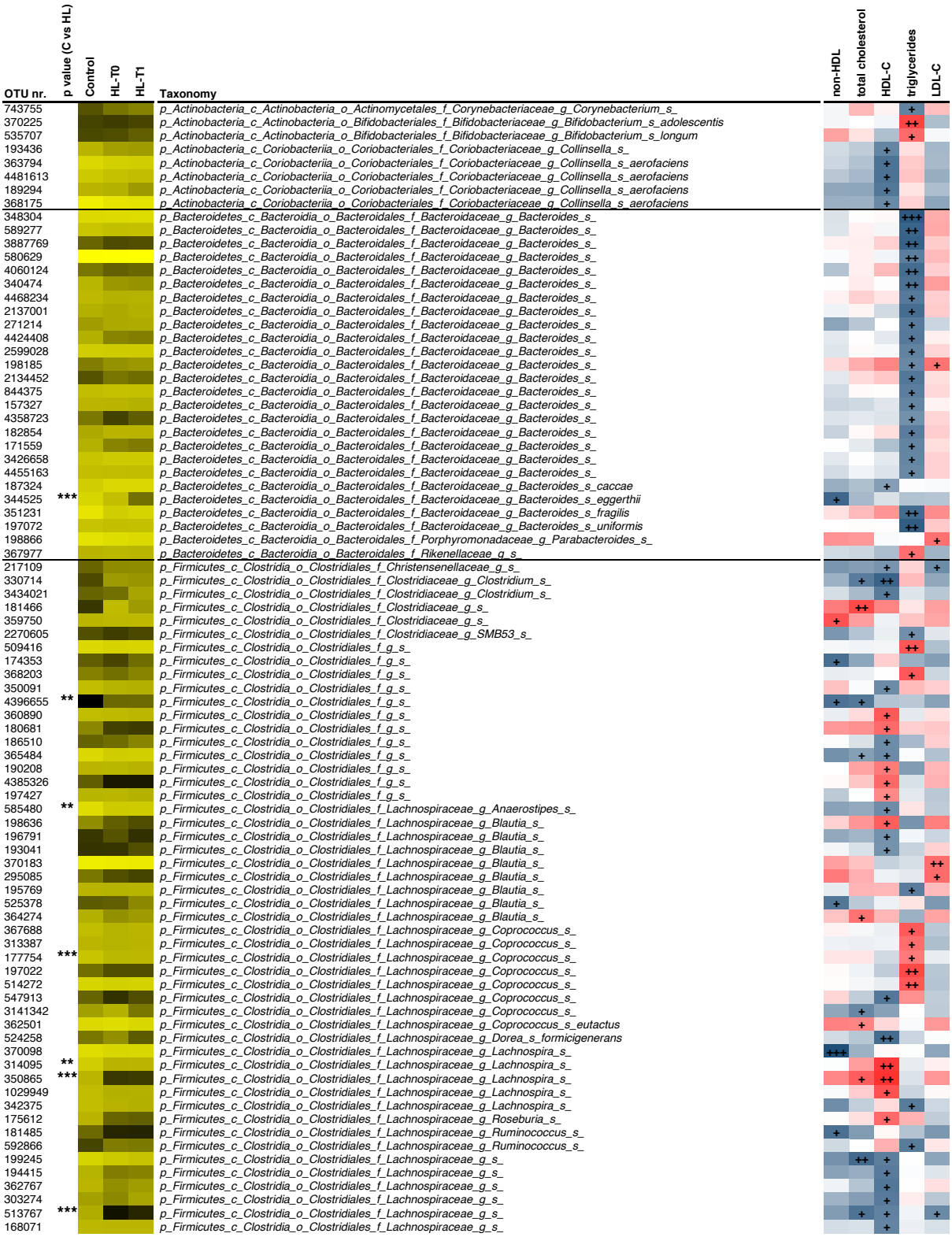
Overall, these results indicate that the eight-week intervention with hazelnut may induce limited changes in the faecal microbiota composition but can significantly modulate the faecal levels of the predominant intestinal SCFAs such as acetate.

3.2.3.2 The modification of several taxa of the faecal microbiota correlates with changes of host's hyperlipidemia biomarkers

We performed correlation analyses between the variations observed in the abundance of bacterial taxa and the changes in the lipid profile of hyperlipidemic subjects. Specifically, we considered the serum levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density

lipoprotein cholesterol (HDL-C), non-HDL cholesterol, and triglycerides (TG). We found that lipidemic parameters changed over the intervention and were associated to variations in 144 OTUs, 19 of which were significantly different between dyslipidemic and control samples (**Figure 5**). We found an inverse correlation between the changes of TC and the variation of several *Ruminococcaceae* OTUs, particularly *Faecalibacterium prausnitzii*. The changes in HDL-C inversely correlated with the fluctuation of the abundance of several *Clostridiales* and *Collinsella* OTUs; on the contrary, a positive correlation for HDL-C was observed with the variation of a smaller number of *Clostridiales*, and mostly *Lachnospiraceae*, including an OTU ascribed to the genus *Roseburia*. Only 15 OTUs were found to be correlated with LDL-C changes, 13 of them (7 positively and 6 negatively correlated) ascribed to the order *Clostridiales*. Notably, trygliceridaemia was inversely correlated with the change of about 20 OTUs ascribed to the genus *Bacteroides*, 9 *Lachnospiraceae* OTUs (including 3 OTUs ascribed to the species *F. prausnitzii*), and 2 *Akkermansia muciniphila* OTUs. On the contrary, trygliceridaemia modifications were positively correlated with changes of 5 *Coprococcus* OTUs.

Overall, these results indicate that lipid profile of the hyperlipidemic subjects here investigated is linked to the modification of the abundance of specific taxa in the intestinal microbiota such as the families *Lachnospiraceae* and *Ruminococcaceae*, and the genera *Akkermansia*, *Bacteroides*, *Roseburia*, and *Faecalibacterium*


OTU nr.
p value (C vs HL)
Control
HL-T0
HL-T1
Taxonomy
non-HDL
total cholesterol
HDL-C
triglycerides
LDL-C
743755 p_Actinobacteria_c_Actinobacteria_o_Actinomycetales_f_Corynebacteriaceae_g_Corynebacterium_s_
370225 p_Actinobacteria_c_Actinobacteria_o_Bifidobacteriales_f_Bifidobacteriaceae_g_Bifidobacterium_s_adolescentis
535707 p_Actinobacteria_c_Actinobacteria_o_Bifidobacteriales_f_Bifidobacteriaceae_g_Bifidobacterium_s_longum
193436 p_Actinobacteria_c_Coriobacteriia_o_Coriobacteriales_f_Coriobacteriaceae_g_Collinsella_s_
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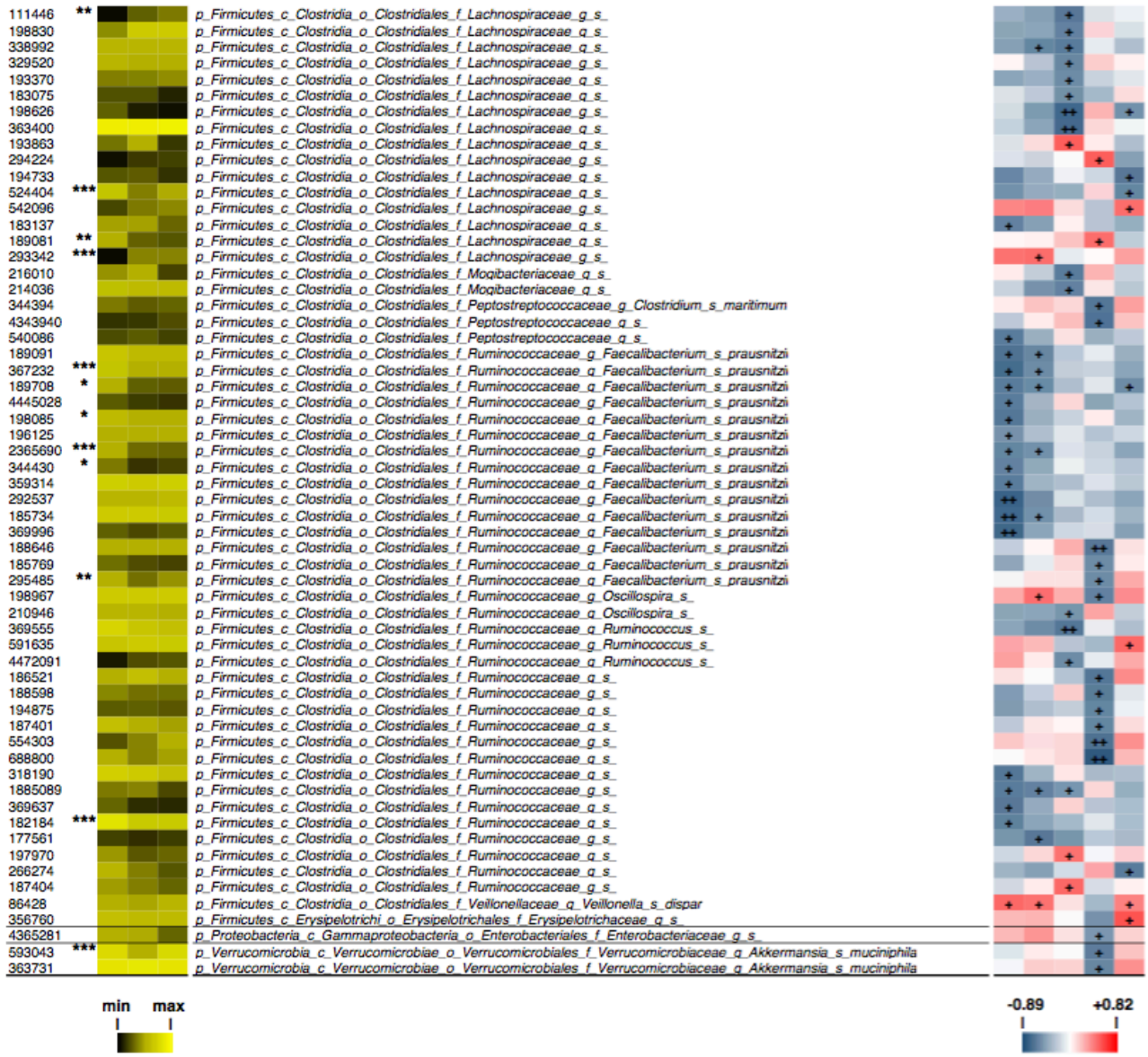

Figure 5. Correlation between lipidemic profile and the changes in faecal microbiota composition. The figure only includes OTUs whose change over the hazelnut intervention significantly correlated with at least one lipidemic parameter according to Kendall's Tau rank correlation. The colors in the left panel represents the mean of DESeq2-normalized abundances of the OTUs in control and hyperlipidemic (HL) samples before (T0) and after (T1) the intervention. p values on the left are according to Wald test on DESeq2-normalized data to indicate significantly different OTUs between control and HL-T0 samples; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. The colors in the right panel represents R-value of Spearman's correlation of the differences over the intervention trial between OTU abundance and lipidemic parameters. Plus signs are according to Kendall's Tau rank correlation: +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$.

3.2.4 Discussion

In this study, we show for the first time that young individuals with inherited hyperlipidemia may possess a dysbiotic gut bacterial ecosystem. We came to this result by comparing the IME of hyperlipidemic children and adolescent (from 7 to 17 years old, mean age of 17) with the IME of age-matched normolipidemic controls (from 5 to 17 years old, mean age of 17). Although the range of age considered was quite wide, spanning from early school-aged childhood to late adolescence, we did not observe age-related differences in the IME of volunteers, in accordance with scientific literature demonstrating that the intestinal microbiota of children reaches the adult state already at around 3 years of age (Lozupone et al. 2013; Matamoros et al. 2013; Rodriguez et al. 2015). Notably, diet can play a leading role in shaping the gut microbiota (Amato et al. 2015; De Filippo et al. 2010), therefore, possible differences in eating behavior between hyperlipidemic and normolipidemic counterparts could have been contributed to IME differences. However, we calculated through food diaries and food frequency questionnaires that the macronutrient contribution to the overall diet was comparable between the two groups of subjects (data not shown).

The results of the present study evidenced that the faecal microbiota of hyperlipidemic subjects is characterized by the alteration of numerous taxonomic units, many of which belong to the *Clostridiales* order. In addition, we observed in hyperlipidemic samples a reduced representation of several *Bacteroidetes* OTUs. Hyperlipidemia is a predisposing factor and an intrinsic feature of several diseases such as obesity, in which a similar alteration of the microbiota has been observed. In fact, a number of studies showed that obesity is associated with dysbiosis supposed to enhance energy extraction from food and increase low-grade inflammation, characterized by an increase of bacteria from the phylum *Firmicutes* (mainly *Clostridiales*) and, particularly, a decrease of *Bacteroidetes* (Armougom et al. 2009, Baothman et al. 2016, Ley et al. 2006, Santacruz et al. 2010). In addition, the enhanced *Firmicutes*/*Bacteroidetes* ratio has been associated with a high-protein, high-fat Western diet (Amato et al. 2015) and distinguished European from African 1–6 y aged children (De Filippo et al. 2010).

Data on type 2 diabetes, which can be a hyperlipidemia-associated disease, indicated only a moderate degree of gut microbial dysbiosis and rather reported a ‘functional dysbiosis’, in which a decrease in the intestinal level of butyrate was observed (Knip and Siljander 2016). The loss of

butyrate-producing bacteria and the decrease of butyrate levels in the gut have been also often reported in intestinal inflammatory conditions (Sokol et al. 2008, Van Immerseel et al. 2010). Accordingly, in our study, compared to normolipidemic controls, we found in hyperlipidemic subjects a significantly reduced abundance of OTUs ascribed to well-recognized butyrate producing bacteria, such as *Faecalibacteirum prausnitzii* and *Roseburia* spp. (Louis and Flint 2009), together with the significantly lower concentration of faecal butyrate.

Butyrate is mostly produced by *Clostridiales* bacteria, whereas acetate and propionate principally derive from the primary metabolism of members of the phylum *Bacteroidetes* (Macfarlane and Macfarlane 2003). In accordance with the observed lack of several *Bacteroidetes* OTUs, we found that the faecal levels of acetate and propionate were significantly decreased in the hyperlipidemic subjects. Acetate and propionate produced by gut microbiota are rapidly absorbed and reach the liver *via* the portal circulation, where they are used as an energy source (Canfora et al. 2015, den Besten et al. 2013) and participate to lipogenesis and gluconeogenesis, respectively (Canfora et al. 2015). There is experimental evidence suggesting that acetate and propionate may regulate cholesterol metabolism by decreasing the activity of hepatic 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS) and reductase (HMGCR) (den Besten et al. 2013); in addition, acetate may increase cholesterol 7-α-hydroxylase (CYP7A1) (Fushimi et al. 2006, Rodwell et al. 1976). Notably, HMGCS and HMGCR are involved in the initial steps of cholesterol biosynthesis (Rodwell et al. 1976), whereas CYP7A1 participates to cholesterol-bile acid conversion (den Besten et al. 2013). It can be then argued that the reduction of the intestinal levels of acetate and propionate in hyperlipidemic subjects may contribute to their altered cholesterol metabolism. Interestingly, in our study, following the dietary intervention with hazelnut, we observed a significant increase of acetate and a trend to rise of propionate levels, with assumed potential benefit for individuals with hyperlipidemia.

In addition to the lower abundance of the predominant intestinal SCFAs (acetate, butyrate and propionate, representing up to 95% of the SCFAs present in the colon), we found in hyperlipidemic faecal samples increased levels of lactate, isobutyrate and pyruvate. Lactate is produced by several members of the intestinal microbiota, such as lactic acid bacteria, bifidobacteria and enterobacterial species (e.g. *E. coli*). However, under normal physiological conditions, lactate does not accumulate in the colon, since it is consumed by other intestinal microorganisms. Particularly, lactate is

converted to butyrate by several gut commensals such as *Eubacterium hallii, Anaerostipes caccae* and *Roseburia intestinalis* (Bourriaud et al. 2005, Duncan et al. 2004, Flint et al. 2015, Van den Abbeele et al. 2013), which are all members of the order *Clostridiales*. Therefore, an accumulation of lactate can be plausibly considered as a microbial metabolic signature of dysbiosis; accordingly, the shifts of bacterial metabolism from short-chain fatty acid to lactate production and the resulting intraluminal pooling of lactate have been associated to pathological conditions (Bustos et al. 1994, Huda-Faujan et al. 2010, Vernia et al. 1988). Reportedly, lactate is present at low concentrations (<3 mmol l^{-1}) or not detected in the feces of healthy individuals (Duncan et al. 2007, Vernia et al. 1988), whereas concentrations up to 100 mmol l^{-1} have been reported in gut disorders (Hove et al. 1994, Vernia et al. 1988). Therefore, considering that in our study lactate exceeded the level of 1 mmol per kg of feces in only 5 out of 48 analyzed samples, the actual physiological significance of the faecal lactate here detected in hyperlipidemic subjects is questionable.

Isobutyrate, which is produced in the gut by degradation of amino acids such as valine (Zarling and Ruchim 1987), has been found to be correlated with behavior changes induced by prebiotics in mice (Burokas et al. 2017), whereas pyruvate, which can derive from bacterial autolysis or exfoliated apical enterocytes, has been associated to inflammatory bowel disease (Huda-Faujan et al. 2010). However, the actual importance of the modification of these organic acids in the human gut is unclear, and literature is too limited yet to allow a complete interpretation of our results.

Although the hazelnut intervention modified significantly the faecal levels of SCFAs, the abundances of the bacterial taxa in the faecal microbiota were only limitedly affected. The observed modifications of SCFA levels that occurred after the hazelnut consumption may be plausibly explained by the intake of fiber derived from the overall diet including the contribution of hazelnuts. However, the elaboration of dietary intake did not support such a hypothesis since fiber intake did not change following the intervention (i.e., about 10 g per day as a mean in this target population) (Deon et al. 2017a). Moreover, despite the energy intake did not change following the regular consumption of hazelnuts, it should be underlined that an increased intake of total fat (about +5%) and monounsaturated fatty acids was observed (Deon et al. 2017a). Finally, children had also an increased intake of phytosterols, tocopherols and polyphenols following hazelnut consumption. Consequently, in light of the recognized activity of phenolic compounds as potential modulator of

the microbiota (Valdes et al. 2015), the contribution of these bioactives on bacterial fermentation in the gut cannot be excluded.

Numerous human trials in recent years included the profiling of the intestinal microbiota, identifying the expansion or depletion of specific taxa as potential markers for pathological conditions or dysfunctions (Knip and Siljander 2016, Miele et al. 2015, Sokol et al. 2008). Nonetheless, only few studies associated specific gut bacteria to defined physiopathological mechanisms (e.g., (Devkota et al. 2012)). Such restricted knowledge on the involvement of bacteria in host physiological processes greatly limits the possibility to understand the actual biological meaning (if any) of several significant correlations we found between specific taxa of the intestinal microbiota and lipidemic profile. However, a few speculations can be done. Interestingly, the variation of OTUs belonging to intestinal bacteria with recognized anti-inflammatory properties such as *Faecalibacterium prausnitzii* (Sokol et al. 2008) and *Akkermansia muciniphila* (Zhao et al. 2017), correlated inversely with the change of TG, TC, LDL-C, and non-HDL cholesterol, suggesting the potential involvement of these bacteria in the link between inflammation and hyperlipidemia (Feingold and Grunfeld 2000, Tall and Yvan-Charvet 2015). Reportedly, *Faecalibacterium prausnitzii* is associated with inflammatory bowel diseases and its supplementation abolished inflammation (Sokol et al. 2008). Furthermore, the abundance of *Akkermansia muciniphila* is lower in obesity and diabetes and the administration of this bacterium has been shown to reduce obesity, fat mass inflammation and also plasma cholesterol and triglycerides (Everard et al. 2013, Plovier et al. 2017). This last example is also in accordance with the present study, where the changes of some plasma lipids are inversely associated with this intestinal commensal. In addition, the change of *Bacteroides fragilis*, which is another species with a reported potential anti-inflammatory role in the gut (Troy and Kasper 2010), was inversely correlated with the modification of trygliceridaemia. On the contrary, we found a direct correlation between the variations of cholesterolaemia and an OTU ascribed to *Veilonella dispar*, which is a potential pathobiont associated with several clinical cases of infection (Houston et al. 1997, Marchandin et al. 2001). *V. dispar* was also found to be enriched in colorectal carcinoma in adenoma (Kasai et al. 2016).

3.2.5 Supplementary material

Figure S1. OTUs distinguishing control and hyperlipidemic subjects determined by using the DESeq2 negative binomial distribution method on 16S rRNA gene profiling data of a single fecal sample per subject. The colors in the heatmap represents the mean of normalized abundances of the reported OTUs. The taxonomic lineage of each taxon is shown; p, phylum; c, class; o, order; f, family; g, genus; s, species. Positive fold changes (shown on a red background) indicate OTU overrepresentation in normolipidemic controls compared to hyperlipidemic samples; negative fold changes (shown on a green background) indicate a decrease of OTU abundance in hyperlipidemic samples compared to normolipidemic controls.

OTU nr.	Taxonomy	Media C	Media HL	log2 Fold Change	pvalue	padj
198774	*p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_Adlercreutzia;s_*			1.48	2.6E-03	1.75E-02
3013444	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_;g_;s_*			-2.42	5.4E-09	2.20E-07
183395	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Barnesiellaceae];g_;s_*			-2.29	3.3E-03	2.03E-02
3500642	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Barnesiellaceae];g_;s_*			-1.64	6.8E-03	3.58E-02
194395	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Barnesiellaceae];g_;s_*			-1.54	1.0E-03	8.29E-03
208479	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Odoribacteraceae];g_Butyricimonas;s_*			-2.02	1.3E-03	1.04E-02
177353	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Odoribacteraceae];g_Odoribacter;s_*			-2.73	2.7E-09	1.36E-07
196947	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Paraprevotellaceae];g_[Prevotella];s_*			-3.17	1.0E-14	2.80E-12
228601	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_*			-2.84	2.6E-11	2.19E-09
4467447	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_*			-1.86	1.9E-04	2.12E-03
326662	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_*			-1.85	8.0E-06	1.55E-04
157748	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_*			-1.57	1.4E-03	1.08E-02
1126638	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_*			-1.14	6.2E-03	3.34E-02
195508	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_caccae*			-2.23	2.4E-03	1.62E-02
184567	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_caccae*			-2.23	1.0E-04	1.42E-03
320120	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_caccae*			-2.18	4.1E-03	2.37E-02
344525	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_eggerthii*			-2.87	9.1E-07	2.28E-05
4387250	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_ovatus*			-1.50	2.2E-03	1.57E-02
585914	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae;g_Parabacteroides;s_distasonis*			-2.88	1.8E-07	5.22E-06
4374084	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae;g_Parabacteroides;s_distasonis*			-1.46	3.6E-04	3.51E-03
336214	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_;s_*			-3.26	9.0E-15	2.80E-12
439437	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_*S24-7*;g_;s_*			-2.72	2.9E-10	2.11E-08
208409	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_*S24-7*;g_;s_*			-2.55	2.0E-09	1.13E-07
339905	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_*S24-7*;g_;s_*			-2.02	1.1E-06	2.63E-05
188735	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_*			1.35	3.1E-03	1.93E-02
583656	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_*			2.47	1.4E-04	1.68E-03
356827	*p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_*			2.77	9.0E-08	2.90E-06
1787644	*p_Cyanobacteria;c_Chloroplast;o_Streptophyta;f_;g_;s_*			-2.63	1.1E-09	6.79E-08
1136443	*p_Deferribacteres;c_Deferribacteres;o_Deferribacterales;f_Deferribacteraceae;g_Mucispirillum;s_schaedleri*			-5.66	6.8E-42	7.47E-39
4431922	*p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus;s_*			-1.24	8.9E-04	7.57E-03
260890	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-3.50	6.6E-17	3.66E-14
848615	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-3.35	2.8E-07	7.79E-06
275139	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-3.18	8.2E-14	1.82E-11
345862	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-2.86	4.3E-12	5.97E-10
198200	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-2.80	6.5E-11	5.13E-09
277265	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-2.60	4.3E-10	2.94E-08
330460	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-2.48	2.8E-09	1.36E-07
272072	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-2.45	1.3E-08	4.96E-07
361727	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-2.45	1.9E-05	3.25E-04
356627	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-2.26	8.6E-08	2.90E-06
188861	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.92	3.4E-03	2.04E-02
180307	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.87	4.0E-05	6.15E-04
198119	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.86	3.3E-06	7.17E-05
192741	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.76	3.8E-03	2.24E-02
1040889	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.74	3.6E-03	2.17E-02
187081	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.73	3.0E-03	1.91E-02
201772	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.68	1.3E-05	2.27E-04
976470	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.64	5.9E-03	3.22E-02
4358921	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.62	1.1E-04	1.42E-03
185034	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.56	3.7E-03	2.18E-02
194095	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.55	7.4E-06	1.46E-04
389371	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.53	5.0E-03	2.81E-02
364736	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.51	8.1E-03	4.11E-02
195799	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.51	4.8E-04	4.40E-03
188832	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.43	2.4E-04	2.51E-03
188596	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.25	4.9E-03	2.77E-02
357389	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_*			-1.21	4.5E-04	4.18E-03
322560	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_SMB53;s_*			-1.19	8.2E-03	4.11E-02
524404	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*			-2.98	4.0E-09	1.69E-07
536910	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*			-2.77	1.1E-05	2.07E-04
561171	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*			-2.52	2.7E-04	2.77E-03
846141	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*			-2.50	1.8E-05	3.17E-04
185486	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*			-2.45	2.2E-06	4.91E-05
336830	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*			-2.38	8.2E-04	7.07E-03
177061	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*			-2.27	6.0E-05	8.83E-04

531888	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-2.26	1.6E-06	3.71E-05
216111	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-2.20	3.5E-05	5.58E-04
513767	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-2.19	2.1E-06	4.89E-05
352733	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-2.06	1.1E-05	2.04E-04
358104	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.85	1.6E-03	1.17E-02
368698	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.74	5.3E-03	2.97E-02
355424	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.73	2.2E-04	2.34E-03
211935	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.63	2.1E-04	2.29E-03
191531	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.56	2.9E-05	4.82E-04
4421998	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.49	1.4E-03	1.08E-02
454363	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.45	1.9E-03	1.38E-02
189081	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.44	4.8E-04	4.37E-03
529740	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.41	2.7E-03	1.77E-02
198270	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.40	2.4E-04	2.51E-03
4412106	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.30	1.3E-03	1.02E-02
194472	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.30	3.0E-03	1.89E-02
178386	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_*	-1.24	4.6E-03	2.62E-02
365256	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Anaerostipes;s_*	-2.02	2.6E-04	2.67E-03
585480	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Anaerostipes;s_*	-1.88	2.7E-03	1.77E-02
184570	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Anaerostipes;s_*	-1.62	1.6E-04	1.85E-03
179593	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-2.68	2.7E-09	1.36E-07
193592	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.95	1.6E-04	1.86E-03
307761	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.80	4.3E-04	4.10E-03
198145	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.79	1.6E-04	1.85E-03
196724	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.75	9.4E-05	1.31E-03
368950	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.74	7.9E-03	4.02E-02
184391	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.64	2.8E-03	1.79E-02
176327	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.62	1.5E-03	1.14E-02
191633	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.55	1.3E-04	1.60E-03
287445	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.46	7.4E-05	1.05E-03
346738	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.41	4.9E-03	2.77E-02
358798	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.41	9.7E-03	4.72E-02
187702	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.35	2.7E-03	1.77E-02
197524	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.24	7.8E-03	4.01E-02
191779	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_*	-1.23	8.2E-03	4.11E-02
182538	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Coprococcus;s_*	-2.24	1.5E-11	1.53E-09
515632	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Coprococcus;s_*	-1.85	4.0E-04	3.90E-03
177754	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Coprococcus;s_*	-1.51	3.4E-04	3.43E-03
181560	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Coprococcus;s_*	-1.15	1.0E-02	4.86E-02
584463	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Lachnobacterium;s_*	-1.25	2.5E-03	1.72E-02
314095	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Lachnospira;s_*	-2.62	1.2E-04	1.51E-03
350865	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Lachnospira;s_*	-2.44	3.2E-06	6.99E-05
3613745	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Lachnospira;s_*	-1.79	2.7E-04	2.77E-03
195719	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_*	-1.69	3.7E-04	3.62E-03
179472	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_*	-1.67	1.1E-03	9.09E-03
365492	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_*	-1.60	3.3E-03	2.03E-02
198542	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_*	-1.40	2.7E-03	1.77E-02
4414044	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_*	-1.09	5.5E-03	3.05E-02
362947	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_faecis*	-2.01	1.7E-03	1.23E-02
186077	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_faecis*	-1.59	1.7E-03	1.25E-02
1624481	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_faecis*	-1.18	5.8E-03	3.14E-02
319262	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.92	1.7E-11	1.53E-09
182184	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.92	1.5E-09	8.70E-08
196935	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.73	7.0E-12	8.62E-10
178015	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.50	4.7E-07	1.27E-05
341024	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.48	8.5E-08	2.90E-06
191393	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.44	9.2E-08	2.90E-06
262258	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.22	9.0E-08	2.90E-06
2686191	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.17	2.7E-07	7.67E-06
350036	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.17	5.9E-06	1.18E-04
355510	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.15	3.9E-06	8.12E-05
194287	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.09	9.5E-07	2.33E-05
353615	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.09	3.2E-08	1.24E-06
187389	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.08	1.8E-07	5.22E-06
191872	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.07	1.5E-04	1.76E-03
175761	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-2.05	2.1E-04	2.29E-03
319687	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-1.99	3.5E-06	7.35E-05
194534	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-1.94	1.0E-04	1.41E-03
367213	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-1.91	6.1E-04	5.32E-03
560535	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-1.90	8.4E-04	7.16E-03
184980	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-1.90	6.6E-07	1.70E-05
206574	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-1.82	2.6E-05	4.33E-04
196458	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-1.75	5.8E-04	5.10E-03
291266	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-1.71	6.8E-05	9.69E-04
575844	*p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_*	-1.69	3.1E-03	1.93E-02

4480359	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.68	1.2E-04	1.51E-03
182911	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.67	7.0E-03	3.66E-02
329668	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.62	5.2E-04	4.64E-03
358639	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.56	6.3E-05	9.10E-04
186963	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.54	6.2E-04	5.40E-03
192323	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.54	1.7E-04	1.88E-03
196518	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.49	1.4E-04	1.68E-03
350681	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.47	1.1E-04	1.42E-03
192046	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.47	2.5E-03	1.72E-02
177695	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.46	4.5E-04	4.18E-03
359175	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.45	1.0E-03	8.44E-03
185814	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.41	2.3E-03	1.60E-02
335041	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.41	1.2E-03	9.51E-03
190562	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.40	1.3E-03	1.04E-02
198947	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.35	1.1E-04	1.43E-03
198221	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.33	3.4E-03	2.05E-02
175520	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.31	3.3E-03	2.01E-02
178485	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.24	1.3E-03	1.00E-02
3530697	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.22	6.7E-03	3.56E-02
190312	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.19	2.2E-03	1.56E-02
187607	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_	-1.06	7.9E-03	4.02E-02
367232	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-2.00	5.6E-06	1.13E-04
525215	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.74	2.9E-04	2.94E-03
208332	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.71	4.5E-05	6.91E-04
173135	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.64	5.9E-05	8.83E-04
2365690	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.41	1.1E-03	8.98E-03
1825542	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.40	4.6E-04	4.22E-03
198085	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.38	1.7E-03	1.23E-02
295485	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.31	9.4E-04	7.90E-03
186392	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.26	2.2E-03	1.57E-02
199145	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.25	3.1E-03	1.93E-02
181422	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.23	1.0E-02	4.96E-02
189984	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.20	4.3E-03	2.48E-02
189708	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.16	6.7E-03	3.56E-02
1943669	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.15	2.7E-03	1.77E-02
344430	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-1.04	2.9E-03	1.87E-02
530327	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii	-0.96	8.8E-03	4.32E-02
275423	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_	-2.90	1.3E-11	1.43E-09
348009	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_	-2.42	2.5E-05	4.32E-04
327808	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_	-2.25	7.5E-08	2.77E-06
519763	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_	-1.25	7.4E-03	3.84E-02
304211	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_	-5.30	1.9E-12	3.02E-10
193755	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_	-3.89	1.4E-07	4.43E-06
210647	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_	-1.87	4.2E-03	2.39E-02
362342	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_	-1.78	7.1E-03	3.74E-02
198062	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_	-1.50	3.8E-03	2.22E-02
2283862	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Dialister;s_	-2.24	1.1E-03	8.92E-03
167270	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Dialister;s_	-1.28	8.9E-03	4.34E-02
369635	p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_;s_	-2.75	1.3E-04	1.55E-03
565289	p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_cc_115;s_	-2.36	5.2E-04	4.63E-03
369763	p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Coprobacillus;s_	-3.40	8.9E-10	5.75E-08
929836	p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Coprobacillus;s_cateniformis	-2.41	1.1E-04	1.43E-03
367215	p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Holdemania;s_	-1.32	9.5E-03	4.62E-02
751601	p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus;s_zeae	2.11	1.7E-04	1.93E-03
292892	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_	1.50	5.7E-03	3.11E-02
4396655	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_	1.52	1.9E-04	2.05E-03
195998	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_	1.67	3.3E-03	2.03E-02
552235	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_Christensenella;s_	1.41	8.2E-03	4.11E-02
325419	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_;s_	1.37	6.4E-03	3.41E-02
843459	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_;s_	1.38	3.1E-03	1.94E-02
4434334	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_;s_	1.41	2.4E-03	1.67E-02
308684	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_;s_	1.49	1.6E-03	1.19E-02
336325	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_SMB53;s_	1.34	1.4E-03	1.07E-02
294324	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Eubacteriaceae;g_Pseudoramibacter_Eubacterium;s_	2.50	2.2E-05	3.76E-04
111446	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_	1.22	4.0E-03	2.32E-02
193174	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_	1.32	2.3E-03	1.58E-02
2762219	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_	1.44	7.5E-03	3.87E-02
329703	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_	1.57	7.5E-03	3.87E-02
293342	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_	1.74	3.5E-05	5.58E-04
192159	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_	2.05	4.7E-05	7.05E-04
211212	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_	2.61	3.3E-05	5.33E-04
198928	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_[Ruminococcus];s_	1.93	9.7E-04	8.05E-03
176211	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_	1.25	1.8E-03	1.28E-02
192746	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_	1.27	2.7E-03	1.77E-02
194015	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_	1.35	5.0E-04	4.50E-03

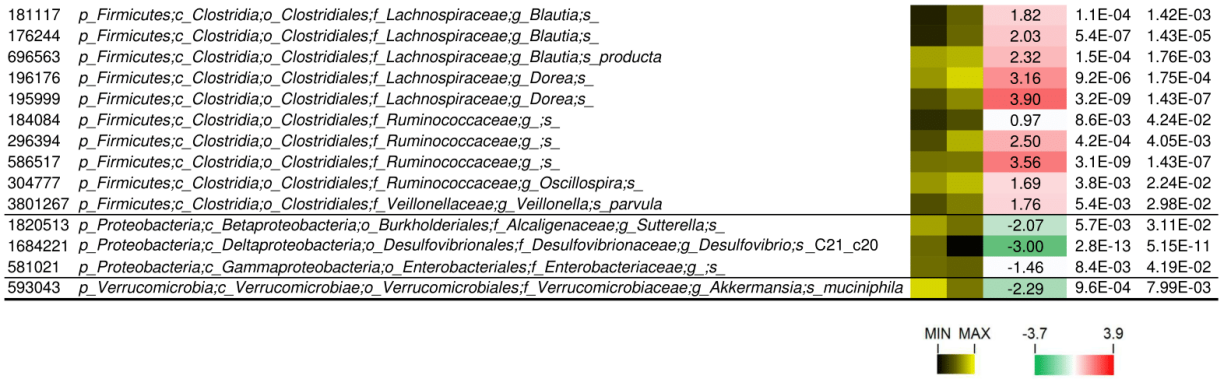

Table S1. Levels of short chain fatty acids in faecal samples at baseline and following hazelnut intervention. Data are reported as mmol per kg of feces. -, not detected.

Subject	Specimen donors	Acetate	Butyrate	Isobutyrate	Isovalerate	Lactate	Propionate	Pyruvate	Succinate	Valerate
C1	Control subjects (n=15)	68.38	27.66	0.18	1.40	1.08	12.11	-	0.30	2.87
C2		42.62	27.24	0.11	0.79	-	13.70	-	0.83	2.73
C3		42.64	11.32	-	1.70	-	9.26	-	0.12	2.62
C4		45.41	6.52	-	1.38	-	11.65	-	0.16	1.39
C5		25.05	1.97	2.11	1.65	-	5.16	-	0.23	0.80
C6		35.67	13.50	0.02	2.28	0.86	9.15	-	0.47	2.81
C7		38.66	15.09	0.03	1.42	-	7.68	-	0.16	1.56
C8		33.11	17.13	-	0.09	-	6.95	-	0.04	0.30
C9		38.44	11.65	-	0.07	-	12.08	-	0.01	3.12
C10		13.25	0.93	0.86	1.56	-	3.06	-	0.02	0.68
C11		39.43	26.21	-	2.94	-	15.46	-	0.21	3.86
C12		32.84	7.77	-	0.67	-	5.68	-	0.23	1.85
C13		12.79	1.09	0.73	0.51	-	2.07	-	0.13	0.36
C14		30.56	28.66	-	7.01	0.73	13.74	-	0.15	4.54
C15		67.07	25.59	-	0.46	7.10	27.22	-	2.33	1.95

Subject	Specimen donors	Acetate	Butyrate	Isobutyrate	Isovalerate	Lactate	Propionate	Pyruvate	Succinate	Valerate
H03	Hyperlipidemics at baseline (n=34)	10.53	16.83	-	0.62	9.12	0.31	9.12	0.14	0.24
H11		11.79	6.32	1.60	0.24	14.21	0.79	14.21	0.06	0.31
H12		14.31	8.33	0.33	0.99	6.47	0.20	6.47	0.12	0.04
H14		13.09	8.06	2.66	2.08	1.16	0.67	1.16	0.30	0.38
H15		7.41	11.54	4.47	3.90	7.50	0.13	7.50	0.14	0.08
H18		16.79	12.36	1.93	1.51	7.26	0.47	7.26	0.17	0.55
H19		15.06	7.04	1.16	1.38	0.85	0.37	0.85	0.34	0.34
H20		7.67	3.54	0.63	0.22	8.89	0.11	8.89	0.21	7.84
H21		13.56	4.47	1.02	0.21	7.17	0.42	7.17	0.34	0.15
H22		14.40	8.29	1.97	1.52	9.21	0.13	9.21	0.08	0.27
H23		13.05	7.78	2.12	1.62	9.64	0.09	9.64	0.17	0.26
H24		12.88	6.14	1.03	0.88	6.42	1.71	6.42	0.21	0.73
H25		12.98	7.07	2.13	1.56	8.69	0.06	8.69	0.60	0.18
H26		16.45	11.59	-	0.21	9.48	1.03	9.48	0.44	0.14
H27		0.07	2.55	0.19	0.85	2.14	0.04	2.14	0.01	0.04
H28		0.07	1.86	0.22	0.50	0.04	0.01	0.04	0.01	0.04
H30		0.07	1.32	0.10	0.17	0.02	0.01	0.02	0.02	0.09
H31		15.01	7.17	0.16	0.24	14.16	9.76	14.16	2.02	0.19
H35		7.82	13.41	0.05	1.30	8.72	0.01	8.72	0.03	0.06
H36		9.98	13.61	0.17	0.20	8.88	-	8.88	0.02	0.04
H37		0.07	-	-	0.05	-	-	-	0.01	0.04
H38		0.21	-	-	0.05	-	0.25	-	0.02	0.14
H39		7.77	4.80	0.15	1.39	3.36	0.03	3.36	0.62	0.12
H41		1.40	0.89	0.05	0.68	0.94	0.02	0.94	0.01	0.04
H42		7.45	6.27	0.18	1.60	2.98	0.01	2.98	0.01	0.05
H46		15.04	8.84	0.22	2.15	5.14	0.17	5.14	0.49	0.09
H47		0.07	4.51	0.10	0.87	0.31	0.00	0.31	0.01	0.04
H48		27.23	23.60	-	1.96	5.95	-	5.95	0.80	0.14
H49		0.19	2.44	0.01	1.46	3.02	0.02	3.02	0.03	0.14
H50		1.17	3.73	0.19	2.61	3.57	0.03	3.57	0.01	0.04
H53		5.63	1.41	0.10	0.15	1.73	0.17	1.73	0.66	0.12
H59		0.96	1.35	1.10	0.91	0.45	0.01	0.45	0.03	0.04
H60		0.07	1.86	1.88	0.80	1.49	0.02	1.49	0.01	0.04
H61		6.50	10.47	3.79	1.99	1.78	-	1.78	0.03	0.04
H03	Hyperlipidemics after hazelnut consumption (n=14)	13.05	7.49	-	0.15	9.25	0.06	9.25	0.07	0.07
H11		12.96	5.23	-	0.66	10.45	0.65	10.45	0.57	0.19
H12		16.65	15.50	0.19	1.32	7.34	0.16	7.34	0.21	0.16
H22		16.49	27.67	0.61	1.58	11.02	0.46	11.02	0.16	0.19
H24		71.70	27.16	1.62	3.13	26.71	6.16	26.71	1.50	2.34
H25		0.07	-	-	0.05	-	-	-	0.03	0.04
H27		14.59	11.41	0.03	1.51	10.13	-	10.13	0.13	0.66
H30		16.67	1.24	0.10	0.26	6.77	7.54	6.77	3.57	0.14
H37		9.21	3.46	0.02	0.13	8.93	0.55	8.93	0.08	0.04
H38		12.41	0.47	0.95	0.49	1.49	0.07	1.49	0.54	0.08
H39		2.65	0.64	0.06	0.57	0.81	0.01	0.81	0.01	0.06
H41		7.27	5.62	0.39	3.32	4.81	0.12	4.81	0.74	0.07
H42		11.25	4.52	0.18	2.36	15.26	0.69	15.26	1.46	0.13
H59		3.18	5.61	3.54	1.20	1.83	0.04	1.83	0.04	0.06
H61	Sample not available for this analysis									

Fig. S2. Fecal levels of short chain fatty acids in hyperlipidemic and control subjects. Black circles refer to control samples analyzed in the present study; white circles refer to data obtained from healthy adults in a previous study (Gargari et al., 2016, doi: 10.1128/AEM.01753-16); grey triangles refer to samples from hyperlipidemic subjects not included in the intervention trial. ***, P<0.001 according to Mann-Whitney U (unpaired) test.

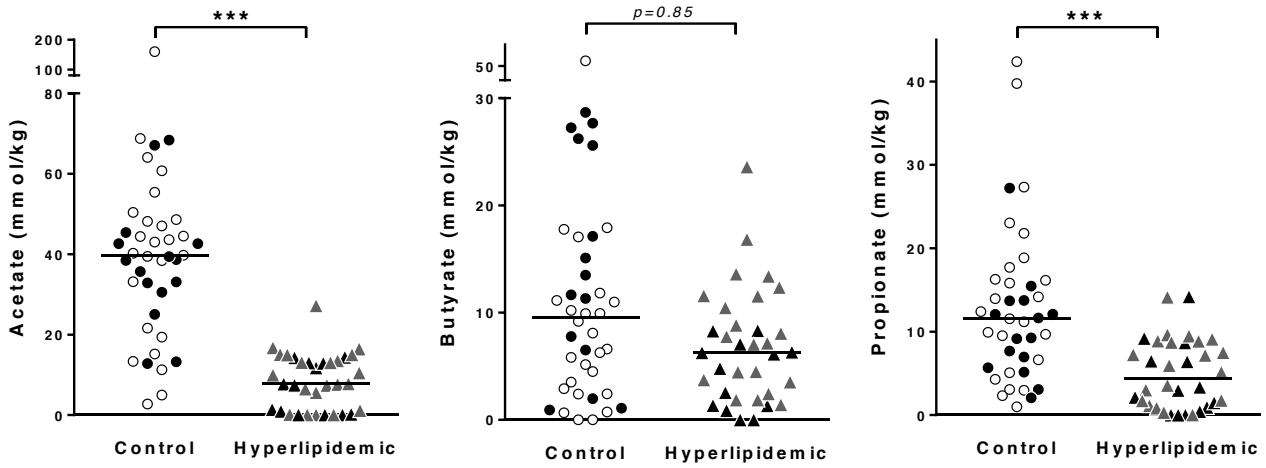

Figure S3. OTUs changed during the hazelnut intervention trial in hyperlipidemic subjects as determined using the DESeq2 negative binomial distribution method on 16S rRNA gene profiling data of a single fecal sample per subject. OTUs have been selected adopting the p value without FDR adjustment with a significance limit of 0.05. The colors in the heatmap represents the mean of normalized abundances of the reported OTUs. The taxonomic lineage of each taxon is shown; p, phylum; c, class; o, order; f, family; g, genus; s, species. Positive fold changes (shown on a red background) indicate OTU increase after the intervention; negative fold changes (shown on a green background) indicate a decrease after the intervention.

OTU ID	Taxonomy	MEAN T0	MEAN T1	log2 Fold Change	pvalue	padj
174278	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_			-1.18	6.1E-05	0.06
195998	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_			-0.60	3.5E-02	1.00
294324	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Eubacteriaceae;g_Pseudoramibacter_Eubacterium;s_			-0.77	8.9E-03	1.00
183137	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_			-0.66	2.7E-02	1.00
180227	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Dorea;s_			-0.98	1.0E-03	0.33
358781	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_			-0.84	4.8E-03	0.95
296394	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_			-0.71	2.1E-02	1.00
370086	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_			-0.70	2.3E-02	1.00
182431	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_			-0.66	3.1E-02	1.00
304777	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_			-0.62	3.2E-02	1.00
210946	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_			-0.56	4.5E-02	1.00
365965	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_			-0.73	1.6E-02	1.00
3235048	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_			-0.61	4.0E-02	1.00
189755	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_			0.51	4.6E-02	1.00
199374	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_			0.58	4.6E-02	1.00
197725	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_			1.09	3.9E-04	0.19
4364746	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_;s_			0.70	1.6E-02	1.00
186289	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_Clostridium;s_			0.70	1.6E-02	1.00
318760	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_			0.63	2.3E-02	1.00
181265	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_			0.64	2.8E-02	1.00
307761	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_			0.67	1.7E-02	1.00
188753	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_			0.85	2.8E-03	0.69
365634	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia;s_			0.65	2.7E-02	1.00
194287	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_			0.54	4.4E-02	1.00
205934	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_			0.57	5.0E-02	1.00
191695	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_			0.59	4.8E-02	1.00
349963	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_			0.61	2.1E-02	1.00
181422	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii			0.57	3.4E-02	1.00
193336	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_			0.65	3.2E-02	1.00
369763	p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Coprobacillus;s_			0.77	8.2E-03	1.00

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3.3 The faecal microbial ecosystem reflects bowel habits in irritable bowel syndrome

3.3.1 Introduction

Irritable bowel syndrome (IBS) is the most prevalent functional gastrointestinal disorder in the Western world. Although it does not have a lethal prognosis, IBS may induce a significant diminution of the quality of life of patients, depending on the severity of symptoms, which characteristically include abdominal pain, bloating, distension and altered bowel habits (Mearin et al. 2016).

IBS is a widely heterogeneous condition in terms of etiology, pathogenesis and clinical presentation. In particular, IBS is conventionally classified in four subtypes according to bowel habits: IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D), IBS alternating constipation and diarrhoea (mixed IBS, IBS-M), and unsubtyped IBS (IBS-U) (Mearin et al. 2016).

Elucidations of the diverse mechanisms underlying the pathophysiology of IBS subtypes are still lacunose; furthermore, conclusively validated biomarkers are not available (Kim et al. 2017). In a recent paper, Collins S. M. proposed to explain the intestinal dysfunctions associated to IBS through a gut-microbiota-centered model (Collins 2014). According to this model, triggers like antibiotics use, infections and/or stress affect host functions such as mucin production, gut motility and hormone secretion, leading to dysbiosis (i.e., compositional and functional alterations of the intestinal microbial ecosystem; IME), which in turn promotes chronic gut dysfunctions. Hence, Collins' model highlights the central role of the intestinal microbiota in IBS, in agreement with clinical evidences of the benefits generated by gut microbiota-targeting strategies such as the use of antibiotics (e.g. rifaximin) (Li et al. 2016) and probiotics (Guglielmetti et al. 2011, O'Mahony et al. 2005). Accordingly, gut dysbiosis was often observed as a common alteration associated to IBS (Taverniti and Guglielmetti 2014, Zhuang et al 2017).

Several possible bacterial signatures have been proposed to distinguish IBS patients from healthy controls, such as the increase in certain *Ruminococcus* phylotypes, the reduction of *Bifidobacteria*, or the expansion of *Proteobacteria* and *Veillonella* spp. (Shukla et al. 2015, Taverniti and Guglielmetti 2014). IBS-subtype specific alterations have been also reported. For instance, the

reduction of *Bifidobacteria*, lactobacilli, and the genus *Desulfovibrio* was observed in IBS-D compared to IBS-C (Malinen et al. 2005); the same study also showed the increase of *Veillonella* in IBS-C (Malinen et al. 2005). The IBS-C subtype was also associated to the expansion of ruminococci (Kassinen et al. 2007, Lyra et al. 2009). Moreover, higher aerobic counts in faecal samples was observed in IBS-D by means of a culture-based analyses (Carroll et al. 2010). In addition, more recently, Tap et al. (Tap et al. 2017) reported that IBS-D patients had more *Methanobacteriales* compared to the other IBS subtypes. However, data concerning the differences in the gut microbiota composition of IBS subtypes are inconsistent, partly contradictory, and often based on methods with low-discriminatory power (Ringel-Kulka et al. 2016, Taverniti and Guglielmetti 2014).

Besides the taxonomic identity of the bacteria constituting the IME, a predominant role for the intestinal microbiota-host interaction is played by bacterial metabolites in the gut and, among them, particularly by the short chain fatty acids (SCFAs) (Koh et al. 2016), which are produced in the intestinal lumen by bacterial fermentation in copious quantity (exceeding hundreds of mmol per kg of feces [Gargari et al. 2016]). Reportedly, acetate, butyrate and propionate, which are the three most abundant intestinal SCFAs, in fact, contribute to host physiology within the gut and in the periphery, affecting the metabolism of several organs (Koh et al. 2016). Although not diffusely taken into consideration in clinical investigations focusing on IBS, available scientific data already indicate that altered levels of the SCFAs may have a significant role in this disorder (Camilleri et al. 2016), suggesting that these metabolites of the intestinal microbiota need further attention in IBS.

Inspired by the above considerations, in this study, we aimed to characterized the IME in IBS subtypes by means of 16S ribosomal RNA (rRNA) gene profiling and SCFAs quantification in faecal samples collected during a multicentre intervention trial that we recently performed to assess the effect of a probiotic preparation on the IBS symptoms of 40 patients (Cremon et al., 2017). In addition, the clinical and immunological data collected during the trial were used to investigate the potential correlations existing in IBS subtypes between the IME and host physiological and clinical parameters, including bowel habits, depression/anxiety scores, and faecal levels of IgA and cytokines.

3.3.2 Materials and Methods

3.3.2.1 Patients

Eligible patients with symptoms meeting Rome III criteria for IBS diagnosis were recruited in five Italian hospitals as previously described (Cremon et al., 2017). In brief, the inclusion criteria comprised a positive diagnosis of all IBS subtypes, age between 18 and 65 years, negative colonoscopy or barium enema examination within the previous 2 years, and negative relevant additional screening or consultation whenever appropriate. Patients were excluded if they were pregnant, breast-feeding, or not using reliable methods of contraception. The exclusion criteria also included intestinal organic diseases, such as celiac disease ascertained by the detection of anti-transglutaminase antibodies, diverticular disease, or inflammatory bowel diseases (IBDs; e.g., Crohn's disease, ulcerative colitis, infectious colitis, ischemic colitis, or microscopic colitis); previous major abdominal surgery; untreated food intolerance, such as ascertained or suspected lactose intolerance as defined by anamnestic evaluation or, if appropriate, lactose breath test; consumption of probiotics or topical and/or systemic antibiotic therapy during the month before study enrolment; frequent consumption of contact laxatives; presence of any relevant organic, systemic, or metabolic disease as assessed by medical history, appropriate consultations, and laboratory tests; or abnormal laboratory values deemed clinically significant on the basis of predefined values.

3.3.2.2 Collected data and missing samples

A total of 40 IBS patients (IBS-C, n=12; IBS-D, n=14; IBS-M, n=3; IBS-U, n=11) were included in the study. Classification into IBS subtypes was according to the Rome III criteria and based on Bristol Stool Form scale characteristics (Longstreth et al. 2006). Information and biological specimens were collected every four weeks at five consecutive time points (visits V1-V5) according to the trial design described by Cremon et al. (2017) and registered at ClinicalTrial.gov (Identifier NCT02371499). One participant (belonging to the IBS-D subgroup) dropped out after visit V3 and, consequently, 198 faecal samples were collected. 16S rRNA gene profiling analyses were performed on all samples, whereas SCFAs were quantified in the 5 faecal samples of 37 patients (i.e. a total of 185 samples; IBS-C, n=12; IBS-D, n=11; IBS-M, n=3; IBS-U, n=11) due to insufficient specimen. Data from Bristol stool, anxiety/depression scales, and IgA and cytokine data were available as described in Cremon et al., 2017. Correlation analyses were performed using data from 150 samples (30 patients).

3.3.2.3 Profiling of faecal microbiota composition

Metagenomics DNA was extracted from about 200 mg of feces using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions. Subsequently, the bacterial community structure was profiled by 16S rRNA gene profiling. In brief, Probio_Uni and Probio_Rev primers were used to amplify a partial region of the 16S rRNA encompassing the V3 variable region (Gargari et al. 2016). Next, amplicons were sequenced using Illumina MiSeq System and the resulting sequence reads were managed by means of the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0 (Caporaso et al. 2010) with the GreenGenes database (version 13.5). Metadata have been deposited in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under accession code PRJEB18753.

3.3.2.4 Quantification of faecal short chain fatty acids (SCFAs)

SCFAs were quantified in the faecal samples as previously described (Gargari et al. 2016). In brief, 100 mg of stools were suspended in 2 ml of 0.001% formic acid, vortexed for 1 min, and centrifuged at 1000 x g for 2 min at 4 °C. Supernatant was recovered and pellet was extracted again as described above. Then, the two supernatants were combined and the volume adjusted to 5 ml with 0.001% formic acid solution. All extracts were stored at -20 °C until analysis, which was performed by UPLC-HR-MS on Acquity UPLC separation module (Waters, Milford, MA, USA) coupled with an Exactive Orbitrap MS through an HESI-II probe for electrospray ionization (Thermo Scientific, San Jose, CA, USA). Column, ion source and interface conditions were reported in (Gargari et al. 2016). Elution was carried out at a flow-rate of 0.2 ml/min with solvents 0.001% HCOOH in MilliQ-treated water (solvent A) and CH_3OH:CH_3CN (1:1 v/v, solvent B), using the following elution gradient: 0% B for 4 min, 0-15% B in 6 min, 15-20% B in 5 min, 20% for 13 min, and then return to initial conditions in 1 min. Subsequently, the UPLC eluate was analyzed in full scan MS in the range 50-130 *m/z* as described elsewhere (Gargari et al 2016). External calibration curves were prepared with reagents from Sigma-Aldrich (Milan, Italy) to quantify acetic, butyric, isobutyric, isovaleric, lactic, propionic, and valeric acids in faecal samples. SCFA concentrations were expressed in mmol per kilogram of wet feces.

3.3.2.5 Statistical analysis

Data concerning the intestinal microbial ecosystem (16S rRNA gene profile and SCFA quantification) were analyzed using R statistic software (version 3.1.2) and QIIME. Statistically significant differences were determined through the Wilcoxon-Mann-Whitney test for unpaired data. Statistically significant differences at OTU level between IBS subtypes were determined by using the differential gene expression analysis based on the negative binomial distribution method (R/Bioconductor DESeq2 package) using *q*-value (FDR adjusted *p*-value) for the threshold (Love et al. 2014). Correlation analyses were performed using the Kendall and Spearman formulas with the items specified in the text as predictors and dependent variables. Significance was set at $P \leq 0.05$; significance in the range $0.05 < P < 0.10$ was accepted as trend. UNIFRAC algorithms were used to study inter-sample diversity of the faecal microbiota composition. Subjects have been clustered using the Jensen–Shannon divergence (JSD) distance and the Partitioning Around Medoids (PAM) clustering algorithm based on microbiota profiling data.

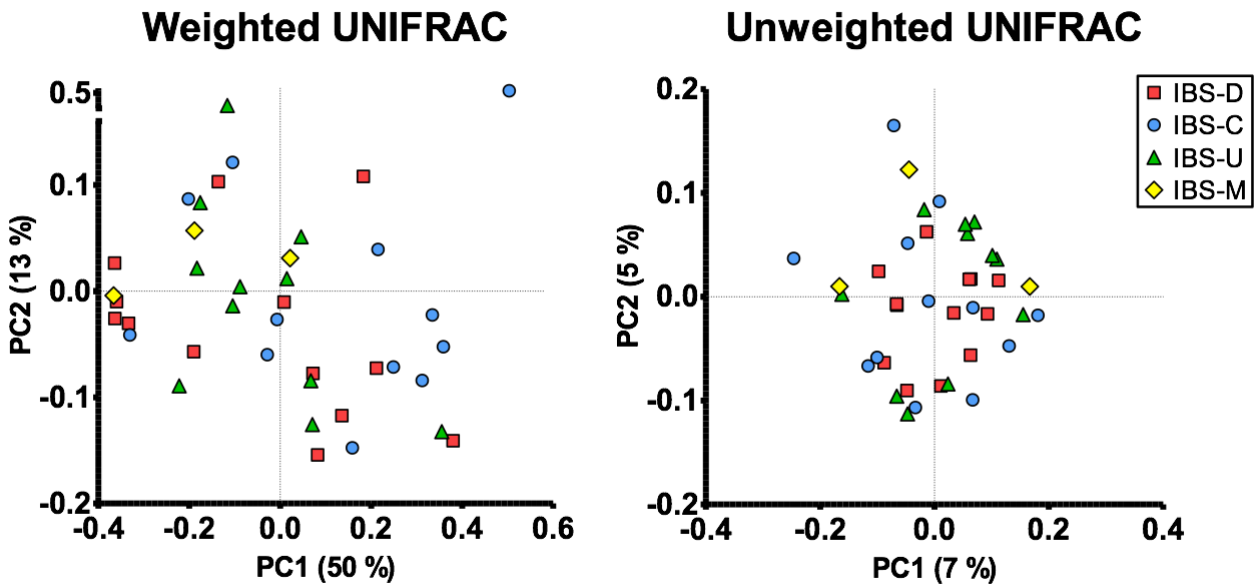
3.3.3 Results

3.3.3.1 The overall bacterial diversity of the faecal microbiota does not discriminate IBS subtypes

16S rRNA gene profiling was performed on 198 faecal samples collected from 40 IBS patients, generating a total of 16'963'222 filtered high-quality sequence reads (average, 138'413 reads per sample). Rarefaction curves demonstrated that most faecal microbiota diversity had been covered (not shown). Unifrac algorithm was used to investigate inter-sample (β-) diversity. According to the two main components extracted, we found that the intra-patient variability observed among the five samples analyzed for each subject was mostly higher than the differences occurring among different subjects (**Supplementary figure S1**). For this reason, besides considering the data of a single 16S rRNA gene profiling determination per subject (single profiling data; n=40), we also performed the analyses with data corresponding to the medians of five 16S rRNA gene profiling determinations per patient (median profiling data; n=39) in order to attenuate the effect of the temporal instability of patients' faecal microbiota.

Afterwards, we investigated the β-diversity of the different types of IBS. This analysis revealed that both weighted and unweighted Unifrac cannot distinguish faecal samples on the basis of the IBS subtypes either with single (**Supplementary Figure S2**) or median (**Figure 1**) profiling data.

Fig. 1



Medians of 5 16S rRNA gene profiling analyses per subject (n = 39)

Figure 1. Ecological β-diversity of the faecal microbiota in IBS subtypes. Principal coordinates analysis of weighted (A) and unweighted (B) Unifrac distances based on the medians of OTU abundances related to five faecal samples per IBS patient (n = 39). The first two coordinates (PC1 and PC2) are displayed with the percentage of variance explained in brackets.

Next, intra-sample taxonomic richness and evenness (α-diversity) were analyzed by means of four algorithms, namely Chao1, Faith's Phylogenetic Diversity, Shannon and Simpson indexes. The α-diversity indexes of the IBS subtypes were not significantly different both with single and median profiling data (**Supplementary Figure S3**).

Microbiota profiling data were then stratified by enterotyping based on the relative abundances of the bacterial genera (Gargari et al 2016). An optimal number of three groups of samples was generated, nonetheless the Silhouette coefficient, which validate the consistency within groups of data, was too low to consider reliable the clustering (**Supplementary Figure S4A**). Notably, the

taxonomic overview of all 198 IBS faecal sample analyzed revealed that the first seven most abundant genera belong to the *Firmicutes* Gram positive order *Clostridiales* (**Supplementary Figure S4B**); in particular, collectively, whereas the *Clostridiales* accounted for about the 75 % of detected bacteria, the relative abundance of members of the order *Bacteroidales* was lower than 10 % (**Supplementary Figure S4B**). On the contrary, in our previous studies, we found that Bacteroidales, and particularly the genera *Bacteroides* and *Prevotella*, were dominant genera of the faecal microbiota of the healthy volunteers (Ferrario et al 2014, Gargari et al 2016). Therefore, in order to assess if the observed expansion of *Clostridiales* compared to *Bacteroidales* is an actual microbiological feature of the investigated IBS patients, we analyzed additional faecal samples collected from 16 healthy adults through 16S rRNA gene profiling adopting the same protocol used for IBS samples. The obtained results indicated that *Clostridiales* are largely dominant also in the feces of control subjects (**Figure S4C**), demonstrating that the alteration of the *Clostridiales*/*Bacteroidales* ratio observed in IBS samples depended on technical issues and, probably, on the protocol used for the extraction of metagenomic DNA from feces. In fact, differently from the present study, in our previous works we extracted the faecal metagenomic DNA with a commercial kit that did not include a cell breaking step with a bead beater, plausibly resulting in the underestimation of the Gram positive bacteria (e.g. *Clostridiales*), which have a stronger cell wall then the Gram negative cells (e.g. *Bacteroidales*).

Overall, these data indicate that the bacterial ecological diversity indexes of the faecal microbiota do not vary significantly among IBS subtypes. The results of this study evidenced a general dominance of *Clostridiales* bacteria in the faecal samples collected from both IBS and control subjects.

3.3.3.2 IBS-C and IBS-D faecal samples are differently enriched of OTUs ascribed to Clostridiales

Subsequently, microbiomic data were examined with the DESeq2 negative binomial distribution method to infer differential relative abundances at the OTU level between IBS subtypes. The analysis was performed both on single and median profiling data, excluding IBS-M subtype because constituted by a too low number of patients (n=3) to allow the identification of significant differences. We found that several OTUs discriminated the three IBS subtypes considered (**Figure 2** and **Supplementary Figure 5**). A summary of the number of significantly different OTUs is shown in

the Venn diagram of **Figure 2A**. In specific, the analysis of median profiling data revealed 26 significantly different OTUs between IBS-U and IBS-C, 11 of which were also found analyzing single profiling data (**Figure 2B** and **Supplementary Figure 5**); 19 OTUs distinguished IBS-U from IBS-D, 6 of which were also found analyzing single profiling data (**Figure 2B** and **Supplementary Figure 5**). The highest number of dissimilarities was found between IBS-C from IBS-D: 85 OTUs had, in fact, a significantly different relative abundance, 39 of which found also analyzing the single profiling data (**Figure 2B** and **Supplementary Figure 5**). Largely most of the discriminating OTUs were taxonomically ascribed to the order *Clostridiales* (**Figure 2B** and **Supplementary Figure 5**); particularly, IBS-C were distinguished from IBS-D by numerous *Clostridiales*-associated OTUs belonging to the families *Ruminococcaceae* (in particular, the genus *Ruminococcus*) and *Lachnospiraceae*. In addition, two OTUs ascribed to *Bifidobacterium adolescentis* were increased in IBS-C whereas OTUs associated to the order *Bacteroidales* and to the *Firmicutes* species *Eubacterium biforme* were enriched in IBS-D samples (**Figure 2B** and **Supplementary Figure 5**).

Overall, these results indicate that the faecal microbiotas of IBS-C and IBS-D are characterized by a different distribution of *Clostridiales* taxonomic units, whereas the faecal microbiota of IBS-U samples possesses compositional features which are, in general, in the intermediate between IBS-C and IBS-D.

Fig. 2A

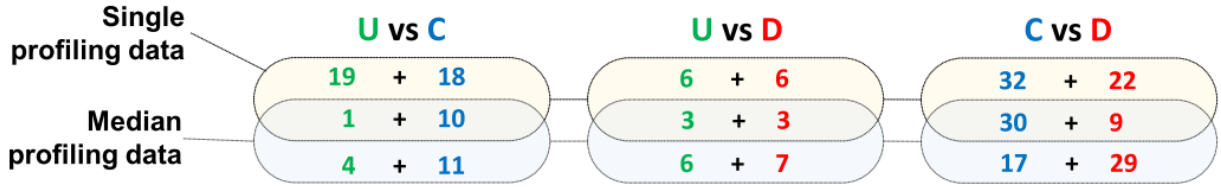
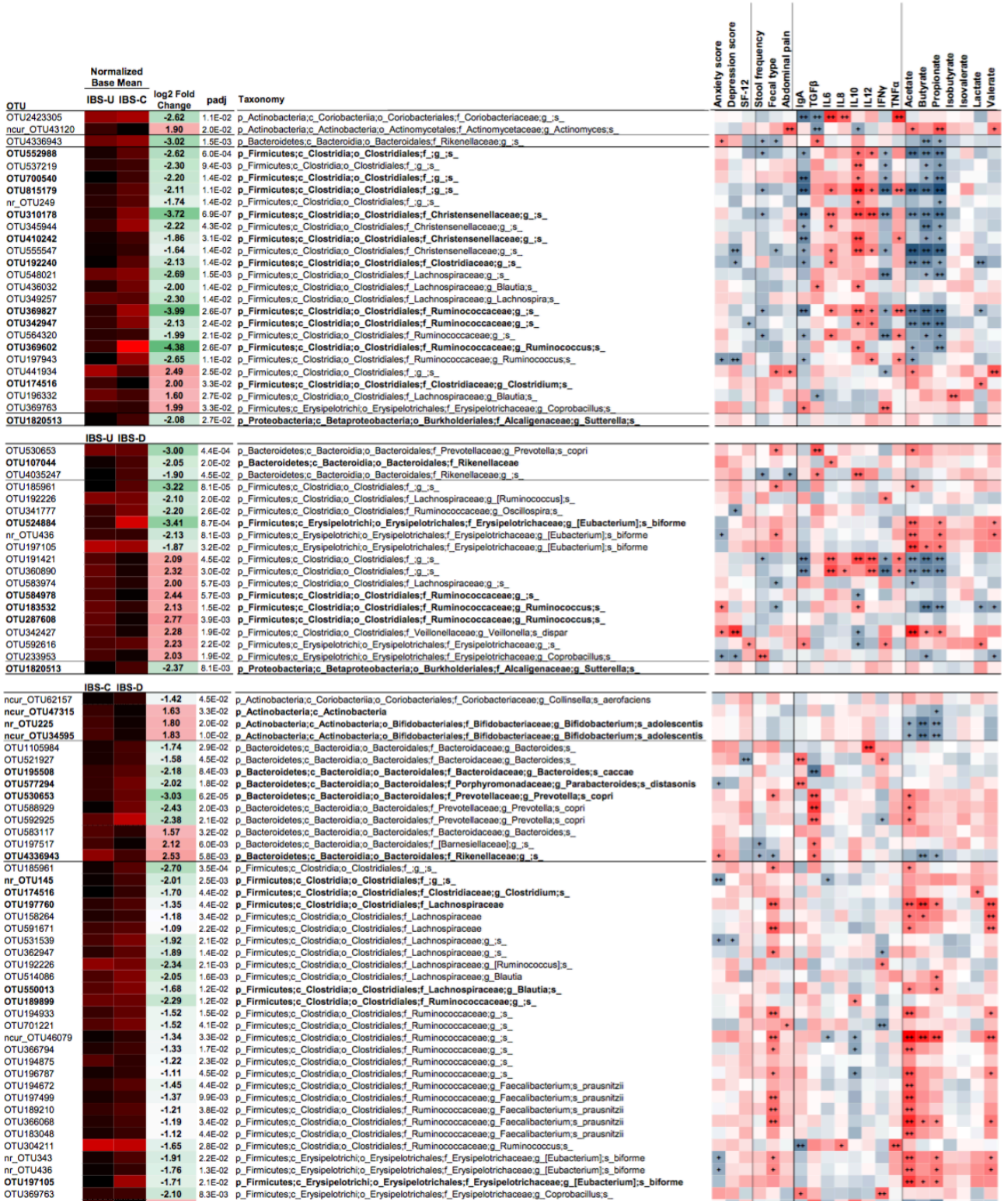


Fig. 2B


Normalized Base Mean
IBS-U IBS-C
OTU
log2 Fold Change
padj
Taxonomy
Anxiety score
Depression score
SF-12
Stool frequency
Fecal type
Abdominal pain
IgA
TGFβ
IL6
IL8
IL10
IL12
IFNγ
TNFα
Acetate
Butyrate
Propionate
Isobutyrate
Isovalerate
Lactate
Valerate
IBS-U IBS-D
IBS-C IBS-D

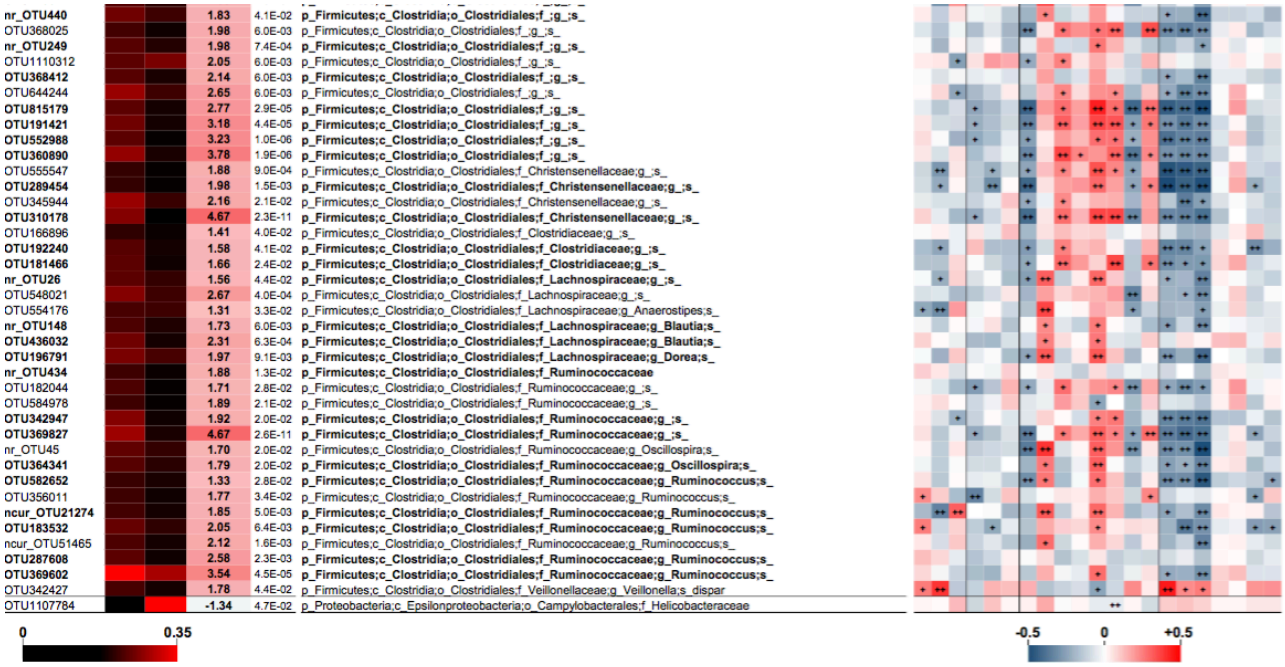


Figure 2. OTUs distinguishing IBS subtypes determined by using the DESeq2 negative binomial distribution method on 16S rRNA gene profiling data of five faecal samples per patient. A, Venn diagrams summarizing the number of OTUs that discriminate IBS subtypes on the basis of 16S rRNA gene profilig data of a single sample (single profiling data) and five samples (median profiling data) per patient. C, IBS with constipation (IBS-C); D, IBS with diharrea (IBS-D); U, unsubtyped IBS (IBS-U). Overrepresented OTUs are reported with the same color of the letter indicanting the IBS subtype. B, IBS subtype-discriminating OTUs according to median profiling data and their correlation with host physiological and clinical parameters. OTUs that distinguished IBS subtypes also according to single profiling data analysis are reported in bold. The heatmap on the left represents the mean of normalized relative abundances of the reported OTUs. The taxonomic lineage of each taxon is shown; p, phylum; c, class; o, order; f, family; g, genus; s, species. Positive fold changes (shown on a red background) designate OTU overrepresentation in the IBS subtype indicated in the column on the left of the Normalized Base Mean; negative fold changes (shown on a green background) designate the OTU overrepresentation in the IBS subtype indicated in the column on the right of the Normalized Base Mean. The heatmap in the right panel represents R-value of Spearman's correlation between the OTU and host's parameters. Plus signs are according to Kendall rank correlation: +, P<0.01; ++, P<0.001.

3.3.3.3 IBS subtypes are characterized by altered faecal levels of short chain fatty acids

The intestinal levels of lactate and the short chain fatty acids (SCFAs) acetate, butyrate, isobutyrate, valerate, isovalerate and propionate were quantified in the IBS faecal samples and used to characterize the IBS subtypes. As for the faecal microbiota composition, the analyses of the SCFAs were carried out considering the levels determined in a single faecal sample per patient (single analysis SCFA levels, n=37; **Supplementary Figure 6**) and the median values of five measurements per patient (median SCFA levels, n=37; **Figure 3A**). The organic acids were also quantified in the IBS-M faecal samples, but this subgroup was excluded from statistical analyses due to the limited number of patients (n=3).

We found that the faecal levels of SCFAs clearly distinguished IBS-C from IBS-D and IBS-U samples. In detail, the levels of acetate, butyrate, propionate and valerate, were significantly higher in IBS-D than IBS-C. In addition, faecal concentrations of acetate, butyrate and propionate were higher in IBS-U than IBS-C. Compared to all IBS samples considered together, the faecal level of acetate was significantly lower in IBS-C, whereas the faecal level of valerate was significantly higher in IBS-D (**Figure 3A**). No significant differences among IBS subgroups were observed for isobutyrate, isovalerate and lactate (**Figure 3A**).

Subsequently, a principal component analysis (PCA) was performed to discriminate samples on the basis of faecal SCFA levels. As evidenced by the PCA bi-plot depicted in **Figure 3B**, increased levels of acetate, butyrate and propionate characterize IBS-D samples and distinguish them from IBS-C; on the other hand, IBS-U samples are located in an intermediate area of the plot or in proximity of IBS-D samples.

Overall, these results indicate that IBS-C patients are characterized by reduced levels of the most abundant faecal SCFAs compared to IBS-U and, more evidently, IBS-D subjects.

Fig. 3A

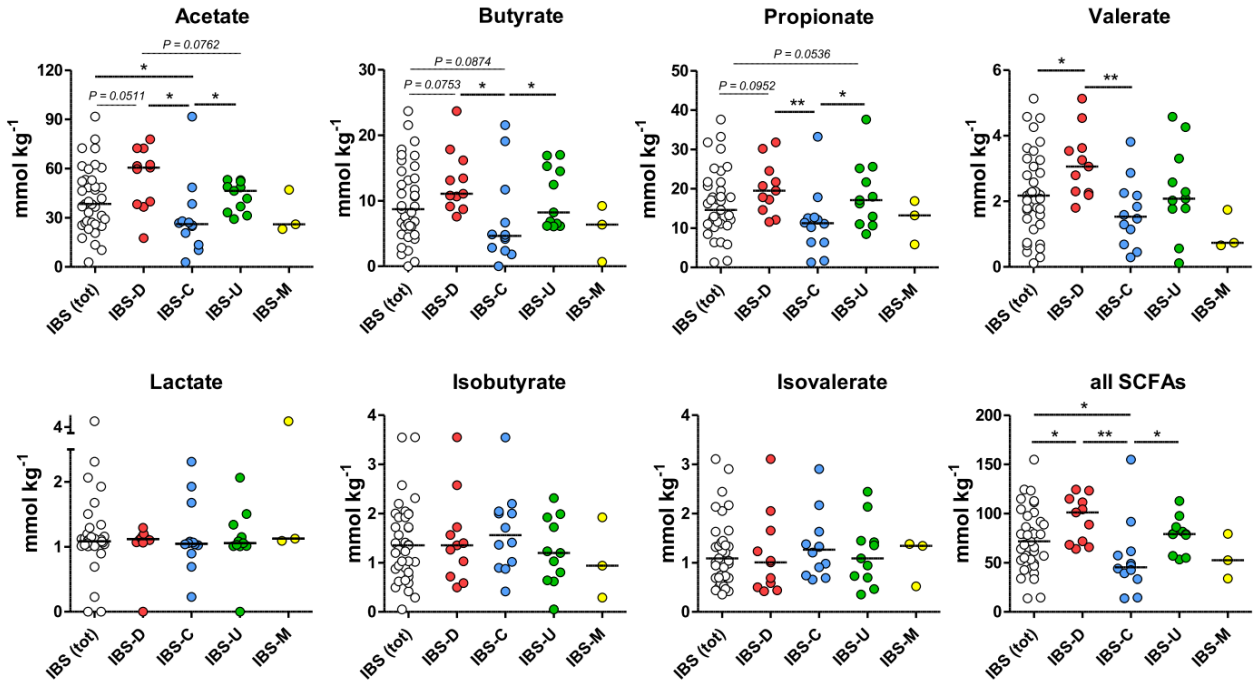


Fig. 3B

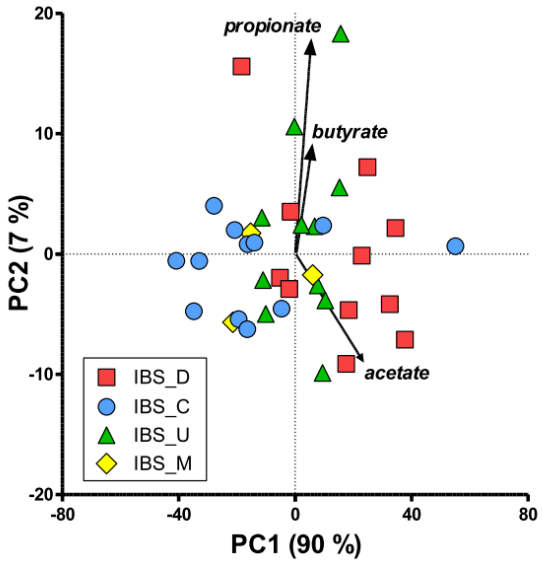


Figure 3. Short chain fatty acids (SCFAs) in faecal samples of IBS patients. A, medians of SCFA concentrations in five faecal samples per IBS patient (n=37). Statistical significances are according to Mann-Whitney test; *, P<0.05; **, P<0.01. B, Principal component analysis (PCA) biplot of SCFAs

(represented by arrows) and IBS patients. The first two coordinates (PC1 and PC2) are displayed with the percentage of variance explained in brackets.

3.3.3.4 The intestinal microbial ecosystem reflects clinical features of IBS subtypes

Finally, we performed correlation analyses between the data of faecal microbial ecology and clinical parameters of the IBS patients. To this aim, we used as predictors the faecal levels of SCFAs or the relative abundances of the OTUs that we found to be significantly different between IBS subtypes; on the other hand, the dependent variables considered were SCFAs, Bristol stool scale data (to assess bowel habits), abdominal pain/discomfort score, faecal levels of IgA and cytokines (TGFβ, IL6, IL8, IL10, IL12, IFNγ, and TNFα), and HADS and SF-12 questionnaire data (to evaluate anxiety and depression, and quality of life, respectively) (Cremon et al., 2017).

We found that host parameters correlated significantly to numerous OTUs (**Figure 2B**). Notably, we found that most *Clostridiales* OTUs enriched in IBS-C negatively correlated with SCFAs and cytokines compared to the *Clostridiales* OTUs increased in IBS-D (**Figure 2B**). In specific, the *Clostridiales* OTUs enriched in IBS-C samples were negatively correlated with the main faecal SCFAs (i.e. acetate, propionate and butyrate), whereas many *Clostridiales* OTUs overrepresented in IBS-D positively correlated with the same SCFAs, particularly acetate (**Figure 2B**). Moreover, most of the IBS-C-enriched OTUs that were inversely linked to SCFAs, at the same time, correlated positively with several cytokines and negatively with IgA and IFNγ. Conversely, great part of the IBS-D-enriched OTUs that were positively associated to SCFAs, were also positively correlated with the faecal type determined through the Bristol stool scale (**Figure 2B**). Accordingly, we also found a positive correlation between the faecal type and acetate, propionate and butyrate, which also inversely correlated with IL10 and IL12. Furthermore, acetate and propionate were positively correlated with IFNγ and depression score (**Figure 4**).

Overall, these results indicate that the differential representation of *Clostridiales* OTUs between IBS subtypes is associated with altered levels of intestinal SCFAs; then, in turn, both OTUs and SCFAs are associated with faecal cytokine levels and stool consistency.

Fig. 4

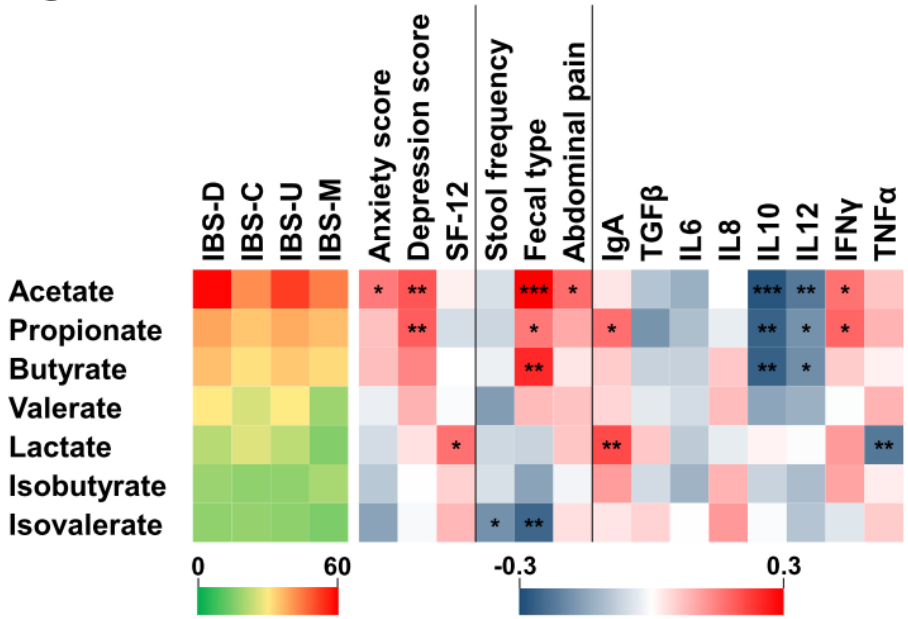


Figure 4. Short chain fatty acids (SCFAs) in IBS subtypes and their correlation with host physiological and clinical parameters. The heatmap on the left represents the median values (reported as mmol per kg of feces) of SCFAs in each IBS subtype. The heatmap in the right panel represents R-value of Spearman's correlation between SCFAs and host's parameters. Astericks are according to Kendall rank correlation: *, P<0.05; **, P<0.01; ***, P<0.001.

3.3.4 Discussion

The primary aim of the present study was to characterize the gut microbiota in IBS subtypes. To this purpose, we carried out 16S rRNA gene profiling and SCFAs quantification in 198 faecal samples obtained from 40 IBS patients enrolled in 5 different Italian hospitals (Cremon et al., 2017).

The temporal instability is a distinguishing feature of the intestinal microbiota associated with IBS (Durban et al. 2013, Matto et al. 2005, Maukonen et al. 2006); for this reason, it was suggested that studies aimed at characterizing the gut microbiota in IBS should include multiple time points (Collins 2014). Accordingly, in this study, we based microbiota analyses on data obtained from five faecal samples collected at 4-week intervals from each patient. These samples derive from a randomized cross-over intervention trial that assessed the clinical efficacy of a probiotic product. Although we

are aware that treatment may have affected the intestinal microbiota of IBS patients, we believe that the benefits of using five different faecal samples per subject are greater than the possible bias and may permit a more reliable identification of gut microbiota biomarkers for IBS subtypes. In specific, all the analyses have been carried out also considering the only data at baseline (single sample data analysis); single sample data analysis implies a mistake due to the great variability of the gut microbiota in IBS subjects, whereas the analysis with the median data of five samples per subject may determine an error due to the subject-dependent response to the probiotic treatment. The two potential errors are compensated by the combined use of the results derived from the analyses of single and median data. We believe, therefore, that those OTUs and SCFAs that resulted significantly different between IBS subtypes with the analysis of both populations of data can be most plausibly considered valid biomarkers.

Several studies focused on the characterization of the microbiota in IBS, with particular attention to the identification of microbial markers distinguishing this dysfunction from healthy condition (Zhuang et al. 2017); however, much less attention has been spent to compare the IMEs of IBS subtypes. In this context, recently, Tap and collaborators reported that neither richness nor variability of the intestinal microbiota differed among IBS groups (Tap et al. 2017). Accordingly, we did not find significant differences in both α- and β-diversity among the different IBS subtype. In a previous study, Jeffery et al. (Jeffery et al. 2012) used pyrosequencing of the 16S rRNA gene to determine the microbiota composition in a faecal specimen from 37 IBS patients. Notably, they identified distinct IBS patients' subsets, which however did not correspond to the traditional IBS subtypes (Jeffery et al. 2012). On the contrary, in the present study, we found that the relative abundance of numerous OTUs were significantly different among IBS subtypes. Particularly, here we report that major differences exist in *Clostridiales* OTUs between IBS-C and IBS-D feces; conversely, IBS-U faecal samples differed much less from IBS-C and IBS-D in terms of OTUs.

In light of the rapidly expanding literature demonstrating the clinical efficacy of dietary patterns based on drastically reduced "Fermentable, Oligo-, Di-, Mono-saccharides and Polyols" (low-FODMAP diet) (Eswaran et al. 2016), we can speculate that *Clostridiales* bacteria in the gut of IBS patients may represent a therapeutic target. FODMAPs, in fact, are preferential fermentation substrates for the intestinal *Clostridiales* bacteria (Flint et al. 2012); accordingly, several trials demonstrated that these bacteria may be affected by reduced FODMAP intake (Chumpitazi et al.

2014, Halmos et al. 2015, McIntosh et al. 2016). Therefore, low-FODMAP diet can be properly considered a *Clostridiales*-modulating intervention.

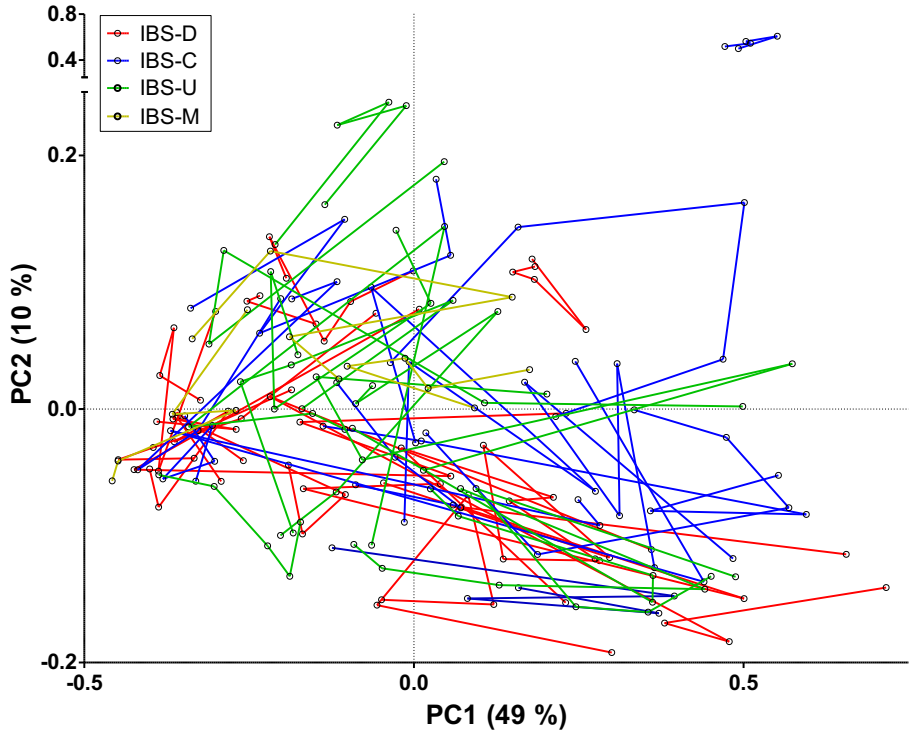
Many OTUs that discriminated IBS-C from IBS-D samples belonged to the *Clostridiales*' families *Ruminococcaceae* and *Lachnospiraceae*. The importance of these gut bacteria in IBS was also evidenced by the study of Tap et al., who defined a composite gut microbial signature for IBS severity constituted by 90 OTUs, among which, at the family level, principally OTUs within *Lachnospiraceae* and *Ruminococcaceae* were found (Tap et al. 2017). *Lachnospiraceae* and *Ruminococcaceae* are the most commonly retrieved families in the active intestinal microbiota (Peris-Bondia et al. 2011) and are considered the principal degraders of plant carbohydrates in the human colon (Flint et al. 2012). For instance, inside the *Ruminococcaceae* family, *Ruminococcus champanellensis* is the only known human intestinal bacterium that can degrade microcrystaline cellulose (Chassard et al. 2007), whereas certain strains of *Faecalibacterium prausnitzii* were reported to catabolize apple pectin; furthermore, bacteria phylogenetically related to *Ruminococcus albus* were demonstrated to utilize galactomannan (Salyers et al. 1977). Pectin can also be degraded by the *species Lachnospira pectinoschiza*, which belongs to the family *Lachnospiraceae* just like *Bryantella formatexigens*, reported to have cellulolytic activity (Wolin et al. 2003), and *Roseburia intestinalis and Butyrivibrio fibrisolvens*, two species that may utilize xylan (Chassard et al 2007). The degradation of plant material by members of the families *Ruminococcaceae* and *Lachnospiraceae* in the human colon directly brings to SCFAs as main products of their energy metabolism (Flint et al. 2012). It is well known, in fact, that these bacterial families include the most important butyrate-producing microorganisms of the human gut such as the genera *Faecalibacterium* and *Roseburia* (Barcenilla et al. 2000, Louis et al. 2010), as well as bacteria that can produce acetate from reductive acetogenesis (Bernalier et al. 1996, Rey et al. 2010), and butyrate or propionate from lactate utilization (Duncan et al. 2004, Rios-Covian et al. 2016).

Considering the above-mentioned literature, the observed differential OTU distribution between IBS-C and IBS-D let presume that the IBS subtypes may have dissimilar faecal levels of SCFAs. Accordingly, we found significantly lower levels of acetate, butyrate, propionate and valerate in IBS-C samples. Notably, in our study, such differences were found to be significant also considering the data calculated as median of five determinations per subject over a period of about 4 months, demonstrating that the observed alterations are stable over time.

Scientific literature on intestinal SCFAs in IBS is quite limited and contradictory, showing no alteration, augmented, or decreased levels compared to healthy controls (Halmos et al. 2014, Mortensen et al. 1987, Rajilic-Stojanovic et al. 2015, Tana et al. 2010, Treem et al. 1996). Nevertheless, our data are in accordance with the study of Ringel-Kulka et al. (Ringel-Kulka et al. 2015), in which IBS-D patients (n=42) were shown to have significantly higher faecal levels of acetate, propionate and butyrate than IBS-C patients (n=26). Interestingly, in this study the authors also found that faecal SCFA concentrations negatively correlated with colon transit time. This result is potentially in agreement with the positive correlation we found between faecal type (determined through the Bristol stool scale) and acetate, propionate and butyrate. The link between colon transit and intestinal SCFAs in IBS subtypes can be explained by two possible opposite mechanisms (Ringel-Kulka et al. 2015): (1) compared to IBS-C, IBS-D patients are characterized by increased colonic fermentation that leads to higher faecal levels of SCFAs, which stimulate the intestinal motility (Fukumoto et al. 2003) and, then, reduce transit time; or (2) decreased transit time in IBS-D patients slows down SCFAs absorption, determining higher SCFAs concentration in the feces compared to IBS-C. Here, we showed that several OTUs significantly enhanced in IBS-D compared to IBS-C correlated positively with the faecal levels of SCFAs (especially acetate) and faecal type; at the same time, a number of OTUs expanded in IBS-C resulted inversely correlated with SCFAs. Nevertheless, both explained scenarios are valid. On one hand, in fact, it is possible that the different distribution of intestinal bacteria is responsible for the dissimilar concentration of SCFAs in IBS subtypes. On the other hand, it can be even speculated that bacteria in the colon may be differently affected by modified intestinal transit (for instance due to variable adhesion ability and/or cell reproduction rate) with the consequent modification in the relative distribution of bacterial taxa in feces. However, a few facts are potentially in support of the first scenario: (i) most of the bacteria that distinguish IBS-C from IBS-D feces belong to taxa known to be SCFA producers; (ii) it is known that SCFAs are stimulators of colonic motility and may also increase the osmotic load leading to diarrhoea (Fritz et al. 2005); (iii) we found that faecal levels of SCFAs are not positively associated to stool frequency, as we would expect if the altered colon transit time was the main trigger of the observed differences between IBS subtypes. In summary, we think it is plausible to hypothesize a self-perpetuating mechanism, in which an initial modified colon transit time (determined by any possible trigger) gives rise to intestinal dysbiosis which, in turn, lead to to altered intestinal levels of SCFAs that may exacerbate or maintain the altered intestinal motility.

3.3.5 Supplementary material

A



B

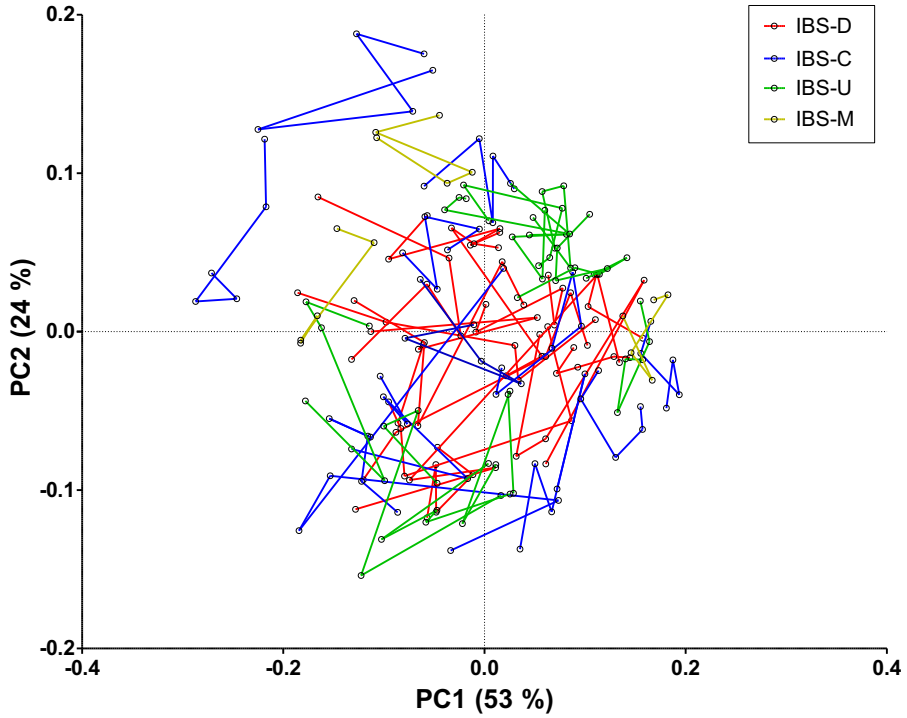


Figure S1. Principal coordinates analysis of weighted (A) and unweighted (B) Unifrac distances based on 16S rRNA gene profiling data. Lines connect samples belonging to the same patient. The first two coordinates (PC1 and PC2) are displayed with the percentage of variance explained in brackets.

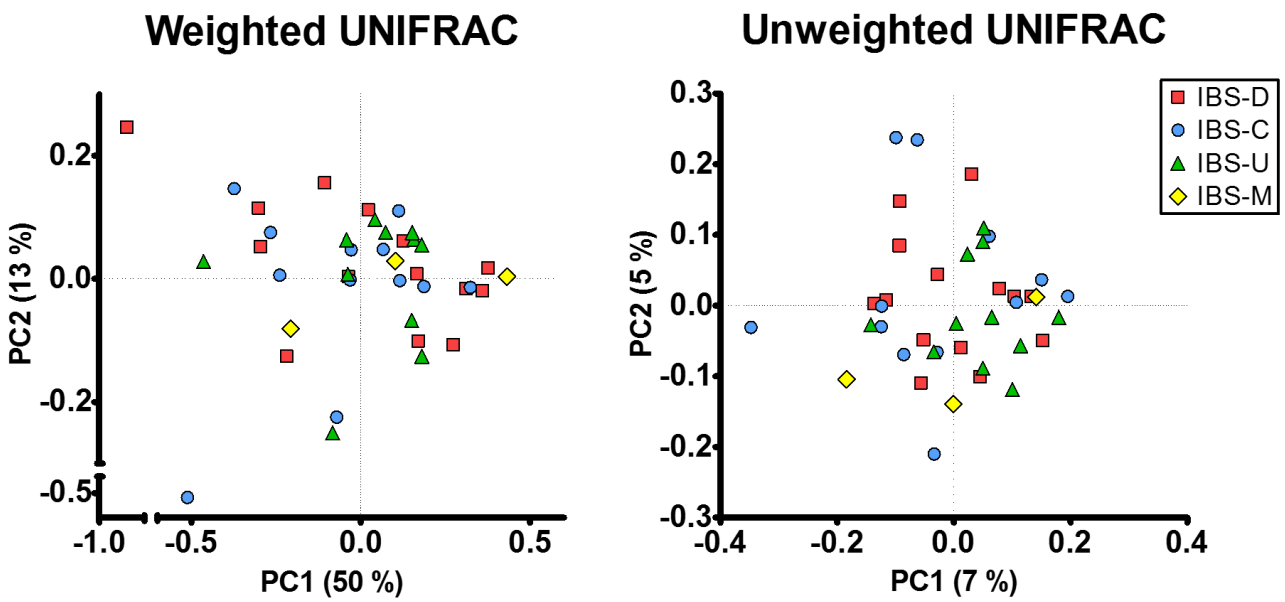
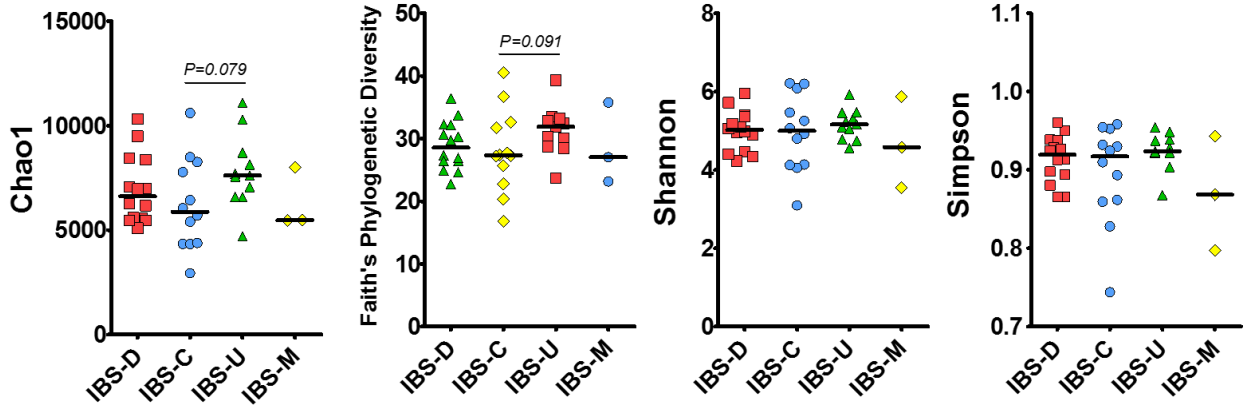


Figure S2. Principal coordinates analysis of Unifrac distances based on 16S rRNA gene profiling data of a single faecal sample collected from 40 IBS patients. The first two coordinates (PC1 and PC2) are displayed with the percentage of variance explained in brackets.

A



B

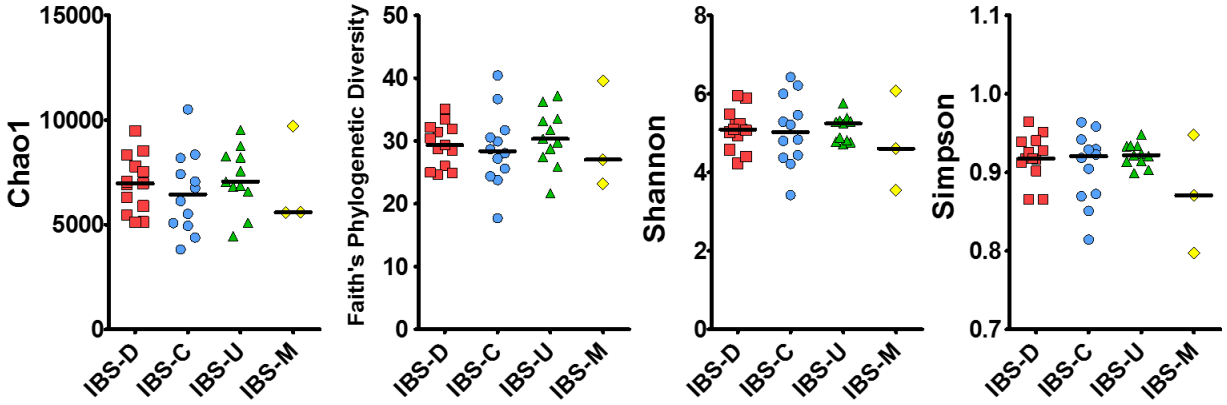
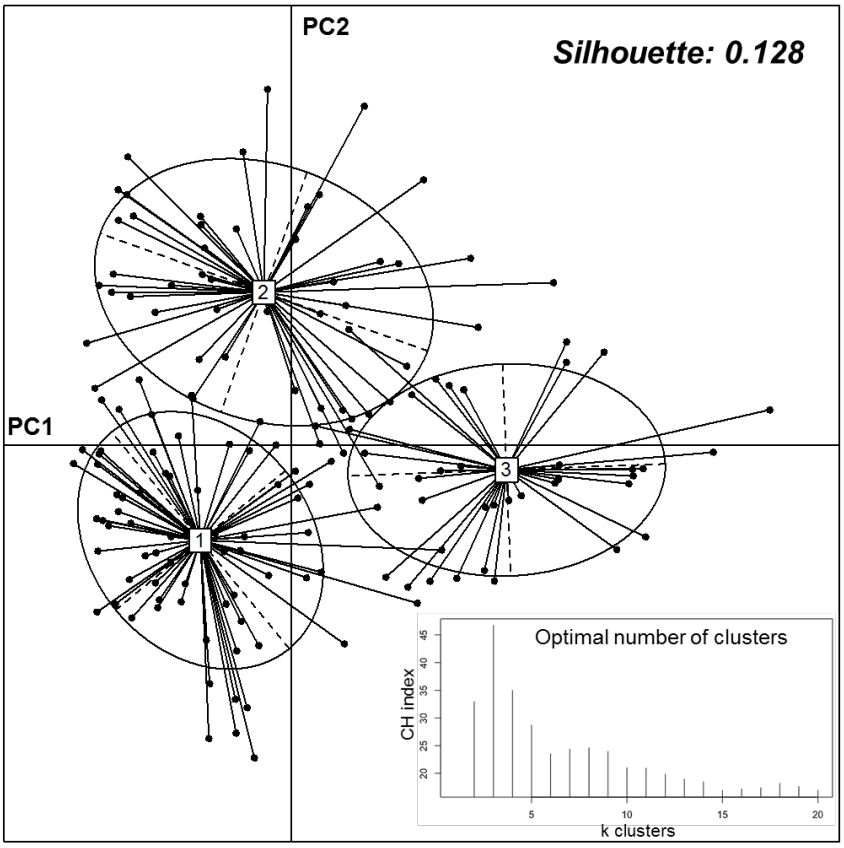
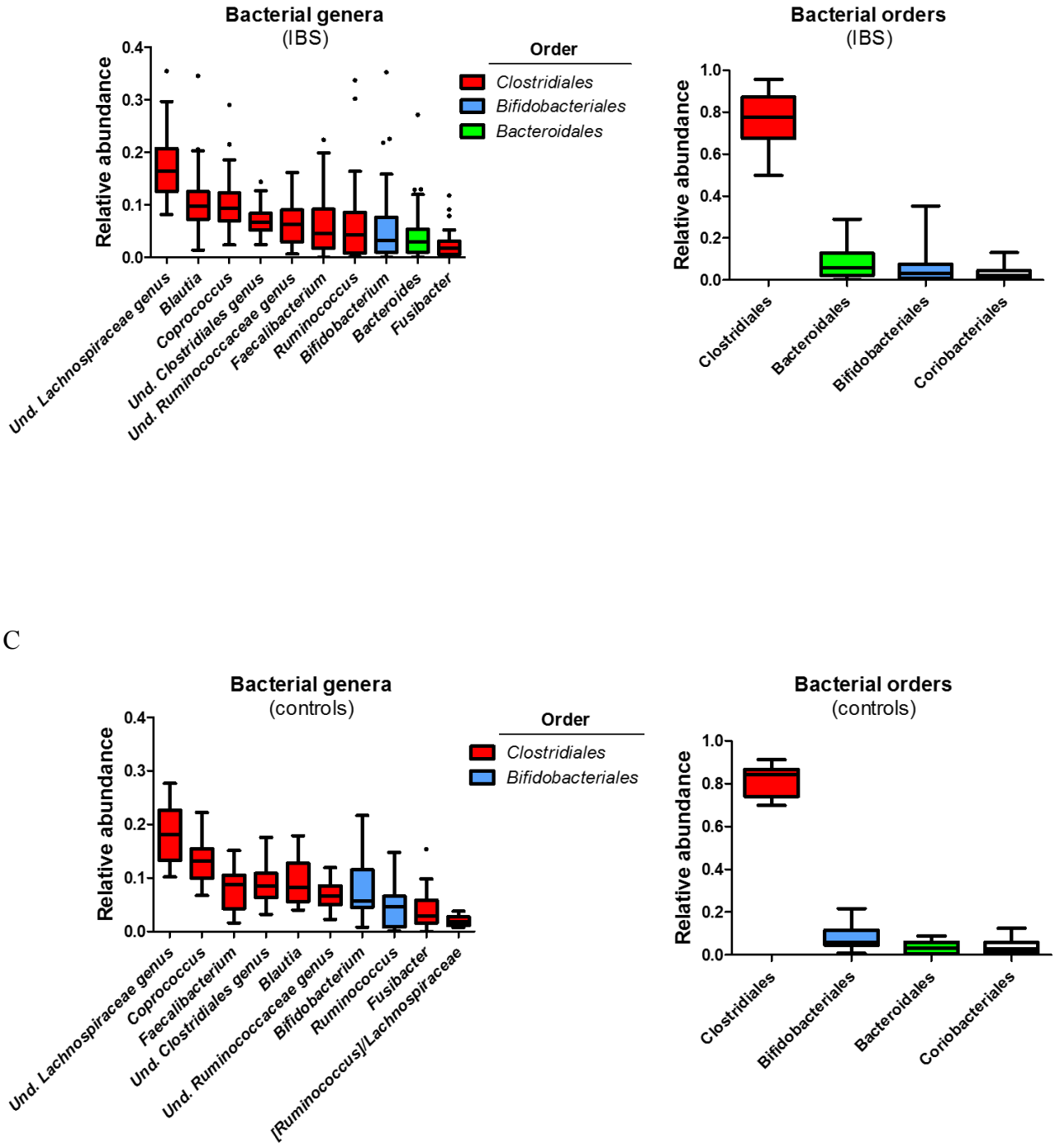


Figure S3. Within-subject (alpha) diversity of faecal samples from IBS patients determined through four different estimators. Scatter dot plots show data from a single faecal sample per subject (panel A; n = 40) and medians of the data from five faecal samples per subject (panel B, n = 39). Statistical significances are according to Mann-Whitney test.

A



B



C

Figure S4. Analyses of enterotypes in IBS faecal samples. A, Principal Coordinates Analysis (PCoA; the first two principal components are shown); clustering was based on genus relative abundance using JSD distance and the Partitioning Around Medoids (PAM) algorithm. The optimal number of

clusters was determined through the Calinski-Harabasz (CH) index and the Silhouette coefficient. B, Tukey boxplots of the dominant bacterial genera and orders in IBS faecal samples.

A

IBS-U *vs* IBS-C	Normalized Base Mean		log2 Fold Change	padj	Taxonomy
OTU	IBS-U	IBS-C			
OTU841419			-2.40	3.5E-02	p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_;s_
OTU560981			-2.32	7.5E-03	p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_Slackia;s_
ncur_OTU34595			-2.21	1.6E-02	p_Actinobacteria;c_Actinobacteria;o_Bifidobacteriales;f_Bifidobacteriaceae;g_Bifidobacterium;s_adolescentis
nr_OTU333			1.80	4.6E-02	p_Actinobacteria;c_Actinobacteria;o_Bifidobacteriales;f_Bifidobacteriaceae;g_Bifidobacterium;s_longum
OTU1646183			2.45	1.9E-02	p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_;s_
OTU355291			-2.70	1.2E-02	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_
OTU197072			2.33	2.6E-02	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_
OTU846127			2.35	1.3E-02	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae;g_Parabacteroides;s_
OTU535549			3.06	7.8E-04	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_fragilis
OTU524318			3.49	1.3E-04	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_
OTU552988			-2.37	1.6E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU1110312			-2.12	3.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU815179			-2.02	2.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU700540			-1.97	4.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU560873			-1.92	3.9E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU310178			-3.80	1.1E-04	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_;s_
OTU410242			-2.40	7.9E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_;s_
OTU192240			-3.49	1.3E-04	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_;s_
OTU181466			-2.05	3.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_;s_
OTU297182			-2.17	4.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU182903			-1.96	3.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU369827			-3.86	1.7E-05	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU342947			-3.05	7.8E-04	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU199374			-2.85	7.9E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU367946			-2.08	1.2E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU147100			-1.97	3.6E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU626544			-1.86	4.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU334215			-2.25	4.7E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_

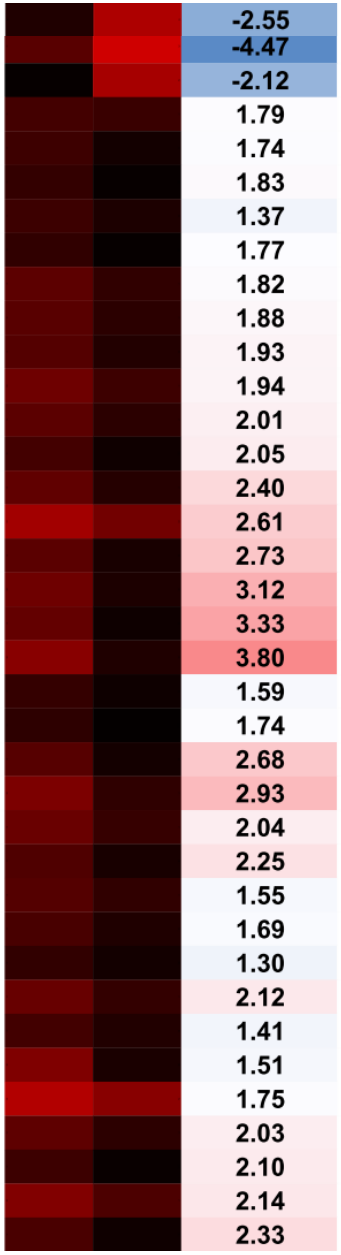
OTU581933			**-1.77**	3.3E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_
OTU369602			**-5.28**	4.2E-07	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_
OTU192079			**-2.34**	4.7E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_
OTU174516			**2.39**	2.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_Clostridium;s_
OTU529740			**2.35**	3.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU197760			**2.35**	7.2E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU345111			**1.95**	3.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_
OTU192226			**2.93**	7.8E-04	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_[Ruminococcus];s_
OTU550013			**3.18**	1.3E-04	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_
ncur_OTU47801			**1.71**	3.2E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
ncur_OTU75122			**2.53**	6.7E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU185659			**2.60**	4.7E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Acidaminococcus;s_
OTU342427			**1.95**	1.4E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Veillonella;s_dispar
OTU1820513			**-2.40**	1.4E-02	p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_Sutterella;s_
OTU791348			**-3.63**	1.7E-05	p_Proteobacteria;c_Deltaproteobacteria;o_Desulfovibrionales;f_Desulfovibrionaceae;g_Desulfovibrio;s_D168
OTU359809			**3.57**	1.3E-04	p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_Sutterella;s_
OTU825033			**2.05**	4.7E-02	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_;s_
OTU1671681			**2.15**	1.1E-02	p_Proteobacteria;c_Gammaproteobacteria;o_Pasteurellales;f_Pasteurellaceae;g_Haemophilus;s_parainfluenzae
OTU968675			**2.82**	2.4E-03	p_Proteobacteria;c_Gammaproteobacteria;o_Pasteurellales;f_Pasteurellaceae;g_Haemophilus;s_parainfluenzae
OTU661526			**-2.40**	1.4E-02	p_Synergistetes;c_Synergistia;o_Synergistales;f_Synergistaceae

B

IBS-U *vs* IBS-D	Normalized Base Mean		log2 Fold Change	padj	Taxonomy
OTU	IBS-U	IBS-D			
OTU841419			-2.98	3.3E-03	p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_;s_
ncur_OTU47315			2.54	3.5E-03	p_Actinobacteria;c_Actinobacteria
OTU568118			-4.07	2.6E-06	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
OTU369449			-2.85	2.5E-02	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_
OTU107044			-2.50	6.4E-03	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae
OTU524318			2.75	5.3E-03	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_
OTU535549			3.16	5.4E-04	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_fragilis
OTU297182			-3.03	1.1E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU199374			-3.36	5.8E-04	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU916143			-3.65	7.4E-05	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Phascolarctobacterium;s_
OTU524884			-5.94	1.2E-10	p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_[Eubacterium];s_biforme
OTU584978			2.68	4.7E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU582089			2.02	2.5E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira;s_
ncur_OTU72889			2.15	1.4E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_
ncur_OTU36690			2.23	1.4E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_
OTU287608			2.77	6.4E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_
OTU183532			3.05	5.8E-04	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus;s_
OTU1820513			-2.33	2.1E-02	p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_Sutterella;s_

C

IBS-C *vs* IBS-D	Normalized Base Mean		log2 Fold Change	padj	Taxonomy
OTU	IBS-C	IBS-D			
ncur_OTU47315			**1.83**	3.9E-02	p_Actinobacteria;c_Actinobacteria
nr_OTU225			**2.01**	2.3E-02	p_Actinobacteria;c_Actinobacteria;o_Bifidobacteriales;f_Bifidobacteriaceae;g_Bifidobacterium;s_adolescentis
ncur_OTU34595			**2.06**	1.1E-02	p_Actinobacteria;c_Actinobacteria;o_Bifidobacteriales;f_Bifidobacteriaceae;g_Bifidobacterium;s_adolescentis
OTU302545			**2.44**	7.1E-03	p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_;s_
OTU560981			**1.88**	1.6E-02	p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_Slackia;s_
OTU850905			**-1.95**	1.7E-02	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Barnesiellaceae];g_;s_
OTU195508			**-2.05**	1.8E-02	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_caccae
OTU577294			**-2.27**	1.2E-02	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae;g_Parabacteroides;s_distasonis
OTU349809			**-2.18**	4.1E-02	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae;g_Parabacteroides;s_distasonis
OTU568118			**-3.90**	5.9E-06	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
OTU530653			**-3.31**	4.6E-04	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella;s_copri
OTU355291			**2.22**	2.6E-02	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_
OTU4336943			**2.92**	6.6E-04	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_;s_
OTU766768			**-2.34**	4.6E-02	p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Enterococcaceae;g_Enterococcus;s_
OTU245625			**-1.42**	4.5E-02	p_Firmicutes;c_Clostridia;o_Clostridiales
nr_OTU145			**-2.02**	1.1E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
ncur_OTU34892			**-1.64**	3.5E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae
OTU174516			**-2.55**	7.3E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_Clostridium;s_
OTU562038			**-2.24**	9.2E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU332349			**-2.01**	1.3E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
nr_OTU48			**-1.98**	1.6E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU584463			**-1.82**	4.8E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU197760			**-1.82**	2.7E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU564400			**-1.73**	4.1E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU508875			**-1.73**	3.5E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
ncur_OTU13517			**-1.86**	1.6E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_
ncur_OTU65094			**-1.61**	4.1E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_
OTU550013			**-1.83**	3.9E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_
nr_OTU390			**-1.89**	2.7E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Peptostreptococcaceae;g_;s_
OTU189899			**-2.92**	3.6E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_;s_
OTU185659			**-2.59**	2.4E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Acidaminococcus;s_
OTU340113			**-1.78**	2.7E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Dialister;s_



OTU916143	-2.55	6.7E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Phascolarctobacterium;s_
OTU524884	-4.47	5.9E-06	p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_[Eubacterium];s_biforme
OTU197105	-2.12	9.4E-03	p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_[Eubacterium];s_biforme
OTU383885	1.79	3.6E-02	p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus;s_
ncur_OTU48808	1.74	2.1E-02	p_Firmicutes;c_Clostridia;o_Clostridiales
ncur_OTU1883	1.83	9.4E-03	p_Firmicutes;c_Clostridia;o_Clostridiales
OTU567011	1.37	2.7E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU311693	1.77	1.4E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
nr_OTU150	1.82	2.0E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU178511	1.88	1.4E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU197427	1.93	4.6E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
nr_OTU440	1.94	4.2E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
nr_OTU249	2.01	3.4E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU416341	2.05	2.2E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU368412	2.40	5.8E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU358439	2.61	6.7E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU815179	2.73	3.6E-04	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU191421	3.12	6.6E-04	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU552988	3.33	6.3E-05	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU360890	3.80	6.8E-05	p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_;s_
OTU552235	1.59	4.1E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_;s_
OTU289454	1.74	1.6E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_;s_
OTU410242	2.68	6.6E-04	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_;s_
OTU310178	2.93	2.7E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_;s_
OTU192240	2.04	3.3E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_;s_
OTU181466	2.25	8.2E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_;s_
nr_OTU61	1.55	1.1E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
OTU368950	1.69	3.3E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae
nr_OTU262	1.30	4.1E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_
nr_OTU26	2.12	8.2E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_;s_
OTU538947	1.41	4.5E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_
OTU436032	1.51	4.0E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_
OTU594227	1.75	2.7E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_
nr_OTU148	2.03	1.3E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_
OTU187112	2.10	6.1E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia;s_
OTU196791	2.14	1.2E-02	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Dorea;s_
nr_OTU434	2.33	8.0E-03	p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae

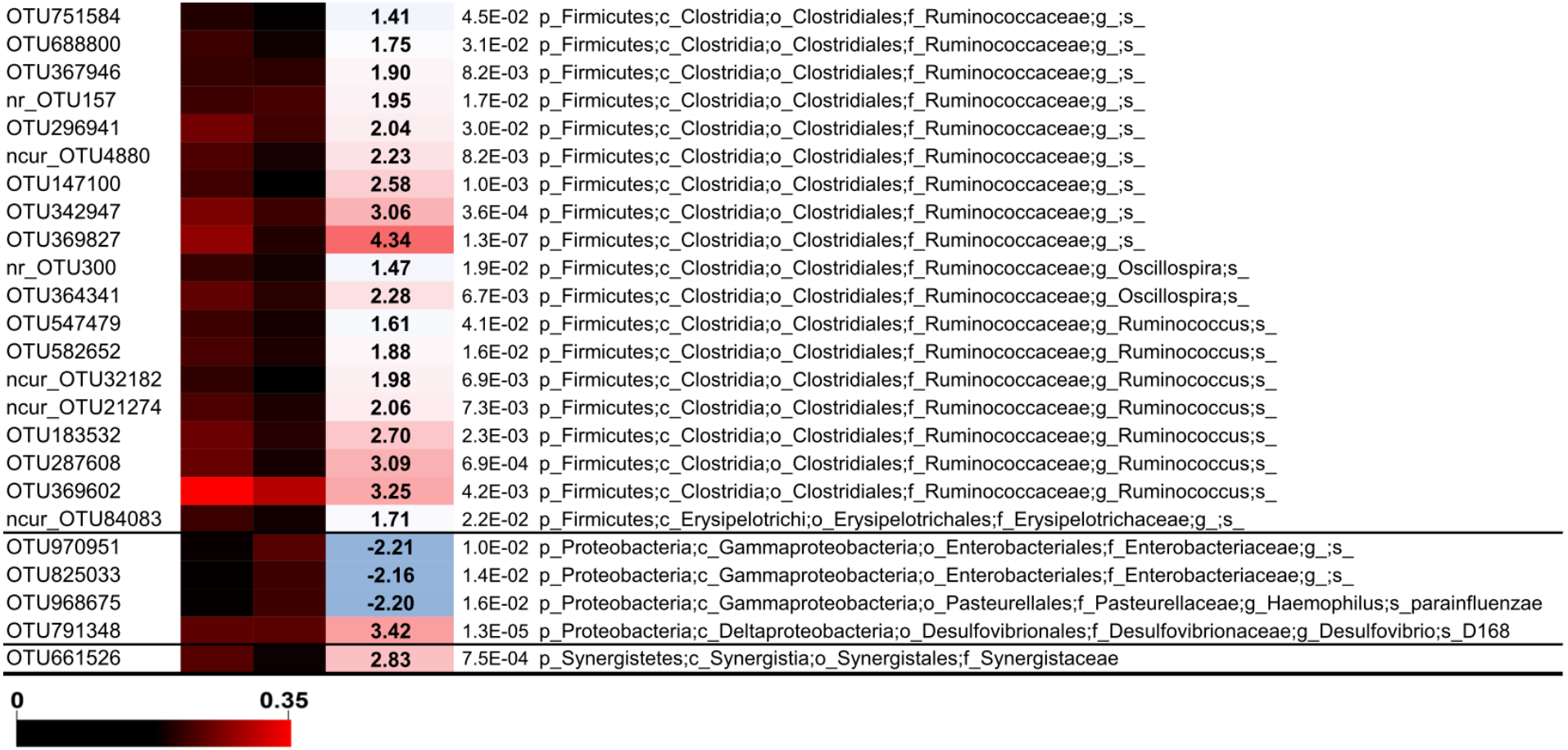
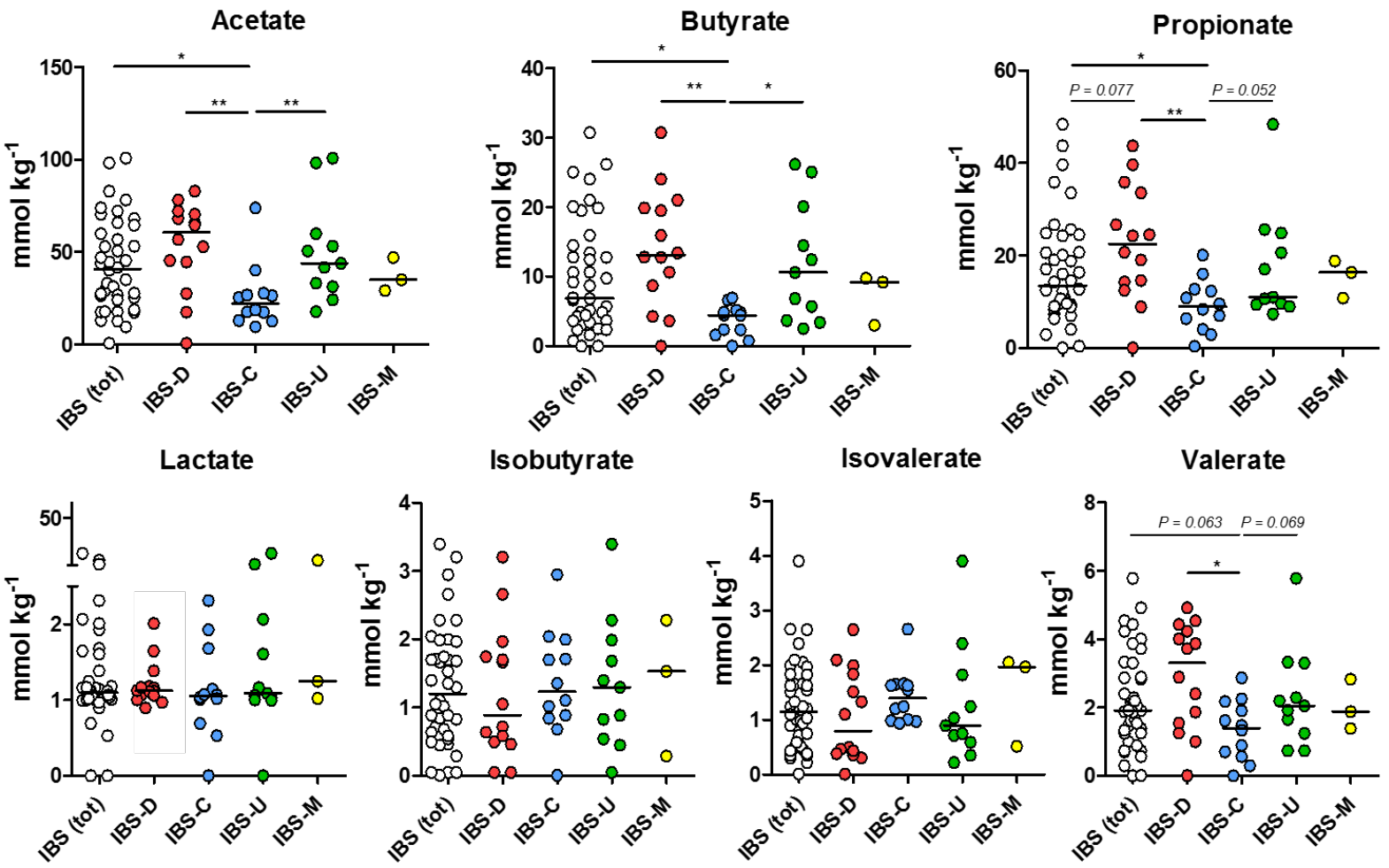


Figure S5. OTUs distinguishing IBS subtypes determined by using the DESeq2 negative binomial distribution method on 16S rRNA gene profiling data of a single faecal sample per patient. The colors in the heatmap represents the mean of normalized relative abundances of the reported OTUs. The taxonomic lineage of each taxon is shown; p, phylum; c, class; o, order; f, family; g, genus; s, species. Positive fold changes (shown on a red

background) indicate OTU overrepresentation in IBS-U (panels A and B) and IBS-C (panel C); negative fold changes (shown on a blue background) indicate an increase of OTU relative abundance in IBS-C (panel A) and IBS-D (panel B and C).

Figure S6. Levels of short chain fatty acids determined in a single faecal sample per IBS patient (n=40). Statistical significances are according to Mann-Whitney test; *, P<0.05; **, P<0.01.


Acetate
Butyrate
Propionate
Lactate
Isobutyrate
Isovalerate
Valerate
mmol kg-1
IBS (tot)
IBS-D
IBS-C
IBS-U
IBS-M
P = 0.077
P = 0.052
P = 0.063
P = 0.069

3.3.6 References

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4. Conclusions

The analysis of the data collected during the three studies presented in this PhD thesis revealed significant correlations between the intestinal microbial ecosystem and specific physiological parameters, characteristic for the physiological conditions under study. Therefore, these findings suggest that the differential abundance of specific OTUs could be used as a biomarker for a specific host condition.

The results obtained and analysed in this PhD work confirmed the interactions among the human organism (host), its microbiota and the surrounding environment, which form the so called "triangle of interaction". Before the introduction of the concept of the holobiont, scientific research approached human health issues omitting to consider the intestinal microorganisms and their products. However, the new and increasing knowledge on the gut microbiota and microbiome is clearly demonstrating the importance of the intestinal microorganisms as mediators of the effects that foods and drugs may have on human health. Each stimulus coming from the environment and each kind of dietary intervention or pharmaceutical treatment does also have an impact on microbiota composition, and on the other hand the microbiota can impact on the intervention effects. This "interfering" activity exerted by the microbiota cannot only amplify or minimize the end effect, but even invert or annul it. The discovery and characterization of these processes will provide the foundation for the creation of new treatments or new behaviour recommendations, and permit the prediction of the efficacy of specific treatments for distinct category of people.

4.1 Conclusion of the probiotic *B. bifidum* Bb crossover intervention trial

The intervention trial based on the administration of *B. bifidum* strain Bb has shown that the probiotic treatment modified the relative abundances of bacterial taxa that have often been associated with healthy conditions. The treatment modulated the faecal levels of butyrate, a microbial metabolite exerting multiple effects on gut health. Therefore, the daily consumption of *B. bifidum* Bb cells may positively affect human health; however, as for most dietary interventions, the

current state of knowledge does not allow us to better define the significance of any taxonomic or metabolite changes of the intestinal microbial ecosystem on the host health.

In a wider perspective, the PROBIOTA-Bb trial contributes to the field of research on probiotics in healthy populations, which is currently attracting significant attention in the context of probiotic health claim assessment by the EFSA. In particular, our study demonstrates that a single daily administration of one bacterial strain approximately at the minimal recommended dose (1 billion CFU [Ministero della salute 2013]) can modify the human intestinal microbial ecology of healthy (not diseased) adults in a significant fashion. These findings emphasize the need to reassess the notion that probiotics do not influence the complex and stable intestinal microbial ecosystem of a healthy individual and the importance of a proper intervention setting coupled with the use of adequate analytical and bioinformatic tools.

4.2 Conclusion of the Children's dyslipidemia single arm intervention trial

The results of this study support the hypothesis that young individuals with primary hyperlipidemia possess an dysbiotic intestinal microbial ecosystem, which could plausibly contribute to the abnormal lipid profile of these subjects. A limitation of this study is the small sample size, which may reduce the potential robustness of the obtained results. Moreover, further studies focusing on the mechanisms involved in such hypothesized association are warranted.

In the last years, several *in vitro* and *in vivo* studies demonstrated the ability of nuts to modulate the abundance of specific microbial taxa of the gut microbiota and change the intestinal concentration of SCFAs (Burns et al. 2016, Liu et al. 2016, Mandalari et al. 2010, Schlörmann et al. 2016, Ukhanova et al. 2014). Nonetheless, the research mostly focused on almonds and, to the best of our knowledge, only one study investigated hazelnuts, showing the increase of butyrate through *in vitro* fermentation by a human faecal sample (Schlörmann et al. 2016). In conclusion, our study is the first human trial investigating the potential role of hazelnuts as IME modulator and, in specific, suggests that a dietary intervention with hazelnut could be an effective and practical strategy to positively modulate the IME of hyperlipidemic subjects.

4.3 Conclusion of the IBS-subtypes observational study

This descriptive study demonstrates that the altered distribution of bacteria within the Gram-positive order *Clostridiales* distinguishes the intestinal microbial ecosystem of IBS subtypes, plausibly contributing to the observed altered faecal levels of the SCFAs acetate, butyrate and propionate. Our study proposes intestinal *Clostridiales* and colonic SCFAs as IBS subtypes biomarkers that can also potentially represent therapeutic targets. In addition, this study supports the notion that distinct therapeutic approaches should be developed for the different IBS subtypes.

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5. Productivity

5.1 List of publications

- Colombo S, Arioli S, Neri E, Della Scala G, **Gargari G**, Mora D. (2017). Viromes As Genetic Reservoir for the Microbial Communities in Aquatic Environments: A Focus on Antimicrobial-Resistance Genes. Front Microbiol. 8:1095.
- Cremon C, Guglielmetti S, **Gargari G**, Taverniti V, Castellazzi AM, Valsecchi C, Tagliacarne C, Fiore W, Bellini M, Bertani L, Gambaccini D, Cicala M, Germanà B, Vecchi M, Pagano I, Barbaro MR, Bellacosa L, Stanghellini V and Barbara G (2017). Effect of *Lactobacillus paracasei* CNCM I-1572 on symptoms, gut microbiota, short chain fatty acids, and immune activation in patients with irritable bowel syndrome: A pilot randomized clinical tria. United European Gastroenterology Journal 0(0) 1–10. DOI: 10.1177/2050640617736478
- Deon V, Del Bò C, Guaraldi F, **Gargari G**, Bosusco A, Simonetti P, Riso P, Guardamagna O. (2017). Serum lipid profile and fatty acid composition of erythrocyte phospholipids in children and adolescents with primary hyperlipidemia. Int J Food Sci Nutr. 68(3):339-348.
- **Gargari G**, Taverniti V, Balzaretti S, Ferrario C, Gardana C, Simonetti P, Guglielmetti S. (2016). Consumption of a *Bifidobacterium bifidum* Strain for 4 Weeks Modulates Dominant Intestinal Bacterial Taxa and Faecal Butyrate in Healthy Adults. Appl Environ Microbiol. 82(19):5850-9.
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- Perruzza L, **Gargari G**, Proietti M, Fosso B, D'Erchia AM, Faliti CE, Rezzonico-Jost T, Scribano D, Mauri L, Colombo D, Pellegrini G, Moregola A, Mooser C, Pesole G, Nicoletti M, Norata GD, Geuking MB, McCoy KD, Guglielmetti S, Grassi F. (2017). T Follicular Helper Cells Promote a Beneficial Gut Ecosystem for Host Metabolic Homeostasis by Sensing Microbiota-Derived Extracellular ATP. Cell Rep. 18(11):2566-2575.

5.2 Papers under submission

- Perruzza L, Strati F, **Gargari G**, D'Erchia A, Fosso B, Pesole G, Guglielmetti S, Grassi F. IgA-targeted gut microbiota in altered host metabolism by defective purinergic control of T-Follicular helper cells.
- **Gargari G**. Deon V, Taverniti V, Gardana C, Marco D, Riso P, Guardamagna, Guglielmetti S. Evidence of dysbiosis in the intestinal microbial ecosystem of children and adolescents with primary hyperlipidemia and potential role of regular hazelnut intake.

5.3 Seminar

- Evidence of dysbiosis in the intestinal microbial ecosystem of children and adolescents with primary hyperlipidemia and potential role of regular halzenut intake, Invited seminar at the Department of Nutritional Sciences University of Toronto, Toronto (Canada). 04/09/2017

5.4 Posters

- **Gargari G.** (2016). Impact of dietary intervention on human intestinal microbial ecology and its characterization in different healthy population conditions. Food system Workshop second year.
- **Gargari G**, Taverniti V, Balzaretti S, Ferrario C, Gardana C, Somonetti P, Mugetti S, Colombo S and Guglielmetti S. (2016). Modulation of the gut microbial ecosystem in healthy people upon probiotic intervention with *Bifidobacterium bifidum* MIMBb75. Gargari G, Taverniti V, Balzaretti S, Ferrario C, Gardana C, Somonetti P, Mugetti S, Colombo S, Guglielmetti S.
- **Gargari G.** (2015). Impact of food and probiotic microorganisms on human intestinal microbial ecology. Food system Workshop first year.

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