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# "Exploring human gut microbiome variations across life: from eubiosis to dysbiosis in Western populations"

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**Abstract**

The human gut microbiome is an extremely dynamic ecosystem, able to establish peculiar configurations in response to several endogenous and exogenous stimuli – ageing, diet, lifestyle, disease. In order to explore the microbiome-host relationship and unravel the gut microbiome variations throughout the human lifespan, we studied specific functional aspects related to eubiosis and dysbiosis in Western diseases, using next-generation sequencing approaches, and developed a versatile murine model of intestinal inflammation to better explore the transition towards dysbiotic layouts.

As for the aspects related to eubiotic microbiota configurations, we characterized the age-related functional changes occurring in the gut microbial ecosystem across life up to extreme longevity (age range, 22-109 years), highlighting life-long adaptive responses potentially supporting a new homeostasis. On the other hand, when compared to traditional populations – whose lifestyle resembles that of our ancestors – the Western gut microbiome is found to be characterized by reduced biodiversity and supposed to contribute to the rising incidence of non-communicable diseases (NCDs). Consequently, we assessed the possibility to modulate the Western GM towards a more 'ancestral' configuration through a dietary intervention with a modern Paleolithic diet.

Focusing on dysbiotic variations associated with NCDs, we investigated the link between diet, gut microbiome and obesity in Western cohorts. In particular, we identified early markers and individual microbiome-host-diet configurations as a potential predictor related to the onset of the disease during childhood, through a 4-year prospective study. Furthermore, we merged metagenomic and metatranscriptomic approaches to unravel specific obese-related gut microbiome layouts at species level and metabolic activities possibly associated with food addiction in obese women.

The shift towards a dysbiotic microbiome structure and its association with several diseases have made the microbiome a strategic therapeutic target, paving the way for the development

of a wide range of microbiome-tailored intervention strategies aimed at the restoration of eubiotic, health-promoting layouts. In this perspective, we developed a murine model that mimics the conditions of inflammation typically associated with inflammatory bowel disease. The development of this murine model may provide researchers with a versatile tool for testing and validating candidate anti-inflammatory agents and/or new microbiome modulators such as classic or next-generation probiotics, before their use in clinical practice.

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Chapter 1 – **INTRODUCTION**



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**ABCs of the human gut microbiome**

Being composed of trillions of microorganisms and their genomes, the human microbiome has recently emerged as an area of great interest for the scientific community. It is a matter of fact that bacteria are commonly found on external and internal surfaces of the human body, e.g. the skin, saliva, oral mucosa, and gastrointestinal tract. Among these body niches, the gastrointestinal tract represents the major reservoir of microorganisms associated with the human body harboring about $10^{13}$ – $10^{14}$ microbial cells, i.e. the gut microbiota (Rajilić-Stojanović *et al*., 2014). Although the ratio between microbial and nucleated human cells has been initially estimated to be 1:10 (Savage 1977), a recent study claims that including non-nucleated human cells in the calculation the ratio drops to 1:1 (Sender *et al*., 2016). In this perspective, the human gastrointestinal tract can be regarded as a dynamic bioreactor, within which resides a complex community that includes all three domains of life – Archaea, Bacteria, Eukarya – and viruses as well as. The human gut microbiome, i.e. the collective genome of the microbial ecosystem, comprises at least 400 times more genes than the 2.85 billion of base pairs found in the human genome (Li *et al*., 2014). Humans can therefore be considered as super-organisms whose genetic makeup is represented by the pool of genes present in human cells and in the genome of intestinal microbiota, considerably increasing the adaptive potential of this hologenome to external perturbations and, ultimately, providing the host with indispensable

extra functions (Qin *et al*., 2010; Sonneburg & Bäckhed, 2016). In this perspective, the microbiome can be considered a diversified ecosystem composed mainly of symbiotic microorganisms – commensals and mutualists – that commonly evolve to compete within the host ecosystem and interact with most, if not all, of the host organs. The mutualistic relationship established between the counterparties involves the acquisition of energy by the host, by absorbing bacterial fermentation end-products (i.e. short-chain fatty acids, SCFAs), while the microbial component can thrive in an optimal environment with controlled temperature and rich of nutrients (Bäckhed *et al*., 2005; Ley *et al*., 2008). A key aspect of this fine-tuned relationship concerns the integration and boosting of the metabolic potential of the host, especially with regard to complex polysaccharides metabolism. Indeed, functional assignments of the gut microbiome highlight a high percentage of sequences assigned to carbohydrate-active enzymes (CAZymes) for overcoming the other KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and COGs (clusters of orthologous groups) involved in energy process and SCFA production (Turnbaugh *et al*., 2009a; Rampelli *et al*., 2013; Soverini *et al*., 2017). Considering the limited repertoire of human genes devoted to polysaccharide degradation, the wide variety of CAZymes encoded by our microbial counterpart is therefore deputed to the metabolism of dietary polysaccharides that reach the colon undigested, becoming metabolically available to gut microbes (recently defined as microbiota-accessible carbohydrates, or MACs) (El Kaoutari *et al*., 2013; Sonnenburg & Sonnenburg 2014), emphasizing the metabolic synergy established between the host and its associated microorganisms. Microbial symbionts perform crucial functions to promote human physiology and health, providing for the extraction, synthesis and absorption of many nutrients and metabolites such as bile acids, lipids, amino acids, vitamins, and short-chain fatty acid (Rakoff-Nahoum *et al*., 2014; Flint *et al*., 2015; Cani *et al*., 2019). In fact, several of our physiological and immunological features depend on the mutualistic association with our intestinal microbial community, which plays an active role in preventing colonization of the gut epithelium by enteropathogens (barrier effect) as well as in

priming, development and regulating the homeostasis and function of innate and adaptive immune cells (Candela *et al*., 2012; Brestoff *et al*., 2013; Marchesi *et al*., 2016; Gensollen *et al*., 2016). Another relevant role played by our microbial counterpart concerns the development and regulation of the central nervous system, also by influencing the endocrine system (Jackson *et al*., 2018; Duvallet *et al*., 2017). Various endogenous and exogenous factors – such as genetics, diet, lifestyle and medication – can influence the gut microbiota composition and function, which in turn modulates the function of several organs via the production of various mediators, i.e. SCFAs, secondary bile acids, neurotransmitters and gut hormones, endotoxins and microbial components (Cani *et al*., 2019; Sen *et al*., 2019). In this scenario, the extremely dynamic crosstalk between the gut microbiota and the host plays a fundamental role in the regulation of human metabolism (Figure 1.1.1).

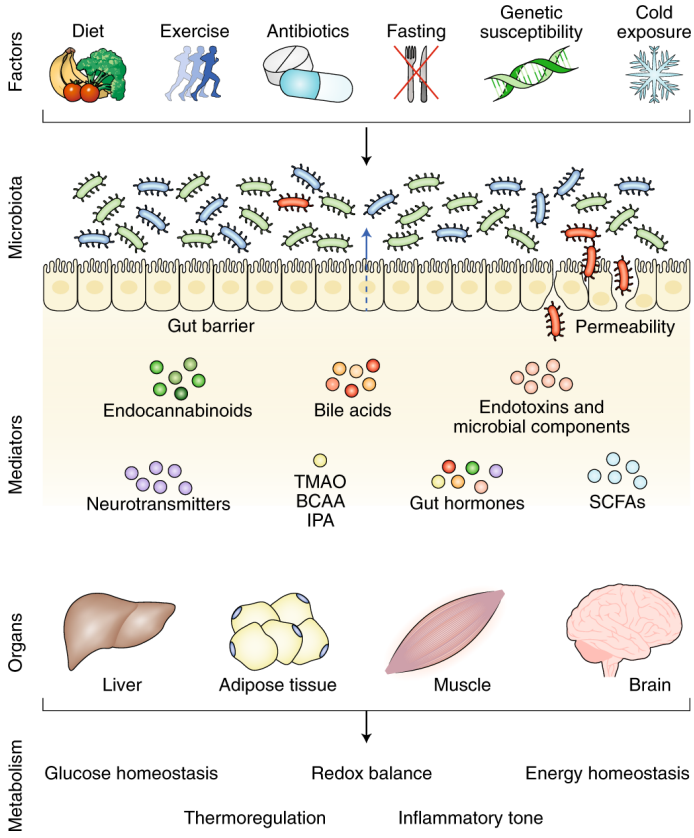


**Figure 1.1.1. Crosstalk between the gut microbiota and the host, and major molecular players involved in the regulation of host metabolism.** TMAO: Trimethylamine *N*-oxide; BCAA: branched-chain amino acid; IPA: indole propionic acid; SCFAs: short-chain fatty acids. This figure is reported by Cani and colleagues (2019).

**The complex structure of the gut microbial ecosystem and its major players**

The phylogenetical characterization of the intestinal microbiota, carried out through the advancement of sequencing techniques of the 16S rRNA gene, highlighted the presence of two dominant phyla: Firmicutes and Bacteroidetes (Ley *et al*., 2006a). Taken together, bacteria belonging to these two divisions represent indeed almost 90% of the phylogenetic types, namely phylotypes, of the colon ecosystem (arounf 65 and 25% of the total community, respectively). While Bacteroidetes are able to produce short-chain fatty acids, the Firmicutes are primarily devoted to energy harvest from ingested food (Turnbaugh *et al*., 2006; Ley *et al*., 2006a cell). The remaining subdominant phylotypes are mainly distributed among Proteobacteria (8%) and Actinobacteria (5%), both normally present in the majority of individuals, in addition to Verrucomicrobia and Fusobacteria (1%) (Costello *et al*., 2009; Muegge *et al*., 2011) (Figure 1.1.2). Despite this phylum level paucity, the gut microbiota biodiversity undergoes a tremendous increase at lower phylogenetic levels. As a matter of fact, up to 1,000 different bacterial species have been detected in the human population (Turnbaugh *et al*., 2007; Garret *et al*., 2010) and every individual possesses a specific subset consisting of around 160 of them, making each layout unique (Qin *et al*., 2010).

Among the archaea commonly found in the human colon, Methanobacteriales is the most abundant order, whose members can produce methane by reducing carbon dioxide or methanol with idrogen as the primary electron donor (Gaci *et al*., 2014). In particular, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* are the main archaea that guide bacterial metabolism by removing $H_2$ from the local environment and thus making the fermentation of polysaccharides more thermodynamically favorable (Eckburg *et al*., 2005; Samuel *et al*., 2007; Lloyd-Price *et al*., 2017).

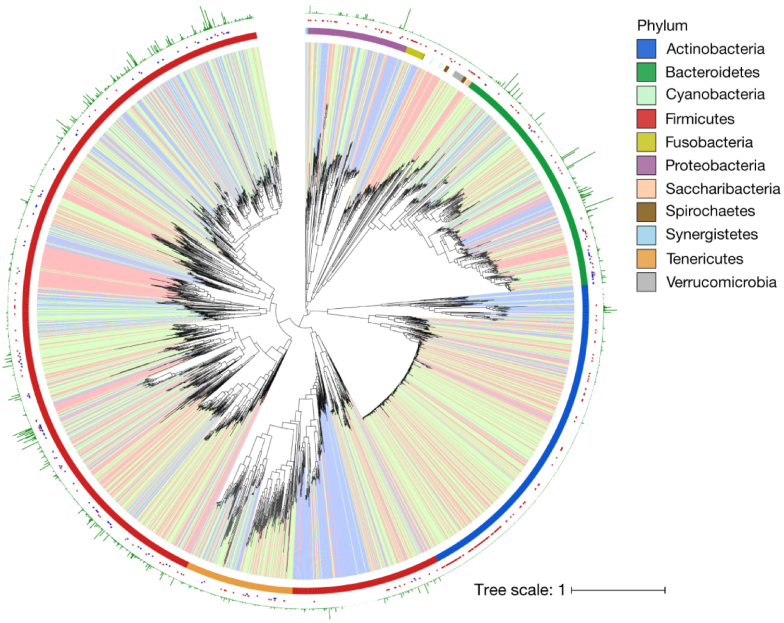


**Figure 1.1.2. Phylogenetic structure of the gut microbiota.** Maximum-likelihood phylogenetic tree including 553 genomes belonging to the HGR (human gut reference) database, composed of 2,468 isolate genomes combined from the Human Microbiome Project (HMP) catalogue and the Human Gastrointestinal Bacteria Genome Collection (HGG) (Forster *et al*., 2019), and 1,952 to UMGS (unclassified metagenomes). Figure adapted from Almeida *et al*., 2019.

Eukaryotic cells, such as fungi, also reside in the lower part of the gastrointestinal tract, contributing to the mass and metabolism of the gut microbiota with an average of $10^6$ fungal cells per gram of colon content (Huseyin *et al*., 2017). Interest in the study of the fungal microbiota, termed mycobiota, has been rising over the past decade resulting in the accumulation of various data sets that describe the potential involvement of this microbial fraction in health and disease (Richard & Sokol, 2019). The first culture-independent analysis of the fungal populations present in the human gastrointestinal tract was conducted in a limited number of patients suffering from pouchitis and undergoing probiotic therapy (Kühbacher *et al*., 2006). Two years later, a complete analysis of the mycobiota in humans was performed combining culture-dependent and culture-independent methods (Scanlan *et al*., 2008). Scanlan and colleagues found little diversity within the mycobiome, with *Gloeotinia* spp., *Paecilomyces* spp., and *Galactomyces* spp. as major components, and only a few *Candida* spp. (mainly *C. parapsilosis* and *C. albicans*) (Scanlan *et al*., 2008). More recent studies have allowed broadening the characterization of the gut mycobiome, identifying two dominant phyla, Ascomycota and Basidiomycota (representing 70% and 30%, respectively), with some studies

also identifying some Zygomycetes (Hallen-Adams *et al*., 2015; Nash *et al*., 2017), and a total of 133 genera: *Penicillium*, *Candida*, *Aspergillus*, *Saccharomyces*, *Cryptococcus*, and *Malassezia* (Hoffman *et al*., 2013; Richard *et al*., 2015; Huseyin *et al*., 2017; Donovan *et al*., 2018). In particular, *Penicillium* constitutes the most diversified genus (15 species), followed by *Candida* (12 species), *Aspergillus* and *Saccharomyces* (5 species each). Despite the individual-specificity of the gut mycobiome layout, the hypothesis of a possible core mycobiome has recently been advanced. In fact, ten genera have been found in the majority of human gastrointestinal tracts, including *Candida* (particularly *C. albicans*), *Saccharomyces* (particularly *S. cerevisiae*), *Penicillium*, *Aspergillus*, *Cryptococcus*, *Malassezia* (particularly *M. restricta*), *Cladosporium*, *Galactomyces*, *Debaryomyces* and *Trichosporon*, ranked in decreasing abundance (Hallen-Adams *et al*., 2017). The intestinal mycobiome can strongly influence the host immune system (response driven by $T_H1$ and/or $T_H17$ cells), but extensive research is still needed to better characterize these interactions and identify additional roles that potentially affect the overall host health. Although *Candida* species are commonly regarded as harmless commensals on many human body sites, within the gastrointestinal tract constitute a major reservoir and source of infections, such as invasive candidiasis (Nucci & Anaissie 2001). When the gut microbiome is disrupted, intestinal mucosal permeability is increased or the host is immune-suppressed, the predominant gut fungus, *C. albicans*, has the ability to invade tissues and disseminate in the body. *Candida* gut overgrowth has been associated with a number of diseases such as diabetes, Crohn's disease and ulcerative colitis, hematologic malignancies and graft vs. host disease (Suhr *et al*., 2015; Richard & Sokol, 2019).

Although mainly consisting of prokaryotic, archaea and eukaryotic cells, the human gut microbiome also includes a viral fraction, named the virome. Nonetheless, unlike bacterial microbiome and the mycobiome, the study of the entire viral fraction present within the human gastrointestinal tract appears to be largely unexplored, even though it contains a highly diverse genetic entity (Lecuit & Eloit 2013; Ogilvie *et al*., 2015). The main critical aspect in this recent

field of research concerns the absence of a gold standard for the bioinformatics pipelines, and the lack of consensus in the methodology and classification of the virus taxonomy underlines significant limitations and challenges in virome analysis (Zuo *et al*., 2019; Sutton *et al*., 2019; Shkoporov *et al*., 2019). Despite the above-listed critical issues, studies conducted so far have revealed the presence of more than $10^9$ viral particles per gram of feces (Schoenfeld *et al*., 2010; Kim *et al*., 2011; Minot *et al*., 2013), comprising viruses infecting each domain of life (Bacteria, Archaea and Eukarya), including the human host. Among the most common viral lineages detected within human fecal samples we find single stranded DNA viruses such as Anelloviruses, Circoviruses, and Parvoviruses, as well as double stranded DNA viruses such as Adenoviruses and Papillomaviridae (Wylie *et al*., 2014; Di Bonito *et al*., 2015; Vetter *et al*., 2015; Rampelli *et al*., 2017). As for the RNA viruses detected in human feces, plant viruses seem to predominate and are assumed to derive from food sources. Finally, the healthy gut virome is also characterized by bacteriophages such as double-stranded DNA Caudovirales and single-stranded DNA Microviridae. Despite the clinical relevance of viruses, their potential role in a healthy human gut ecosystem is still largely unexplored (Reyes *et al*., 2010; Shkoporov *et al*., 2019).

**Adaptive flexibility and plasticity of the human gut microbiome**

The human gut microbiome can be considered an extremely dynamic ecosystem characterized by a significant degree of plasticity, being able to adjust its compositional structure in response to a wide variety of endogenous and exogenous stimuli (Candela *et al*., 2012; Cani *et al*., 2019). The development of the gut microbiome is indeed regulated by a complex interplay between the host and environmental factors, including lifestyle and diet, ethnicity and geographical localization (Candela *et al*., 2012; Rothschild *et al*., 2018; Cani *et al.*, 2019). In a eubiotic context, fluctuations in our microbial counterpart allow the host to quickly adapt its metabolic and immunological functions in response to changes that occur in the surrounding environment. In a complex microbial community such as the intestinal microbiota, lateral gene transfer plays a decisive role in shaping the ecosystem itself during its assembly and allowing rapid adaptation to environmental changes (Tamames *et al*., 2010; Zhao *et al*., 2019; Song *et al*., 2019). The gene flow within the members of the microbial community could therefore be responsible for the phenotypic plasticity and functional redundancy present in the various microbial lineages, resulting in the typical gut microbiome of the adult: variable in the phylogenetic composition but preserved in the main functional traits. Confirming this assumption, a pioneering study focused on comparing the gut microbiome layouts of individuals with different health status allowed the researchers to identify a core microbiome, defined by a constant and shared pool of gene functions essential to support the mutualistic relationship established with the host over millennia of coevolution (Turnbaugh *et al*., 2009a). Specifically, by examining the gut microbiomes of six adult twin pairs and their mothers, Turnbaugh and colleagues suggested the presence of a gene-level core microbiome. The enrolled individuals shared >93% of the enzyme-level functional groups, but no bacterial phylotypes were present at >0.5% in all samples. Shortly after, the international MetaHIT (Metagenomes of the Human Intestinal Tract) project annotated a comprehensive gene catalogue derived from the metagenomic analysis of fecal samples collected from 124 individuals (Qin *et al*., 2010). Interestingly, microbial genes

included within the catalogue and 18 species were found to be shared among the gut microbiome of all individuals. In order to confirm these findings, several large-population studies were subsequently carried out. The Human Microbiome Project (HMP) Consortium underlined that samples collected from the same individual are more similar to one another than those from different individuals, suggesting that each person has a microbiota that is distinct and stable (HMP Consortium 2012; Lloyd-Price *et al*., 2017). Furthermore, two pivotal studies have estimated that the variable fraction covers 40% of this complex ecosystem, and that its structural stability can be preserved and maintained for up to five years (Faith *et al*., 2013; Rajilić-Stojanović *et al*., 2014).

One of the clearest examples of the human gut microbiome plasticity concerns its ability to quickly respond to dietary changes (Walker *et al*., 2011; Wu *et al*., 2011; Muegge *et al*., 2011; Zmora *et al*., 2018). The dietary contribution to microbiome modulation is evident from the beginning of life, when the human milk oligosaccharides (HMO) participate in the maturation of the microbiota in early childhood (Charbonneau *et al*., 2016). With the introduction of solid foods follows an increase in bacterial richness, typically associated with adulthood (Koenig *et al*., 2011; Dominguez-Bello *et al*., 2011; Laursen *et al*., 2017), and which undergoes a gradual decrease along with age. The decrease in diversity of the gut ecosystem has been found to be particularly evident in frail elderly populations that age in long-stay care, probably due to reduced food diversity (Claesson *et al*., 2012) (Figure 1.1.3). Nutrients introduced with diet can exert a direct impact on the relative abundance of gut bacteria, interacting with microorganisms to promote or inhibit their growth and, ultimately, conferring a direct competitive advantage to select members of the microbial community at the expenses of less-adapted members (Korem *et al*., 2015). In a milestone study by Wu and colleagues (2011), a cross-sectional analysis of 98 healthy volunteers and a short-term controlled-feeding experiment were performed to test the stability of the gut microbiome. A subset of 10 individuals was randomized to high-fat/low-fiber or low-fat/high-fiber diets, and fecal samples were collected over 10 days. Remarkably,

changes in microbiome composition were detectable within 24 hours of strictly controlled feeding. The comparison of dietary data response allowed the authors to identify bacterial taxa influenced exclusively by short- or long-term dietary habits, pointing towards a dynamic dimension of the human gut microbiome (Wu *et al*., 2011).

In addition to diet, even ethnicity and geographical location play an important role in modulating the structure and defy the extraordinary plasticity of the human gut microbiome. A pivotal comparative analysis of the intestinal microbiota of individuals enrolled in Korea, China, Japan and USA showed a clear clustering of the microbiota structure according to the geographic origin (Nam *et al*., 2011).

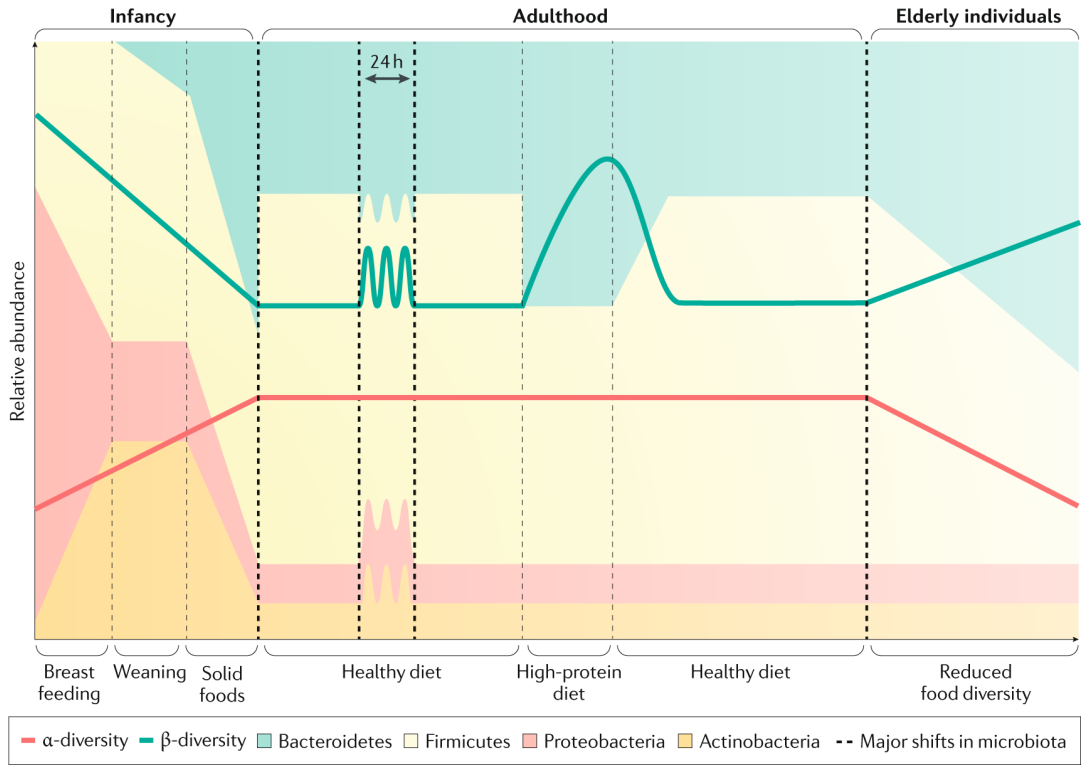


**Figure 1.1.3. Diet influence on the gut microbiota structure throughout the human lifespan.** The blue line indicates the trend of microbiota configuration in resembling an arbitrary homeostatic configuration during adulthood (β-diversity). The red line indicates the gut microbiota richness (α-diversity). Background colors indicate typical taxa abundances. Figure from Zmora and colleagues (2018).

Furthermore, De Filippo and colleagues (2010) contributed to the characterization of country-related differences in the gut microbiota of children from Europe and Burkina Faso. The peculiarities of the latter regard an enrichment of Bacteroidetes and Actinobacteria, at the expense of Firmicutes and Proteobacteria, in comparison with European gut ecosystems (De Filippo *et al*., 2010). However, the studies conducted so far have focused on small groups, mostly comparing individuals living in different geographical areas with marked lifestyle differences (Yatsunenko *et al*., 2012; Schnorr *et al*., 2014; Gupta *et al*., 2017). To disentangle the impact played by geography and ethnicity on the composition of the fecal microbiota, Deschaseaux and colleagues have recently enrolled 2,084 participants with varied ethnic backgrounds but living in the same city: 439 Dutch, 367 Ghanaians, 280 Moroccans, 197 Turks, 443 African Surinamese, and 358 South-Asian Surinamese. The authors conclude that the influence on α- and β-diversity are independent of metabolic health and only partly explained by ethnic-related characteristics, including sociodemographic, lifestyle, or diet factors. Nonetheless, the ethnic origin could constitute a marker for differences in the composition of the gut microbiota (Deschasaux *et al*., 2018). Another recent study conducted by He and colleagues provided the characterization of the gut microbiota of 7,009 individuals from 14 districts within one province in China. This unique cohort, composed of individuals with highly homogenous ancestry (99% Han), allowed the authors to isolate the effect of geography without ethnicity as a confounding factor, underlining a strong associations of host location with microbiota variations (He *et al*., 2018). Taken together, all these findings support the strong impact exerted by lifestyle, ethnicity, and geography on the gut microbiome, suggesting that the differences observed within the gut ecosystem profile may reflect specific adaptations to environmental conditions (Schnorr *et al*., 2014; Rampelli *et al*., 2015; Rothschild *et al*., 2018; Ayeni *et al*., 2018).

Finally, several studies have focused on gene-environment interactions to explore the host-microbe cross-talk, aiming at uncovering the mechanisms related to different effects played by

the genetic background when the environmental context changes (e.g. the lactase gene, LCT, and *Bifidobacterium*, where diary intake serves the environmental background) (Blekhman *et al*., 2015; Goodrich *et al*., 2016; Bonder *et al*., 2016). Although the addition of microbiome data to host genetics data aid prediction accuracy for several host phenotypes, a recent study conducted by Rothschild and colleagues suggests that the environment plays a substantially greater role than host genetics in shaping the human gut microbiome structure (Rothschild *et al*., 2018). The authors demonstrate that previously identified heritable bacteria represent only a small fraction of the entire ecosystem (overall microbiome heritability between 1.9% and 8.1%), and that the reported associations between specific single nucleotide polymorphisms (SNPs) and bacterial taxa are either weak or population-dependent (Rothschild *et al*., 2018). Future studies with larger sample sizes will probably identify additional heritable taxa and SNP associations, but are unlikely to change the overall conclusion that microbiome composition is predominantly shaped by non-genetic factors.

Chapter 1 – **INTRODUCTION**

*1.1 The human gut microbiome*

**1.2 Assembly and maturation of the gut microbiome across different stages of life**

*1.3 Dysbiotic variations of the gut microbiome in Western diseases*

*1.4 Microbial ecology assessment: from next-generation sequencing to culturomics*

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**Establishment of the gut microbiome symbiosis and microbial succession in early life**

Although the exact time the first colonization occurs is still an open question, most scientists support the hypothesis that the fetus develops in a virtually sterile environment and that most of our initial microbiota is acquired during and immediately after birth. On the other hand, a few studies have found traces of bacterial DNA in the placenta, in the amniotic fluid surrounding the fetus, as well as in meconium (Aagaard *et al*., 2014; Collado *et al*., 2016). These results suggest the advent of prenatal colonization, but are still under debate as they could be the result of contamination as well.

Several perinatal conditions, host and external factors (i.e. mode of delivery, type of feeding, antibiotic use, lifestyle, and geography) can influence the highly dynamic process of development and maturation of the gut microbiota (Koenig et al., 2011; Planer *et al*., 2016; Pabst *et al*., 2016; Korpela *et al*., 2018). Among these, the delivery mode represents one of the main factors driving the first colonization of the gastrointestinal tract of the newborn (Wampach *et al*., 2018). The microbiota of babies born vaginally tends to be enriched with bacteria similar to the maternal vaginal microbiota (i.e. *Lactobacillus* spp.), in addition to *Bifidobacterium*, *Escherichia*, *Bacteroides* and *Parabacteroides*. On the contrary, babies born with cesarean section (C-section) generally lack these taxa and are instead enriched in skin commensals and bacteria associated with the hospital environment, i.e. *Enterococcus*, *Staphylococcus*,

*Streptococcus*, and *Propionibacterium* spp. (Dominguez-Bello *et al*., 2010; Chu *et al*., 2017; Yassour *et al*., 2018; Shao *et al*., 2019). Over time the differences initially observed between children born with vaginal delivery and C-section are gradually reduced (Stewart *et al*., 2018; Vatanen *et al*., 2018). Nonetheless, Stewart and colleagues revealed the presence of bacteria associated with C-section up to two years of age, suggesting that the delivery mode could exert long-term effects on the composition of the microbiota, thus reflecting potential detrimental effects on host health. Within this extensive longitudinal study, Stewart and colleagues enrolled 903 infants from four countries (Germany, Finland, Sweden, USA) and collected fecal samples monthly for up to 3 years of life. By monitoring the development of the intestinal microbiota, the authors revealed three distinct evolutionary phases, depending on the dynamics of prevalent phyla and changes in ecosystem biodiversity: (i) a developmental phase (3-14 months) dominated by *Bifidobacterium* spp. in which the α-diversity and the phyla detected are gradually modified; (ii) a transitional phase (15-30 months) in which α-diversity continues to evolve and only Bacteroidetes and Proteobacteria continue to change; (iii) a stable phase (≥31 months) with a predominance in Firmicutes, in which the phyla detected and the high levels of α-diversity achieved do not undergo further modifications (Stewart *et al*., 2018). Over the course of these phases, the infant gut microbiome adapts over time, shaped by the availability of different nutrients and numerous postnatal factors (Wopereis *et al*., 2014) (Figure 1.2.1). According to a healthy developmental trajectory, the gut microbiota of breastfed infants is largely dominated by bacterial species that metabolize human milk homopolysaccharides (HoPS) to produce lactate (i.e. *Bifidobacterium* spp.), and shifts in microbial composition are observed following the introduction of increasingly complex dietary substrates, as well as an enrichment of bacterial functions related to carbohydrates metabolism and the biosynthesis of amino acids and vitamins (Koenig *et al*., 2011; Dominguez-Bello *et al*., 2011; Backhed *et al*., 2015; Laursen *et al*., 2017). Particularly noteworthy is the impact of breast milk, which covers not only a prebiotic function, but contains a complex microbial community – dominated by

*Bifidobacteriaceae*, *Streptococcaceae* and *Staphylococcaceae* – which could contribute to seed the infant gut microbiota as well (Biagi *et al*., 2017). The dietary regimen of the newborn dramatically influences the maturation of the gut microbiota. Studies conducted on malnourished infants have revealed a different developmental trajectory of the gut microbiota from that observed in healthy newborns, suggesting that a malnourished microbiome in childhood may compromise the maturation of the adult-like microbiota structure and ultimately perpetuate the growth impairments and health deficits later in life (Smith *et al*., 2013; Subramanian *et al*., 2014; Tamburini *et al*., 2016). In particular, Subramanian and colleagues observed that the microbiota of malnourished children (6.5 – 26 months of age) appears to be particularly enriched with opportunistic pro-inflammatory bacteria belonging to *Entrobactericeae* family and *Streptococcus* genus, as well as depleted of immune-modulatory *Bifidobacterium* spp. Noteworthy is also the trend observed during weaning, a period in which the microbiota of these children does not undergo a progressive increase of bacteria essential for the development of the adult-like structure, such as *Clostridium* and *Ruminococcus* genera (Subramanian *et al*., 2014). Although essential to treat serious bacterial infections, antibiotics use can disrupt the stability and diversity of the developing microbiota in infants, resulting in long-lasting health implications (Bokulich *et al*., 2016). Antimicrobial treatments constitute an obstacle to the growth of specific taxa, whose abundances remain reduced for years after treatment. Furthermore, their use in early life has been linked to increased risk of developing different diseases, including asthma, inflammatory bowel disease and allergies (Tamburini *et al*., 2016; Bokulich *et al*., 2016). Obesity, metabolic syndrome and atopy are other disorders that have been associated with microbiota disturbance in early childhood (Milani *et al*., 2017; Bernstein *et al*., 2019; Roth *et al*., 2019). As for the potential role of host genetics in influencing the composition of the infant GM, a study conducted on a cohort of 1,126 UK twin pairs has allowed the identification of associations between inheritance of specific taxa and host genes (Goodrich *et al*., 2016). In particular, the association between the LCT gene and the presence

of *Bifidobacterium* has been observed and successfully replicated in different populations (Goodrich *et al*., 2016; Rothschild *et al*., 2018). Considering the crucial role of this initial phase of life in our long-term development, an in-depth characterization of the postnatal developmental trajectory of the gut microbiota in larger cohorts is mandatory.

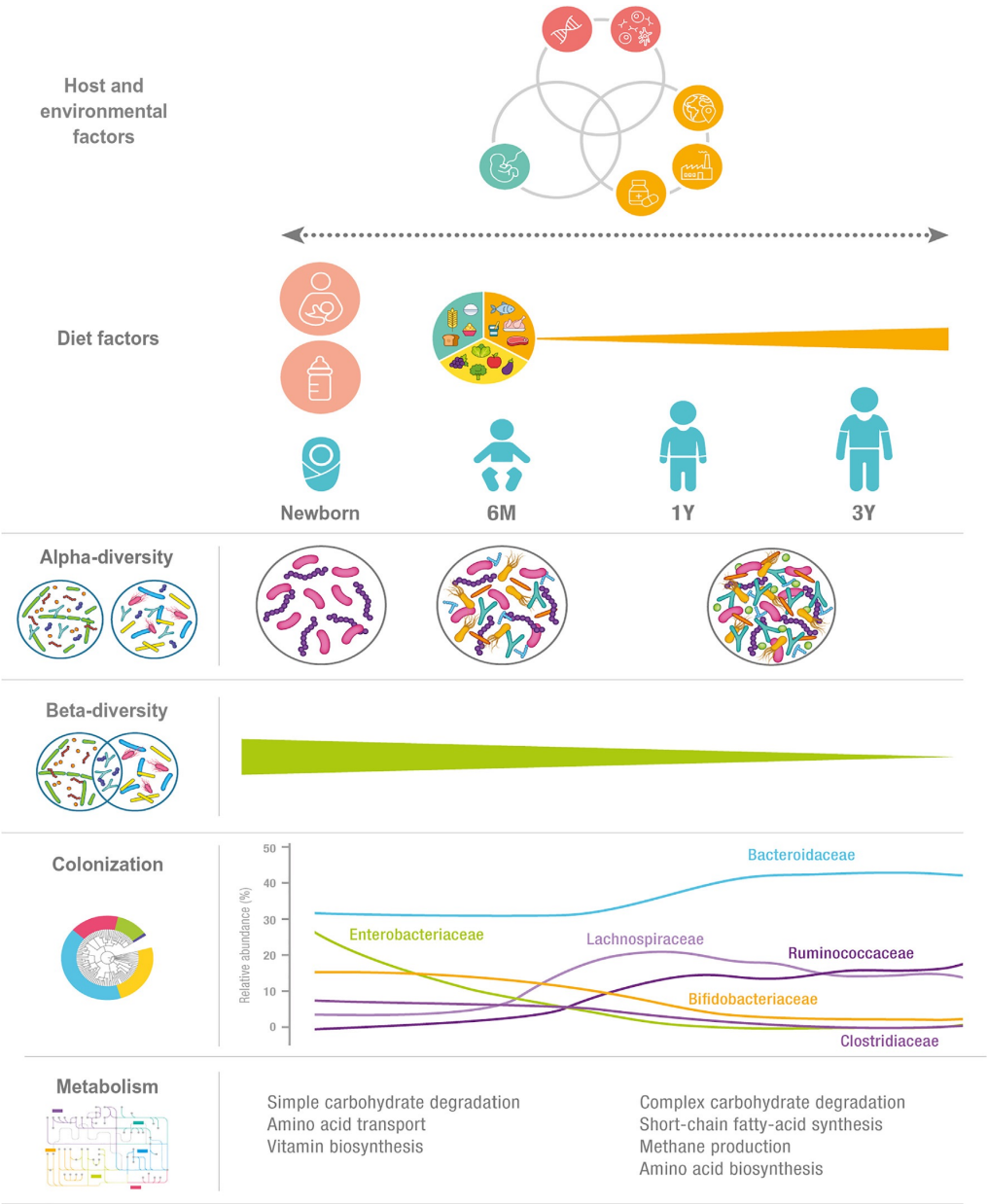


**Figure 1.2.1. The gut microbiota assembly and development within the first 3 years of life.** Bacterial α-diversity and functional complexity increase with age, while β-diversity decreases. Colonization pattern is based on Yassour *et al*., 2018, and the figure is taken from Derrien *et al*., 2019.

**Maturation of the gut microbiota up to the adult-like configuration**

Although researchers generally agree that the infant microbiome reaches a stable adult-like configuration at the age of 3 years, recent studies have suggested that the process of developing a mature gut microbiota may take longer. In a recent publication, the dynamics of the gut microbiota in healthy pre-school and school-age children were assessed in 61 Dutch children (2-18 years old) by collecting fecal samples in the short and long term (weekly sampling for 6 weeks and a follow-up sample after 18 months, respectively) (de Meij *et al*., 2016). The authors found a strong association between higher α-diversity and higher stability between the ages of 2 and 18 years, while observing a greater stability over time of bacterial phyla such as Bacteroidetes and Proteobacteria. On the other hand, the largest cross-sectional analysis of the gut microbiota from 281 school-age children (6-9 years old) enrolled in the KOALA Birth Cohort Study (Zhong *et al*., 2019) showed that the functional layout and the overall structure of the ecosystem ($\beta$-diversity) mirror those observed in adults, with an enrichment of Bacteroidetes and Actinobacteria. In a recent meta-analysis of the limited numbers of studies performed so far, Derrien and colleagues suggest that the gut microbiota of pre-school and school-age children is similar to that of adults in terms of overall composition, and that some features may develop more slowly in some children compared to others (Derrien *et al*., 2019). Coherently, the gut microbiota is recognized to undergoes most of its development very early in life but to continue to evolve after the age of 3 years, with a peculiar and continuous decrease in *Bifidobacterium* levels until adulthood (Agans *et al.*, 2011). Once the configuration typically found in adult individuals is reached, it remains relatively stable and resilient in the long term (Faith *et al*., 2013; Rajilić-Stojanović *et al*., 2014; Franzosa *et al*., 2015; Palleja *et al*., 2018). In a milestone study by David and colleagues, fecal samples were daily collected from 2 healthy volunteers over the course of one year. The analysis of the microbiota has revealed the alternation of periods of apparent stability – or stationary dynamics – that reflect daily fluctuations in diet and other host factors, and to abrupt compositional variations attributable to

significant dietary and/or environmental changes (David *et al*., 2014). It is therefore necessary to conduct longitudinal studies on large cohorts to follow the evolution of the GM during development, with the ultimate goal of bridging the gap in our knowledge of the microbiota dynamics during childhood and puberty, and attempting to define 'normal' layouts and trajectories.

**Elder microbiome and extreme longevity**

Bidirectional interactions with the gut microbiota remain overall stable during adulthood, and begin to suffer interference in the elderly. The ageing process occurs following different passages of dysfunction in the body, and is characterized by a gradual loss of functionality and physiological impairment of locomotion and circulation, metabolism and immune functions (López-Otín *et al*., 2013; An *et al*., 2018) (Figure 1.2.2). Both the host genetics and environmental factors, such as lifestyle, diet, exercise and stress, contribute to the ageing process, with a respective estimated impact of 25% and 75% (Biagi *et al*., 2010, 2013; Claesson *et al*., 2012; Brooks-Wilson 2013; Giuliani *et al*., 2018). The impaired immune system, the increased permeability and the alterations of gastrointestinal function associated with ageing influence the quantity and type of nutrients that reach the colon, induce alterations in the composition and functionality of the intestinal microbiota and negatively affect the microbiota-host interactions. In frail individuals, the homeostatic relationship in the human superorganism undergoes a deterioration along with ageing, moving towards a dysbiotic configuration of the gut ecosystem that undermines the beneficial effects of the microbiota on the host physiology, potentially inducing pro-inflammatory innate immunity and triggering pathological conditions (Biagi *et al*., 2013; An *et al*., 2018; Finlay *et al*., 2019). Being characterized by compromised stability and high inter-individual variability (Claesson *et al*., 2011), it is therefore difficult to define a typical layout of the intestinal microbiota of the elderly. Several recent studies report

conflicting results regarding the α-diversity of the intestinal ecosystem of the elderly compared to young adults, emphasizing the presence of a wide range of host and/or lifestyle confounding factors. Higher levels of α-diversity were reported in the microbiota of community dwelling elderly (Odamaki *et al*., 2016; Falony *et al*., 2016; Jackson *et al*., 2016; Bian *et al*., 2017), while other studies reported no significant differences (Biagi *et al*., 2010; Maffei *et al*., 2017; O'Toole *et al*., 2015) or reduced levels of biodiversity (Mueller *et al*., 2006; Biagi *et al*., 2012). Also in centenarians, the picture is not yet entirely clear, with reports of higher α-diversity than that of elderly (Biagi *et al.*, 2016; Kong *et al*., 2016; Wang *et al*., 2015) or no differences (Biagi *et al*., 2010; Odamaki *et al*., 2016; Wang *et al*., 2015). As for the phylogenetic composition of the elder microbiome, recent studies reported country-related peculiarities ascribable to dissimilarities in diet and lifestyle (Mueller *et al*., 2006; Biagi *et al*., 2011; Lozupone *et al*., 2013). In the elderly population of Japan, Finland and Italy, an increase in Firmicutes members, such as *Eubacterium*, *Clostridium*, *Ruminococcus*, *Dorea*, *Roseburia*, *Lachnospira* and *Butyrivibrio*, as well as a notable reduction in the health-promoting genus *Faecalibacterium*, have been observed (Hayashi *et al*., 2003; Mueller *et al.,* 2006; Biagi *et al*., 2010; Makivuokko *et al*., 2010), while an opposite trend was found in German elderly (Mueller *et al*., 2006). Furthermore, the increase in Bacteroidetes observed in the elderly populations of Austria, Finland, Germany and Ireland (Bartosch *et al*., 2004; van Tongeren *et al*., 2005; Tiihonen *et al*., 2008; Zwielehner *et al*., 2009) has not been found in the Italian population (Mueller *et al*., 2006; Biagi *et al*., 2010). The gut microbiota of elderly generally comprises lower levels of *Bifidobacterium* and an enrichment of *Streptococcaceae*, a trend that is more pronounced especially in comorbid and frail elderly (Mueller *et al*., 2006; Biagi *et al*., 2010; Makivuokko *et al*., 2010). Generally recognized as pathobionts – commensals that may become detrimental in particular conditions – bacteria belonging to the latter two families are able to provoke and sustain inflammation processes. Considering the concomitant depletion of the immunomodulatory symbiont *Faecalibacterium* and relatives, the hypothesis of the

establishment of a pro-inflammatory loop in the elder microbiome has been advanced, ultimately contributing to the development of inflammatory disorders and challenging the host-bacteria equilibrium (Biagi *et al*., 2010). In a recent study focused on the gut microbiome and longevity, Biagi and colleagues analysed the fecal profiles of 24 semi-supercentenarians (105-109 years old), identifying a peculiar microbial layout mainly composed of health-associated taxa, such as *Bifidobacterium*, *Akkermansia*, and *Christensenellaceae*, positively supporting the ageing process in these extremely long-lived individuals (Biagi *et al.*, 2016). However, longitudinal studies are needed to verify whether these alleged 'useful microbiome species' are present throughout life, or whether they are lost during the ageing process and regained only later by people who reach extreme longevity. To date, the metabolic potential of the elder microbiome through metagenomics analysis is still largely underexplored. In a pivotal study, Rampelli and colleagues have compared the gut microbiome of non-institutionalized elderly with young adults, reporting an increased proteolytic potential at the expense of the saccharolytic potential, with lower relative abundance of genes devoted to SCFA production in the elder microbiome (Rampelli *et al*., 2013).

Although the typical elderly microbiota profile is still difficult to define, recent studies have added important elements to the understanding of microbiota variations in healthy individuals, and have paved the way for the design of microbiome-targeted interventions in this delicate phase of our life (Biagi *et al*., 2016). In this perspective, longitudinal studies on cohorts of elderly individuals with the aim of assessing the potential beneficial effects of prebiotics, probiotics and synbiotic administration for modulating the elder gut microbiome are increasingly necessary.

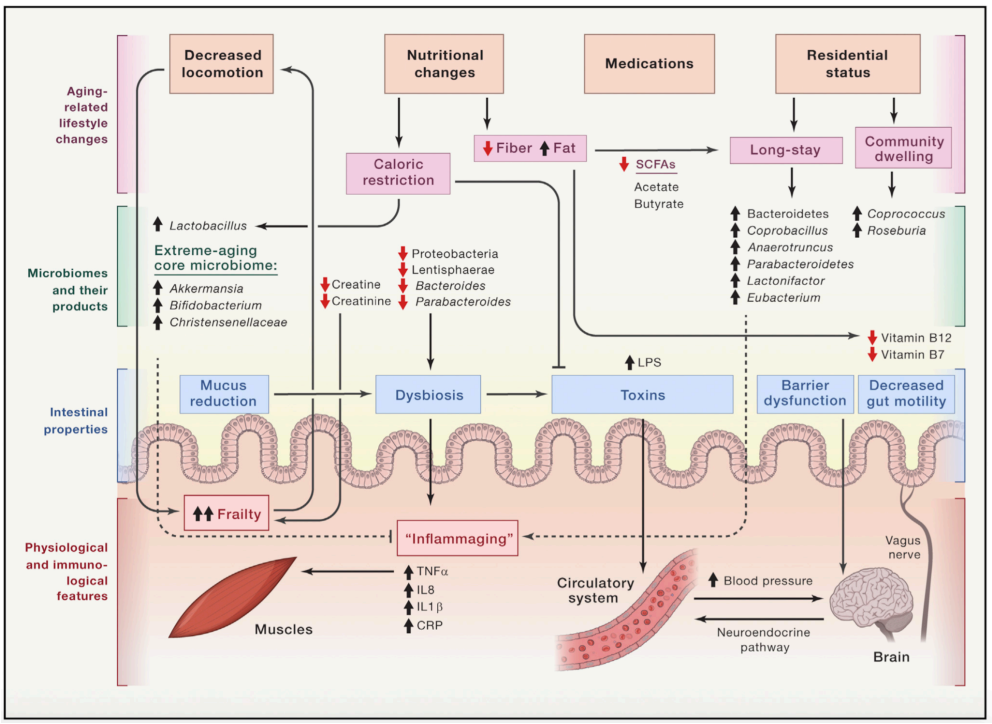


**Figure 1.2.2. Key changes in gastrointestinal function and composition and functionality of gut microbiome observed during the ageing process.** The main factors able to influence the intestinal ecosystem in the elderly, as well as the involved microbiome shifts are also reported. TNF: tumor necrosis factor; IL: interleukin; CRP: C-reactive protein. The figure is taken from Kundu and colleagues (2019).

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**Deviations from mutualism in the Western microbiome**

As discussed above, from a mutualistic point of view the homeostatic interaction between the host and its associated microbes can shift between different configurations. However, in the presence of persistent and prolonged environmental stressors, such as infection, and endogenous factors, such as inflammation or ageing, the mutualistic relationship with our microbial counterpart could break, pushing the microbiota layout toward dysbiotic configurations – generally characterized by lower biodiversity levels – often associated with various gastrointestinal, neurodegenerative, metabolic and oncological diseases states (Lynch & Pedersen 2016). In a recent meta-analysis, Duvallet and colleagues collected 28 published case–control gut microbiome data sets spanning ten different disease states, and investigated patterns of disease-associated shifts in the human gut microbiome (Duvallet *et al*., 2017). The authors provided a more nuanced insight into dysbiosis, revealing distinct types of alterations that more precisely describe the disease-associated microbiome changes. While some diseases (e.g. colorectal cancer, CRC) are indeed characterized by a bloom of commensals that may become detrimental or infectious, others (e.g. inflammatory bowel disease, IBD) largely show a depletion of health-promoting microbes. In this framework, the connection between a dysbiotic gut microbiome layout and multiple diseases has made the microbiome a strategic therapeutic target, paving the way for the development of a series of microbiome-tailored

intervention strategies aimed at the rehabilitation of a health-promoting layout (Jia *et al*., 2008; Wilson & Nicholson 2016; Duvallet *et al*., 2017; Jackson *et al*., 2018). Dietary supplementation with classic (e.g. *Lactobacillus* and *Bifidobacterium*) or next-generation probiotics (e.g. *Faecalibacterium prausnitzii*), prebiotics or synbiotics (i.e. a combination of pre- and probiotics) are the most commonly used nutritional approaches to beneficially alter the microbiota, but special attention should be paid when administering these treatments to subjects with impaired immune function. Among other strategies to manipulate the intestinal microbiota, the use of fecal microbiota transplantation (FMT) has also been proposed. However, it is absolutely necessary to adopt stringent criteria and in-depth screening in selecting stool donors, in order to avoid the potential transmission of pathogens or antibiotic resistance genes.

In order to better understand the specificities of the human microbiome assembly from an evolutionary point of view, extensive meta-analyses of human and non-human primate microbiomes have been recently carried out (Moeller *et al*., 2014; Davenport *et al*., 2017). This comparative approach has led to the identification of several compositional changes along with a progressive reduction of biodiversity as the main distinctive features of the human gut microbiome along the evolutionary history (Moeller *et al*., 2014). Interestingly, these hallmarks have been found to be exacerbated in Western urban populations compared to traditional and rural counterparts (De Filippo *et al*., 2010; Yatsunenko *et al*., 2012; Schnorr *et al*., 2014; Obregon-Tito *et al*., 2015). In particular, consistent with the disappearing microbiota hypothesis (Blaser *et al*., 2017), the dramatic shrinkage of individual gut microbiome diversity in Western urban populations is deemed to depict a maladaptive microbiome state that may contribute to the rising incidence of chronic non-communicable diseases, such as obesity, diabetes, asthma and inflammatory bowel disease (IBD) (Sonnenburg & Sonnenburg 2014; Mosca *et al*., 2016; Zuo *et al*., 2018; Cani *et al*., 2018; Durack and Lynch, 2018). Consequently, in recent years, a large body of research has been devoted to understanding the mechanisms

leading to the dysbiotic alterations in the Western urban gut microbiome. It is in this scenario that the multiple-hit hypothesis has been advanced (Sonnenburg & Sonnenburg 2014). According to this theory, the progressive changes in the human gut microbiome and especially the reduction of biodiversity have occurred at multiple stages along the recent transition to modern urban societies, and several aspects typical of the urbanization process – such as sanitation, antibiotics, C-section and Western diet – have been pointed out as contributing factors. In particular, the reduction in quantity and diversity of MACs in the diet has been considered one of the leading causes of the disappearing gut microbiome in Western urban populations (Sonnenburg & Sonnenburg 2014). Moreover, food additives, emulsifiers and xenobiotics–ubiquitous in industrially processed foods–have recently been shown as important additional drivers of gut microbiome diversity shrinkage (Danchin *et al*., 2018).

In healthy individuals, the microbiome varies over time within a 'healthy plane of variation', defined by a discrete number of stable eubiotic microbiome configurations. On the other hand, the personal dysbiotic trajectory associated with common Western disease (e.g. IBD) are characterized by a great variability over time, with random and rapid shifts between several unstable states, moving to and from the healthy plane of variation. Therefore, to fully address Western diseases, it is essential to thoroughly evaluate the microbiome shifts associated with a dysbiotic state (e.g. bloom of specific microbes, polymicrobial infections, altered consortium interactions, strong environmental filters) as well as the identification of specific perturbations that lead to dispersion effects (e.g. autoimmunity and immunosuppression). This approach can therefore be summarized by the 'Anna Karenina principle' – *all healthy microbiomes are similar; each dysbiotic microbiome is dysbiotic in its own way* – stressing that dysbiotic individuals vary more in microbial community composition than healthy individuals. Accumulating evidence have recently highlighted the importance of integrating microbiome information into personalized medicine, and personalized prediction of drug pharmacokinetics and pharmacodynamics, in understanding which disorders tend to destabilize microbiomes or

driving them to new stable states. Ultimately, recovering this valuable information may help to shape screening regimes for microbiome-based personalized medicine.

**Metabolic disorders and low-grade inflammation**

The incidence of many metabolic disorders associated with chronic low-grade inflammation, such as obesity and its related comorbidities, type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD), is rapidly increasing worldwide (Tilg & Moschen 2008; Donath & Shoelson 2011; Hotamisligil *et al*., 2017; NCD-RisC 2019). In addition to exhibiting profound compositional and functional alterations in the intestinal microbiota (Greiner *et al*., 2011; Levy *et al*., 2017), obesity, T2D and NAFLD are characterized by a compromised and defective intestinal barrier, which allows microbes or their components (e.g. endotoxins) to translocate into the blood stream and cause low-grade inflammation (Volynets *et al*., 2012; Bischoff *et al*., 2014). Although the first evidence for a role of the gut microbiota in host metabolism came from colonization studies in germ-free mice (Hooper *et al*., 2001; Backhed *et al*., 2004), pioneering human studies have allowed researchers to suggest the existence of a peculiar microbiome signature of obesity (Ley *et al*., 2006b; Turnbaugh *et al*., 2009a,b). In fact, it has been proposed that obesity, or the propensity to gain weight, as well as dyslipidemia, insulin resistance and low-grade inflammation, are associated with reduced microbial diversity (Le Chatelier *et al*., 2013; Aron-Wisnewsky *et al*., 2019). Additionally, an enrichment in pro-inflammatory bacterial taxa – *Ruminococcus gnavus* or *Bacteroides* spp. – and a concomitant depletion in anti-inflammatory strains – *F. prausnitzii* – have been observed in the microbiome profiles of obese individuals compared to lean ones (Cotillard *et al*., 2013). In a milestone study, Turnbaugh and colleagues compared the functional profiles of obese microbiomes to those of lean individuals, showing a decreased functional biodiversity and an enrichment in genes devoted to carbohydrate, lipid and amino acid metabolism (Turnbaugh *et al*., 2009a). The

authors suggest that the gut microbiome of obese individuals is more efficient at extracting energy from the diet than the microbiota of lean individuals, named 'energy harvest hypothesis'. Many studies support this theory, showing an increase in body weight and fat in in germ-free mice after transplanting gut microbiome derived from wild as well as from obese-mice (Turnbaugh *et al*., 2009a; Ridaura *et al*., 2013). In particular, the weight gain has been ascribed to several microbial related mechanisms, including the generation of SCFAs subsequently converted to complex lipids in the liver. Furthermore, recent findings in murine models suggest that a certain intestinal microbiome layout may also contribute to weight regain in obese mice after successful dieting (Thaiss *et al*., 2016). Taken together, evidences from animal models strongly support the presence of a specific gut microbiome signature of obesity, although peculiar obese-related dysbiotic layouts are still to be demonstrated in human beings.

As for T2D and NAFLD, several milestone studies have reported a peculiar microbiome signature also (Qin *et al*., 2012; Karlsson *et al.*, 2013; Loomba *et al*., 2017). In the first landmark human study from China, Qin and colleagues describe the gut microbiome dysbiosis of T2D. In addition to decreased relative abundances of *Roseburia intestinalis* and *F. prausnitzii*, the dysbiotic ecosystem was characterized by an increase in branched-chain amino acid (BCAA) and genes devoted to sugar transport, as well as a depletion in pathways assigned to butyrate biosynthesis. In addition, the link between the altered microbiome composition and the instauration of an inflammatory state was supported by the enrichment in genes involved in oxidative stress signaling (Qin *et al.*, 2012). Concordant results were observed in the first European study in T2D women conducted by Karlsson and colleagues, who also showed a decrease in the abundance of *Lactobacillus* spp. (Karlsson *et al.*, 2013). A recent study also suggests that subjects with prediabetes present an altered gut microbiota composition, characterized by reduced levels of *Akkermansia muciniphila* (Allin *et al*., 2018). Furthermore, the relative abundance of this mucin-degrading bacteria has been inversely correlated to several disease states (e.g. obesity, diabetes, cardiometabolic diseases and low-grade inflammation),

making it a potential next-generation probiotic candidate (Cani *et al*., 2017).

The beneficial modulatory effects on the gut microbiota by the supplementation of dietary fiber in individuals with T2D have been recently demonstrated by Zhao and colleagues (2018). Following consumption of a high fiber diet, the overgrowth of SCFA-producing bacteria and the upregulation of glucagon-like peptide 1 (GLP-1) led to improved glycemic control, reflected by reduced levels of glycated hemoglobin levels (Zhao *et al*., 2018).

As for NAFLD, the first landmark study conducted on a large population described the gut microbiome signature of this metabolic disorder (exacerbated in cases associated with advanced fibrosis), which is characterized by increased relative abundances of *Eubacterium rectale*, *Bacteroides vulgatus*, and *Escherichia coli*, as well by decreased levels of Firmicutes members (Loomba *et al*., 2017). The presence of dysbiotic bacterial consortia and the consequent aberrant intestinal signaling may contribute to barrier disruption and priming of metabolic inflammation (Thaiss *et al*., 2018). These events may in turn trigger a chronic systemic inflammation response, which ultimately affects the function of the end-organ and further exacerbates metabolic diseases. In animal models, different probiotic therapies have been shown to be effective in reducing intestinal permeability and, consequently, the use of next-generation probiotics in the treatment of metabolic diseases has recently been advanced. Remarkable is the impact of *A. muciniphila* supplementation in a mouse model of alcoholic liver disease. This next-generation probiotic species was found to protect from gut leakiness by enhancing the expression of tight junctions and increasing mucus thickness (Grander *et al*., 2017). The administration of the same bacterium or one of its purified membrane proteins was also tested in obese and diabetic mice. Among the beneficial effects, the researchers observed an enhancement of the gut barrier integrity, with attenuations of dyslipidemia and improved glucose tolerance, as well as a reduction in body weight and fat-mass gain (Plovier *et al*., 2017).

**Inflammatory bowel disease**

Involving a dysregulated immune activation towards the intestinal microbiota in genetically sensitive individuals, IBD is considered a chronic inflammatory disorder of the gastrointestinal tract (Podolsky *et al*., 1991). IBD includes ulcerative colitis (UC) and Crohn's disease (CD) as major forms, both leading to substantial morbidity and health care costs (Love *et al*., 1992; Sands *et al*., 2002). In recent decades, the emergence of IBD in regions undergoing rapid urbanization, particularly Asia, the Middle East and South America (Ng *et al*., 2013a, b; 2015; Park *et al*., 2014), has mirrored the patterns observed in the Western world at the beginning of the 20$^{th}$ century: an increasing prevalence of CD followed by the development of UC (Kirsner *et al*., 2001; Kaplan *et al*., 2015). IBD has traditionally been considered a disease of European origin (Sartor *et al*., 2017) known to occur in genetically sensitive individuals in whom exposure to triggering environmental factors elicits an unbalanced immune response to intestinal microorganisms (Kostic *et al*., 2014; Paun *et al*., 2015; Gensollen *et al*., 2016). Consistent with the disappearing microbiota hypothesis and its association with the incidence of chronic diseases (Blaser *et al*., 2017), the progressive loss of ecosystem diversity during urbanization could largely explain the increased incidence of IBD in the Western world. Indeed, a wide range of Western-like environmental factors, i.e. diet, pollution, antibiotic treatments and the absence of an adequate microbial exposure in early childhood, have been demonstrated to implement the loss of key symbionts of our microbiota, ultimately predisposing to the onset of IBD and its rising incidence in urban contexts (Molodecky *et al*., 2010; Ng *et al*., 2015). In particular, the use of antibiotic during early life has recently been linked to peculiar gut microbiome dysbiosis associated with IBD, atopic and autoimmune diseases (Card *et al*., 2004; Ramakrishna *et al*., 2012; Yamamoto-Hanada *et al*., 2017). In addition, recent studies conducted in both murine models and humans have underlined the role of Western diet in influencing the onset and/or pathogenesis of various non-communicable diseases, including IBD (Agus *et al*., 2016; Statovci *et al*., 2017). In particular, the Western-like diets – high in fat

and refined sugar – have been associated with greater susceptibility to adherent-invasive infection with *E. coli* (AIEC), whose presence in the gut has in turn been associated with the pathogenesis of IBD (Simpson *et al*., 2011; Negroni *et al*., 2012; Agus *et al*., 2016). In particular, low amounts of dietary fiber have been shown to associate with enhanced growth and activity of mucin-degrading bacteria, ultimately promoting the onset of pathogen-induced colitis (Pituch-Zdanowska *et al*., 2015; Chiba *et al*., 2015; Desai *et al*., 2016).

The specific microbiota signatures of IBD have been recently described (Manichanh *et al*., 2012). Being characterized by high heterogeneity, CD might virtually affect any part of the gastrointestinal tract, causing a wide variety of inflammatory lesions endowed with peculiar phenotypic characteristics (e.g. inflammatory, structuring or penetrating). In addition to a reduced complexity of the gut ecosystem, the common features in patients with CD concern the interaction of gut microbes with the mucosal immune compartments (Sartor *et al*., 2008; Guarner *et al*., 2008). The peculiar dysbiosis observed in CD patients include the disappearance of health-associated taxa such as *Faecalibacterium* and *Roseburia*, and an increased abundance of the mucin-degrader *R. gnavus*, capable of thrive on mucus layer and influence the gut barrier integrity, ultimately increasing the intestinal permeability (Willing *et al*., 2010).

Furthermore, an enrichment of *Enterobacteriaceae* (*E. coli* in particular), has been observed and this tendency has been found to be particularly evident in mucosal biopsies compared to stool samples (Chassaing *et al*., 2011). On the other hand, bacterial species able to invade the epithelium, such as *Fusobacterium varius* and *F. nucleatum*, have been identified in cultures of inflamed tissue biopsy from UC patients (Ohkusa *et al*., 2002; Strauss *et al*., 2011). Martínez and colleagues also reported an increased load of *Desulfovibrio* spp., sulphate-reducing bacteria involved in UC pathogenesis due to their capacity to generate pro-inflammatory sulphides (Martínez *et al*., 2008; Roediger *et al*., 1997; Rowan *et al*., 2010).

Considering the recent advances in the field of GM modulation and the development of microbiome-tailored therapies, several health benefits have been associated with the

administration of classical probiotics, i.e. *Lactobacillus* and *Bifidobacterium*, in IBD patients (Borruel *et al*., 2002; Llopis *et al*., 2009). Furthermore, being able to downregulate the expression of key pro-inflammatory cytokines and chemokines, as well as neutralize the pro-inflammatory effects of *E. coli* by stimulating the production of anti-inflammatory cytokines (IL-10), the next-generation probiotic *F. prausnitzii* also proved to be particularly promising in the treatment of IBD (Sokol *et al*., 2008; Martin *et al*., 2017a).

Chapter 1 – **INTRODUCTION**

*1.1 The human gut microbiome*

*1.2 Assembly and maturation of the gut microbiome across different stages of life*

*1.3 Dysbiotic variations of the gut microbiome in Western diseases*

**1.4 Microbial ecology assessment: from culturomics to next-generation sequencing**

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## Exploration of microbial diversity through culture-dependent approaches and culturomics

Conventionally, different (selective and non-selective) media have been used for the isolation and growth of bacteria. However, such artificial cultural conditions provide a less favorable environment for previously uncultivated bacteria, and predominant bacteria are likely to mask the growth of less abundant ones. The classical culture-dependent approaches for the study of human intestinal microbiota allow to detect only 30-50% of the microorganisms actually present in this complex ecosystem (Eckburg *et al*., 2005). As a part of the renaissance of culture techniques in microbiology, culturomics has been developed precisely for the cultivation and identification of unknown bacteria that inhabit the human intestine, by combining multiple culture conditions with rapid identification of bacterial species (through matrix assisted laser desorption ionization-time of flight mass spectrometry, MALDI-TOF MS, and 16S rRNA sequencing) (Lagier *et al*., 2012) (Figure 1.3.1). To better mimic the growth conditions present in the original habitat, culture media have been enriched with blood, rumen fluid, and sterile extracts of feces. Acting as natural stimulant, these components facilitate the isolation of previously uncultured bacteria (Lagier *et al*., 2015, 2016; Browne *et al*., 2016; Lau *et al*., 2016). In less than 5 years, the combination of prolonged incubation and multiple culture conditions have allowed the isolation of hundreds of bacterial species from the intestine. In particular,

Lagier and colleagues have expanded the repertoire of human gut microbiota by identifying a total of 1,057 prokaryotic species, 146 of which previously not reported in the gut, 187 bacterial and 1 archaeal (*Haloferax alexandrines*) not previously isolated from humans, and 197 potentially representing new species (Lagier *et al*., 2016). Culturomics has not only significantly increased our knowledge of the repertoire of species associated with humans (bringing the total to 2,671 species), but also has a pivotal role in for the development of future microbiome-based intervention strategies. We are indeed, currently witnessing a paradigm shift in which the manipulation of the human microbiota is promising for treating infections and dysbiosis-related/associated diseases, such as autoimmune and inflammatory diseases and cancer (Sokol *et al*., 2008; Lagier *et al*., 2012; Vetizou *et al*., 2015; Daillere *et al*., 2016; Zitvogel *et al*., 2017). In this scenario, culturomics can provide bacterial strains that can be used in *in vitro* experiments, and animal models to confirm their role in disease pathogenesis, as well as provide new next-generation probiotics candidates to promote host health.

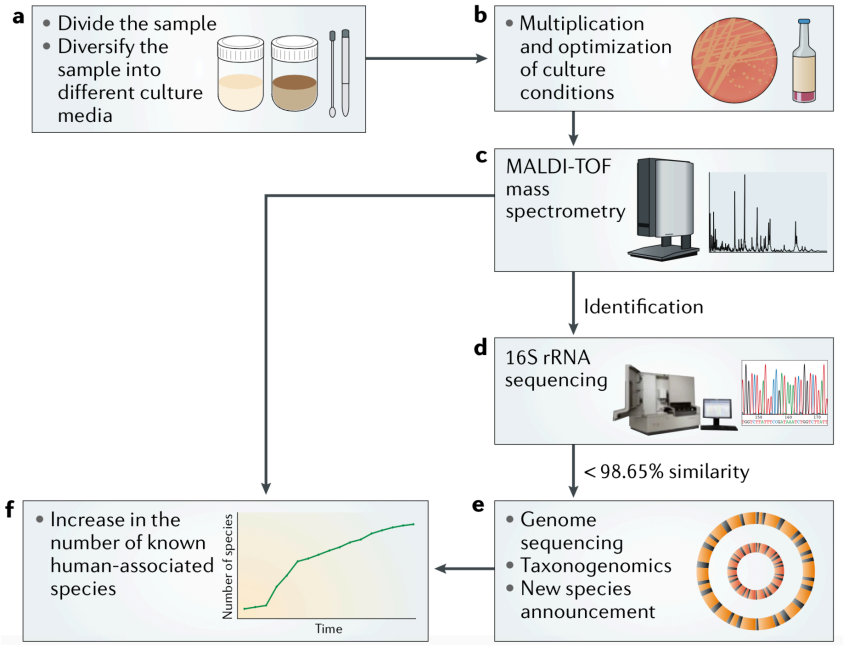


**Figure 1.3.1. The culturomic workflow.** Samples are divided into different culture conditions (a) to suppress dominant populations and promote subdominant growth (b). Isolates identification by matrix assisted laser desorption ionization-time of flight mass spectrometry, MALDI-TOF MS (c), and 16S rRNA sequencing (d). New taxa discovery is confirmed by genome sequencing (e), and taxonogenomics. Through culturomics our knowledge of bacterial species associated with humans has undergone a notable increase (f). Figure taken from Lagier *et al*., 2018.

**Culture-independent approaches and next-generation sequencing**

Overcoming the limits related to culture-dependent techniques, DNA sequencing technologies have transformed our ability to study the composition and functions of complex microbial communities inhabiting various environments, including the gastrointestinal tract of mammals. To investigate bacterial taxa and their phylogeny, in the 1970s Carl Woese and colleagues introduced Sanger sequencing of the 16S rRNA gene, in which conserved regions are alternated with 9 hypervariable regions (V1-V9) (Sanger *et al*., 1977). In the following 30 years, numerous methods based on polymerase chain reaction (PCR) have been developed, such as denaturation/temperature gradient gel electrophoresis (DGGE/TGGE), restriction fragment length polymorphism (RFLP), and quantitative PCR (qPCR). In parallel, fluorescence in situ hybridization (FISH) and microarrays have also been developed (Metzker *et al*., 2005; Centanni *et al*., 2013). In order to overcome the challenges of Sanger method in terms of cost and time, while improving sequencing performance, Metzker and colleagues paved the way for next-generation sequencing (NGS) techniques (Metzker *et al.*, 2005). In particular, during the Illumina sequencing process DNA flanked by two adapters passes over a layer of complementary oligonucleotides bounded to a flow cell. The bound DNA fragment undergoes a bridge amplification process, generating a cluster of clonal populations. During amplification, each fluorescent labeled deoxyribonucleotide triphosphate is detected by fluorophore excitation in a parallel and massive way. Sequencing of marker genes, as in the case of bacterial 16S rRNA, eukaryotic 18S rRNA, or fungal internal transcribed spacer (ITS), constitutes a fast and convenient method to obtain a high-level but low-resolution overview of the phylogenetic composition of a microbial community. By sequencing the entirety of microbial genomes present within a sample (including viral and eukaryotic DNA), shotgun metagenomics provides taxonomic resolution to species or strain level (Scholz *et al*., 2016; Mukherjee *et al*., 2016), and allows to retrieve detailed information on molecular functions. Thanks to this NGS technique, an integrated gene catalog (IGC) of 9.8 million non-redundant microbial genes was published

from metagenomic analysis of 1,267 human stool samples, including 760 Europeans from MetaHIT, 139 from the US, and 368 from China (Li *et al*., 2014). Although the assembly of whole microbial genomes provides more detailed genomic information than marker gene sequencing, samples preparation, sequencing and analysis remain relatively expensive. Unlike marker gene and metagenomics approaches, the metatranscriptomic sequencing of total RNA can be used to characterize gene expression in microbial communities, ultimately providing valuable information on the active functional output of the microbiome. However, host RNA contamination can be a hindrance to sequencing, and methods to deplete the particularly highly abundant rRNA fraction should be implemented in samples preparation (Giannoukos *et al*., 2012). Recently developed, third-generation DNA sequencing techniques have shown substantial progress over second-generation ones. Exploiting the properties of zero-mode waveguides, Pacific Biosciences (PacBio) has developed a single molecule real-time sequencing platform, while Oxford Nanopore technology (MinION) is based on a nanoscale pore structure and involves the measurement of base-dependent changes in the electric field surrounding the pore during DNA transit. Through the production of longer reads compared to NGS, these technologies can easily cover entire genomes bypassing the computational challenges of genome assembly and transcription reconstruction. In addition, they require minimal pre-processing, and epigenetic modifications (i.e. CpG island methylation) can be directly detected. Therefore, data collection and analysis can be operated in real-time, allowing efficient diagnosis and rapid corrective actions for several microbiome-related diseases.

**Bioinformatics pipelines for NGS data analysis**

In recent years, NGS technologies have allowed the development of different methods for surveying microbial communities, producing varying results depending on the sequencing approach adopted. Marker gene sequences (i.e. 16S rRNA) provide a high-level, but low-resolution overview of complex microbial ecosystems. On the other hand, metagenomic sequencing provides more detailed information by analysing the total DNA in a sample, allowing strain-level resolution and detection of genes to collect information on molecular functions. To characterize gene expression in microbial communities, the metatranscriptomic sequencing of total RNA is required. Each of the abovementioned methods requires a dedicated bioinformatics workflow, reported in Figure 1.3.2.

Although the sequencing error rates declared by the main companies are very low, ranging from 0.1 to 0.5% per nucleotide in Illumina and Roche 454 sequencing, respectively (Glenn *et al*., 2011; Luo *et al*., 2012), the very first step in the analysis of marker gene amplicon data consists of removing sequencing errors. In fact, most of the apparent diversity between sequences has been demonstrated to arise from sequencing errors (Reeder *et al*., 2009). By using software packages for the analysis of microbiomes, such as QIIME (Caporaso *et al*., 2010; Bolyen et al., 2019) and Mothur (Schloss *et al*., 2009), this problem is tackled by grouping 97% similar sequences in operational taxonomic units (OTUs) through OTU picking, consolidating them into individual features. Nonetheless, this method does not allow to discriminate subtle and real variations in biological sequences, such as single nucleotide polymorphisms (SNPs) (Callahan *et al*., 2017). Recently developed algorithms, such as Deblur (Amir *et al*., 2017) and DADA2 (Callahan *et al*., 2016), use error profiles to resolve sequence data into exact sequence features named amplicon sequence variants (ASV). The resulting output consists of a table of DNA sequences and their counts or OTUs per sample, subsequently used for the taxonomic assignment. For this purpose, machine learning approaches such as the RDP classifier (Wang *et al*., 2007), a naïve Bayesian classifier trained on oligonucleotide frequencies at genus level,

are used to operate an exact match of the sequences against reference databases (i.e. Greengenes and SILVA). Predictive functional profiling is a technique based on evolutionary models, such as PICRUSt (Langille *et al*., 2013), linking 16S rRNA data with available microbial genomes to make predictions on the metagenomic content, and therefore infer putative biological functions, of a microbial community (Okuda *et al*., 2012; Langille *et al*., 2013; Asshauer *et al*., 2015). Although promising, these methods provide confidence intervals on gene content prediction accuracy based on the availability of reference genomes, which represents a tremendous limiting factor.

On the other hand, metagenomic and metatranscriptomic sequencing allow to investigate the complete nucleic acid profile of a sample, yielding accurate information that can be used to address a broad range of taxonomic, functional and evolutionary aspects of microbial communities as a whole. The bioinformatics pipelines developed for retrieve information from the enormous amount of data generated by metagenomic and metatranscriptomic sequencing combine different analytical approaches, each with several good aspects, but also many defects. Samples can contain thousands of species – many of which are unknown – at dramatically different abundance levels, and sequence data include short reads with a high error rate that may contain artifacts and experimental biases (Niu *et al*., 2011). To overcome these problems, several methods can be used, including taxonomy binning, diversity analysis, sequence clustering, open reading frame (ORF) calling, assembly, and so on. Therefore, the optimal strategy involves merging different analytical approaches, directly using reads and adopting an assembly approach (Davenport *et al*., 2013).

Read-based profiling approaches compare unassembled DNA or mRNA sequence against reference databases to assign taxonomy or annotate genes, continuously refined to improve the profiling accuracy. In particular, MetaPhlAn2 (Truong *et al*., 2015) and TIPP (Nguyen *et al*., 2014) exploit specific genomic regions to perform the taxonomic assignment, focusing on universal single-copy elements such as marker genes. Other tools, for example HUMAnN2

(Abubucker *et al*., 2012) and MEGAN (Huson *et al.*, 2016) can be used to annotate taxonomy, genes and metabolic pathways at the same time. To increase the annotation accuracy, it is essential to select a curated genome database, such as MetaHit for human gut samples (Qin *et al*., 2010) or RefSeq (O'Leary *et al*., 2016). Finally, in order to efficiently compare sample profiles, it is necessary to carry out a normalization of the reading counts (e.g. counts per million) and convert the data in relative abundances. To perform data normalization, tools such as edgeR (Robinson *et al*., 2010) and DESeq2 (Anders *et al*., 2010) can be used.

Nowadays, machine learning plays an increasingly important role in exploiting microbiome data to stratify samples or patients based on the current state (i.e. health versus disease) (Knights *et al*., 2011; Yazdani *et al*., 2016), or to predict future microbiome configurations (Huang *et al*., 2014; Teng *et al*., 2015). For example, the machine learning technique based on random forest regression has been effectively applied to build up a microbiota maturity index in child development (Subramanian *et al*., 2014). Furthermore, the Bayesian estimator of microbial sources forming an unknown community, SourceTracker (Knights *et al*., 2011), represents a useful tool to classify microbial samples based on the environment of origin (Lax *et al*., 2014).

The integration of different data types – obtained from marker gene sequencing, metagenomics, metatranscriptomics, metabolomics and other analytic techniques – is crucial to fully understand the function of microbial communities. This multi-omics approach indeed allows to obtain a holistic outline of the biological system under study.

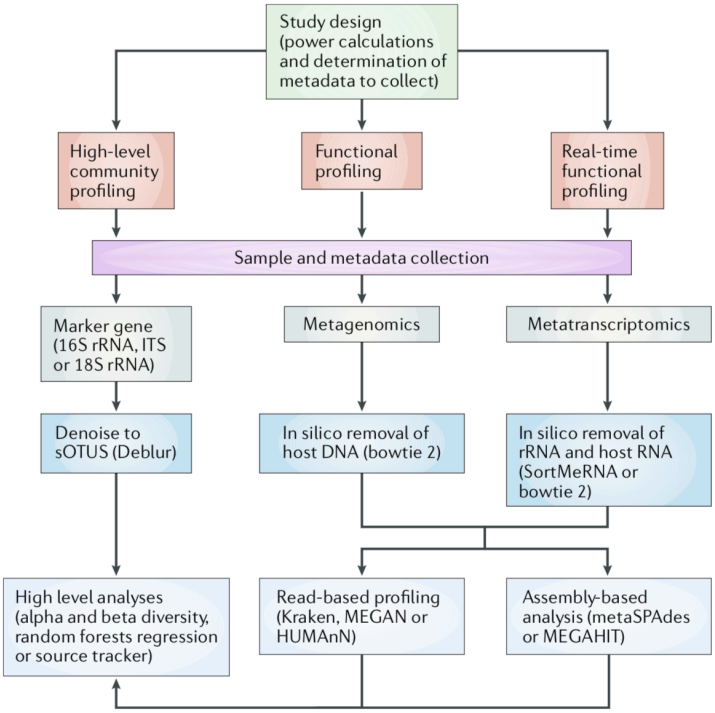


**Figure 1.3.2. Optimized workflow for the bioinformatics analysis of 16S rRNA, metagenomics and metatranscriptomic sequencing data.** Figure taken from Knight *et al*., 2018.

# Chapter 2 – **PROJECT OUTLINE**

The key role played by the gut microbiome in human biology has been extensively investigated in the last years, deepening our knowledge on the microbiome-host interactions during all phases of human life, and instrumental to maintaining a homeostatic physiological balance. Starting from newborn colonization to the late stages of our life, the gut microbiome constitutes a plastic and extremely dynamic ecosystem able to provide the host with extra metabolic functions and adapt to a wide range of endogenous and exogenous stimuli – e.g. diet, lifestyle and medication. Nonetheless, a dramatic shrinkage of individual gut microbiome diversity has been observed in Western populations as compared to worldwide traditional hunter-gatherer populations, and interpreted as a maladaptive microbiome state that may contribute to the rising incidence of chronic non-communicable diseases (NCDs), including obesity and inflammatory bowel disease (IBD). The shift towards a dysbiotic microbiome structure and its association with several diseases have made the microbiome a strategic therapeutic target, paving the way for the development of a wide range of microbiome-tailored intervention strategies aimed at the restoration of eubiotic, health-promoting layouts. In this perspective, murine models that mimic the conditions of inflammation typically associated with NCDs could represent primary tools for screening the potential benefits of innovative microbiome modulators, including next-generation probiotics candidates.

The present work is structured in three main chapters focused on the exploration of human gut microbiome variations throughout life, with the aim of shedding light especially on the functional aspects related to eubiosis (Chapter 3) and dysbiosis in Western diseases (Chapter 4), and on the development of a versatile murine model of intestinal inflammation to better explore the transition towards dysbiotic layouts.

The functional changes that occur in the gut microbiome during the human lifespan up to extreme longevity are still largely unexplored. In order to provide some glimpses in this

direction, the fecal microbiomes of 62 individuals, with age ranging from 22 to 109 years, were characterized by shotgun metagenomics. The obtained profiles showed a peculiar remodeling in microbial gene functions with the ageing process. In particular, the increase in reads count of genes involved in xenobiotic degradation (i.e. ethylbenzene, chlorobenzene, chlorocyclohexane and toluene), as well as the rearrangement in metabolic pathways related to carbohydrate, amino acid and lipid metabolism were found to be even boosted in centenarians (99-104 years) and semi-supercentenarians (105-109 years). These microbiome features probably represent the result of a life-long adaptive response to xenobiotic exposure, ultimately reflecting the progressive changes that occur in diet and lifestyle along with ageing in Western urban areas. In modern anthropic societies, where strong selective pressure is exerted on our microbial counterpart, our findings once again underline its key role as a plastic ecosystem, capable of adapting to external conditions while supporting host homeostasis.

Tracing back to aspects related with human evolution and dietary habits, the modern gut microbiomes are characterized by a remarkably diminished biodiversity – a hallmark of healthy gut – compared to traditional modern populations, whose lifestyle resembles that of our ancestors. Western diets, low in fiber while rich in industrialized and processed foods, are indeed considered one of the leading cause of maladaptive gut microbiome changes along human evolution. In this scenario, the modern Paleolithic diet (MPD) is gaining substantial public attention because of its potential multiple health benefits. With the aim of shedding some light on the possibility to modulate the Western gut microbiome towards a more 'ancestral' configuration through dietary approaches, the fecal profiles of urban Italians adhering to MPD were characterized and compared with other urban Italians following a Mediterranean diet, as well as worldwide traditional hunter-gatherer populations. Notwithstanding a strong geography effect on the gut microbiome structure, our results show an unexpectedly high degree of biodiversity in MPD subjects, which well approximates that of traditional populations, suggesting that this dietary pattern may contribute to partially rewild the microbial ecosystem.

Focusing on the dysbiotic variations associated with NCDs, the link between diet, intestinal microbiome and obesity, as well as with its associated metabolic disorders (e.g. type 2 diabetes and inflammatory bowel disease, IBD), is gaining increasing attention in Western societies. In particular, food addiction is a fundamental neuro-endocrine factor able to ultimately influence lifestyle. The potential involvement of gut-brain axis alterations in mechanisms related to food addiction could in turn be partially responsible for the onset of diet-related disorders. Stool and blood samples were collected from 35 obese women with diagnosed food addiction, 28 obese women without diagnosed food addiction, and 37 healthy normal-weight women, with the aim of unraveling specific bacterial groups and metabolic activities involved in the development of obesity and associated comorbidities, and possibly related to food addiction, through metagenomics and metatranscriptomic analysis. Following the integration of microbiome data with information gathered from dietary habits, hematological parameters and the absorbed x-ray energy scan, the structure of the gut microbiota was stratified into distinct groups. In particular, two microbiome configurations were characterized by lower diversity levels and associated with higher energy intake. Focusing the analysis on a species level, the configurations associated with high food addiction were depleted of the well-known health-promoting *Faecalibacterium prausnitzii*, while enriched in *Ruminococcs torques* and *Akkermansia muciniphila*, both of them being potentially associated with alteration of the intestinal barrier. Furthermore, in these configurations, functional analysis revealed an increased contribution of pathways involved in amino acid metabolism, supporting the previously observed obesity-associated variations in short-chain fatty acid (SCFA) levels.

Considering the increasing prevalence of obesity in children and the related risks to develop cardiovascular risks factors (i.e. hypertension, insulin resistance, and dyslipidemia) during adulthood, it is essential to identify early markers related to the onset of the disease. In this scenario, the microbiome structure of 70 children was monitored in a perspective study collecting samples at the baseline and following 4 years. Despite all children had normal weight

at the beginning, 36 of them gained excessive weight at the subsequent time point. Microbiome data were integrated with dietary information, physical activity, and inflammatory parameters, allowing the stratification of the gut microbiota structures into a discrete number of groups. Regardless of age, gender, and body weight, these microbiome groups were characterized by different biodiversity and correlated with inflammatory markers and dietary habits. It is interesting to note that the microbiome groups of normal-weight children who subsequently gained weight were found to be characterized by lower biodiversity levels and associated with inflammatory profiles. Collectively, our data underline the importance of the individual microbiome-host-diet configuration as a possible predictor of obesity.

Murine colitis models are valuable tools for better understanding intestinal homeostasis and inflammation, even though current models utilize highly inbred mouse strains and only one sex to limit bias. With the aim to develop a more realistic murine model that reflects the high heterogeneity of genetic diversity and the sex-related differences observed in humans, while mimicking the chronic nature of colitis forms as those occurring with IBD, we chemically induced colon inflammation in an outbred strain of both female and male mice (RjOrl_SWISS [CD-1]). Our results showed that intrarectal administrations of dinitrobenzene sulfonic acid (DNBS) effectively causes colitis in both female and male CD-1 mice in a dose-dependent manner, as reflected by loss of body mass, macroscopic scores and histological scores. Furthermore, colon cytokine levels and mesenteric lymph node characteristics indicate that this model involves immune system activation. Although some variables were sex-specific, most of the results support including both females and males in the model. The development of this murine model may provide researchers with a versatile tool for studying the role of the gut microbiome in the onset and progression of NCDs, as well as for testing and validating candidate anti-inflammatory agents and/or new microbiome modulators such as classic or next-generation probiotics, before their use in clinical practice.

# Chapter 3 – **EUBIOTIC GUT MICROBIOME TRAJECTORY ACROSS THE HUMAN LIFESPAN**

In modern anthropic societies, where a strong selective pressure is exerted on our microbial counterpart, the human gut microbiome plays a key role as a plastic ecosystem capable of adapting to external conditions while supporting host homeostasis. Nonetheless, the functional changes that occur in the gut microbiome of extremely long-lived hosts are still largely unexplored. In order to provide some glimpses in this direction, we shotgun sequenced stool samples from healthy individuals aged from 22 to 109 years, highlighting life-long adaptive responses potentially supporting a new homeostasis. On the other hand, when compared to traditional populations, whose lifestyle resembles that of our ancestors, the Western gut microbiome is found to be characterized by reduced biodiversity and supposed to contribute to the rising incidence of non-communicable diseases (NCDs). By providing limited fiber intake and high consumption of industrialized and processed foods, Western diets are in fact considered one of the leading cause of maladaptive – or dysbiotic – gut microbiome changes along human evolution. In this scenario, the modern Paleolithic diet (MPD) is gaining substantial public attention because of its potential multiple health benefits. With the aim of shedding some light on the possibility to modulate the Western gut microbiome towards a more 'ancestral' configuration through dietary approaches, the fecal profiles of urban Italians adhering to the MPD were characterized and compared with other urban Italians following a Mediterranean diet, as well as worldwide traditional hunter-gatherer populations.

## 3.1 Shotgun metagenomics of human gut microbiota up to extreme longevity and the increasing role of xenobiotics degradation

### 3.1.1 Brief introduction

*3.1.2 Materials and Methods*

*3.1.3 Results and Discussion*

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Longevity has been described as the result of a complex combination of variables of endogenous and exogenous origin, related to genetics, lifestyle and the environment (Franceschi *et al*., 2018a; Giuliani *et al*., 2018). In this scenario, the human gut microbiome (GM) has been proposed as a possible mediator of healthy ageing by counteracting inflammageing (a condition characterized by elevated levels of blood inflammatory markers that carries high susceptibility to chronic morbidity, disability, frailty, and premature death) (Biagi *et al*., 2010; Franceschi *et al*., 2018b), intestinal permeability (Nicoletti *et al*., 2015), and deterioration of cognitive and bone health (Nicoletti *et al*., 2015; Villa *et al*., 2017). Correlations have indeed been found between age-related GM dysbioses and levels of pro-inflammatory cytokines, as well as hospitalization, poor diet and frailty in the elderly (Claesson *et al*., 2012). More recently, the longest trajectory of human GM in the course of ageing has been reconstructed, by comparing the fecal bacterial taxa of healthy adults and older individuals, including semi-supercentenarians – persons aged 105 or older (Rampelli *et al*., 2013; Biagi *et al*., 2016). However, to date the functional rearrangements that occur in the GM along with age remain largely unexplored. In an attempt to provide some insight in this direction, advancing our knowledge on whether and how the GM may support the maintenance of health in extreme ageing, the fecal microbiome of 62 individuals, with age ranging from 22 to 105 years, was characterized by shotgun metagenomics.

## 3.1 Shotgun metagenomics of human gut microbiota up to extreme longevity and the increasing role of xenobiotics degradation

*3.1.1 Brief introduction*

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**Subjects and study groups**

The genomic DNA extracted from 62 fecal samples collected by Biagi *et al*. (2016) was used in the study. Individuals were enrolled in Emilia Romagna region (Italy) and categorized into four study groups: Y, 11 young adults (6 females and 5 males; aged 22-48 years, mean age 32.2); K, 13 younger elderly (6 females and 7 males; aged 65-75 years, mean age 72.5); C, 15 centenarians (14 females and 1 male; aged 99-104 years, mean age 100.4); S, 23 semi-supercentenarians (17 females and 6 males; aged 105-109 years, mean age 106.3). The study protocol was approved by the Ethics Committee of Sant'Orsola-Malpighi University Hospital (Bologna, Italy) as EM/26/2014/U (with reference to 22/2007/U/Tess).

**Library preparation and shotgun sequencing**

DNA libraries were prepared using the Qiagen QIAseq FX DNA Library Kit, following the manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, total microbial DNA was quantified by Qubit fluorometer (Invitrogen, Waltham, MA, USA) and 100 ng of each sample were fragmented to 450-bp size, end-repaired and A-tailed using FX Enzyme Mix with the following thermal cycle: 4°C for 1 min, 32°C for 8 min and 65°C for 30 min. Samples were then incubated at 20°C for 15 min in the presence of DNA ligase and Illumina adapter barcodes for adapter ligation. After two purification steps with Agencourt AMPure XP magnetic beads

(Beckman Coulter, Brea, CA, USA), 10-cycle PCR amplification and a further step of purification as above, the final library was obtained by pooling the samples at equimolar concentration of 4 nM. Sequencing was performed on Illumina NextSeq platform using a 2×150 bp paired-end protocol, following the manufacturer's instructions (Illumina, San Diego, CA, USA).

**Bioinformatics and biostatistics**

The functional annotation of the sequences deriving from the 62 genomic DNA samples (Biagi *et al*., 2016) was conducted as described by Rampelli and colleagues (2013). In brief, shotgun reads were first filtered by quality and human sequences by means of the human sequence removal pipeline and the whole genome sequencing (WGS) read processing procedure of Human Microbiome Project (HMP) Consortium (Turnbaugh *et al*., 2007). The obtained reads were taxonomically characterized at species level by MetaPhlAn2 (Truong *et al*., 2015) and assigned for functionality at different levels of the Kyoto Enciclopedia of Genes and Genomes (KEGG) database (Wixon *et al*., 2000) using Metagenome Composition Vector (MetaCV) with default parameters (Liu *et al*., 2013). Principal Coordinate Analysis (PCoA) was carried out using vegan (https://cran.r-project.org/web/packages/vegan/index.html) in R. Data separation in the PCoA was tested using a permutation test with pseudo-*F* ratios (function adonis in the vegan package). When appropriate, P values were adjusted for multiple comparisons using the Benjamini-Hochberg correction. A false discovery rate (FDR) <0.05 was considered statistically significant. Where present, the species-level classification of MetaCV (Liu *et al*., 2013) was retrieved, and the taxon ID in the NCBI taxonomy database was obtained using the web interface of the NCBI Taxonomy Browser tool (https://www.ncbi.nlm.nih.gov/Taxonomy/TaxIdentifier/tax_identifier.cgi). In order to retrieve the entire phylogeny of the assignment, we transformed the NCBI taxonomy IDs into the full lineage by using the ETE3 toolkit (Huerta-Cepas *et al*., 2016).

## 3.1 Shotgun metagenomics of human gut microbiota up to extreme longevity and the increasing role of xenobiotics degradation

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Shotgun metagenomics was applied to 62 DNA samples from the same study, revealing functional and species-level taxonomic connections between the GM and extreme ageing. Specifically, the GM from 11 young adults (group Y, 6 females and 5 males; aged 22-48 years, mean age 32.2), 13 younger elderlies (group K, 6 females and 7 males; aged 65-75 years, mean age 72.5), 15 centenarians (group C, 14 females and 1 male; aged 99-104 years, mean age 100.4), and 23 semi-supercentenarians (group S, 17 females and 6 males; aged 105-109 years, mean age 106.3) was characterized. The sequencing yielded a total of 1.3 billion sequences, with an average of 20 million (± 5 M, sd) reads per subject.

First, it was confirmed that in all age groups the GM is dominated by a few bacterial families (i.e. *Bifidobacteriaceae*, *Bacteroidaceae*, *Lachnospiraceae* and *Ruminococcaceae*), whose relative abundance trend is inversely proportional to age (mean relative abundance ± sd: Y, 73% ± 3%; K, 65% ± 4%; C, 62% ± 4%; S, 58% ± 6%). Focusing on the species level, the major contribution to fecal profiles is represented by 13 bacterial species: *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Bacteroides uniformis*, *F. prausnitzii*, *Ruminococcus bromii*, *Subdoligranulum* spp., *Blautia obeum*, *R. torques*, *Coprococcus comes*, and *Roseburia* spp.

Bray-Curtis PCoA of species-level relative abundance profiles provided evidence of an age-related segregation (p value < 0.0001, permutation test with pseudo-*F* ratios) (Figure 3.1.1).

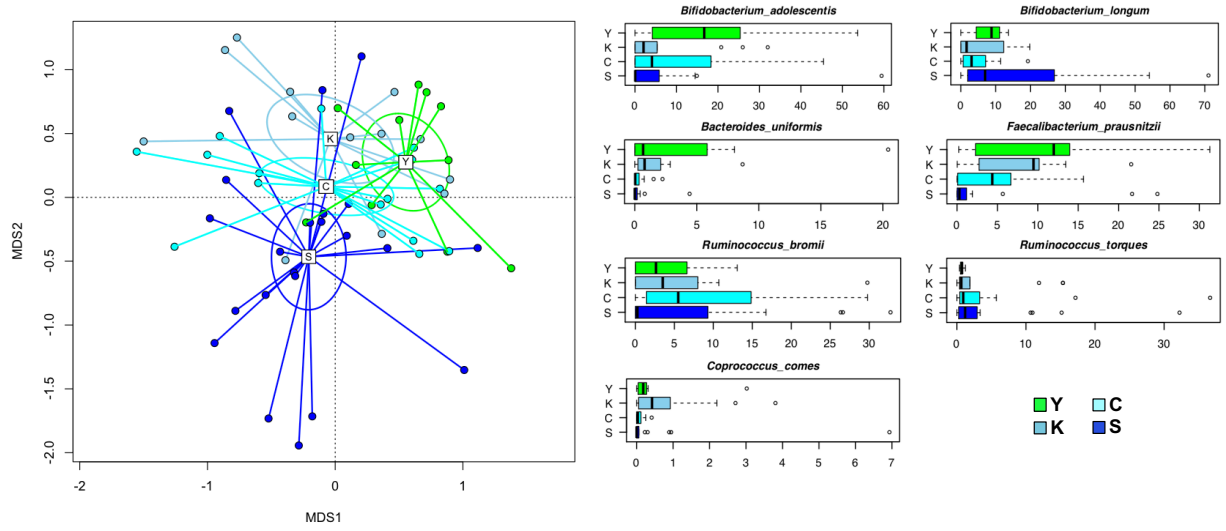


**Figure 3.1.1. Gut microbiome variation along ageing**. Left, PCoA plot of Bray-Curtis dissimilarity between the microbiome species-level relative abundance datasets of the four age groups. Right, Boxplots of the normalized relative abundance of bacterial species differentially represented among the four age groups (Y, young adults; K, younger elderly; C, centenarians; S, semi-supercentenarians) (p value < 0.05, Kruskal-Wallis test)

However, it should be noted that the species-level compositional structure of the GM from younger elderly overall matched that from young adults (p value = 0.2), suggesting that the physiology of the ageing process may not involve coarse changes in GM species and their relative abundance. On the other hand, centenarians and semi-supercentenarians are featured by a distinctive rearrangement in their GM taxonomic configuration. Compared with younger individuals, long-living people show a decreased contribution of *B. uniformis*, *E. rectale*, *C. comes* and *F. prausnitzii*, along with a progressive increase of *E. coli*, *M. smithii*, *A. muciniphila* and *Eggerthella lenta* (p value < 0.05, Wilcoxon test). These trends had already been reported in previous 16S rRNA gene-based microbiome works in the same subjects (Biagi *et al*., 2010; Biagi *et al*., 2016) as well as in Chinese centenarians (Wang *et al*., 2015), further strengthening that the observed GM variations could be part of the extreme ageing process, regardless of environmental variables (i.e. geographical origin and cultural habits, such as diet and lifestyle) (Santoro *et al*., 2018).

By focusing the analysis on a functional scale, it is interesting to note a segregation of the relative abundances of KEGG pathways according to age groups (Figure 3.1.2). In particular, a significant positive correlation was found between the pathways distribution along the first axis of the PCoA plot, suggesting a functional rearrangement within the GM coherently with increasing age.

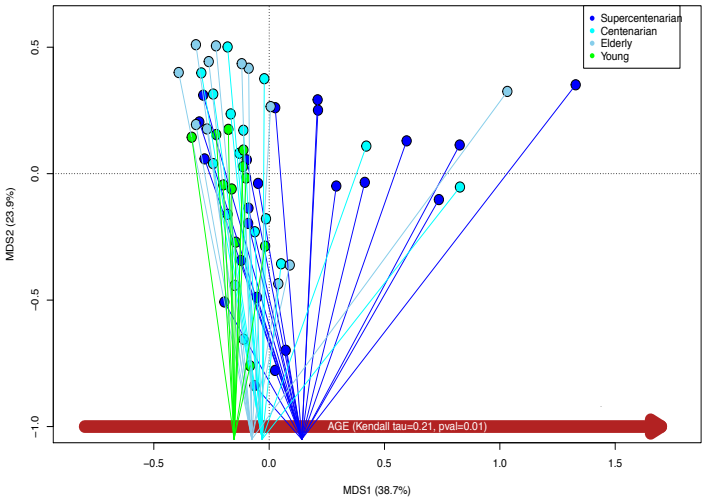


**Figure 3.1.2. Functional structure of the gut microbiome along ageing.** PCoA analysis based on the Bray-Curtis distances of the KEGG pathway relative abundances. Age shows a significant positive correlation with MDS1 axis (p value = 0.01, tau = 0.21; Kendall correlation test).

Furthermore, a progressive age-related increase in the number of reads for genes devoted to xenobiotic biodegradation and metabolism, as well as a simultaneous decrease in genes involved in carbohydrate metabolism have been found (Figure 3.1.3). This functional rearrangement is even more penetrating in the GM of centenarians and semi-supercentenarians, being characterized by a reduced contribution of pathways for starch and sucrose (KEGG pathway, ko00500), pentose phosphate (ko00030) and amino sugar and nucleotide sugar (ko00520) metabolism, and a concomitant increase in toluene (ko00623), ethylbenzene (ko00642), caprolactam (ko00930) and chlorocyclohexane and chlorobenzene (ko00361) degradation pathways. While the changes related to carbohydrate metabolism have already been reported in previous studies and suggested to be associated with age-related changes in dietary habits (Claesson *et al.*, 2012; Rampelli *et al.*, 2013), the peculiar increase in genes for xenobiotic metabolism had not yet been observed previously.

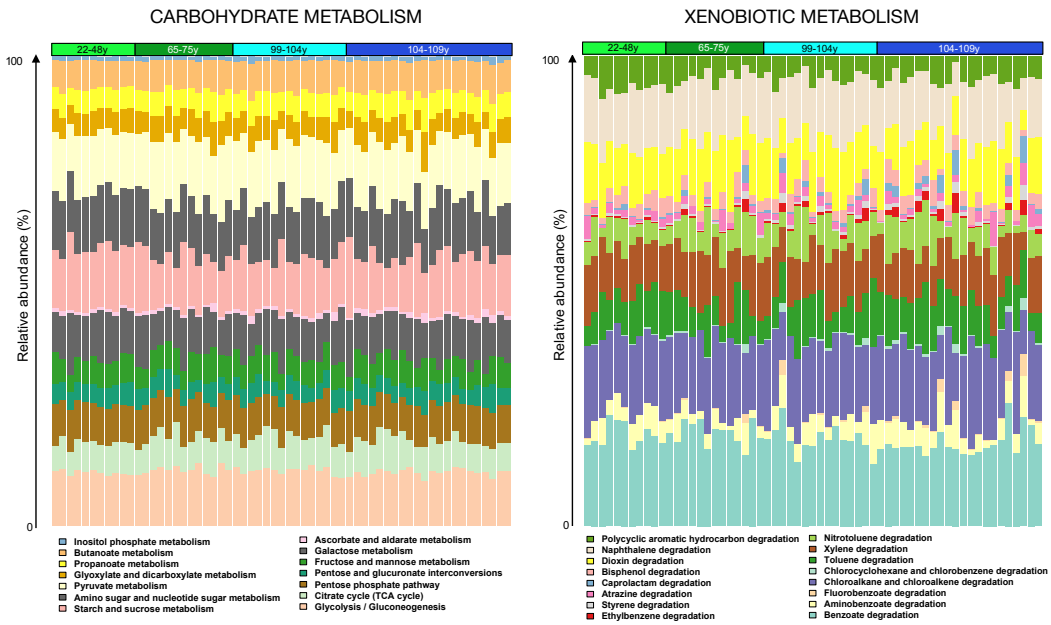


**Figure 3.1.3. Ageing-related configurations of selected gut microbiome functional pathways.** Bar plots of the KEGG pathway-classified metabolic configurations for carbohydrate and xenobiotic metabolism, as the mean relative contribution of each pathway to the total normalized number of reads assigned to each specific metabolism.

Ethylbenzene, chlorobenzene, chlorocyclohexane and toluene are pervasive chemicals mainly deriving from industrial manufacturing and municipal discharges, under monitoring all over the world as part of the main environmental contaminants of the atmosphere, due to their toxic effects (Bruno *et al*., 2008; Buczynska *et al*., 2009; Leusch *et al*., 2010). These molecules are known to be abundant in emissions from motor vehicle and cigarette smoke, as well as being generated during the processing of refined petroleum products (e.g. plastics), and contained in common consumer products, such as paints and lacquers, thinners and rubber products (Leusch *et al*., 2010). Furthermore, caprolactam is the raw material of nylon, used for the production of many indoor products, such as synthetic fibers, resins, synthetic leather and plasticizers. Previous studies have demonstrated the higher indoor burden of these molecules compared to the outdoor environment, underlining the outstanding importance of indoor exposure on human health (Massolo *et al*., 2010; Esplugues *et al*., 2010). It is a matter of fact that living in environments under strong anthropic pressures – such as the Emilia Romagna region in Italy

(Larsen *et al*., 2012; Zauli Sajani *et al*., 2018) – results in the continuous and constant exposure to these pervasive xenobiotic substances, favoring their maintenance and progressive accumulation in body tissues, including the gut (Heinrich-Ramm *et al*., 2000; Galloway *et al*., 2015; Sutic *et al*., 2016; Wright *et al*., 2017). In anthropic environments, therefore, appropriate conditions could be established for the selection of GM components capable of detoxifying such chemical compounds, with a mutual benefit in terms of microbiome and host fitness. Indeed, recent works suggests that the human-associated microbial communities of urban Western populations are functionally suited to the degradation of xenobiotic molecules, including caprolactam (Wu *et al*., 2016; Rampelli *et al*., 2015; Lee *et al*., 2019). Our results demonstrated that this adaptive microbiome feature becomes more prevalent with ageing, probably matching the cumulative exposure to these xenobiotic substances during the course of life in anthropic environments. Further supporting the importance of human microbiomes in providing an adaptive response to xenobiotic exposure, in a recent work the upper airway microbiome of non-asthmatic individuals was found to possess greater ability to metabolize caprolactam than asthmatic people (Lee *et al*., 2019). According to the authors, the selection of caprolactam-degrading microbes in the airway microbiome would decrease host exposure to indoor air pollutants, providing an ultimate impact on human health. Centenarians and semi-supercentenarians are long-living individuals who as such, could claim an important history of exposure to xenobiotic stressors. Furthermore, considering their reduced mobility, these subjects tend to spend more time in their own houses than younger people, with increased exposure to indoor pollutants. It is thus tempting to speculate that their microbiome is better equipped for the degradation of these xenobiotics as a result of an adaptive process driven by the more lasting and assiduous exposure to these chemicals. This raises important open questions on the biological mechanisms that lead to the consolidation and enrichment of xenobiotic-degrading abilities in the centenarian and semi-supercentenarian GM. Our results suggest that the highest contribution to xenobiotic degradation by commensals in long-living

people might mainly be the result of a top-down selection process related to the lifestyle habits of these exceptionally old individuals, i.e. stable and constant living settings within their own homes, together with a longer exposure and consequent accumulation of these chemicals in the host tissues due to their longer life.

Besides xenobiotic-degrading genes and those involved in carbohydrate metabolism, age-related differences in other metabolic pathways, including those associated with lipid metabolism were also found. In particular, centenarians and semi-supercentenarians showed more reads for alpha-linoleic acid (ko00592) and glycerolipid metabolism (ko00561); on the other hand, younger people show a greater contribution of genes involved in sphingolipid (ko00600) and glycerophospholipid metabolism (ko00564). These profiles possibly reflect the higher intake of plant-derived fats than animal ones by centenarians and semi-supercentenarians, given that glycerophospholipids and sphingolipids are known to be more abundant in animal-derived foods (Vesper *et al*., 1999; Castro-Gomez *et al*., 2015), while alpha-linoleic acid is mainly derived from plant foods (Stark *et al*., 2008). Moreover, regarding the functional pathways involved in amino acid metabolism, a progressive increase in genes for the metabolism of tryptophan (ko00380), tyrosine (ko00350), glycine, serine and threonine (ko00260) were observed in the GM of older individuals. On the other hand, genes for alanine, aspartate and glutamate metabolism (ko00250) were found to be more abundant in younger individuals. This evidence is in agreement with our previous study (Rampelli *et al*., 2013), in particular with regard to the metabolism of tryptophan and tyrosine as an indicator of enhanced proteolytic metabolism. Furthermore, these findings fit with metabolite measures in the centenarians of our cohort, i.e. the decrease of the bioavailability of tryptophan in serum (Collino *et al*., 2013) as well as the increased urinary levels of phenolic metabolites, derived from the metabolism of tyrosine (Moco *et al*., 2014). Finally, the progressive increase with ageing of genes for lipopolysaccharide biosynthesis (ko00540), which can be associated with the presence of pathobionts (i.e. members of the *Enterobacteriaceae* family) and the low levels

of chronic inflammation (i.e. inflammageing), is consistent with previous findings in long-lived people (Biagi *et al*., 2010; Rampelli *et al*., 2013; Biagi *et al*., 2016).

In conclusion, the present study describes for the first time the adaptive metagenomics changes of the human GM along ageing, up to extreme longevity (>105 years of age). In addition to confirming the known taxonomic features of an ageing microbiota down to species level, an accurate depiction of the functional changes occurring along with age is provided. The results suggest the fascinating hypothesis that ageing in Western urban environments progressively selects for commensal GM strains with metabolic abilities towards specific xenobiotics. This selective pressure could represent an adaptive response of the human holobiont to the increased exposure to – and accumulation of – xenobiotic substances along the ageing process. Future studies should be aimed at better understanding the interplay between xenobiotics exposure and the human GM. In particular, long-term longitudinal studies must be conceived, with the aim of highlighting the mechanisms underlying the GM adaptive variation, as a result of a top-down selection process of microbial functions for xenobiotic detoxification, and the ultimate impact in terms of host health protection.

## 3.2 Gut microbiome response to a modern Paleolithic diet in a Western lifestyle context

### 3.2.1 Brief introduction

*3.2.2 Materials and Methods*

*3.2.3 Results and Discussion*

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In recent years, extensive meta-analyses of human and non-human primate microbiomes have been carried out to understand the specificities of the human microbiome assembly (Moeller *et al*., 2014; Davenport *et al.*, 2017). This comparative approach has highlighted the importance of individual biodiversity reduction among the distinctive features of the human gut microbiome (GM) along the evolutionary history (Moeller *et al*., 2014). It is interesting to note that this hallmark has been found to be exacerbated in Western urban populations, which show an even more marked compression of GM diversity than traditional and rural counterparts (De Filippo *et al.*, 2010; Yatsunenko *et al.*, 2012; Schnorr *et al.*, 2014; Obregon-Tito *et al.*, 2015). This dramatic shrinkage of GM diversity in Western urban populations portrays a maladaptive microbiome state that has been supposed to contribute to the rising incidence of chronic NCDs – such as obesity, diabetes, asthma and IBD – (Sonnenburg & Sonnenburg 2014; Mosca *et al*., 2016; Zuo *et al*., 2018; Cani & Jordan 2018) which fully agrees with the hypothesis of the disappearing microbiota (Blaser *et al*., 2017). In recent years, a large body of research has been indeed devoted to understanding the mechanisms that lead to the loss of diversity in the Western urban GM. It is in this scenario that the multiple-hit hypothesis has been advanced (Sonnenburg & Sonnenburg 2014). According to this theory, the progressive reduction of human GM diversity has occurred at multiple stages along the recent transition to modern urban societies, and several aspects typical of the urbanization process – such as sanitation, antibiotics, cesarean section and Western diet – have been pointed out as contributing factors. In particular, the

reduction in quantity and diversity of dietary microbiota-accessible carbohydrates (MACs) – coming from a variety of sources including plants, animal tissues or food-borne microbes – has been considered one of the leading causes of the disappearing GM in Western urban populations (Sonnenburg & Sonnenburg 2014). Moreover, the importance of food additives, emulsifiers and xenobiotics – ubiquitous in industrially processed foods – in reducing the GM diversity has recently been demonstrated, identifying an additional driver that contributes to biodiversity shrinkage (Danchin *et al*., 2018). However, all currently available studies exploring the disappearing GM are based on the comparison between Western urban and traditional rural populations (De Filippo *et al.*, 2010; Yatsunenko *et al.*, 2012; Schnorr *et al.*, 2014; Obregon-Tito *et al.*, 2015; He *et al*., 2018; Deschasaux *et al*., 2018; Ayeni *et al*., 2018). Consistently, the observed GM differences are likely to be the result of the combined action of several covariates in addition to the diet – i.e. ethnicity, geographical origin, climate, subsistence, medication, hygiene and life sharing – and do not allow to weight the importance of the single determinants. In the last few years, the modern Paleolithic diet (MPD), with high intake of vegetables, fruit, nuts, seeds, eggs, fish and lean meat, while excluding grains, dairy products, salt and refined sugar, attracted substantial public attention in the Western world because of its potential multiple health benefits (Lindeberg *et al*., 2007; Jonsson *et al*., 2009; Whalen *et al*., 2016; Otten *et al*., 2018; Genoni *et al*., 2016, 2019). In the present work, the GM structure of 15 Italian subjects who followed the MPD for at least one year was profiled and compared with that of urban Italian individuals largely adhering to the Mediterranean diet (MD) from our previous works (Schnorr *et al*., 2014; De Filippis *et al*., 2016). Notwithstanding the small sample size, our GM exploratory study gave us the unique opportunity to assess to what extent the consumption of MACs deriving from plant-based foods – but not grains – along with the exclusion of industrially processed food, might counteract the GM diversity reduction observed in Western urban populations. Indeed, the comparison between MPD and Western diets in subjects living in the same country allowed excluding the impact of confounding drivers of GM

variation, such as geography, ethnicity, medication and hygiene (De Filippis *et al*., 2016; He *et al*., 2018; Deschasaux *et al*., 2018). In order to extend the GM comparison at the meta-population level, publicly available microbiome data from traditional hunting and gathering populations showing high GM diversity, such as the Hadza from Tanzania, from our previous publication (Schnorr *et al*., 2014), the Matses from Peru (Obregon-Tito *et al*., 2016), and the Inuit from the Canadian Arctic (Girard *et al*., 2016) were also included in our analysis. Although the mechanisms underlying the human-microbiome interactions are still far from being fully understood, the possibility of rewilding the modern microbiota through the diet could be the key to restore evolutionarily important functionality to the gut, ultimately improving our health.

## 3.2 Gut microbiome response to a modern Paleolithic diet in a Western lifestyle context

*3.2.1 Brief introduction*

### 3.2.2 Materials and Methods

*3.2.3 Results and Discussion*

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**Subjects and sample collection**

Fifteen healthy individuals following a MPD for at least one year were recruited from different urban areas across Italy (Lombardia, Piemonte, Emilia-Romagna, Toscana, Umbria, Lazio, Campania, Molise, Puglia and Calabria regions). Exclusion criteria included: age below 18 and over 65 years; BMI <18.5 and >24.9 $kg \cdot m^{-2}$; habitual intake of drugs and nutritional and pharmacological supplements of pre- and probiotics; taking antibiotics in the last three months; presence of intestinal and metabolic disorders (i.e. IBD, bacterial contamination syndrome, irritable bowel syndrome, constipation, celiac disease, type 1 and 2 diabetes, cardio- and neurovascular diseases, rheumatoid arthritis, allergies, neurodegenerative diseases, cancer). Written informed consent was obtained from all volunteers. All work was approved by the Ethics Committee of the Sant'Orsola-Malpighi Hospital, University of Bologna (ref. number, 118/2015/U/Tess).

Each subject was asked to fill in a 7-day weighted food intake record (7D-WR), with the total food and beverage consumption (including methods of preparation whenever possible) for 7 days representing their usual intake, as previously described (Dall'Asta *et al*., 2012). Daily total calorie intake as well as that of macro- and micro-nutrients were assessed through the MètaDieta software version 3.7 (METEDA). The participants were also asked to fill in two questionnaires, one regarding their socio-economic status (according to the guidelines of the Health Survey for England – 2013, http://www.hscic.gov.uk/catalogue/PUB16076) and the

other on physical activity (based on the Global Physical Activity Questionnaire – GPAQ – developed by World Health Organization, http://www.who.int/chp/steps/resources/GPAQ_ Analysis_Guide.pdf). A single fecal sample was self-collected by each participant after completing the 7D-WR (i.e. on day 7) and immediately frozen at -20°C. All specimens were delivered to the laboratory of the Microbial Ecology of Health Unit (Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy) where they were stored at -80°C until processing. Data and fecal samples were collected between March and April 2017.

**Microbial DNA extraction**

Total bacterial DNA was extracted from each stool sample using the DNeasy Blood and Tissue kit (Qiagen) with the modifications previously described by Biagi *et al.* (2016). In brief, 250 mg of fecal sample were suspended in 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA, 4% (w/v) SDS), added with four 3-mm glass beads and 0.5 g of 0.1-mm zirconia beads (BioSpec Products) and homogenized using a FastPrep instrument (MP Biomedicals) with three bead-beating steps at 5.5 movements/sec for 1 min, and 5-min incubation in ice between treatments. After incubation at 95°C for 15 min, stool particles were pelleted by centrifugation at 14,000 rpm for 5 min. Nucleic acids were precipitated by adding 260 µl of 10 M ammonium acetate and one volume of isopropanol. The pellets were then washed with 70% ethanol and suspended in TE buffer. RNA was removed by treatment with 2 µl of DNase-free RNase (10 mg/ml) at 37°C for 15 min. Protein removal and column-based DNA purification were performed following the manufacturer's instructions (Qiagen). DNA was quantified with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

**16S rRNA gene sequencing**

For each sample, the V3-V4 region of the 16S rRNA gene was amplified using the S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 primers (Klindworth *et al*., 2013) with Illumina overhang adapter sequences. PCR reactions were performed in a final volume of 25 μl, containing 12.5 ng of genomic DNA, 200 nM of each primer, and 2X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Roche), in a Thermal Cycler T (Biometra GmbH) with the following gradient: 3 min at 95°C for the initial denaturation, 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, and a final extension step at 72°C for 5 min. PCR products of around 460 bp were purified using a magnetic bead-based system (Agencourt AMPure XP; Beckman Coulter) and sequenced on Illumina MiSeq platform with the 2×250 bp paired-end protocol, according to the manufacturer's guidelines (Illumina). Briefly, each indexed library was prepared by limited-cycle PCR using Nextera technology, and further purified as described above. The libraries were subsequently pooled at equimolar concentrations, denatured with NaOH 0.2 N, and diluted to 6 pM before loading onto the MiSeq flow cell. Sequencing reads were deposited in MG-RAST (project ID, mgp89161).

**Bioinformatics and statistics**

Raw sequences were processed using a pipeline that combines PANDAseq (Masella *et al*., 2012) and QIIME (Caporaso *et al*., 2010). The UCLUST software (Edgar *et al*., 2010) was used to bin high-quality reads into OTUs at 0.97 similarity threshold through an open-reference strategy. Taxonomy was assigned through the RDP classifier, using the Greengenes database as a reference (release May 2013). Chimera filtering was performed by using ChimeraSlayer (Haas *et al*., 2011). All singleton OTUs were discarded.

16S rRNA gene sequencing data of our cohort were compared with publicly available data from the following previous studies: De Filippis *et al*. (2016) (127 Italians; NCBI Sequence Read Archive (SRA) accession number: SRP042234), Schnorr *et al*. (2014) (16 Italians and 27 Hadza

hunter-gatherers from Tanzania; MG-RAST ID: 7058), Obregon-Tito *et al.* (2015) (25 Matses hunter-gatherers from Peru; NCBI SRA: PRJNA268964), and Girard *et al*. (2016) (21 Inuit from the Canadian Arctic; Qiita ID: 10439). Genus-level community composition was generated for all cohorts combined. Alpha diversity was assessed using the Shannon and Simpson indices. Beta diversity was evaluated using the Bray-Curtis dissimilarity measure. All statistical analysis was performed in R 3.3.2, using R Studio 1.0.44 and the libraries vegan, made4 and stats. The significance of data separation in the PCoA of Bray-Curtis distances was tested using a permutation test with pseudo-*F* ratios (function adonis of vegan package) and ANOSIM test. Superimposition of bacterial genera on the PCoA plot was performed using the envfit function of vegan. Wilcoxon test was used to assess significant differences between groups (for intra- and inter-individual diversity), while Kruskal–Wallis test was used for multiple comparisons. P values were corrected for FDR (Benjamini-Hochberg) and p value < 0.05 is considered statistically significant.

## 3.2 Gut microbiome response to a modern Paleolithic diet in a Western lifestyle context

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**Diet, socio-economic context and gut microbiome structure in Italian adults following the modern Paleolithic diet**

Fifteen healthy individuals, 12 males and 3 females, who followed the MPD for at least one year were recruited from different urban areas across Italy. The average age of the enrolled subjects was 39.2 years (range, 26-57), and the average body mass index (BMI) 22.1 $kg \cdot m^{-2}$ (range, 19.4-25.7). The MPD adopted by the 15 subjects is mainly based on the consumption of unprocessed foods, with high intake of vegetables, fruit, nuts and seeds, eggs, fish and lean meat, while excluding grains, dairy products, salt and refined sugar. The average daily energy intake of the enrolled cohort is 1,843.45 kcal (range, 1,563-2,186 kcal). The percentage of macronutrients is distributed as follows: fat, 51.02%; protein, 30.14%; carbohydrate, 18.84% (Figure 3.2.1A). With regard to lipids, 51.65% of total calories are from monounsaturated fatty acids (MUFAs), 30.93% from saturated fatty acids (SFAs) and 17.42% from polyunsaturated fatty acids (PUFAs) (Figure 3.2.1B). The average daily fiber intake is 14.64 g/1,000 kcal. Based on the data collected through a questionnaire on the socio-economic status, one third of the subjects lived in highly urbanized areas, more than half in semi-urbanized areas (8/15) and only one individual in a rural setting. Two thirds lived in apartments and the remainder in independent houses. Eight out of 15 subjects declared they had pets and daily contact with nature (defined as 2 to 15 hours a week spent in a green area). According to a questionnaire on physical activity (the Global Physical Activity Questionnaire - GPAQ), 12 individuals reported

practicing moderate to intense fitness activities for an average of 1 hour a day for at least 3 days a week.

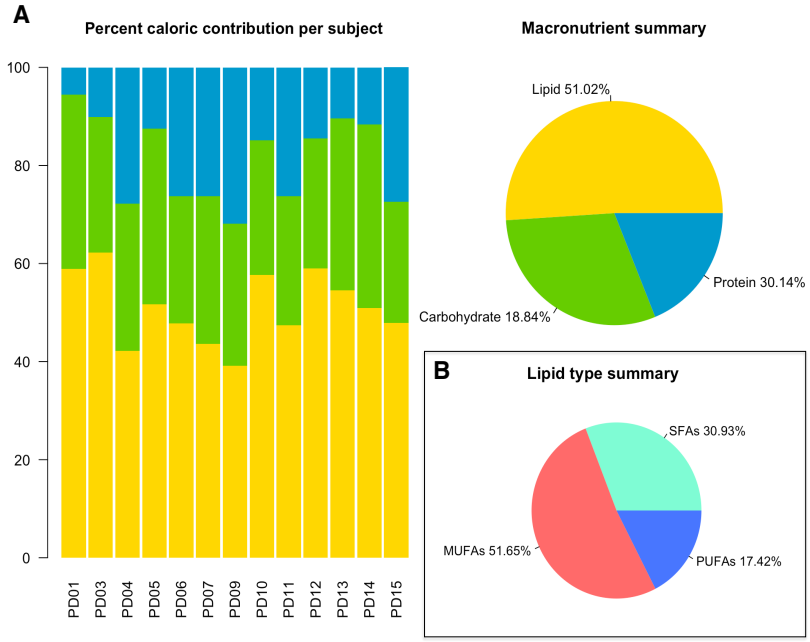


**Figure 3.2.1. Macronutrient composition of the modern Paleolithic diet.** (A) Bar plots of the percent caloric contribution of fat, protein and carbohydrate per subject, based upon weighted food intake records over 7 days. The pie chart shows the summary of the average macronutrient intake for the entire cohort. (B) Pie chart of the lipid type summary. PUFAs: polyunsaturated fatty acids; MUFAs: monounsaturated fatty acids; SFAs: saturated fatty acids.

The GM structure of MPD Italian adults was profiled through 16S rRNA gene sequencing of fecal DNA. A total of 864,439 high-quality reads (mean ± sd, 57,629 ± 19,752; range, 25,142 - 95,924) were generated and clustered in 7,483 OTUs. The phyla Firmicutes (relative abundance, mean ± sem, 65.1 ± 2.1%) and Bacteroidetes (24.6 ± 2.2%) dominate the gut microbial ecosystem, with Proteobacteria (4.4 ± 1.6%), Actinobacteria (3.4 ± 0.8%) and Verrucomicrobia (1.2 ± 0.5%) as minor components. At family level, *Ruminococcaceae* (26.7 ± 1.7%), *Lachnospiraceae* (18.7 ± 1.4%), *Bacteroidaceae* (13.7 ± 1.8 %) and *Prevotellaceae* (7.4 ± 2.4%) are the dominant GM constituents. The most abundant (≥ 5%) bacterial genera are *Bacteroides*, *Prevotella*, and *Faecalibacterium*, while *Coprococcus*, *Ruminococcus*, *Blautia*, *Lachnospira*, *Phascolarctobacterium*, *Streptococcus*, *Roseburia*, *Akkermansia*, *Oscillospira* and *Eubacterium* (family *Erysipelotrichaceae*) represent minor components of the microbial ecosystem (range, 1.0 ± 0.4% - 4.4 ± 0.7%) (Figure 3.2.2).

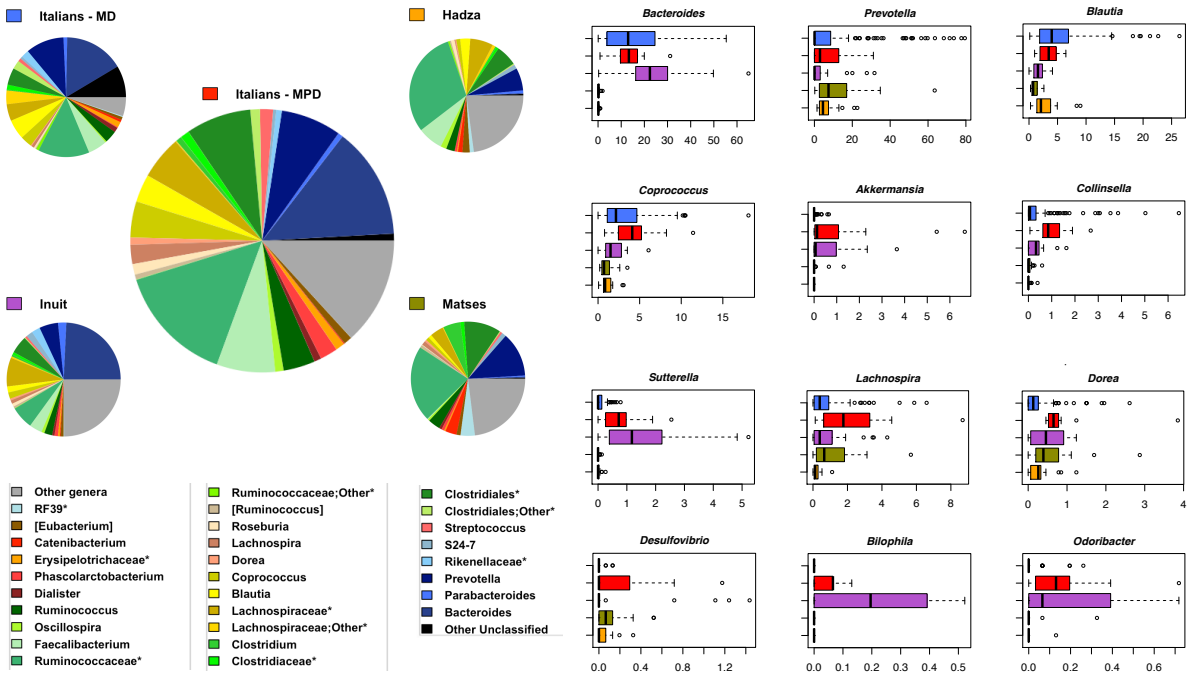


**Figure 3.2.2. Genus-level phylogenetic structure of the gut microbiome of Italian adults adhering to the modern Paleolithic diet and major differences among study groups.** Pie charts show the average relative abundance of bacterial genera represented in the GM of the enrolled study groups. Only bacterial genera with relative abundance >0.5% are shown. Boxplots show the relative abundance distribution of significantly different bacterial genera among study groups. *, unclassified OTU reported at higher taxonomic level.

**Gut microbiome diversity in MPD Italian adults and comparison with other Western urban populations and traditional communities**

In order to investigate whether the adherence to the MPD is sufficient to promote a more diverse GM ecosystem – even in a Western urban context – the GM diversity of the 15 MPD subjects has been compared to that of 43 urban Italians with different level of adherence to the MD, whose GM composition has been described in De Filippis *et al*. (2016) (n = 127) and Schnorr *et al*. (2014) (n = 16). Moreover, to extend the comparative analysis to a global level, the GM structural profiles of the following traditional hunter-gatherer populations have been also included: 27 Hadza from Tanzania (Schnorr *et al*., 2014), 25 Matses from Peru (Obregon-Tito *et al*., 2015), and 21 Inuit from Canada (Girard *et al*., 2016). According to our findings, significant differences in the GM biodiversity among the study groups have been detected (Simpson index, p value = $2.6\times10^{-15}$; Shannon index, p value = $2.2\times10^{-16}$; Kruskal-Wallis test) (Figure 3.2.3). Interestingly, the GM diversity observed for MPD subjects far exceeds that of urban Italians adhering to the MD (Simpson index, p value = $2.5\times10^{-7}$; Shannon index, p value = $6.1\times10^{-9}$; Wilcoxon test), is comparable to that of the Hadza (p value = 0.39; 0.26), and even greater than Matses (p value = 0.0082; 0.0039) and Inuit (p value = 0.00075; 0.0027). As recently discussed, a high species diversity could promote healthy competition among microbial symbionts and modulate bacterial interactions, ultimately maintaining the overall ecosystem stability (Coyte *et al.*, 2015).

The PCoA based on Bray-Curtis distances was next performed to assess overall genus-level compositional differences in the GM structure between study groups. Our data show clear separation of GM profiles by provenance and, within the Italian cohort, by dietary pattern (adonis: p value < $1\times10^{-5}$, $R^2 = 0.27$; ANOSIM: p value < $1\times10^{-5}$, R = 0.48) (Figure 3.2.4A). Interestingly, MPD subjects show a low level of interpersonal GM variation (Bray-Curtis distances, mean ± sd, 0.42 ± 0.095), approximating that observed for the Hadza (0.36 ± 0.092) (Figure 3.2.4B).

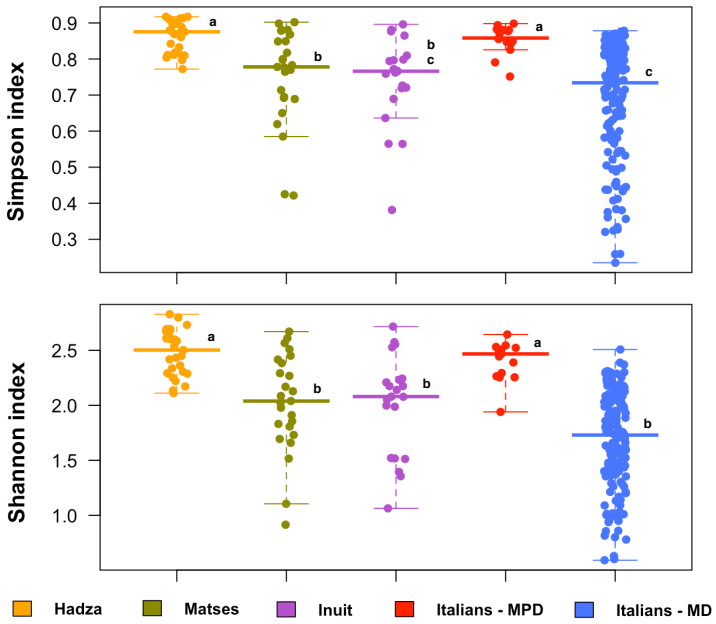


**Figure 3.2.3. The gut microbiome of Italian subjects following the modern Paleolithic diet shows intermediate biodiversity between Western urban and traditional populations.** Box and scatter plots showing the alpha diversity values, measured with Simpson and Shannon indices, for each study population. Different letters in the boxplots indicate significant differences (p value < 0.05, Wilcoxon test). MPD: Modern Paleolithic Diet; MD: Mediterranean Diet.

In order to identify the bacterial drivers with a statistically significant contribution (permutation correlation test, p value < 0.001) to the sample ordination, we superimposed the genus relative abundance on the PCoA plot (Figure 3.2.5). According to our data, the microorganisms characterizing the Italian cohort are *Bacteroides*, *Collinsella*, *Coprococcus* and *Blautia*, bacterial genera commonly found within Western healthy microbiomes (De Filippo *et al.*, 2010; Yatsunenko *et al.*, 2012; Schnorr *et al.*, 2014; Obregon-Tito *et al.*, 2015). The genera *Clostridium*, *Prevotella*, [*Prevotella*], *Catenibacterium* and *Oscillospira* have been found to be associated with Hadza and Matses, while *Sutterella* and *Parabacteroides* with Inuit. According to the literature, the separation due to geography seems to be less evident among the traditional populations, with Matses and Hadza sharing a high abundance of *Prevotella* (Schnorr *et al.*, 2014; Obregon-Tito *et al.*, 2015).

It is worth noting that the MPD microbiome shows several compositional differences with respect to the other cohorts including urban Italians and hunter-gatherers, which well match the peculiar macronutrient intake (Figure 3.2.1). In particular, compared with all other populations, except for the Inuit (as expected based on available dietary information), the MPD fecal profiles

are enriched in asaccharolytic genera, such as *Sutterella* and *Odoribacter* (Rajilic-Stojanovic *et al*., 2014), and in bile-loving microorganisms such as *Bilophila*, typically associated with animal protein and saturated fat consumption (Cotillard *et al*., 2013; David *et al*., 2014), as well as in *Akkermansia*, known to be associated with the consumption of unsaturated fat (Dao *et al*., 2016) ($p$ value $< 0.02$; Wilcoxon test). Although *Akkermansia* has recently been identified as potential next-generation probiotics, its role in inflammatory contexts is still controversial and requires further investigation (Caesar *et al*., 2015; Dao *et al*., 2016; Cani *et al*., 2017; Groves *et al*., 2018). Moreover, when compared to hunter-gatherer populations (whose subsistence, at least during sampling, was mainly based on tubers and other plant foods), the microbiome of MPD subjects shows increased relative abundance of the bile tolerant *Bacteroides*, *Collinsella* and *Dorea* ($p$ value $< 0.003$). *Bacteroides* is indeed typically associated with Western-type animal-based diets (David *et al*., 2014), the genus *Collinsella* is known to comprise bacterial species capable of deconjugating bile acids and positively correlated with plasma cholesterol levels (Lahti *et al*., 2013), and *Dorea* has recently been suggested to be involved in the production of the secondary bile acid, deoxycholic acid (Martin *et al*., 2018). In light of the known associations between changes in the bile acid pool, in particular with increased production of secondary bile acids, and increased risk of non-infectious bowel disease and colorectal cancer (Wirbel *et al*., 2019), the increased presence of bile-loving bacteria could constitute a red flag for human health, worthy of being further explored possibly in long-term studies.

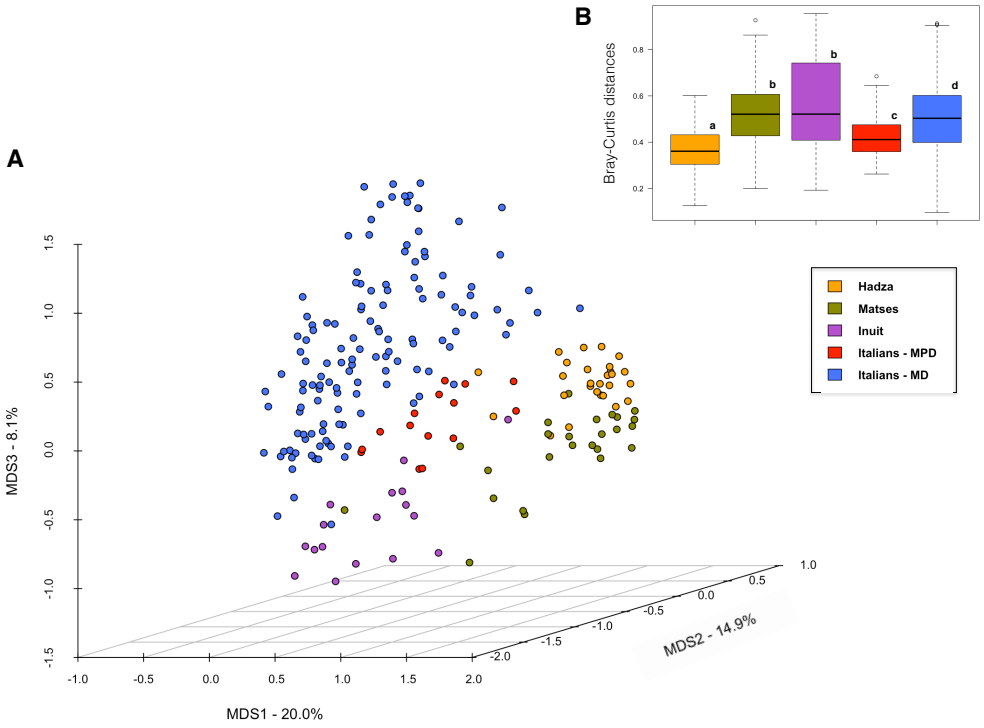


**Figure 3.2.4. Beta diversity of the fecal microbiome of Italian subjects following the modern Paleolithic diet compared with other Western urban populations and traditional communities.** (A) The PCoA plot shows the Bray-Curtis distances between the genus-level microbiota profiles of each study population, highlighting a significant segregation among groups (p value < $1\times10^{-5}$; permutation test with pseudo-*F* ratios). (B) Boxplots show the interpersonal variation, in terms of Bray-Curtis distances between the genus-level microbiota profiles, for each study group. Different letters in the boxplots indicate significant differences (p value < 0.05, Wilcoxon test). MPD: modern Paleolithic diet; MD: Mediterranean diet.

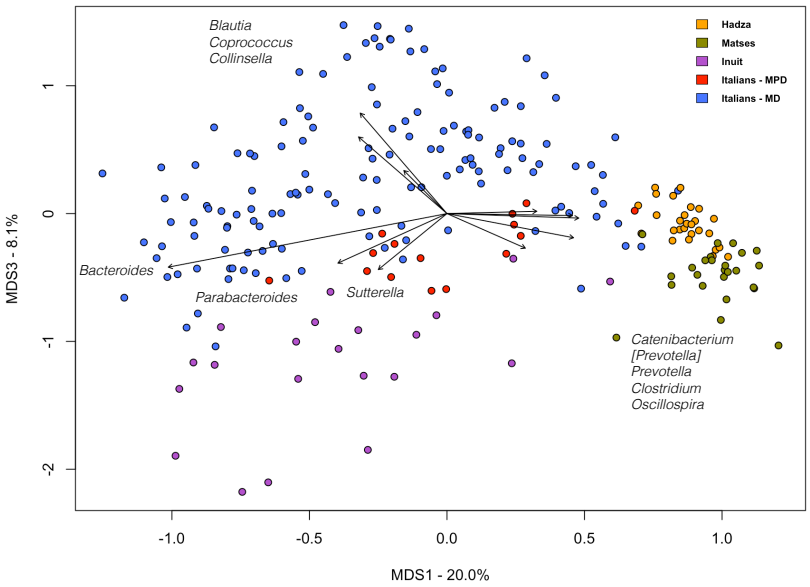


**Figure 3.2.5. Superimposition of the genus relative abundance on the PCoA plot.** Arrows represent the direction of significant correlations (p value < 0.001, permutation correlation test). A significant segregation among study populations was found (p value < $1x10^{-5}$, permutation test with pseudo-*F* ratios). MPD: Modern Paleolithic Diet; MD: Mediterranean Diet.

On the other hand, it should be noted that, compared to traditional populations, MPD profiles show greater proportions of the SCFA producers *Lachnospira* and *Coprococcus* (p value < 0.008). Furthermore, the levels of fiber-degrading SCFA producers, such as *Faecalibacterium*, *Ruminococcus*, *Lachnospira* and *Coprococcus*, are comparable between MPD subjects and other Italians, suggesting that even excluding grains and legumes, the high serves of fruit, vegetables, nuts and seeds in the MPD could ensure adequate supply of MACs to the GM.

We also evaluated the *Prevotella* ratio, i.e. the ratio of *Prevotella* to the sum of *Prevotella* and *Bacteroides* (Gorvitovskaia *et al*., 2016) (Figure 3.2.6). These genera are indeed recognized as biomarkers of diet and lifestyle, with *Bacteroides* typically associated with high-protein high-fat Western diets and *Prevotella* with carbohydrate/fiber-based diets typical of more agrarian societies (Gorvitovskaia *et al*., 2016; Smits *et al*., 2017). Although no detailed dietary information is available for traditional populations, Hadza and Matses diets are known to be heavily based on the consumption of highly fibrous tubers and vegetal foods (Schnorr *et al*., 2014; Girard *et al*., 2016). On the other hand, the fiber intake of MPD individuals (29.5 ± 20.5 g/day) does not exceed by far that reported for urban MD Italians (range, 10.4-21.0 g/day) (De Filippis *et al*., 2016). Consistent with this, a significantly lower *Prevotella* ratio was observed for MPD individuals as well as for other urban Italians compared to Hadza and Matses (p value < $6.6 \times 10^{-7}$).

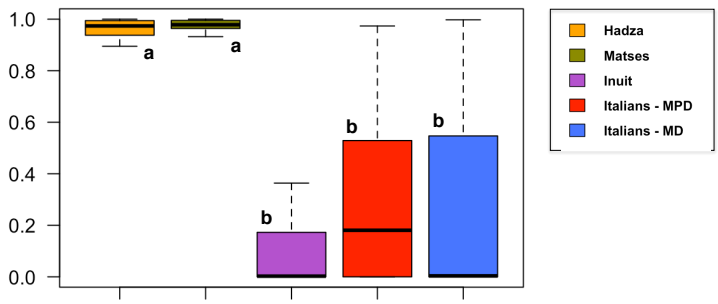


**Figure 3.2.6. *Prevotella-Bacteroides* ratio.** Different letters in the boxplots indicate significant differences (p value < 0.05, Wilcoxon test).

Taken together, these findings seem to suggest that even in extremely different geographic locations, with disparate cultural practices, environmental exposure, economic development and other lifestyle factors, the ancestral microbiome could be at least partly restored. Since the Italian subjects of our cohort share the provenance and all that it entails, including the lifestyle, it can be hypothesized that the MPD-associated bloom in GM diversity is accounted for by the peculiarities of the MPD compared to the MD. Though the two diets are similar in many respects – i.e. high intake of fruit, vegetables, fish and nuts, as well as low glycemic load – the MPD is in fact distinguished by: (i) consumption of MACs from plant foods but excluding grains and legumes; (ii) total exclusion of industrially processed products; (iii) higher intake of unsaturated fatty acids, especially MUFAs, from olive oil, nuts and meat; (iv) no consumption of foods containing refined sugars (Lindeberg *et al*., 2007; Jonsson *et al*., 2009; Whalen *et al*., 2016; Otten *et al*., 2018). It is, therefore, tempting to speculate that these MPD distinctive features may be sufficient to support the consolidation of a highly-diversified GM layout, thus counteracting the loss of GM biodiversity, typically observed in Western urban populations as compared to traditional communities (De Filippo *et al.*, 2010; Yatsunenko *et al.*, 2012; Schnorr *et al.*, 2014; Obregon-Tito *et al.*, 2015). However, at least two important considerations must be made in relation to biodiversity: i) simplifying the GM to a measure of biodiversity has obvious limitations as it does not reflect its compositional structure, including the complex ecological interactions existing among its members (Lozupone *et al*., 2012); ii) a reduced diversity is not necessarily detrimental to the host, especially when it is a consequence of the selective enrichment of health-promoting symbionts (Lozupone *et al*., 2012; Coyte *et al*., 2015; Johnson *et al*., 2016).

In conclusion, the present work has shed some light on the effects of the MPD on the GM structure and diversity in Western urban populations. Despite the limitations of this observational study (i.e. cross-sectional nature and small sample size), our findings suggest that the MPD could be a means to counteract the risk of losing the bacterial memory that has

accompanied our ancestors throughout human evolutionary history. The consumption of MACs from plant-based foods – but not grains – at the expense of refined sugars, and the minimization of the intake of processed foods, both hallmarks of the MPD, could indeed act synergistically in maintaining an eubiotic level of GM diversity. The high intake of MUFAs, as found in the MPD, suggests that these fatty acids could play a role in supporting high GM diversity, which is worthy of being further explored in larger cohorts. However, we cannot exclude that other genetic or lifestyle-related factors not considered in the present study are involved. On the other hand, we do not know how this high-diverse GM will behave over time in a context so different from that of our ancestors. Furthermore, the presence of some red flags, such as the overrepresentation of bile- and fat-loving microbes, requires attention for potential long-term health effects. Albeit several studies have suggested intriguing potential benefits of the MPD in obese and type 2 diabetes patients in the medium and long term (i.e. increase in insulin sensitivity, glycemic control and leptin levels, and lowering of total fat mass and triglyceride levels) (Jonsson *et al*., 2009; Mellberg *et al*., 2014; Otten *et al*., 2017), particular caution must be taken when following Paleolithic diets for a long time with percentages of macronutrients so far from nutritional recommendations, at least until more comprehensive longitudinal studies in larger cohorts, including randomized controlled trials, fully assess the MPD impact on host health.

Chapter 4 – **DYSBIOTIC VARIATIONS IN NON-COMMUNICABLE DISEASES: A FOCUS ON OBESITY AND FOOD ADDICTION**



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Obesity and associated metabolic diseases are linked to diet and gut microbiome (GM) in an intimate way (Sonnenburg and Backhed, 2016). Prevalence rates of obesity have increased dramatically in the past decades. In 2014, 1.9 billion adults worldwide were overweight and 600 million of them were obese (World Health Organization, 2016; http://www.who.int/mediacentre/factsheets/fs311/en).

Obesity, deriving from a positive energy balance that results from a surplus in ingested with respect to the expended energy, is considered a major risk factor for health, with important consequences on quality of life, life expectancy, and healthcare costs (Stevens *et al*., 2015). The GM is a pivotal emerging factor that can affect human metabolic homeostasis and promote the risk of metabolic complications connected to obesity. Even if there is a lack of consensus on the obese-type microbiome configuration, several taxonomic and functional alterations have been suggested to contribute to the pathogenesis of obesity in both humans and animal models (Turnbaugh *et al*., 2006; Candela *et al*., 2012; Ridaura *et al*., 2013). The altered microbial profile occurring in obese people is considered as an extreme deviation from the microbiota-host mutualism, resulting from the response to a high-fat high-sugar diet (Candela *et al*., 2012). The obesity-related GM is generally characterized by a low degree of biodiversity and enrichment in pathobiont bacteria, such as members of the family *Enterobacteriaceae*, as well as *Erysipelotrichaceae* and the sulphate reducer species *Bilophila wadsworthia* (Ridaura *et al*.,

2013; Turnbaugh *et al*., 2009b). This dysbiotic microbial structure is probably involved in the manifestation of obesity in a multifactorial way (Cox *et al*., 2013). Coherently with the energy harvest hypothesis (Turnbaugh *et al*., 2006), the GM of obese individuals possesses higher efficiency in energy extraction from the diet, providing an extra supply of calories to the host (Musso *et al*., 2010; Schwiertz *et al*., 2010; Patil *et al*., 2012). Furthermore, the concomitant overload of the intestinal microbial ecosystem with pro-inflammatory *Enterobacteriaceae* and sulphate-reducing bacteria may consolidate the obesity-associated inflammation and insulin resistance (Cani *et al*., 2009).

The prevalence of obesity is increasing worldwide, particularly, in children (Ahrens *et al*., 2011), and this has been closely associated with cardiovascular risk factors, such as hypertension, insulin resistance, and dyslipidemia, during adulthood (Ahrens *et al*., 2014). One of the common explanations for the increase in obesity over recent decades is the environment and, in particular, the availability of highly varied, palatable and fattening foods – which have been considered to be addictive (Schulte *et al*., 2015). While many individuals manage to maintain a healthy weight, obese individuals have been shown to have a preference for such energy-dense foods compared to normal-weight individuals (Drewnowski *et al*., 1992; Blundell *et al*., 1993). Although food addiction has not yet been recognized in the Diagnostic and Statistical Manual of Mental Disorders (DSM), similarities between some feeding and eating disorders and substance-use disorders (SUDs) have been acknowledged, including the experience of cravings, reduced control over intake, increased impulsivity and altered reward-sensitivity. Binge eating disorder (BED) and bulimia nervosa (BN) have been proposed as phenotypes that may reflect these similarities to the greatest extent (Meule *et al*., 2014; Shell *et al*., 2017). Focusing on the analogy in patterns of regional brain activation among substance abusers and obese individuals, Gearhardt and colleagues have underlined the potential mechanism through which addictive processes might be involved in the etiology of obesity (Gearhardt *et al*., 2011). In particular, the release of dopamine in mesolimbic regions has been

associated with the reward dependent on both food and drug use. A reduced activation of the same reward circuits suggests that obese patients, similarly to substance abusers, may consume excessive food to compensate a reward deficit. Most of the studies conducted so far have focused on the definition of obesity as a phenotype, both at metabolic and brain response level, while little efforts have been devoted to the identification of different brain activation patterns in response to food. Moreover, links among diet, microbiome structure, child health and food addiction, are still unclear.

First, to test the hypothesis that the composition and/or the diversity of the GM had an impact on the onset of obesity, we explored the fecal microbiota structure in 70 children in a prospective study, at a baseline survey and a follow-up after 4 years (IDEFICS/I.Family cohort). All children were normal weight at baseline, but 36 developed an excessive weight gain until follow-up. Second, we explored the GM structure and functional activity in 72 obese women, stratified according to BMI and food addiction severity (diagnosed through the Yale Food Addiction Scale, YFAS) and 28 normal-weight women (NeuroFAST cohort), and performed metagenomic and metatranscriptomic surveys in a subset of 45 women, with the specific aim of identifying potential microbial signatures of food addiction. Comprehensive data on lifestyle, such as dietary intake and physical activity, as well as medical history, anthropometry, measures of physiological, immunological, psychological parameters, and socioeconomic status were also collected.

Chapter 4 – **DYSBIOTIC VARIATIONS IN NON-COMMUNICABLE DISEASES: A FOCUS ON OBESITY AND FOOD ADDICTION**

*4.1 Brief introduction*

**4.2 Materials and Methods**

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**The IDEFICS/I.Family cohort**

The sample comprised children from the surveys of the 'Identification and Prevention of Dietary- and Lifestyle-Induced Health Effects in Children and Infants' (IDEFICS) cohort study and from the project 'Investigating the determinants of food choice, lifestyle and health in European children, adolescents and their parents' (I.Family). The IDEFICS study is a prospective cohort of 16,228 children aged 2-9 years from eight European countries (Belgium, Cyprus, Estonia, Germany, Hungary, Italy, Spain, and Sweden), from kindergartens and schools. The IDEFICS study consisted of one baseline survey (T0) performed from September 2007 to May 2008 and one follow-up survey (T1), which was conducted 2 years later (September 2009 to July 2010). The surveys provided information about dietary habits, physical activity, socio-demographic factors, clinical and physical examinations, and health outcomes. The follow-up project I.Family represents an extension of the IDEFICS study (T3) and was conducted in 2013-2014, in which children who participated in T0 and/or T1 were followed up for the third time complemented with information from their parents and siblings. Details of the design and methods of these surveys have been described elsewhere (Ahrens *et al*., 2017).

The present study is based on a subgroup of IDEFICS/I.Family children who provided stool samples. The first stool samples were collected in 2010 during the second survey of IDEFICS (T1) in five of the eight participating countries (Cyprus, Estonia, Germany, Hungary, and

Sweden). A second stool sample was collected in these countries during the follow-up at T3. None of the children took antibiotics in the 14 days before sample collection. All applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. Approval by the appropriate ethics committees (Cyprus National Bioethics Committee, Cyprus, 12/Jul/2007, No. EEBK/EM/2007/16 and 21/Feb/2013, No. EEBK/ETI/2012/33; Tallinn Medical Research Ethics Committee (TMREC), Estonia, 14/Jun/2007, No. 1093 and 17/Jan/2013, No. 128; Ethic Commission of the University of Bremen, Germany, 16/Jan/2007 and 11/Dec/2012; Medical Research Council, Hungary, 21/Jun/2007, 22-156/2007-1018EKU and 18/Dec/2012, 4536/2013/EKU; Regional Ethics Research Board in Gothenburg, Sweden, 30/Jul/2007, No. 264-07 and 10/Jan/2013, No. 927-12) was obtained by each of the centers doing the fieldwork. The parents or guardians as well as children from the age of 12 years gave their written informed consent and younger children expressed their oral consent for the examinations and data collection.

**The NeuroFAST cohort**

The NeuroFAST project aims to provide insights into food addiction in a cohort of obese women, by characterizing the GM compositional and functional layouts and integrating GM data with BMI and stress levels as well as metabolic, emotional-affective and hormonal abnormalities, and alterations in brain responses. Study population included women aged > 18 years in a premenopausal state, with BMI ranging between 24.9 and 40.0 $kg \cdot m^{-2}$. Exclusion criteria were the presence of acute/chronic diseases (e.g. central nervous system illness and cancer), neurological or psychiatric disease (e.g. anorexia and bulimia) and use of psychiatric medication, endogenous hypercortisolism or corticosteroid therapy, diabetes, drug or alcohol abuse, post-menopausal state, pregnancy or nursing. In order to exclude any country-related effects on the GM profile, all women were enrolled in Italy. Stool, blood, urine and saliva samples were collected from 72 obese women and 28 normal-weight women. Psychological,

psychiatric and nutritional questionnaires were administered to the subjects. Briefly, patients underwent a visit with a fully trained psychologist from the Department of Psychology (University of Bologna), aimed at investigating the previous or current presence of psychopathological disorders, use of psychotropic agents, and/or psychotherapies (through the Mini-International Neuropsychiatric Interview). Several validated tests were used to score perception of stress (Perceived Stress Scale), binge eating (Binge Eating Scale) and the presence of bulimic symptoms (Bulimic Investigatory Test). Nutritional questionnaires were administered to obtain information on the frequency of consumption (no. of portions per week) and the portion size (small, medium or large) of every category of food (Food Frequency Test), to measure cognitive and behavioral components of eating (The Three Factor Eating Questionnaire) and patient's confidence in her own capacity to control and/or change some aspects of food habits (Confidence Rating Questionnaire). Food addiction (FA) was assessed using the Yale Food Addiction Scale (YFAS), a questionnaire developed by Gearhardt *et al*. (2009) to operationalize FA, including 25 sub-items to address eating habits over the past 12 months. Following the authors' suggestions, women were stratified into a 'high' FA group (with 3 or more symptoms) or 'low' FA group (with 2 or fewer symptoms) (Gearhardt *et al*., 2011). The YFAS is extensively used as a reliable psychometric tool, showing internal consistency, as well as convergent and incremental validity. Elevated YFAS scores have been associated with higher neural activation in reward circuitry in response to food cues and reduced activation of inhibitory regions in response to food intake (Gearhardt *et al*., 2011). Finally, the GM of the enrolled cohort was profiled by 16S rRNA gene-based next-generation sequencing of fecal samples from 72 obese women and 28 normal-weight women. In order to deeply investigate the FA-related GM structural and functional layouts, a subset of fecal samples from the three subject groups (i.e. obese women with high and low FA, and healthy normal-weight women), underwent metagenomic and metatranscriptomic sequencing.

**Sample collection**

The IDEFICS and I.Family examinations of children, as well as the NeuroFAST examinations of obese women with high or low FA and normal-weight women, included the collection of biological samples (blood, urine, saliva, feces). Venous blood was drawn after an overnight fast using standardized procedures (Peplies *et al*., 2010) by all survey centers, and stored at -80°C. Feces from the IDEFICS/I.Family cohort were collected with the PSP Spin Stool DNA PLUS Kit (Stratec Molecular) at home. The stool collection kit included a collection tube with a DNA stabilizer, an illustrated description of how to collect the stool samples, a short questionnaire and a paper stool collector. The participant had to collect one spoon of the middle of the fecal sample and to mix the sample by shaking.

Screw-top containers were used to collect one spoon of stool sample from the NeuroFAST cohort. Venous blood, urine, and saliva specimens were also collected for each NeuroFast woman. All samples were stored at -20°C on the day of collection and then transferred to -80°C upon arrival in the laboratory.

**Collection of clinical, behavioral, and nutritional data**

Examinations of the enrolled subjects included anthropometric data (i.e. body weight, height, BMI, waist and hip circumferences, and waist-to-hip ratio), systolic and diastolic blood pressure, accelerometry, genetic data from saliva, and physiological markers in blood and urine. Dietary intake and behavior were measured in detail using a validated semi-quantitative food frequency questionnaire (FFQ) (Lanfer *et al*., 2011; Huybrechts *et al*., 2011) and a self-administered computer-assisted 24-h dietary recall, which is linked to a tailor-made European database of food composition tables (Hebestreit *et al*., 2014; Heberstreit *et al*., 2017) as described below. Data from dual energy x-ray absorptiometric (DXA) scan, the Three-Factor Eating Questionnaire (TFEQ; Karlsson *et al*., 2000) and YFAS (Gearhardt *et al*., 2011) questionnaire were also collected for each NeuroFAST woman, along with information about

personal/familiar anamnesis, menstrual history and pregnancies, body weight curve (recall of body weight values since the age of 18 to the present, in order to identify the occurrence of 'stress-related weight gain'), and prior and current medications.

**Microbial DNA extraction and sequencing**

Total microbial DNA was extracted from fecal samples by the repeated bead-beating plus column method (Yu and Morrison, 2004) with some additional steps as reported by Barone *et al*. (2019). The V3-V4 hypervariable region of the 16S rRNA gene was amplified using the 341F and 805R primers with added Illumina adapter overhang sequences as previously reported (Barone *et al*., 2019). Indexed libraries were prepared by limited-cycle PCR using Nextera technology and the final library was diluted to 6 pM with 20% PhiX control. Sequencing was performed on Illumina MiSeq platform using a 2×300 bp paired-end protocol, according to the manufacturer's instructions.

Metagenomic DNA libraries were prepared using the QIAseq FX DNA Library Kit, following the manufacturer's instructions (Qiagen). Briefly, for each sample, 100 ng of DNA were fragmented to 450-bp size, end-repaired and A-tailed using FX Enzyme Mix with the following thermal cycle: 4°C for 1 min, 32°C for 8 min and 65°C for 30 min. Samples were then incubated at 20°C for 15 min in the presence of DNA ligase and Illumina adapter barcodes for indexing and adapter ligation. After two purification steps with Agencourt AMPure XP magnetic beads (Beckman Coulter), 10-cycle PCR amplification and a further step of purification as above, samples were pooled at equimolar concentration of 4 nM. Sequencing was performed on Illumina NextSeq platform using a 2×150 bp paired-end protocol, following the manufacturer's instructions (Illumina).

RNA extraction was carried out using the RNeasy PowerMicrobiome kit (Qiagen), according to the manufacturer's instructions. Minor adjustments were made for the homogenization step, performed using a FastPrep instrument (MP Biomedicals), with one treatment at 5.5

movements/sec for 1 min. For each sample, rRNA was depleted using the RiboMinus Transcriptome Isolation Kit for bacteria (Thermo Fisher Scientific). RNA libraries were prepared using the QIAseq Stranded Total RNA Lib Kit (Qiagen), according to the manufacturer's instructions and pooled at equimolar concentration of 4 nM. Sequencing was performed on Illumina NextSeq 500 platform using a 2x150 bp paired-end protocol, following the manufacturer's instructions (Illumina).

**Bioinformatics and biostatistics**

Paired-end reads from 16S rRNA gene-based sequencing were processed combining PANDAseq (Masella *et al*., 2012) and QIIME (Caporaso *et al*., 2010). High-quality sequences were clustered into OTUs at 97% sequence similarity by UCLUST (Edgar *et al*., 2010). Taxonomy was assigned with the RDP classifier against the Greengenes database (May 2013 release). Chimeric OTUs were identified using ChimeraSlayer (Haas *et al*., 2011) and then filtered out. All singleton OTUs were discarded. Alpha diversity was evaluated using three different metrics: Shannon, Phylogenetic Diversity (PD) whole tree, and number of observed OTUs. Beta diversity was estimated by computing weighted and unweighted UniFrac distances, which were used as input for Principal Coordinates Analysis (PCoA). PCoA, heatmap, and bar plots were built using the R packages made4 (Culhane *et al.,* 2005) and vegan (http://www.cran.r-project.org/package=vegan). Microbiome steady states were identified through hierarchical Ward linkage clustering based on the Spearman correlation coefficients of the proportion of OTUs, filtered for OTU subject prevalence of at least 20%. It was then verified that each cluster showed significant correlations between samples within the group (multiple testing using the Benjamini-Hochberg method) and that the clusters were statistically significantly different from each other (permutational MANOVA using the Spearman distance matrix as input, function adonis of the vegan package in R). The R packages stats and vegan were used for statistical analysis. In particular, to compare the macronutrient intake and the α-

diversity of the GM structures among different groups for, we used the Kruskal-Wallis test was used. The significance of data separation in the PCoA was assessed by a permutation test with pseudo-*F* ratios (function adonis in vegan). Cluster separation in hierarchical clustering analysis was tested using Fisher's exact test. Significant differences in bacterial relative abundance at different phylogenetic levels among groups were assessed by Wilcoxon or Kruskal-Wallis test was used. P values were corrected for multiple comparisons using the Benjamini-Hochberg method when appropriate. FDR $\leq 0.05$ was considered as statistically significant.

Functional annotation of sequences from shotgun metagenomics sequencing was conducted as previously described by Rampelli and colleagues (2015). In brief, shotgun reads were first filtered by quality and human sequences by means of the human sequence removal pipeline and the WGS read processing procedure of the HMP Consortium (Turnbaugh *et al*., 2007). The obtained reads were taxonomically characterized at species level by MetaPhlAn2 (Truong *et al*., 2015) and assigned for functionality at different levels of the KEGG database (Wixon *et al*., 2000) using MetaCV with default parameters (Liu *et al*., 2013).

Metatranscriptomic reads passed through the KeadData quality control pipeline to remove low-quality bases, reads of human origin and reads encoding for rRNA. Metatrascriptomes were functionally profiled using HUMAnN2 (Abubucker *et al*., 2012) to quantify expression levels of genes and pathways. Reads were aligned to sample-specific pangenomes, i.e. all gene families detected in a given sample, using Bowtie and the UniRef90, MinPath and KEGG databases. Hits were counted per gene family and normalized for length and alignment quality score. HUMAnN2 RNA-level outputs (transcript abundance) were then normalized by the corresponding DNA-level outputs (from metagenomic results) to quantify microbial expression regardless of gene copy number. To investigate functional differences among microbiota configurations associated or not with food addiction, the frequency of the reads mapped to the KEGG Orthology database was assessed by applying the MetaCV pipeline to the metatranscriptomic sequences (Liu *et al*., 2013).

**Correlation analysis of clinical data and gut microbiota**

Correlations between microbiota composition and host metadata, including inflammatory markers and other health parameters were analyzed using quantile (median) regression tests, adjusted for age. Median regression is less influenced by outliers than the classical linear regression because it gives less relevance to extreme values. As for the IDEFICS/I.Family cohort, the potential impact of gender and maturation stage according to Tanner classification (Duke *et al*., 1980) – whose information was available only at T3 for children who were 8 years old or older, i.e. 68 out of 70 – on the microbiota structure was also evaluated. The analysis was carried out by using the R package quantreg, as already performed by Claesson *et al*. (2012).

**Analysis of nutritional data**

Dietary data was collected through a semi-quantitative food frequency questionnaire (FFQ), weighted by 7-day consumption frequencies. Regarding the IDEFICS/I.Family cohort, 46 items were in common between FFQs at T1 and T3. Additional four items were obtained from questions about the type of milk and yoghurt consumed (skimmed or full-fat). For all FFQs the lowest frequency option was 'never or less than once a week', for foods with the highest frequency, the option was '4 or more times per day' (Lanfer *et al*., 2011; Huybrechts *et al*., 2011). As for the NeuroFAST cohort, women were asked to compile 24-h dietary recalls, to retrieve more details on the composition of their diet (Heberstreit *et al*., 2014, 2017). The dietary questionnaire was designed *ad hoc* for the study, to complete the information collected by dietary interview. After considering several methodological approaches to quantify food frequency, we elected to convert the frequency of consumption assessed with the FFQ to a continuous scale of daily consumption (e.g. if the food was eaten 2 times per day, then the daily consumption was 2). When the frequency was reported as a range (e.g. eaten 1-3 times per week), the mean of the range (e.g. 2) was used to calculate the daily consumption.

The superimposition of the food frequencies on the microbiota PCoA space was conducted using the envfit function of the vegan package of R. A corrected p value ≤ 0.01 was considered as statistically significant. Macronutrient data were taken from dietary recalls, in particular in I.Family (T3), dietary intake of the previous 24 h was assessed using the validated web-based SACANA (Self-Administered Children, Adolescents and Adult Nutrition Assessment) 24-h dietary recall tool, which is based on the validated SACINA (Self-Administered Children, Infants and Adult Nutrition Assessment) offline version (Hebestreit *et al*., 2014) used in the IDEFICS study (T1). Children reported their diet and entered the type and amount (g) of all drinks and foods consumed during the previous day. While in SACINA all information was reported by the parents, in SACANA, children reported for themselves with the help of their parents (Livingstone *et al*., 2000) or from a dietician or trained study nurse during the survey examinations.

**Co-abundance analysis**

Co-abundance groups (CAGs) were identified as previously described (Claesson *et al*., 2012). In brief, associations among bacterial genera, present in at least two samples with relative abundance >0.1%, were evaluated by the Kendall correlation test, displayed using hierarchical Ward clustering with the Spearman correlation-based distance metrics and utilized to determine co-abundant groups of bacterial genera. Significant associations were controlled for multiple testing using the q-value method (FDR ≤ 0.05) (Dabney *et al*., 2013). Permutational MANOVA was used to determine whether the CAGs were significantly different from each other. Wiggum plot networks were created using the Cytoscape software (http://www.cytoscape.org/), as previously reported (Claesson *et al*., 2012). The circle size represents the bacterial abundance and connections between nodes represent positive and significant Kendall correlations among genera (FDR ≤ 0.05).

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**The IDEFICS/I.Family cohort**

**Microbiota structure and healthy growth**

Within the present work, the fecal microbial composition in 70 children at two time points (T1, T3), within a 4-year window, was assessed to investigate links between the gut microbiota and obesity, health, diet, and other lifestyle factors. Children were stratified by timing and weight status: at T1, all children were normal weight of which 34 are referred to as T1_N who remained normal weight (mean ± sd, age: 7 ± 2 years; BMI: 16 ± 1 $kg \cdot m^{-2}$), and 36 as T1_O (age: 8 ± 2; BMI: 16 ± 2), who gained excessive weight; accordingly, at T3, 34 subjects had maintained their normal weight (T3_N; age: 11 ± 2; BMI: 17 ± 2), while 36 had gained excessive weight (T3_O; age: 12 ± 2; BMI: 20 ± 3).

The sequencing yielded a total of 7.9 million sequence reads from 16S rRNA gene V3-V4 amplicons, with an average of 56,485 (± 22,321, sd) paired-end reads per sample, for 20,360 OTUs grouped at 97% of sequence identity. When examining OTUs abundance, we identified four subject clusters, one of which (C3) included the majority of obese subjects, before and after they had developed obesity. For 18 out of the 70 children, the most relevant variable that drove the separation was the sample origin, with samples T1 and T3 from the same individual clustering together in the dendrogram (p value = 0.0001, Fisher's test) (data not showed).

To identify trends in the GM, we established co-abundance associations of genera, and then clustered correlated bacterial taxa into four co-abundance groups (CAGs), describing the microbiota structures found across the whole dataset. The dominant (i.e. the most abundant) genera in these CAGs were *Bacteroides* (green), *Prevotella* (yellow), *Dorea* (violet), and *Bifidobacterium* (blue). The CAG relationships are termed Wiggum plots, where genus abundance is represented as a disc proportional to normalized over-abundance (Figure 4.1). The four subject divisions, as identified by OTU clustering, were superimposed on the unweighted (Figure 4.1), allowing defining four clusters, C1-C4. Within a spectrum of microbiota structures, each of these clusters constitutes a steady state, representing groups of individuals who have a significantly different microbiota layout from each other, as defined by the permutation multivariate analysis of variance (MANOVA) test on UniFrac data ($p$ value $< 0.001$). Wiggum plots for the GM for each of the 4 groups were constructed (Figure 4.1). The microbiota variation from the groups dominated by normal-weight children (C1/C2) to the groups dominated by obese children (C3/C4) was accompanied by distinctive CAG dominance, specifically by abundances of *Prevotella* CAG (C1) and *Dorea* and *Bacteroides* CAGs (C4). In particular, steady states C3 and C4 were more heterogeneous, with the first showing the concomitant presence of all the four CAGs (*Prevotella*, *Bacteroides*, *Bifidobacterium*, and *Dorea*), and the second lacking only the *Bifidobacterium* CAG. Significant associations between several health/inflammation parameters and the major axes from unweighted UniFrac PCoA analysis are shown in Table 4.1. In particular, when considering the whole cohort, a shift of the microbiota structure towards positive values of the PCo1 axis was associated with inflammation, i.e. increased serum levels of C-reactive protein (CRP) and IL-6, as well as with C3 and C4. Interestingly, other inflammatory markers, such as IL-15, tumor necrosis factor α (TNFα), interferon gamma-induced protein 10 (IP-10), IL- 6, and IL-8, correlated only with the microbiota profiles from children developing obesity. As expected, there was minimal variability amongst normal-weight subjects. It should be noted that the education level score

and physical activity score (i.e. time spent in moderate to vigorous physical activity, MVPA) were also associated with the microbiota structure, but in an independent way with respect to inflammatory parameters and the lean/obese phenotype. Furthermore, the microbial biodiversity was inversely associated with the inflammatory status. Indeed, we observed a gradual change of the level of biodiversity along PCo1, from the highest level in the samples belonging to the C2 cluster to the lowest values in the C4 microbiomes (p value < 0.000001, Kruskal-Wallis test). On the other hand, when comparing the biodiversity of the child microbiota among the original groupings (T1_N, T3_N, T1_O, T3_O), a significant difference was detected only by using the Shannon index. In particular, the T1_N microbiome displayed a higher biodiversity than T1_O samples (p value < 0.01, Wilcoxon test), but this evidence was not confirmed with the other metrics. Moreover, samples from pre-obese (T1_O) and obese (T3_O) children were largely more prevalent in the low-diversity clusters (C3 and C4). It should be noted that both the unweighted and weighted UniFrac PCoA space was not correlated with either the child's age (p value = 0.7 for unweighted UniFrac; p value = 0.6 for weighted UniFrac, permutational correlation test), gender (p value = 0.2 for unweighted UniFrac; p value = 0.4 for weighted UniFrac; permutational MANOVA) or maturation stage according to Tanner classification (Duke *et al*., 1980) (p value = 0.2 and 0.6 for unweighted and weighted UniFrac, respectively), meaning that the associations described above were irrespective of these variables.

Taken together, these results indicate that the low-diversity GM configurations C3 and C4 might represent obesogenic GM layouts, predisposing children to metabolic inflammation and obesity. GM may exist under a number of configurations, which are associated with host metabolic and immunological factors and, in the context of other individual lifestyle and genetic variables, may be involved (or not) in the development of the multifactorial obese phenotype.

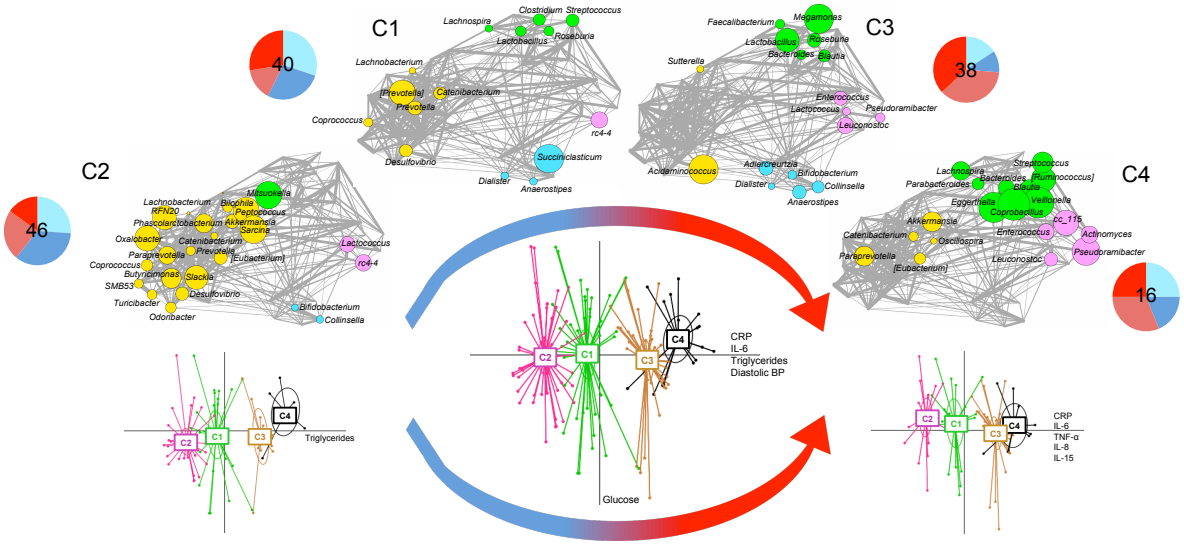


**Figure 4.1. Variation of the gut microbiota structure across normal weight and obese children is mirrored by changes in health indices.** The PCoA plots show four significantly different groups of subjects (C1–C4, p value < 0.001), as defined by unweighted UniFrac microbiota analysis of normal weight children (T1_N, T3_N; left), the whole cohort (center) and obese children (T1_O, T3_O; right). At the top, Wiggum plots corresponding to the four groups from the whole cohort analysis, in which disc sizes indicate genus over-abundance compared to the average relative abundance in the whole cohort. Pie charts show the proportion and number of subjects per group (pink, T1 normal weight children that will develop obesity (T1_O); red, T3 obese children (T3_O); cyan, T1 normal weight children (T1_N); light blue, T3 normal weight children (T3_N)). Curved arrows indicate a transition from health (blue) to an inflammatory state (red), as defined by the increase in several inflammatory markers (CRP, IL-6, IL-8, IL-15, TNFα), as well as in triglycerides and diastolic blood pressure. Please see also Table 4.1.

| Parameter | PCo1 | | | PCo2 | | | PCo3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | RC range | RC sd | *p* | RC range | RC sd | *p* | RC range | RC sd | *p* |
| **(a) Unweighted UniFrac PCoA for all subjects** | | | | | | | | | |
| ISCED (education level score) | −0.95934 | −0.15989 | 0.3 | 0.369546 | 0.10869 | 0.04 | 0.350038 | 0.13463 | 0.3 |
| Evenson MVPA (moderate to vigorous physical activity) score | 0.64632 | 0.10772 | 0.5 | −0.415378 | −0.12217 | 0.05 | 0.387634 | 0.14909 | 0.2 |
| CRP | 3.10902 | 0.51817 | 0.0003 | 0.000374 | 0.00011 | 0.9 | −0.05109 | −0.01965 | 0.9 |
| Triglyceride | 1.83048 | 0.30508 | 0.04 | 0.071706 | 0.02109 | 0.7 | 0.149942 | 0.05767 | 0.6 |
| IL-6 | 2.34798 | 0.39133 | 0.03 | 0.082212 | 0.02418 | 0.8 | −0.284232 | −0.10932 | 0.5 |
| Diastolic blood pressure | 2.24544 | 0.37424 | 0.04 | 0.32045 | 0.09425 | 0.3 | 0.33124 | 0.1274 | 0.4 |
| Glucose | 0.2517 | 0.04195 | 0.8 | 0.131818 | 0.03877 | 0.7 | −0.951886 | −0.36611 | 0.02 |
| **(b) Unweighted UniFrac PCoA for developing obesity-only subjects (T1_O, T3_O)** | | | | | | | | | |
| Evenson MVPA (moderate to vigorous physical activity) score | −0.23454 | −0.03909 | 0.8 | −0.440368 | −0.12952 | 0.04 | 0.076778 | 0.02953 | 0.8 |
| CRP | 3.18672 | 0.53112 | 0.007 | −0.097478 | −0.02867 | 0.7 | −0.327028 | −0.12578 | 0.2 |
| TNF-α | 2.78556 | 0.46426 | 0.04 | −0.09656 | −0.0284 | 0.8 | −0.691912 | −0.26612 | 0.3 |
| IP-10 | 2.99568 | 0.49928 | 0.05 | 0.250036 | 0.07354 | 0.4 | −0.479206 | −0.18431 | 0.4 |
| IL-8 | 3.16782 | 0.52797 | 0.04 | −0.031246 | -0.00919 | 0.9 | −0.313976 | −0.12076 | 0.6 |
| IL-6 | 3.00882 | 0.50147 | 0.04 | 0.181254 | 0.05331 | 0.6 | −0.701142 | −0.26967 | 0.3 |
| IL-15 | 2.92746 | 0.48791 | 0.04 | 0.16286 | 0.0479 | 0.7 | −0.434798 | −0.16723 | 0.6 |
| **(c) Unweighted UniFrac PCoA for normal weight-only subjects (T1_N, T3_N)** | | | | | | | | | |
| Weight | −0.81234 | −0.13539 | 0.4 | −0.646952 | −0.19028 | 0.05 | −0.317382 | −0.12207 | 0.5 |
| Triglyceride | 1.94376 | 0.32396 | 0.04 | 0.462876 | 0.13614 | 0.2 | 0.41379 | 0.15915 | 0.4 |

**Table 4.1. Associations between clinical variables and microbiota composition.** Quantile (median) regression tests of associations between metadata measurements and microbiota composition as measured by unweighted UniFrac PCoA across all groups. RC range, regression coefficients scaled to the full variation along each PCoA axis, thus indicating direction and magnitude of the association; RC sd, regression coefficients scaled to one standard deviation; p, quantile regression p value.

**Impact of diet on the gut microbiota**

To identify the food types with the most significant contribution (p value < 0.05, permutational correlation test) to the microbiota ordination, we superimposed the food data from FFQs on the unweighted UniFrac PCoA plot of Figure 4.1 (Figure 4.2a). Remarkably, a higher consumption of milk, fish, seeds, and whole meal bread was associated with the GM configurations C1 and C2. On the other hand, the microbiota configurations C3 and C4 were associated with a higher consumption of dairy products, pizza, sausages, and sweetened drinks. In line with the available literature on the diet as a major driver of the microbiota structure (Zmora *et al.*, 2018), differences in food consumption may contribute to differences in microbiota diversity between groups. In particular, by focusing on macronutrients, we found that the most discriminant category was carbohydrate, whose consumption increased in a gradual manner along the first axis contrarily to the GM biodiversity (Figure 4.2b). In light of the fact that diet, in terms of excess energy intake, is a major cause of obesity (Sonnenburg and Backhed 2016), it is important to note that the microbiota configurations (C1-C4) were independent of the total daily caloric intake, and that an increase of caloric intake was observed at T3, compared to the first time point, in accordance with the growth of children.

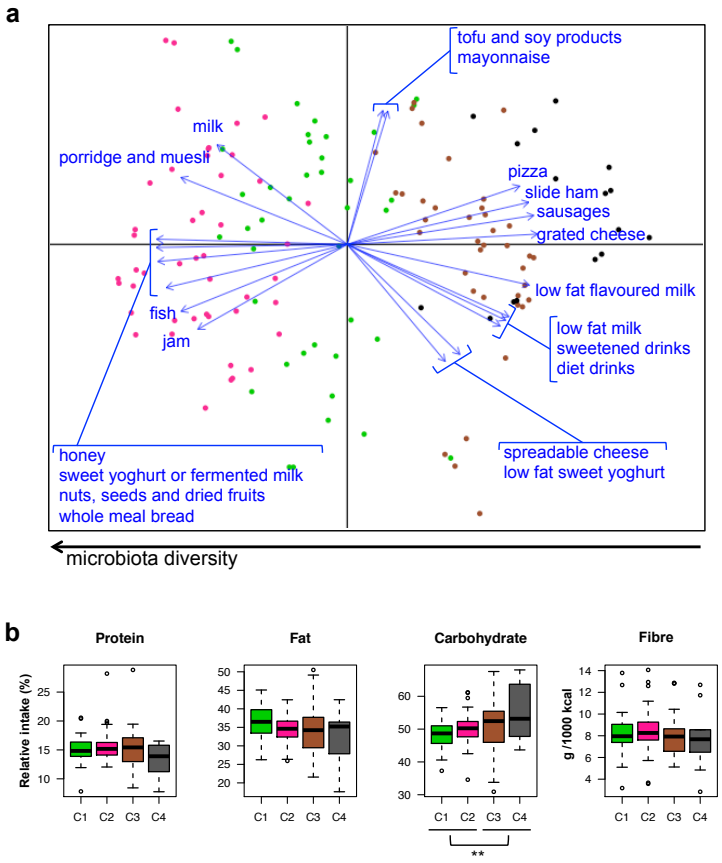


**Figure 4.2. Dietary contribution to the microbiota ordination.** (a) PCoA based on unweighted UniFrac distances of the fecal microbiota. The biplot of the average food coordinates weighted by frequency of consumption per sample was superimposed on the PCoA plot to identify the foods contributing to the ordination space (blue arrows). Only the food categories showing a highly significant correlation with the sample separation (p value < 0.005, permutational correlation test) were displayed. Samples are colored by subject group (C1-C4), as in Figure 4.1. The black arrow at the bottom indicates the direction of the microbiota diversity gradient along PCo1. (b) Summary of the macronutrient intake, expressed as a percentage of kilocalories consumed per day, and fiber consumption, as grams of fiber intake per 1000 kilocalories consumed. **: p value < 0.05, Wilcoxon test.

**Microbiota, diet, and physical activity relation to obesity**

FFQ data were further explored in a Correspondence Analysis, where the first axis, describing over 9% of the dataset variance, contained most of the discriminating food types identified in the previous correlation analysis of FFQ data on the microbiota PCoA, such as milk, pizza, and sweetened drinks. Application of Ward linkage clustering and Euclidean distance metrics to this axis allowed identifying five dietary groups (p value < 0.001, Fisher's test): D1 ('low protein/low carbohydrate'), D2 ('high carbohydrate/high fat'), D3 ('high carbohydrate/high fiber'), D4 ('low protein/low fat'), and D5 ('high protein/high fat') (Figure 4.3a). Furthermore, the Healthy Food Diversity (HFD) index, an index used to measure dietary diversity, based on the evidence that a diverse diet promotes health (Drescher *et al*., 2007) was calculated. By analysing samples by dietary group rather than microbiota group, it was found that the least diversified diet was D2, while D1 and D3 showed the highest diversity, reflecting a high potential to promote health (p value = 0.0002, Kruskal-Wallis test; Figure 4.3b).

Focusing on the sample distribution in a longitudinal way, the T1 and T3 samples fell in the same dietary group for 16 out of 70 children. Twelve children changed their diet group from D1 to D3 or *vice versa*, thus maintaining a high HFD index. Only four children (2 T1_O/ T3_O and 2 T1_N/T3_N) modified their dietary group in the worst way, i.e. from diets with the highest HFD index (D1, D3) to the least diverse diet, D2. By matching the stratifications of subjects in dietary and microbiota groups, redundant combinations associated with the obese phenotype were sought. In particular, in the light of the obtained results, the combinations D2 diet and C3/C4 microbiota were more prevalent in pre-obese (T1_O) and obese children (T3_O) compared to the other subjects, and were exclusively associated with a disease-promoting and inflammation status. Seven obese children out of 36 were classified with the combination D2-C3/C4, whereas none of T1_N/T3_N children possessed this configuration (p value = 0.0006, Fisher's exact test).

It is important to note that the only T1_N child who fell in the D2 group (C2 microbiota group) showed a high MVPA score (time spent in moderate to vigorous physical activity higher than 75% of T1 subjects), suggesting a protective role of physical activity in children consuming a diet associated with a low Healthy Food Diversity (HFD) index. This was also confirmed for the combination D5 diet and C3/C4 microbiota, in which fell seven obese children and only one T1_N child with high MVPA score (p value = 0.008). However, it should be pointed out that the combination D2 diet and C3/C4 microbiome steady state was exclusively observed in obese children, while seven of the eight children that showed the combination D5 and C3/C4 were obese. It is thus tempting to speculate that the differences in food intake may contribute to the observed microbiota differences. These findings stress that obesity is a complex mosaic, in which several endogenous and exogenous factors, including host genetics, contribute to health decline. Interestingly, when looking at T1 samples in a prospective manner, these hypotheses were confirmed by detecting D2/D5-C3/C4 configurations exclusively in normal-weight children who showed excessive weight gain at T3. Our results support the relevance of the predictive potential of the microbiome-host-diet configuration, even if the model clearly needs to be implemented with more subjects, sampling points and other omics data to increase statistical power.

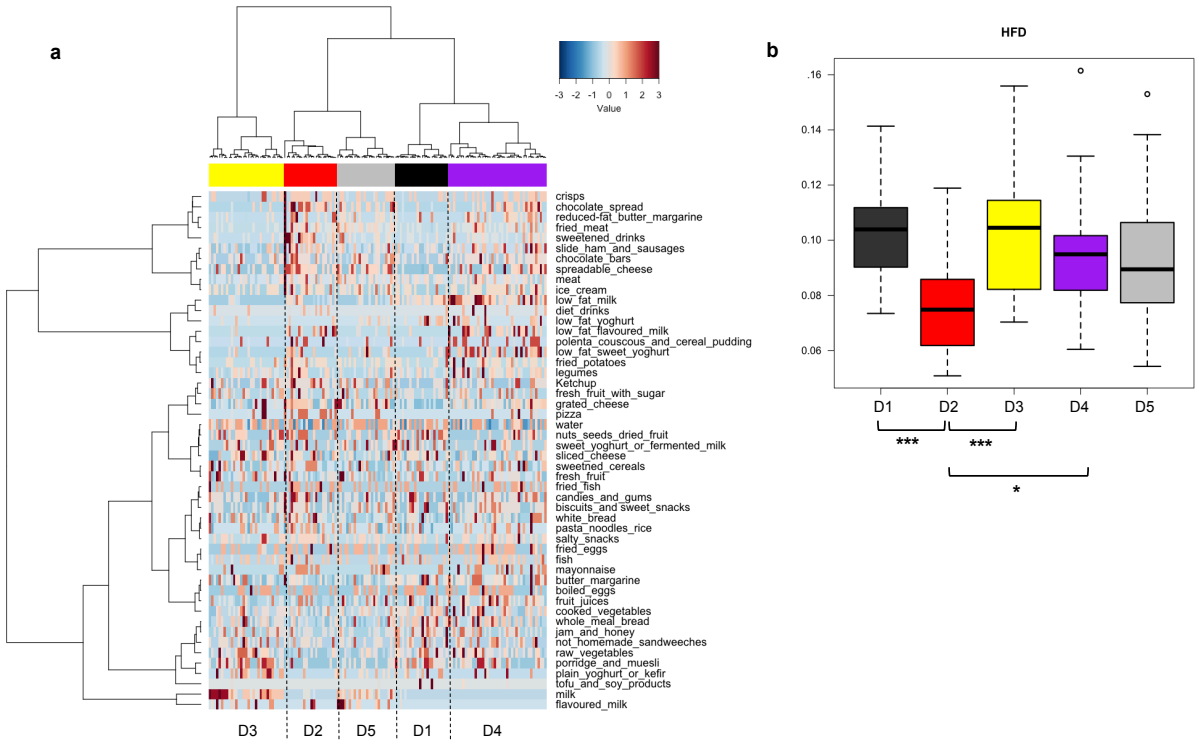


**Figure 4.3. Dietary patterns discriminate children for the Healthy Food Diversity index.** (a) Five dietary groups (D1–D5) revealed through Ward linkage clustering using Euclidean distances applied to the first eigenvector in a Correspondence Analysis of data from Food Frequency Questionnaires. (b) Comparison of the Healthy Food Diversity (HFD) index (Drescher *et al*., 2007) across the five dietary groups identified in a). *: p value < 0.01; ***: p value < 0.0001, Wilcoxon test.

**Microbiota signatures of obesity**

UniFrac PCoA analysis showed weak but significant separation between subjects with (T3_O) or without obesity (T1_N, T3_N, T1_O), according to both unweighted (p value = 0.02, permutation test with pseudo-*F* ratios; Figure 4.4a) and weighted (p value = 0.05; Figure 4.4b) distance metrics. Family-level microbiota assignment highlighted a readjustment within the phylum Bacteroidetes, with a higher proportion of *Bacteroidaceae* and a lower proportion of *Prevotellaceae* in obese children when compared to the normal-weight counterparts (Figure 4.4c). In addition, obese children showed a higher contribution of the genus *Lachnospira* compared to normal-weight children at the same time point (T3_N). When looking at T1_O/T3_O children in a longitudinal way, an increase in the relative abundance of Proteobacteria emerged, as well as a decrease in the proportions of the families *Clostridiaceae* and *Ruminococcaceae* after the onset of obesity. On the other hand, when focusing on normal weight children, only a sensible reduction in Proteobacteria at the second time point (T3_N) compared to the baseline was detected. A complete summary of the significant differences in the GM between groups is reported in Table 4.2. It is important to note that differences between obese (T3_O) and non-obese (T1_O and T3_N) children involved major microbiota components, whereas differences between non-obese children (T1_N, T3_N, and T1_O) involved only minor components, proving that obesity is associated with certain GM profiles, although alone cannot be used as unique predictive tool.

Consistent with the GM arrangements reported in other studies, we observed higher levels of *Bilophila* in children that consumed more milk (Turnbaugh *et al*., 2012), and higher contribution of *Prevotella* in children with higher intake of whole meal bread (Vitaglione *et al*., 2015). On the other hand, we found more *Bacteroides* and *Oscillospira* in children who ate more ham and sausages, as already described in adults following an animal-based, low-fiber diet (David *et al*., 2014). As demonstrated by Zhernakova and colleagues (2016), we also found evidence of an inverse correlation between microbiota diversity and consumption of sugar-

sweetened drinks. It is worth noting that a high diversity in the GM ecosystem, together with high levels of SCFA production were reported in rural children of Burkina Faso, whose diet is rich in complex carbohydrates and fiber (De Filippo *et al*., 2010). Similarly, a high-diverse GM, with enrichment of genes involved in the metabolism of complex polysaccharides, was found in the Hadza, a hunter-gatherer population following a heavily plant-based diet (Rampelli *et al*., 2015). In line with these findings, our results showed that the microbiota diversity was higher in children who ate more foods containing oligosaccharides, such as honey and whole milk (Zivkovic *et al*., 2011), with the latter being also a source of fat-soluble vitamin D, whose deficiency is associated with obesity in children and adolescents (Plesner *et al*., 2018). The microbiota diversity was also higher in children with high consumption of complex polysaccharides, such as whole meal bread, nuts, and seeds. The link between diet and microbiota also clearly involves human physiology. Indeed, it has been demonstrated that the dietary fat increases the endotoxins level in the blood (Erridge *et al*., 2007), and that circulating endotoxin levels are associated with elevated TNFα, IL-8, and IL-6 concentrations (Ghanim *et al*., 2009; Boulangé *et al*., 2016). In agreement with these data, we found higher plasmatic levels of IL-6, IL-8, and TNFα associated with an overabundance of gram-negative bacteria, such as *Veillonella*, *Akkermansia*, *Bacteroides*, and *Parabacteroides*, in the C3/C4 configurations. On the other hand, we found that the consumption of fish was directly connected to a microbiota configuration with low inflammatory grade, as it has been reported for lard-consuming mice transplanted with the microbiota of fish oil-consuming mice (Caesar *et al*., 2015). Importantly, the health-microbiota associations were statistically significant even when the model was adjusted for age, and robust to gender and maturation stage according to Tanner classification (Duke *et al*., 1980).

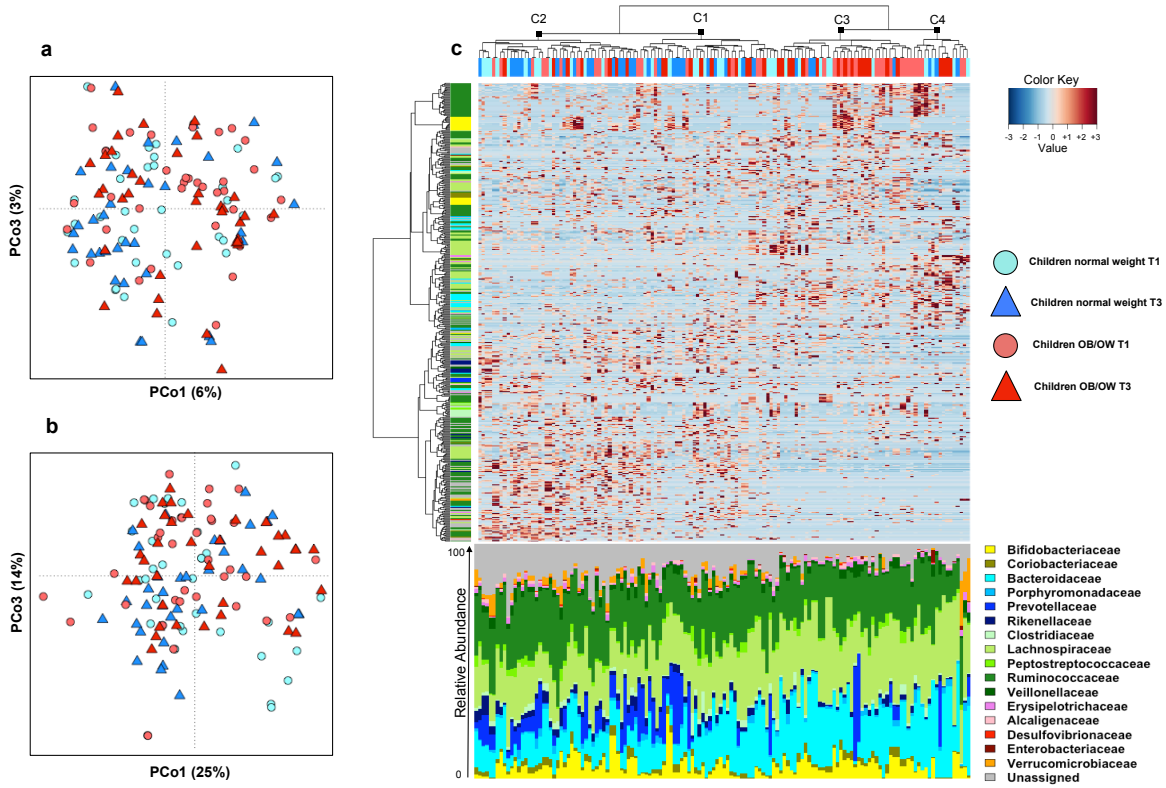


**Figure 4.4. Microbiota analysis separates children based on obesity.** (a) Unweighted and (b) weighted UniFrac PCoA of the fecal microbiota, at two different time points. (c) Hierarchical Ward linkage clustering based on the Spearman correlation coefficients of the relative abundance of OTUs, filtered for OTU presence in at least 20% of the subjects. Labelled groups in the top tree (basis for the four groups in Figure 4.1) are highlighted by black squares. OTUs are color-coded by family assignment in the vertical tree. Bacteroidetes phylum, blue gradient; Firmicutes, green; Proteobacteria, red; and Actinobacteria, yellow. Four hundred fifty-six OTUs classified to the family level are visualized. The bar plot shows the relative abundance of the family-classified microbiota profiles.

**Differences between normal weight children at T1**

| Taxon | Level | Mean T1_N | SEM T1_N | Mean T1_O | SEM T1_O | *p* |
|---|---|---|---|---|---|---|
| Cyanobacteria | Phylum | 0.19 | 0.07 | 0.14 | 0.07 | 0.05 |
| *S24-7* | Family | 0.7 | 0.25 | 0.11 | 0.08 | 0.05 |
| *Slackia* | Genus | 0.05 | 0.01 | 0.03 | 0.01 | 0.008 |
| *[Prevotella]* | Genus | 0.3 | 0.18 | 0 | 0 | 0.05 |
| *Lactococcus* | Genus | 0.12 | 0.04 | 0.1 | 0.07 | 0.04 |

**Differences between normal weight children at T1 and T3**

| Taxon | Level | Mean T1_N | SEM T1_N | Mean T3_N | SEM T3_N | *p* |
|---|---|---|---|---|---|---|
| Proteobacteria | Phylum | 1.51 | 0.19 | 1.08 | 0.11 | 0.04 |
| Tenericutes | Phylum | 0.6 | 0.18 | 0.18 | 0.08 | 0.03 |
| *[Mogibacteriaceae]* | Family | 0.14 | 0.02 | 0.18 | 0.02 | 0.03 |
| *Enterobacteriaceae* | Family | 0.32 | 0.17 | 0.04 | 0.02 | 0.01 |
| *Catenibacterium* | Genus | 0.07 | 0.04 | 0.23 | 0.1 | 0.04 |

**Differences between children before and after the onset of obesity**

| Taxon | Level | Mean T1_O | SEM T1_O | Mean T3_O | SEM T3_O | *p* |
|---|---|---|---|---|---|---|
| Proteobacteria | Phylum | 1.1 | 0.13 | 1.56 | 0.24 | 0.02 |
| *Lactobacillaceae* | Family | 0.2 | 0.13 | 0.11 | 0.07 | 0.02 |
| *Clostridiaceae* | Family | 1.5 | 0.2 | 1.14 | 0.17 | 0.03 |
| *Ruminococcaceae* | Family | 24.82 | 1.16 | 21.55 | 0.98 | 0.02 |
| *Alcaligenaceae* | Family | 0.66 | 0.09 | 0.86 | 0.09 | 0.03 |
| *Ruminococcus* | Genus | 5.92 | 0.86 | 3.88 | 0.47 | 0.05 |
| *Sutterella* | Genus | 0.66 | 0.09 | 0.86 | 0.09 | 0.03 |

**Differences between normal weight and obese children at T3**

| Taxon | Level | Mean T3_N | SEM T3_N | Mean T3_O | SEM T3_O | *p* |
|---|---|---|---|---|---|---|
| *Bacteroidaceae* | Family | 15.76 | 1.86 | 19.62 | 1.68 | 0.03 |
| *Prevotellaceae* | Family | 6.16 | 1.45 | 5.23 | 1.74 | 0.02 |
| *Christensenellaceae* | Family | 0.49 | 0.11 | 0.29 | 0.10 | 0.05 |
| *[Mogibacteriaceae]* | Family | 0.18 | 0.02 | 0.11 | 0.02 | 0.001 |
| *[Tissierellaceae]* | Family | 0.02 | 0 | 0.01 | 0 | 0.04 |
| *[Cerasicoccaceae]* | Family | 0.10 | 0.06 | 0 | 0 | 0.002 |
| *Slackia* | Genus | 0.05 | 0.01 | 0.02 | 0.01 | 0.008 |
| *Bacteroides* | Genus | 15.76 | 1.86 | 19.62 | 1.68 | 0.03 |
| *Prevotella* | Genus | 6.16 | 1.45 | 5.23 | 1.74 | 0.02 |
| *[Prevotella]* | Genus | 0.26 | 0.12 | 0.03 | 0.03 | 0.02 |
| *Lachnospira* | Genus | 0.74 | 0.10 | 1.44 | 0.21 | 0.02 |
| *Roseburia* | Genus | 0.13 | 0.02 | 0.23 | 0.04 | 0.03 |
| *Oscillospira* | Genus | 0.80 | 0.05 | 0.66 | 0.05 | 0.04 |

**Table 4.2. Microbial taxa significantly different across the four groups of children.** Only taxa found in at least 20% of the samples were considered. Differences in relative abundance were assessed by Wilcoxon test, paired or unpaired as needed. SEM: standard error mean; p: p value.

In conclusion, our data highlight the importance of the individual microbiome configuration as a mediator of the dietary impact on the metabolic and immunological homeostasis. In particular, the individual GM configuration – in terms of steady state – together with the long-term dietary habit could be considered as a predictive tool for the development of obesity, particularly in children (Figure 4.5). Hence, our data pave the way for a new perspective, where dietary recommendations to reduce the obesity risk in children are specifically tailored based on the individual microbiome structure, with the precise purpose of avoiding combinations of diet and microbiome configuration that are likely to favor the onset of obesity (Figure 4.5). Our data also stress the multifactorial nature of obesity, where GM dysbioses and interacting factors (e.g. diet) are only a part of the complex mosaic of determinants of this phenotypic trait. Future studies on larger cohorts, based on shotgun metagenomics and possibly providing for more extensive sampling, are needed to better unravel the contribution of the GM, as well as of specific species and/or strains, to this complex mosaic.

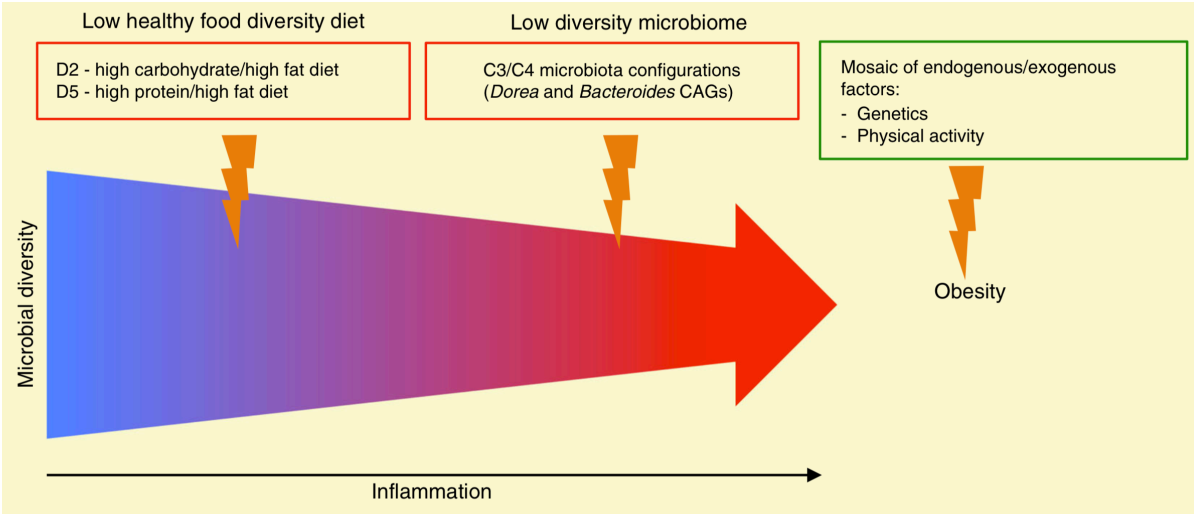


**Figure 4.5. The mosaic etiology of obesity.** The gut microbiota diversity is likely altered at multiple stages by the diet. Unhealthy diets may promote an inflammatory state that, in turn, is strictly interconnected with the gut microbial configuration. The combination of these three factors (unhealthy diets, inflammation and a dysbiotic, low-diverse and pro-inflammatory microbial layout) may favor the onset of obesity. High physical activity may protect the human host from obesity, even when diet and microbiota are in a low-diversity and pro-inflammatory configuration. However, human genetics can lead the host to develop obesity, regardless of the microbiome configuration.

**The NeuroFAST cohort**

**Microbiota structure and food addiction**

To investigate links between the GM, obesity and FA, the fecal microbial composition in 100 women was analysed. The enrolled individuals were stratified according to weight status and food addiction, diagnosed according to Yale Food Addiction Scale, YFAS (Gearhardt *et al*., 2011) into three study groups: 35 obese women with high FA (referred to as HFA), 28 obese women with low FA (LFA), and 37 normal-weight women.

The sequencing yielded a total of 6.5 million sequence reads from 16S rRNA gene V3-V4 amplicons, with an average of 73,152 (± 38,578, sd) paired-end reads per sample, for 11,874 OTUs grouped at 97% of sequence identity. When examining OTUs abundance, we identified four subject clusters, two of which (C2 and C3) included the majority of obese women (p value < 0.001, Fisher's test). In particular, C3 included the majority of obese women with high FA (i.e. HFA).

To identify trends in the GM, we established co-abundance associations of genera, and then clustered correlated bacterial taxa into five co-abundance groups (CAGs), describing the microbiota structures found across the whole dataset. The dominant (i.e. the most abundant) genera in these CAGs were *Bifidobacterium* (violet), *Ruminococcus* (blue), *Dorea* (green), *Prevotella* (light blue), and *Bacteroides* (pink). In Figure 4.6 Wiggum plots are shown, representing genus abundance as a disc proportional to normalized over-abundance. The four subject divisions, as identified by OTU clustering, were superimposed on the unweighted (Figure 4.6), allowing defining four clusters, C1–C4. As specified above, within a spectrum of microbiota structures, each of these clusters constitutes a steady state, representing groups of individuals who have a significantly different microbiota layout from each other, as defined by MANOVA test on UniFrac data (p value < 0.001). The microbiota variation from the group dominated by normal weight women (C1) to the groups dominated by obese women (C2/C3/C4) was accompanied by distinctive CAG dominance, specifically by abundances of

*Prevotella* CAG (C1) and *Ruminococcus*, *Dorea* and *Bifidobacterium* CAGs (C2/C3/C4). In particular, the steady state C2 was the most heterogeneous, showing the concomitant presence of four CAGs (*Prevotella*, *Bacteroides*, *Bifidobacterium*, and *Dorea*). On the other hand, C3 lacked the *Bifidobacterium* and *Bacteroides* CAGs, the latter being not represented also within C4. Significant associations of several measurements related to the available hematological parameters, DXA and eating behavior surveys with the major axes from unweighted UniFrac PCoA analysis are shown in Table 4.3. In particular, when considering the whole cohort, a shift of the microbiota structure towards negative low values of PCo1 was associated with higher BITE symptoms and TFEQ UE – indicative of binge-eating and a greater cognitive restraint, uncontrolled, or emotional eating, respectively. On the contrary, positive values of PCo1 were associated with higher android BMC – bone mineral content within the android region, an indicator of the potential risk of fractures and osteoporosis in the post-menopausal period. When considering the microbiota structure of obese women with low FA, the majority of samples were included within the C2 configuration and associated with higher BITE severity, therefore reflecting an abnormal eating behavior. On the other hand, obese women with high FA were more associated with the C3 and C4 configurations, in addition to being characterized by higher YALE scores. Furthermore, a lower insulin secretion rate was observed in these women, suggesting metabolic imbalance and impaired blood glucose management. Glucose and insulin levels have been associated with altered brain activity in regions involved in reward processing, such as the mesolimbic system. In particular, insulin increases dopamine reuptake in the presynaptic membrane and suppresses food-motivated behavior (Figlewicz *et al*., 2008). Furthermore, Anthony and colleagues demonstrated that brain insulin resistance exists in regions that mediate appetite and reward, diminishing the link between intake control and energy balance (Anthony *et al*., 2006).

With specific regard to biodiversity, our results confirm the tendency to reduced microbial biodiversity in obese individuals as observed previously (Le Chatelier *et al*., 2013; Aron-

Wisnewsky *et al*., 2019). In particular, a gradual change of the level of biodiversity was observed along PCo1, from the highest level in the samples belonging to the C1 and C3 clusters to the lowest values in C2 and C4 microbiomes (p value < $8x10^{-4}$, Kruskal-Wallis test). Furthermore, when comparing the biodiversity of the women microbiota among the original groupings (HFA, LFA, and normal-weight), a significant difference was also detected, with the normal-weight group displaying the highest biodiversity level with respect to the LFA group (p value < 0.03, Wilcoxon test), while HFA individuals showed intermediate values.

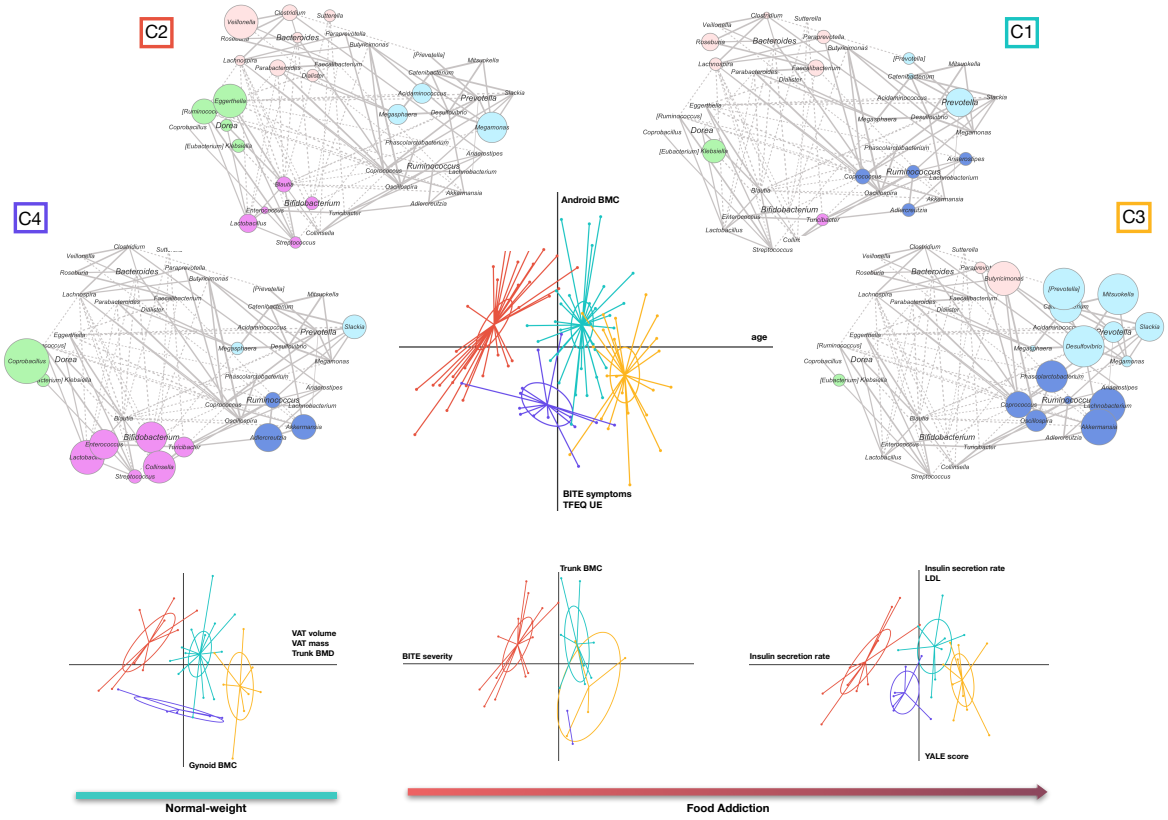


**Figure 4.6. Variation of the gut microbiota structure across normal weight and obese women with high and low food addiction is mirrored by changes in eating habits and insulin secretion rate.** The PCoA plots show four significantly different groups of subjects (C1–C4, p value < 0.001), as defined by unweighted UniFrac microbiota analysis of the whole cohort (center), normal weight women (bottom, left), and obese women with low or high food addiction (bottom, right). At the top, Wiggum plots corresponding to the four groups from the whole cohort analysis, in which disc sizes indicate genus over-abundance compared to the average relative abundance in the whole cohort. The arrow indicates a transition from low to high food addiction. Please see also Table 4.2. BMC: body mineral component; BITE: bulimic investigatory test; TFEQ UE: three factor eating questionnaire, uncontrolled eating; VAT: visceral adipose tissue; BMD: bone mineral density; LDL: low density lipoprotein.

| Parameter | PCo1 | | | PCo2 | | | PCo3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | RC range | RC sd | p-value | RC range | RC sd | p-value | RC range | RC sd | p-value |
| **I. Unweighted UniFrac PCoA for all women** | | | | | | | | | |
| Age | 2.87438 | 0.38843 | 0.007 | -0.03924 | -0.00981 | 0.9 | 1.02296 | 0.30087 | 0.01 |
| Android BMC | 1.70030 | 0.22977 | 0.02 | 1.57268 | 0.39317 | 0.00002 | -0.47066 | -0.13074 | 0.1 |
| BITE symptoms | 1.02542 | 0.13857 | 0.3 | -1.61860 | -0.40465 | 0.007 | -0.60743 | -0.16873 | 0.2 |
| TFEQ UE | 0.78270 | 0.10577 | 0.5 | -1.37016 | -0.34254 | 0.01 | -0.52106 | -0.14474 | 0.3 |
| BITE severity | 0.78240 | 0.10573 | 0.6 | -0.05700 | -0.01425 | 0.9 | 1.08313 | 0.30087 | 0.01 |
| **II. Unweighted UniFrac PCoA for obese women with high food addiction** | | | | | | | | | |
| Insulin secretion rate | -2.32715 | -0.31448 | 0.02 | 1.76392 | 0.44098 | 3.40E-10 | -0.44604 | -0.12390 | 0.7 |
| LDL | 1.12154 | 0.15156 | 0.5 | 2.08916 | 0.52229 | 0.01 | -0.17078 | -0.04744 | 0.7 |
| YALE score | 0.92337 | 0.12478 | 0.7 | -1.61428 | -0.40357 | 0.01 | 1.05340 | 0.29261 | 0.3 |
| TFEQ CR | -1.30366 | -0.17617 | 0.5 | -1.55760 | -0.38940 | 0.2 | -2.01888 | -0.56080 | 0.002 |
| Android BMC | 2.95645 | 0.39952 | 0.03 | 1.65680 | 0.41420 | 0.2 | -1.16039 | -0.32233 | 0.007 |
| **III. Unweighted UniFrac PCoA for obese women with low food addiction** | | | | | | | | | |
| BITE severity | -2.07259 | -0.28008 | 0.01 | 0.81664 | 0.20416 | 0.4 | -0.17770 | -0.04936 | 0.9 |
| Trunk BMC | 3.02490 | 0.40877 | 0.04 | 2.21476 | 0.55369 | 0.01 | -0.65894 | -0.18304 | 0.6 |
| BDI | -0.48921 | -0.06611 | 0.8 | 1.64160 | 0.41040 | 0.3 | -2.45358 | -0.68155 | 0.0004 |
| Ghrelin | -0.35002 | -0.04730 | 0.6 | -0.23056 | -0.05764 | 0.7 | -1.18174 | -0.32826 | 0.001 |
| WB BMC | 1.22366 | 0.16536 | 0.05 | 0.43260 | 0.10815 | 1 | -1.01783 | -0.28273 | 0.002 |
| **IV. Unweighted UniFrac PCoA for normal weight-only women** | | | | | | | | | |
| VAT volume | 2.00466 | 0.27090 | 0.001 | -0.34744 | -0.08686 | 0.9 | -1.29449 | -0.35958 | 0.00004 |
| VAT mass | 1.99763 | 0.26995 | 0.001 | -0.34752 | -0.08688 | 1 | -1.29542 | -0.35984 | 0.00004 |
| Trunk BMD | 3.48015 | 0.47029 | 0.002 | -0.93356 | -0.23339 | 0.3 | -0.71802 | -0.19945 | 0.3 |
| Gynoid BMC | 2.51667 | 0.34009 | 0.05 | -2.52892 | -0.63223 | 0.006 | -0.08892 | -0.02470 | 0.9 |
| Gynoid fat mass | 2.17812 | 0.29434 | 0.3 | -0.12524 | -0.03131 | 0.9 | -1.38046 | -0.38346 | 0.00003 |
| Gynoid lean mass | 2.34906 | 0.31744 | 0.1 | -0.12800 | -0.03200 | 0.9 | -1.36724 | -0.37979 | 0.00009 |
| Trunk fat mass | 2.05424 | 0.27760 | 0.3 | -0.33604 | -0.08401 | 0.9 | -1.22764 | -0.34101 | 0.0001 |
| Trunk lean mass | 2.67961 | 0.36211 | 0.07 | -0.38936 | -0.09734 | 0.8 | -1.46333 | -0.40648 | 0.0004 |

**Table 4.3. Associations between clinical variables and microbiota composition.** Quantile (median) regression tests of associations between metadata measurements and microbiota composition as measured by unweighted UniFrac PCoA across all groups. RC range, regression coefficients scaled to the full variation along each PCoA axis, thus indicating direction and magnitude of the association; RC sd, regression coefficients scaled to one standard deviation; p-value: quantile regression p value.

## Impact of diet on the gut microbiota

The data from FFQs were superimposed on the unweighted UniFrac PCoA plot of Figure 4.6, with the aim of identifying the food types with the most significant contribution (p value < 0.05, permutational correlation test) to the microbiota ordination (Figure 4.7a). Remarkably, a higher consumption of seasonings and condiments (i.e. butter, margarine, pesto and ragù sauces), olive oil, fried potatoes and sausages, as well as sweetened drinks, milk and yogurt was associated with the configurations C2. The C4 configuration was characterized by an increased consumption of cheese, while C1 and C3 displayed lower consumption of the above-mentioned food categories. As discussed above, the microbiota diversity was directly correlated to the first

PCoA axis, similar to fiber intake, while the total energy intake (kcal/day) showed an opposite trend. By focusing on macronutrients, we found several differences between the four clusters identified according to GM configurations. Specifically, compared to C1, i.e. the configuration that includes the majority of normal-weight women, a significant reduction in the daily fiber intake was observed in C2, which mainly includes low FA women (p value = 0.03, Wilcoxon). Furthermore, significantly reduced fat intake (p value < 0.039) was found in the C3 and C4 configurations, and the latter also showed higher carbohydrate consumption (p value = 0.05) (Figure 4.7b). As expected, the C4 microbiota configurations (including high FA obese women) was characterized by a significantly higher energy intake compared to C1 (normal-weight individuals) (p value = 0.04).

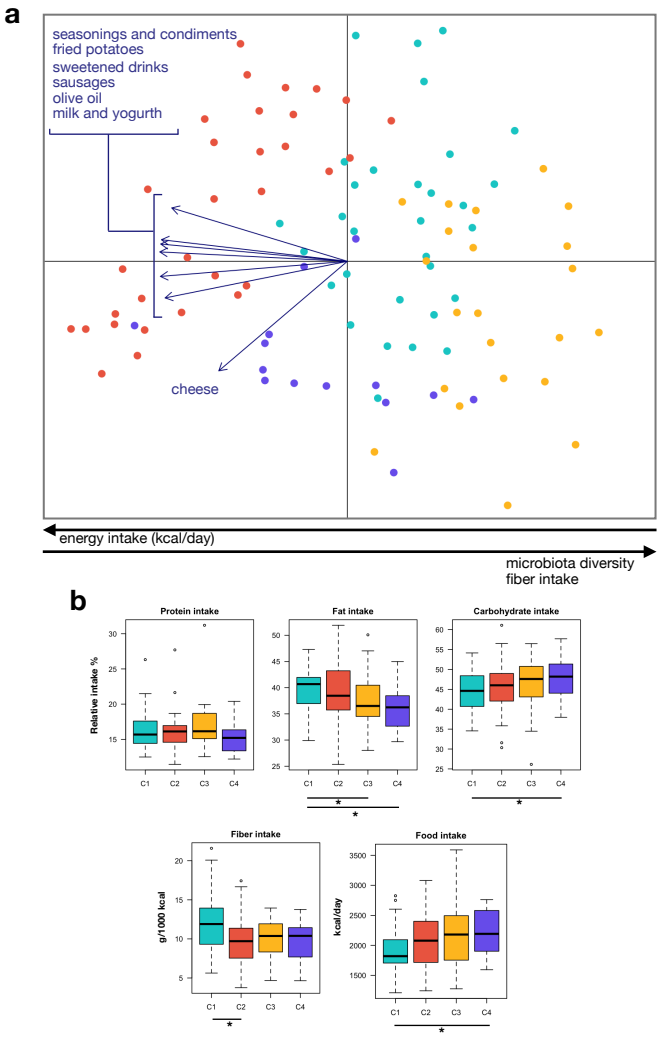


**Figure 4.7. Dietary contribution to the microbiota ordination.** (a) PCoA based on unweighted UniFrac distances of the fecal microbiota. The biplot of the average food coordinates weighted by frequency of consumption per sample was superimposed on the PCoA plot to identify the foods contributing to the ordination space (blue arrows). Only the food categories showing a highly significant correlation with the sample separation (p value < 0.005, permutational correlation test) were displayed. Samples are colored by subject group (C1–C4), as in Figure 4.2. The black arrows at the bottom indicates the direction of the microbiota diversity, energy and fiber intake gradient along PCo1. (b) Summary of the macronutrient intake, expressed as a percentage of kilocalories consumed per day, and fiber consumption, as grams of fiber intake per 1,000 kilocalories consumed. *, p value < 0.05, Wilcoxon test.

**Microbiota and diet in relation to obesity and food addiction**

FFQ data were further explored in a Correspondence Analysis, where the first axis, describing over 13.7% of the dataset variance, contained most of the discriminating food types identified in the previous correlation analysis of FFQ data on the microbiota PCoA, such as cheese, sweetened drinks, seasonings and condiments. Application of Ward linkage clustering and Euclidean distance metrics to this axis allowed identifying three dietary groups (p value < 0.001, Fisher's test): D1 ('low protein/high carbohydrate'), D2 ('low carbohydrate/high protein'), D3 ('high fat/high protein') (Figure 4.8a). By comparing the Healthy Food Diversity (HFD) index between dietary groups it emerged that D2 and D3 were the most diversified diets, while D1 showed the lowest values (p value < 0.02, Wilcoxon test) (Figure 4.8b). In particular, D1 includes a greater consumption of biscuits and sweet snacks, D2 of sliced ham and homemade sandwiches, while D3 involves a greater consumption of cheese, milk and yogurt. By matching the stratifications of women in dietary and microbiota groups, redundant combinations associated with the obese phenotype were sought. In particular, the less diversified diet D1 was mainly consumed by the C2 and C4 configurations, both associated with obesity and characterized by lower levels of microbial diversity, as well as by higher energy intake. Focusing the analysis on food addiction, most of the women classified as high FA were represented by the C2 configuration, while the women with low FA were mainly associated with the C4 configuration. On the contrary, the configuration C1 was composed mainly of normal-weight women and was not associated with a particular dietary group.

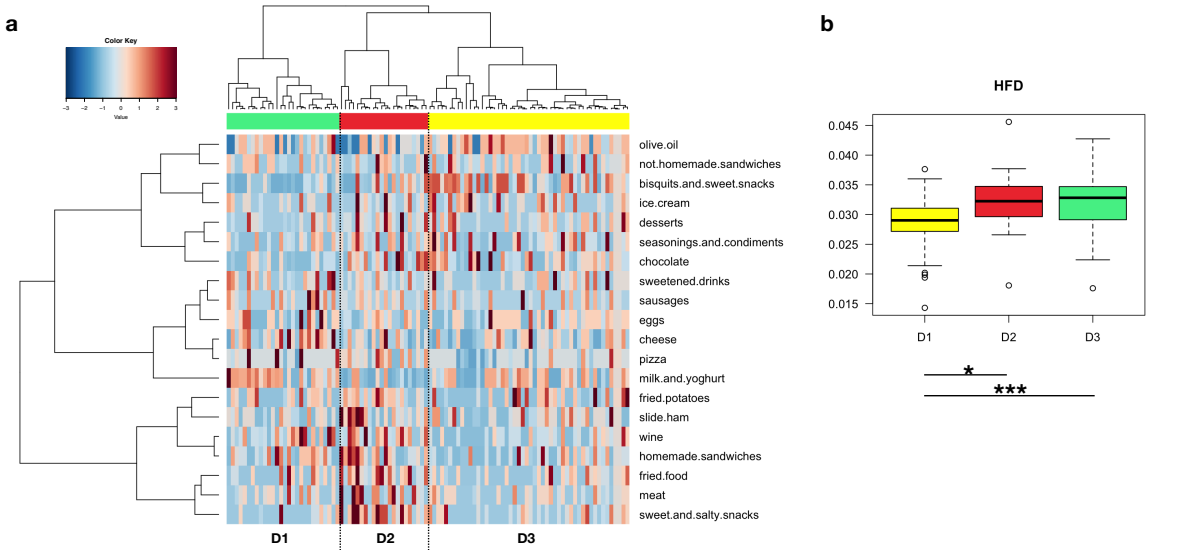


**Figure 4.8. Dietary patterns discriminate women for the Healthy Food Diversity index.** (a) Three dietary groups (D1–D3) revealed through Ward linkage clustering using Euclidean distances applied to the first eigenvector in a Correspondence Analysis of data from Food Frequency Questionnaires. (b) Comparison of the Healthy Food Diversity (HFD) index (Drescher *et al*., 2007) across the three dietary groups identified in a). *: p value = 0.02; ***: p value < 0.00001, Wilcoxon test.

## Microbiota signatures of obesity and food addiction

Unweighted UniFrac PCoA analysis showed weak but significant separation between women with normal weight and obese women with either high or low FA, (p value = 0.03, permutation test with pseudo-*F* ratios; Figure 4.9b). On the other hand, no significant differences were found according to weighted UniFrac distances (p value = 0.1) (Figure 4.9c), suggesting only a few differences in subdominant components of the GM. Phylum-level microbiota assignment showed comparable levels of Firmicutes (mean % ± sem; obese, O: 69.5 ± 1.5; normal-weight, N: 72.4 ± 1.4), Bacteroidetes (O: 10.3 ± 1.2; N: 11.8 ± 1.4), Actinobacteria (O: 13.2 ± 1.3; N: 11.4 ± 1.2), and Verrucomicrobia (O: 2.5 ± 0.7; N: 3.2 ± 0.8). Conversely, at family level, *Turicibacteraceae* were significantly lower in obese women compared to normal-weight women (O: 0.15 ± 0.05; N: 0.25 ± 0.07; p value = 0.003, Wilcoxon test), with an ever more marked reduction in obese women with high FA (0.04 ± 0.07; p value = 0.002) (Figure 4.9a). Furthermore, obese women showed lower levels of the genera *Roseburia*, *Adlercreutzia*,

*Turicibacter* and *Coprococcus*, in addition to higher levels of *Dorea*, when compared to normal-weight women (p value < 0.02). When looking at differences in microbiota profiles of women with high and low FA, the increase in relative abundance of *Dorea* was even more exacerbated in the latter group. A complete summary of the significant differences in the GM between groups is reported in Table 4.4. Taken together, our data raise the hypothesis in favour of a possible role for these gut microorganisms in eating behaviours, opening the way to further investigation particularly on the role of *Turicibacter* and *Dorea* in FA.

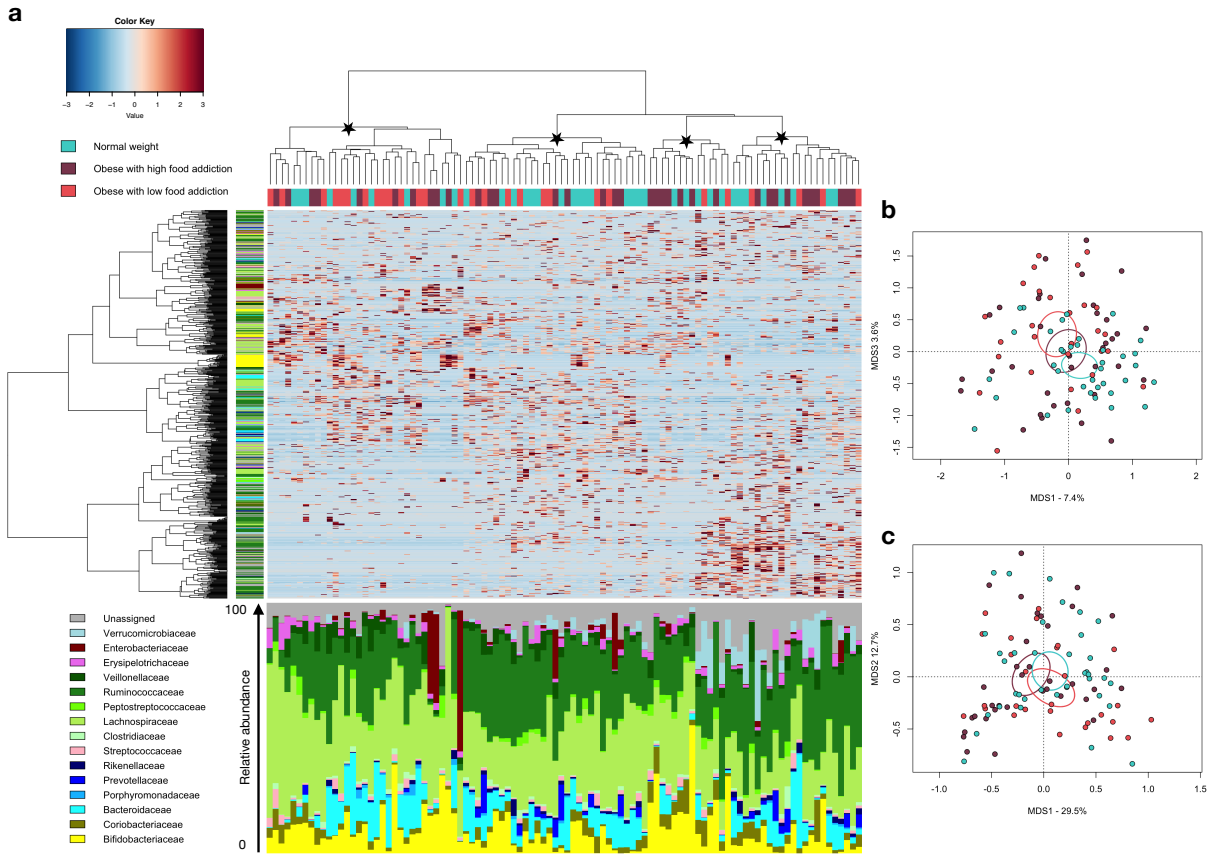


**Figure 4.9. Microbiota analysis separates women based on obesity and food addiction.** (a) Hierarchical Ward linkage clustering based on the Spearman correlation coefficients of the relative abundance of OTUs, filtered for OTU presence in at least 20% of the subjects. Labelled groups in the top tree (basis for the four groups in Figure 4.2) are highlighted by black stars. OTUs are color-coded by family assignment in the vertical tree. Bacteroidetes phylum, blue gradient; Firmicutes, green; Proteobacteria, red; and Actinobacteria, yellow. The bar plot shows the relative abundance of the family-classified microbiota profiles. (b) Unweighted and (c) weighted UniFrac PCoA of the fecal microbiota of the enrolled women.

**Differences between normal weight and obese women**

| | Taxon | Mean NW | SEM NW | Mean OB | SEM OB | P-value |
|---|---|---|---|---|---|---|
| phylum | Synergistetes | 0.06 | 0.03 | 0 | 0 | 0.0008 |
| | Tenericutes | 0.02 | 0.01 | 0.02 | 0.01 | 0.01 |
| | Euryarchaeota | 0.03 | 0.01 | 0.01 | 0 | 0.04 |
| family | Synergistaceae | 0.06 | 0.03 | 0 | 0 | 0.0008 |
| | Turicibacteraceae | 0.25 | 0.07 | 0.15 | 0.05 | 0.003 |
| | [Barnesiellaceae] | 0.32 | 0.06 | 0.25 | 0.06 | 0.03 |
| | Methanobacteriaceae | 0.03 | 0.01 | 0.01 | 0 | 0.04 |
| genus | *Roseburia* | 0.69 | 0.12 | 0.28 | 0.04 | 0.001 |
| | *Cloacibacillus* | 0.03 | 0.02 | 0 | 0 | 0.003 |
| | *Adlercreutzia* | 0.22 | 0.05 | 0.12 | 0.02 | 0.003 |
| | *Turicibacter* | 0.25 | 0.07 | 0.15 | 0.05 | 0.003 |
| | *Coprococcus* | 1.88 | 0.34 | 0.97 | 0.18 | 0.005 |
| | *Ruminococcus* | 5.62 | 0.65 | 4.19 | 0.5 | 0.03 |
| | *[Ruminococcus]* | 1.06 | 0.15 | 2.01 | 0.34 | 0.03 |
| | *Methanobrevibacter* | 0.03 | 0.01 | 0.01 | 0 | 0.03 |

**Differences between normal weight and obese women with high food addiction**

| | Taxon | Mean NW | SEM NW | Mean OB_HA | SEM OB_HA | P-value |
|---|---|---|---|---|---|---|
| phylum | Synergistetes | 0.06 | 0.03 | 0 | 0 | 0.004 |
| family | Turicibacteraceae | 0.25 | 0.07 | 0.1 | 0.04 | 0.002 |
| | Synergistaceae | 0.06 | 0.03 | 0 | 0 | 0.004 |
| | Pasteurellaceae | 0.02 | 0.01 | 0.01 | 0.01 | 0.03 |
| | [Barnesiellaceae] | 0.32 | 0.06 | 0.23 | 0.08 | 0.04 |
| genus | *Turicibacter* | 0.25 | 0.07 | 0.1 | 0.04 | 0.002 |
| | *Roseburia* | 0.69 | 0.12 | 0.29 | 0.05 | 0.01 |
| | *Adlercreutzia* | 0.22 | 0.05 | 0.12 | 0.03 | 0.01 |
| | *Coprococcus* | 1.88 | 0.34 | 0.96 | 0.25 | 0.02 |
| | *Cloacibacillus* | 0.03 | 0.02 | 0 | 0 | 0.03 |
| | *Haemophilus* | 0.02 | 0.01 | 0.01 | 0.01 | 0.03 |

**Differences between normal weight and obese women with low food addiction**

| | Taxon | Mean NW | SEM NW | Mean OB_LA | SEM OB_LA | P-value |
|---|---|---|---|---|---|---|
| phylum | Synergistetes | 0.06 | 0.03 | 0 | 0 | 0.04 |
| | Proteobacteria | 0.68 | 0.1 | 3.21 | 1.99 | 0.04 |
| | Euryarchaeota | 0.03 | 0.01 | 0.01 | 0.01 | 0.04 |
| family | Eubacteriaceae | 0.01 | 0 | 0 | 0 | 0.02 |
| | Synergistaceae | 0.06 | 0.03 | 0 | 0 | 0.04 |
| | Methanobacteriaceae | 0.03 | 0.01 | 0.01 | 0.01 | 0.04 |
| genus | *Roseburia* | 0.69 | 0.12 | 0.27 | 0.07 | 0.002 |
| | *Dorea* | 1.12 | 0.11 | 2.11 | 0.35 | 0.007 |
| | *Adlercreutzia* | 0.22 | 0.05 | 0.11 | 0.04 | 0.01 |
| | *Ruminococcus* | 5.62 | 0.65 | 3.4 | 0.55 | 0.01 |
| | *Coprococcus* | 1.88 | 0.34 | 0.99 | 0.26 | 0.02 |
| | *cc_115* (Erysipelotrichaceae) | 0.04 | 0.01 | 0.01 | 0 | 0.02 |
| | *[Ruminococcus]* | 1.06 | 0.15 | 2.76 | 0.71 | 0.03 |
| | *Methanobrevibacter* | 0.03 | 0.01 | 0.01 | 0.01 | 0.04 |
| | *Cloacibacillus* | 0.03 | 0.02 | 0 | 0 | 0.05 |

**Differences between obese women with high and low food addiction**

| | Taxon | Mean OB_HA | SEM OB_HA | Mean OB_LA | SEM OB_LA | P-value |
|---|---|---|---|---|---|---|
| family | Pasteurellaceae | 0.01 | 0.01 | 0.05 | 0.03 | 0.02 |
| genus | *Haemophilus* | 0.01 | 0.01 | 0.05 | 0.03 | 0.02 |
| | *Dorea* | 1.18 | 0.12 | 2.11 | 0.35 | 0.02 |

**Table 4.4. Microbial taxa significantly different across the study groups.** Only taxa found in at least 20% of the samples were considered. Differences in relative abundance were assessed by Wilcoxon test, paired or unpaired as needed. NW: normal-weight; OB: obese; SEM: standard error mean; P-value: p value.

To investigate differences among microbiota configurations C1-C4 (Figure 4.6) at species level, the frequency of reads mapped to the KEGG Orthology database was investigated by applying the MetaCV pipeline to shotgun metagenomic sequences (Liu *et al*., 2013). In particular, 15 Gb of bacterial DNA from 45 samples belonging to 15 obese women with high FA, 16 obese women with low FA, and 14 normal-weight women were sequenced. Focusing on the species level characterization of the four microbiome configurations (C1-C4), several differences were found, mainly involving 7 bacterial species: *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis* and *B. bifidum*, *Subdoligranulum* spp., *Ruminococcus bromii*, *Eubacterium rectale*, and *Akkermansia muciniphila* (Figure 4.10). Particularly noteworthy is the trend observed for *F. prausnitzii*, well-known for its anti-inflammatory properties (Santoru *et al.*, 2017), whose relative abundance was particularly high in C1 – the microbiome configuration comprising mostly normal-weight women – and underwent a progressive decrease in relation to the severity of FA. On the other hand, *Ruminococcus torques*, a mucolytic bacterial species known to decrease gut barrier integrity (Cani et al., 2014), appeared to be significantly higher in C2 – including obese women with low FA – with respect to C1, and also widespread in obese women with high FA included within the configurations C3 and C4. Furthermore, the mucin degrader *A. muciniphila* was found to be particularly abundant in C3 and almost completely absent in C2, with C1 and C4 showing comparable levels of its relative abundance. The configuration C4 displayed also higher relative abundances of *R. bromii*, *B. adolescentis* and *B. bifidum*, the last two probably related to the greater consumption of cheese and milk derivatives as emerged from the analysis of dietary surveys.

With the aim of investigating functional differences among microbiome metatranscriptomes with regards to the configurations C1-C4 (Figure 4.6), the frequency of reads mapped to the KEGG Orthology (KO) genes for amino acid and carbohydrate metabolism was investigated by applying the HUMAnN2 pipeline (Abubucker *et al*., 2012) to the same samples that underwent to metagenomic shotgun sequencing.

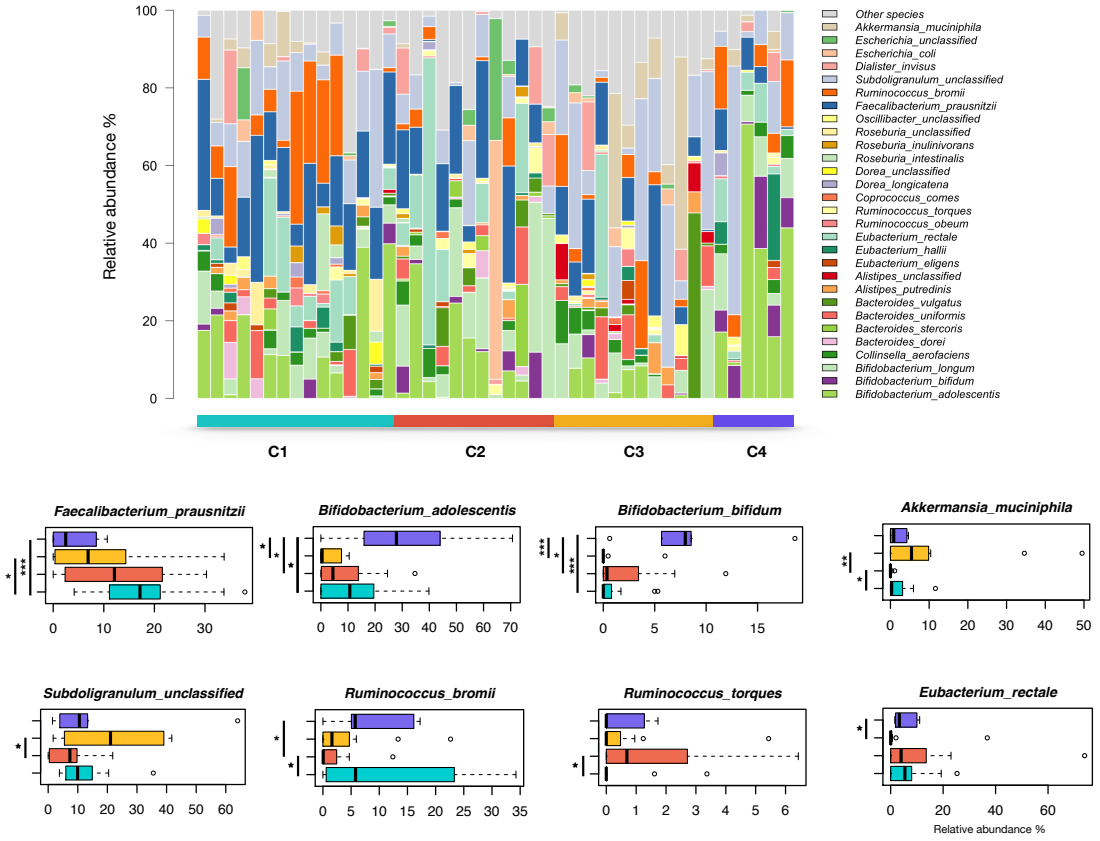


**Figure 4.10. Species-level signatures of the microbiome configurations.** Top, Barplots of the metagenomics profiles at the species level of the gut microbiome of the enrolled women, stratified according to the microbiome configurations (C1-C4) identified through hierarchical Ward linkage clustering (Figure 4.6). Bottom, Boxplots showing the distribution of the relative abundances of significantly enriched or depleted bacterial species between the study groups (Wilcoxon test). *: p value < 0.05; **; p value < 0.005; ***: p value < 0.0005.

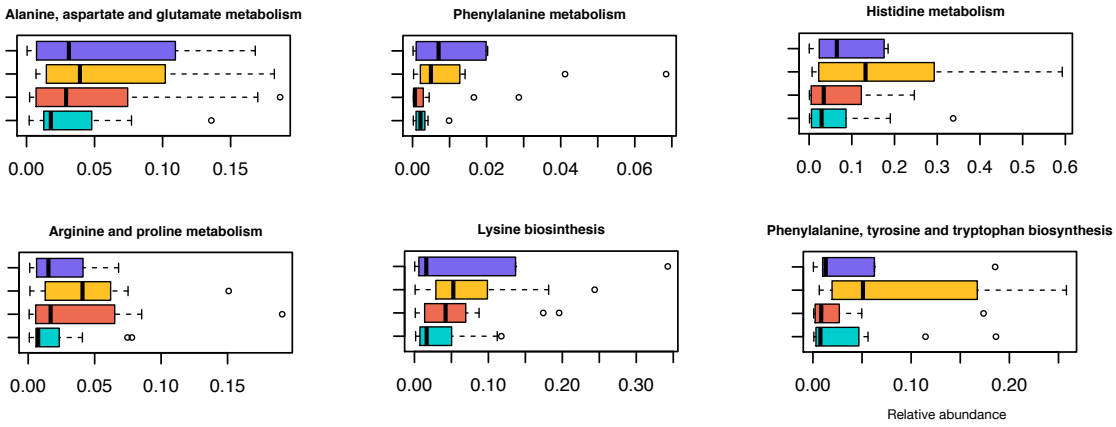


**Figure 4.11. Metatrascriptome configuration of the genes involved in amino acid metabolism for samples belonging to the NeuroFAST cohort.** The strong dependence of transcript number on underlying genomic copy number, was corrected producing and using the 'relative expression' measurements 'RNA abundance/DNA abundance' for each specific KO gene. The pathway abundances used to build these boxplots were computed for each sample considering the mean relative expression of the KO genes involved in each pathway.

Although no significant differences were found between the four microbiome configurations (p value > 0.05, Kruskal-Wallis test), an obesity-related increase in the number of reads for genes devoted to amino acids metabolism and biosynthesis with respect to normal-weight women was observed (Figure 4.11). In particular, these functional rearrangements provide an increased contribution of pathways involved in alanine, aspartate and glutamate metabolism in obese women, regardless of the FA severity. On the other hand, the configurations C3 and C4 – which mainly include obese women with high FA – showed a greater contribution of genes involved in phenylalanine, histidine, arginine and proline metabolism, as well as of genes devoted to lysine, phenylalanine, tyrosine and tryptophan biosynthesis. In particular, the catabolism of phenylalanine can produce trans-cinnamic acid and phenylethylamine by decarboxylation. Although little is known about these phenylalanine-derived metabolites, phenylethylamine is a neurotransmitter that functions as an 'endogenous amphetamine' (Marcobal *et al*., 2012). In terms of its production in the gut, phenylethylamine has been positively associated with Crohn's disease and negatively correlated with *F. prausnitzii* (Santoru *et al*., 2017). It is interesting to note that the latter trend is consistent with our previous observations on obese women following metagenomic analysis, especially for C4 configuration.

Many studies have proposed several mechanisms by which changes in the composition of the GM could contribute to the development of obesity (Backhed *et al*., 2007; Samuel *et al*., 2008). One of these mechanisms concerns the modulation of energy expenditure through the oxidation of fatty acids and the accumulation of energy in the form of triglycerides. In fact, intestinal bacteria have been shown to alter fatty acid metabolism by promoting fat accumulation in the liver and fatty tissue of mice (Backhed *et al*., 2007). Other studies suggest a role for the GM in the modulation of nutrient absorption through the SCFA signaling action on two G-protein-coupled receptors – Gpr41 and Gpr43 – expressed by intestinal epithelial and enteroendocrine cells, but also in adipocytes (Xiong *et al*., 2004; Samuel *et al*., 2008). These receptors, activated by SCFAs, induce the secretion of the intestinal hormones glucagon-like peptide-1 (GLP-1)

and peptide YY (PYY). PYY regulates intestinal motility that can influence the absorption of nutrients from the intestine, while GLP-1 regulates satiety (De Silva *et al*., 2012). Recent studies have also shown that overweight and obese individuals have higher fecal concentrations of SCFAs than their lean counterparts on a similar diet, confirming that colon fermentation also differs based on body weight in humans (Schwiertz *et al*., 2010; Teixeira *et al*., 2012).

In conclusion, our data highlight the importance of the individual microbiome configuration as a mediator of the dietary impact on metabolic homeostasis and potentially reflecting eating behaviors. Our results pave the way for a new perspective, in which dietary recommendations and interventions for the treatment of obesity in subjects with food addiction can be specifically tailored based on the individual microbiome structure, with the specific aim of avoiding combinations of diet and microbiome configuration that could lead to altered metabolic functionalities and potentially stress altered eating behaviors.

Chapter 5 – **A VERSATILE NEW MODEL OF CHEMICALLY INDUCED CHRONIC COLITIS USING AN OUTBRED MURINE STRAIN**



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Murine colitis models are crucial tools for understanding intestinal homeostasis and inflammation (Martin *et al*., 2017b). Their use over recent years has resulted in an exponential growth of knowledge on host–bacteria interactions. The most common *in vivo* models use rodents, mimicking different types of colitis with the aim of testing how the microbiota affects colon inflammation. Based on their disease induction method, models can be classified as: (i) chemically induced colitis; (ii) bacterially induced colitis; (iii) spontaneous colitis (including congenital and genetically engineered forms); and (iv) adoptive-cell-transfer colitis (Martin *et al*., 2017b). All these models have advantages and disadvantages. For instance, the intrinsic similarities and differences between mice and humans as well as external factors (e.g. living conditions and diet) might influence the ability of murine models to represent disease-related changes that occur in human microbiota (Nguyen *et al*.,2015). Here, we focus on chemically induced colitis models, which recreate the morphological, histopathological, and clinical features of human inflammatory bowel diseases (IBD) by orally or intrarectally administering various chemical compounds (Randhawa *et al*., 2014). For example, colitis can be induced by giving rodents drinking water containing dextran sodium sulfate (DSS) (Wirtz *et al*., 2007), causing the complete loss of the surface epithelium in the intestine through its cytotoxic effects (Randhawa *et al*., 2014). The integrity of the mucosal barrier is therefore affected and large molecules can pass through, provoking colitis (Ni *et al*., 1996). Colitis can also be induced by

rectally injecting a haptenating agent dissolved in ethanol, which allows the agent to pass through the mucosal barrier. The agent is then thought to act upon autologous or microbial proteins in the colon, making them immunogenic to the host immune system (Wirtz *et al*., 2007). The most commonly used haptenating agents are trinitrobenzene sulfonic acid (TNBS) and dinitrobenzene sulfonic acid (DNBS). Both TNBS and DNBS produce isolated points of inflammation and necrosis, as well as self-antigens that provoke immune responses (Elson *et al*., 2005). Although the models are similar, they are not identical: model functionality may vary, depending on host species identity and genetic background (Mizoguchi and Mizoguchi, 2008; Mizoguchi, 2012). Traditionally, acute protocols are used, in which the DNBS/TNBS injection or DSS period is performed just once and the recovery phase is optional. However, because inflammation can be chronic, a more realistic model would employ a protocol in which colitis is reactivated at least once, thus mimicking flare-ups and relapses. Colitis development is evaluated using changes in body mass, clinical symptoms (e.g. diarrhea, constipation, and bloody feces), colon morphology, and histological features. Furthermore, because this form of colitis is clearly tied to the immune system, colon cytokine concentrations, lymphocyte levels, and myeloperoxidase (MPO) activity (indicator of neutrophil infiltration that reflects the local immune response) are helpful markers of colitis severity (Wirtz *et al*., 2007; Martin *et al*., 2014a). Researchers use these models to identify and characterize candidate anti-inflammatory agents and test their effects on different IBD or other forms of intestinal mucosal inflammation. Such anti-inflammatory agents include for instance different type of molecules and also microorganisms known as probiotics. Probiotics are ‘live microorganisms that, when administered in adequate amounts, confer a health benefit on the host’ (Hill *et al*., 2014). At present, thanks to our improved knowledge of the human microbiota, candidate probiotics have been identified from among the dominant members of the gastrointestinal tract (GIT) microbiota found in healthy adults. They are referred to as next-generation probiotics (NGPs) and were originally identified as commensal bacteria species that can reestablish or enhance

colonization resistance (Pamer, 2016). However, this definition has been expanded rapidly to include more potential health benefits, overlapping with the emerging concept of live biotherapeutics (O'Toole *et al*., 2017). NGPs must be shown to be safe for the host; able to survive production, storage, and GIT transit; and elicit a positive host response that confers demonstrable health benefits (Martin *et al*., 2014b). Since these properties are strain specific, each candidate will have to be tested individually (Pineiro and Stanton, 2007; Hill *et al*., 2014; Miquel *et al*., 2015). In the normal sequence of events, these functional analyses involve preliminary *in vitro* testing and then preclinical *in vivo* testing in murine models, with the ultimate goal of performing clinical trials in humans.

There are two main challenges in this process. First, it is necessary to reproduce the *in vitro* results in the *in vivo* models and, second, reproduce the *in vivo* results in clinical trials. The use of several *in vitro* markers and models has been proposed with the aim of linking *in vitro* results with *in vivo* results. For instance, it was recently suggested that the ratio of anti-inflammatory and pro-inflammatory cytokines (interleukin IL-10 and IL-12, respectively) produced by peripheral blood mononuclear cells (PBMCs) upon *in vitro* exposure to probiotic strains could be a predictor of protective effects *in vivo* in a chemically induced murine colitis model (Foligne *et al*., 2007). Nevertheless, *in vivo* interactions are much more complex than *in vitro* ones, and it is challenging to identify the best *in vitro* test for predicting the impact that a candidate anti-inflammatory agent will have *in vivo*. The most widely accepted scientific strategy is to employ a combination of several *in vitro* tests. However, transferring murine results onto a human framework is a separate challenge because success depends upon how well effects in rodents translate into effects in humans. Indeed, past studies found that humans did not experience the beneficial effects of anti-inflammatory agents that were observed in a murine model of colon inflammation mimicking IBD. For example, a *Lactococcus lactis* strain secreting IL-10 was found to decrease DSS-induced colitis by 50%; however, humans treated with a biocontained

strain (thyA-/hIL-10+) did not experience beneficial effects in a phase II-A trial (Steidler *et al*., 2000, 2003, 2009).

Although this discordance in the results obtained in murine vs. humans could be due to their intrinsic differences (Nguyen *et al*., 2015), it could also be that researchers failed to carefully consider model suitability. At present, most models use an inbred strain of mice (individuals are genetically identical because of extensive inbreeding); furthermore, often only one sex is utilized to limit bias. However, this targeted approach itself introduces bias because it ignores natural genetic diversity and sex-related differences. As a result, it becomes even more difficult to extrapolate any knowledge gleaned from murine models to human populations. Here, our aim is to describe a versatile model of chemically induced chronic colitis that utilizes an outbred strain of mice and both females and males. The ultimate aim is establishing a more realistic model for effectively testing anti-inflammatory agents, for example probiotics, to better translate effects in rodents to effects in humans.

Chapter 5 – **A VERSATILE NEW MODEL OF CHEMICALLY INDUCED CHRONIC COLITIS USING AN OUTBRED MURINE STRAIN**

*5.1 Brief introduction*

**5.2 Materials and Methods**

*5.3 Results and Discussion*

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**Animals, experimental design, and sampling procedure**

We performed two trials looking at chronic colitis development in RjOrl:SWISS (CD-1) mice (Janvier, Le Genest Saint Isle, France) using C57BL/6JRj (Black-6) mice as control in the first trial (Table 5.1). The experiment was carried by duplicate in two different periods for each trial. In each period, 5 weeks-old mice were distributed into eight cages based on strain and sex (5 mice/cage) and evenly assigned to control or treatment groups (one cage/experimental group). For each trial a total of 40 females and 40 males were used (two cage/experimental group, n=10 mice per group) for a total of 160 mice used in all the study including both trials. Mice were maintained in the animal facilities of the French National Institute of Agricultural Research (IERP, INRA Jouy-en-Josas, France) under specific pathogen free (SPF) conditions at 21°C and housed in cages of 5. They were given food and water *ad libitum* and experienced a 12:12h light-dark cycle. Before the experiments began, animals were kept under these conditions for 1 week to allow them time to acclimate.

| Name | RjOrl:SWISS (CD-1) | C57BL/6JRj (Black-6) |
|---|---|---|
| Type | Outbred mouse (guaranteed to display less than 1% inbreeding per generation) | Inbred mouse (guaranteed to display autosomal pair homozygosity) |
| Distributor and origin | Janvier Labs CSAL (Orleans)—1965 | Janvier Labs CSAL (Orleans)—1993 |
| Color and related genotype | Albino mouse—Tyrc/Tyrc | Black mouse, an (a/a) non-agouti MHC: Haplotype H2b |
| Breeding | Good breeder, strong maternal instinct | Good breeder but difficult to rear due to environmental sensitivity, pup cannibalism |

**Table 5.1. Murine strains used in this study.**

The experimental protocol for inducing chronic inflammation is illustrated in Figure 5.1. At week 6, mice were anesthetized using an intraperitoneal (i.p.) injection of 0.1% ketamine (Imalgene 1000, Merial) and 0.06% xylazine (Rompun, Bayer) (Figure 5.1B1). Colitis was induced using DNBS (Sigma-Aldrich) resuspended in 50 µl of 30% ethanol (EtOH) in PBS. In the first experiment, we wanted to compare inflammation between the two mouse strains (Black-6 is the classical inbred murine strain typically used in these types of experiments). Animals in the treatment group were injected twice with 200 mg/kg of DNBS, which corresponds to 2.7, 3, 4.1, and 4.3 mg/mouse for Black-6 females and males and CD-1 females and males, respectively (Table 5.2). In the second experiment, in order to obtain different degrees of colitis severity in CD-1 mice, the DNBS dose was therefore modulated the in the treatment groups accordingly. Doses were fixed at 1.5, 2.5, and 3.5 mg/mice, irrespective of mouse mass or sex (Table 5.2).

| Experiments | DNBS Dose |
|---|---|
| Trial 1: Inflammation patterns in CD-1 mice vs. Black-6 mice | 200 mg/kg<br>2.7 mg—Black-6 females<br>3 mg—Black-6 males<br>4.1 mg—CD-1 females<br>4.3 mg—CD-1 males |
| Trial 2: Protocol optimization using CD-1 mice | 3.5 mg/2.5 mg/1.5 mg |

**Table 5.2. Dinitrobenzene sulfonic acid doses employed in this study.**

In both experiments, the DNBS solution was administered on day 1 by injection with a tuberculin syringe (Terumo) and a flexible plastic probe (model V0104040, ECIMED) inserted 3.5 cm into the colon (Figure 5.1B2). Control groups were injected with equivalent amounts of the 30% EtOH solution. All mice received a subcutaneous injection of 1 ml of saline solution (0.9% NaCl) to prevent dehydration (Figure 5.1B3). Mice were kept in a horizontal position until they awoke (Figure 5.1B4). These saline injections were repeated daily for the first 3 days (no anesthesia). In this model, colitis develops in the first 3 days following the DNBS injection.

During this activation period, the mice lost significant body weight. Mice were allowed to recover for 18 days and then received a second DNBS injection at day 21, reactivating inflammation. During this reactivation period, mice lost weight until the experiment's endpoint; no saline injections were performed because they could have affected body mass values at the endpoint. Mice were constantly monitored for the duration of the experiment, but especially so during the first 3 days after the DNBS injections. The model we employed in this study is a chronic colitis model because we used two DNBS injections: the first injection induces colitis, a recovery period follows, and then the second injection initiates a reactivation period. Classical acute models utilize a single injection, and colitis induction may or may not be followed by a recovery period. On day 24, blood samples were collected from the submandibular vein, and mice were euthanized by cervical dislocation. The abdomen was then sterilized with 70% EtOH, the abdominal cavity was opened to collect the spleen and the mesenteric lymph nodes (MLNs), and the entire large intestine was removed. Bowel length was measured, and a small portion of distal colon was immediately placed in a 4% paraformaldehyde (PFA, Prolabo) PBS solution for later histological analyses. The intestine was then cut open longitudinally, and the tissue was washed with saline solution after removing the contents. Colon sections of 1 cm were collected and immediately frozen in liquid nitrogen. All procedures were performed in accordance with European Union (EU) rules on ethical animal care (Directive 2010/63/EU) and were approved by the French Ministry of Research and COMETHEA, the animal ethics committee at INRA Jouy-en-Josas (authorization #3445-2016010615159974).

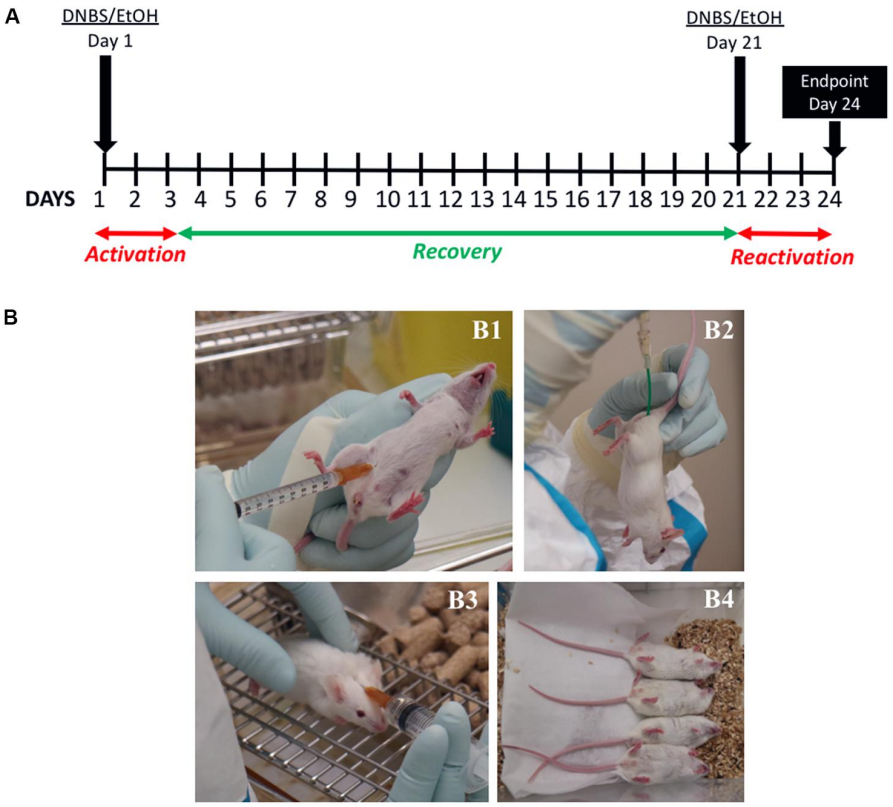


**Figure 5.1. Experimental design (A) and key methodological steps (B).** Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine and xylazine (B1). The DNBS solution or the control solution was administered by injection, using a tuberculin syringe and a flexible plastic tube inserted 3.5 cm into the colon (B2). Following the treatment, all mice received a subcutaneous injection of 1 ml of saline solution to prevent dehydration (B3); they were kept in a horizontal position until they awoke (B4).

## Weight trend and survival rate

In both trials, mice were carefully monitored. Their body mass was measured daily throughout the entire experimental period. Saline solution was administered when there was significant loss of body mass to prevent dehydration. In accordance with EU regulations (Directive 2010/63/EU), if mice lost 20% or more of their initial mass and/or showed signs of severe distress, they were euthanized and their id numbers were recorded. Percentage loss of body mass was calculated 3 days after each DNBS injection to compare results among groups.

## Macroscopic scores

Dinitrobenzene sulfonic acid-induced chronic inflammation is usually visible at the macroscopic level, and inflammation intensity can be evaluated by measuring different parameters, like mucosal damage in colon tissue and stool consistency. In both trials, macroscopic scores were determined using Wallace's score (Wallace *et al*., 1989), with the following modifications: tissue sections from each mouse were scored by evaluating ulcerations (score of 0-5), adhesions (presence/absence: 0/1), hyperemia (presence/absence: 0/1), altered transit, such as diarrhea or constipation (presence/absence: 0/1), and increases in colon wall thickness (presence/absence: 0/1; measured using an electronic caliper, Control Company, WVR, United States) (Table 5.3). Although colon length is not typically part of the macroscopic score in these types of models, it was also recorded (see above).

**Table 5.3. Macroscopic score.**

| Characteristic | Score |
|---|---|
| Ulcers | Absence: 0<br>1 ulcer smaller than 0.5 cm: 1<br>1 ulcer between 0.5 and 1 cm: 2<br>2 ulcers smaller than 1 cm<br>or 1 ulcer larger than 1 cm: 3<br>2 ulcers larger than 1 cm: 4<br>More than 2 ulcers: 5 |
| Adhesions | Presence: 1; Absence: 0 |
| Hyperaemia | Presence: 1; Absence: 0 |
| Altered transit | Presence: 1; Absence: 0 |
| Colon wall thickening | Presence: 1; Absence: 0 |

## Histological scores

In both trials, the tissues collected for the histological analyses were fixed for 24 h in a 4% paraformaldehyde (PFA) solution and then transferred to 70% EtOH. After 24-48 h, the tissues were gradually dehydrated by soaking for 1 h each in 80% EtOH, 90% EtOH, 100% EtOH, and xylene in an automated tissue processer (Leica Biosystem). Samples were embedded in paraffin using a tissue embedding system (Leica), cut into 5-µm sections using a microtome (UC6, Reicher E - Leica UC6), and then stained with hematoxylin and eosin (HE) for histological scoring using an automated staining system (Leica). All these procedures were performed

following conventional methodologies by the histological platform of the GABI Joint Research Unit (INRA, Jouy-en-Josas). Tissues were visualized using a high-capacity digital slide scanner (3DHISTECH Ltd.) and Panoramic and Case software (3DHISTECH Ltd.). For each animal, at least three tissue sections were evaluated to characterize alterations in mucosal architecture, the degree of immune cell infiltration, and Goblet cell depletion (Ameho score: 0-6) (Ameho *et al*., 1997).

**Myeloperoxidase activity and cytokine levels**

In the second trial, to measure myeloperoxidase (MPO) activity, a 1-cm section of colon tissue from each mouse was weighed and homogenized with Precellys (Bertin Corp.) in 300 µl of a 0.5% hexadecyltrimethyl-ammonium bromide (HTAB, Sigma-Aldrich) solution in 50 mM potassium phosphate buffer (PPB, pH 6.0); 0.35-0.40 mg of 1.4 and 2.8 mm ceramic beads (Ozyme) were added. Each sample was then vortexed for 10 s, centrifuged at 13,000 × g and 4°C for 10 min, and then transferred to a 96-well plate. To assay MPO activity, 50 µl of each aliquot was mixed with 200 µl of 50 mM PPB (pH 6.0) containing 0.167 mg/ml of o-dianisidine-dihydrochloride (Sigma-Aldrich) and 0.0005% hydrogen peroxide ($H_2O_2$, Sigma-Aldrich). The colorimetric reaction was measured by reading absorbance at 405 nm with a spectrophotometer (Infinite M200, Tecan) at two-time points: immediately and after 1 h. MPO activity was characterized by comparison with a standard (MPO activity of human polymorphonuclear leukocytes, Merck Chemicals) and then expressed in units/mg of tissue. One activity unit represents the conversion of 1 µM of $H_2O_2$ to water in 1 min at room temperature. To measure cytokine levels, 25 µl of each aliquot or 25 µl of serum were transferred to a 96-well plate. We quantified concentrations of IFN-γ, IL-5, TNFα, IL-2, IL-6, IL-4, IL-10, IL-9, IL-17A, IL-17F, IL-21, IL-22, and IL-13 using a cytometric bead array system, the Mouse Th Cytokine Panel (13-plex; BioLegend), in accordance with manufacturer instructions.

**Lymphocyte populations in the spleen and mesenteric lymph nodes**

In the second trial, cell suspensions were obtained by mechanically extruding the spleen and MLNs using the plunger end of a syringe and a 75-µm nylon cell strainer (BD). Cells were washed through the strainer using 1 ml of Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (PS, Lonza). The red blood cell lysing buffer Hybri-Max (Sigma-Aldrich) was used to lyse the erythrocytes present in the cell suspension isolated from spleen, in accordance with manufacturer instructions. For each sample, aliquots of 106 cells were transferred to two 96-well plates (Greiner). Following standard protocols, cells were stained with anti- CD4-FITC, anti-CD3e-PE, anti-T-bet-APC, and anti-Gata3-PerCP as well as with anti-CD4-FITC, anti-CD3e-PerCP, and anti-Foxp3-PE, both stainings were performed in the presence of CD16/CD32 (all products came from eBioscience) to avoid unspecific staining. In brief, the cells were washed with PBS and incubated for 30 min with 0.5 µg of purified anti-mouse CD16/CD32 and surface antibodies (anti-CD4, anti-CD3) in PBS with 10% FBS and 1% sodium azide (Sigma-Aldrich). Intracellular staining was performed as follows using the Foxp3 Transcription Factor Staining Buffer Kit (eBioscience) in accordance with manufacturer instructions. Briefly, samples were washed with PBS and incubated for 20 min with a permeabilization/fixation buffer. They were then stained with intracellular antibodies (anti-T-bet-APC and anti-Gata3-PerCP or anti-Foxp3-PE) in permeabilization buffer over a period of 30 min. Samples were subsequently washed in permeabilization buffer, resuspended in PBS, and analysed using an Accuri C6 cytometer (BD). The data obtained from the cytofluorimetric analysis were processed using CFlow Sampler software (BD).

**Gastrointestinal tract permeability**

In the second trial, 0.6 mg/g of fluorescein isothiocyanate-dextran 4 (FITC-dex 4; Sigma-Aldrich) dissolved in PBS was administered intragastrically to each mouse. Blood samples were collected after 3.5 h as described above, and 80 µl of serum was transferred to a 96-well black plate (Greiner). The concentration of FITC-dex 4 was determined using fluorescence spectrophotometry (excitation: 488 nm; emission: 520 nm; Infinite M200, Tecan); serially diluted FITC-dextran was the standard (range: 0–12,000 µg/ml).

**Statistics**

Statistical analyses were performed using GraphPad (GraphPad Software, San Diego, CA, United States). Survival curves analyses have been performed by Logrank test (Mantel Cox). For weight curves, a multiple unpaired T-test was performed per day with fewer assumptions corrected for multiple comparison with Holm-Sidak method. Normality and variance analysis were performed using Shapiro-Wilk normality test and one-way ANOVA (Brown-Forsythe test), respectively. For normal samples (Gaussian distribution) with equal variances two-way ANOVA has been performed to compare the effect of the strain and the dose for the first trial and of the sex and the dose for the second trial; multiple comparisons were carried out using Tukey's test. For non-normal samples or/and with unequal variances non-parametric tests have been performed inside the groups (Kruskal-Wallis test); multiple comparisons were carried out using Dunn's test. P value $< 0.05$ were considered statistically significant.

Chapter 5 – **A VERSATILE NEW MODEL OF CHEMICALLY INDUCED CHRONIC COLITIS USING AN OUTBRED MURINE STRAIN**



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**CD1 mice are susceptible to DNBS-induced chronic colitis**

To design a murine model that will better predict results in humans, it is crucial to consider the real-life context of the target disease. IBD, including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by an abnormal activation of the gut immune system, which results in local chronic inflammation. Throughout their lives, patients with these diseases display active and inactive phases of variable duration that result in successive periods of relapse and quiescence. A good murine model must account for these disease dynamics. To trigger immune-mediated inflammation, it is possible to use haptenating agents, chemical compounds typically dissolved in ethanol. The ethanol allows the compounds to pass through the mucosal barrier. They then act upon either autologous or microbial proteins in the colon, rendering them immunogenic and thus provoking the abnormal activation of the immune system (Wirtz *et al*., 2007). As mentioned above, DNBS is one of the most common haptenating agents (Martin *et al*., 2017b); it consistently induces chronic inflammation (Martin *et al*., 2014a). Most murine models of colitis use inbred strains, such as C57BL/6JRj (Black-6), and only employ males or females with the purported goal of limiting bias. However, this approach makes it problematic to transfer results to humans because representation of natural diversity in the mouse population is poor. Here, we wished to develop a more realistic murine colitis model, and we thus focused on three improvements to classical models: (i) mimicking the chronic nature of the disease; (ii)

accounting for normal genetic variability by using outbred mice; and (iii) employing both female and male mice. More specifically, we used DNBS to chemically induce chronic inflammation in females and males of an outbred murine strain (RjOrl:SWISS [CD-1]) following the protocol described in Figure 5.1.

In our first trial, we compared inflammation patterns in CD-1 and Black-6 mice; the latter is the inbred murine strain traditionally used in colitis models. We induced initial inflammation and then relapse by sequential injections of 200 mg/kg of DNBS; the dosage was thus mass calibrated. We observed that, although CD-1 mice were heavier than Black-6 mice (mean body mass: 29.9 g and 20.1 g, respectively), they were also more sensitive to inflammation in a significant way as observed in the survival curves (p value = 0.0048, Log-rank test). In fact, the mortality rate was 5% for Black-6 mice (0% for females and 10% for males) and 45% for CD-1 mice (50% for females and 40% for males) (Figure 5.2A). Survival curves analyses using Log-rank test also showed that the differences were also significant when sex differences were considered (p value = 0.03). Nevertheless, no statistical significant sex-related differences were found inside the different strains (p value = 0.7 and 0.3 for CD-1 and Black-6 mice, respectively), supporting the accurateness of pooling female and male individuals. The pattern was the same when evaluating body mass (Figures 5.2B-D). CD-1 mice lost more body mass after the first and second injections than did Black-6 mice, being this effect more persistent during reactivation (Figure 5.2C). This effect was stronger in CD-1 females than in CD-1 males, indicating they are more sensitive to DNBS-induced colitis (Figures 5.2B,C). In Black-6 mice, the pattern was reversed: females lost less body mass than did males (Figures 5.2C,D). Two-way ANOVA analyses of inflamed mice showed the presence of strain effect (p value = 0.0002) as well as interaction between sex and strain factors (p value = 0.0021), confirming the differences observed. Furthermore, a delay at the beginning of the recovery period was observed in CD-1 mice, while Black-6 mice started to recover at days 2-3, CD-1 mice began at day 4 (Figures 5.2B,D). Three days after the second DNBS injection, all the mice were

sacrificed, and their colons were recovered for sampling and scoring. The macroscopic scores, which took into account the presence of ulcers, adhesions, hyperemia, altered transit, and colon wall thickness, provided complementary evidence that CD-1 mice were more sensitive than Black-6 mice to inflammation (Figure 5.3A). The sex-specific patterns in macroscopic scores mirrored those seen for body mass: CD-1 females had higher scores than did CD-1 males, indicating greater sensitivity, and Black-6 females had lower scores than did Black-6 males, indicating lesser sensitivity or a failure of colitis induction (see next paragraph). Histological scoring yielded similar results (Figure 5.3B). Of note, levels of eosinophils were higher in CD-1 mice than in Black-6 mice (Figures 5.3C,D). Traditionally, the dosage of the haptenating substance is based on body mass. However, because Black-6 females and males differed dramatically in mass (mean body mass: 17.8 and 22.3 g, respectively), this approach may have been inappropriate. Black-6 females received lower doses of DNBS because of their lighter mass, and that dose might have been too low to trigger inflammation. Nonetheless, it is difficult to conclude if the lack of inflammation was due to the low DNBS dose and/or to a possible difference in sensitivity between females and males. However, significant sex-specific differences in body mass were also observed in CD-1 mice (mean body mass for females and males: 27.6 and 32.2 g, respectively), and females were clearly more sensitive than males to inflammation. Because standard deviation values were not very large, it was possible to pool females and males for most of the characteristics analysed (Figures 5.2C, 5.3A,B).

Ultimately, one of the goals of murine colitis models is to test the efficacy of candidate anti-inflammatory agents, including probiotics. Using the model described here, we would expect effective treatments to result in an improvement in inflammation-related symptoms. More specifically, mortality rates should decline, body mass should recover more quickly, and macroscopic and histological scores should be lower. The degree of improvement would be proportional to agent efficacy, but it would not necessarily reveal the mechanisms involved. An important caveat is that the underlying mechanism for DNBS-induced colitis is abnormal

stimulation of the immune system. As a result, the model is not suitable for testing certain anti-inflammatory agents. For instance, probiotics, molecules or others that provide functional benefits unrelated to inflammation, such as excluding pathogens or modulating metabolic processes, could not be properly tested using this model.

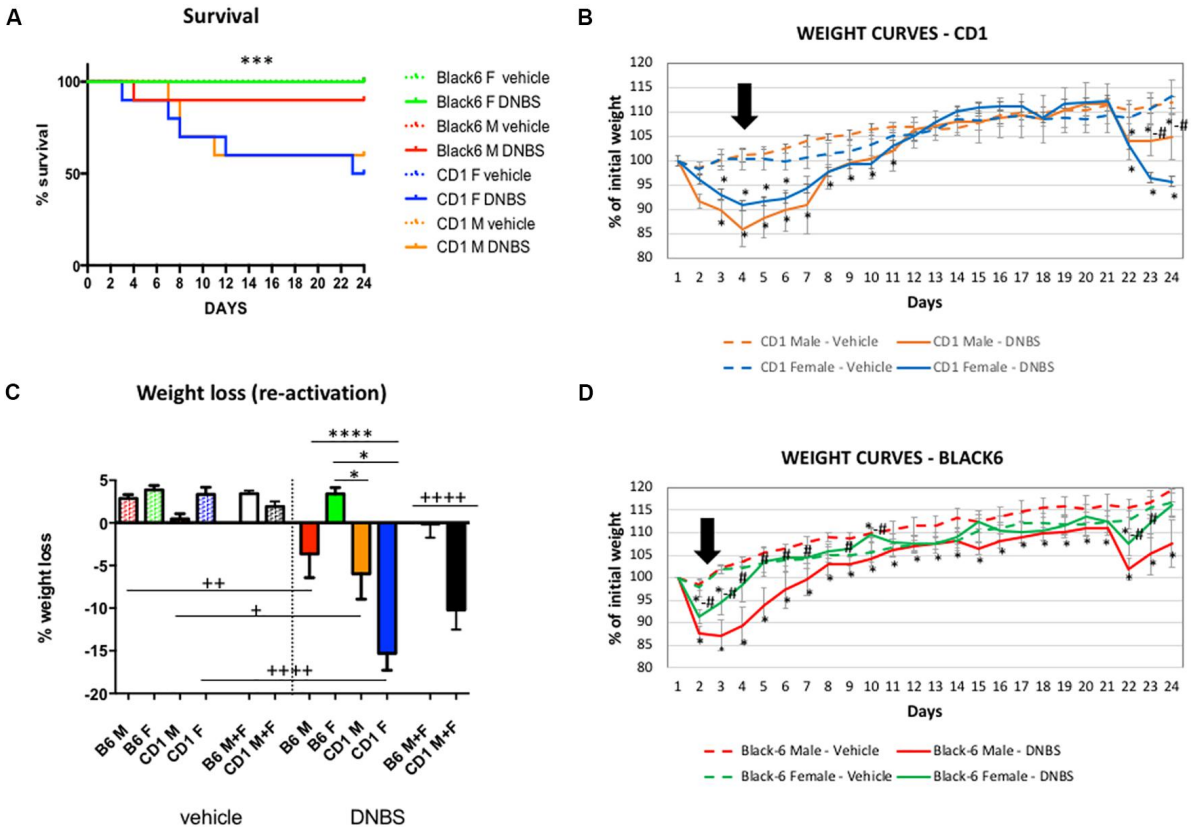


**Figure 5.2. Survival rate (A) and body mass trends in CD-1 (B) and Black-6 (D) mice, and loss of body mass after second DNBS injection (C).** For the survival rate analysis Logrank test (Mantel Cox) was performed. For weight curves, a multiple unpaired T-test was performed per day with fewer assumptions corrected for multiple comparison with Holm–Sidak method, (∗) indicates significance vs. vehicle group and (#) significance between female and male individuals in DNBS treated groups. n = 10; p value < 0.05. For the weight loss analyses, due to the lack of uniform variances when included the vehicle groups, two-way ANOVA was performed only in inflamed groups with strain and sex as factors followed by a Tukey test (results indicated as ∗). In order to compare the effect of the DNBS vs. the vehicle groups, a non-parametric Kruskal–Wallis test followed by a Dunn's test was performed inside CD-1 and Black 6 groups separately (results indicated as +). n = 10; *: p value < 0.05; ****: p value < 0.0001; +: p value < 0.05; ++: p value < 0.01; ++++: p value < 0.0001. The black arrows indicate the moment when mice started to recover weight after the first DNBS injection. B6M, Black-6 males; B6F, Black-6 females; CD1M, CD-1 males; CD1F, CD-1 females; B6 M+F, Black-6 mice; CD-1 M+F, CD-1 mice.

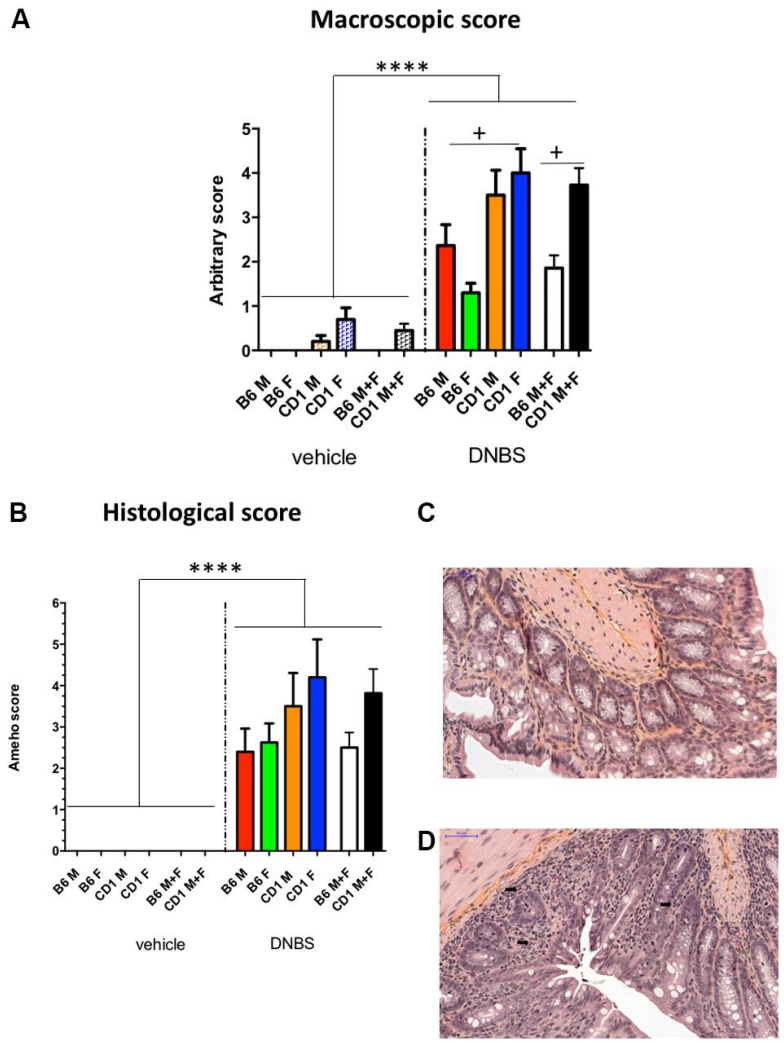


**Figure 5.3. Macroscopic (A) and histological scores (B) and representative images of Black-6 mice (C) and CD-1 mice (D).** As both scores do not follow a Gaussian distribution, in order to compare the effect of the DNBS vs. the vehicle groups, a non-parametric Kruskal–Wallis test followed by a Dunn's test was performed inside CD-1 and Black 6 groups separately (results indicated as ∗). The same test was performed for testing differences among inflamed groups (results indicated as +). n = 10; ****: p value < 0.0001; +: p value < 0.05. The black arrows indicate eosinophils. B6M, Black-6 males; B6F, Black-6 females; CD1M, CD-1 males; CD1F, CD-1 females; B6 M+F, Black-6 mice; CD-1 M+F, CD-1 mice.

**Chronic colitis severity in CD-1 mice can be modulated using DNBS dosage**

Once we had verified that CD-1 mice were good candidates for developing a murine model of chronic colitis, we performed a second experiment in which we modulated DNBS dose to obtain different degrees of colitis severity. This experiment allowed us to better characterize the model and to gather data that, in future studies, will clarify the appropriate DNBS dosage depending on agent type, presumed agent efficacy, and target disease. We tested three different doses of DNBS – 1.5, 2.5, and 3.5 mg per mouse – based on findings from comparative experiments with Black-6 mice in the first trial, where a dose of around 4 mg per mouse resulted in a high mortality rate. Furthermore, the doses were not calibrated for body mass because the results from the first experiment showed that smaller doses might not produce sufficient inflammation, and that there are probably sex-related differences in DNBS sensitivity.

In the second experiment, mortality rates were lower: only two female mice, given a dose of 3.5 mg, died. As expected, after both DNBS injections, a dose-dependent effect on body mass was observed (Figures 5.4A,B). It is worth noting that loss of body mass was similar in females and males given the same dose. Taken together, these results suggest that CD-1 females are more sensitive to severe and severe-to-moderate inflammation (50% mortality at 4 mg of DNBS and 20% mortality at 3.5 mg); however, this sensitivity was not manifest when inflammation was moderate or low. A similar dose-dependent effect was seen in the macroscopic and histological scores (Figures 5.4C,D). Furthermore, the observed standard deviations were small, indicating that both sexes could be pooled in analyses.

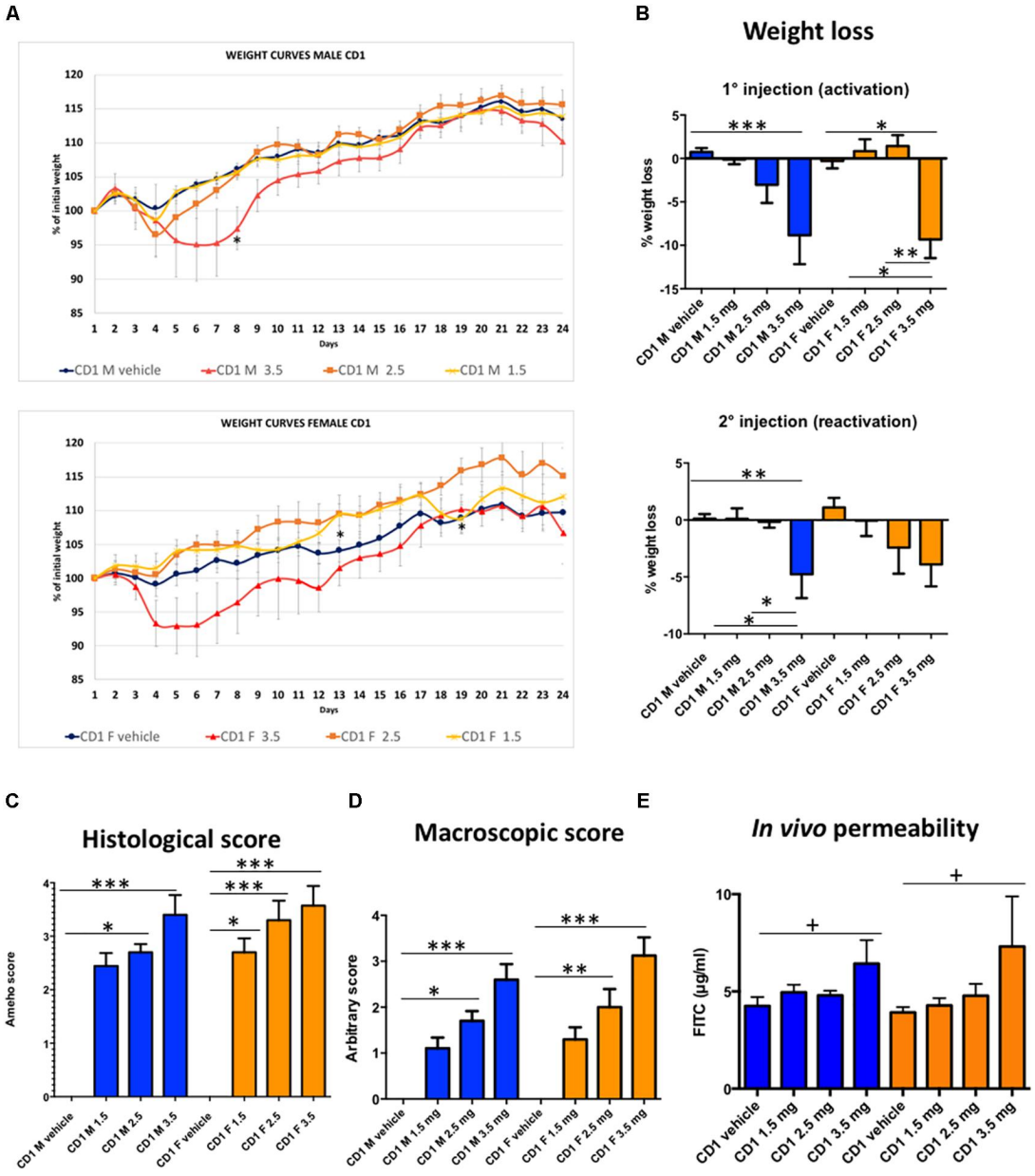


**Figure 5.4. Response of CD-1 mice to colitis induced by different doses of DNBS.** Body mass trends (A), loss of body mass after the first DNBS injection and the second DNBS injection (B), macroscopic (C) and histological scores (D) and in vivo permeability (E). For weight curves, a multiple unpaired T-test was performed per day with fewer assumptions corrected for multiple comparison with Holm–Sidak method, (*) indicates significance vs. vehicle group. n = 10; p value < 0.05. No Gaussian data comparisons (loss of body mass and macro and histological scores) were performed using a non-parametric Kruskal-Wallis test followed by a Dunn's test (results indicated as *). For the permeability analyses, two-way ANOVA was performed with dose and sex as factors followed by a Tukey test (results indicated as +). CD-1 males (M, in blue) and CD-1 females (F, in orange). n = 10; *: p value < 0.05; **: p value < 0.01; ***: p value < 0.001; +: p value < 0.05.

**DNBS-induced chronic colitis in CD-1 mice modifies intestinal permeability, colon cytokine levels, and lymphocyte populations in the spleen and mesenteric lymph nodes**

As mentioned above, this murine model can be useful in two ways. First, the model's general metrics such as loss of body mass, macroscopic scores, and histological scores could reveal the efficacy of potential treatments (e.g. anti-inflammatory compounds or probiotic strains). Second, the model could also help decipher the mechanisms underlying any positive effects. Because our model induces colitis using DNBS, it is best suited for examining immunomodulatory properties. For example, DNBS-provoked inflammation in Black-6 mice appears to arise from such mechanisms as altered gut barrier permeability and the activation of specific immune responses (Martin *et al*., 2014a). Consequently, this model could be used by researchers to study the specific effects of candidate anti-inflammatory agents on gut permeability and the immune system. Nevertheless, it is not possible to describe the expected results to be obtained when testing an anti-inflammatory agent as they will depend on their mechanisms of action. Such permeability alterations are also present in CD-1 mice with DNBS-induced colitis (Figure 5.4E). Dysfunction of the epithelial barrier is a hallmark of inflammatory intestinal diseases. GIT permeability can be characterized by orally administering the paracellular tracer FITC-dextran. This technique reveals the degree of colon permeability and has been successfully linked to directly measure alterations in local permeability in colon tissues employing Ussing chambers (Martin *et al*., 2015). In this study, GIT permeability was modified in CD-1 mice challenged with different doses of DNBS (trial 2). Two-way ANOVA analysis showed that there is a dose effect (p value = 0.03), although no sex effect or interaction between both factors have been found (p value = 0.9, respectively). Even if there was a clear response in males and females at the highest DNBS concentration tested (p value < 0.005), basal permeability appears to be high, necessitating a strong dose of DNBS to obtain results (Figure 5.4E). These findings suggest that it may be problematic to use CD-1 mice to characterize permeability using moderate or low-grade inflammation models. However, we

must interpret the results with caution since there is the possibility that permeability might be altered at other points along the GIT.

DNBS-induced chronic colitis might change levels of several cytokines in CD-1 mice (Figures 5.5, 5.6), confirming the generally dose-dependent nature of the inflammation response. We observed IL-9, IL-10, IL-17A, TNFα, IL-2, IL-17F, IL-6, and IL-4 variations in the colon samples (Figure 5.5) and TNFα and IL-6 in the serum samples (Figure 5.6). Two-way ANOVA analyses revealed dose-dependent responses for IL-2, IL-9, IL-10, TNFα IL-6, IL-17A, and IL-17-B and sex influence on IL-4, IL-10, and serum IL-6 ($p$ value $< 0.005$). IL-10 is an important immunoregulatory cytokine that reduces inflammation by suppressing the exaggerated mucosal immune response in the colon (Schreiber *et al*., 2000), thus preserving the mucus barrier (Hasnain *et al*., 2013), and represent a cytokine of reference in almost all murine colitis models. Similarly, we observed a decline in IL-9, IL-2, and IL-4 levels. IL-9 controls intestinal barrier function (Gerlach *et al*., 2015), IL-2 is a potent inducer of T-cell proliferation and drives T-helper 1 (Th1) and Th2 effector T-cell differentiation (Hoyer *et al*., 2008), and IL-4 has exhibit anti-inflammatory properties through stimulation of alternative macrophages (M2s). In contrast to classical macrophages (M1s), M2s participate in a Th1-polarized response and enhance the production of pro-inflammatory cytokines, ultimately counteracting inflammation and promoting tissue repair (Goerdt *et al*., 1999; Gordon, 2003; Mantovani *et al*., 2007). In this sense, neutrophil activation, measured by myeloperoxidase (MPO) activity, reveal a week activation of neutrophils as MPO activity was similar for CD-1 mice that those in Black-6 mice treated with low doses of DNBS (Martin *et al*., 2015; data not shown). As neutrophils are involved in inflammation, macrophage recruitment and M2s differentiation this result point also for a slight Th1 response in CD-1 mice. On the other hand, we saw an increase in IL-17A, IL-17F, IL-6, and TNFα, underscoring that pro-inflammatory responses were occurring as well (Gabay, 2006; Bradley, 2008; Jin and Dong, 2013). The results for IFN-γ highlight that mouse strain matters: IFN-γ is a pro-inflammatory cytokine that plays a central role in DNBS-induced

inflammation in Black-6 mice (Martin *et al*., 2014a), but that seemed to have the opposite effect in CD-1 mice (Figures 5.5, 5.6). Consequently, it appears that DNBS and TNBS can elicit a Th1-mediated immune response (Randhawa *et al*., 2014) but that model functionality may vary depending on the host species and its genetic background (Mizoguchi and Mizoguchi, 2008; Mizoguchi, 2012). For instance, when treated with these compounds, SJL/J mice displayed a major Th1-mediated response (Neurath *et al*., 1995, 1996), while IFN-γ-/- mice with a Balb/c background showed a Th2-mediated response (Dohi *et al*., 1999). In our study, to identify the major Th cell lines involved in the response of the CD-1 mice, T-cells from the spleen and MLNs were isolated and analysed using flow cytometry. Several differences were found between male and female mice (Figure 5.7). CD-1 males had a weak response, and a slight increase in CD3/CD4 cells in both the spleen and MLNs was observed.

CD-1 females had a different, stronger response: CD3/CD4 cells decreased in the spleen and increased in the MLNs (Figure 5.7). Furthermore, CD-1 females had diminished Th2 and Treg levels in both the spleen and MLNs (revealed by GATA-3 and Fox-p3 staining, respectively). In contrast, males displayed slightly increased Th2 levels in the spleen alone (Figure 5.7). These results, taken in tandem with the high levels of eosinophils (Figure 5.3) suggest that the Th2 response played a major role in CD-1 mice. The Th1 response, measured using T-bet staining, was not strong enough to be detected (data not shown). These findings indicate that, in future studies, it may be better to use females when testing for immunomodulation by candidate probiotics *in vivo*, especially if *in vitro* trials indicate that the mechanism of action involves changes in IL-10 production; IL-10 is produced by Treg cells, among others. However, it is necessary to broadly examine cytokine production and lymphocyte levels to fully clarify the mechanisms of action of any anti-inflammatory agent.

Overall, our findings allow us to recommend this model to test anti-inflammatory agents, including probiotics. We strongly recommend performing the experiment at least in duplicate with a minimum of 10 mice per group. Nevertheless, this is a minimum, as the efficacy of the

agent tested will determine the number of mice required to have statistical significant results. The use of 3.5 mg seems the better choice, however, as the dose-effect observed is usually animal facility-dependent, a preliminary study to optimize the dose is mandatory.

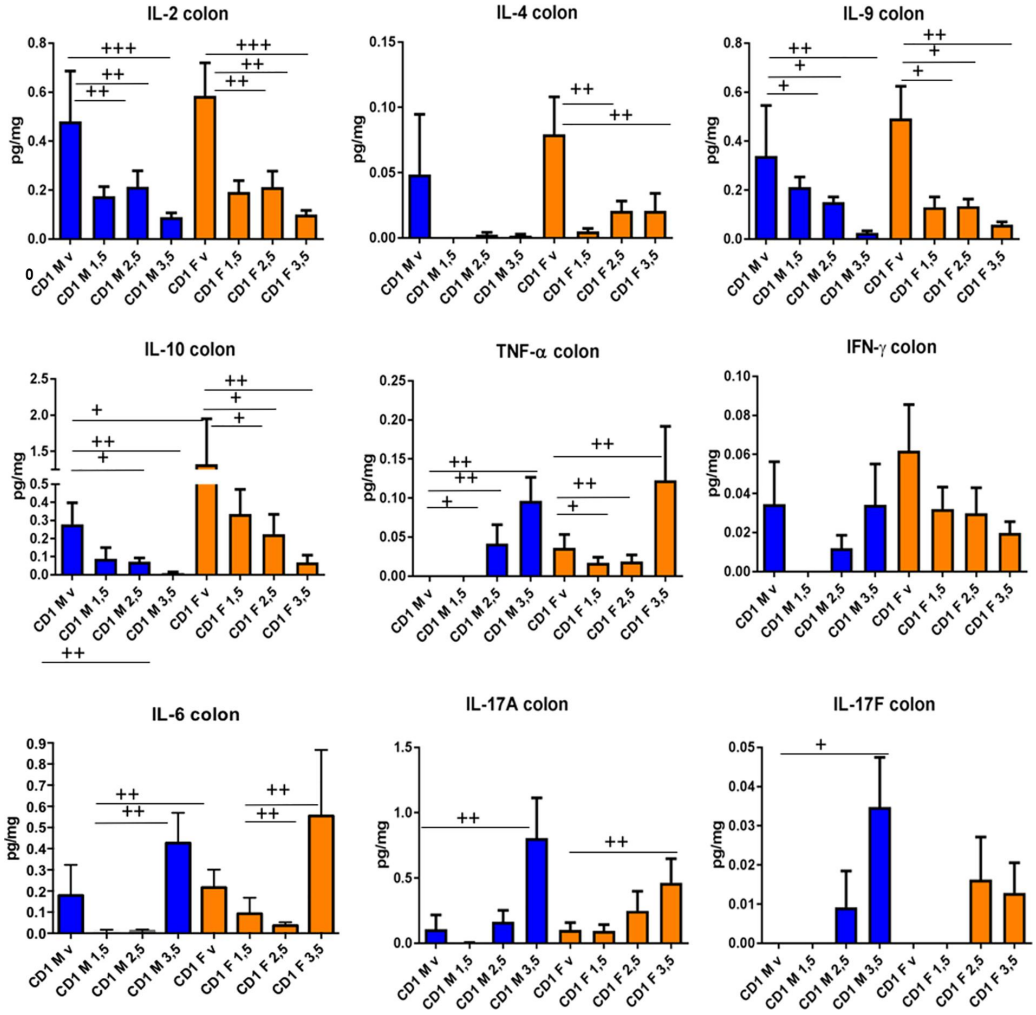


**Figure 5.5. Colon levels of cytokines induced by different doses of DNBS in CD-1 mice.** Analyses were performed by two-way ANOVA with dose and sex as factors followed by a Tukey test. CD-1 males (M, in blue) and CD-1 females (F, in orange) injected with vehicle (v), 1.5 mg (1.5), 2.5 mg (2.5), or 3.5 mg (3.5) of DNBS. n = 10; +: p value < 0.05, ++: p value < 0.01, +++: p value < 0.001.

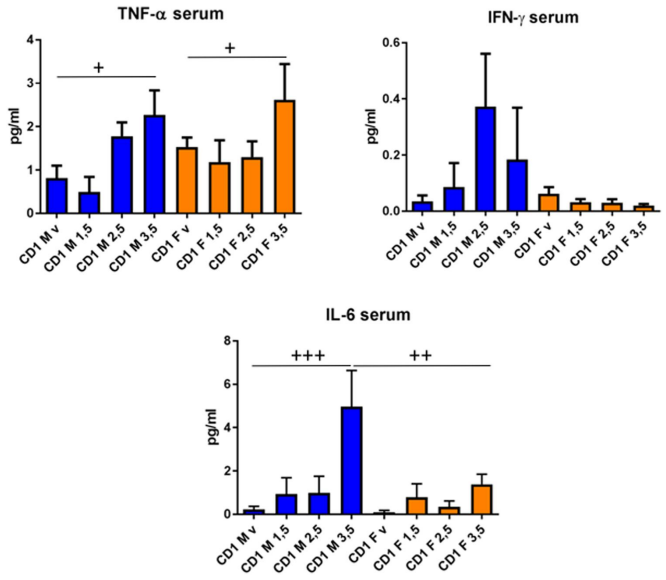


**Figure 5.6. Serum levels of cytokines induced by different doses of DNBS in CD-1 mice.** Analyses were performed by two-way ANOVA with dose and sex as factors followed by a Tukey test. CD-1 males (M, in blue) and CD-1 females (F, in orange) injected with vehicle (v), 1.5 mg (1.5), 2.5 mg (2.5), or 3.5 mg (3.5) of DNBS. n = 10; +: p value < 0.05; ++: p value < 0.01; +++: p value < 0.001.

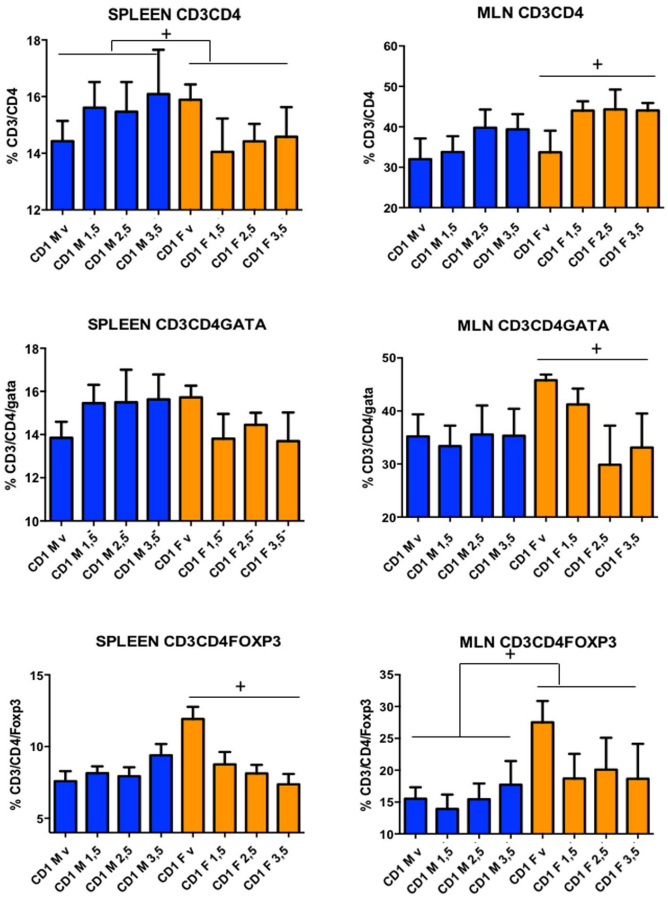


**Figure 5.7. Levels of lymphocytes induced by different doses of DNBS in CD-1 mice.** Lymphocyte populations were characterized using flow cytometry. Analyses were performed by two-way ANOVA with dose and sex as factors followed by a Tukey test. CD-1 males (M, in blue) and CD-1 females (F, in orange) injected with vehicle (v), 1.5 mg (1.5), 2.5 mg (2.5), or 3.5 mg (3.5) of DNBS. n = 10; +: p value < 0.05; ++: p value < 0.01; +++: p value < 0.001.

In the present work, we describe a murine model of chronic colitis in which inflammation was induced by the intrarectal administration of DNBS. This versatile and innovative model involves the use of an outbred murine strain, CD-1, employing both male and female mice. Ultimately, our aim is to make this model available to researchers who are testing the efficacy of anti-inflammatory agents, including probiotics (mainly NGP), with immunomodulatory properties. The model could also serve to identify the potential mechanisms of action of the anti-inflammatory agent. Indeed, our goal is to provide the scientific community with a realistic alternative model to test the efficacy of anti-inflammatory agents, such as candidate probiotics – a model that can be customized based on agent type and target disease. We showed that it is possible to use an outbred murine strain without reproducibility problems, and that females and males can be grouped to yield more representative results for some features. However, combining results for the two sexes should be performed with caution, as evidence of sex-specific sensitivity to severe inflammation protocols, as well as sex-specific differences in some of the measured characteristics were observed.

# Chapter 6 – **CONCLUDING REMARKS**

The present work focused on the exploration of gut microbiome variations throughout the human life, with the aim of shedding light especially on the functional aspects related to eubiosis and dysbiosis in Western diseases, and on the development of a versatile murine model of intestinal inflammation to better explore the transition towards dysbiotic layouts.

In order to provide some glimpses on the functional changes that occur in the human gut microbiome across life up to extreme longevity, we characterized by shotgun sequencing the fecal microbiomes of centenarians and semi-supercentenarians in comparison to younger individuals. The obtained profiles showed a peculiar remodeling in microbial gene functions along with the ageing process, characterized by an increased potential for xenobiotic degradation and a rearrangement in metabolic pathways related to carbohydrate, amino acid and lipid metabolism. Being particularly exacerbated in later stages of life, these microbiome features probably reflect the progressive changes occurring in diet and lifestyle along with ageing in Western urban areas.

Tracing back to aspects related to human evolution and dietary habits, we unravel the potential multiple health benefits of adopting a modern Paleolithic diet (MPD) to modulate the Western gut microbiome towards a more 'ancestral' configuration, counterbalancing the effects of diets low in fiber while rich in industrialized and processed foods. Interestingly, the gut microbiome of individuals following MPD were characterized by an unexpectedly high degree of biodiversity, which well approximates that of traditional populations, suggesting the contribution of this peculiar dietary intervention in partially rewild the microbial ecosystem – a hallmark of healthy gut.

Focusing on the dysbiotic variations associated with non-communicable diseases (NCDs), we investigated the link between diet, intestinal microbiome and obesity in Western cohorts. Considering the increasing prevalence of obesity in children and the related risks of developing

cardiovascular risks factors during adulthood, we aimed at identifying early markers and individual microbiome-host-diet configurations as a potential predictor related to the onset of the disease during childhood, through a 4-years prospective study.

While many adult individuals manage to maintain a healthy weight, obese individuals have been shown to have a preference for energy-dense palatable and fattening foods, ultimately considered to be addictive. Moreover, similarities between some feeding and eating disorders and substance-use disorders (SUDs) have been acknowledged, including the experience of cravings, reduced control over intake, increased impulsivity and altered reward-sensitivity. With the aim of unravel specific bacterial groups and metabolic activities potentially involved in the development of obesity and possibly related to food addiction, we characterize the microbiome configurations of 100 women, stratified according to body mass index (BMI) and the severity of food addiction. Metagenomics and metatranscriptomic approaches were adopted for describing obese-related GM layouts at species level, as well as providing functional information on the active fraction of the microbial ecosystem.

The transition towards a dysbiotic microbiome structure and its association with various diseases have made the microbiome a strategic therapeutic target, laying the basis for the development of a wide range of microbiome-tailored intervention strategies aimed at restoring eubiotic, health-promoting layouts. Considering the suitability of murine colitis models as tools for better understanding intestinal homeostasis and inflammation, we developed a versatile murine model that reflects the high heterogeneity of genetic diversity and sex-related differences observed in humans. In particular, we chemically induced colon inflammation in an outbred strain of female and male mice (RjOrl_SWISS [CD-1]), mimicking the chronic nature of colitis forms as those occurring with infalammatory bowel disease (IBD). The development of this murine model may provide researchers with a versatile tool for studying the role of the gut microbiome in the onset and progression of NCDs, as well as for testing and validating

candidate anti-inflammatory agents and/or new microbiome modulators such as classic or next-generation probiotics, before their use in clinical practice.

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Chapter 8 – **ACKNOWLEDGEMENTS**

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## Chapter 9 – **LIST OF AUTHOR'S PUBLICATIONS**

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