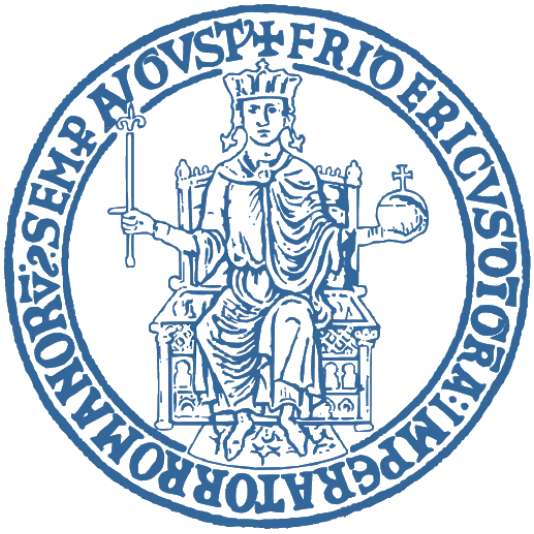
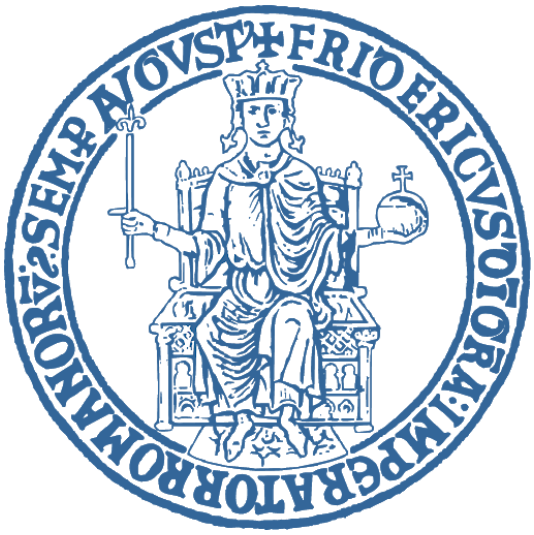


DIETARY INTERVENTIONS AND HOST-MICROBIOME INTERACTION

Manolo Laiola

PhD Supervisor
Prof. Danilo Ercolini
Dept. of Agricultural Sciences
University of Naples Federico II, Naples, Italy

PhD Coordinator
Prof. Guido D'Urso
Dept. of Agricultural Sciences
University of Naples Federico II, Naples, Italy



DIETARY INTERVENTIONS AND HOST-MICROBIOME INTERACTION

Manolo Laiola

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BAs; Bile Acids

BCAAs; Branched-Chain Amino Acids

CMA; Cow's Milk Allergy

CVD; Cardiovascular Diseases

EHCF; Extensively Hydrolyzed Casein Formula

FA; Food allergy

GIT; Gastrointestinal Tract

MedDiet; Mediterranean diet

RCT; Randomized Clinical Trials

SCFAs; Short-Chain Fatty Acids

T2D; Type II Diabetes

TMAO; trimethylamine N-oxide

CHAPTER 1

Brief thesis presentation

1.1 Overview

The term microbiome refers to the whole community of living microorganisms in a sample along with their potential activities that might influence the metabolic capabilities and functioning of such micro-environment. In particular, the human gut microbiome includes symbiotic bacteria, viruses, fungi and archaea located in the last part of our gastrointestinal tract. Each individual has a unique gut microbial composition as a peculiar fingerprint and it has been agreed that the development of several types of diseases in humans might be linked to gut microbiome perturbation in its microbial equilibrium, such condition known as dysbiosis.

To this regard, in the last decades a worrisome increase in food allergy prevalence linked to a defect in immune tolerance mechanisms has been observed. The onset of such multifactorial disease is in turn associated to a gut microbiota alteration and mediated by both genetic and environmental risk factors, especially in pediatric age. Moreover, it's known that the immune system may control microbial composition and diversity. It should be considered that most of the knowledge on the associations between gut microbiota and immunity system derives from animal model studies. For this reason, the understanding of the relationship between food allergies and intestinal dysbiosis could be translated into advances regarding i) knowledge in prevention of onset of such diseases and ii) clinical practice with diet-directed therapeutic interventions using pro- or prebiotics, aimed at modulating the compromised immune system indirectly through gut microbiota activities.

Indeed, the role of diet in influencing the gut microbiota composition and functions is widely recognized and the existence of the axis diet-microbiota-health is nowadays well established. The food-human interplay is of interest because the most recent trends are oriented towards a profitable use of diet to provide benefits to human health. In this respect, the Mediterranean diet received great attention as an appropriate strategy for the prevention and improvement of human health status. In fact, it has been demonstrated such dietary pattern being beneficial for

the treatment of obesity, type II diabetes, inflammatory diseases, colorectal cancer and cardiovascular diseases. The significant and tangible evidence of the link between the Mediterranean diet and gut microbiota opens encouraging paths towards the establishments of diet-based health care and disease prevention.

Despite such evidences, many challenges still exist since most of the studies assessed the impact of diet over human microbiome through correlation as well as association researches. Further efforts are necessary to understand the complicate host-microbiome interaction and to contribute to shed light on novel and different dietary strategies in order to beneficially impact human microbiome as well as human health.

1.2 Aims and outline

The overall aim of this thesis is to contribute to the evidences regarding the effects of dietary interventions in modulating the human microbiome which might in turn significantly impact human health, as to support the improvement of knowledge in such context. This general aim has been addressed through the accomplishment of three randomized clinical trials (RCTs). In particular, the evaluation of the dietary treatments efficacy over gut microbiota composition and butyrate production of i) a fermented milk product by probiotic *Lactobacillus paracasei* CBA L74 (FM-CBAL74) in healthy schoolchildren, ii) an extensively hydrolyzed casein formula enriched with the probiotic *Lactobacillus rhamnosu*s GG in children with diagnosticated non-IgE cow's milk allergy and iii) the overall effects exerted by a nutritional intervention based on the health-promoting Mediterranean dietary pattern over gut and salivary microbiota composition in overweight/obese subjects. These objectives were achieved making use of a research plan structured according to the following thesis outline.

The first RCT regarded the assessment of a dietary treatment using a cow's milk fermented product with the probiotic *Lactobacillus paracasei* CBA L74 (FM-CBAL74) in young children attending school (**Chapter 3).** Dietary intervention with FM-CBAL74 was conducted in order to figure out specific signatures in gut microbiota composition induced by such dietary supplementation, the potential stimulation of microbiota-related butyrate producers as well as the impact over levels of butyrate concentration. Moreover, the correlation of microbial changes with levels of innate and acquired immunity biomarkers was also tested to evaluate potential health outcomes, driven by dietary treatment and mediated by gut microbiota composition.

The effect of a dietary treatment with an extensively hydrolyzed casein formula (EHCF) alone or in combination with the probiotic *Lactobacillus rhamnosus* GG (LGG) was tested in children with a non-IgE-mediated cow's milk allergy (CMA) (**Chapter 4**). In this RCT, we evaluated gut microbiota changes and fecal butyrate levels in children affected by non-IgE-mediated CMA and treated with such formula. At this purpose, the level of gut dysbiosis was also compared to IgE-mediated CMA patients from a previously published study.

Chapter 5 reports the effects of an 8-week randomized controlled trial through an isocaloric Mediterranean diet (MedDiet) intervention among overweight/obese subjects with metabolic disease risk due to unhealthy lifestyle. Dietary adherence, metabolic parameters, gut

microbiome and systemic metabolome changes were monitored over the study period along with microbial-derived metabolites production. This RCT aimed at monitoring the beneficial effects of dietary changes in line with an increased level of adherence to the Mediterranean diet in subjects with cardiometabolic risk for low level of physical activity. Similarly, **Chapter 6** describes results regarding the effects of such Mediterranean-based dietary intervention over salivary microbiota composition in the same study population. Altogether, the adoption of nutritionally recommended dietary pattern such as the Mediterranean diet was considered for clinical outcomes amelioration beyond individual probiotics employments.

The last chapter (**Chapter 7)** is a general discussion of the overall findings as well as future perspectives based on knowledge generated.

All the studies described in the above chapters are multidisciplinary and carried out in collaboration with other researchers with multiple different expertises. My role in all the studies was the evaluation of the microbiota structure, bioinformatics analyses and statistical analysis/modelling of the microbiological as well as other types of metadata.

CHAPTER 2

Literature Review

2.1 Introduction

The human body harbors 10-100 trillion of symbiotic microbial cells and the term 'microbiota' refers to a collection of all taxa constituting microbial communities, most of them located in the human gut (Ursell *et al*., 2012). Similarly, the term microbiome refers to the whole community of living microorganisms living in a specific matrix along with its potential genome activities that might influence the metabolic capabilities and functioning of such micro-environment. It was estimated that the genes of our microbiome outnumber 100 times the number of the genes represented in our genomes (Zhao, 2010; De Filippis *et al*., 2018). In particular, each individual has a unique gut microbial composition as a peculiar fingerprint and it has been agreed that the development of several types of diseases in humans might be linked to gut microbiome perturbation, such condition known as dysbiosis. In fact, the gut microbiota is an organ which is in symbiosis with the human host, a mutual association established due to a long story of coevolution and the homeostasis of such strictly anaerobic ecosystem is necessary for the maintenance of human health (Ley *et al*., 2006). Moreover, the human oral cavity is a complex and open ecosystem, harboring a whole community of microorganisms and it is considered the second most complex symbiont microbiota in the human body after the gut (Dewhirst *et al*., 2010). Several publications appeared in recent years documenting that alterations in salivary microbial communities are associated to both oral and non-oral diseases. Accordingly, the oral microbiota dysfunction has been linked to atherosclerosis and cardiovascular disorder (Acharya *et al*., 2017). Hence, oral symbionts may indirectly elicit the immune dysregulation leading to the progressive inflammation associated with cardiovascular diseases (Slocum *et al*., 2016). Notably, the oral homeostasis is an important factor in order to avoid the growth of opportunistic pathogens, potentially causing both oral inflammation and systemic infection (Kodukula *et al*., 2017).

Overall, the human microbiome is recognized as our second genome and the understanding of its composition and modulation could be used as therapeutic target for personalized microbiome-directed interventions in order to prevent the onset of human diseases.

In particular, the gut microbiota is made of hundreds of species and while its composition could vary in the early stages of life, it has been observed an overall microbial stability during adulthood (Zoetendal *et al*., 2002). Due to endogenous and exogenous factors, the composition of gut microbiota may slightly change. Among others, antibiotics, diet and/or food supplements as well as pathological conditions are able to provisionally modulate the microbial structure. Therefore the resilience, or rather the gut ability to resist to perturbations depends on the responsive capabilities of the core taxa. Such condition plays a role in maintaining gut homeostasis, its normal composition and functioning, affecting in turn the microbiota-host interaction (Uhr *et al*., 2019).

In adults, it has been observed that the most dominant bacteria belong to Bacteroidetes and Firmicutes phyla, with proportions that vary across the population (Marchesi *et al*., 2016). Despite the stability of such phylogenetic groups, diversity in both specific species and strains characterize the inter-individual variability. This led to the observation that different individuals host different structural and functional gut profiles, as representing a personal signature (Tierney *et al*., 2019). Nevertheless, the Gram-positive Firmicutes phylum is the most abundant, which the most represented members belong to *Eubacterium, Clostridium* and *Ruminococcus* genera, followed by Bacteroidetes (*Bacteroides* and *Prevotella* among others) which are Gram-negative bacteria (Rajilić-Stojanović *et al*., 2007; Marchesi *et al*., 2016). The remaining percentage of dominance is represented by the Actinobacteria phylum (including the *Bifidobacterium* and *Collinsella* spp.) and Verrucomicrobia (*Akkermansia* spp. among others) (Rinninella *et al*., 2019). A stratification in enterotypes according to three bacterial taxa was initially proposed, independently of age, gender and geography, namely *Bacteroides*, *Prevotella* and *Ruminoccous* in the attempt to simplify gut microbiome diversity and to partially reduce complexity (Arumugam *et al*., 2011). This is illustrated in Figure 2.1. The proposed stratification resulted in functional and ecological differences between enterotypes varying in microbial community and richness diversity (Costea *et al*., 2018).

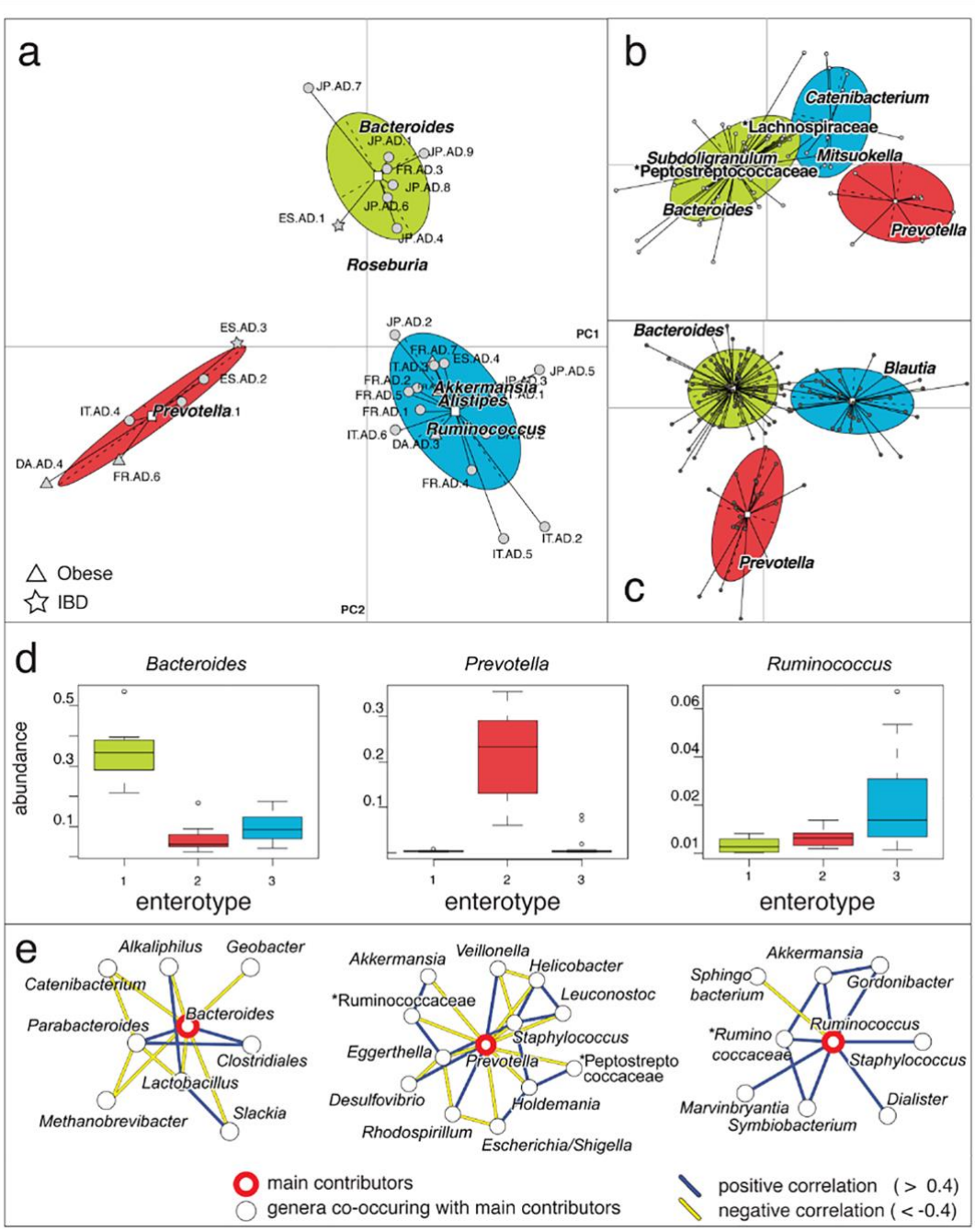


Figure 2.1 Phylogenetic differences between enterotypes. Principal Component Analysis and clustering of the genus compositions from three different metagenomes datasets reveal three robust clusters, called enterotypes. IBD, inflammatory bowel disease (**a**, **b**, **c**). Abundances of the main contributors (**d**) and Co-occurrence networks of each enterotype (**e**). Image from Arumugam *et al.*, 2011.

It was demonstrated that under the influence of diet, drugs and other factors in a given individual it was possible to observe shifts and movements of gut microbiota composition through the space of these three configurations, even though each enterotype had a specific ecological stability and a lower propensity to switch to another one (Knights *et al.*, 2014). However, this paradigm was overtaken since among individuals considerable variations in microbiota composition were described (Falony *et al.*, 2016; Gilbert *et al.*, 2018). Despite

being core taxa contributing to enterotype clustering, it has been shown a large variance in terms of relative abundances (Falony *et al.*, 2016). Morevover, species- and strain-level variations were neglected and several studies highlithted the important contribution of such taxa to functional differences between individuals in clinical contexts (Schloissnig *et al.*, 2013; Zhu *et al.*, 2015). In addition, recent meta-analyses made progress in understanding the strain-level diversity within the human gut microbiome and gene-level variation across strains was found to be related to gut microbiome divesity in human health and disease (Almeida *et al.*, 2019; Pasolli *et al.*, 2019; Zeevi *et al.*, 2019). Despite less prevalent species in well-studied populations are newly identified, the bacterial diversity remains uncultured and the complete bacterial and functional repertoire of the human gut microbiome remains still to be undefined (Almeida *et al.*, 2019). However, the identification of the complex gut ecosystem may help us understanding human health and disease status.

Individual microbiome contains rare microbial strains and genes in a metagenomic sample are individual-specific, thus may explaining variations in both microbiome composition among people and microbiome-associated human diseases (Tierney *et al.*, 2019; Sandoval-Motta *et al.*, 2017). Moreover, it has been observed that variations in the gut microbiome composition induce metabolic shifts resulting in phenotype alterations (Visconti *et al.*, 2019) and certain host genetic variants define the composition of the gut microbiome predisposing towards microbiome dysbiosis (Gilbert *et al.*, 2016; Hall *et al.*, 2017). Indeed, the gut microbiome composition is affected by both genetic and physiological predispositions or environmental factors and together are involved in maintaining gut homeostasis. In turn, a healthy gut microbiota composition is substantially pivotal to maintain both intestinal and distal proper host metabolic functioning. Such interactions between intestinal microbiota and host metabolism are depicted in the following Figure 2.2.

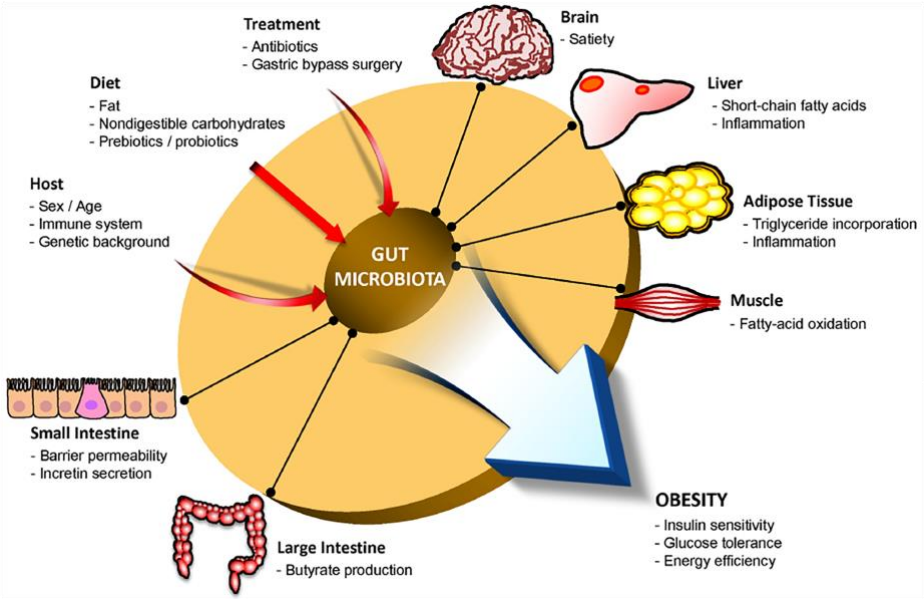


Figure 2.2 Interactions between gut microbiota and host metabolism. A number of external factors such as host background, diet, and medical treatments could influence gut microbiota composition. An imbalance of intestinal microbiota structure can lead to severe metabolic disorders by altering host insulin sensitivity or energy homeostasis, among others. Image from Yang and Kweon, 2016.

Among host factors, genetics, geographical location and circadian rhythm seemed to be modulating the gut microbiome composition (Mueller *et al.*, 2006; Yatsunenko *et al.*, 2012; Thaiss *et al.*, 2014). Beyond genetic components, Wu and colleagues showed that long-term dietary habits may have the strongest impact on gut microbiome distribution and composition. Thus, different diets are associated to bacterial species diversification in the gut. Accordingly, they reported the *Bacteroidetes* enterotype to be dominant in subjects adopting a Western diet richer in sugars, proteins and fats of animal origin while conversely, a diet rich in fruits and vegetables promoted a *Prevotella* enterotype (Wu *et al.*, 2011).

In conclusion, a comprehensive understanding of the human microbiome seems challenging. The key message is that due to the resilience property, each microbiome is heterogeneous and adapted to each host while several and still poorly understood regulatory mechanisms are necessary to maintain gut homeostasis and human wellness (Lloyd-Price *et al.*, 2017; Zhao *et al.*, 2019). To this regard, understanding the association of gut ecological patterns with the pathophysiological phenotypes could result in the identification of health/disease signatures and host features. These could represent in turn targets for microbiota-directed therapeutic intervention aimed at preserving and restoring the microbiota-host symbiosis. Many factors could modulate gut ecological composition and such changes could pave the way of possibly modulate the gut microbiota for therapeutic purposes thorough dietary interventions.

2.2 The concept of dysbiosis

Microbial consortia harbored in our bowels might be used as biomarker for both health condition and diseases risk factors (Blottière *et al*., 2013). Each microbiota is a high-specific individual fingerprinting and poised interconnections between gut microbiota, host cells and organs are required for a healthy host ecosystem. In particular, the development and the maintenance of the microbiota depend on host and conversely, the host depends on gut microbial composition for its physiological functions (Thursby and Juge, 2017). Being structured as an ecosystem, a healthy gut microbiota configuration in terms of richness and biodiversity promotes stability, resistance and resilience to the host. (Huttenhower *et al*., 2012; Marteau and Doré, 2017). However, such established symbiosis between host and its microbiota may be lost through environmental insults and endogenous factors affecting gut ecology. Indeed, the term dysbiosis is an anomaly in the commensal microbial structure likely associated with a disease condition. Such intestinal disorder is related to the lack of usual aspects of a balanced microbiota, thought as loss of diversification in term of species and important functions. Hence, a microbiota enriched in pathobionts or depleted in protective microorganisms as well as biodiversity and gene richness restrictions provoke a homeostasis alteration, making the host susceptible to and increasing the risk of disease development (Tap *et al*., 2009; Le Chatelier *et al*., 2013; Blottière *et al*., 2013). This breakdown of the microbiota-host symbiosis is now commonly recognized as a sign of dysbiosis. (Marteau and Doré, 2017). To this regard, a perturbation in microbial functions may also be assumed in such definition. In particular, biomarkers of metabolic signatures in disease condition could be identified. Alteration of usual capabilities like digestion, modulation of absorption, intestinal permeability variation, short chain fatty acids (SCFAs) depletion or bile acids (BAs) production are some examples, due to their consequences on inflammation status. (Weiss and Hennet, 2017). A fine-tuned dysbiosis description might be used in practical and medical perspectives, using the detection of microbial features, immune markers and metabolic signatures as diagnostic tools for disorders strongly associated to dysbiosis.

In conclusion, new diagnostic strategies may be proposed aimed at modulating and correcting the host-symbionts alterations, improving in turn host disease condition. Thus, beyond widely used antibiotic treatments, new therapeutic practice could conceive the use of microbiota-targeted new generation prebiotics, probiotics and whole dietary pattern.

2.2.1 Obesity

Obesity is a clinical condition recognized as a social health disease and it is acknowledged as an independent risk factor for metabolic-driven chronic diseases (Cox *et al.*, 2014; De Lorenzo *et al.*, 2019). Obesity and the increasing prevalence of obesity-associated conditions, including cardiovascular disease and type 2 diabetes, are widespread health concerns worldwide. They have an economic impact on the health system and social implications. However, obesity is a preventable and reversible condition and innovative strategies like optimal dietary interventions and promising metabolic therapies are urgently needed, in order to easily control both patients' weight and obesity-related disease throughout life, beyond primary lifestyle prevention (Lean *et al.*, 2019).

The etiology of obesity is complicate, and it includes both genetical and environmental factors. In high-income countries, sedentary lifestyle, high-fat food consumption and low-grade systemic inflammation are the key components of obesity (Cox *et al.*, 2014; Choi *et al.*, 2013). Moreover, gut microbial alterations have been associated to obesity pathophysiology, behavioral and eating disorders, energy disregulation and fat storage (Ley *at al.*, 2005; Rosenbaum *et al.*, 2015; Patterson *et al.*, 2016; Torres-Fuentes *et al.*, 2017; Liu *et al.*, 2017). Indeed, the involvement of intestinal microbiota in energy balance has been assessed in studies with animal models. Despite a higher calories' consumption, germ-free mice were shown to be protected against obesity. They were significantly leaner compared to control mice, conventionalized with normal microbiota (Bäckhed *et al.*, 2004). Additionally, higher levels of Firmicutes taxa were found in obese compared to lean mice. Conversely, Bacteroidetes phylum was found to be more abundant in normal weight mice and notably, some members are responsible of plant starch and fiber breakdown for energy harvest (Turnbaugh *et al.*, 2006; Ley *et al.*, 2005). These findings were the first tangible proof of obesity-associated microbiota signatures. Consistently, changes in the human microbiome were also linked to obesity, further confirming the straight connection between gut microbiota composition and such disease condition (Turnbaugh and Gordon, 2009; Ridaura *et al.*, 2013). Further investigations are needed to exploit the possible existence of obesity-associated microbial biomarkers. Beyond phylum-changes, microbial taxa at lower taxonomic levels have also been associated with obesity, although with a strain-dependent effect (Gérard 2016). For instance, *Lactobacillus casei* and *L. plantarum* were found to be associated to weight loss in animals and humans along with some strains of bifidobacterial species, exerting anti-obesogenic effects. Interestingly, the relative abundance of *Faecalibacterium prausnitzii* was found to be decreased in obese patients and *A. muciniphila* was found to be inversely related

to fat accumulation and adipocyte diameter in obese humans (Furet *et al*., 2010; Dao *et al*., 2015). Overall, these findings suggest that some species may directly contribute to obesity development or protection. Conversely, the wide human gut heterogeneity and confounding factors affecting intestinal ecology make the obese-microbiota profile difficult to define. However, functions and the production of secondary metabolites produced by symbionts may contribute to obesity development. These molecules regulate host appetite and food reward, which in turn have roles in obesity. Accordingly, microbial-derived bioactive metabolites produced by gut microorganisms in a diet-dependent manner, exert peripheral and central effects on the hypothalamus. The gut bacterial fermentation activity of complex dietary plant polysaccharides results in the production of SCFAs, principally acetate, propionate, and butyrate. These molecules represent an important host energy source but also fine tune signals influencing energy intake and human metabolism (Conterno *et al*., 2011). Such SCFAs, γ-aminobutyric acid (GABA), serotonin (5-HT), and other neurotransmitters (NTs) modify host metabolism via vagal stimulation or through immune–neuroendocrine mechanisms. Hence, after a meal the presence of nutrients in the gastrointestinal tract (GIT) causes a complex network of gut-brain signaling in order to regulate appetite and energy balance. Moreover, an increase of BAs production and an altered BAs metabolism was associated to low-grade inflammation (Ridlon *et al*., 2014). Nevertheless, one of a major function of the gut symbionts is to contribute to energy harvest from non-digestible dietary starches. The presence of an obesity-related microbiota increases the calorie uptake providing more energy to the host. Accordingly, the contribution of the intestinal microbiota to regulate fat storage and energy harvest and its associations with body composition was demonstrated (Ding *et al*., 2010; Cani *et al*., 2007; Turnbaugh *et al*., 2006; Jumpertz *et al*., 2011).

Despite the observations reported in the scientific literature, the possible association between dysbiosis and obesity is still debated. Interestingly, evidences from gut microbiota transplant experiments in mice demonstrated a causal role of the gut microbiota in obesity. These findings showed the transmissibility of metabolic abnormalities and obese phenotype *via* gut microbiota transfer along with intestinal bacterial species responsible of lean status, addressed to have the capacity to protect from obesity (Turnbaugh *et al*., 2008; Turnbaugh *et al*., 2009; Ridaura *et al*., 2013). Hence, obesity is characterized by a gut microbiota different from that of a lean individual in terms of composition, diversity, metabolic activity and bacterial gene richness (Le Chatelier *et al*., 2013). However, further translational researches conducted in humans should be promoted to validate assumptions from animal experiments.

A healthy gut microbiota is substantially pivotal to maintain proper host metabolic functioning, intestinal homeostasis and central appetite mechanisms. Gut microbiota is a potentially effective therapeutic target for anti-obesity therapies, through dietary interventions and/or dietary patterns recommendations.

2.2.2 Food allergy

The gut microbiota is an extremely complex ecosystem and constantly interacts with host cells and physiological functions. Thus, it is not surprising that the pivotal role of the microbiota dysbiosis as a pathophysiological risk factor for the development of several diseases. Indeed, the understanding of the molecular mechanisms underlying the commensal bacteria-humans symbiosis would shape *ad hoc* strategies for immunologic treatment of diseases. Hence, microbiome-targeted therapeutic interventions including dietary modulation and the use of pre- and probiotics could potentially allow the development of new strategies to improve clinical outcomes.

The host immune system should be able to distinguish between beneficial and pathogenic microorganisms, since both share similar molecular patterns. The ability of recognize is an adaptive immune system skill, which is in turn driven by an efficient co-evolved gut microbiota. Data from several studies highlight how the commensal bacteria and microbial-derived metabolites program the T cells differentiation, promoting the development and shaping the maturation of the adaptive immunity, influencing the wellbeing and pathogens clearance (Jacobson *et al*., 2018; Ludwig *et al*., 2018; Ansaldo *et al*., 2019; Skelly *et al*., 2019). The ingested nutrients are metabolized by the gut microbiota and the derived metabolites exert the modulation of host immunity. To this regard, fiber-degrading species such as *F. prausnitzii* and *Roseburia intestinalis* taxa through the metabolization of indigestible carbohydrates produce fermentation products (SCFAs). Acetate, propionate and butyrate are involved in a sophisticated host–microbiome network participating in regulatory T cell (Tregs) development, B cell proliferation, the release of anti-inflammatory cytokines and antibody production (Zhang *et al*., 2019). (Figure 2.3)

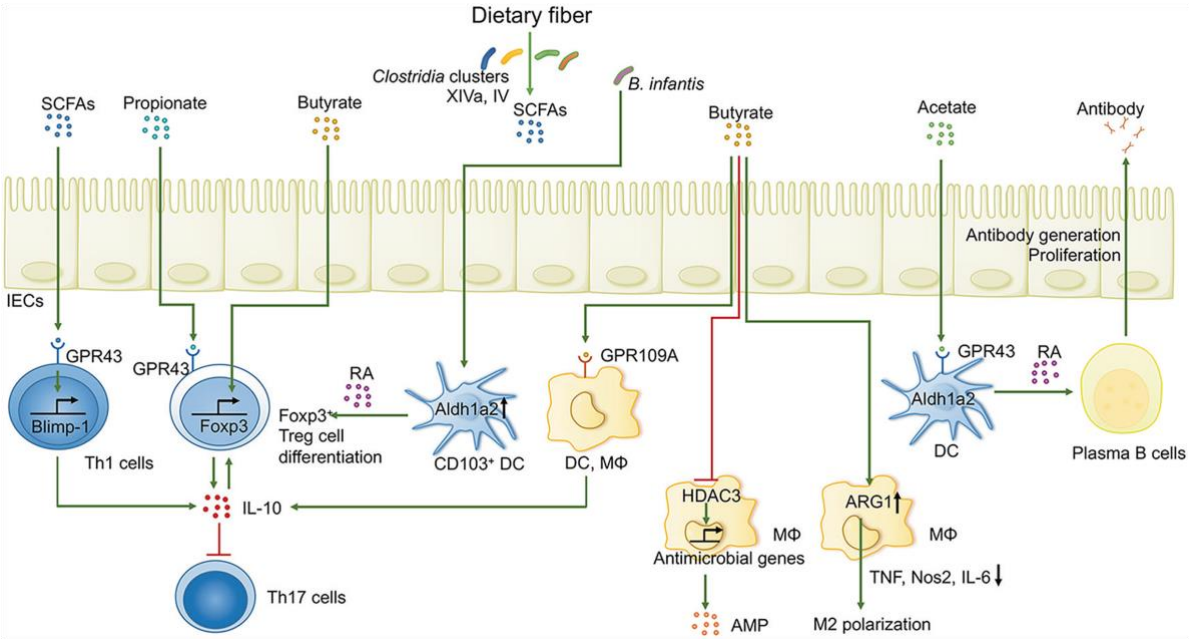


Figure 2.3 Mechanisms of signaling from microbial-derived SCFAs to multiple immune cells in the gut. Image from Zhang *et al*., 2019.

In particular, it has been discovered that our commensals provide signals inducing the CD4+ T cell differentiation into pro- and anti-inflammatory cells to contrast microbial infections, along with the inhibition of the proinflammatory Th17 cell expansion to enhance immunity (Atarashi *et al*., 2008). Accordingly, SCFAs produced by commensal bacteria such as *Bifidobacteria infantis* and *F. prausnitzii* induce the anti-inflammatory Foxp3+ Tregs development and interleukin IL-10 production in the gut (O'Mahony *et al*., 2008; Sokol *et al*., 2008; Mariño *et al*., 2017). *Bacteroides fragilis* has been shown to prevent colitis through the bacterial molecule Polysaccharide A (PSA) and the *Akkermansia muciniphila* species induces intestinal adaptive immune responses (Mazmanian *et al*., 2008; Ansaldo *et al*., 2019). These findings suggest that host cell differentiation in the colon is promoted by signals from the host genome and gut microbiota, which is in turn pivotal for the maintenance of host homeostasis. Moreover, an efficient immune system along with the gut microbiota will be able to contrast pathogens proliferation. SCFAs depletion and antibiotic-induced gut dysbiosis induce the expansion of proinflammatory Th1 cells, undermining the pH homeostasis and promoting the loss of the intestinal barrier function. Hence, the host is exposed to damages caused by *Clostridium difficile* toxins as well as susceptible to pathogens infection such as the invasion of *Clostridium rodentium* (Byndloss *et al*., 2017; Scott *et al*., 2018; Gillis *et al*., 2018; Fachi *et al*., 2019; Desai *et al*., 2016).

Microbiota composition and diversity are in turn controlled by the immune system and a loss of the interplay between these two can determine onset of disease. In addition, the immune

system as well as the commensal microorganisms are sensitive to the nutritional status of the host. Evidences now exist concerning the bidirectional interactions between diet, immune system and microbiota (Figure 2.4.

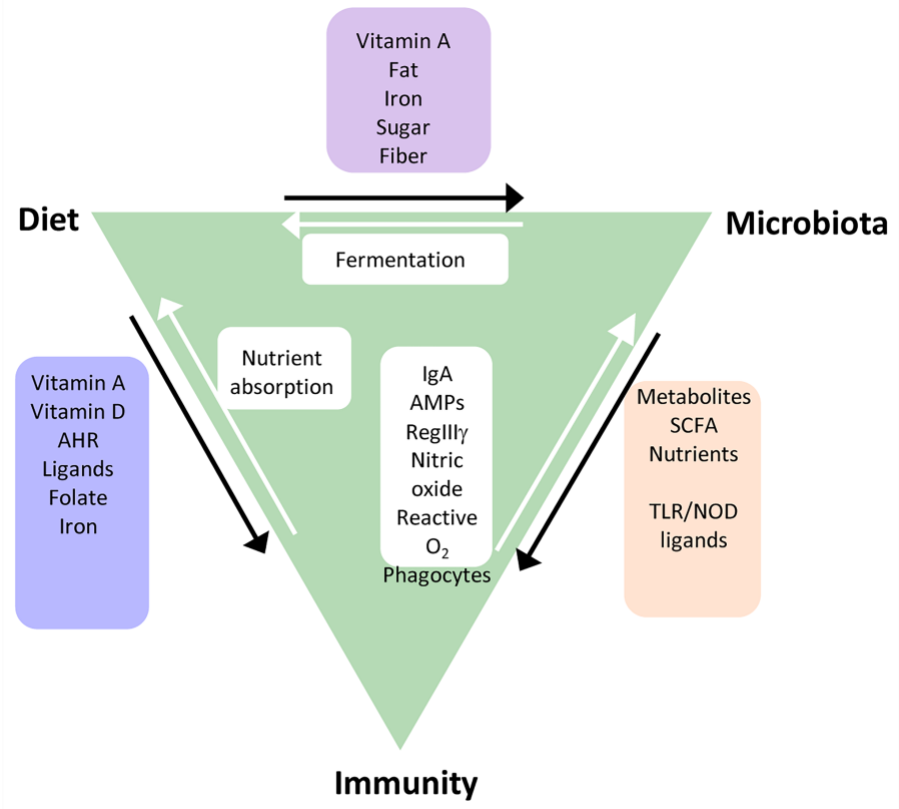


Figure 2.4 Interdependence of Diet, Immune, and Microbiota Interactions. Image from Belkaid and Hand, 2014.

A dysbiosis during childhood increases the risk of onset of food allergies (Prince *et al.*, 2015; Azad *et al.*, 2015; Savage *et al.*, 2018). Food allergy (FA) is a common allergic disorder in the pediatric age, a global health problem particularly in industrialized world (Boyce *et al.*, 2010). This condition derives from a breakdown of immune tolerance mechanisms involved in the activation of specific Tregs cells to dietary antigens exposure (Noval Rivas *et al.*, 2013). Multiple risk factors could influence the FA onset, including sex, C-section, ethnicity, genetic background and epigenetic modulation of gene expression (Trompette *et al.*, 2014; Greenwood *et al.*, 2014; Berni Canani *et al.*, 2019). However, recent evidences suggest the pivotal influence of gut microbiome dysbiosis as responsible for the occurrence of FA, being involved in the alterations of the intestinal barrier and immune system function (Prince *et al.*, 2015; Berni Canani *et al.*, 2019). Due to its early introduction, cow's milk allergy (CMA) is one of the earliest and most common FA (Agostoni *et al.*, 2014). In fact, children affected by CMA in the first year of life have an increased risk to develop other atopic manifestations in

their later life (Branum and Lukacs, 2009; Boyce *et al*., 2011), as well as other chronic immune-mediated disorders such as inflammatory bowel diseases (Virta *et al*., 2013).

Summarizing the studies reviewed, it appears that promoting the development of a beneficial microbiota with pre- and/or probiotic supplementation in children may be an important step to prevent FA. This indicates a need to translate the information emerging from descriptive researches and controlled clinical interventions aimed at establishing the gut microbiome as causative factor for FA onset. However, data on gut microbiota features in FA seem still preliminary because the general small number of observations. The proper interpretation of such findings might be the starting point for appropriate approaches of personalized medicine strategies (Bashiardes *et al*., 2018; De Filippis *et al*., 2018). To this regard, the screening of gut microbial diversity in FA patients of larger cohorts may be useful in identifying microbiome signatures responsible of specific FA conditions. Furthermore, microbiome biomarkers and microbial-derived metabolites inferred with multi-omics approaches in such complex network might be meaningful in targeted intervention against FA conditions using post-biotic strategies (Figure 2.5).

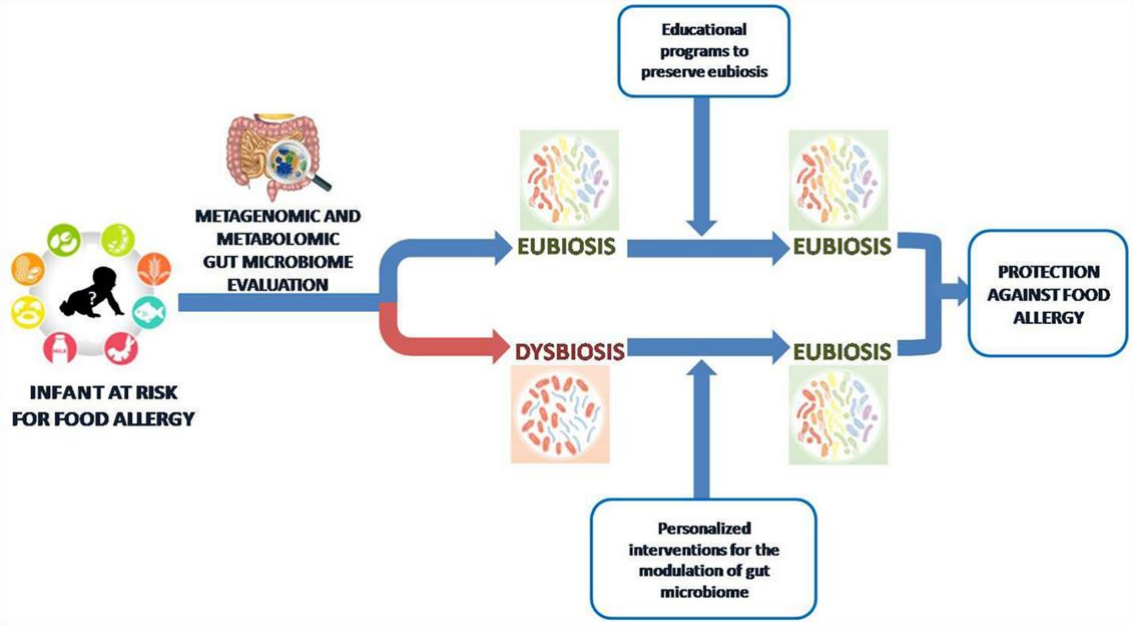


Figure 2.5 The scheme for a gut microbiome-based precision medicine against food allergy. Image from Berni Canani *et al*., 2019.

In conclusion, targeting the diet-gut microbiome–immune system axis might be a promising target to restore an "eubiosis" state and for FA care. Thus, understanding how through dietary intervention and/or supplementation we could influence gut bacteria communities in school children will led to encouraging findings for innovative clinical practice, nutritional counselling and educational programs.

2.3 Modulation of the microbiota

Data from scientific literature demonstrated the straight connection between diet, gut microbiota and human health (De Vos *et al*., 2012). In particular, the microbiota processes nutrients and specific food components reaching the colon. For this reason, diet has a key role impacting microbial composition and functions mostly depending on proteins and non-digestible carbohydrates. This mutual interaction is established since early childhood during the first years of life. The human milk is rich in oligosaccharides and these are the main factor affecting breastfeed infants' gut microbiota, mainly colonized by *Bifidobacterium* taxa, and promoting development of an optimal host immune system (Derrien *et al*., 2019). Currently, newborn feed formulas are nowadays enriched with prebiotics as well as probiotics in order to positively impact the well-being and health especially for bottle-fed infants (Hemarajata and Versalovic, 2013). Following weaning, diet assortment is related to a diversification of bacterial species (McDonald *et al*., 2018). Most notably, different diets and eating habits related to socio-economic status, lifestyle, ethnicity and different cultural practices formed over a long period, directly and profoundly shape gut microbiota composition between populations acting as a long-term nutritional regulator (De Filippo *et al*., 2010; Yatsunenko *et al*., 2012; Tyakht *et al*., 2013). Despite such observations and inter-individual genetic differences, short-term nutritional interventions were demonstrated to be effective in rapidly changing the biodiversity of gut microbial composition, even though in a transient manner (David *et al*., 2014; Leeming *et al*., 2019). In fact, rapid and meaningful gut microbial changes were achieved through either short-term or long-term human dietary interventions. Besides, several studies displayed that the gut microbiota reverts back to baseline composition after the treatments, with long-term diet emerging as key driver (Wilson *et al*., 2017). Core microbial taxa appear to be resilient to most outside influences and possibly responsible of inter-individual variation to dietary interventions (Wu *et al*., 2011; Schmidt *et al*., 2018). However, the foods absence/reduction or the supplementation of components in habitual diet and dietary choices may beneficially and temporarily influence symbionts leading in turn to a positive effect on human health.

Hence, the interplay between diet, gut microbiota and human health is becoming clear and has been extensively reported. For this reason, modulation of the gut microbiota through diet will be a potential therapeutic target for both the prevention and treatment of specific diseases.

Plant-based foods contain dietary fiber. Specific dietary fiber types including inulin, fructo-oligosaccharides and galacto-oligosaccharides are considered to be prebiotic, "a substrate that is selectively used by host microorganisms conferring a health benefit" (Gibson *et al*., 2017).

Several studies reported the effect of dietary components such as non-digestible carbohydrates to qualitatively impact gut microbiota composition, consequently promoting the growth of beneficial bacteria such as an enrichment in fiber-degrading taxa (Everard *et al*., 2014; Bendall *et al*., 2018). To this regard, gut bacteria are rapidly capable of adapting their metabolism to changes in nutrients intake. Hence, a transient increase of fiber intake is related to an increase in glycosidases and amylase gene expression (Tap *et al*., 2015). Therefore, the degradation and fermentation of indigestible sugars in the colon leads to a higher abundance of taxa belonging to *Bifidobacterium*, *Ruminoccous*, *Eubacterium* and *Lactobacillus* spp. among others and to the production of vitamins and SCFAs, whose anti-inflammatories properties are widely known (Torres-Fuentes *et al*., 2015; Pryde *et al*., 2002; LeBlanc *et al*., 2017). At the same time, such microbiota modulation confers health benefits to the host by causing anti-obesity effects and decreasing diabetes severity in animal models and human studies as well as reducing circulating pro-inflammatory cytokines (Kellow *et al*., 2014; Chassaing *et al*., 2015; Torres-Fuentes *et al*., 2015). In addition, recent researches focused on the effects of whole grains cereal products, rich in dietary fibers and resistant starch and known to be related to protection against chronic diseases. The results suggested a prebiotic-like outcome over inflammation by such fiber consumption despite no significant changes over the gut microbiota composition were detected (Costabile *et al*., 2008; Vanegas *et al*., 2017; Roager *et al*., 2019). However, Tap *et al*. demonstrated that a 5-days supplementation of dietary fibers was associated with a higher richness and diversity of gut microbiota composition. Higher proportions of *Prevotella* and *Coprococcus* levels were achieved as well as a modulation in the expression of many metabolic pathways and fermentable activities (Tap *et al*., 2015). Altogether, these findings further corroborated such beneficial effects over human health exerted by short-term dietary interventions, indirectly mediated by microbiome activities.

Analogously, the term probiotics as defined by the Food and Agricultural Organization and the World Health Organization refers to "live microorganisms which when administered in adequate amounts, confer a beneficial health effect on the host" (Guarner *et al*., 2011). Probiotics are currently available as drugs, foods or foods supplements. Several publications appeared demonstrating the beneficial effects exerted by probiotics ameliorating obesity or metabolic disorders both in animal models and in human studies (Yin *et al*., 2010; Derwa *et al*., 2017; Kunk, 2019). Probiotics alter gut microbiota composition inducing the production of beneficial microbial-derived metabolites, although it was shown that outcomes may vary depending on individuals, probably due to the competition with host symbionts for substrates

(Licciardi *et al*., 2010). Commonly, the most used probiotics contain strains belonging to *Bifidobacterium* and *Lactobacillus* genera. In particular, several strains of *Lactobacillus* such as *L. rhamnosus, L. plantarum*, *L. paracasei, L. reuteri, L. acidophilus, L. fermentum* were widely used in animal models showing positive effects on health, reducing body fat mass and improving lipid profiles and glucose homeostasis in obese mice (Gérard, 2016). Similarly, strains of *L. gasseri* in controlled studies in overweight human subjects were associated with weight loss. More recently, the administration of *Akkermansia muciniphila* in overweight/obese insulin-resistant volunteers improved blood metabolic parameters for liver dysfunction and inflammation (Depommier *et al*., 2019). This opens up a new concept regarding *Akkermansia muciniphila* as a promising candidate among the next-generation beneficial microbes (Cani and de Vos, 2017). Moreover, probiotics play an important role in the maintenance of immunologic homeostasis in the GIT through a direct interaction with host immune cells and they have been widely studied in a variety of gastrointestinal-related diseases (Wilkins *et al*., 2017). Indeed, several probiotics-derived genes and specific compounds mediate immunoregulatory effects, by regulating the functioning of systemic and mucosal immune cells and intestinal epithelial cells, by stimulating the production of interleukins from peripheral blood cells as well as by modulating innate immunity response (Yan and Polk, 2011). In addition, several studies were performed in order to evaluate the effects of probiotics in the prevention and treatment of allergy (Gourbeyre *et al*., 2011; Schiavi *et al*., 2011). These results provided evidences of the complexity and functionality of probiotics targeting the GI tract and its microbiota composition.

In conclusion, probiotics efficacy has to be assessed through the highest level of evidence such as double-blind RCTs and more should be carried on in the field of food allergies. Thus, due to encouraging results from therapeutic and *in vivo* experiments it seems clear that probiotics supplementation and administration might be an appropriate approach to modulate and maintain a healthy microbiota and further studies are requested in this field. The Chapter 3 addressed this topic.

2.4 Mediterranean Diet and human health

Long-term dietary patterns and habitual food intake play a major role in shaping an individual and resilient microbiota profile more than acute dietary treatments. The adoption of whole and nutritionally recommended dietary pattern should also be considered for clinical outcomes amelioration, beyond individual components. Thus, the gut homeostasis may be achieved with beneficial implications for human health mediated by the synergistic effects within foods of an appropriate dietary-model.

The Mediterranean diet (MedDiet) is a dietary model adopted by countries neighbouring the Mediterranean Sea. It consists of a high intake of plant-based compared to animal-based food products, such as legumes and vegetables, fruits and nuts, whole grains cereal products and vegetable oils (mainly olive oil), a moderate consumption of fish, eggs and dairy products and a limited intake of processed cereals and red meat (Mitrou *et al*., 2007). This historical dietary pattern established between food availability and humans surrounding the Mediterranean basin has been recognized as intangible cultural heritage by UNESCO (Lăcătușu *et al*., 2019; Turmo, 2012). Several studies proved the beneficial effects of such dietary pattern in lowering the risk of overall incidence and mortality of cardiovascular diseases (CVD), CVD-related morbidities and neurodegenerative disease (Dinu *et al*., 2018). Moreover, scientific literature has identified robust evidences for a higher adherence to MedDiet to be efficient in the treatment of obesity risk factors, type II diabetes (T2D), colorectal cancer as well as inflammatory parameters (Del Chierico *et al*., 2014*a*; Martínez-González *et al*., 2016; Eleftheriou *et al*., 2018).

Despite a growing body of knowledge assessing the human health improvement due to the MedDiet exposure, further epidemiological studies and clinical trials are needed. Optimistically, cost-effective strategies such as a proper diet adoption may be able to advancing our understanding of such MedDiet dietary pattern efficacy upon health outcomes and to implement preventative medicine.

Conversely, the molecular and metabolic mechanisms underlying the complex diet–microbiome–host interactions have not been completely elucidated. Nevertheless, the evidences of the correlation between the dysbiosis condition in a wide variety of pathologies and the dynamic response of the gut microbial ecology to dietary interventions sustained the hypothesis of a direct modulation of the gut microbiome through diet. Hence, the study of host-enteric microbial ecology has been defined as a reliably therapeutic target affecting human health and challenging in nutrition studies. In addition, delineating the effectiveness of a nutritionally recommended dietary pattern such the MedDiet upon both human metabolome

and microbiome will facilitate the discovery of microbial signatures and diet-specific biomarkers in health/disease status. These findings will lead to the development of personalized dietary guidelines in the era of 'precision medicine' for the prevention but mostly the treatment of specific type of diseases.

Several recent researches in nutritional metabolomics focused on discovery of metabolic biomarkers for the assessment of the MedDiet adherence (Vázquez-Fresno *et al*., 2015; González-Guardia *et al*., 2015; Almanza-Aguilera *et al*., 2017) along with metabolites correlated to health outcomes following a MedDiet-based dietary intervention. To this regard, Guasch-Ferré and coworkers found positive associations of protein degradation products such as aromatic amino acids (tyrosine and phenylalanine) and branched-chain amino acids (BCAAs) such as leucine, isoleucine, and valine with type II diabetes incidence. Similarly, it has been estimated that acylcarnitines and dicarboxylacylcarnitines generally linked to meat consumption were associated with increased CVD risk along with TMAO, different classes of lipids, ceramides, tryptophan and phosphatidylethanolamine, further highlighting the cardioprotective effects of the MedDiet (Ruiz-Canela *et al*., 2017; Wang *et al*., 2017; Jin *et al*., 2019).

Up to now, recent advances on culture-independent methods and high-throughput sequencing aimed at analyzing the microbial composition carried out several evidences, suggesting the pivotal role of host gut microbiome on metabolic functions, immunity, and health outcomes (Del Chierico *et al*., 2014*b*; Singh *et al*., 2017). In addition, as stated before long-term diet is the major environmental factor affecting human microbiome (Zhang *et al*., 2010; De Filippis *et al*., 2016). With respect to the MedDiet, existing studies assessed the impact of single components and individual foods particularly beneficial to health over microbial ecology composition and metabolome features (Widmer *et al*., 2105; Roager *et al*., 2019; Garcia-Aloy *et al*., 2019) but fewer analyzed the effects of the overall MedDiet-dietary pattern over clinical outcomes. Conventionally, the *Prevotella* spp. have been associated to a MedDiet style and accordingly to higher levels of consumption of vegetables and grain cereal products. Conversely, taxa belonging to *Bacteroides* spp. correlated with a Westernized dietary pattern (Wu *et al*., 2011; Del Chierico *et al*., 2014*a*; De Filippis *et al*., 2019). Moreover, Gutiérrez-Díaz *et al*. recently found that higher level of adherence to MedDiet increased the relative abundances of *Faecalibacterium prausnitzii* (a butyrate producer), *Akkermansia* spp., whose strain *A. muciniphila* has been defined as the next-generation beneficial microorganism (Cani and de Vos, 2017) and *Bifidobacterium* spp. These results suggest a beneficial impact of such dietary style on gut microbial ecology and indirectly impacting metabolites production

(Garcia-Mantrana *et al.*, 2018). Therefore, additional clinical researches are warranted to define MedDiet-adherence gut biomarker along with MedDiet-derived effects on microbiome-mediated disease outcome.

The impact of the MedDiet on gut microbial composition associated to health and disease is currently an area of active investigation. It was recently figured out that a MedDiet consumption is associated with higher gut microbial diversity and a restored gut dysbiosis in a cohort of people with diagnosed T2D or obesity (Haro *et al.*, 2016*a*; Haro *et al.*, 2016*b*). In addition, increased levels of bacteria known to be associated to health in humans were found after a MedDiet-based intervetion, among others the fibre-degrading *Bacteroides thetaiotaomicron* and *F. prausnitzii*, the butyrate-producing genus *Roseburia* spp., the probiotics taxa *B. adolescentis* and *B. longum*. (see Figure 2.6).

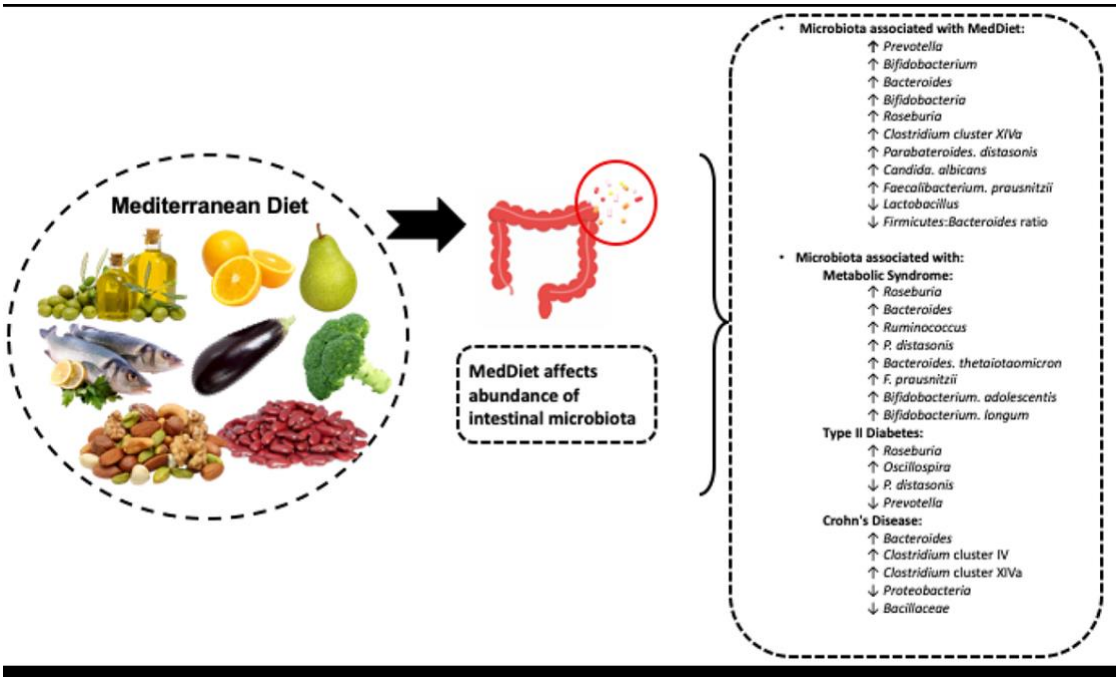


Figure 2.6 MedDiet-related effects upon microbiota composition in health and disease conditions. ⇓: decreased levels through MedDiet consumption. ⇑: increased levels through MedDiet consumption. Image from Jin *et al.*, 2019.

The restoration of gut eubiosis linked to the increase in the relative abundance of taxa with saccharolytic activity are associated with an increase in fermentation capacity, leading to microbial-derived metabolites such as SCFAs or urolithins for which health benefits have been described (De Filippis *et al.*, 2016; Selma *et al.*, 2018).

However, an extensive variability in MedDiet-derived signatures were proved, despite the well-known clinical outcomes in which the MedDiet is involved, like lowering risk factors for diseases onset through metabolome and microbiome alterations (Jin *et al.*, 2019). These discrepancies may result from different analytical methods, laboratory variability techniques,

heterogeneity of dietary assessment methods (because of a wide variety of dietary indices) but mostly the complexity of the diet–disease relationship. Such induced biases might result in inconsistent results on MedDiet-based dietary interventions in cohort studies. In addition, the worryingly globalization and a spread westernized food-culture are now driving a continuously growing prevalence of eating-related chronic diseases in the Mediterranean-neighboring populations, beyond several territories of the world. A Western diet is characterized by an excessive intake of foods with a high energy density and that are rich in fats, sugars, and animal proteins, as well as a very low intake of fruits and vegetables. Such a dietary style, accompanied by low levels of physical activity, promotes inflammation and predisposes individuals to obesity, CVD, type 2 diabetes and metabolic syndrome (Myles, 2014; Minihane *et al*., 2015; Mozaffarian *et al*., 2011).

In conclusion, it is undeniable that multi-omics approaches will improve our understanding of the complex diet–microbiota-health relationships. Future researches are needed to validate previous findings in westernized countries as well as conclusive evidences and proof-of-concept of MedDiet-based dietary intervention upon clinical outcomes (Cani, 2018). Thus, integrating the observations deriving from microbiome analysis and metabolomic data will help in i) discovering biomarkers related to the whole MedDiet-adherence pattern and ii) comprehensively elucidating several aspects of dysbiosis-related human diseases. The adoption of a nutritionally recommended dietary pattern was considered for clinical outcomes amelioration and the findings related to a MedDiet-based RCT are presented in the Chapters 5 and 6.

2.5 References

Acharya A, Chan Y, Kheur S, Jin LJ, Watt RM, Mattheos N. 2017. Salivary microbiome in non-oral disease: A summary of evidence and commentary. Arch Oral Biol 83:169–173.

Agostoni C, Goulet O, Kolacek S, Koletzko B, Moreno L, Puntis J, Rigo J, Shamir R, Szajewska H, Turck D. 2007. Fermented infant formulae without live bacteria. J Pediatr Gastroenterol Nutr 44:392–397.

Almanza-Aguilera E, Urpi-Sarda M, Llorach R, Vázquez-Fresno R, Garcia-Aloy M, Carmona F, Sanchez A, Madrid-Gambin F, Estruch R, Corella D. 2017. Microbial metabolites are associated with a high adherence to a Mediterranean dietary pattern using a 1H-NMR-based untargeted metabolomics approach. J Nutr Biochem 48:36–43.

Almeida A, Mitchell AL, Boland M, Forster SC, Gloor GB, Tarkowska A, Lawley TD, Finn RD. 2019. A new genomic blueprint of the human gut microbiota. Nature 568:499.

Ansaldo E, Slayden LC, Ching KL, Koch MA, Wolf NK, Plichta DR, Brown EM, Graham DB, Xavier RJ, Moon JJ. 2019. *Akkermansia muciniphila* induces intestinal adaptive immune responses during homeostasis. Science (80-) 364:1179–1184.

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto J-M. 2011. Enterotypes of the human gut microbiome. Nature 473:174.

Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, Yagita H, Ishii N, Evans R, Honda K. 2008. ATP drives lamina propria T H 17 cell differentiation. Nature 455:808.

Azad MB, Konya T, Guttman DS, Field CJ, Sears MR, HayGlass KT, Mandhane PJ, Turvey SE, Subbarao P, Becker AB. 2015. Infant gut microbiota and food sensitization: associations in the first year of life. Clin Exp Allergy 45:632–643.

Bäckhed F, Ding H, Wang T, Hooper L V, Koh GY, Nagy A, Semenkovich CF, Gordon JI. 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci 101:15718–15723.

Bashiardes S, Godneva A, Elinav E, Segal E. 2018. Towards utilization of the human genome and microbiome for personalized nutrition. Curr Opin Biotechnol 51:57–63.

Belkaid Y, Hand TW. 2014. Role of the microbiota in immunity and inflammation. Cell 157:121–141.

Bendall CL, Mayr HL, Opie RS, Bes-Rastrollo M, Itsiopoulos C, Thomas CJ. 2018. Central obesity and the Mediterranean diet: A systematic review of intervention trials. Crit Rev Food Sci Nutr 58:3070–3084.

Berni Canani R, Paparo L, Nocerino R, Di Scala C, Della Gatta G, Maddalena Y, Buono A, Bruno C, Voto L, Ercolini D. 2019. Gut microbiome as target for innovative strategies against food allergy. Front Immunol 10.

Blottière HM, de Vos WM, Ehrlich SD, Doré J. 2013. Human intestinal metagenomics: state of the art and future. Curr Opin Microbiol 16:232–239.

Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, Plaut M, Cooper SF, Fenton MJ, Arshad SH. 2011. Guidelines for the diagnosis and management of food allergy in the United States: summary of the NIAID-sponsored expert panel report. J Am Acad Dermatol 64:175–192.

Branum AM, Lukacs SL. 2009. Food allergy among children in the United States. Pediatrics 124:1549–1555.

Byndloss MX, Olsan EE, Rivera-Chávez F, Tiffany CR, Cevallos SA, Lokken KL, Torres TP, Byndloss AJ, Faber F, Gao Y. 2017. Microbiota-activated PPAR-γ signaling inhibits dysbiotic *Enterobacteriaceae* expansion. Science (80-) 357:570–575.

Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C. 2007. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 56:1761–1772.

Cani PD, de Vos WM. 2017. Next-generation beneficial microbes: the case of *Akkermansia muciniphila*. Front Microbiol 8:1765.

Cani PD. 2018. Human gut microbiome: hopes, threats and promises. Gut 67:1716–1725.

Chassaing B, Miles-Brown J, Pellizzon M, Ulman E, Ricci M, Zhang L, Patterson AD, Vijay-Kumar M, Gewirtz AT. 2015. Lack of soluble fiber drives diet-induced adiposity in mice. Am J Physiol Liver Physiol 309:G528–G541.

Choi J, Joseph L, Pilote L. 2013. Obesity and C-reactive protein in various populations: a systematic review and meta-analysis. Obes Rev 14:232–244.

Conterno L, Fava F, Viola R, Tuohy KM. 2011. Obesity and the gut microbiota: does up-regulating colonic fermentation protect against obesity and metabolic disease? Genes Nutr 6:241.

Costabile A, Klinder A, Fava F, Napolitano A, Fogliano V, Leonard C, Gibson GR, Tuohy KM. 2008. Whole-grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double-blind, placebo-controlled, crossover study. Br J Nutr 99:110–120.

Costea PI, Hildebrand F, Arumugam M, Bäckhed F, Blaser MJ, Bushman FD, De Vos WM, Ehrlich SD, Fraser CM, Hattori M. 2018. Enterotypes in the landscape of gut microbial community composition. Nat Microbiol 3:8.

Cox AJ, West NP, Cripps AW. 2015. Obesity, inflammation, and the gut microbiota. lancet Diabetes Endocrinol 3:207–215.

Dao MC, Everard A, Aron-Wisnewsky J, Sokolovska N, Prifti E, Verger EO, Kayser BD, Levenez F, Chilloux J, Hoyles L. 2016. *Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. Gut 65:426–436.

David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling A V, Devlin AS, Varma Y, Fischbach MA. 2014. Diet rapidly and reproducibly alters the human

gut microbiome. Nature 505:559.

De Filippis F, Pasolli E, Tett A, Tarallo S, Naccarati A, De Angelis M, Neviani E, Cocolin L, Gobbetti M, Segata N. 2019. Distinct genetic and functional traits of human intestinal *Prevotella copri* strains are associated with different habitual diets. Cell Host Microbe 25:444–453.

De Filippis F, Pellegrini N, Vannini L, Jeffery IB, La Storia A, Laghi L, Serrazanetti DI, Di Cagno R, Ferrocino I, Lazzi C. 2016. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut 65:1812–1821.

De Filippis F, Vitaglione P, Cuomo R, Berni Canani R, Ercolini D. 2018. Dietary interventions to modulate the gut microbiome - how far away are we from precision medicine. Inflamm Bowel Dis 24:2142–2154.

De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci 107:14691–14696.

De Lorenzo A, Romano L, Di Renzo L, Di Lorenzo N, Cenname G, Gualtieri P. 2019. Obesity: a preventable, treatable, but relapsing disease. Nutrition 110615.

De Vos WM, Engstrand L, Drago L, Reid G, Schauber J, Hay R, Mendling W, Schaller M, Spiller R, Gahan CG. 2012. Human microbiota in health and disease. SelfCare 3:1–68.

Del Chierico F, Gnani D, Vernocchi P, Petrucca A, Alisi A, Dallapiccola B, Nobili V, Lorenza P. 2014. Meta-omic platforms to assist in the understanding of NAFLD gut microbiota alterations: tools and applications. Int J Mol Sci 15:684–711.*b*

Del Chierico F, Vernocchi P, Dallapiccola B, Putignani L. 2014. Mediterranean diet and health: food effects on gut microbiota and disease control. Int J Mol Sci 15:11678–11699.*a*

Depommier C, Everard A, Druart C, Plovier H, Van Hul M, Vieira-Silva S, Falony G, Raes J, Maiter D, Delzenne NM. 2019. Supplementation with *Akkermansia muciniphila* in overweight and obese human volunteers: a proof-of-concept exploratory study. Nat Med 25:1096–1103.

Derrien M, Alvarez A-S, de Vos WM. 2019. The Gut Microbiota in the First Decade of Life. Trends Microbiol.

Derwa Y, Gracie DJ, Hamlin PJ, Ford AC. 2017. Systematic review with meta-analysis: the efficacy of probiotics in inflammatory bowel disease. Aliment Pharmacol Ther 46:389–400.

Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, Pudlo NA, Kitamoto S, Terrapon N, Muller A. 2016. A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. Cell 167:1339–1353.

Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu W-H, Lakshmanan A, Wade WG. 2010. The human oral microbiome. J Bacteriol 192:5002–5017.

Ding S, Chi MM, Scull BP, Rigby R, Schwerbrock NMJ, Magness S, Jobin C, Lund PK. 2010. High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. PLoS One 5:e12191.

Dinu M, Pagliai G, Casini A, Sofi F. 2018. Mediterranean diet and multiple health outcomes: an umbrella review of meta-analyses of observational studies and randomised trials. Eur J Clin Nutr 72:30.

Eleftheriou D, Benetou V, Trichopoulou A, La Vecchia C, Bamia C. 2018. Mediterranean diet and its components in relation to all-cause mortality: meta-analysis. Br J Nutr 120:1081–1097.

Everard A, Lazarevic V, Gaïa N, Johansson M, Ståhlman M, Backhed F, Delzenne NM, Schrenzel J, Francois P, Cani PD. 2014. Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. ISME J 8:2116.

Fachi JL, de Souza Felipe J, Pral LP, da Silva BK, Corrêa RO, de Andrade MCP, da Fonseca DM, Basso PJ, Câmara NOS, e Souza ÉL de S. 2019. Butyrate Protects Mice from *Clostridium difficile*-Induced Colitis through an HIF-1-Dependent Mechanism. Cell Rep 27:750–761.

Falony G, Joossens M, Vieira-Silva S, Wang J, Darzi Y, Faust K, Kurilshikov A, Bonder MJ, Valles-Colomer M, Vandeputte D. 2016. Population-level analysis of gut microbiome variation. Science (80-) 352:560–564.

Furet J-P, Kong L-C, Tap J, Poitou C, Basdevant A, Bouillot J-L, Mariat D, Corthier G, Doré J, Henegar C. 2010. Differential adaptation of human gut microbiota to bariatric surgery–induced weight loss: links with metabolic and low-grade inflammation markers. Diabetes 59:3049–3057.

Garcia-Aloy M, Hulshof PJM, Estruel-Amades S, Osté MCJ, Lankinen M, Geleijnse JM, De Goede J, Ulaszewska M, Mattivi F, Bakker SJL. 2019. Biomarkers of food intake for nuts and vegetable oils: an extensive literature search. Genes Nutr 14:7.

Garcia-Mantrana I, Selma-Royo M, Alcántara Baena C, Collado MC. 2018. Shifts on gut microbiota associated to mediterranean diet adherence and specific dietary intakes on general adult population. Front Microbiol 9:890.

Gérard P. 2016. Gut microbiota and obesity. Cell Mol life Sci 73:147–162.

Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, Scott K, Stanton C, Swanson KS, Cani PD. 2017. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol 14:491–502.

Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch S V, Knight R. 2018. Current understanding of the human microbiome. Nat Med 24:392.

Gilbert JA, Quinn RA, Debelius J, Xu ZZ, Morton J, Garg N, Jansson JK, Dorrestein PC, Knight R. 2016. Microbiome-wide association studies link dynamic microbial consortia to disease. Nature 535:94.

Gillis CC, Hughes ER, Spiga L, Winter MG, Zhu W, de Carvalho TF, Chanin RB, Behrendt CL, Hooper L V, Santos RL. 2018. Dysbiosis-associated change in host metabolism generates lactate to support *Salmonella* growth. Cell Host Microbe 23:54–64.

González-Guardia L, Yubero-Serrano EM, Delgado-Lista J, Perez-Martinez P, Garcia-Rios A, Marin C, Camargo A, Delgado-Casado N, Roche HM, Perez-Jimenez F. 2014. Effects of the Mediterranean diet supplemented with coenzyme q10 on metabolomic profiles in elderly men and women. Journals Gerontol Ser A Biomed Sci Med Sci 70:78–84.

Gourbeyre P, Denery S, Bodinier M. 2011. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. J Leukoc Biol 89:685–695.

Greenwood C, Morrow AL, Lagomarcino AJ, Altaye M, Taft DH, Yu Z, Newburg DS, Ward D V, Schibler KR. 2014. Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of *Enterobacter*. J Pediatr 165:23–29.

Guarner F, Khan AG, Garisch J, Eliakim R, Gangl A, Thomson A, Krabshuis J, Lemair T, Kaufmann P, De Paula JA. 2012. World gastroenterology organisation global guidelines: probiotics and prebiotics october 2011. J Clin Gastroenterol 46:468–481.

Guasch-Ferré M, Hruby A, Toledo E, Clish CB, Martínez-González MA, Salas-Salvadó J, Hu FB. 2016. Metabolomics in prediabetes and diabetes: a systematic review and meta-analysis. Diabetes Care 39:833–846.

Gutiérrez-Díaz I, Fernández-Navarro T, Salazar N, Bartolomé B, Moreno-Arribas MV, de Andres-Galiana EJ, Fernández-Martínez JL, de los Reyes-Gavilan CG, Gueimonde M, González S. 2017. Adherence to a Mediterranean diet influences the fecal metabolic profile of microbial-derived phenolics in a Spanish cohort of middle-age and older people. J Agric Food Chem 65:586–595.

Hall AB, Tolonen AC, Xavier RJ. 2017. Human genetic variation and the gut microbiome in disease. Nat Rev Genet 18:690.

Haro C, Garcia-Carpintero S, Alcala-Diaz JF, Gomez-Delgado F, Delgado-Lista J, Perez-Martinez P, Zuñiga OAR, Quintana-Navarro GM, Landa BB, Clemente JC. 2016. The gut microbial community in metabolic syndrome patients is modified by diet. J Nutr Biochem 27:27–31.*b*

Haro C, Montes-Borrego M, Rangel-Zúñiga OA, Alcalá-Díaz JF, Gómez-Delgado F, Pérez-Martínez P, Delgado-Lista J, Quintana-Navarro GM, Tinahones FJ, Landa BB. 2016. Two healthy diets modulate gut microbial community improving insulin sensitivity in a human obese population. J Clin Endocrinol 101:233–242.*a*

Hemarajata P, Versalovic J. 2013. Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. Therap Adv Gastroenterol 6:39–51.

Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, Creasy HH, Earl AM, FitzGerald MG, Fulton RS. 2012. Structure, function and diversity of the healthy human microbiome. Nature 486:207.

Jacobson A, Lam L, Rajendram M, Tamburini F, Honeycutt J, Pham T, Van Treuren W, Pruss K, Stabler SR, Lugo K. 2018. A gut commensal-produced metabolite mediates colonization resistance to *Salmonella* infection. Cell Host Microbe 24:296–307.

Jin Q, Black A, Kales SN, Vattem D, Ruiz-Canela M, Sotos-Prieto M. 2019. Metabolomics and microbiomes as potential tools to evaluate the effects of the Mediterranean diet. Nutrients 11:207.

Jumpertz R, Le DS, Turnbaugh PJ, Trinidad C, Bogardus C, Gordon JI, Krakoff J. 2011. Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. Am J Clin Nutr 94:58–65.

Kellow NJ, Coughlan MT, Reid CM. 2014. Metabolic benefits of dietary prebiotics in human subjects: a systematic review of randomised controlled trials. Br J Nutr 111:1147–1161.

Knights D, Ward TL, McKinlay CE, Miller H, Gonzalez A, McDonald D, Knight R. 2014. Rethinking "enterotypes." Cell Host Microbe 16:433–437.

Kunk D. Probiotics: elixir or empty promise? Lancet Gastroenterol Hepatol 4:81.

Lăcătușu C-M, Grigorescu E-D, Floria M, Onofriescu A, Mihai B-M. 2019. The Mediterranean Diet: from an environment-driven food culture to an emerging medical prescription. Int J Environ Res Public Health 16:942.

Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto J-M, Kennedy S. 2013. Richness of human gut microbiome correlates with metabolic markers. Nature 500:541.

Lean MEJ, Leslie WS, Barnes AC, Brosnahan N, Thom G, McCombie L, Peters C, Zhyzhneuskaya S, Al-Mrabeh A, Hollingsworth KG. 2019. Durability of a primary care-led weight-management intervention for remission of type 2 diabetes: 2-year results of the DiRECT open-label, cluster-randomised trial. Lancet Diabetes Endocrinol 7:344–355.

LeBlanc JG, Chain F, Martín R, Bermúdez-Humarán LG, Courau S, Langella P. 2017. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. Microb Cell Fact 16:79.

Leeming ER, Johnson AJ, Spector TD, Le Roy CI. 2019. Effect of Diet on the Gut Microbiota: Rethinking Intervention Duration. Nutrients 11:2862.

Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. 2005. Obesity alters gut microbial ecology. Proc Natl Acad Sci 102:11070–11075.

Ley RE, Peterson DA, Gordon JI. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837–848.

Licciardi P V, Wong S-S, Tang MLK, Karagiannis TC. 2010. Epigenome targeting by probiotic metabolites. Gut Pathog 2:24.

Liu R, Hong J, Xu X, Feng Q, Zhang D, Gu Y, Shi J, Zhao S, Liu W, Wang X. 2017. Gut microbiome and serum metabolome alterations in obesity and after weight-loss intervention. Nat Med 23:859.

Lloyd-Price J, Mahurkar A, Rahnavard G, Crabtree J, Orvis J, Hall AB, Brady A, Creasy HH, McCracken C, Giglio MG. 2017. Strains, functions and dynamics in the expanded Human Microbiome Project. Nature 550:61.

Ludwig IS, Broere F, Manurung S, Lambers TT, van der Zee R, van Eden W. 2018. *Lactobacillus rhamnosus* GG-Derived soluble Mediators Modulate adaptive immune cells. Front Immunol 9.

Marchesi JR, Adams DH, Fava F, Hermes GDA, Hirschfield GM, Hold G, Quraishi MN, Kinross J, Smidt H, Tuohy KM. 2016. The gut microbiota and host health: a new clinical frontier. Gut 65:330–339.

Mariño E, Richards JL, McLeod KH, Stanley D, Yap YA, Knight J, McKenzie C, Kranich J, Oliveira AC, Rossello FJ. 2017. Gut microbial metabolites limit the frequency of autoimmune T cells and protect against type 1 diabetes. Nat Immunol 18:552.

Marteau P and Doré J. 2017. Gut Microbiota: A Full-fledged Organ. John Libbey Eurotext.

Martínez-González MÁ, Ruiz-Canela M, Hruby A, Liang L, Trichopoulou A, Hu FB. 2015. Intervention trials with the Mediterranean diet in cardiovascular prevention: understanding potential mechanisms through metabolomic profiling. J Nutr 146:913S-919S.

Mazmanian SK, Round JL, Kasper DL. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 453:620.

McDonald D, Hyde E, Debelius JW, Morton JT, Gonzalez A, Ackermann G, Aksenov AA, Behsaz B, Brennan C, Chen Y. 2018. American gut: an open platform for citizen science microbiome research. mSystems 3:e00031-18.

Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, Teeling JL, Blaak EE, Fenech M, Vauzour D. 2015. Low-grade inflammation, diet composition and health: current research evidence and its translation. Br J Nutr 114:999–1012.

Mitrou PN, Kipnis V, Thiébaut ACM, Reedy J, Subar AF, Wirfält E, Flood A, Mouw T, Hollenbeck AR, Leitzmann MF. 2007. Mediterranean dietary pattern and prediction of all-cause mortality in a US population: results from the NIH-AARP Diet and Health Study. Arch Intern Med 167:2461–2468.

Mitsou EK, Kakali A, Antonopoulou S, Mountzouris KC, Yannakoulia M, Panagiotakos DB, Kyriacou A. 2017. Adherence to the Mediterranean diet is associated with the gut microbiota pattern and gastrointestinal characteristics in an adult population. Br J Nutr 117:1645–1655.

Mozaffarian D, Hao T, Rimm EB, Willett WC, Hu FB. 2011. Changes in diet and lifestyle and long-term weight gain in women and men. N Engl J Med 364:2392–2404.

Mueller S, Saunier K, Hanisch C, Norin E, Alm L, Midtvedt T, Cresci A, Silvi S, Orpianesi C, Verdenelli MC. 2006. Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl Environ Microbiol 72:1027–1033.

Myles IA. 2014. Fast food fever: reviewing the impacts of the Western diet on immunity. Nutr J 13:61.

O'Mahony C, Scully P, O'Mahony D, Murphy S, O'Brien F, Lyons A, Sherlock G, MacSharry J, Kiely B, Shanahan F. 2008. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-κB activation. PLoS Pathog 4:e1000112.

Pasolli E, Asnicar F, Manara S, Zolfo M, Karcher N, Armanini F, Beghini F, Manghi P, Tett A, Ghensi P. 2019. Extensive unexplored human microbiome diversity revealed by over 150,000 genomes from metagenomes spanning age, geography, and lifestyle. Cell 176:649–662.

Patterson E, Ryan PM, Cryan JF, Dinan TG, Ross RP, Fitzgerald GF, Stanton C. 2016. Gut microbiota, obesity and diabetes. Postgrad Med J 92:286–300.

Prince BT, Mandel MJ, Nadeau K, Singh AM. 2015. Gut microbiome and the development of food allergy and allergic disease. Pediatr Clin 62:1479–1492.

Pryde SE, Duncan SH, Hold GL, Stewart CS, Flint HJ. 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiol Lett 217:133–139.

Rajilić-Stojanović M, Smidt H, De Vos WM. 2007. Diversity of the human gastrointestinal tract microbiota revisited. Environ Microbiol 9:2125–2136.

Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR. 2013. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science (80-) 341:1241214.

Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. 2014. Bile acids and the gut microbiome. Curr Opin Gastroenterol 30:332.

Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, Mele MC. 2019. What is the healthy gut microbiota composition? a changing ecosystem across age, environment, diet, and diseases. Microorganisms 7:14.

Rivas MN, Burton OT, Wise P, Zhang Y, Hobson SA, Lloret MG, Chehoud C, Kuczynski J, DeSantis T, Warrington J. 2013. A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis. J Allergy Clin Immunol 131:201–212.

Roager HM, Vogt JK, Kristensen M, Hansen LBS, Ibrügger S, Mærkedahl RB, Bahl MI, Lind MV, Nielsen RL, Frøkiær H. 2019. Whole grain-rich diet reduces body weight and systemic low-grade inflammation without inducing major changes of the gut microbiome: a randomised cross-over trial. Gut 68:83–93.

Rosenbaum M, Knight R, Leibel RL. 2015. The gut microbiota in human energy

homeostasis and obesity. Trends Endocrinol Metab 26:493–501.

Ruiz-Canela M, Hruby A, Clish CB, Liang L, Martínez-González MA, Hu FB. 2017. Comprehensive metabolomic profiling and incident cardiovascular disease: a systematic review. J Am Heart Assoc 6:e005705.

Sandoval-Motta S, Aldana M, Martínez-Romero E, Frank A. 2017. The human microbiome and the missing heritability problem. Front Genet 8:80.

Savage JH, Lee-Sarwar KA, Sordillo J, Bunyavanich S, Zhou Y, O'connor G, Sandel M, Bacharier LB, Zeiger R, Sodergren E. 2018. A prospective microbiome-wide association study of food sensitization and food allergy in early childhood. Allergy 73:145–152.

Schiavi E, Barletta B, Butteroni C, Corinti S, Boirivant M, Di Felice G. 2011. Oral therapeutic administration of a probiotic mixture suppresses established Th2 responses and systemic anaphylaxis in a murine model of food allergy. Allergy 66:499–508.

Schloissnig S, Arumugam M, Sunagawa S, Mitreva M, Tap J, Zhu A, Waller A, Mende DR, Kultima JR, Martin J. 2013. Genomic variation landscape of the human gut microbiome. Nature 493:45.

Schmidt TSB, Raes J, Bork P. 2018. The human gut microbiome: from association to modulation. Cell 172:1198–1215.

Scott NA, Andrusaite A, Andersen P, Lawson M, Alcon-Giner C, Leclaire C, Caim S, Le Gall G, Shaw T, Connolly JPR. 2018. Antibiotics induce sustained dysregulation of intestinal T cell immunity by perturbing macrophage homeostasis. Sci Transl Med 10:eaao4755.

Selma M V, González-Sarrías A, Salas-Salvadó J, Andrés-Lacueva C, Alasalvar C, Örem A, Tomás-Barberán FA, Espín JC. 2018. The gut microbiota metabolism of pomegranate or walnut ellagitannins yields two urolithin-metabotypes that correlate with cardiometabolic risk biomarkers: Comparison between normoweight, overweight-obesity and metabolic syndrome. Clin Nutr 37:897–905.

Singh RK, Chang H-W, Yan D, Lee KM, Ucmak D, Wong K, Abrouk M, Farahnik B, Nakamura M, Zhu TH. 2017. Influence of diet on the gut microbiome and implications for human health. J Transl Med 15:73.

Skelly AN, Sato Y, Kearney S, Honda K. 2019. Mining the microbiota for microbial and metabolite-based immunotherapies. Nat Rev Immunol 19:305–323.

Slocum C, Kramer C, Genco CA. 2016. Immune dysregulation mediated by the oral microbiome: potential link to chronic inflammation and atherosclerosis. J Intern Med 280:114–128.

Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux J-J, Blugeon S, Bridonneau C, Furet J-P, Corthier G. 2008. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci 105:16731–16736.

Tap J, Furet J, Bensaada M, Philippe C, Roth H, Rabot S, Lakhdari O, Lombard V, Henrissat B, Corthier G. 2015. Gut microbiota richness promotes its stability upon increased dietary fibre intake in healthy adults. Environ Microbiol 17:4954–4964.

Tap J, Mondot S, Levenez F, Pelletier E, Caron C, Furet J, Ugarte E, Muñoz-Tamayo R, Paslier DLE, Nalin R. 2009. Towards the human intestinal microbiota phylogenetic core. Environ Microbiol 11:2574–2584.

Thaiss CA, Zeevi D, Levy M, Zilberman-Schapira G, Suez J, Tengeler AC, Abramson L, Katz MN, Korem T, Zmora N. 2014. Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. Cell 159:514–529.

Thursby E, Juge N. 2017. Introduction to the human gut microbiota. Biochem J 474:1823–1836.

Tierney BT, Yang Z, Luber JM, Beaudin M, Wibowo MC, Baek C, Mehlenbacher E, Patel CJ, Kostic AD. 2019. The landscape of genetic content in the gut and oral human microbiome. Cell Host Microbe 26:283–295.

Torres-Fuentes C, Schellekens H, Dinan TG, Cryan JF. 2015. A natural solution for obesity: bioactives for the prevention and treatment of weight gain. A review. Nutr Neurosci 18:49–65.

Torres-Fuentes C, Schellekens H, Dinan TG, Cryan JF. 2017. The microbiota–gut–brain axis in obesity. lancet Gastroenterol Hepatol 2:747–756.

Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL. 2014. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 20:159.

Turmo IG. 2012. The Mediterranean diet: consumption, cuisine and food habits. Int Cent Adv Mediterr Agron Stud 115.

Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. 2008. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe 3:213–223.

Turnbaugh PJ, Gordon JI. 2009. The core gut microbiome, energy balance and obesity. J Physiol 587:4153–4158.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444:1027.

Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. 2009. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med 1:6ra14-6ra14.

Tyakht A V, Kostryukova ES, Popenko AS, Belenikin MS, Pavlenko A V, Larin AK, Karpova IY, Selezneva O V, Semashko TA, Ospanova EA. 2013. Human gut microbiota community structures in urban and rural populations in Russia. Nat Commun 4:2469.

Uhr GT, Dohnalová L, Thaiss CA. 2019. The dimension of time in host-microbiome interactions. MSystems 4:e00216-18.

Ursell LK, Metcalf JL, Parfrey LW, Knight R. 2012. Defining the human microbiome. Nutr Rev 70:S38–S44.

Vanegas SM, Meydani M, Barnett JB, Goldin B, Kane A, Rasmussen H, Brown C, Vangay P, Knights D, Jonnalagadda S. 2017. Substituting whole grains for refined grains in a 6-wk randomized trial has a modest effect on gut microbiota and immune and inflammatory markers of healthy adults. Am J Clin Nutr 105:635–650.

Vázquez-Fresno R, Llorach R, Urpi-Sarda M, Lupianez-Barbero A, Estruch R, Corella D, Fitó M, Arós F, Ruiz-Canela M, Salas-Salvadó J. 2014. Metabolomic pattern analysis after mediterranean diet intervention in a nondiabetic population: a 1-and 3-year follow-up in the PREDIMED study. J Proteome Res 14:531–540.

Virta LJ, Ashorn M, Kolho K-L. 2013. Cow's milk allergy, asthma, and pediatric IBD. J Pediatr Gastroenterol Nutr 56:649–651.

Visconti A, Le Roy CI, Rosa F, Martin TC, Mohney RP, Weizhong L, de Rinaldis E, Bell JT, Venter JC, Nelson KE. 2019. Interplay between the human gut microbiome and host metabolism. Nat Commun 10:4505.

Wang DD, Toledo E, Hruby A, Rosner BA, Willett WC, Sun Q, Razquin C, Zheng Y, Ruiz-Canela M, Guasch-Ferré M. 2017. Plasma ceramides, Mediterranean diet, and incident cardiovascular disease in the PREDIMED trial (Prevencion con Dieta Mediterranea). Circulation 135:2028–2040.

Weiss GA, Hennet T. 2017. Mechanisms and consequences of intestinal dysbiosis. Cell Mol Life Sci 74:2959–2977.

Widmer RJ, Flammer AJ, Lerman LO, Lerman A. 2015. The Mediterranean diet, its components, and cardiovascular disease. Am J Med 128:229–238.

Wilkins T, Sequoia J. 2017. Probiotics for gastrointestinal conditions: a summary of the evidence. Am Fam Physician 96:170–178.

Willson K, Situ C. 2017. Systematic review on effects of diet on gut microbiota in relation to metabolic syndromes. J Clin Nutr Metab 1:1–12.

Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R. 2011. Linking long-term dietary patterns with gut microbial enterotypes. Science (80-) 334:105–108.

Yan F, Polk DB. 2011. Probiotics and immune health. Curr Opin Gastroenterol 27:496.

Yang J-Y, Kweon M-N. 2016. The gut microbiota: a key regulator of metabolic diseases. BMB Rep 49:536.

Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris

M, Hidalgo G, Baldassano RN, Anokhin AP. 2012. Human gut microbiome viewed across age and geography. Nature 486:222.

Zeevi D, Korem T, Godneva A, Bar N, Kurilshikov A, Lotan-Pompan M, Weinberger A, Fu J, Wijmenga C, Zhernakova A. 2019. Structural variation in the gut microbiome associates with host health. Nature 568:43.

Zhang C, Zhang M, Wang S, Han R, Cao Y, Hua W, Mao Y, Zhang X, Pang X, Wei C. 2010. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. ISME J 4:232.

Zhang Z, Tang H, Chen P, Xie H, Tao Y. 2019. Demystifying the manipulation of host immunity, metabolism, and extraintestinal tumors by the gut microbiome. Signal Transduct Target Ther 4:1–34.

Zhao L. 2010. Genomics: The tale of our other genome. Nature 465:879.

Zhao S, Lieberman TD, Poyet M, Kauffman KM, Gibbons SM, Groussin M, Xavier RJ, Alm EJ. 2019. Adaptive evolution within gut microbiomes of healthy people. Cell Host Microbe 25:656–667.

Zhu A, Sunagawa S, Mende DR, Bork P. 2015. Inter-individual differences in the gene content of human gut bacterial species. Genome Biol 16:82.

Zoetendal EG, von Wright A, Vilpponen-Salmela T, Ben-Amor K, Akkermans ADL, de Vos WM. 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl Environ Microbiol 68:3401–3407.

CHAPTER 3

Probiotic dietary supplementation correlates with gut microbiota changes and immunity biomarkers

3.1 Introduction

Common infectious diseases, affecting the respiratory and gastrointestinal tracts, are an important problem for young children attending preschool or day care centers (Maldonado *et al*., 2012). Young children are especially prone to infection, and this susceptibility is thought to be driven by immaturity in organ function, immune response, and also potentially in the gut microbiota (Prodeus *et al*., 2016). Functional foods, based on the fermentation of cow's milk with probiotics, have been proposed as an effective strategy to reduce the incidence of infectious diseases in children, but the results are still conflicting (Merenstein *et al*., 2010; Campeotto *et al*., 2011; Nagata *et al*., 2011; Thibault *et al*., 2004; Mullié *et al*., 2004; Agostoni *et al*., 2007). These discrepancies could derive mainly from different study designs and population studies and from different functional properties of the investigated fermented foods. The efficacy of fermented foods is believed to be strain specific and dose dependent. Therefore, additional research is required to understand the mode of action and the impact of each product, and clinical trials are needed to determine efficacy of claims in human populations.

Bacteria associated with fermented foods may influence gut-associated microbial composition and function by direct competition, by metabolic interaction, through direct immune activation, or via the production of bioactive molecules, such as the short-chain fatty acids (SCFAs), that are able to influence the host health regulating a number of immune and non-immune protective mechanisms (Marco *et al*., 2017; Tamang *et al*., 2016; Unno *et al*., 2015; Derrien and van Hylckama Vlieg, 2015; Corrêa-Oliveira *et al*., 2016).

Many fermented foods are processed such that viable bacteria are inactivated at the time of consumption (Marco *et al*., 2017). Postbiotics containing dead bacterial cells have been

shown to exert biological effects on the host immune system and to stimulate the production of anti-inflammatory cytokines (Vieira *et al*., 2015; Vieira *et al*., 2016; Asama *et al*., 2016). We previously demonstrated that a fermented cow's milk product with heat-killed *Lactobacillus paracasei* CBA L74 (FM-CBAL74) efficiently protects schooled children against respiratory and gastrointestinal tract infections and that this protective effect is associated with a significant stimulation of innate and acquired immunity (Nocerino *et al*., 2015).

In order to assess the possible association of these effects with the structure of the gut microbiota, we designed this study to determine the effects of FM-CBAL74 treatment on gut microbiota composition and butyrate production.

3.2 Materials and Methods

Study subjects

Detailed description of screening and recruitment of study population has been provided elsewhere (Nocerino *et al*., 2015). Briefly, consecutive healthy children (12 to 48 months of age) attending day care or preschool at least 5 days a week, were invited to participate to the study. The exclusion criteria were as follows: age ≤ 12 months or ≥ 48 months, concomitant chronic systemic diseases, congenital cardiac defects, gastrointestinal or urinary or respiratory tract surgery, active tuberculosis, autoimmune diseases, immunodeficiency, chronic inflammatory bowel diseases, cystic fibrosis, metabolic diseases, history of suspected or challenge-proved food allergy, lactose intolerance, malignancy, chronic pulmonary diseases, malformations of gastrointestinal or urinary or respiratory tract, severe malnutrition (z score for weight-for-height < 3 SD scores), and the use of pre/pro/symbiotics, antibiotics, or immune stimulating products in the 2 weeks before the enrollment. From the original study population enrolled (Nocerino *et al*., 2015), we randomly selected 10 subjects per group through a random number generator (Randomness and Integrity Services, Ltd., Dublin, Ireland (https://www.random.org). Anamnestic, demographic, and clinical features, including innate and acquired immunity biomarkers data, as well as information regarding dietary habits, assessed by a 3-day food diary collected every week for the entire study duration, were available for the entire cohort (Nocerino *et al*., 2015). The sample size was calculated, taking into account the size effect estimated from our previous data on butyrate levels (Berni Canani *et al*., 2016). We calculated that 10 children per group were needed to detect an increase of at

least 50% above baseline mean fecal butyrate level with a power of 0.80 at an alpha level of 0.05 (t test for two independent samples with common variance two-tailed test). This study was approved by the Ethics Committee of the University of Naples Federico II and was registered in the Clinical Trials Protocol Registration System (ClinicalTrials.gov) with the identifier NCT01909128.

Intervention

The investigators were blinded to the treatment at all times, i.e., allocation, intervention, laboratory analysis, and statistical analysis (Nocerino *et al*., 2015). The study subjects were distributed into two groups according to a computer-generated randomization list. The investigators assigned each child the next available number on entry into the trial. Investigators, parents, and children were not aware of the dietary treatment assigned. Subjects were supplemented daily for 3 months with either a dietary product deriving from cow's milk fermented with *L. paracasei* CBA L74 (MILK) or a placebo (PL). The composition of the dietary products used is reported in Table 3.1. They were provided in powder by Heinz Italia SpA, Latina, Italy, an affiliate of H. J. Heinz Company, Pittsburgh, PA. The fermented milk was prepared from skimmed milk fermented by *L. paracasei* CBA L74. The fermentation was started in the presence of 10^6 bacteria, reaching 5.9 X 10^9 CFU/g after a 15-h incubation at 37°C. After heating at 85°C for 20 s in order to inactivate the live bacteria, the formula was spray-dried. Thus, the final fermented milk powder contained only bacterial bodies and fermentation products and no living microorganisms. The placebo consisted of maltodextrins with similar energy content of the fermented milk. Study products were provided in tins containing 400 g of powder, and the packaging was similar. Study products were stored at room temperature and in a dry environment.

The investigators instructed parents about the daily amount of the assigned study product and the method of preparation. All subjects received 7 g/day of study products diluted in a maximum of 150 ml of cow's milk or water. Parents were encouraged to contact the investigators if necessary and to maintain the habitual diet of the child, but to exclude prebiotics, probiotics, postbiotics, symbiotics, and immune stimulating products during the 3-month study period.

Compliance was defined as the consumption of at least 80% of the assigned treatment during the study and was evaluated by counting and weighing the returned tins and by the notes on

the diary compiled by parents. At the enrollment and at the end of the trial, a stool sample (3 g) was obtained from all study subjects and stored at -80°C prior to further analysis.

Table 3.1 Composition of the study dietary products

Component	**Composition (per 100 g of product) for each treatment group**	
	MILK	**PL**
Energy (kcal)	367	388
Protein (g)	24.0	0
Carbohydrate (g)	66.4	97
Fat (g)	0.6	0
L. paracasei CBA L74 (CFU)***a***	$5.9 \times 10_{11}$	

a The CFU of killed bacteria

DNA extraction and 16S rRNA gene sequencing

Fecal samples (~1 g) were fully homogenized in STE buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and centrifuged (1,000 rpm X 1 min) in order to pellet debris. The supernatant was centrifuged again (12,000 X g, 2 min), and the pellet was used for DNA extraction by using a PowerFecal DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The V3-V4 region of the 16S rRNA gene was amplified by using the primers S-D-Bact-0341F5'-CCTACGGGNGGCWGCAG and S-D-Bact-0785R5'-GACTACHVGGGTATCTAATCC (Klindworth *et al.*, 2013). Library multiplexing, pooling, and sequencing were carried out according to the Illumina 16S metagenomic sequencing library preparation protocol on a MiSeq platform and using the MiSeq Reagent kit v2, yielding 2X250-bp, paired-end reads.

Bioinformatics and statistical analysis

Demultiplexed, forward, and reverse reads were joined by using FLASH (Magoč and Salzberg, 2011). Joined reads were quality trimmed (Phred score < 20) and short reads (<250 bp) were discarded by using Prinseq (Schmieder and Edwards, 2010). High-quality reads were then imported into QIIME (Caporaso *et al.*, 2010). Operational taxonomic units (OTU) were picked using a de novo approach and the uclust method, and taxonomic assignments were obtained by using the RDP classifier and the Greengenes (McDonald *et al.*, 2012) database, following a pipeline previously reported (De Filippis *et al.*, 2014). In order to avoid biases due to the different sequencing depth, OTU tables were rarefied to the lowest number

of sequences per sample. Bray-Curtis distance matrix and alpha diversity indices were computed by QIIME on rarefied OTU tables. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (http://picrust.github.io/picrust) (Langille *et al*., 2013) was used to predict the functional profiles of the samples, as recently reported (De Filippis *et al*., 2016*a*). Statistical analyses and plotting were carried out in an R environment (https://www.r-project.org). Permutational multivariate analysis of variance (nonparametric MANOVA) based on Jaccard and Bray-Curtis distance matrices was carried out using 999 permutations to detect significant differences in the overall microbial community or oligotype patterns, by using the *adonis* function in the *vegan* package. The Bioconductor statistical package *DeSeq2* (Love *et al*., 2014) was used to find taxa differentially abundant between the groups. Spearman's pairwise correlations were computed between OTU and other quantitative variables (the *corr.test* function in the *psych* package) and plotted by using the *heatplot* function in the *made4* package. P values were corrected for multiple testing using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

Oligotyping analysis.

Reads assigned to *Bacteroides* and to genera within *Ruminococcaceae* and *Lachnospiraceae* with an abundance of >5% in at least 10% of the samples were extracted, and entropy analysis and oligotyping were carried out (Eren *et al*., 2013). Only *Bacteroides*, *Roseburia*, and *Blautia* oligotype patterns were significantly affected by treatment. After the initial round of oligotyping, high-entropy positions were chosen (-C option): 2, 27, 30, 31, 32, 94, 114, 120, and 291 (*Bacteroides*); 1, 2, 12, 27, 28, 30, 56, 57, 58, 61, 82, 101, 103, 157, 160, 170, 172, 174, 176, 184, 191, 213, 215, 220, 235, 237, 273, 274, 293, 343, 347, 371, and 409 (*Blautia*); and 2, 28, 57, 215, 272, 369, 370, 409, and 410 (*Roseburia*). To minimize the impact of sequencing errors, we required an oligotype to be represented by at least 100 reads (-M option). Moreover, rare oligotypes present in fewer than 10 samples were discarded (-s option). These parameters led to 56, 59, and 52 samples and 90,195, 298,288, and 24,503 sequences left in the *Bacteroides*, *Blautia*, and *Roseburia* data sets, respectively. BLASTn was used to query the representative sequences against the NCBI nr database, and the top hit was considered for taxonomic assignment. Statistical analyses and plotting were carried out in R.

Fecal butyrate analysis

One gram of frozen feces was diluted with saline buffer, vortexed, and centrifuged (12,000 X g) for 10 min in 2-ml tubes. The supernatant was filtered (0.45 μm pore size) and stored at 20°C until analysis. Frozen fecal extracts were acidified with 20 μl of 85% (wt/vol) phosphoric acid and 0.5 ml of ethyl acetate, mixed and centrifuged (12,000 X g) for 1h and extracted in duplicate. About 0.5 ml of the pooled extract containing the acidified butyrate was transferred into a 2-ml glass vial and loaded onto an Agilent Technologies (Santa Clara, CA) 7890 gas chromatograph (GC) system with an automatic loader/injector. The GC column was a J&W DB-FFAP (Agilent Technologies) of 30 m, with an internal diameter of 0.25 mm and a film thickness of 0.25 μm. The GC was programmed to achieve the following run parameters: an initial temperature of 90°C, a hold for 0.5 min, and ramp of 20°C min^{-1} up to a final temperature of 190°C; a total run time of 8.0 min; a gas flow of 7.7 ml min^{-1} split less to maintain 3.26 lb/in^2 column head pressure; and a septum purge of 2.0 ml min^{-1}. Detection was achieved using a flame ionization detector. Peaks were identified using a mixed external standard and quantified by using a peak height/internal standard ratio.

Assessment of innate and acquired immunity biomarkers

For all study subjects, data related to the fecal levels of α-defensin (HNP1-3), β-defensin 2 (HBD-2), cathelicidin (LL-37), and sIgA were available. The determinations were performed as previously described (Nocerino *et al*., 2015). The results are expressed as ng/ml for α-defensin, β-defensin, and LL-37 and as μg/ml of supernatants for sIgA.

Accession number(s)

The 16S rRNA gene sequences produced in this study are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under accession number SRP100769.

3.3 Results

Study subjects

The main features of the study populations are reported in Table 3.2. All children were from families of middle socioeconomic status from the same urban area. The dietary habits were very similar between the two study groups -energy (kcal ± the standard deviations [SD]), 1,420 ± 51 versus 1,388 ± 59; carbohydrate (in grams ± the SD), 225.9 ± 7.3 versus 215.1 ± 8; protein, 31.8 ± 2.7 versus 30.6 ± 2.9; fat, 51.28 ± 4.3 versus 51.46 ± 4; and fiber, 9.3 ± 3.6 versus 11.9 ± 2.7—in MILK (dietary product deriving from cow's milk fermented with *L. paracasei* CBA L74)- and placebo (PL)-treated groups, respectively. All children were nonfebrile at inclusion, and none was suffering from respiratory tract or gastrointestinal symptoms. The vaccination status was identical among the two groups. The interventions were well accepted by the children, and the compliance was good in all study subjects. No differences were detected in the daily intake of the active and placebo products between the study groups. No modifications in blood sugar and insulin levels were observed upon treatment. Significant increases in α-defensin (HNP1-3), β-defensin 2 (HBD-2), and cathelicidin (LL-37) were observed only in children treated with FM-CBAL74, as well as an increasing trend in secretory IgA (sIgA) (Supplemental Material from Berni Canani *et al.*, 2017). No differences in the average weighted Unifrac distances were detected between the MILK and PL groups at the beginning of the treatment ($P < 0.05$).

Table 3.2 Main anamnestic, demographic, and immunological features of the study population

Parameter		Mean ± SD*a*	
		MILK	**PL**
Demographic data			
	No. of subjects	10	10
	No. (%) of male subjects	8 (80)	5 (50)
	Age (mo)	34.3 ± 8.9	37.2 ± 8.7
	No. (%) breastfeeding	10 (100)	7 (70)
	Duration (mo) of breastfeeding	6.2 ± 5.9	10 ± 6.6
Wt (kg)			
	t0	15.7 ± 3.3	14.9 ± 2.2
	t3	16.1 ± 2.8	15.6 ± 2.4
Ht (cm)			
	t0	95.9 ± 8.5	95.7 ± 6.2
	t3	97.6 ± 7.6	98 ± 6.1
Level			
	Alpha-defensin at t0 (ng/ml)	1.5 ± 1.4	1.2 ± 1.2
	Alpha-defensin at t3 (ng/ml)	4.2 ± 1.9	1.6 ± 1.3
	Beta-defensin 2 at t0 (ng/ml)	28.7 ± 25.1	32.3 ± 13.2
	Beta-defensin 2 at t3 (ng/ml)	46.8 ± 21.1	38.6 ± 15
	LL-37 at t0 (ng/ml)	13.3 ± 6.9	16 ± 9.3
	LL-37 at t3 (ng/ml)	32 ± 16.3	19.4 ± 14.5
	sIgA at t0 (μg/ml)	24.1 ± 9.9	30.8 ± 18.2
	sIgA at t3 (μg/ml)	42.8 ± 14	32.5 ± 16.4

Values are reported as means ± the standard deviations, except as noted otherwise in column 1. Treatment groups: MILK, cow's milk fermented with *L. paracasei* CBA L74; PL, placebo.

Effects of FM-CBAL74 on gut microbiota composition

We did not observe significant differences in alpha and beta diversities between children belonging to the two groups at the baseline (data not shown). Treatment with FM-CBAL74 affected gut microbiota composition. Although a great variability was observed, multivariate analysis of variance (MANOVA) showed significant differences in the gut microbiota composition between the two study groups following intervention ($P < 0.05$). Nevertheless, principal-coordinate analysis did not show any clustering of the subjects according to the treatment received (data not shown). The relative proportion of *Lactobacillus* and

Ruminococcaceae significantly increased following FM-CBAL74 treatment ($P < 0.05$), with specific significant increases in *Oscillospira* and *Faecalibacterium* (Figure 3.1, $P < 0.05$). In addition, we found positive correlations between the relative abundance of several genera belonging to *Ruminococcaceae* and fecal LL-37 level, whereas *Lachnospira* and *Ruminococcus* (*Lachnospiraceae*) correlated with HBD-2 levels (Figure 3.2). The gut microbiotas of PL subjects were significantly different from those of FM-CBAL74-treated subjects after 3 months of treatment, with significantly higher levels of *Bacteroides* (data not shown, $P < 0.05$).

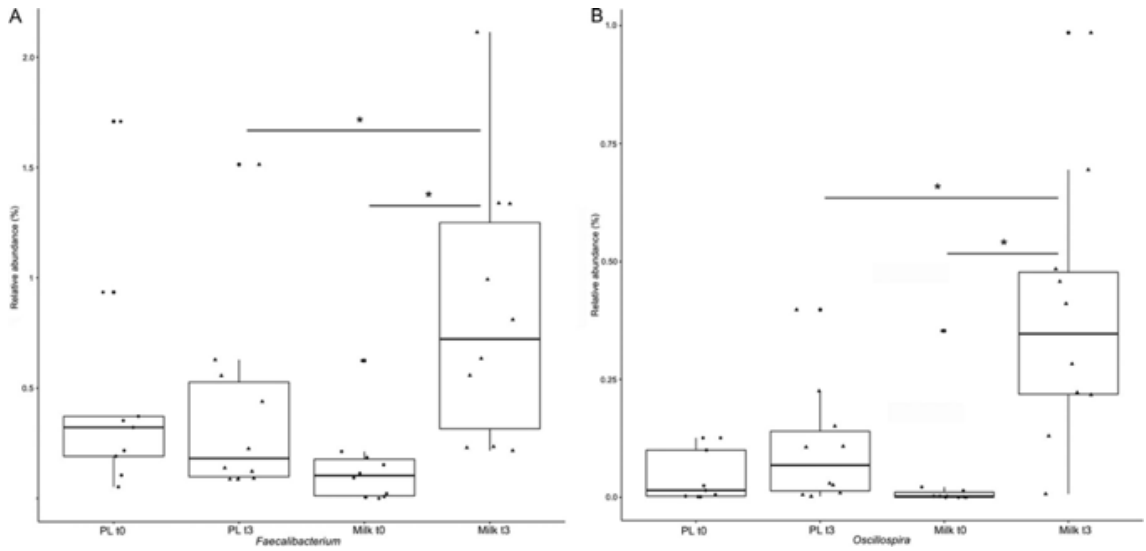


Figure 3.1 *Oscillospira* and *Faecalibacterium* levels. Box plots show the abundance of *Oscillospira* and *Faecalibacterium* in the studied population at baseline (t0) and after 3 months of treatment (t3) with fermented milk (MILK) and placebo (PL). Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (second quartile). Whiskers denote the lowest and highest values within 1.5 × IQR from the first and third quartiles, respectively. Asterisks indicate a significant difference as obtained by a pairwise Wilcoxon test ($P < 0.05$).

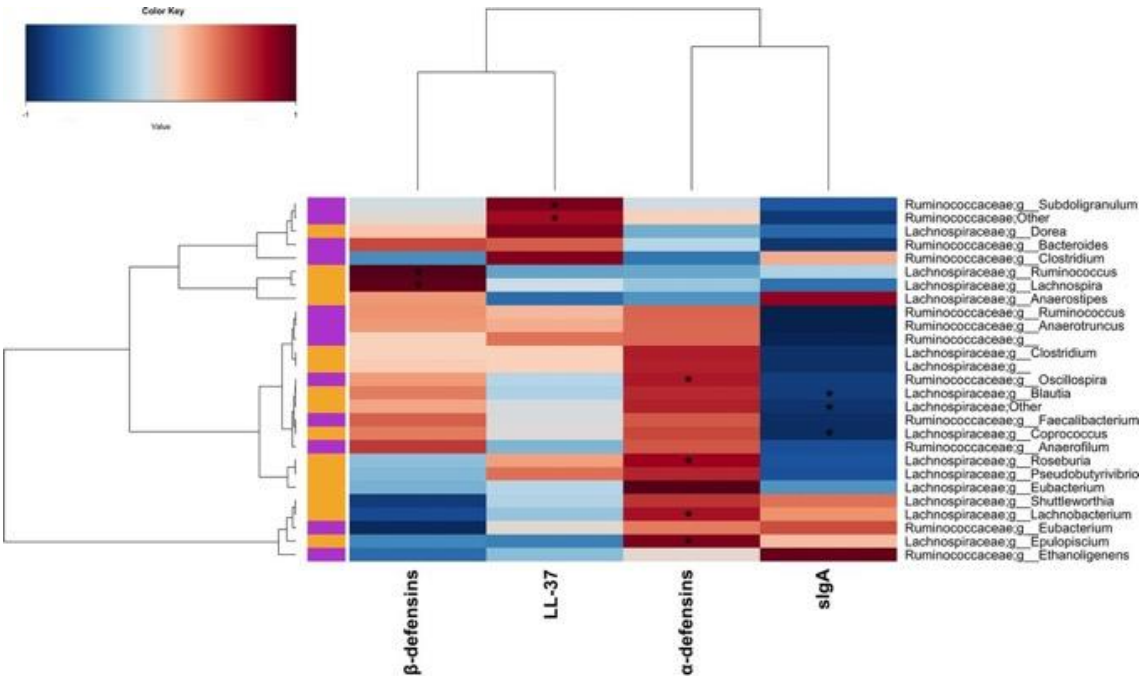


Figure 3.2 *Lachnospiraceae* and *Ruminococcaceae* abundance correlates with innate and acquired immunity. A heatplot shows the Spearman correlations between genera belonging to *Lachnospiraceae* and *Ruminococcaceae* and the levels of immunity biomarkers. Rows and columns are clustered by Euclidean distance and Ward linkage hierarchical clustering. The intensity of the colors represents the degree of association, as measured by Spearman correlations. Only genera occurring in at least 20% of the samples were included. Asterisks indicate a significant correlation after Benjamini-Hochberg correction.

Effect of fermented milk on gut microbiota at subgenus level

In order to explore the possible effect of FM-CBAL74 at a subgenus level, we carried out oligotyping on sequences of *Bacteroides* and genera belonging to *Ruminococcaceae* and *Lachnospiraceae*, since these bacterial groups are well-known butyrate producers. Only *Bacteroides*, *Blautia*, and *Roseburia* oligotype patterns showed significant changes after dietary intervention, as shown by MANOVA ($P < 0.05$). Specific *Roseburia* oligotypes were promoted by FM-CBAL74 treatment (*Roseburia* oligotype 1) and showed positive correlations with sIgA (Rho= 0.63, $P < 0.038$) and β-defensin (Rho= 0.87, $P < 0.023$). *Blautia* oligotypes 5 and 13 also increased with FM-CBAL74 treatment and were positively correlated with α-defensin (Rho= 0.84, $P < 0.007$; Rho= 0.58, $P < 0.040$). Finally, *Bacteroides* oligotypes 12 and 19 increased after FM-CBAL74 treatment, but only Bac12 (Rho= 0.67, $P < 0.042$) was positively correlated with α-defensin.

FM-CBAL74 treatment promotes butyrate production in the gut

FM-CBAL74 treatment resulted in an increase in the relative abundance of predicted genes involved in butyrate synthesis (PICRUSt-predicted metagenomes), especially genes encoding butyryl coenzyme A (butyryl-CoA) transferase (EC 2.8.3.8) and butyrate kinase (EC 2.7.2.7) (Supplemental Material from Berni Canani *et al*., 2017, $P < 0.05$). Consistently, a significant increase in fecal butyrate levels in children consuming FM-CBAL74 was observed (Figure 3.3, $P < 0.05$).

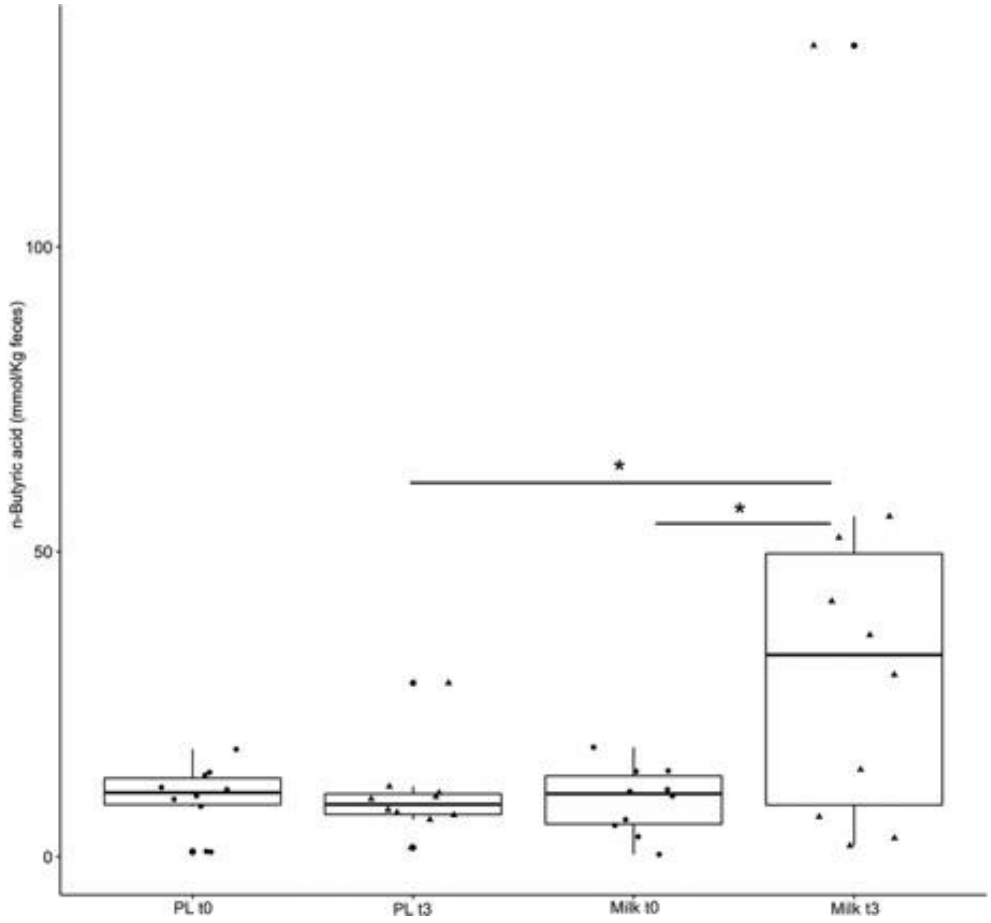


Figure 3.3 Fecal butyrate concentration. Box plots show the abundance of fecal butyrate in the study population at baseline (t0) and after 3 months of treatment (t3) with fermented milk (MILK) and placebo (PL). Asterisks indicate a significant difference. as obtained by pairwise Wilcoxon test ($P < 0.05$). See the legend to Figure 3.1 for a description of the box plots.

3.4 Discussion

It is increasingly understood that the outcomes of microbial fermentation provide additional properties to fermented foods beyond basic nutrition (Marco *et al*., 2017). During fermentation, the metabolic activity of microorganisms can change the nutritional and

bioactive properties of food matrices with possible beneficial consequences for human health (Marco *et al*., 2017). The gut microbiota plays a key role in the development and function of the immune system (Gensollen *et al*., 2016) and some of the health benefits of fermented foods could derive also from their impact on gut microbial composition and function (Marco *et al*., 2017). In a randomized controlled trial, we demonstrated that a cow's milk fermented product containing the heat-killed probiotic strain *L. paracasei* CBA L74 is effective in reducing the incidence respiratory and gastrointestinal tract infections in young children (Nocerino *et al*., 2015). Interestingly, these results have been recently confirmed by a multicenter trial with a similar study design (Corsello *et al*., 2017). Here, we tested the effect of this specific fermented product on gut microbiota composition and butyrate production. Distinctive traits of the gut microbiota, with an increase in genera known as butyrate producers such as *Oscillospira* and *Faecalibacterium* (Konikoff and Gophna, 2016; Ríos-Covián *et al*., 2016), were observed in children receiving the fermented product. It has been previously shown that *Faecalibacterium* strains exert a stimulating effect on the immune system and on T cell differentiation (Rossi *et al*., 2016). In addition, an increase in *Lactobacillus* abundance was observed upon treatment with the fermented product, which is unlikely due to the presence of heat-killed lactobacilli in the fermented product and more probably derives from a stimulatory effect of this product on such populations of lactic acid bacteria.

We also demonstrated an effect of the fermented milk product beyond the genus level. In fact, specific oligotypes of *Roseburia* and *Blautia* were boosted by the treatment, suggesting an effect at the subgenus level and highlighting a possible different effect of the fermented product on species and strains belonging to these genera, as previously pointed out for other common members of the gut microbiota (De Filippis *et al*., 2016*b*; Ley, 2016). The dietary treatment resulted in an increase in the relative abundance of predicted genes involved in butyrate synthesis, especially genes encoding butyryl-CoA transferase (EC 2.8.3.8) and butyrate kinase (EC 2.7.2.7), and an associated significant increase in fecal butyrate levels likely deriving from lactate catabolism, one of the primary pathways for butyrate production by gut bacteria (Duncan *et al*., 2004). Altogether, these results suggest that a shift in the relative proportion of certain bacterial genera and oligotypes may be associated with an enhanced butyrate synthesis. Butyrate regulates several non-immune and immune defense mechanisms against infections, including the regulation of luminal pH in the gut, mucus production, cell growth and differentiation, the modulation of gut permeability and of transepithelial ion transport, the modulation of the inflammatory response, and the stimulation

of innate and acquired immunity (Ríos-Covián *et al*., 2016; Berni Canani *et al*., 2011; Keku *et al*., 2015; Macfarlane and Macfarlane, 2012). Although we investigated only butyrate, it cannot be excluded that also the production of other SCFAs could be regulated by this particular fermented product.

We found positive correlations between specific gut microbiota signatures and the fecal levels of innate and acquired immunity biomarkers. These data are in line with the results obtained using a fermented milk formula containing two heat-inactivated probiotic strains in preterm infants (Campeotto *et al*., 2011).

The results of this study support the concept of a mutualistic interaction occurring between gut microbiota and immune system, where gut microbiota influences immune system development and function, and the immune system shapes gut microbiota composition (Honda, 2015; Jenke *et al*., 2013). It is possible to speculate that the effect of this particular fermented food on gut microbiota could derive at least in part by a modulation of innate immunity peptides. Indeed, evidence on the positive correlations between specific members of the gut microbiota and immunity peptides has been obtained in this study. The final result is the establishment and consolidation of a microbiota composition that can be responsible for a protective action against infectious diseases. In line with this view, we have recently demonstrated that, through a direct interaction with human enterocytes, FM-CBAL74 stimulates the synthesis of β-defensin 2 and LL-37 (Paparo *et al*., 2015). Future studies are necessary to define the components of this particular fermented product that are involved in these effects. In this light, comparison with different types of placebo, such as milk without the addition of bacteria or fermented by other lactobacilli, would provide useful information.

It is important to recognize that this study investigated a dietary product fermented with a specific probiotic strain, a well-defined dose, and age group and that our findings cannot be extrapolated for other products containing different probiotic strains. Indeed, it cannot be excluded that similar results could be obtained with milk products fermented by phylogenetically close lactic acid bacteria. In this study, the similar dietary habits of treated and placebo groups strongly suggest that the effects observed on gut microbiota composition and butyrate production derive from the administration of the fermented milk used. In this study, children enrolled in the placebo group, received maltodextrins. Maltodextrins, even at higher doses, are commonly used as placebos in clinical trials (Kolida *et al*., 2007; Abrams *et al*., 2005). Contrasting data suggest that maltodextrins could influence gut microbiota composition and immune system (Costabile *et al*., 2010; Nickerson *et al*., 2015; Kredier *et al*., 2007). Here, we did not observe significant changes in the gut microbiota composition,

fecal butyrate levels, and innate and acquired immunity biomarkers in children enrolled in the placebo group, which supports the use of maltodextrins in placebo treatments.

3.5 Conclusion

In conclusion, although additional research should be focused on the specific molecular mechanisms involved, we have shown that FM-CBA L74 induces positive regulation of the mutual interaction between the immune system and gut microbiota. The use of a fermented milk product containing the heat-killed probiotic strain *Lactobacillus paracasei* CBAL74 induces changes in the gut microbiota, promoting the development of butyrate producers. These changes in the gut microbiota composition correlate with increased levels of innate and acquired immunity biomarkers.

3.6 Notes

This chapter reports the content of paper entitled "Specific Signatures of the Gut Microbiota and Increased Levels of Butyrate in Children Treated with Fermented Cow's Milk Containing Heat-Killed *Lactobacillus paracasei* CBA L74" by Berni Canani R, De Filippis F, Nocerino R, Laiola M, Paparo L, Calignano A, De Caro C, Coretti L, Chiariotti L, Gilbert JA and Ercolini D (2017) published on Applied and Environmental Microbiology 83:e01206-17.

3.7 References

Abrams SA, Griffin IJ, Hawthorne KM, Liang L, Gunn SK, Darlington G, Ellis KJ. 2005. A combination of prebiotic short-and long-chain inulin-type fructans enhances calcium absorption and bone mineralization in young adolescents–. Am J Clin Nutr 82:471–476.

Agostoni C, Goulet O, Kolacek S, Koletzko B, Moreno L, Puntis J, Rigo J, Shamir R, Szajewska H, Turck D. 2007. Fermented infant formulae without live bacteria. J Pediatr Gastroenterol Nutr 44:392–397.

Asama T, Kimura Y, Kono T, Tatefuji T, Hashimoto K, Benno Y. 2016. Effects of heat-killed *Lactobacillus kunkeei* YB38 on human intestinal environment and bowel movement: a pilot study. Benef Microbes 7:337–344.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 57:289–300.

Berni Canani R, Di Costanzo M, Leone L, Pedata M, Meli R, Calignano A. 2011. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol WJG 17:1519.

Berni Canani R, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, Calignano A, Khan AA, Gilbert JA, Nagler CR. 2016. *Lactobacillus rhamnosus* GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. ISME J 10:742.

Berni Canani R, De Filippis F, Nocerino R, Laiola M, Paparo L, Calignano A, De Caro C, Coretti L, Chiariotti L, Gilbert JA. 2017. Specific signatures of the gut microbiota and increased levels of butyrate in children treated with fermented cow's milk containing heat-killed *Lactobacillus paracasei* CBA L74. Appl Environ Microbiol 83:e01206-17.

Campeotto F, Suau A, Kapel N, Magne F, Viallon V, Ferraris L, Waligora-Dupriet A-J, Soulaines P, Leroux B, Kalach N. 2011. A fermented formula in pre-term infants: clinical tolerance, gut microbiota, down-regulation of faecal calprotectin and up-regulation of faecal secretory IgA. Br J Nutr 105:1843–1851.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335.

Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MAR. 2016. Regulation of immune cell function by short-chain fatty acids. Clin Transl Immunol 5:e73.

Corsello G, Carta M, Marinello R, Picca M, De Marco G, Micillo M, Ferrara D, Vigneri P, Cecere G, Ferri P. 2017. Preventive effect of cow's milk fermented with *L. paracasei* CBA L74 on common infectious diseases in children: a multicenter randomized controlled trial. Nutrients 9: 669.

Costabile A, Kolida S, Klinder A, Gietl E, Bäuerlein M, Frohberg C, Landschütze V, Gibson GR. 2010. A double-blind, placebo-controlled, cross-over study to establish the bifidogenic effect of a very-long-chain inulin extracted from globe artichoke (*Cynara scolymus*) in healthy human subjects. Br J Nutr 104:1007–1017.

De Filippis F, Pellegrini N, Vannini L, Jeffery IB, La Storia A, Laghi L, Serrazanetti DI, Di Cagno R, Ferrocino I, Lazzi C. 2016. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut 65:1812–1821.*a*

De Filippis F, Pellegrini N, Laghi L, Gobbetti M, Ercolini D. 2016. Unusual sub-genus associations of faecal *Prevotella* and *Bacteroides* with specific dietary patterns. Microbiome 4:57.*b*

De Filippis F, Vannini L, La Storia A, Laghi L, Piombino P, Stellato G, Serrazanetti DI, Gozzi G, Turroni S, Ferrocino I. 2014. The same microbiota and a potentially discriminant metabolome in the saliva of omnivore, ovo-lacto-vegetarian and vegan individuals. PLoS One 9:e112373.

Derrien M, van Hylckama Vlieg JET. 2015. Fate, activity, and impact of ingested bacteria within the human gut microbiota. Trends Microbiol 23:354–366.

Duncan SH, Louis P, Flint HJ. 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl Environ Microbiol 70:5810–5817.

Eren AM, Maignien L, Sul WJ, Murphy LG, Grim SL, Morrison HG, Sogin ML. 2013. Oligotyping: differentiating between closely related microbial taxa using 16S rRNA gene data. Methods Ecol Evol 4:1111–1119.

Gensollen T, Iyer SS, Kasper DL, Blumberg RS. 2016. How colonization by microbiota in early life shapes the immune system. Science (80-) 352:539–544.

Honda K. 2015. TFH-IgA responses keep microbiota in check. Cell Host Microbe 17:144–146.

Jenke AC, Postberg J, Mariel B, Hensel K, Foell D, Däbritz J, Wirth S. 2013. S100A12 and hBD2 correlate with the composition of the fecal microflora in ELBW infants and expansion of *E. coli* is associated with NEC. Biomed Res Int 2013.

Keku TO, Dulal S, Deveaux A, Jovov B, Han X. 2014. The gastrointestinal microbiota and colorectal cancer. Am J Physiol Liver Physiol 308:G351–G363.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41:e1–e1.

Kolida S, Meyer D, Gibson GR. 2007. A double-blind placebo-controlled study to establish the bifidogenic dose of inulin in healthy humans. Eur J Clin Nutr 61:1189.

Konikoff T, Gophna U. 2016. *Oscillospira*: a central, enigmatic component of the human gut microbiota. Trends Microbiol 24:523–524.

Kreider RB, Earnest CP, Lundberg J, Rasmussen C, Greenwood M, Cowan P, Almada AL. 2007. Effects of ingesting protein with various forms of carbohydrate following resistance-exercise on substrate availability and markers of anabolism, catabolism, and immunity. J Int Soc Sports Nutr 4:18.

Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RLV, Knight R. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31:814.

Ley RE. 2016. Gut microbiota in 2015: *Prevotella* in the gut: choose carefully. Nat Rev Gastroenterol Hepatol 13:69.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550.

Macfarlane GT, Macfarlane S. 2012. Bacteria, colonic fermentation, and gastrointestinal health. J AOAC Int 95:50–60.

Magoč T, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27:2957–2963.

Maldonado J, Cañabate F, Sempere L, Vela F, Sánchez AR, Narbona E, López-Huertas E, Geerlings A, Valero AD, Olivares M. 2012. Human milk probiotic *Lactobacillus fermentum*

CECT5716 reduces the incidence of gastrointestinal and upper respiratory tract infections in infants. J Pediatr Gastroenterol Nutr 54:55–61.

Marco ML, Heeney D, Binda S, Cifelli CJ, Cotter PD, Foligné B, Gänzle M, Kort R, Pasin G, Pihlanto A. 2017. Health benefits of fermented foods: microbiota and beyond. Curr Opin Biotechnol 44:94–102.

McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J 6:610.

Merenstein D, Murphy M, Fokar A, Hernandez RK, Park H, Nsouli H, Sanders ME, Davis BA, Niborski V, Tondu F. 2010. Use of a fermented dairy probiotic drink containing *Lactobacillus casei* (DN-114 001) to decrease the rate of illness in kids: the DRINK study A patient-oriented, double-blind, cluster-randomized, placebo-controlled, clinical trial. Eur J Clin Nutr 64:669.

Mullié C, Yazourh A, Thibault H, Odou M-F, Singer E, Kalach N, Kremp O, Romond M-B. 2004. Increased poliovirus-specific intestinal antibody response coincides with promotion of *Bifidobacterium longum-infantis* and *Bifidobacterium breve* in infants: a randomized, double-blind, placebo-controlled trial. Pediatr Res 56:791.

Nagata S, Asahara T, Ohta T, Yamada T, Kondo S, Bian L, Wang C, Yamashiro Y, Nomoto K. 2011. Effect of the continuous intake of probiotic-fermented milk containing *Lactobacillus casei* strain Shirota on fever in a mass outbreak of norovirus gastroenteritis and the faecal microflora in a health service facility for the aged. Br J Nutr 106:549–556.

Nickerson KP, Chanin R, McDonald C. 2015. Deregulation of intestinal anti-microbial defense by the dietary additive, maltodextrin. Gut Microbes 6:78–83.

Nocerino R, Paparo L, Terrin G, Pezzella V, Amoroso A, Cosenza L, Cecere G, De Marco G, Micillo M, Albano F. 2017. Cow's milk and rice fermented with *Lactobacillus paracasei* CBA L74 prevent infectious diseases in children: A randomized controlled trial. Clin Nutr 36:118–125.

Paparo L, Aitoro R, Nocerino R, Maddalena Y, Pezzella V, Amoroso A, Di Scala C, Siciiano E, Buono B, Berni Canani R. 2015. Direct effects of fermented cow's milk with *Lactobacillus paracasei* CBA L74 on human enterocytes. J Pediatr Gastroenterol Nutr 60:732.

Prodeus A, Niborski V, Schrezenmeir J, Gorelov A, Shcherbina A, Rumyantsev A. 2016. Fermented milk consumption and common infections in children attending day-care centers: a randomized trial. J Pediatr Gastroenterol Nutr 63:534.

Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M, de los Reyes-Gavilán CG, Salazar N. 2016. Intestinal short chain fatty acids and their link with diet and human health. Front Microbiol 7:185.

Rossi O, Van Berkel LA, Chain F, Khan MT, Taverne N, Sokol H, Duncan SH, Flint HJ, Harmsen HJM, Langella P. 2016. *Faecalibacterium prausnitzii* A2-165 has a high capacity to induce IL-10 in human and murine dendritic cells and modulates T cell responses. Sci Rep 6:18507.

Schmieder R, Edwards R. 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27:863–864.

Tamang JP, Watanabe K, Holzapfel WH. 2016. Review: Diversity of microorganisms in global fermented foods and beverages. Front Microbiol. 2016; 7: 377.

Thibault H, Aubert-Jacquin C, Goulet O. 2004. Effects of long-term consumption of a fermented infant formula (with *Bifidobacterium breve* c50 and *Streptococcus thermophilus* 065) on acute diarrhea in healthy infants. J Pediatr Gastroenterol Nutr 39:147–152.

Unno T, Hisada T, Takahashi S. 2015. Hesperetin modifies the composition of fecal microbiota and increases cecal levels of short-chain fatty acids in rats. J Agric Food Chem 63:7952–7957.

Vieira AT, Fukumori C, Ferreira CM. 2016. New insights into therapeutic strategies for gut microbiota modulation in inflammatory diseases. Clin Transl Immunol 5:e87.

Vieira AT, Rocha VM, Tavares L, Garcia CC, Teixeira MM, Oliveira SC, Cassali GD, Gamba C, Martins FS, Nicoli JR. 2016. Control of *Klebsiella pneumoniae* pulmonary infection and immunomodulation by oral treatment with the commensal probiotic *Bifidobacterium longum* 51A. Microbes Infect 18:180–189.

CHAPTER 4

Probiotic dietary treatment influences gut dysbiosis in children with non-IgE cow's milk allergy

4.1 Introduction

Food allergy (FA) results from an abnormal immune-mediated reaction against food antigens, such as cow's milk proteins (Sicherer and Sampson, 2014; Renz *et al.*, 2014). Due to its early introduction, cow's milk allergy (CMA) is one of the earliest and most common FA (Agostoni *et al.*, 2014). The immune mechanism of CMA can be IgE-mediated or non-IgE-mediated (cell mediated) and it is recognized as a first indicator of a dysregulated immune response in the pediatric age (Fiocchi *et al.*, 2010). In fact, children affected by CMA in the first year of life have an increased risk to develop other atopic manifestations in their later life (Branum and Lukacs, 2009; Boyce *et al.*, 2011), as well as other chronic immune-mediated disorders such as inflammatory bowel diseases (Virta *et al.*, 2013). Therefore, understanding CMA pathogenesis is important in order to effectively prevent and manage the disease and its later life consequences. The intestinal microbiota plays a critical role in the maturation and continued education of the host immune system (Fulde and Hornef, 2014). Evidence suggests that selected bacterial species and their metabolites from healthy gut microbiota, in particular the short-chain fatty acid butyrate, may positively modulate immune tolerance mechanisms (Feehley *et al.*, 2015; Atarashi *et al.*, 2011; Atarashi *et al.*, 2013; Smith *et al.*, 2013; Wesemann and Nagler, 2016; Berni Canani *et al.*, 2016*d*). On the contrary, emerging data suggest that gut microbiota dysbiosis, characterized by imbalanced composition and function of the intestinal microbes, could be associated to the development of FA (Lynch and Pedersen, 2016; Ling *et al.*, 2014; Fazlollahi *et al.*, 2018; Bunyavanich *et al.*, 2016). Data on gut microbiota features in FA seem still preliminary because the general small number of observations, difference in the experimental tools used, poor characterization of the study subjects and lack of adequate matched controls (Berni Canani *et al.*, 2015*c*). We recently

demonstrated that gut microbiota in IgE-mediated CMA infants shows significantly higher diversity than that of healthy controls. Bacterial families chacteristic of the healthy infant gut, such as *Bifidobacteriaceae* were significantly decreased in the IgE-mediated CMA gut (Berni Canani *et al.*, 2016*d*). Butyrate-producing bacteria were significantly enriched by dietary treatment with extensively hydrolyzed casein formula (EHCF) with the probiotic *Lactobacillus rhamnosus* GG (LGG) (Berni Canani *et al.*, 2016*d*).

In about one third to half of CMA patients a non-IgE-mediated mechanism is recognizable (Nowak-Węgrzyn *et al.*, 2015). Gut microbiota features in children affected by non-IgE-mediated CMA are still poorly characterized. We aimed to comparatively evaluate gut microbiota composition and butyrate production in children affected by non-IgE-mediated CMA and in healthy controls. The impact of treatment with EHCF alone or in combination with LGG was also investigated, and a comparative evaluation of gut microbiota features in IgE- and non-IgE mediated CMA was also performed.

4.2 Materials and Methods

Study subjects

From March to September 2014, 52 consecutive children (age range 1–26 months) visiting our tertiary pediatric allergy center for recent occurrence (last 2–4 weeks) of signs or symptoms of suspected non-IgE-mediated CMA, or for follow up visit after 6 months of exclusion diet upon a confirmed diagnosis of non-IgE-mediated CMA were evaluated and invited to participate in a cross sectional study. The exclusion criteria were: use of pre- or probiotic products and/or antibiotics in the previous 4 weeks; history of cow's milk-induced anaphylaxis and/or other IgE-mediated signs of food allergy; concomitant presence of other food allergies or allergic diseases, eosinophilic disorders of the gastrointestinal tract, chronic systemic diseases, congenital cardiac defects, active tuberculosis, autoimmune diseases, immunodeficiency, chronic inflammatory bowel diseases, celiac disease, cystic fibrosis, metabolic diseases, lactose intolerance, malignancy, chronic pulmonary diseases or malformations of the gastrointestinal tract. Written informed consent was obtained from the parents/guardians of each subject. The diagnosis of non-IgE-mediated CMA was based on clinical history, negative result of skin prick test, and/or negative level of IgE serum-specific anti-cow's milk proteins, and the results of a double blind placebo-controlled oral food challenge (DBPCFC) (Berni Canani *et al.*, 2012*a*; Berni Canani *et al.*, 2017*b*). All DBPCFC

were performed in a double-blind, placebo-controlled manner in the outpatient clinic on 2 separate days with a 1-week interval. Parents of patients taking antihistamines were advised to withhold these medications for 72 hours before and during the challenge. Randomization and preparation of the challenges were performed by experienced dietitians who were not directly involved in the procedures. In detail, every 20 minutes, increasing doses (0.1, 0.3, 1, 3, 10, 30, and 100 mL) of fresh pasteurized cow's milk containing 3.5% of fat or an amino acid formula were administered. Full emergency equipment and medications (epinephrine, antihistamines, and steroids) were available. The results were assessed simultaneously by experienced pediatric allergists. Study subjects were scored for 9 items divided into 4 main categories on a 0 to 3-point scale (0, none; 1, light; 2, moderate; and 3, severe): (1) general (decreased blood pressure plus tachycardia); (2) skin (rash and urticaria/angioedema); (3) gastrointestinal (nausea or repeated vomiting, crampy- like abdominal pain, and diarrhea); and (4) respiratory (sneezing or itching, nasal congestion or rhinorrhea, and stridor deriving from upper airway obstruction or wheezing). If at least 2 of the 3 physicians independently scored one item at level 3 or 2 (or more) items at level 2, the test result was considered positive. Children were observed for up to 4 hours after the final dose and then discharged. In case of a positive DBPCFC result at any testing dose, the patient remained under observation until symptom resolution. If the patient did not show any symptoms within the first 24 hours, parents were advised to provide a single feed of 100 mL of the tested formula (verum or placebo) every day at home for 7 days. If any symptoms occurred during this period, the patients returned to the outpatient clinic on the same day. After 7 days of verum or placebo administration, the patients were examined, and the parents were interviewed at the center. Parents were asked to contact the center if any symptoms occurred in the 7 days after the DBPCFC procedures to rule out false-negative challenge results. The challenge result was considered negative if the patient tolerated the entire challenge, including the observation period. Fifty-two CMA patients were evaluated. Four patients were excluded because of the presence of exclusion criteria, and 2 were excluded for the lack of informed consent. Therefore, 46 CMA patients were included in this study. According to disease state and dietary treatment, CMA patients were divided in three groups: group 1 included patients with non-IgE-mediated CMA at diagnosis, before any therapeutic intervention and receiving standard formula (n = 23); group 2 (n = 9) included patients with diagnosis of non-IgE-mediated CMA after treatment for 6 months with an extensively hydrolyzed casein formula (EHCF; Nutramigen, Mead Johnson Nutrition, Evansville IN, US); group 3 (n = 14) included patients with diagnosis of non-IgE-mediated CMA after treatment for 6 months with EHCF added with the probiotic *L.*

rhamnosus GG (EHCF + LGG; Nutramigen LGG, Mead Johnson Nutrition, Evansville IN, US). The specific formula use was prescribed and adherence was checked according to the standard procedure adopted at our Center. Briefly, the parents received written instructions regarding the commercial name of the product and the formula preparation procedure. Then, the adherence to the treatment was checked monthly during the first 3 months of treatment and then every 6 months. Formula use was evaluated at each time visit by dietitians, counseling parents about issues that could arise during the elimination diet and on how to reach the daily recommended intake for Italian children. This allowed the study staff to evaluate compliance with the formula and to ensure that the patients received an appropriate quantity of formula to meet their nutritional requirements. During the same study period, consecutive healthy children (group 4, n = 23), with negative clinical history for any allergic condition visiting our center because of minimal surgical procedures or vaccination program were also enrolled. Anamnestic, demographic, anthropometric and clinical data were obtained from the parents of each subject and recorded in a clinical database. The 3-day dietary diary was collected from all study subjects at enrolment. All diaries were assessed using a specific software (Winfood, Medimatica srl, Colonnella Teramo, Italy). For all study subjects, a stool sample (3 g) was collected to evaluate gut microbiota composition and fecal butyrate concentration and stored at −80 °C until analyses.

The study was approved by the Ethics Committee of the University of Naples Federico II and was registered in the Clinical Trials Protocol Registration System on March 14, 2014 (https://clinicaltrials.gov - ID number: NCT02087930).

DNA extraction and 16S sequencing

Fecal samples (about 1 g) were fully homogenized in STE buffer (100 mMNaCl, 10 mMTris-Cl pH 8.0, 1 mM EDTA pH 8.0) and centrifuged (500 × g, 1 min) in order to pellet debris. The supernatant was centrifuged again (12,000 × g, 2 min) and the pellet was used for DNA extraction with the PowerFecal DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA). V3-V4 region of the 16S rRNA gene was amplified by using primer and PCR conditions recently described (Berni Canani *et al.*, 2017*e*). PCR products were purified with the Agencourt AMPure XP beads (Beckman Coulter) and quantified using a Plate Reader AF2200 (Eppendorf). Amplicon multiplexing, pooling and sequencing were carried out following the Illumina 16S Metagenomic Sequencing Library Preparation protocol, on a MiSeq platform and using the MiSeq Reagent kit v2, leading to 2 × 250 bp, paired-end reads.

Fecal butyrate analysis

One gram of frozen feces was diluted with saline buffer, vortexed and centrifuged (12,000 × g) for 10 min in 2 ml tubes. The supernatant was filtered (0.45 μm) and stored at −20 °C until analysis. Frozen fecal extracts were acidified with 20 μl of 85% (w/v) phosphoric acid and 0.5 ml of ethyl acetate, mixed, centrifuged (12,000 × g) for 1 h, and extracted in duplicate. About 0.5 ml of the pooled extract containing the acidified butyrate was transferred into a 2 ml glass vial and loaded onto an Agilent Technologies (Santa Clara, CA, USA) 7890 gas chromatograph (GC) system with automatic loader/injector. The GC column was an Agilent J&W DB-FFAP (Agilent Technologies) of 30 m, internal diameter 0.25 mm and film thickness 0.25 μm. The GC was programmed to achieve the following run parameters: initial temperature 90 °C, hold 0.5 min, ramp of 20 °C min^{-1} up to a final temperature of 190 °C, total run time 8.0 min, gas flow 7.7 ml min^{-1} split less to maintain 3.26 p.s.i. column head pressure, septum purge 2.0 ml min^{-1}. Detection was achieved using a flame ionization detector. Peaks were identified using a mixed external standard and quantified by peak height/internal standard ratio.

Statistical and bioinformatics analysis

All data were collected in a dedicated database and analysed by a statistician with IBM SPSS Statistics version 19.0 for Windows (SPSS Inc, Chicago, IL). The $\chi 2$ test and Fisher's exact test were used for categorical variables. The level of significance for all statistical tests was 2-sided, $P < 0.05$.

Raw sequence quality filtering and pre-processing was carried out as recently reported (Berni Canani *et al*., 2017*e*). Briefly, demultiplexed, forward and reverse reads were joined by using FLASH (Magoč and Salzberg, 2011). Joined reads were quality trimmed (Phred score < 20) and short reads (<250 bp) were discarded by using Prinseq (Schmieder and Edwards, 2011). High quality reads were then imported in QIIME (Caporaso *et al*., 2010). OTUs were picked through *de novo* approach and uclust method and taxonomic assignment was obtained by using the RDP classifier and the Greengenes database (McDonald *et al*., 2012), following a pipeline previously reported (Berni Canani *et al*., 2017*e*). In order to avoid biases deriving from different sequencing depth, OTU tables were rarefied to the lowest number of sequences

per sample. Statistical analyses and visualization were carried out in R environment (https://www.r-project.org).

To discriminate the microbial profiles as a function of disease, a model based on projection on latent structures (PLS) in its discriminant (DA) version was built, based on the normalized abundance (log_{10}) of the microbial genera identified. The R package *mixOmics* was used. Permutational Multivariate Analysis of Variance (non-parametric (PER)MANOVA) based on Jaccard and Bray Curtis distance matrices was applied with 999 permutations to detect significant differences in the overall microbial community composition, by using the *adonis* function in *vegan* package. Non-parametric Kruskal-Wallis and pairwise Wilcoxon tests were carried out in order to find OTUs differentially abundant between the groups. A Generalized Linear Model (R function *glm*) was built in order to test the importance of continuous or discrete variables available for the subjects (mode of birth, age at weaning, age at sampling, sex, months of exclusive breastfeeding, average daily consumption of proteins and fat, health status – that is, healthy or CMA) on the relative abundance of bacterial genera significantly different between healthy and CMA subjects. Spearman's pairwise correlations were computed between OTUs or oligotypes and short-chain fatty acid abundance (*corr.test* function in *psych* package). Correction of p-values for multiple testing was performed (Benjamini and Hochberg, 1995). Differences in fecal butyrate levels between the groups were evaluated by non-parametric Kruskal-Wallis and pairwise Wilcoxon tests. In order to compare the gut microbiota composition in children with non-IgE (analyzed in the present study) and IgE-mediated CMA from our previous study (Berni Canani *et al*., 2016*d*), quality filtered reads of the previous study were downloaded from MG-RAST. Since the reads from the previous study included only V4 region of the 16S rRNA gene, they were aligned to those produced in this study, that were trimmed in 5′ direction to the same length. Reads from both the studies were re-analyzed as described above.

Sub-genus diversity of Bacteroides

Reads assigned to *Bacteroides* genus were extracted and entropy analysis and oligotyping (Eren *et al*., 2013) were carried out as described previously (De Filippis *et al*., 2016). After the initial round of oligotyping, high entropy positions were chosen (−C option): 2, 30, 94, 104, 106, 107, 109, 114, 302, 380. To minimize the impact of sequencing errors, we required an oligotype to be represented by at least 100 reads (−M option). Moreover, rare oligotypes present in less than 10 samples were discarded (−s option). These parameters led 70,142

sequences left in the dataset. BLASTn was used to query the representative sequences against the NCBI nr database, and the top hit was considered for taxonomic assignment. Statistical analyses and visualization were carried out in R environment as described above.

Data availability

The 16S rRNA gene sequences produced in this study are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), under accession number SRP092171.

4.3 Results

Study subjects

During a six-month study period, 52 non-IgE-mediated CMA subjects were evaluated for the study. Four were excluded because of the presence of exclusion criteria and 2 were excluded because the lack of informed consent, thus 46 patients were enrolled in the study. According to disease state and dietary treatment, the CMA patients were subdivided in three groups: Group 1 (CMA patients at diagnosis before any dietary intervention) (n = 23); Group 2 (CMA patients treated for 6 months with extensively hydrolyzed casein formula, EHCF) (n = 9); Group 3 (CMA patients treated for 6 months with EHCF containing the probiotic *L. rhamnosus* GG, LGG) (n = 14).

During the same study period, consecutive healthy children, with negative clinical history for any allergic condition visiting our center because of minimal surgical procedures or vaccination program were also enrolled in the study, Group 4 (n = 23).

Main demographic and clinical features of the study subjects and p-value of paired comparisons are reported in Table 4.1. In particular, the age at enrolment, when stool sampling was performed, was similar among groups. All study subjects were weaned. Study subjects enrolled in Group 1 (CMA at baseline before any dietary intervention) were on standard formula at the time of enrolment. The adherence to treatment was optimal in all subjects. Dietary habits were similar among the four groups, with the exception of the type of hypoallergenic formula used for CMA treatment in subjects enrolled in Groups 2 and 3. The hypoallergenic formula was previously prescribed by physicians when CMA diagnosis was confirmed.

The median (minimun-maximum) formula intake was 480 ml (400–500 ml) in Group 2 and 465 ml (400–500 ml) in Group 3. The protein (daily intake of 1–2 g/kg) and fat (daily intake of 2.5–6.0 g/kg) intakes were similar into the 4 study groups. All study subjects were caucasian and were from an urban area. All subjects were single child. Information about exposure to pets and/or history of maternal/infant dietary supplements were reported. Clinical manifestations in all CMA patients enrolled in Groups 1, 2 and 3 were limited to the gastrointestinal tract.

Table 4.1 Main demographic and clinical features of the study population

	Subjects with non IgE-mediated CMA			
	At diagnosis	**Treated with EHCF**	**Treated with EHCF + LGG**	**Healthy subjects**
	Group 1	**Group 2**	**Group 3**	**Group 4**
N.	23	9	14	23
Male, n (%)	12 (52.2)	6 (66.7)	8 (57.1)	9 (39.1)
Age at enrolment, months (SD)	11.4 (7.2)	11.3 (1)	14.1 (5.8)	12.9 (7.4)
Age at diagnosis, months (SD)	11.4 (7.2)	5.3 (1)	8.1 (5.8)	—
Vaginal delivery, n (%)	11 (47.8)	6 (66.7)	5 (35.7)	10 (43.5)
Birth weight, kg (SD)	3.1 (0.3)	2.9 (0.5)	2.9 (0.5)	3.1 (0.4)
Breastfeeding for at least 1 month, n (%)	19 (82.6)	8 (88.9)	9 (64.3)	14 (60.4)
Duration of breastfeeding, months (SD)	4.1 (2.7)	2.12 (2.03)	4.55 (4.1)	3.1 (2.05)
Age at weaning, month (SD)	5 (0.8)	4.9 (0.8)	4.9 (1.2)	4.7 (1)

	p-value					
		Group 1 *vs*		**Group 2 *vs***		**Group 3 *vs***
	Group 2	**Group 3**	**Group 4**	**Group 3**	**Group 4**	**Group 4**
Male, n (%)	0.694	0.769	0.375	1.000	0.243	0.286
Age at enrolment, months (SD)	0.967	0.255	0.483	0.179	0.521	0.634
Age at diagnosis, months (SD)	0.018	0.149	—	0.179	—	—
Vaginal delivery, n (%)	0.444	0.471	0.767	0.214	0.433	0.641
Birth weight, kg (SD)	0.171	0.095	0.937	0.942	0.201	0.114
Breastfeeding for at least 1 month, n (%)	1.000	0.255	0.102	0.340	0.210	0.835
Duration of breastfeeding, months (SD)	0.079	0.733	0.246	0.150	0.309	0.260
Age at weaning, month (SD)	0.724	0.661	0.323	0.944	0.681	0.741
Familiar allergy risk, n (%)	1.000	1.000	0.522	1.000	0.685	0.713

p-values of paired t-test were reported for all variables.

Gut microbiota of children with non-IgE-mediated CMA differs from that of healthy controls

Non-IgE-mediated CMA children at diagnosis, before dietary treatment, presented significant differences in gut microbial composition when compared to healthy controls, while the alpha diversity of the microbiota was not associated with the health status (data not shown). A PLS-DA model was able to discriminate healthy from non-IgE-mediated CMA subjects (Figure 4.1). Only one bacterial *phylum*, Bacteroidetes, was significantly enriched in non-IgE-mediated CMA patients (Wilcoxon pairwise tests, $p < 0.05$, data not shown). However, at the level of genus, two Bacteroidetes genera, *Bacteroides* and *Alistipes*, and a single Firmicutes,

Sarcina, were significantly enriched in non-IgE-mediated CMA when compared to healthy controls (Wilcoxon pairwise tests, $p<0.05$, data not shown).

We applied a Generalized Linear Model (GLM) for *Bacteroides* abundance against eight features, including protein and fat consumption, mode of delivery, sex, age, age at weaning, breastfeeding duration and health status, to compare between non-IgE-mediated CMA (group 1) and healthy controls (group 4). Health status (healthy or non-IgE-mediated CMA) described the majority of the variance in the relative abundance of *Bacteroides* between these cohorts (Figure 4.2).

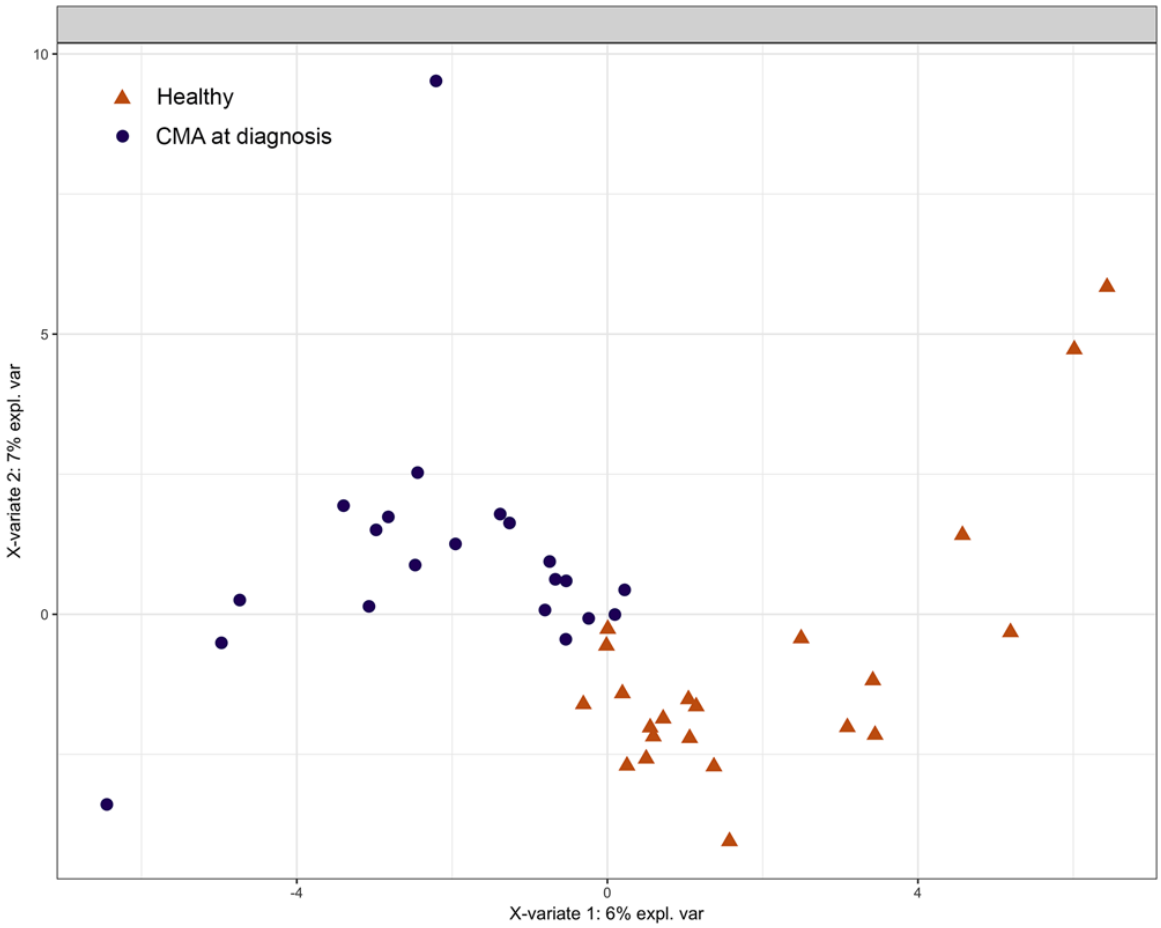


Figure 4.1 Score plot of the sPLS-DA model based on the microbiota composition at genus level of healthy and non-IgE mediated CMA subjects.

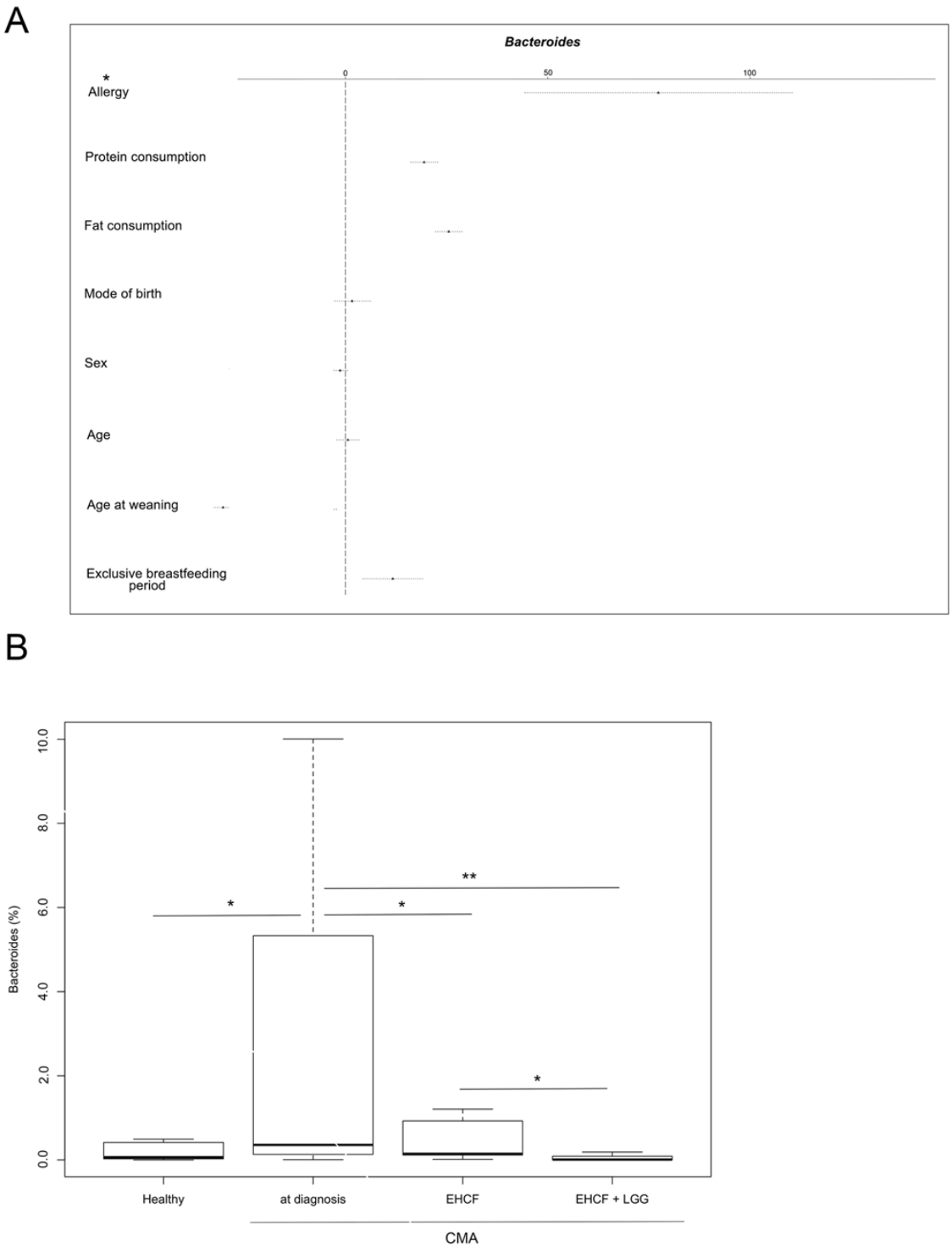


Figure 4.2 Generalized linear model fitting of patient demographic information across relative abundance of *Bacteroides* (A) and box plots showing the abundance of *Bacteroides* (B). In panel A, parallel x axis represents the relative contribution value of every factor, as predicted by the GLM model (*$p < 0.05$). In panel B, boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.5 x IQR from the first and third quartiles, respectively. Asterisks indicate a significant difference as obtained by pairwise Wilcoxon test ($p < 0.05$).

Dietary management and gut microbiota composition in children with non-IgE-mediated CMA

The abundance of *Bacteroides* and *Alistipes* significantly decreased with both dietary supplementation (data not shown) compared to initial non-IgE mediated CMA samples at diagnosis. However, the relative abundance of both *Bacteroides* and *Alistipes* was significantly lower in the samples from patients treated with EHCF + LGG (Wilcoxon pairwise tests, $p < 0.05$; Figure 4.2). In addition, EHCF + LGG treated patients showed a significantly greater relative abundance of *Lachnospira*, *Ruminococcus*, *Oscillospira* compared to patients given EHCF alone ($p < 0.05$, Supplementary Material). Finally, *Lactobacillus* was observed at a greater relative abundance in EHCF + LGG treated children (data not shown).

Sub-genus diversity of Bacteroides differentiates healthy and non-IgE-mediated CMA subjects

As *Bacteroides* had the strongest statistical association with non-IgE-mediated CMA, we further stratified the sequences annotated to this genus using oligotyping analysis. A total of 29 *Bacteroides* oligotypes were identified, and the diversity in oligotype composition was not associated to the relative abundance of the genus (data not shown). CMA children maintained a greater average number of *Bacteroides* oligotypes compared to healthy subjects (11.9 vs 4.4, respectively; Wilcoxon test, $p < 0.001$), and the oligotypes that were enriched substantially differentiated healthy versus CMA children (Fig. 3). In particular, oligotypes Bac10 and Bac12 were significantly enriched and Bac8 and Bac9 were significantly reduced in CMA at diagnosis ($p < 0.05$). Both dietary interventions altered the oligotype diversity of *Bacteroides*, but EHCF + LGG resulted in a *Bacteroides* diversity pattern similar to that seen in healthy controls (Figure 4.3). Indeed, the abundance of oligotypes associated with CMA (Bac10 and Bac12) was significantly reduced compared with CMA at diagnosis upon both the treatments ($p < 0.05$), but only EHCF + LGG resulted in an abundance of oligotype Bac8 similar to that found in the healthy controls ($p > 0.05$). Oligotype Bac9 also increased but was still lower than the controls ($p < 0.05$). Oligotype representative sequences were queried against the NCBI nr database and 11 different *Bacteroides* species were identified, some showing exact match (100% identity on the whole length), with sequences in the database (data not shown). Overall 11 of the oligotypes were most similar to sequences of species belonging to *B. fragilis* group (data not shown).

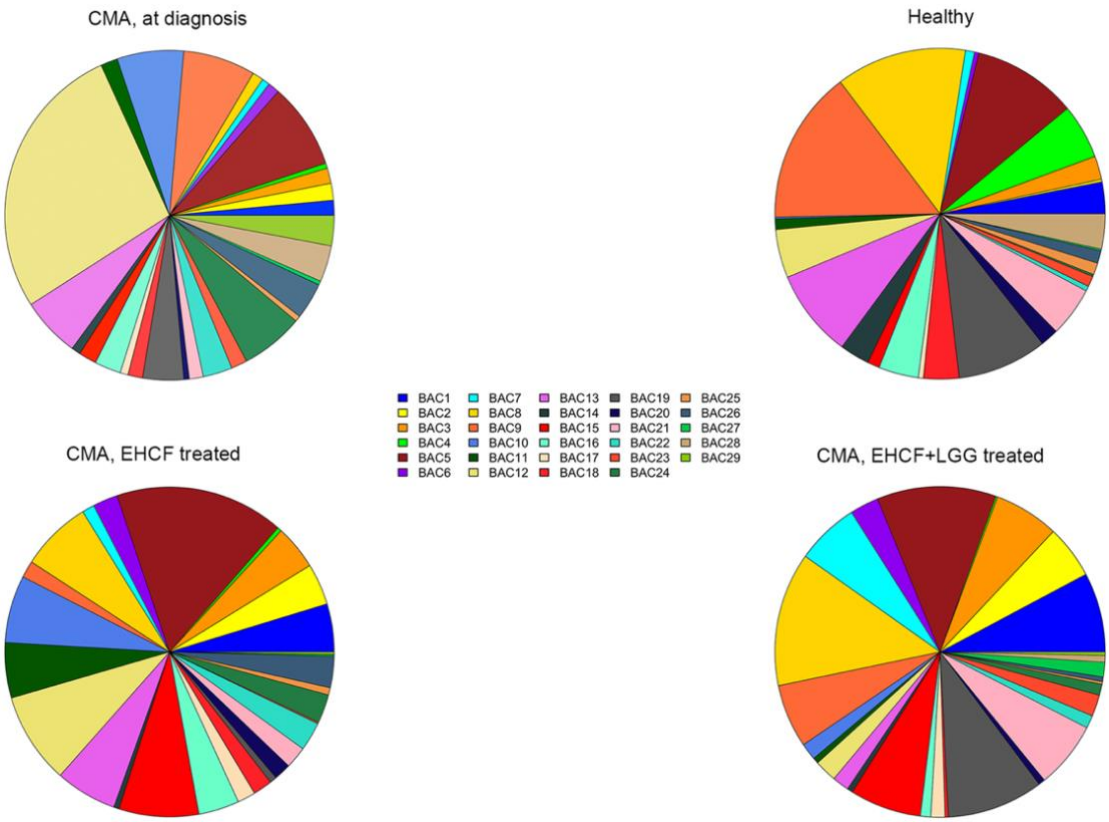


Figure 4.3 Pie charts showing the abundance of *Bacteroides* oligotypes in the different subject categories.

Dietary treatments, fecal butyrate concentration and correlation with specific gut bacteria

Children with non-IgE-mediated CMA had a significantly lower fecal concentration of butyrate compared to healthy controls (pairwise Wilcoxon tests, $p < 0.05$). While both dietary regimens were associated to a significant increase in butyrate concentrations, the result was more evident in children treated with EHCF + LGG (Figure 4.4). Butyrate concentration was significantly correlated to the relative abundance of *Lachnospira* and two *Bacteroides* oligotypes (Bac7 and Bac8) that were enriched in EHCF + LGG treated children. On the contrary, the relative abundance of oligotype Bac12, which was enriched in the CMA group, was negatively correlated to butyrate concentration.

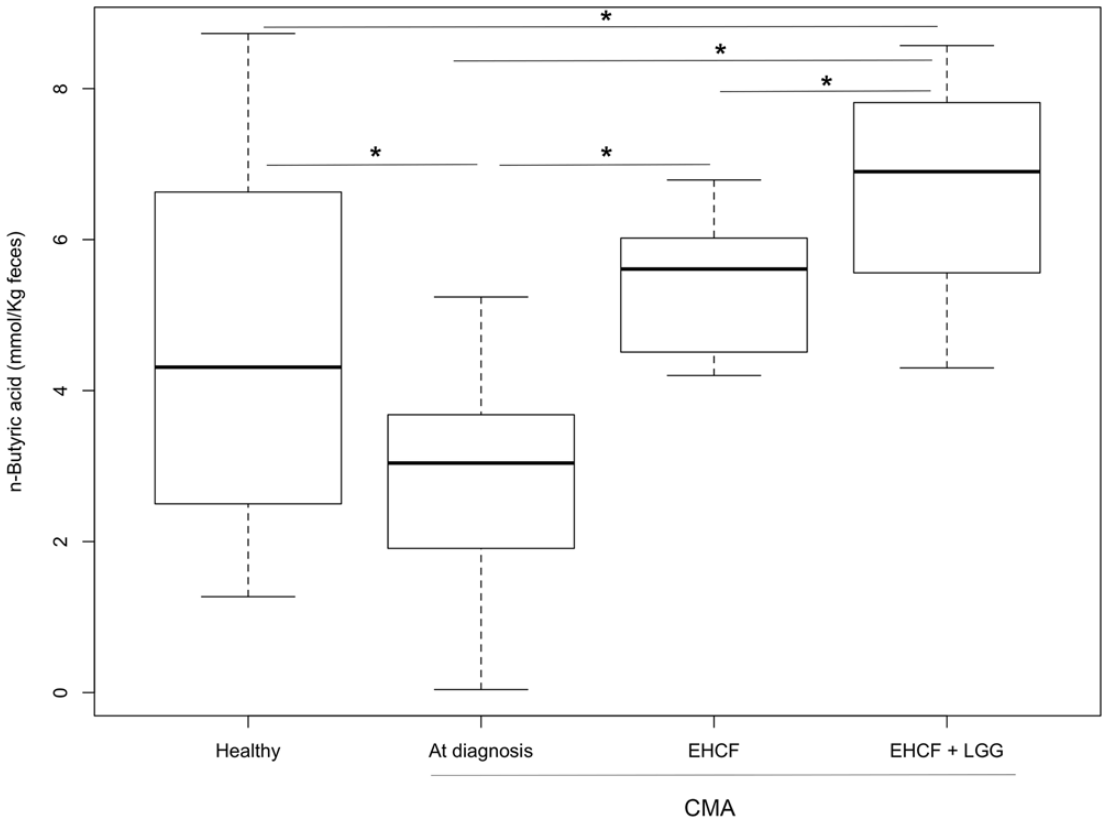


Figure 4.4 Box plots showing faecal butyrate concentration in CMA, healthy and treated children (*p < 0.05). For a description of the box plots, see Figure 4.2 legend.

Gut microbiota features overlaps in IgE and non-IgE-mediated CMA children

The sequence data from this study were re-analyzed alongside data produced in a previous study to compare the microbiota in non-IgE-mediated CMA *vs*. IgE-mediated CMA patients (Berni Canani *et al*., 2016*d*). Healthy subjects from both studies clustered together in a hierarchical clustering based on Ward distance (Figure 4.5). IgE-mediated CMA children at diagnosis and after treatment clearly clustered apart, indicating strong differences in gut microbiota composition, while non-IgE-CMA patients (with or without treatment) were more similar to healthy subjects (Figure 4.5). This progressive gradient of dysbiosis was also clear in the PLS-DA model, where non-IgE-CMA subjects were closer to the healthy controls and separated from IgE-mediated CMA children (data not shown). Accordingly, the average weighted Unifrac distance between IgE-mediated CMA and healthy subjects was significantly higher than that between non-IgE-CMA and healthy controls (0.68 ± 0.04 and 0.49 ± 0.08, respectively; $p < 0.05$). Interestingly, overlapping features characterized the gut microbiota dysbiosis in the two forms of CMA. In particular, a significant enrichment in *Bacteroides* was observed from healthy to non-IgE-mediated, and then to IgE-mediated CMA profiles (Figure 4.6). *Alistipes, Fusobacterium* and *Bilophila* were significantly enriched in IgE-mediated

compared to non-IgE-mediated CMA subjects (Wilcoxon test, $p < 0.05$; Supplementary Material), while *Eubacterium*, *Blautia*, *Akkermansia* and *Raoultella* resulted increased in non-IgE-mediated CMA patients (data not shown).

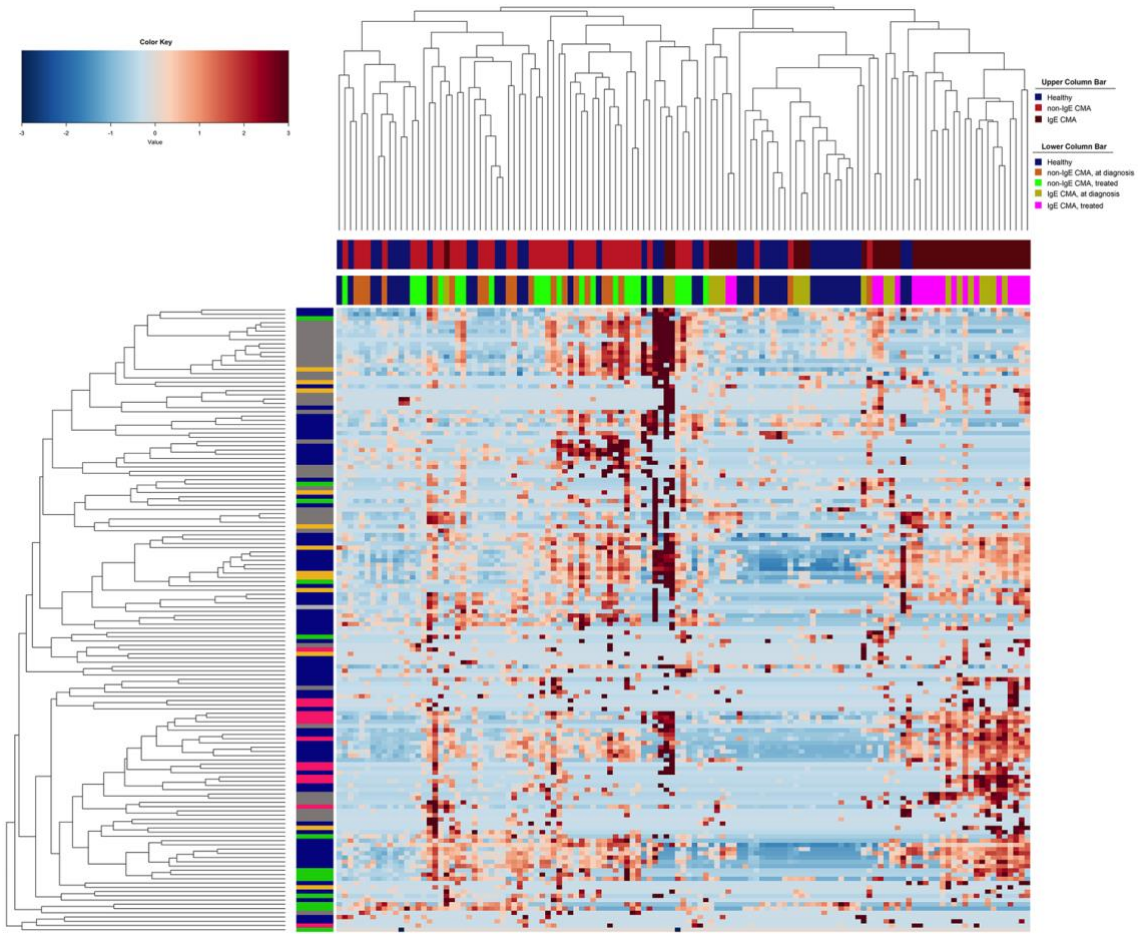


Figure 4.5 Hierarchical McQuitty-linkage clustering of the samples based on the Pearson's correlation coefficient of the abundance of OTUs present in at least 10% of the samples. Subjects from a previously published study (Berni Canani *et al*., 2016*d*) were included. The color scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance and blue indicating low abundance. Column bars are colored according to the subject categories. Row bar is colored according to the *phylum*: Actinobacteria, green; Bacteroidetes, red; Firmicutes, navy blue; Proteobacteria, grey; others, orange.

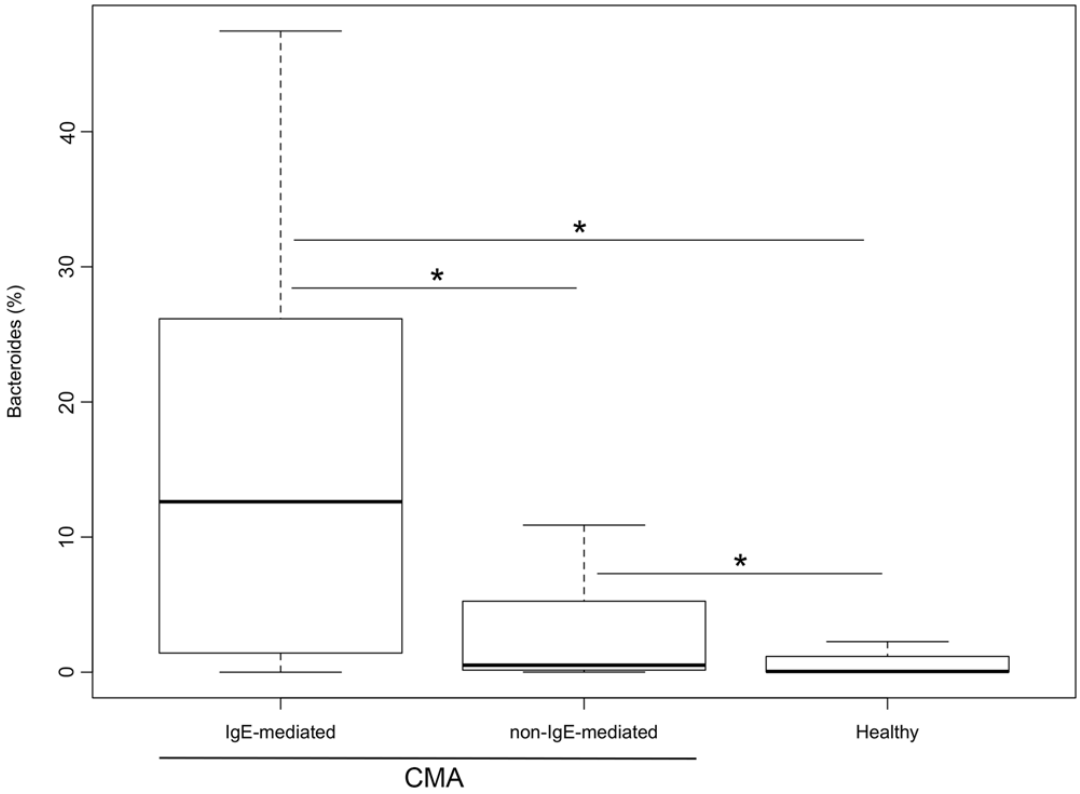


Figure 4.6 Box plots showing the abundance of *Bacteroides* in healthy, non-IgE mediated and IgE mediated CMA subjects (*p < 0.05). Subjects from a previously published study (Berni Canani *et al*., 2016*d*) were included. For a description of the box plots, see Figure 4.2 legend.

4.4 Discussion

We are witnessing a dramatic and apparently ongoing increase in the prevalence of FA (Wood, 2015), but the cause of this increase is still largely undefined. Recent evidence has emphasized the role of intestinal bacteria in the prevention or treatment of FA, and there is mounting evidence that microbial dysbiosis early in life represents a critical factor underlying FA development (Prince *et al*., 2015; Di Costanzo *et al*., 2016). We observed that children with non-IgE-mediated CMA had elevated relative abundances of *Bacteroides* and *Alistipes*. Different sub-genus patterns of *Bacteroides* were associated with CMA. An increase in *Bacteroides* has been associated with peanut and tree nut allergy and other atopic manifestations (Hua *et al*., 2016; Odamaki *et al*., 2008; Kirjavainen *et al*., 2002), and *Bacteroides* species are reported to alter gut permeability (Hua *et al*., 2016; Odamaki *et al*., 2008; Kirjavainen *et al*., 2002; Curtis *et al*., 2014). Conversely, Ling and co-workers (Ling *et al*., 2014) reported a decrease in Bacteroidetes in a cohort of Chinese children characterized by different types of FA. These discrepancies may be due to different variable regions of 16S rRNA gene targeted, to the low number of children evaluated in the study (non-IgE-mediated CMA children, n=4), and to different dietary patterns or ethnicity (Ling *et al*., 2014). We

found that the relative abundance of *Bacteroides* was higher in children with IgE-mediated CMA compared to patients with non-IgE-mediated CMA and healthy controls, suggesting a key role of this genus in CMA pathogenesis and pointing to potential common pathways predisposing to both non-IgE- and IgE-mediated FA. Interestingly, a transition to IgE serum level positivity has been demonstrated in up to 30% of non-IgE-mediated FA subjects (Nowak-Węgrzyn *et al*., 2015).

Both EHCF and EHCF + LGG treatments influenced gut dysbiosis in non-IgE-mediated CMA children, but the result was more pronounced in patients treated with EHCF + LGG. Remarkably, the treatment with EHCF + LGG appeared to restore the *Bacteroides* sub-genus composition and structure, which exhibited diversity similar to that shown by the healthy controls.

Bacterial metabolites are an important communication tool between the commensal microbiota and the host immune system and establish a broad basis for mutualism (Smith *et al*., 2013). Short chain fatty acids (SCFAs) are among the most abundant microbial metabolites and play a critical role in mucosal integrity, local and systemic metabolic function and regulation of immune response (Wesemann and Nagler, 2016; Geuking *et al*., 2013; Furusawa *et al*., 2013; Maslowski and Mackay; 2011). In agreement with previous findings (Berni Canani *et al*., 2016*d*), EHCF + LGG treatment significantly increased butyrate production. This increase correlated with an enrichment of potential SCFA-producers as well as selected *Bacteroides* oligotypes. Previous clinical findings showed that dietary management with EHCF + LGG results in a higher rate of tolerance acquisition in infants with non-IgE-mediated CMA (Berni Canani *et al*., 2012*a*; Berni Canani *et al*., 2013*b*).

The use of a well characterized and homogeneous study population without ethnic diversities and with similar environmental influences (all weaned and living in urban area, similar breastfeeding rate, single child, no pets and no history of maternal/infant dietary supplements) represents a major strength of this study. Conversely, the relatively small number of subjects and the cross-sectional design are the major limitations. Longitudinal cohort studies in children with CMA are advocated and could better assess the development of gut microbiota during the disease course, and also in response to different therapeutic dietary strategies for CMA treatment. Moreover, although the age at enrolment (when faecal samples collection was done) was similar among the groups, we detected a significant difference in the age at diagnosis between Group 1 and 2, that might have affected the differences observed in the gut microbiota.

4.5 Conclusion

Our data support the hypothesis that gut microbiota dysbiosis could be a relevant target of treatment in CMA and that EHCF + LGG-based diet can be an efficient strategy for microbiome-targeted intervention. Integrating these data with data generated through transcriptome, epigenome, and metabolome investigations, will facilitate our understanding of FA and might drive the development of new preventive and therapeutic strategies.

4.6 Notes

This chapter reports the content of paper entitled "Gut microbiota composition and butyrate production in children affected by non-IgE-mediated cow's milk allergy" by Berni Canani R, De Filippis F, Nocerino R, Paparo L, Di Scala C, Cosenza L, Della Gatta G, Calignano A, De Caro C, Laiola M, Gilbert JA and Ercolini D (2018) published in Scientific reports 8.1:12500

4.7 References

Agostoni C, Goulet O, Kolacek S, Koletzko B, Moreno L, Puntis J, Rigo J, Shamir R, Szajewska H, Turck D. 2007. Fermented infant formulae without live bacteria. J Pediatr Gastroenterol Nutr 44:392–397.

Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, Deroos P, Liu H, Cross JR, Pfeffer K, Coffer PJ. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 504:451.

Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y. 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. Science (80-) 331:337–341.

Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K. 2013. T reg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature 500:232.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 57:289–300.

Berni Canani R, Nocerino R, Terrin G, Coruzzo A, Cosenza L, Leone L, Troncone R. 2012. Effect of *Lactobacillus* GG on tolerance acquisition in infants with cow's milk allergy: a randomized trial. J Allergy Clin Immunol 129:580–582.*a*

Berni Canani R, Nocerino R, Terrin G, Frediani T, Lucarelli S, Cosenza L, Passariello A, Leone L, Granata V, Di Costanzo M. 2013. Formula selection for management of children

with cow's milk allergy influences the rate of acquisition of tolerance: a prospective multicenter study. J Pediatr 163:771–777.*b*

Berni Canani R, Gilbert JA, Nagler CR. 2015. The role of the commensal microbiota in the regulation of tolerance to dietary allergens. Curr Opin Allergy Clin Immunol 15:243.*c*

Berni Canani R, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, Calignano A, Khan AA, Gilbert JA, Nagler CR. 2016. *Lactobacillus rhamnosus* GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. ISME J 10:742.*d*

Berni Canani R, De Filippis F, Nocerino R, Laiola M, Paparo L, Calignano A, De Caro C, Coretti L, Chiariotti L, Gilbert JA. 2017. Specific signatures of the gut microbiota and increased levels of butyrate in children treated with fermented cow's milk containing heat-killed *Lactobacillus paracasei* CBA L74. Appl Environ Microbiol 83:e01206-17.*e*

Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, Plaut M, Cooper SF, Fenton MJ, Arshad SH. 2011. Guidelines for the diagnosis and management of food allergy in the United States: summary of the NIAID-sponsored expert panel report. J Am Acad Dermatol 64:175–192.

Branum AM, Lukacs SL. 2009. Food allergy among children in the United States. Pediatrics 124:1549–1555.

Bunyavanich S, Shen N, Grishin A, Wood R, Burks W, Dawson P, Jones SM, Leung DYM, Sampson H, Sicherer S. 2016. Early-life gut microbiome composition and milk allergy resolution. J Allergy Clin Immunol 138:1122–1130.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335.

Curtis MM, Hu Z, Klimko C, Narayanan S, Deberardinis R, Sperandio V. 2014. The gut commensal *Bacteroides thetaiotaomicron* exacerbates enteric infection through modification of the metabolic landscape. Cell Host Microbe 16:759–769.

De Filippis F, Pellegrini N, Laghi L, Gobbetti M, Ercolini D. 2016. Unusual sub-genus associations of faecal *Prevotella* and *Bacteroides* with specific dietary patterns. Microbiome 4:57.

Di Costanzo M, Amoroso A, Canani RB. 2016. Gut microbiota as a target for food allergy. J Pediatr Gastroenterol Nutr 63:S11–S13.

Eren AM, Maignien L, Sul WJ, Murphy LG, Grim SL, Morrison HG, Sogin ML. 2013. Oligotyping: differentiating between closely related microbial taxa using 16S rRNA gene data. Methods Ecol Evol 4:1111–1119.

Fazlollahi M, Chun Y, Grishin A, Wood RA, Burks AW, Dawson P, Jones SM, Leung DYM, Sampson HA, Sicherer SH. 2018. Early-life gut microbiome and egg allergy. Allergy 73:1515–1524.

Feehley T, Aitoro R, Stefka A, Nagler C. 2015. Bacterial metabolites reduce intestinal permeability to protect against food allergen sensitization (MUC8P. 724). Am Assoc Immnol.

Fiocchi A, Brozek J, Schünemann H, Bahna SL, Von Berg A, Beyer K, Bozzola M, Bradsher J, Compalati E, Ebisawa M. 2010. World Allergy Organization (WAO) diagnosis and rationale for action against cow's milk allergy (DRACMA) guidelines. World Allergy Organ J 3:57.

Fulde M, Hornef MW. 2014. Maturation of the enteric mucosal innate immune system during the postnatal period. Immunol Rev 260:21–34.

Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T. 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 504:446.

Geuking MB, McCoy KD, Macpherson AJ. 2013. Metabolites from intestinal microbes shape Treg. Cell Res 23:1339.

Hua X, Goedert JJ, Pu A, Yu G, Shi J. 2016. Allergy associations with the adult fecal microbiota: analysis of the American Gut Project. EBioMedicine 3:172–179.

Kirjavainen P V, Arvola T, Salminen SJ, Isolauri E. 2002. Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning? Gut 51:51–55.

Ling Z, Li Z, Liu X, Cheng Y, Luo Y, Tong X, Yuan L, Wang Y, Sun J, Li L. 2014. Altered fecal microbiota composition associated with food allergy in infants. Appl Environ Microbiol 80:2546–2554.

Lynch S V, Pedersen O. 2016. The human intestinal microbiome in health and disease. N Engl J Med 375:2369–2379.

Magoč T, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27:2957–2963.

Maslowski KM, Mackay CR. 2011. Diet, gut microbiota and immune responses. Nat Immunol 12:5–9.

McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J 6:610.

Nowak-Węgrzyn A, Katz Y, Mehr SS, Koletzko S. 2015. Non–IgE-mediated gastrointestinal food allergy. J Allergy Clin Immunol 135:1114–1124.

Odamaki T, Xiao J-Z, Sakamoto M, Kondo S, Yaeshima T, Iwatsuki K, Togashi H, Enomoto T, Benno Y. 2008. Distribution of different species of the *Bacteroides fragilis* group in individuals with Japanese cedar pollinosis. Appl Environ Microbiol 74:6814–6817.

Prince BT, Mandel MJ, Nadeau K, Singh AM. 2015. Gut microbiome and the development of food allergy and allergic disease. Pediatr Clin 62:1479–1492.

Renz H, Allen KJ, Sicherer SH, Sampson HA, Lack G, Beyer K, Oettgen HC. 2018. Food allergy. Nat Rev Dis Prim 4:17098.

Schmieder R, Edwards R. 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27:863–864.

Sicherer SH, Sampson HA. 2014. Food allergy: epidemiology, pathogenesis, diagnosis, and treatment. J Allergy Clin Immunol 133:291–307.

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS. 2013. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science (80-) 341:569–573.

Virta LJ, Ashorn M, Kolho K-L. 2013. Cow's milk allergy, asthma, and pediatric IBD. J Pediatr Gastroenterol Nutr 56:649–651.

Wesemann DR, Nagler CR. 2016. The microbiome, timing, and barrier function in the context of allergic disease. Immunity 44:728–738.

Wood RA. 2016. Advances in food allergy in 2015. J Allergy Clin Immunol 138:1541–1547.

CHAPTER 5

Impact of a Mediterranean diet intervention on gut microbiome and metabolome

5.1 Introduction

Diet is a fundamental factor affecting gut health. Mounting evidence highlights that diets richer in plant- rather than animal-based foods could represent healthier choices to prevent disease (Kelly *et al.*, 2017; Chiavaroli *et al.*, 2019). The Mediterranean diet (MD) is a recommended nutritional pattern with evidence of beneficial effects including the prevention of several types of disease, such as cardiovascular disease (CVD), type 2 diabetes, obesity, inflammatory diseases, degenerative diseases and cancer (Bendall *et al.*, 2018; Eleftheriou *et al.*, 2018; Martínez-González *et al.*, 2019).

The microbiome partly but significantly affects individual metabolism and how one responds to changes in dietary habits (David *et al.*, 2014; Cani, 2018). Host health is influenced by microbiome composition and by microbial metabolites that can be produced from host metabolic intermediates or from dietary precursors (Roager and Dragsted, 2019). Therefore, current trends in personalized nutrition suggest that diet can be used to modulate microbiome composition and function (Bashiardes *et al.*, 2018; Johnson *et al.*, 2019). Indeed, the production of beneficial microbial metabolites can be increased, and the production of detrimental metabolites can be reduced by modulating nutrient intake and supplying a beneficial pattern of key precursors to the microbiome.

The current knowledge of the role of diet on microbiome-mediated health outcomes in humans mainly relies on observational studies in which confounding affects the conclusions (Grosso *et al.*, 2017). Intervention studies to address the causal effects of diet on microbiome functions are still scarce or have been performed in animal models, and this lack of knowledge also applies to the MD (De Filippis *et al.*, 2018; Bailey and Holscher, 2018). Despite their cost and labour-intensiveness, randomized controlled trials (RCTs) are the gold standard for evidence-based medicine and are an appropriate tool for identifying a causal

relationship of a specific nutrient/diet on a health outcome in humans (Harris *et al*., 2001; Blumberg *et al*., 2010).

A Western diet is characterized by an excessive intake of foods with a high energy density and that are rich in fats, sugars, and animal proteins, as well as a very low intake of fruits and vegetables. Such a dietary style, accompanied by low levels of physical activity, promotes inflammation and predisposes individuals to obesity, CVD, type 2 diabetes and metabolic syndrome (Myles, 2014; Minihane *et al*., 2015; Mozaffarian *et al*., 2011). Because obesity is highly prevalent worldwide and is recognized as an independent risk factor for metabolic-driven chronic diseases, efforts need to be made urgently to provide evidence-based recommendations for healthy dietary patterns.

The aim of this study was to evaluate the effect of an individually tailored MD intervention in subjects at increased risk of cardiovascular disease.

5.2 Materials and Methods

Study design and population

We investigated the gut microbiome, faecal, blood and urinary metabolomic profiles in 82 overweight/obese subjects in response to an 8-week isocaloric dietary intervention with a MD or a control diet. The trial was conducted at the University of Naples Federico II and was approved by the related Ethics Committee (Protocol number: 108/16). Each participant provided written informed consent and received no financial compensation. The trial was registered at ClinicalTrials.gov (number NCT03071718). The protocol ended when the last group of participants completed the protocol (Study Start Date: June 2016; Actual Primary Completion Date: July 2017; Actual Study Completion Date: February 2019).

The study design, selection criteria and participant flow throughout the study are reported in the online Supplementary Materials (Supplementary Figure 1). Plasma lipids (including plasma cholesterol and triglycerides) and faecal levels of short-chain fatty acids (SCFAs) were registered as primary outcomes of the study, while changes in gut microbiota and some intermediate markers of metabolic disease, such as blood pressure, fasting blood glucose, serum high sensitivity C-reactive protein (hs-CRP), urinary and plasma trimethylamine oxide (TMAO), plasma gastrointestinal peptides, and urinary polyphenols, were secondary outcomes (detection methods described in the Supplementary material). Briefly, 334 potentially eligible adults were screened on the basis of the inclusion/exclusion criteria, including medical and lifestyle conditions (*i.e.*, habitual diet and physical activity) (detailed

criteria are in the Supplementary Materials). Adherence to the MD was estimated by using the 11-unit dietary score and is reported as the Italian Mediterranean Index (MD index) (Agnoli *et al*., 2011).

Eighty-two subjects (43 female and 39 male, average BMI 31.1±4.5 kg/m2, age 43±12 y, further baseline features in the Supplementary Table 1) were selected, enrolled and randomised between the two intervention arms of the parallel study design, *i.e.* MedD or ConD.

Dietary intervention

Each participant in the MedD group consumed an individually tailored diet that maintained the daily energy and macronutrient intake of the habitual diet and guaranteed a dietary pattern typical of the MD. Participants in the ConD group were asked to maintain their habitual diet. Individual compliance with the protocol was assessed every 2 weeks by self-recorded 7-d food diaries and physical activity questionnaires. Visits and sample collection were performed at baseline, 4 weeks and 8 weeks (full details reported in the Supplementary Material).

Metabolomics

Untargeted urine, serum and faeces metabolomics as well as targeted quantification of bile acids (BAs) and SCFAs in the faeces were performed by ultra-high-performance liquid chromatography mass spectrometry (UHPLC-MS) (Andersen *et al*., 2014; Barri *et al*., 2013; Christiansen *et al*., 2018; Hjerpsted *et al*., 2016). Trimethylamine N-oxide (TMAO), carnitine, choline, creatinine, betaine in plasma and urine as well as urinary urolithins were also determined by targeted metabolomics using liquid chromatography tandem mass spectrometry (LC-MS/MS). Details are available in the Supplementary Materials. Metabolomics untargeted data were yielded at the University of Copenaghen (Denmark).

Metagenomics

A full description of the sampling, sequencing and data analysis procedures is reported in the Supplementary Materials. DNA libraries were sequenced using the Ion Proton Sequencer (ThermoFisher Scientific, Waltham, US), with a minimum of 20 million 150-bp high-quality reads generated per library. Metagenomic species pangenome (MSP) was used to identify and

quantify species associated with the 9.9-million-gene integrated reference catalogue (Plaza Oñate *et al*., 2018). The functional potentials of the intestinal gut microbiota were determined by using the in-house FAnToMet pipeline as described in the Supplementary Materials. High throughput sequencing, MSP and functional potentials determination were performed at MetaGenoPolis center (INRAE, Jouy-en-Josas, France)

Statistical analysis

Statistical analysis and visualization were carried out in R environment version 3.4.2 (https://www.r-project.org). *ggpubr* and *PMCMR* R packages were used to assess significant differences. Variations in dietary and clinical variables at specific timepoints compared to baseline values between the MedD and ConD groups were evaluated by 2-way ANOVA with repeated measures and Tukey's *post hoc* test. Non-parametric Wilcoxon signed-rank test (*testRelations* function of *momr* R package) was performed to compare means between ConD and MedD subjects at each time point, while the *post hoc* Nemenyi test for multiple comparisons following the Friedman test was used within each group.

Pairwise Spearman's rank correlations were used to estimate the overall similarity of the microbiome and metabolome within the MedD and ConD groups and between time points (baseline vs 4 weeks and 4 weeks vs 8 weeks). The same test was applied to the microbiome, dietary variables, clinical markers and targeted metabolome datasets. Adjustments were performed using the Benjamini-Hochberg procedure. Correlations were visualised using the ComplexHeatmap package (Gu *et al*., 2016).

Machine learning-based classification (Pasolli *et al*., 2016) of metabolomics data and further details on data analysis and visualization are provided in the Supplementary Materials from Meslier *et al*., 2020).

5.3 Results

MD lowered plasma cholesterol in the overall population

No significant differences in anthropometric measures or clinical variables monitored in blood and urine samples were observed between the ConD (n=39) and MedD (n=43) groups at baseline (Supplementary Table 1). Regarding the primary outcomes, as a consequence of the intervention, the participants in the MedD showed a significant decrease in total plasma cholesterol (Figure 5.1) and HDL-cholesterol after 4 weeks compared to the ConD group

(Supplementary Table 1). No changes in any of the secondary outcomes such as blood glucose, serum hs-CRP, plasma insulin, TMAO or any intermediate markers of metabolic disease (glucagon, ghrelin, GIP, GLP-1, leptin, C-peptide, resistin, visfatin and PAI-1) were observed (Supplementary Table 1).

Compliance throughout the intervention and MD adherence-based analysis

Adherence to the MD significantly increased in the MedD group at 4 weeks and 8 weeks compared to the baseline (Figure 5.1) and was highly correlated with the Healthy Food Diversity (HFD) index (Supplementary Figure 2) (Drescher *et al*., 2007). Significant percentage changes in dietary and metabolic variables are shown in Figure 1B. Participants in the MedD group significantly increased their daily intake of dietary fibre by 2-fold and their dietary vegetable:animal protein ratio by 2.5-fold over the intervention compared to the ConD group ($p<0.005$, Figure 5.1). A significant reduction in saturated fat intake and an increase in polyunsaturated fat intake was also achieved ($p<0.005$, Figure 5.1). These changes in nutrient intake in the MedD vs the ConD group were due to increased consumption of fruits, vegetables, nuts, wholegrain cereals and fish products concurrent with reduced consumption of refined cereals, dairy and meat products. The reduced consumption of meat products was confirmed by the reduction in the biomarker of the intake of these foods in the MedD vs ConD group, *i.e.*, the concentration of carnitine in the plasma (14% and 11% reductions after 4 and 8 weeks, $p<0.05$ and $p<0.005$, respectively, Figure 5.1) and urine (75% and 51% reductions after 4 and 8 weeks, $p<0.01$ and $p<0.005$, respectively, Figure 5.1).

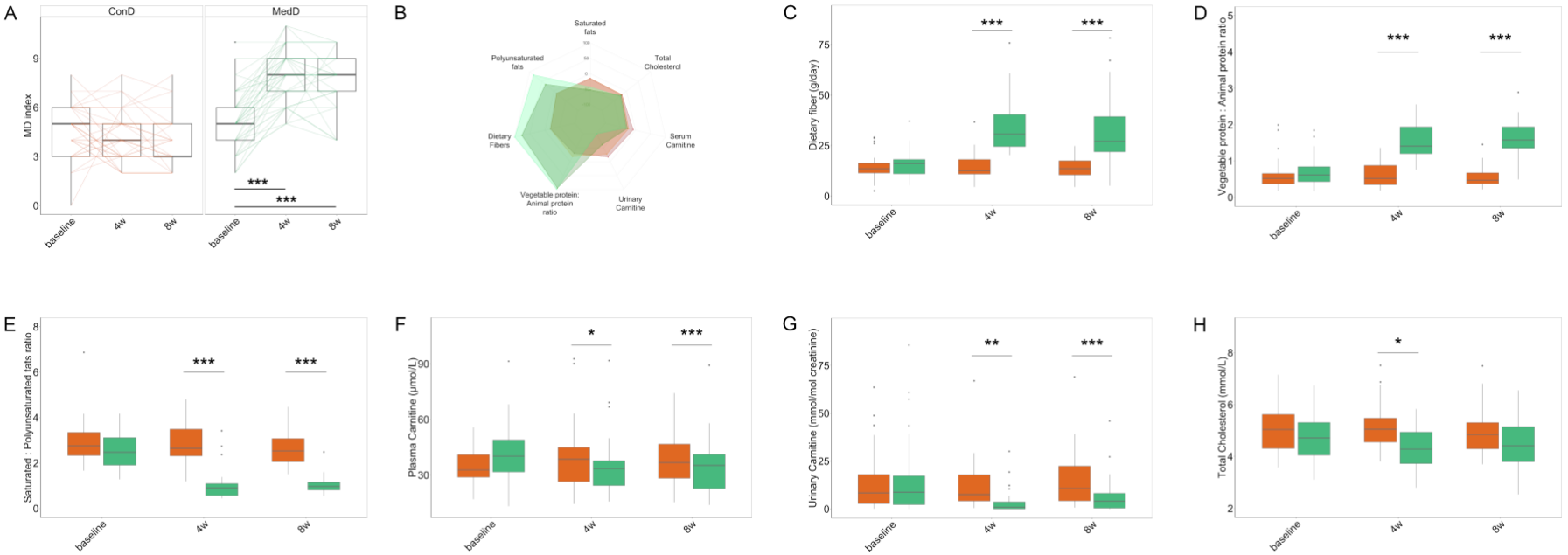


Figure 5.1 Adherence to the Mediterranean diet (MD) and changes in dietary and metabolic variables. (A) Box plots showing MD index score for controls (ConD) or treated subjects (MedD) during the intervention, the significance was tested by applying the *post hoc* Friedman-Nemenyi test for pairwise test of multiple comparisons within each group. (B) Percentage changes in dietary and metabolic variables are represented as spider chart. Changes in levels of dietary components consumption including (C) dietary fibre, (D) vegetable proteins/animal proteins ratio, (E) saturated to polyunsaturated fats ratio. Reduction in serum and urinary markers such as (F) plasma carnitine, (G) urinary carnitine and (H) total cholesterol. The significance was tested by applying unpaired Wilcoxon rank-sum tests for variation at the specific timepoint compared to baseline in MedD vs ConD. Orange boxes refer to controls and green boxes to Mediterranean subjects, respectively. Baseline, 0 weeks; 4w, 4 weeks; 8w, 8 weeks of nutritional intervention. (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

During the run-in period, some subjects (12 in the MedD group and 6 in the ConD group) undesirably increased their intake of fruit and vegetables above 3 servings/day compared to their consumption at the time of enrolment. We decided to strictly focus on the population who maintained a baseline dietary intake of fruits and vegetables <3 servings/day. In addition, two subjects were not considered because not all the faecal samples were available. Therefore, the subsequent data analyses were carried out with a subgroup of 62 subjects, 32 in the ConD group and 30 in the MedD group. High compliance with the intervention was confirmed in both groups. Changes in dietary intake of nutrients from several food categories are shown in Supplementary Figure 3. The effect of MedD on plasma cholesterol was confirmed in this subgroup. Indeed, following 4 weeks of intervention with a MD, a significant reduction (p=0.03) in plasma LDL-cholesterol from 2.90±0.13 mmol/L at baseline to 2.66±0.12 mmol/L at 4 weeks was observed in the MedD group compared to the change in the ConD group (3.24±0.13 mmol/L at baseline to 3.25±0.12 mmol/L at 4 weeks), and a significant reduction (p=0.02) in plasma HDL-cholesterol from 1.26±0.05 mmol/L at baseline to 1.18±0.04 mmol/L at 4 weeks was observed in the MedD group compared to the change in the ConD group (1.21±0.05 mmol/L at baseline to 1.25±0.05 mmol/L at 4 weeks). Interestingly, a significant inverse correlation was found between cholesterol levels and the MD index. By applying a linear model, it was found that each unit increase in the MD index corresponded to ≃2% reduction in total plasma cholesterol (p=0.003, Supplementary Figure 4), a 2% reduction in plasma LDL-cholesterol (p=0.01) and 1% reduction in plasma HDL-cholesterol (p=0.04) after adjustment for age, sex, BMI and energy intake.

MD-mediated metabolome changes highlight several biomarkers of the MD and compliance with the intervention

We measured approximately 11,000 molecular features in all our participants during the intervention (2,200 in faeces, 4,125 in blood and 4,645 in urine). A list of annotated metabolites is provided in Supplementary Table 4 and the evidence substantiating the annotation of diet-responsive metabolites is provided in Supplementary Figures 5-25. Clear shifts in the metabolomic profiles were observed in the MedD group after the intervention compared to the baseline conditions and to the ConD group (Figure 5.2). Decreasing Spearman's correlation coefficients (4 weeks vs baseline; 8 weeks vs baseline) indicated a significant change in the urine metabolic profiles after 4 (p=0.01) and 8 weeks (p=0.01) of intervention in the MedD group vs the ConD group. In order to validate the robustness of the shifts observed, we used a machine learning-based classification approach (area under the

curve (AUC) = 0.88 and 0.87 between the ConD and MedD groups at 4 weeks and 8 weeks, respectively; as a control, AUC = 0.52 was observed at baseline), which supported the metabolome changes found. In agreement with the replacement of refined cereal with wholegrain products and the replacement of meat, eggs and dairy products with fishery products, legumes and provided nuts, we found increased levels of the biomarkers of wholegrains (3-(3,5-dihydroxyphenyl) propanoic acid-glucuronide), legumes (tryptophan betaine), vegetables/berries (oxindole-3-acetic acid), and nuts (urolithins) in the MedD group, while biomarkers of meat (carnitine), BAs, leucine and isoleucine were more closely linked to the ConD group (Figure 5.2) (Roager *et al*., 2019; Keller *et al*., 2013; Cuparencu *et al*., 2016; Tulipani *et al*., 2012; Dragsted, 2010). Notably, no change in urine or serum TMAO was observed, possibly due to contrasting effects of increasing fish intake and lowering intakes of animal proteins in the MedD group.

MD-mediated increase in biomarkers of wholegrain (benzoxazinoids, pipecolic acid betaine), vegetable (oxindole-3-acetic acid), legume (tryptophan betaine, pyrogallol-sulphate), nuts (urolithins) and fish (3-carboxy-4-methyl-5-propanyl-2-furanpropionic acid) consumption and decrease in meat (carnitine) and protein degradation products such as branched-chain amino acids (BCAAs), aromatic amino acids, N-acetylcadaverine and microbial-derived proteolysis products (p-cresol sulphate, indoxyl sulphate, phenylacetylglutamine) was further confirmed by their significant associations with the MD index (Supplementary Figure 5). Finally, a range of host-derived short- and medium-chain acylcarnitines was significantly reduced in the urine following the MD intervention, indicating a shift in substrates for energy metabolism from fat to complex carbohydrates and protein (Khakimov *et al*., 2016).

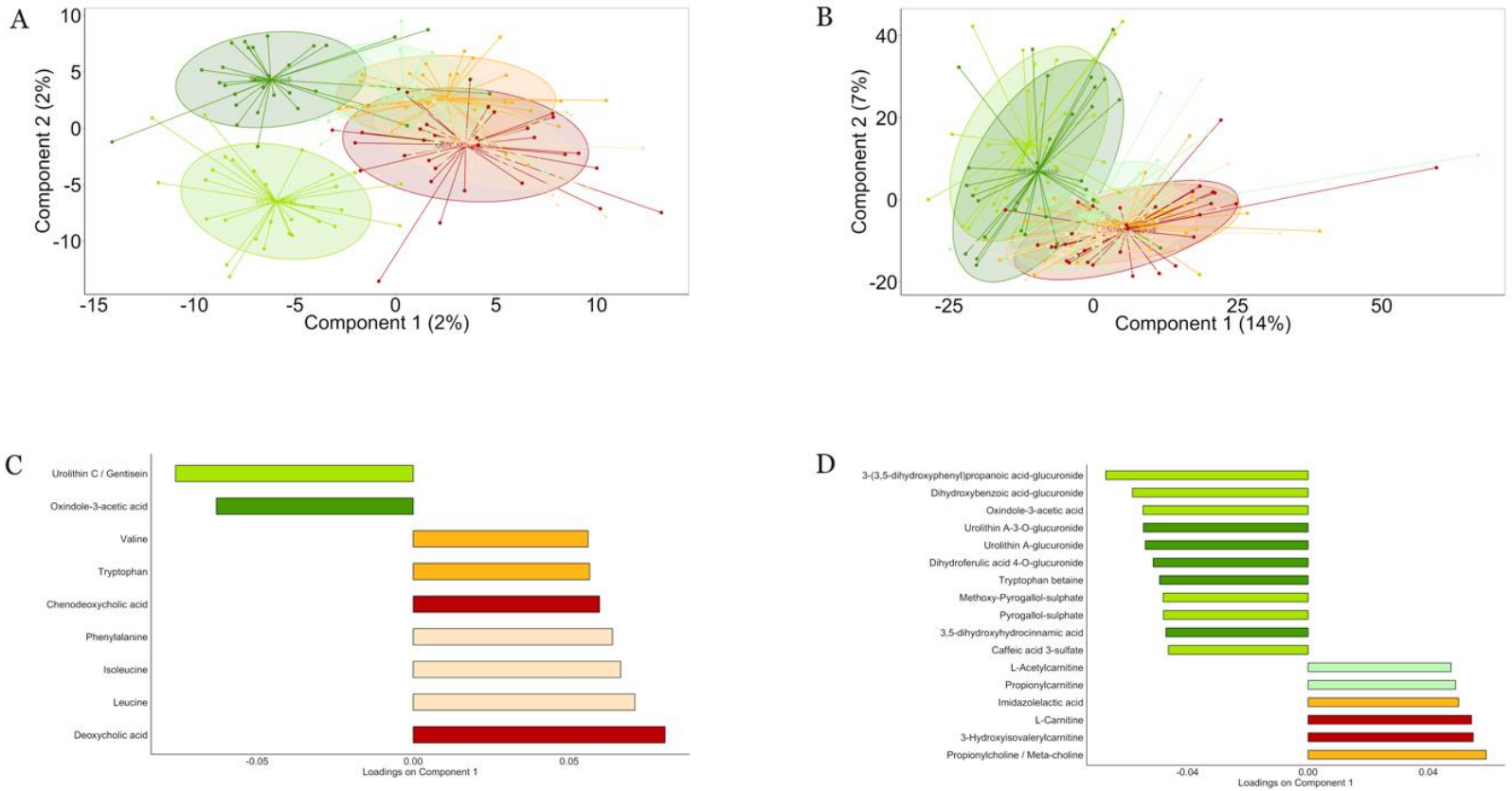


Figure 5.2 Mediterranean diet changes the intestinal and systemic metabolome. Partial Least Squares Discriminant Analysis (PLS-DA) plots based on molecular features detected in (A) faeces and (B) urine. Subjects belonging to different categories were coloured according to diet and timepoints: MedD subjects at baseline (light green), after 4 (green) and 8 weeks of intervention (dark green). ConD subjects at baseline (light orange), after 4 (orange) and 8 weeks (dark orange) of intervention. The loading plots display vectors that contributed the most to variability of individual dataset; variables explaining the variance between the groups in (C) faecal and (D) urine metabolome are reported as bar plots. RT, Retention Time.

Gut microbiome composition is modulated by adherence to the MD

Gene (average = 555,131.5 ± 120,191) and MSP richness (average = 230.9 ± 53.1) metrics were maintained during the intervention. However, a significant inverse correlation was found between the variation in gut microbial gene richness and individual inflammatory status evaluated by serum hs-CRP variations (Figure 5.3). Subjects showing increased gene richness displayed significantly lower levels of serum hs-CRP after 8 weeks of the dietary intervention (Figure 5.3).

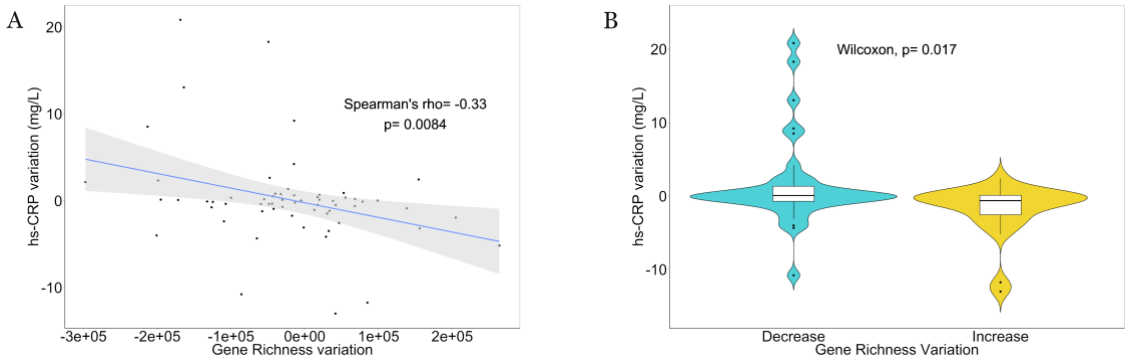


Figure 5.3 Microbial diversity richness anti-correlates with inflammation. (A) Spearman's correlation between variation of gut microbial gene richness and individual inflammatory status (serum hs-CRP) variation at the end of trial; n observation=62. (B) Violin plot showing differences in serum hs-CRP variation between subjects increasing (n=25, yellow) compared to subjects decreasing (n=37, light blue) gene richness at the end of trial. Statistical differences between groups were determined using unpaired Wilcoxon rank-sum tests.

The increased adherence to the MD in the first 4 weeks corresponded to a decrease in the microbiome similarity in the MedD group during the same time-interval, suggesting a MD-induced rearrangement of the gut microbiome composition. This change was not observed either in the ConD group over the entire intervention or in the MedD group between 4 weeks and 8 weeks, *i.e.* in intervention conditions when participants did not change their adherence to the MD (data not shown).

While a negligible number of differentially abundant MSPs was found at baseline between the ConD and MedD groups (n=27 MSPs, Supplementary Figure 6 and Supplementary Table 5), more contrasting species were observed at 4 (n=77 MSPs) and 8 weeks (n=44 MSPs, Supplementary Figures 7-8 and Supplementary Table 5), with the proportion of contrasting species consistently linked to the MD adherence evaluated by MD index (Supplementary Tables 5-6).

During the increasing MD adherence phase (baseline-4 weeks), *Ruthenibacterium lactatiformans, Flavonifractor plautii*, *Parabacteroides merdae, Ruminococcus torques* and *R. gnavus* were significantly reduced in the MedD compared to the ConD group, along with *Streptococcus thermophilus*, a well-known marker of dairy product consumption. In contrast, 5 members of the *Faecalibacterium prausnitzii* clade were enriched in the MedD compared to the ConD group at either 4 or 8 weeks (Supplementary Table 5-6), along with several members of the *Roseburia* and *Lachnospiraceae* taxa. Consistently, MSPs enriched in the MedD group after 4 weeks were significantly linked to MD food biomarkers (Supplementary Figure 9).

While only 5 gut metabolic modules (GMMs) were different between the diets (4% of functional potential variation; Supplementary Table 7) at baseline, 18% variation in the metabolic potential captured by GMM was observed after 4 weeks. Several GMMs (n=19) were enriched in the MedD group, mainly including pathways related to amino acid and carbohydrate degradation. The pathways also included triglyceride and glycoprotein degradation and conversion of acetyl-CoA and glutamate degradation, both leading to crotonyl-CoA, a possible precursor of butyrate metabolism (Supplementary Table 7). Although only 6% variation was observed after 8 weeks, enrichment in glutamate degradation to crotonyl-CoA was maintained in the MedD group. This pathway was significantly linked to the levels of *Faecalibacterium prausnitzii* msp_0388 (Spearman's rho= 0.73, p< 10e-6, Supplementary Figure 9).

Altogether, by integrating the three meta-omics datasets, we observed a separation of the ConD and MedD groups on the basis of microbiome diversity, functional modules and metabolomic profiles (Hotelling T^2=40.95, p<7.038e-12; Supplementary Figure 10) corroborating the changes induced by the MD intervention. (Singh *et al*., 2019)

MD intervention affects microbiome activities

We measured a number of metabolites associated with gut microbial metabolism to investigate the effect of the MD dietary intervention upon health-related microbial activities. Urinary levels of urolithin glucuronides increased in the MedD compared the ConD group (Table 5.1). Such increase was consistently linked with the levels of urolithin producers in the microbiome, including, among others, members of the *Eggerthellaceae* family (Supplementary Table 8), and with the consumption of nuts that were the sole dietary source of ellagitannins significantly increased in the MedD group (FDR<0.05, Supplementary Figure

11). Interestingly, urolithin production was negatively correlated with serum hs-CRP, triglycerides, body fat mass, body weight, BMI and urinary carnitine (FDR<0.05, Supplementary Figure 11).

Compared to the baseline values, a significant reduction in faecal concentrations of total BAs, including both primary and secondary BAs, was observed in the MedD group upon the MD intervention (Figure 5.4). In addition, faecal deoxycholic acid was significantly reduced after 4 ($p<0.01$) and 8 weeks of the intervention ($p<0.01$) along with faecal lithocholic acid ($p<0.05$ and $p<0.01$ after 4 and 8 weeks, respectively) within the MedD group. Paired Wilcoxon rank-sum tests of faecal BA concentrations within each intervention group are shown in Supplementary Figure 12. A comparison of faecal BA concentrations between the MedD and ConD groups after 8 weeks showed a significant reduction in faecal chenodeoxycholic acid ($p<0.05$). Accordingly, primary and secondary BAs in the faeces were positively linked to proteins and fats from animal-based food products as well as systolic blood pressure, BMI, body weight and urinary carnitine (FDR<0.05, Supplementary Figure 11).

We also noticed that subjects showing the highest reduction in total BAs and the secondary/primary BA ratio had higher baseline levels of *Bilophila wadsworthia*, which decreased significantly after 4 weeks of the intervention ($p<0.05$, Figure 5.4).

Despite the 2-fold increase in dietary fibre intake, no changes in faecal concentrations of the main SCFAs acetate, butyrate and propionate were observed. However, significant reductions in branched-chain fatty acids (BCFAs), such as valerate, isovalerate, isobutyrate and 2-methylbutyrate, were observed in the faeces of the participants in the MedD group over the intervention (Figure 5.5), and these changes mirrored the increased intake of plant-based foods (FDR<0.05, Supplementary Figure 11). Moreover, subjects in the quartile of the highest faecal butyrate increase at 4 weeks showed consistently higher levels of *F. prausnitzii* and *Lachnospiraceae* taxa (Figure 5.5).

Table 5.1 Urinary urolithins-glucuronides levels (ng/µmol creatinine) detected over the study period.

	MedD			**ConD**			**P values**	
	baseline	4w	8w	baseline	4w	8w	$\Delta_{(4w - baseline)}$	$\Delta_{(8w - baseline)}$
Urolithin-A-glucuronide	30.8±37.7	139.8±296.8	214.4±358	5.4±30.5	6.9±35.7	5±28.3	**0.013**	**0.025**
Urolithin-B-glucuronide	0.1±0.7	21.7±60.6	74.1±243.4	0.2±0.9	0.1±0.5	5.4±30.6	**0.0073**	0.086
Urolithin-C-glucuronide	1.6±8.8	46.8±107.2	43.2±176.6	0±0	0±0	0±0	**0.021**	0.16
Total Urolithins (A+B+C)	32.6±91.8	208.3±373.8	336.7±594.3	5.5±30.5	7±35.7	10.4±41	**0.00034**	**0.033**

Data are expressed as mean±standard deviation (SD).

P values refer to variation at the specific timepoint compared to baseline in MedD vs ConD measured by unpaired Wilcoxon rank-sum tests.

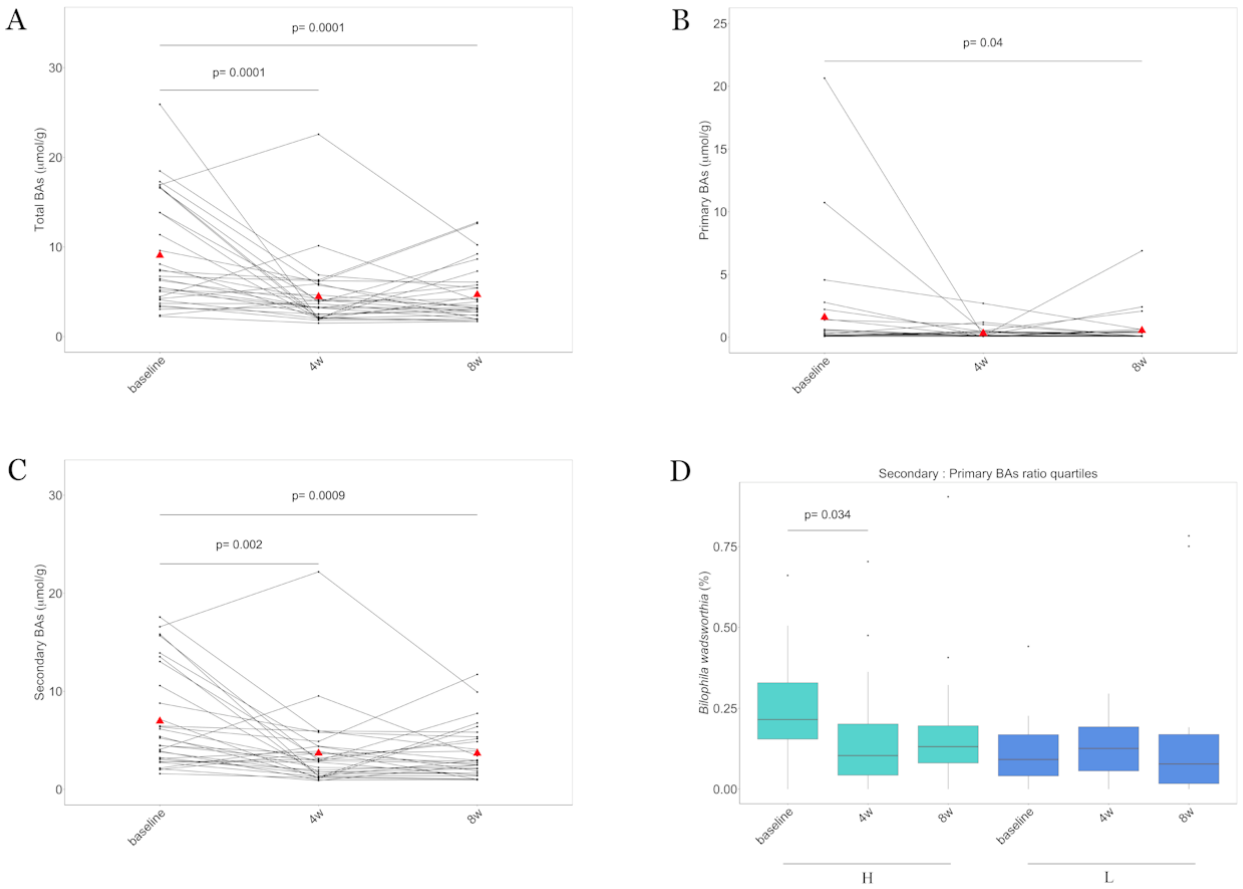


Figure 5.4 Faecal bile acids (BAs) concentrations over the nutritional intervention. Parallel coordinates plot showing variations of faecal (A) primary, (B) secondary and (C) total BAs concentrations within the MedD group during the intervention. The red triangles indicate mean values, the lines connecting dots are used to indicate the same sample at each time point. The significance was tested by applying the *post hoc* Friedman-Nemenyi test for pairwise test of multiple comparisons within each group. (D) In the box plot the relative abundances of *Bilophila wadsworthia* are compared considering subjects falling in the highest quartile (n=16, green) and in the lowest quartile of reduction (n=16, blue) of secondary to primary BAs ratio after 4 weeks of treatment. Baseline, 0 weeks; 4w, 4 weeks; 8w, 8 weeks of nutritional intervention. H, highest quartile of reduction; L, lowest quartile of reduction; BAs, bile acids.

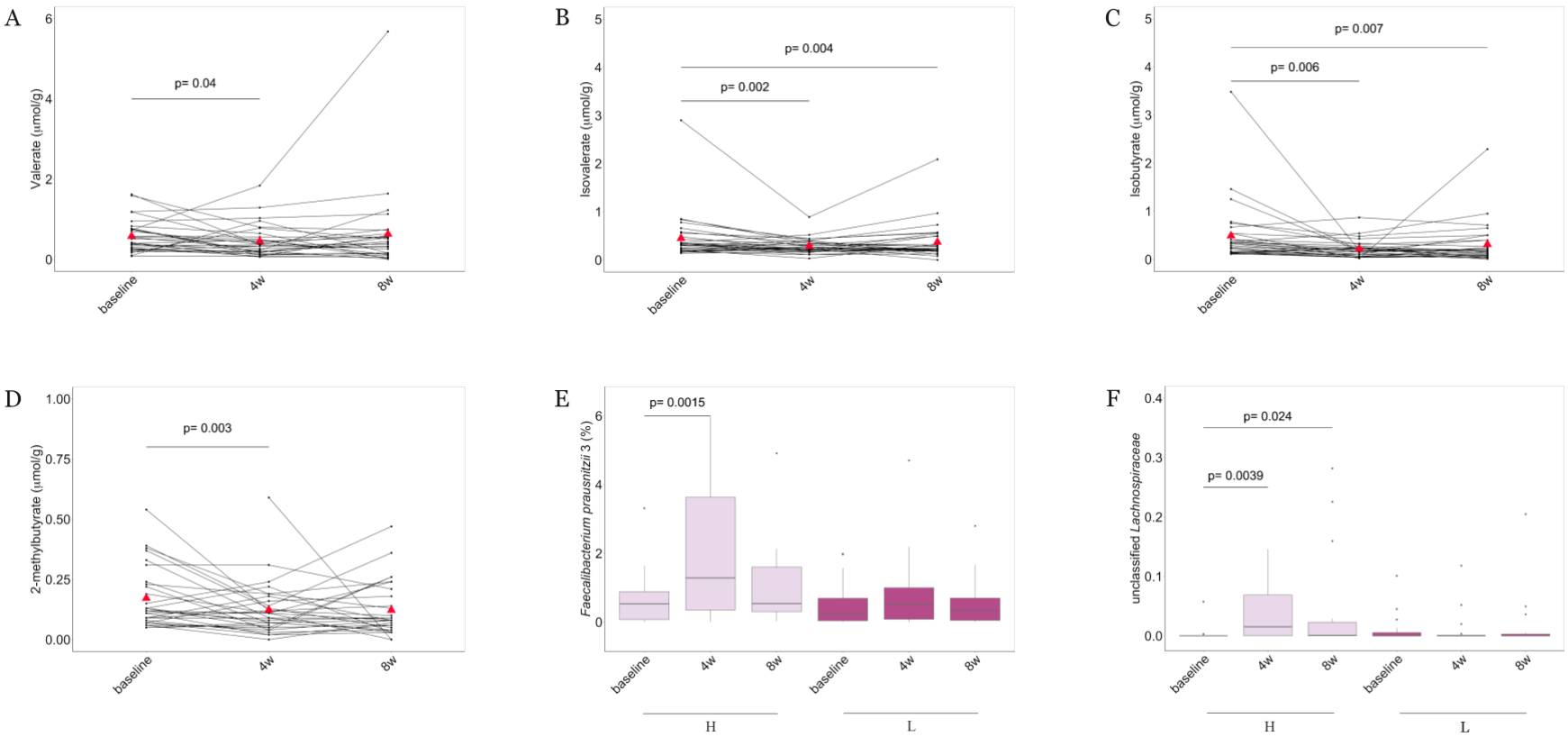


Figure 5.5 MD intervention determines a reduction of faecal branched-chain fatty acids (BCFAs) concentrations and higher levels of *Faecalibacterium prausnitzii* and *Lachnospiraceae* taxa. Parallel coordinates plot showing variations of (A) valerate, (B) isovalerate, (C) isobutyrate and (D) 2-methylbutyrate faecal concentrations within MedD population. The red triangles indicate mean values, the lines connecting dots are used to indicate the same sample at each time point. The significance was tested by applying the *post hoc* Friedman-Nemenyi test for pairwise test of multiple comparisons within each group. In the box plots, the relative abundances of (E) *Faecalibacterium prausnitzii* 3 and (F) *Lachnospiraceae* family are compared considering subjects falling in the highest quartile (n=16, violet) and in the lowest quartile (n=16, purple) of faecal butyrate increase after 4 weeks of treatment. Statistical differences between groups were determined using Wilcoxon rank-sum tests. Baseline, 0 weeks; 4w, 4 weeks; 8w, 8 weeks of intervention. H, highest quartile of increase; L, lowest quartile of increase.

Microbiome composition, MD intervention and variation in insulin resistance

The Homeostatic Model Assessment for Insulin Resistance (HOMA) was calculated as a measure of insulin resistance, and it did not change as a result of the intervention (Supplementary Table 1). However, by stratifying the subjects by the variation in HOMA at 4 weeks compared to baseline, we found that subjects who reduced their HOMA upon the MD intervention had significantly higher baseline levels of several *Bacteroides* species (including *B. uniformis* and *B. vulgatus*, $p<0.05$) and lower *Prevotella* sp. and *P. copri* levels ($p<0.05$) than subjects who did not exhibit changes in HOMA over time (Supplementary Figure 13). Interestingly, *P. copri* baseline levels showed a positive correlation with HOMA variation over the intervention (Spearman's rho=0.28; p=0.031).

Consistently, when we computed co-abundance groups (CAGs) from 16S rRNA gene sequencing analysis, we found significantly lower levels of CAG2 (including *Prevotella* as the most abundant genus) in subjects who exhibited reduced HOMA, while levels of CAG4 (including *Faecalibacterium*, *Roseburia, Bacteroides*, other Clostridia) were significantly higher at baseline in participants who exhibited reduced HOMA and increased upon dietary treatment (Supplementary Figure 14).

The pangenome of the HOMA- and serum hs-CRP-associated species (*P. copri, F. prausnitzii, B. uniformis, B. vulgatus*) was further investigated. No clear differences were found according to intervention, increase in dietary fibre consumption or decrease in HOMA indicating a high subject-specificity at the strain level (Supplementary Figure 15).

5.4 Discussion

The results of this study clearly show that a change from a Western diet to a Mediterranean dietary pattern, without any concomitant change in energy intake, macronutrient intake or physical activity, modulates individual clinical outcomes, the gut microbiome and metabolome after 4 weeks of the intervention in a population with cardiometabolic risk due to unhealthy lifestyle habits.

Each participant in the MedD group received a diet that was tailored to his/her habitual energy and macronutrient intake to increase the adherence to a typical MD pattern. In other words, each subject was instructed on the exact replacements of foods so that specific amounts of Western diet foods were exactly replaced by foods typical of a MD. At the best of

our knowledge, this approach has not been previously used in intervention studies with the MD. This ensured that changes in metabolic markers, the gut microbiome, and systemic metabolome were not biased by variation in energy intake over the nutritional intervention.

From a clinical perspective, the data show that within a short period, a MD can lower total, LDL- and HDL- cholesterol in plasma independently of energy intake. The decrease in LDL-cholesterol (by 0.24 mmol/L, -8.3% vs baseline) associated with MD in this study is far from the reduction of 1 mmol/L that has been indicated as clinically relevant towards a reduction in heart disease risk (Lewington *et al.*, 2008). However, it is higher than the average reductions (between 0.11 and 0.23 mmol/L) that have been found in RCTs comparing meat-based diets with plant-protein based diets including nuts or legumes separately as well as those achievable (~0.1 mmol/L) with diets including ellagitannins or anthocyanins (García-Conesa *et al.*, 2018; Guasch-Ferré *et al.*, 2019). Interestingly, in our study, the cholesterol-lowering effect was linearly associated with individual adherence to the MD. The relationship between the MD index and plasma cholesterol highlights the importance of the whole MD pattern and of individual dietary compliance in eliciting the hypolipidaemic effect of the MD. We hypothesize that the lower dietary intakes of cholesterol ($p<0.0001$ at 4 weeks and 8 weeks vs baseline) and saturated fats ($p=0.005$ at 4 weeks and at 8 weeks vs baseline) upon the MD intervention are the main factors responsible for that effect (Wolff *et al.*, 2011).

Adherence to the MD was confirmed by comprehensive untargeted metabolic profiling of faeces, serum and urine, as well as targeted quantification of selected biomarkers. In agreement with the MD pattern, we found increased levels of biomarkers of wholegrains, legumes, vegetables and nuts, as well as reduced levels of biomarkers of meat and protein degradation products after the MD intervention. These objective measures substantiated the dietary records obtained by the FFQ and the 7-day food diary. The MD-dependent metabolome shift was particularly evident in the urine metabolome due to the accumulation of diet-derived metabolites of wholegrains, nuts and vegetables. In addition, a range of short- and medium-chain acylcarnitines were consistently reduced in urine following the MD intervention, suggesting a diet-induced shift in energy production from beta-oxidation to glycolysis in the mitochondria, probably due to an extended period of carbohydrate availability due to a steady release from fibre degradation. In agreement with these findings, plasma short-chain acylcarnitines have been associated with a Western diet and have been found in higher concentrations in meat eaters than in vegetarians and vegans, and urine levels of acylcarnitines were reduced with increased wholegrain intake (Bouchard-Mercier *et al.*, 2013; Schmidt *et al.*, 2015; Ross *et al.*, 2013). Since acylcarnitines have been associated with

an increased risk of CVD, the reduction in acylcarnitines in urine suggests a beneficial MD-induced effect on energy metabolism caused by increased intake of dietary fibre (Guasch-Ferré *et al*., 2016).

Overall, the differences in the faecal metabolome associated with the intervention reflect the replacement of foods of animal origin with plant-based foods following MD adherence. Oxindole-3-acetic acid, a naturally occurring auxin in plants, as well as the BCAAs leucine and isoleucine and BAs appeared to be the main drivers (Korver *et al*., 2018). BAs can be implicated in atherosclerosis, diabetes, and other cardiometabolic diseases (Chávez-Talavera *et al*., 2017). Targeted quantification of faecal BAs confirmed a significant reduction in their concentrations within the MedD group coherently with the reduced intake of meat products. In line with these findings, a vegan diet has been found to reduce plasma BCAAs and BAs in comparison with the levels associated with an animal-based diet (Draper *et al*., 2018).

High adherence to a MD has also been associated with increased faecal concentrations of SCFAs (De Filippis *et al*., 2016). Despite the fact that participants doubled their intake of dietary fibre, the MD intervention did not significantly increase the faecal concentrations of SCFAs. Stool SCFAs represents the difference between the production and absorption or utilization of SCFAs in the colon and rectum. We speculate that a possible improved gut epithelial function may have increased SCFAs utilization and absorption thus hampering the observation of their increase due to higher fibre intake. This result was corroborated by recent findings that dysbiosis is associated with increased faecal SCFA excretion (de la Cuesta-Zuluaga *et al*., 2019). The MD decreased faecal concentrations of BCFAs, including valerate, which is in agreement with previous studies reporting faecal valerate as linked to the consumption of protein-rich animal foods and not to MD adherence (De Filippis *et al*., 2016; Mitsou *et al*., 2017). These results suggest an altered colonic proteolytic fermentation caused by the replacement of animal-based products with plant-based foods. This finding was substantiated by microbial-derived proteolytic products being reduced with increased MD adherence. An interesting increase in urolithins was observed in the MedD group. Urolithins are gut microbial metabolites of ellagitannins (Cerdà *et al*., 2005). Dietary sources of these polyphenols are berries, pomegranate and walnuts. However, our data indicated that only nuts consumption significantly increased over the intervention with MD. Therefore, increase in urinary urolithin glucuronides were most likely attributed to the intake of walnuts in our study as previously reported by others (Garcia-Aloy *et al*., 2019).

Recently, urolithin A has been shown to improve intestinal barrier function in a pre-clinical model and has also been associated with lower cardiometabolic risk (Singh *et al*., 2019;

Selma *et al.*, 2018). In addition, urolithin A has been demonstrated to be involved in the prevention of prostate, endometrial and breast cancer *in vitro* (Stanisławska *et al.*, 2019; Zhang *et al.*, 2016; Teixeira *et al.*, 2017). Interestingly, in our intervention study urolithins levels were negatively correlated to cardiometabolic risk factors such as triglycerides and BMI and these observations further corroborate the hypothesis that a MD dietary pattern might beneficially impacts human health status through gut microbiota metabolism.

It was recently reported that microbiome composition is more associated with specific food choices than with nutritional patterns, that food-microbe interactions are highly personalized, and that these factors might limit the observation of overall microbiome responses to specific diets (Johnson *et al.*, 2019). Interestingly, despite such insightful evidence, we observed clear microbiome shifts following our dietary intervention protocol.

Gut microbial taxonomic and functional composition in our isocaloric MD intervention revealed that the overall microbial richness was maintained, which is consistent with recent studies showing similar trends after increased consumption of wholegrain (Roager *et al.*, 2019; Haro *et al.*, 2016*a*). However, we observed that the MD dynamically modulates the intestinal microbiome composition and that the microbiome variations are proportional to the increase in MD adherence rates.

Even though prior studies addressed the link between diet, gene richness and inflammation markers, interventional studies aimed at describing variation of the microbial genetic richness following a MD dietary pattern have not previously been described (David *et al.*, 2014; Roager et al., 2019; Haro *et al.*, 2016*a*; Haro *et al.*, 2016*b*; Vanegas *et* al., 2017). Interestingly, here MD improves the inflammatory status of individuals experiencing an increase in gut microbiome gene richness during controlled energy and modified macronutrient intakes, further supporting the idea that MD might be an efficient dietary strategy to reduce inflammation (Vitaglione *et* al., 2014; Bailey and Holscher, 2018).

The MD intervention protocol determined a decline in *Ruminococcus torques* and *R. gnavus.* This latter has been recently demonstrated as a proinflammatory species due to secretion of a polysaccharide that induces tumor necrosis factor alpha (TNFα) in dendritic cells, whereas possible involvement of *R. torques* in inflammation remains largely uncertain and is currently based on associations (Le Chatelier *et al.*, 2013; Brahe *et al.*, 2015; Henke *et al.*, 2019).

We noticed that subjects with the highest reduction in faecal BAs consistently also exhibited reduced relative abundance of *Bilophila wadsworthia*, which was previously linked to higher BA levels, animal-based and high fat diets, as well as irritable bowel diseases (IBDs) (Devkota *et al.*, 2012; David *et al.*, 2014; Natividad *et al.*, 2018). This decline was

accompanied by an increase in several potentially beneficial species, including the fibre-degrading *Faecalibacterium prausnitzii*, *Roseburia,* and members of the *Clostridiales* and *Lachnospiraceae* taxa, linked to butyrate precursor functional pathways. These favourable species were previously documented for their anti-inflammatory properties and their role in the development of the intestinal barrier and were, in the present study, found to be boosted with foods recommended as part of a healthy MD nutritional pattern (Sokol *et al.*, 2008; Vital *et al*., 2017).

Our data also show that a MD-tailored dietary intervention might be helpful in ameliorating insulin sensitivity in individuals harbouring higher levels of several *Bacteroides* species and lower levels of *Prevotella* sp. and *P. copri*. The association of *P. copri* with insulin resistance was already reported by Pedersen *et al.* and it was recently demonstrated to be strain-dependent and correlated with the occurrence of genes involved in BCAA biosynthesis (Pedersen *et al*., 2016; De Filippis *et al*., 2019).

5.5 Conclusion

These findings are in line with the concept of personalized responses of individuals to similar diets, and they are of importance for clinical practice in the era of precision medicine and personalized nutrition (De Filippis *et al*., 2018; Johnson *et al*., 2019).

Taken together, our findings indicate that a MD may remodel the intestinal microbiome towards a state that promotes metabolic and cardiovascular health. In addition, our results can be useful to plan baseline stratifications of subjects based on microbiome composition to select specific metabotypes that could be involved in *ad hoc* nutritional interventions to potentiate the clinical outcomes.

5.6 Notes

This chapter reports the content of manuscript entitled “Mediterranean diet intervention in overweight and obese subjects lowers plasma cholesterol and leads to multiple shifts in the gut microbiome and metabolome independently of energy intake” by Victoria Meslier, Manolo Laiola, Henrik M. Roager, Francesca De Filippis, Hugo Roume, Benoit Quinquis, Rosalba Giacco, Ilario Mennella, Rosalia Ferracane, Nicolas Pons, Edoardo Pasolli, Angela A. Rivellese, Lars O. Dragsted, Paola Vitaglione, Dusko S. Ehrlich, Danilo Ercolini (2019) published in Gut. doi:10.1136/gutjnl-2019-320438.

5.7 References

Agnoli C, Krogh V, Grioni S, Sieri S, Palli D, Masala G, Sacerdote C, Vineis P, Tumino R, Frasca G. 2011. A priori–defined dietary patterns are associated with reduced risk of stroke in a large Italian cohort. J Nutr 141:1552–1558.

Andersen M-BS, Kristensen M, Manach C, Pujos-Guillot E, Poulsen SK, Larsen TM, Astrup A, Dragsted L. 2014. Discovery and validation of urinary exposure markers for different plant foods by untargeted metabolomics. Anal Bioanal Chem 406:1829–1844.

Barri T, Dragsted LO. 2013. UPLC-ESI-QTOF/MS and multivariate data analysis for blood plasma and serum metabolomics: effect of experimental artefacts and anticoagulant. Anal Chim Acta 768:118–128.

Bashiardes S, Godneva A, Elinav E, Segal E. 2018. Towards utilization of the human genome and microbiome for personalized nutrition. Curr Opin Biotechnol 51:57–63.

Bailey MA, Holscher HD. 2018. Microbiome-mediated effects of the Mediterranean diet on inflammation. Adv Nutr 9:193–206.

Bendall CL, Mayr HL, Opie RS, Bes-Rastrollo M, Itsiopoulos C, Thomas CJ. 2018. Central obesity and the Mediterranean diet: A systematic review of intervention trials. Crit Rev Food Sci Nutr 58:3070–3084.

Blumberg J, Heaney RP, Huncharek M, Scholl T, Stampfer M, Vieth R, Weaver CM, Zeisel SH. 2010. Evidence-based criteria in the nutritional context. Nutr Rev 68:478–484.

Bouchard-Mercier A, Rudkowska I, Lemieux S, Couture P, Vohl M-C. 2013. The metabolic signature associated with the Western dietary pattern: a cross-sectional study. Nutr J 12:158.

Brahe LK, Le Chatelier E, Prifti E, Pons N, Kennedy S, Hansen T, Pedersen O, Astrup A, Ehrlich SD, Larsen LH. 2015. Specific gut microbiota features and metabolic markers in postmenopausal women with obesity. Nutr Diabetes 5:e159.

Cani PD. 2018. Human gut microbiome: hopes, threats and promises. Gut 67:1716–1725.

Cerdá B, Periago P, Espín JC, Tomás-Barberán FA. 2005. Identification of urolithin A as a metabolite produced by human colon microflora from ellagic acid and related compounds. J Agric Food Chem 53:5571–5576.

Chávez-Talavera O, Tailleux A, Lefebvre P, Staels B. 2017. Bile acid control of metabolism and inflammation in obesity, type 2 diabetes, dyslipidemia, and nonalcoholic fatty liver disease. Gastroenterology 152:1679–1694.

Chiavaroli L, Viguiliouk E, Nishi SK, Mejia SB, Rahelić D, Kahleová H, Salas-Salvadó J, Kendall CWC, Sievenpiper JL. 2019. DASH dietary pattern and cardiometabolic outcomes: An umbrella review of systematic reviews and meta-analyses. Nutrients 11:338.

Christiansen CB, Gabe MBN, Svendsen B, Dragsted LO, Rosenkilde MM, Holst JJ. 2018. The impact of short-chain fatty acids on GLP-1 and PYY secretion from the isolated perfused rat colon. Am J Physiol Liver Physiol 315:G53–G65.

Cuparencu CS, Andersen M-BS, Gürdeniz G, Schou SS, Mortensen MW, Raben A, Astrup A, Dragsted LO. 2016. Identification of urinary biomarkers after consumption of sea buckthorn and strawberry, by untargeted LC–MS metabolomics: a meal study in adult men. Metabolomics 12:31.

David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling A V, Devlin AS, Varma Y, Fischbach MA. 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature 505:559.

de la Cuesta-Zuluaga J, Mueller N, Álvarez-Quintero R, Velásquez-Mejía E, Sierra J, Corrales-Agudelo V, Carmona J, Abad J, Escobar J. 2019. Higher fecal short-chain fatty acid levels are associated with gut microbiome dysbiosis, obesity, hypertension and cardiometabolic disease risk factors. Nutrients 11:51.

De Filippis F, Pellegrini N, Vannini L, Jeffery IB, La Storia A, Laghi L, Serrazanetti DI, Di Cagno R, Ferrocino I, Lazzi C. 2016. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut 65:1812–1821.

De Filippis F, Vitaglione P, Cuomo R, Berni Canani R, Ercolini D. 2018. Dietary interventions to modulate the gut microbiome—how far away are we from precision medicine. Inflamm Bowel Dis 24:2142–2154.

De Filippis F, Pasolli E, Tett A, Tarallo S, Naccarati A, De Angelis M, Neviani E, Cocolin L, Gobbetti M, Segata N. 2019. Distinct genetic and functional traits of human intestinal *Prevotella copri* strains are associated with different habitual diets. Cell Host Microbe 25:444–453.

Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, Antonopoulos DA, Jabri B, Chang EB. 2012. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10−/− mice. Nature 487:104.

Dragsted LO. 2010. Biomarkers of meat intake and the application of nutrigenomics. Meat Sci 84:301–307.

Draper CF, Vassallo I, Di Cara A, Milone C, Comminetti O, Monnard I, Godin J, Scherer M, Su M, Jia W. 2018. A 48-Hour Vegan Diet Challenge in Healthy Women and Men Induces a BRANCH-Chain Amino Acid Related, Health Associated, Metabolic Signature. Mol Nutr Food Res 62:1700703.

Drescher LS, Thiele S, Mensink GBM. 2007. A new index to measure healthy food diversity better reflects a healthy diet than traditional measures. J Nutr 137:647–651.

Eleftheriou D, Benetou V, Trichopoulou A, La Vecchia C, Bamia C. 2018. Mediterranean diet and its components in relation to all-cause mortality: meta-analysis. Br J Nutr 120:1081–1097.

Garcia-Aloy M, Hulshof PJM, Estruel-Amades S, Osté MCJ, Lankinen M, Geleijnse JM, De Goede J, Ulaszewska M, Mattivi F, Bakker SJL. 2019. Biomarkers of food intake for nuts and vegetable oils: an extensive literature search. Genes Nutr 14:7.

García-Conesa M-T, Chambers K, Combet E, Pinto P, Garcia-Aloy M, Andrés-Lacueva C, de Pascual-Teresa S, Mena P, Konic Ristic A, Hollands W. 2018. Meta-analysis of the effects of foods and derived products containing ellagitannins and anthocyanins on cardiometabolic biomarkers: Analysis of factors influencing variability of the individual responses. Int J Mol Sci 19:694.

Grosso G, Micek A, Godos J, Pajak A, Sciacca S, Galvano F, Boffetta P. 2017. Health risk factors associated with meat, fruit and vegetable consumption in cohort studies: A comprehensive meta-analysis. PLoS One 12:e0183787.

Gu Z, Eils R, Schlesner M. 2016. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics 32:2847–2849.

Guasch-Ferré M, Zheng Y, Ruiz-Canela M, Hruby A, Martínez-González MA, Clish CB, Corella D, Estruch R, Ros E, Fitó M. 2016. Plasma acylcarnitines and risk of cardiovascular disease: effect of Mediterranean diet interventions. Am J Clin Nutr 103:1408–1416.

Guasch-Ferré M, Satija A, Blondin SA, Janiszewski M, Emlen E, O'Connor LE, Campbell WW, Hu FB, Willett WC, Stampfer MJ. 2019. Meta-analysis of randomized controlled trials of red meat consumption in comparison with various comparison diets on cardiovascular risk factors. Circulation 139:1828–1845.

Haro C, Montes-Borrego M, Rangel-Zúñiga OA, Alcalá-Díaz JF, Gómez-Delgado F, Pérez-Martínez P, Delgado-Lista J, Quintana-Navarro GM, Tinahones FJ, Landa BB. 2016. Two healthy diets modulate gut microbial community improving insulin sensitivity in a human obese population. J Clin Endocrinol 101:233–242.*a*

Haro C, Garcia-Carpintero S, Alcala-Diaz JF, Gomez-Delgado F, Delgado-Lista J, Perez-Martinez P, Zuñiga OAR, Quintana-Navarro GM, Landa BB, Clemente JC. 2016. The gut microbial community in metabolic syndrome patients is modified by diet. J Nutr Biochem 27:27–31.*b*

Harris RP, Helfand M, Woolf SH, Lohr KN, Mulrow CD, Teutsch SM, Atkins D, Preventive MWGTUS, Force ST. 2001. Current methods of the US Preventive Services Task Force: a review of the process. Am J Prev Med 20:21–35.

Henke MT, Kenny DJ, Cassilly CD, Vlamakis H, Xavier RJ, Clardy J. 2019. *Ruminococcus gnavus*, a member of the human gut microbiome associated with Crohn's disease, produces an inflammatory polysaccharide. Proc Natl Acad Sci 116:12672–12677.

Hjerpsted JB, Dragsted LO, Tholstrup T. 2016. Cheese intake lowers plasma cholesterol concentrations without increasing bile acid excretion. J Nutr Intermed Metab 3:12–17.

Johnson AJ, Vangay P, Al-Ghalith GA, Hillmann BM, Ward TL, Shields-Cutler RR, Kim AD, Shmagel AK, Syed AN, Students PMC. 2019. Daily Sampling Reveals Personalized Diet-Microbiome Associations in Humans. Cell Host Microbe 25:789–802.

Keller BO, Wu BTF, Li SSJ, Monga V, Innis SM. 2013. Hypaphorine is present in human milk in association with consumption of legumes. J Agric Food Chem 61:7654–7660.

Kelly JT, Palmer SC, Wai SN, Ruospo M, Carrero J-J, Campbell KL, Strippoli GFM. 2017. Healthy dietary patterns and risk of mortality and ESRD in CKD: a meta-analysis of cohort studies. Clin J Am Soc Nephrol 12:272–279.

Khakimov B, Poulsen SK, Savorani F, Acar E, Gürdeniz G, Larsen TM, Astrup A, Dragsted LO, Engelsen SB. 2016. New nordic diet versus average danish diet: a randomized controlled trial revealed healthy long-term effects of the New Nordic Diet by GC–MS blood plasma metabolomics. J Proteome Res 15:1939–1954.

Korver RA, Koevoets IT, Testerink C. 2018. Out of shape during stress: A key role for auxin. Trends Plant Sci 23:783–793.

Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto J-M, Kennedy S. 2013. Richness of human gut microbiome correlates with metabolic markers. Nature 500:541.

Lewington S, Whitlock G, Clarke R, Sherliker P, Emberson J, Halsey J, Qizilbash N, Peto R, Collins R. 2008. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55000 vascular deaths (vol 370, pg 1829, 2007). Lancet 372.

Martínez-González MA, Gea A, Ruiz-Canela M. 2019. The Mediterranean Diet and Cardiovascular Health: A Critical Review. Circ Res 124:779–798.

Meslier V, Laiola M, Roager HM, De Filippis F, Roume H, Quinquis B, Giacco R, Mennella I, Ferracane R, Pons N. 2020. Mediterranean diet intervention in overweight and obese subjects lowers plasma cholesterol and causes changes in the gut microbiome and metabolome independently of energy intake. Gut. doi:10.1136/gutjnl-2019-320438.

Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, Teeling JL, Blaak EE, Fenech M, Vauzour D. 2015. Low-grade inflammation, diet composition and health: current research evidence and its translation. Br J Nutr 114:999–1012.

Mitsou EK, Kakali A, Antonopoulou S, Mountzouris KC, Yannakoulia M, Panagiotakos DB, Kyriacou A. 2017. Adherence to the Mediterranean diet is associated with the gut microbiota pattern and gastrointestinal characteristics in an adult population. Br J Nutr 117:1645–1655.

Mozaffarian D, Hao T, Rimm EB, Willett WC, Hu FB. 2011. Changes in diet and lifestyle and long-term weight gain in women and men. N Engl J Med 364:2392–2404.

Myles IA. 2014. Fast food fever: reviewing the impacts of the Western diet on immunity. Nutr J 13:61.

Natividad JM, Lamas B, Pham HP, Michel M-L, Rainteau D, Bridonneau C, Da Costa G, van Hylckama Vlieg J, Sovran B, Chamignon C. 2018. *Bilophila wadsworthia* aggravates high fat diet induced metabolic dysfunctions in mice. Nat Commun 9:2802.

Pasolli E, Truong DT, Malik F, Waldron L, Segata N. 2016. Machine learning meta-analysis of large metagenomic datasets: tools and biological insights. PLoS Comput Biol 12:e1004977.

Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BAH, Forslund K, Hildebrand F, Prifti E, Falony G. 2016. Human gut microbes impact host serum metabolome and insulin sensitivity. Nature 535:376.

Plaza Oñate F, Le Chatelier E, Almeida M, Cervino ACL, Gauthier F, Magoulès F, Ehrlich SD, Pichaud M. 2018. MSPminer: abundance-based reconstitution of microbial pan-genomes from shotgun metagenomic data. Bioinformatics 35:1544–1552.

Roager HM, Dragsted LO. 2019. Diet-derived microbial metabolites in health and disease. Nutr Bull 44:216–227.

Roager HM, Vogt JK, Kristensen M, Hansen LBS, Ibrügger S, Mærkedahl RB, Bahl MI, Lind MV, Nielsen RL, Frøkiær H. 2019. Whole grain-rich diet reduces body weight and systemic low-grade inflammation without inducing major changes of the gut microbiome: a randomised cross-over trial. Gut 68:83–93.

Ross AB, Pere-Trépat E, Montoliu I, Martin F-PJ, Collino S, Moco S, Godin J-P, Cléroux M, Guy PA, Breton I. 2013. A whole-grain–rich diet reduces urinary excretion of markers of protein catabolism and gut microbiota metabolism in healthy men after one week. J Nutr 143:766–773.

Schmidt JA, Rinaldi S, Ferrari P, Carayol M, Achaintre D, Scalbert A, Cross AJ, Gunter MJ, Fensom GK, Appleby PN. 2015. Metabolic profiles of male meat eaters, fish eaters, vegetarians, and vegans from the EPIC-Oxford cohort. Am J Clin Nutr 102:1518–1526.

Selma M V, González-Sarrías A, Salas-Salvadó J, Andrés-Lacueva C, Alasalvar C, Örem A, Tomás-Barberán FA, Espín JC. 2018. The gut microbiota metabolism of pomegranate or walnut ellagitannins yields two urolithin-metabotypes that correlate with cardiometabolic risk biomarkers: Comparison between normoweight, overweight-obesity and metabolic syndrome. Clin Nutr 37:897–905.

Singh A, Gautier B, Shannon CP, Vacher M, Rohart F, Tebbutt SJ, Le Cao K-A. 2016. DIABLO–an integrative, multi-omics, multivariate method for multi-group classification. BioRxiv 67611.

Singh R, Chandrashekharappa S, Bodduluri SR, Baby B V, Hegde B, Kotla NG, Hiwale AA, Saiyed T, Patel P, Vijay-Kumar M. 2019. Enhancement of the gut barrier integrity by a microbial metabolite through the Nrf2 pathway. Nat Commun 10:89.

Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux J-J, Blugeon S, Bridonneau C, Furet J-P, Corthier G. 2008. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci 105:16731–16736.

Stanisławska IJ, Granica S, Piwowarski JP, Szawkało J, Wiązecki K, Czarnocki Z, Kiss AK. 2019. The Activity of Urolithin A and M4 Valerolactone, Colonic Microbiota Metabolites of Polyphenols, in a Prostate Cancer In Vitro Model. Planta Med 85:118–125.

Teixeira L de L, Costa GR, Dörr FA, Ong TP, Pinto E, Lajolo FM, Hassimotto NMA. 2017. Potential antiproliferative activity of polyphenol metabolites against human breast cancer

cells and their urine excretion pattern in healthy subjects following acute intake of a polyphenol-rich juice of grumixama (Eugenia brasiliensis Lam.). Food Funct 8:2266–2274.

Tulipani S, Urpi-Sarda M, García-Villalba R, Rabassa M, López-Uriarte P, Bulló M, Jáuregui O, Tomás-Barberán F, Salas-Salvadó J, Espín JC. 2012. Urolithins are the main urinary microbial-derived phenolic metabolites discriminating a moderate consumption of nuts in free-living subjects with diagnosed metabolic syndrome. J Agric Food Chem 60:8930–8940.

Vanegas SM, Meydani M, Barnett JB, Goldin B, Kane A, Rasmussen H, Brown C, Vangay P, Knights D, Jonnalagadda S. 2017. Substituting whole grains for refined grains in a 6-wk randomized trial has a modest effect on gut microbiota and immune and inflammatory markers of healthy adults. Am J Clin Nutr 105:635–650.

Vitaglione P, Mennella I, Ferracane R, Rivellese AA, Giacco R, Ercolini D, Gibbons SM, La Storia A, Gilbert JA, Jonnalagadda S. 2014. Whole-grain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: role of polyphenols bound to cereal dietary fiber. Am J Clin Nutr 101:251–261.

Vital M, Karch A, Pieper DH. 2017. Colonic butyrate-producing communities in humans: an overview using omics data. Msystems 2:e00130-17.

Wolff E, Vergnes M-F, Portugal H, Defoort C, Amiot-Carlin M-J, Lairon D, Nicolay A. 2011. Cholesterol-Absorber Status Modifies the LDL Cholesterol–Lowering Effect of a Mediterranean-Type Diet in Adults with Moderate Cardiovascular Risk Factors. J Nutr 141:1791–1798.

Zhang W, Chen J, Aguilera-Barrantes I, Shiau C, Sheng X, Wang L, Stoner GD, Huang Y. 2016. Urolithin A suppresses the proliferation of endometrial cancer cells by mediating estrogen receptor-α-dependent gene expression. Mol Nutr Food Res 60:2387–2395.

CHAPTER 6

Impact of a Mediterranean diet intervention on salivary microbiota

6.1 Introduction

The human oral cavity is a complex and open ecosystem, harboring a whole community of microorganisms. More than 700 bacterial species inhabit the site making up the oral microbiota (Paster *et al*., 2006) and it is considered the second most complex symbiont microbiota in the human body after the gut (Dewhirst *et al*., 2010).

Different individuals display a vast genetic variation in the oral microbial ecology. This dynamic ecosystem is influenced by both biological host parameters including age (Peterson *et al*., 2009), health status (Francavilla *et al*., 2014) and genetic predisposition (Maukonen *et al*., 2008) and local environmental factors such as diet (De Filippis *et al*., 2014), geographical environment (Eriksson *et al*., 2017), antibiotics consumption (Costello *et al*., 2013) and smoking (Wu *et al*., 2016). In addition, the oral microbiota composition varies among different oral sites (Segata *et al*., 2012). Therefore, a healthy composition of the oral microbiota cannot be easily defined (Idris *et al*., 2017).

Surprisingly, despite remarkable inter- and intra-individual variability, the microbial composition of the oral cavity is stable over time and in particular, saliva exhibits high evenness in terms of microbial diversity (Zhou *et al*., 2013; Cameron *et al*., 2015).

Several publications appeared in recent years documenting that alterations in salivary microbial communities are associated to both oral and non-oral disease (Acharya *et al*., 2017) and specific components of the salivary microbiota were proposed as predictive biomarkers for different type of pathologies (Hajishengallis *et al*., 2011; Cockburn *et al*., 2012; Zhang *et al*., 2015).

Recently, the oral microbiota dysfunction has been linked to atherosclerosis and cardiovascular disorder. Hence, oral symbionts may indirectly elicit the immune dysregulation leading to the progressive inflammation associated with cardiovascular diseases (Slocum *et al*., 2016).

Beyond host's health, data from several studies highlight that diet is able to affect and to restore the homeostasis of a particular microenvironment concerning microbial ecology. Although in a recent paper (De Filippis *et al*., 2014) it has been observed that the salivary metabolome could categorize individuals depending on their dietary habits, more than the occurrence of specific bacterial signatures, studies on both the connection and the effect of long-term diet and the salivary microbiota composition are still lacking.

Mediterranean diet (MD) is a healthy dietary pattern useful for both prevention and treatment of diet-related diseases. High-level of adherence to the MD is significantly and directly correlated to protection against the major chronic inflammatory diseases (Sofi *et al*., 2010), and it has been recently shown to impact gut microbiota and metabolome (De Filippis *et al*., 2015). Several researches have been focused on the effect of dietary interventions on gut microbiota composition, but little is known about the possible effects on salivary microbial ecology. In order to further explore the effects of MD on human health, in this study we investigated the global changes in the salivary microbial communities in overweight subjects after a MD intervention.

6.2 Materials and Methods

Study design and population

We investigated the salivary microbiota profiles in 50 overweight/obese subjects in response to an 8-week isocaloric dietary intervention with a MD or a control diet. A selection of participants was made from the original study population of the trial number NCT03071718.

Dietary intervention

Subjects were randomly distributed into two groups: Mediterranean (MedD, n=30) and control diet (ConD, n=20). The MedD group underwent to an individual dietary intervention based on a MD model for 8 weeks, preserving both the usual energy and the individual intake of macronutrients. The Mediterranean-based dietary pattern involved high-level intake of fruit, vegetables, legumes, nuts, olive oil and minimally processed cereals, moderately high consumption of fish, low intake of saturated fat, meat and dairy products and low-to-moderate consumption of alcohol. On the contrary, subjects in the

ConD group continued with their habitual diet and low physical activity. The compliance was assessed with weekly food diaries. Saliva samples were collected during the intervention, at the baseline (t0), after 4 (t1) and 8 weeks (t2).

DNA extraction and 16s rRNA gene sequencing

Saliva samples were processed as previously described (De Filippis *et al*., 2014); aliquots of 2 ml were centrifuged (10,000 x g; 1 min) and the pellet was used for DNA extraction by using the QIAamp BiOstic Bacteremia DNA Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The V3-V4 region of the 16S rRNA gene was amplified by using primers and PCR conditions recently described (Canani Berni *et al*., 2017). Amplicon libraries were sequenced on a MiSeq platform, leading to 2x250bp paired-end reads.

Bioinformatics and statistical analysis

De-multiplexed forward and reverse reads were joined by using FLASH (Magoč *et al*., 2011). Bases with a Phred score < 30 were trimmed by Prinseq (Schmieder and Edwards, 2011) and those shorter than 250 bp were discarded. High-quality reads were analyzed by QIIME 1.9 (Caporaso *et al*., 2010), with a pipeline recently described (Canani Berni *et al*., 2017). Statistical analysis and visualization was carried out using R environment (https://www.r-project.org). The principal component analysis (PCA) was assessed on log10 transformed OTU tables by using *dudi.pca* function (library *made4*). To separate groups based on microbial profiles, a Partial Least Squares Discriminant Analysis (PLS-DA) was performed with the *plsda* function (library *mixOmics*). The non-parametric Wilcoxon-Mann-Whitney test was performed (*pairwise.wilcox.test*) to discriminate MedD and ConD conditions at the baseline and after treatment and only microbial features differentially abundant with an adjusted p-value <0.05 were considered (Benjamini-Hochberg correction).

In order to identify discriminant taxa avoiding incorrect identification, all picked OTUs found to be significantly different between groups were double-checked against the human oral bacterial 16S rRNA gene sequences available at HOMD platform (https://www.homd.org).

The sub-genus diversity of *Streptococcus* was investigated as recently reported (De Filippis *et al*., 2016). Reads assigned to this genus were sorted and entropy analysis and oligotyping were carried out (Eren *et al*., 2013). The –C option was set to assess high-

entropy nucleotides from Streptococcus reads (16,17,19,22,23,93,84,127,192,230,269 and 299). The result of the analysis led to 18 oligotypes representative sequences, subsequently blasted (BLASTn) against the HOMD 16S rRNA RefSeq database for the identification and the top hit was considered for taxonomic assignment.

6.3 Results

Effects of the dietary treatment on salivary microbiota composition

In this study, we analysed the bacterial composition of one hundred fifty saliva samples from overweight/obese subjects. The overall microbial diversity was not significantly different between MedD and control groups and after the dietary intervention. Likewise, the weighted and unweighted Unifrac distance was unchanged after the treatment. Accordingly, we did not observe any clustering of the subjects according to the dietary intervention nor the group (MedD or ConD), suggesting no remarkable differences in the overall microbiota composition after the dietary treatment (Figure 6.1). A core microbiota with thirteen genera (*Actinomyces*, *Atopobium*, *Rothia*, *Porphyromonas*, *Prevotella*, *Granulicatella*, *Gemella*, *Streptococcus*, *Moryella*, *Veillonella*, *Neisseria*, *Haemophilus*, *Leptotrichia*) shared by the 99% of saliva samples was identified (Figure 6.2). These genera belonged to the phyla Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Fusobacteria. Moreover, these microbial taxa were not affected by the dietary intervention although their relative abundances showed great inter-individual variability. There was no significant difference in the average abundance of the core microbial genera between the two intervention groups, both at baseline (t0) and after the treatment with the Mediterranean (MedD) or control diet (ConD) for 4 (t1) or 8 (t2) weeks. In all the samples, *Prevotella* occurred at highest levels concerning the relative abundance (22.6% ± 9.1), followed by *Streptococcus* (16.5% ± 6.3), *Veillonella* (10.4% ± 4.3), *Neisseria* (8.9% ± 6.5), *Porphyromonas* (6.2% ± 4.8), *Rothia* (5.3% ± 4), *Actinomyces* (4% ± 2.1), *Haemophilus* (4% ± 4), *Granulicatella* (2.7% ± 1.2), *Leptotrichia* (2.4% ± 2.2), *Gemella* (2% ± 1.2).

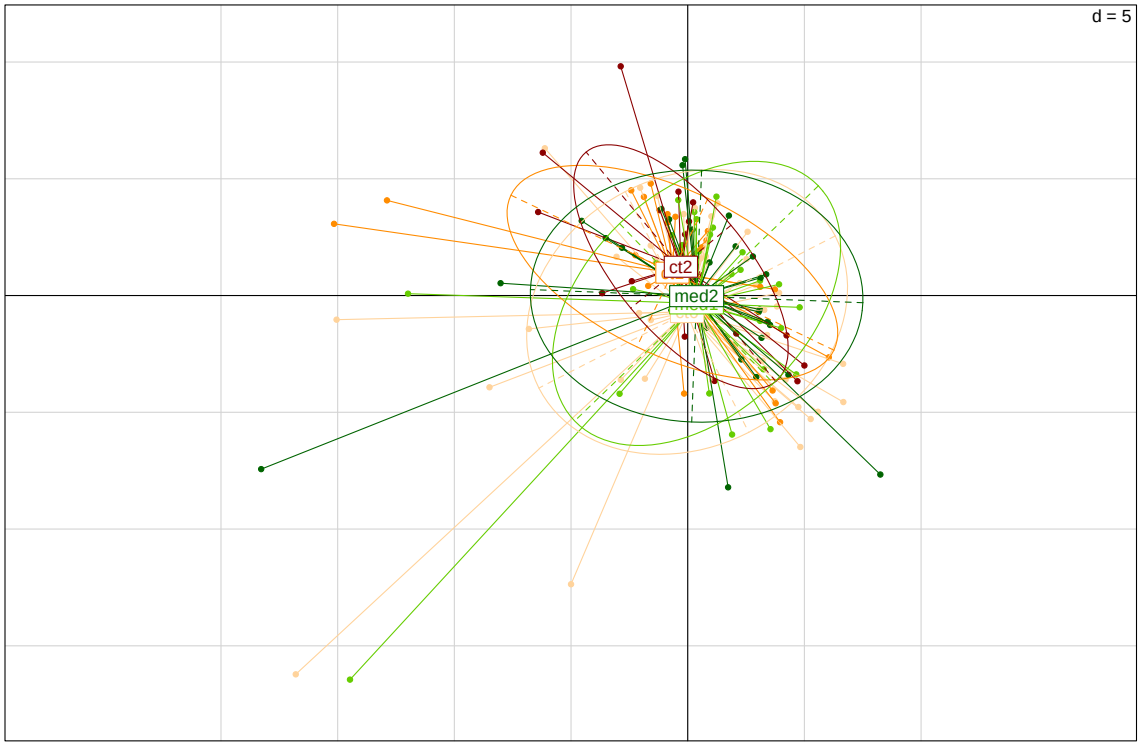


Figure 6.1 Principal Component Analysis (PCA) based on log_{10} transformed OTU tables at genus level. The two principal components were plotted using the *dudi.pca* function. Subjects belonging to different categories were colored according to diet and timepoints: MedD subjects at baseline (Med0, light green), after 4 (Med1, green) and 8 weeks of intervention (Med2, dark green). ConD subjects at baseline (Ct0, light orange), after 4 (Ct1, orange) and 8 weeks (Ct2, dark orange) of intervention.

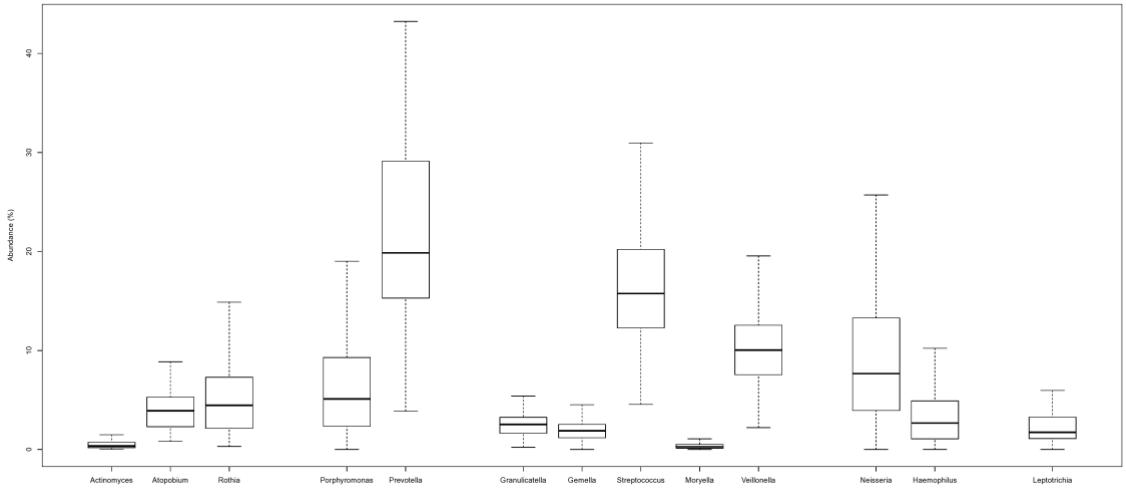


Figure 6.2 Relative abundance of the microbial genera identified as core salivary microbiota in this study. Occurrence of the core genera was set at 99% in all subjects. The bottom and top of the box indicate the first and third quartiles, the line inside the box the median. The boxes are grouped according to phylum.

The Mediterranean diet and microbial features

Although there were no significant differences in the salivary microbiota composition between treated and untreated subjects, we found specific microbial signatures varying in relative abundance upon the dietary intervention. In particular, a significant decrease in the relative abundance of the genus *Subdoligranulum* (Wilcoxon-Mann-Whitney test, $p<0.05$) and the species *Treponema denticola* ($p<0.05$), *Porphyromonas gingivalis* ($p<0.005$) and *Prevotella intermedia* ($p<0.05$) was observed after 8 weeks of treatment with the MedD (Figure 6.3). In addition, lower levels of *Streptococcus*, *Filifactor* and *Lactobacillus* genera were registered after both 4 and 8 weeks of dietary treatment with MD, although such differences failed to reach significance.

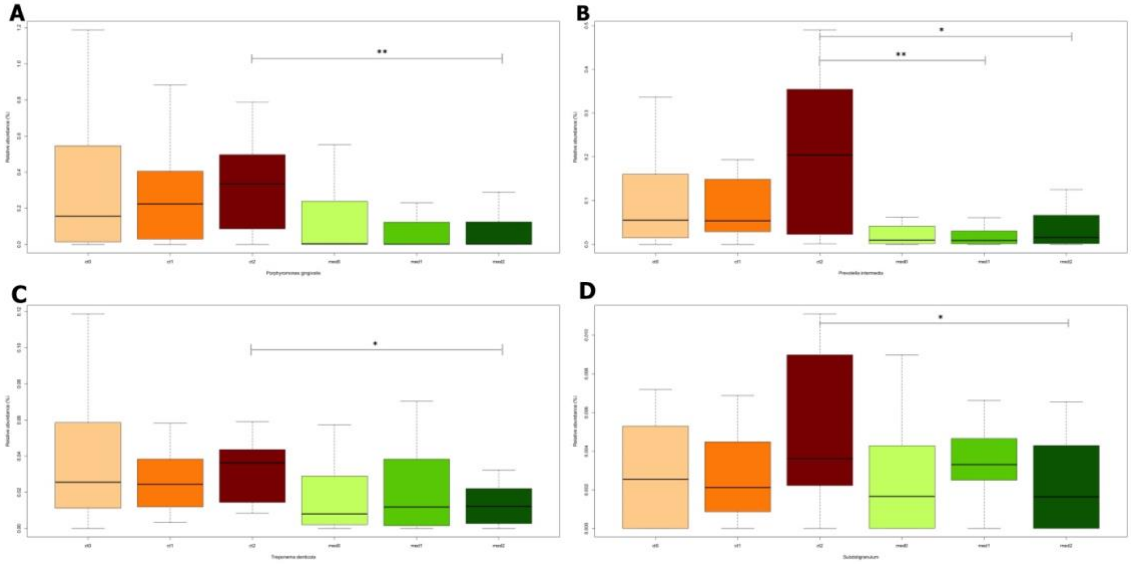


Figure 6.3 Microbial features significantly impacted by Mediterranean Diet. Boxplots showing the relative abundance of *P. gingivalis* (A), *P. intermedia* (B), *T. denticola* (C) and *Subdoligranulum* spp. (D) from saliva samples. Boxplots describe median, lower/upper quartile and standard deviation. A Wilcoxon-Mann-Whitney test was used to analyze differences between MedD and ConD groups and the time points (*$p < 0.05$ and **$p < 0.005$). For a description of subjects and categories, see Figure 6.1 legend.

Sub-genus diversity of Streptococcus genus

The oligotyping analysis of *Streptococcus* genus led to 18 different oligotypes. The diversity in oligotypes composition did not separate subjects belonging to different groups. Indeed, the dietary intervention did not affect the *Streptococcus* diversity since all the oligotypes were closely related in a hierarchical clustering analysis based on distance matrix. Surprisingly, *Streptococcus* oligotype S3 showed a significant increase in its relative abundance within treated group compared to controls after 8 weeks of dietary

treatment, as shown in Figure 6.4. (Wilcoxon-Mann-Whitney test, $p < 0.05$). We blasted the representative sequences against the HOMD 16S rRNA RefSeq database for the identification and further labelled S3 oligotype as *Streptococcus cristatus* (ID 99.3%).

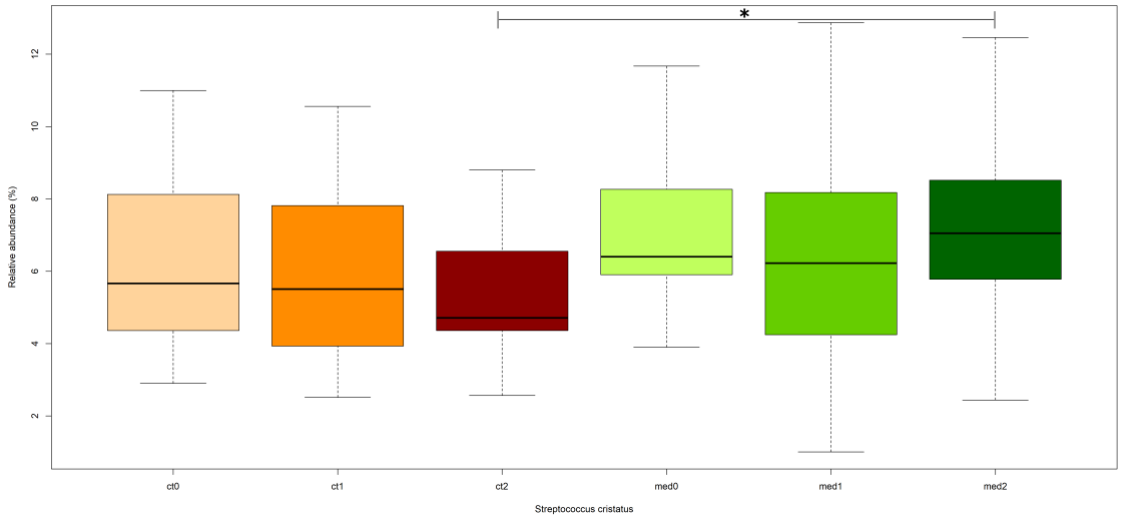


Figure 6.4 Box plots showing the relative abundance of *Streptococcus cristatus* oligotype in the categories analyzed in this study. Boxes represent the medians and the interquartile ranges (IQRs), the whiskers indicate the lowest and highest values that were within 1.5 times the IQR from the first and third quartiles (*p<0.05). For a description of subjects and categories, see Figure 6.1 legend.

6.4 Discussion

The human oral microbiota is a complex ecosystem and its homeostasis is important in order to avoid growth of opportunistic pathogens and dysbiosis, potentially resulting in both oral inflammation and systemic infection (Kodukula *et al*., 2017).

Here we described the effect of a well-known health-promoting dietary model on the oral microbial ecology in overweight subjects. Overweight is a worldwide public health syndrome, a condition associated to systemic low-grade inflammation and correlated to gut microbiota dysbiosis (Bendall *et al*., 2017; Ley *et al*., 2005). It has been reported that overweight status is positive associated with an increased risk of oral chronic inflammatory diseases such as periodontitis (Suvan *et al*., 2011; Chaffee and Weston, 2010), which in turn is caused by oral dysbiosis and facilitates oral pathogens colonization (Kodukula *et al*., 2017). On the other hand, possible mechanisms by which oral bacteria could affect body weight and contribute to overweight condition have been proposed (Goodson *et al*., 2009). The aim of this paper is to explore changes in salivary microbial community of overweight subjects upon a Mediterranean diet-based intervention. The

results may provide a profitable therapeutic diet-based approach leading to an oral eubiosys condition, which in turn could positively affect host's biology.

Detected taxa in 99% of saliva samples largely coincided with those reported in previous studies. Recently, De Filippis *et al.* (2014) observed that the salivary microbiota was not significantly linked to specific dietary habits, although a core microbiota was identified, which included 6 out of the 13 core genera identified here. In line with our results, other detected members of the core (*Veillonella*, *Gemella*, *Actynomices* and *Rothia*) coincided with those found in other saliva samples (Segata *et al.*, 2012). However, these shared genera seem to be as part of common oral commensals, although reported studies investigated salivary microbial communities in healthy cohorts whereas our work focused on overweight subjects. In addition, the overall microbiota composition showed to be lately resistant to external perturbations as well as dietary intervention. This finding is consistent with other researches finding that human salivary microbiome is stable in adulthood as a result of lifestyle factors and environmental events (Stahringer *et al.*, 2012).

Despite inter-individual variability, host and environmental effects, understanding the oral microbiome could have a potential role for the prevention and the management of diseases (Acharya *et al.*, 2017). In particular, we detected the effects of diet on genera well known to be associated to both oral and systemic diseases. Interestingly, after 8 weeks there was a significant decrease in the relative abundance of some oral pathogens such as *Porphyromonas gingivalis*, *Prevotella intermedia* and *Treponema denticola* in MedD subjects. These microorganisms were classified as periodontopathogenic bacteria and named as "red bacterial complex" (Holt and Ebersole, 2005). Due to their virulence factors, they are responsible of periodontitis disease and following oral tissue destruction, subgingival pathogens colonization and host defence immunomodulation (Bodet *et al.*, 2007). These periodontal pathogens have a proteolytic pool of enzymes involved in adhesion and nutrition phase. In addition, they are non-saccharolytic microorganisms and they require peptides and amino acids to grow (Eley and Cox, 2003). The Mediterranean diet is a vegetarian-oriented dietary pattern that entails a high intake of fibre and carbohydrates and a lower intake of proteins, especially of animal origin. Indeed, the lower levels of protein nitrogen might explain the lower levels of the potential periodontal pathogens. Mechanical treatments and locally applied antimicrobial agents are currently used in periodontal therapy. Since the chronic oral infection of periodontitis may be a risk factor for systemic pathologies (Pereira *et al.*, 2017; Acharya *et al.*, 2017; Zhang *et al.*,

2015), the effect of diet could be relevant in reducing both periodontal bacteria and risks factors in the oral cavity, resulting in an improved oral health. In addition, the most interesting finding is that we observed a significant increase in the relative abundance of *Streptococcus cristatus* oligotype in MedD group compared to ConD. In particular, this observation is in agreement with that obtained by Ho *et al*. (2017) which demonstrated how *Streptococcus cristatus* inhibits virulence genes expression in *P. gingivalis* with a direct interaction. Hence, the observed decrease in the relative abundance of *P. gingivalis* within treated subjects could be attributed to an antagonistic presence of *S. cristatus* (Xie *et al*., 2012). Our findings suggest that Mediterranean diet may have a protective effect against the occurrence of the 'red bacterial complex' responsible of periodontal lesions. Clearly, further research will be required to validate a Mediterranean-based diet as an additional and useful tool to manage and to treat periodontitis disease.

It's well known that salivary dysbiosis is related to non-oral pathologies and that saliva could be used as a non-invasive biomarker in diagnostics. In our cohort, we found an increase in the relative abundance of the genus *Leptotrichia* between groups after 4 and 8 weeks, although not significant ($p>0.05$). It has been reported that a greater abundance of *Leptotrichia* in oral samples was associated with a decreased risk of pancreatic cancer (Fan *et al*., 2016). On similar lines, subjects in MedD tended to have a lower prevalence of *Lactobacillus* and *Streptococcus*. The occurrence of these genera in saliva samples seems to be related to HNSCC (head and neck squamous cell carcinoma) as reported by Guerrero-Preston *et al*. (2016).

After 8 weeks, a significant decrease of *Subdoligranulum* levels ($p<0.05$) was assessed after treatment. As reported in literature, *Subdoligranulum* genus is correlated to inflammatory parameters in human type 1 diabetes (De Groot *et al*., 2017), although it was detected in human stool samples. This is not surprising if we consider the emerging link between oral and gut microbiomes. Recent evidences suggest the hypothesis that the oral microbiome is linked to the gut microbiome. Indeed, metabolic disorders could be enhanced by swallowed oral pathogens as shown in mice model (Arimatsu *et al*., 2014) and gut microbiota alterations in diabetes condition could cause a pathogenic shift in the oral microbiome leading to an oral dysbiosis (Xiao *et al*., 2017). Therefore, reducing or suppressing the systemic dissemination of oral bacteria might be crucial in managing diabetes disease.

6.5 Conclusion

The oral microbiota is resistant to diet as environmental insult seemingly to be not affected by dietary habits or dietary intervention. In the present intervention study, the overall structure of the oral microbial community was minimally disturbed by treatment, although the results highlighted the impact of a Mediterranean-based diet model reducing the relative abundance of both oral and non-oral pathogens. Currently, the concept of "Precision Nutrition" is rising up as a new topic of personalized medicine and a health-promoting diet could play an essential role for the prevention of diseases. In this case, MD might be used to positively influence oral microbial ecology, to prevent the onset of diet-related pathologies and to pursue host homeostasis.

6.6 References

Acharya A, Chan Y, Kheur S, Jin LJ, Watt RM, Mattheos N. 2017. Salivary microbiome in non-oral disease: A summary of evidence and commentary. Arch Oral Biol 83:169–173.

Arimatsu K, Yamada H, Miyazawa H, Minagawa T, Nakajima M, Ryder MI, Gotoh K, Motooka D, Nakamura S, Iida T. 2014. Oral pathobiont induces systemic inflammation and metabolic changes associated with alteration of gut microbiota. Sci Rep 4:4828.

Bendall CL, Mayr HL, Opie RS, Bes-Rastrollo M, Itsiopoulos C, Thomas CJ. 2018. Central obesity and the Mediterranean diet: A systematic review of intervention trials. Crit Rev Food Sci Nutr 58:3070–3084.

Bodet C, Chandad F, Grenier D. 2007. Pathogenic potential of *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, the red bacterial complex associated with periodontitis. Pathol Biol (Paris) 55:154–162.

Cameron SJS, Huws SA, Hegarty MJ, Smith DPM, Mur LAJ. 2015. The human salivary microbiome exhibits temporal stability in bacterial diversity. FEMS Microbiol Ecol 91.

Canani RB, De Filippis F, Nocerino R, Laiola M, Paparo L, Calignano A, De Caro C, Coretti L, Chiariotti L, Gilbert JA. 2017. Specific signatures of the gut microbiota and increased levels of butyrate in children treated with fermented cow's milk containing heat-killed *Lactobacillus paracasei* CBA L74. Appl Environ Microbiol 83:e01206-17.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335.

Chaffee BW, Weston SJ. 2010. Association between chronic periodontal disease and obesity: a systematic review and meta-analysis. J Periodontol 81:1708–1724.

Cockburn AF, Dehlin JM, Ngan T, Crout R, Boskovic G, Denvir J, Primerano D, Plassman BL, Wu B, Cuff CF. 2012. High throughput DNA sequencing to detect differences in the subgingival plaque microbiome in elderly subjects with and without dementia. Investig Genet 3:19.

Costello EK, Carlisle EM, Bik EM, Morowitz MJ, Relman DA. 2013. Microbiome assembly across multiple body sites in low-birthweight infants. MBio 4:e00782-13.

De Filippis F, Vannini L, La Storia A, Laghi L, Piombino P, Stellato G, Serrazanetti DI, Gozzi G, Turroni S, Ferrocino I. 2014. The same microbiota and a potentially discriminant metabolome in the saliva of omnivore, ovo-lacto-vegetarian and vegan individuals. PLoS One 9:e112373.

De Filippis F, Pellegrini N, Vannini L, Jeffery IB, La Storia A, Laghi L, Serrazanetti DI, Di Cagno R, Ferrocino I, Lazzi C. 2016. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut 65:1812–1821.

De Filippis F, Pellegrini N, Laghi L, Gobbetti M, Ercolini D. 2016. Unusual sub-genus associations of faecal *Prevotella* and *Bacteroides* with specific dietary patterns. Microbiome 4:57.

De Groot PF, Belzer C, Aydin Ö, Levin E, Levels JH, Aalvink S, Boot F, Holleman F, Van Raalte DH, Scheithauer TP. 2017. Distinct fecal and oral microbiota composition in human type 1 diabetes, an observational study. PLoS One 12:e0188475.

Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu W-H, Lakshmanan A, Wade WG. 2010. The human oral microbiome. J Bacteriol 192:5002–5017.

Eley BM, Cox SW. 2003. Proteolytic and hydrolytic enzymes from putative periodontal pathogens: characterization, molecular genetics, effects on host defenses and tissues and detection in gingival crevice fluid. Periodontol 2000 31:105–124.

Eren AM, Maignien L, Sul WJ, Murphy LG, Grim SL, Morrison HG, Sogin ML. 2013. Oligotyping: differentiating between closely related microbial taxa using 16S rRNA gene data. Methods Ecol Evol 4:1111–1119.

Eriksson L, Holgerson PL, Johansson I. 2017. Saliva and tooth biofilm bacterial microbiota in adolescents in a low caries community. Sci Rep 7:5861.

Fan X, Alekseyenko A V, Wu J, Peters BA, Jacobs EJ, Gapstur SM, Purdue MP, Abnet CC, Stolzenberg-Solomon R, Miller G. 2018. Human oral microbiome and prospective risk for pancreatic cancer: a population-based nested case-control study. Gut 67:120–127.

Francavilla R, Ercolini D, Piccolo M, Vannini L, Siragusa S, De Filippis F, De Pasquale I, Di Cagno R, Di Toma M, Gozzi G. 2014. Salivary microbiota and metabolome associated with celiac disease. Appl Environ Microbiol 80:3416–3425.

Goodson JM, Groppo D, Halem S, Carpino E. 2009. Is obesity an oral bacterial disease? J Dent Res 88:519–523.

Guerrero-Preston R, Godoy-Vitorino F, Jedlicka A, Rodríguez-Hilario A, González H, Bondy J, Lawson F, Folawiyo O, Michailidi C, Dziedzic A. 2016. 16S rRNA amplicon sequencing identifies microbiota associated with oral cancer, human papilloma virus infection and surgical treatment. Oncotarget 7:51320.

Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA, McIntosh ML, Alsam A, Kirkwood KL, Lambris JD. 2011. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. Cell Host Microbe 10:497–506.

Ho M-H, Lamont RJ, Xie H. 2017. Identification of *Streptococcus cristatus* peptides that repress expression of virulence genes in *Porphyromonas gingivalis*. Sci Rep 7:1413.

Holt SC, Ebersole JL. 2005. *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia*: the 'red complex', a prototype polybacterial pathogenic consortium in periodontitis. Periodontol 2000 38:72–122.

Idris A, Hasnain SZ, Huat LZ, Koh D. 2017. Human diseases, immunity and the oral microbiota—Insights gained from metagenomic studies. Oral Sci Int 14:27–32.

Kodukula K, Faller D V, Harpp DN, Kanara I, Pernokas J, Pernokas M, Powers WR, Soukos NS, Steliou K, Moos WH. 2017. Gut microbiota and salivary diagnostics: the mouth is salivating to tell us something. Biores Open Access 6:123–132.

Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. 2005. Obesity alters gut microbial ecology. Proc Natl Acad Sci 102:11070–11075.

Magoč T, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27:2957–2963.

Maukonen J, Mättö J, Suihko M-L, Saarela M. 2008. Intra-individual diversity and similarity of salivary and faecal microbiota. J Med Microbiol 57:1560–1568.

Paster BJ, Olsen I, Aas JA, Dewhirst FE. 2006. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontol 2000 42:80–87.

Pereira PAB, Aho VTE, Paulin L, Pekkonen E, Auvinen P, Scheperjans F. 2017. Oral and nasal microbiota in Parkinson's disease. Parkinsonism Relat Disord 38:61–67.

Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, Bonazzi V, McEwen JE, Wetterstrand KA, Deal C. 2009. The NIH human microbiome project. Genome Res 19:2317–2323.

Schmieder R, Edwards R. 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27:863–864.

Segata N, Haake SK, Mannon P, Lemon KP, Waldron L, Gevers D, Huttenhower C, Izard J. 2012. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. Genome Biol 13:R42.

Slocum C, Kramer C, Genco CA. 2016. Immune dysregulation mediated by the oral microbiome: potential link to chronic inflammation and atherosclerosis. J Intern Med 280:114–128.

Sofi F, Abbate R, Gensini GF, Casini A. 2010. Accruing evidence on benefits of adherence to the Mediterranean diet on health: an updated systematic review and meta-analysis. Am J Clin Nutr 92:1189–1196.

Stahringer SS, Clemente JC, Corley RP, Hewitt J, Knights D, Walters WA, Knight R, Krauter KS. 2012. Nurture trumps nature in a longitudinal survey of salivary bacterial communities in twins from early adolescence to early adulthood. Genome Res 22:2146–2152.

Suvan J, D'Aiuto F, Moles DR, Petrie A, Donos N. 2011. Association between overweight/obesity and periodontitis in adults. A systematic review. Obes Rev 12:e381–e404.

Wu J, Peters BA, Dominianni C, Zhang Y, Pei Z, Yang L, Ma Y, Purdue MP, Jacobs EJ, Gapstur SM. 2016. Cigarette smoking and the oral microbiome in a large study of American adults. ISME J 10:2435.

Xiao E, Mattos M, Vieira GHA, Chen S, Corrêa JD, Wu Y, Albiero ML, Bittinger K, Graves DT. 2017. Diabetes enhances IL-17 expression and alters the oral microbiome to increase its pathogenicity. Cell Host Microbe 22:120–128.

Xie H, Hong J, Sharma A, Wang B. 2012. *Streptococcus cristatus* ArcA interferes with *Porphyromonas gingivalis* pathogenicity in mice. J Periodontal Res 47:578–583.

Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, Wu X, Li J, Tang L, Li Y. 2015. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nat Med 21:895.

Zhou Y, Gao H, Mihindukulasuriya KA, La Rosa PS, Wylie KM, Vishnivetskaya T, Podar M, Warner B, Tarr PI, Nelson DE. 2013. Biogeography of the ecosystems of the healthy human body. Genome Biol 14:R1.

CHAPTER 7

General discussion

Nowadays, the perturbation in the gut equilibrium among different members of the gut microbiota has been extensively correlated to the development of several types of diseases. Moreover, the modulation of microbial composition and activity through dietary interventions is a fascinating and promising research topic for both prevention and treatment of diseases. To this regard, further clinical interventions need to be based on evidences-derived and proof-of-concept provided by microbiome-directed RCTs, thought to identify the underlying relationship between diet, gut symbionts and clinical outcomes. Therefore, in order to accomplish this aim a multidisciplinary approach is necessary. This strategy could provide stronger evidences and produce valuable applications in the fields of personalized nutrition, preventive and precision medicine as well as functional food development (De Filippis *et al*., 2018).

In this thesis, the potential of microbiome-targeted dietary interventions was investigated. These studies demonstrated the potential of beneficial dietary manipulations over human microbiome for patients care in health and disease. Importantly, a probiotic-enriched formula induces significant changes in gut microbiota composition, promoting the development of butyrate producers in healthy children. Moreover, such changes correlate with increased levels of innate and acquired immunity biomarkers, as described in the **Chapter 3**. Similarly, as presented in the **Chapter 4**, a gut microbiota dysbiosis related to non-IgE-mediated CMA condition is attenuated by a probiotic dietary treatment. Such findings further highlight the microbiota as a relevant target for innovative therapeutic strategies in children affected by FA. Interestingly, the results in the **Chapters 5** and **6** clearly show that an isocaloric 8 weeks intervention with a MedDiet-based pattern modulates individual clinical outcomes, the oral microbiota and gut microbiome composition along with its metabolome in obese and overweight subjects with cardiometabolic risk for unhealthy lifestyle. These findings provide

insights into profitable therapeutic diet-based approach leading to eubiosys condition, positively affecting host biology.

Despite the relevant improvements toward the useful application of RCTs to health amelioration, some challenges still need to be addressed for clinical practice and to further study the complex nature of the diet-microbe-host interactions in order to achieve the ultimate goal of precision medicine. Before adopting microbiome-directed interventions there is a need for an improved understanding of the crucial role of microbiome related to the onset, maintenance and progression of disease (Figure 7.1).

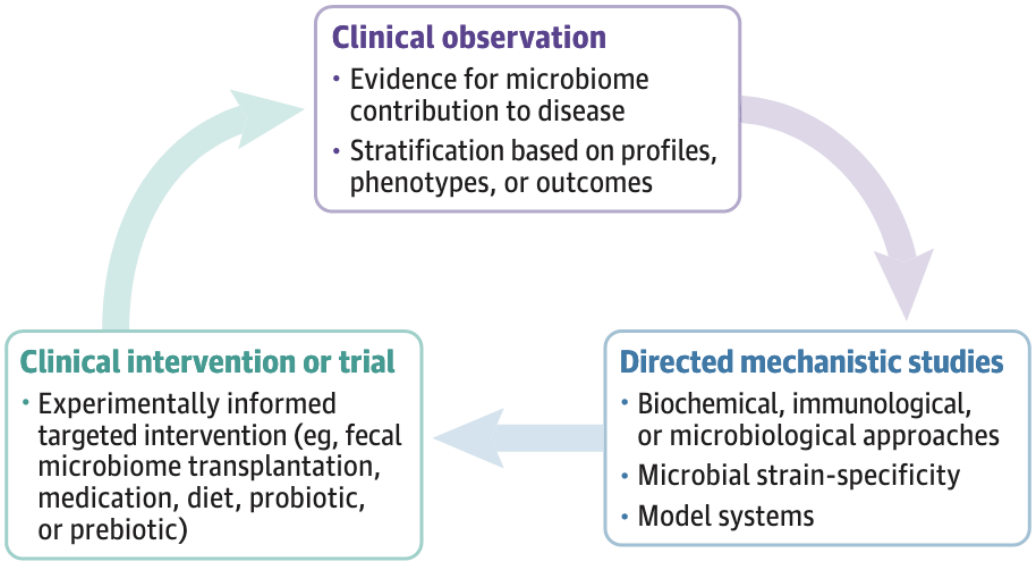


Figure 7.1 Translating microbiome science into clinical practice. Image from Harkins *et al*., 2019.

To this end, further clinical and nutritional trials interventions on ever larger cohorts are needed. Hence, collected evidences and observations will be helpful to clearly identify biological mechanisms and to elucidate the causal relationships between diet/food and microbiome changes underlying clinical outcomes. Moreover, data carried out will be functional to stratify population for responsive phenotypes or metabotypes in the attempt to develop *ad hoc* personalized nutrition approaches. Later, the proper management of critical factors (*e.g.*, study design and population, biomarker selection, compliance, data analysis) will lead to more sensible data used for specific mechanistic studies. Sorted observations from such evidences will underpin machine-learning models i) to fuel microbial or gene-based signatures identification as health/disease biomarkers and ii) to predict individual metabolic responses for personalized microbiota-targeted therapeutic approaches. Accordingly, it was recently reported that different subjects may have very different metabolic responses to the

same meals (Lynch and Pedersen, 2016). In fact, in order to successfully modify blood glucose levels, personalized diets might be used as predicted by individual postprandial glycemic responses (Zeevi *et al*., 2015). Consequently, intra-individual variability of the baseline microbiome composition should be also considered regarding responsiveness to dietary intervention to intensively modulate gut ecology and microbial metabolic potential in a highly personalized manner (Doré and Blottière, 2015; Visconti *et al*., 2019). The stratification in enterotypes/metabotypes/phenotypes will have potential clinical benefits contributing to diagnosis, monitoring the disease etiology and progression as well as guiding treatments (Costea *et al*., 2018). Lastly, meta-analyses correlating microbiome features with host genomics, transcriptomics, epigenomics and metabolomics data should be performed, representing another exciting step for investigating human outcomes. Despite such long workflow and huge amount of data are in the process of being validated, the results presented in this thesis well fit the scientific request of rationally modulating host-microbiome interactions through dietary interventions for clinical management.

In conclusion, as literally Hippocrates stated in "De Alimento" more than 2000 years ago "In nutriment purging excellent, in nutriment purging bad; bad or excellent according to circumstances". Specifically, "In food excellent medicine can be found, in food bad medicine can be found; good and bad are relative", we are now looking at and contributing to the start of the precision medicine era, heralding diet as a practical tool to promote host health by managing human microbial ecology.

References

Costea PI, Hildebrand F, Arumugam M, Bäckhed F, Blaser MJ, Bushman FD, De Vos WM, Ehrlich SD, Fraser CM, Hattori M. 2018. Enterotypes in the landscape of gut microbial community composition. Nat Microbiol 3:8.

De Filippis F, Vitaglione P, Cuomo R, Berni Canani R, Ercolini D. 2018. Dietary interventions to modulate the gut microbiome—how far away are we from precision medicine. Inflamm Bowel Dis 24:2142–2154.

Doré J, Blottière H. 2015. The influence of diet on the gut microbiota and its consequences for health. Curr Opin Biotechnol 32:195–199.

Harkins CP, Kong HH, Segre JA. 2019. Manipulating the Human Microbiome to Manage Disease. JAMA.

Haro C, Garcia-Carpintero S, Alcala-Diaz JF, Gomez-Delgado F, Delgado-Lista J, Perez-Martinez P, Zuñiga OAR, Quintana-Navarro GM, Landa BB, Clemente JC. 2016. The gut microbial community in metabolic syndrome patients is modified by diet. J Nutr Biochem 27:27–31.

Lynch S V, Pedersen O. 2016. The human intestinal microbiome in health and disease. N Engl J Med 375:2369–2379.

Visconti A, Le Roy CI, Rosa F, Martin TC, Mohney RP, Weizhong L, de Rinaldis E, Bell JT, Venter JC, Nelson KE. 2019. Interplay between the human gut microbiome and host metabolism. Nat Commun 10:4505.

Zeevi D, Korem T, Zmora N, Israeli D, Rothschild D, Weinberger A, Ben-Yacov O, Lador D, Avnit-Sagi T, Lotan-Pompan M. 2015. Personalized nutrition by prediction of glycemic responses. Cell 163:1079–1094.

CURRICULUM VITAE

Name/Surname.	Manolo Laiola
Address	Via Spinelli, 82 – Boscoreale (NA)
Phone	+39 3663325952
e-mail	laiola.manolo@gmail.com
Date of birth	May 28th 1989

Education and training

February 2017 up now

PhD student in Agricultural and Food Sciences (XXXII). Activities carried out at the Dept. of Agricultural Sciences – Section of Microbiology, University of Naples Federico II. Advisor Prof. Danilo Ercolini (ercolini@unina.it)

January 2018 – December 2018

Internship during PhD studies at MetaGenoPolis unit (Domaine de Vilvert, Bâtiment 325, 78350 Jouy-en-Josas - INRA - France)

January 2017

Specialization course in "La piattaforma R per l'analisi statistica di dati biomedici" at University of Naples Federico II. Advisor Prof. Dario Bruzzese (dario.bruzzese@unina.it)

October 2016

Master's degree in Plant biotechnology (LM-7) obtained at the University of Naples "Federico II" (110/110 *cum laude*). Experimental thesis on "Effect of probiotics on gut microbiota of infants". Tutor Prof. Danilo Ercolini (ercolini@unina.it)

September 2014

Bachelor's degree in Biotechnology (L-2, undergraduate degree of first level) obtained at the University of Naples "Federico II" (102/110). Experimental thesis on "RNAi targeting NIMIN-1 gene in *A. thaliana*". Tutor Prof. Giandomenico Corrado (giandomenico.corrado@unina.it).

July 2008

High school degree.

Foreign languages

English and French

Technical skills

- DNA/RNA extraction from plant, microbial and environmental samples;
- Microbiological analyses, through culture- independent techniques (qPCR, PCR);
- Preparation of libraries for 16S rRNA and shotgun sequencing for metagenomic analysis;
- Knowledge of the protocols for next-generation sequencing, in particular Illumina sequencing.

Bioinformatics skills

- Good knowledge of QIIME software for the analysis of next-generation sequencing data (from amplicon-based and shotgun sequencing);
- Bash scripting;
- Good knowledge of R software for biostatistical analysis, plotting and clustering;
- Multi-omics data analysis and clinical data integration;
- Microsoft Office for data manipulation and presentation.

Participation to international research projects

February 2017 up now

European project under the Joint Action "Healthy diet for a heathy life" (HDHL), JPI- Intestinal Microbiomics. Project titled "Diet-INduced Arrangement of the gut Microbiome for Improvement of Cardiometabolic health " - Acronym "DINAMIC". Project Coordinators: Prof. Thomas Clavel and Dirk Haller.

Grants and Awards

September 2019

Winner of an Early Carrier Scientific meeting grant from The MD2019 FEMS Grant Committee for the presentation of the poster "Mediterranean based dietary intervention affects oral microbial ecology in overweight subjects" **Laiola M**., De Filippis F., Vitaglione P., Ercolini D. at the 5th International Conference Microbial Diversity. "Microbial diversity as a source of novelty: function, adaptation and exploitation"

Publications in peer-reviewed international journals

1. De Filippis, F., **Laiola, M**., Blaiotta, G., & Ercolini, D. (2017). Different amplicon targets for sequencing-based studies of fungal diversity. *Appl. Environ. Microbiol., 83*(17), e00905-17.
2. Berni Canani, R., De Filippis, F., Nocerino, R., **Laiola, M**., Paparo, L., Calignano, A., ... & Ercolini, D. (2017). Specific signatures of the gut microbiota and increased levels of butyrate in children treated with fermented cow's milk containing heat-killed Lactobacillus paracasei CBA L74. *Appl. Environ. Microbiol.*, *83*(19), e01206-17.
3. Berni Canani, R., De Filippis, F., Nocerino, R., Paparo, L., Di Scala, C., Cosenza, L., Della Gatta, G., Calignano, A., De Caro, C., **Laiola, M**., Gilbert, J. A., Ercolini, D. (2018). Gut microbiota composition and butyrate production in children affected by non-IgE-mediated cow's milk allergy. *Scientific reports*, *8*(1), 12500.
4. Meslier, V.,§ **Laiola, M**.,§ Roager, H.M.,§ De Filippis, F., Roume, H., Quinquis, B., Giacco, R., Mennella, I., Ferracane, R., Pons, N., Pasolli, E., Rivellese, A.A., Dragsted, L.O., Vitaglione, P., Ehrlich, D.S., Ercolini E. (2019). Mediterranean diet intervention in overweight and obese subjects lowers plasma cholesterol and leads to multiple shifts in the gut microbiome and metabolome independently of energy intake. *Gut*, doi: 10.1136/gutjnl-2019-320438. (§) These authors contributed equally to this work.
5. Vitale, M., Giacco, R. **Laiola, M**., Della Pepa, G., Luongo, D., Mangione, A., Salomone, D., Vitaglione, P., Ercolini, D., Rivellese, A.A. (2019). Acute and chronic improvement in postprandial glucose metabolism by a diet resembling the traditional Mediterranean dietary pattern: may SCFAs have a role?. submitted to *Clin. Nutr*.
6. **Laiola, M**., De Filippis, F., Vitaglione, P., Ercolini E. (2019). A Mediterranean diet intervention reduces the levels of salivary periodontopathogenic bacteria in overweight and obese subjects. submitted to *Appl. Environ. Microbiol*.

Abstracts and oral presentations in international conferences proceedings

- **Laiola M**., De Filippis F., Nocerino R., Di Scala C., Cosenza L., Calignano A., De Caro C., Paparo L., Berni Canani R., Ercolini D., "Gut microbiota dysbiosis in children with cow's milk allergy is partly restored by a hydrolysed casein formula treatment", 4th International Conference On Microbial Diversity 2017, pp. 310-311, Bari (Italy).
- **Laiola M**., De Filippis F., Nocerino R., Paparo L., Calignano A., De Caro C., Berni Canani R., Ercolini D., "A *Lactobacillus paracasei* CBA L74 fermented cow's milk impacts gut microbiota composition and butyrate levels", 4th International Conference On Microbial Diversity 2017, pp. 312-

313, Bari (Italy).

- **Laiola M**., De Filippis F., Mennella I., Vitaglione P., Ercolini E., "Changes in the salivary microbiota after a Mediterranean diet-based intervention in overweight subjects", 7th International Human Microbiome Congress 2018, p. 50, Killarney (Ireland).
- De Filippis F., **Laiola M**., Nocerino R., Di Scala C., Cosenza L., Calignano A., De Caro C., Paparo L., Berni Canani R., Ercolini1 E., "Overlapping signatures in the gut microbiota dysbiosis in children with IgE- and non-IgE-mediated cow's milk allergy", 7th International Human Microbiome Congress 2018, p. 37, Killarney (Ireland).
- De Filippis F., **Laiola M**., Mennella I., Vitaglione P., Ercolini E., "Dietary intervention with a Mediterranean-style diet modulates gut microbiome in healthy obese subjects", 7th International Human Microbiome Congress 2018, p. 35, Killarney (Ireland).
- **Laiola M**., De Filippis F., Vitaglione P., Ercolini E., "Mediterranenan-based dietary intervention affetcs oral microbial ecology in overweight subjects", 5th International Conference On Microbial Diversity 2019, pp. 336-337, Catania (Italy).
- De Filippis F., **Laiola M**., Gallo M.A., Giacco R., Rivellese A.A., Vitaglione P., Ercolini E., "Mediterranean-style dietary intervention promotes the production of beneficial metabolites and modulates gut microbiome in healthy obese subjects", 5th International Conference On Microbial Diversity 2019, pp. 88-89, Catania (Italy).