Università degli Studi di Cagliari

PhD DEGREE

Molecular and Translational Medicine

Cycle XXXIII

TITLE OF THE PhD THESIS

Gut microbiota alterations associated with obesity and impact of a weight-loss intervention based on a hypocaloric balanced diet

Scientific Disciplinary Sector(s)

MED/07

PhD Student:	Silvia Pisanu
Supervisor	Prof.ssa Roberta Loddo
Co-Supervisor	Prof. Aldo Manzin

Final exam. Academic Year 2019 – 2020
Thesis defence: January 2021 Session


Silvia Pisanu gratefully acknowledges Sardinian Regional Government for the financial support of her PhD scholarship (P.O.R. Sardegna F.S.E. –Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2014-2020- Axis III Educational and training. Thematic goal 10. Investiment Priority 10ii), Specific goal 10.5.

Index

Legend of Figures

Legend of Tables

Acknowledgements

I would first like to thank my advisor, Prof. Aldo Manzin, for providing me the opportunity to join his team as a Ph.D. student and for including me in several research projects. I also thank him for the supervision and for the revision of my Ph.D. thesis.

I would also like to express my warmest thanks to Prof.Fernanda Velluzzi and Prof. Andrea Loviselli for the opportunity to conduct this research project, which was based on the collaboration with the Obesity clinic, and mostly for all the support received during the Ph.D. and before. I thank them both for making themselves always available for any help or suggestion.

I would also like to acknowledge the large contribution of Dr.Vanessa Palmas and Dr. Veronica Madau, with which I have worked side by side during these years. I am grateful for the cooperation and the mutual help.

My sincere thank also goes to all the staff of the Obesity Clinic, for the help in patients' recruitment, and in particular to the nutritionist, Dr. Andrea Deledda, and to the nurse Elisabetta Loi. I would also like to thank the technician Roberto Murru for the assistance in the recruitment of healthy controls.

I also thank my Ph.D. colleague and friend Emanuela Casula for the help with data collection and above all for the laughs and the goodtime spent together.

During my Ph.D., I was hosted at the International Agency forResearch on Cancer for a period of eight months. Therefore, I would like to thank my supervisor, Dr. Inge Huybrecths, for everything I learned during the period spent abroad, and for all the opportunities of networking and personal growth.

I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement throughout my years of study and my life in general.

I also wish to thank my best friends for the unconditional support. You were there for me during the Master, the Bachelor, and for many years before the starting of the university. So the time has come to thank you for growing up with me, for being a constant presence in my life, for the understanding and complicity.

I would also like to thank Sara for our precious friendship,which becomes more and more solid year after year, and for being, literally, always, there for me.

Finally, I would like to thank Diego for being, without any doubt, the best part of these three years.

Abstract

Several scientific pieces of evidence confirm that the metabolic activity of the intestinal microbiota can play an important role in the pathogenesis of obesity. Here, the gut microbiota (GM) from overweight/obese patients (OB) of Sardinian origin was characterized and compared to normal-weight controls from the same geographical area (NW), matched for sex, age and smoking status. Fecal samples were analyzed by Illumina MiSeq sequencing of the 16S rRNA gene. The gut microbial community of OB patients exhibited a significant decrease in the relative abundance of several Bacteroidetes taxa (i.e. Flavobacteriaceae, Porphyromonadaceae, Sphingobacteriaceae, *Flavobacterium, Bacteroides* spp.) when compared to NW; instead several Firmicutes taxa were significantly increased in the same subjects (Lachnospiraceae, Gemellaceae, Streptococcaceae, *Gemella*, *Megamonas*, *Mitsuokella*, *Streptococcus*, *Eubacterium* spp., *Ruminococcus* spp., *Megamonas* spp., *Megasphaera* spp. and *Veillonella* spp.). Correlation analysis indicated that body fatness negatively correlated with Bacteroidetes taxa, while Firmicutes taxa positively correlated with body fatness and negatively correlated with muscle mass and/or physical activity level. Furthermore, the relative abundance of several bacterial taxa belonging to Enterobacteriaceae family, known to exhibit endotoxic activity, was increased in the OB group compared to NW. A secondary aim of the study was to evaluate the impact of a moderately hypocaloric Mediterranean diet, lasted three months, on the GM of OB patients. After nutritional intervention, patients presented a reduction in body weight and fat mass, along with changes in the relative abundance of many microbial patterns. In fact, an increase in the abundance of several Bacteroidetes taxa and a depletion of many Firmicutes taxa were observed. In addition, the genus *Sutterella*, within Proteobacteria, decreased after the intervention. The present study extends our knowledge of the GM profiles in OB, highlighting the potential benefit of moderate caloric restriction in counteracting the gut dysbiosis.

1. Introduction

1.1 The human microbiota

The human body is colonized by a vast number of microbes, collectively referred to as the human microbiota. The human microbiota consists of the 10-100 trillion symbiotic microbial cells harbored by each person and includes bacteria, viruses, archaea, protozoans, and fungi (1). This community contains at least 1000 different species of known bacteria and carries 150 times more microbial genes than are found in the entire human genome (2,3).

This community co-evolved with the host over thousands of years to establish an intricate and mutually beneficial relationship (4). In fact, this collection of microorganisms holds functional features that are not presented in humans. The microbiota is intimately involved in numerous aspects of normal host physiology, from nutritional status to behavior and stress response (5). The human microbiota, especially the gut microbiota (GM), has even been considered to be an "essential organ"(6), due to its crucial role in development and homeostasis in adult life.

The term "microbiota" refers to the microbial taxa associated with humans, while "microbiome" is the catalog of these microbes and their genes (1). In common usage, the term microbiome has evolved, reflecting the state of the identification methods. Initially, all characterization of the various microbial members was performed through classical microbiological methods: culturing through a series of selective and differential media, re-culturing of colony isolates, standardized biochemical tests on isolates, and observation of the isolates under the microscope. These methods allow us to obtain information on the types of microbiota but do not characterize the genomics. This goal can be achieved by current PCR-based amplification and sequencing methods (7). The availability of novel tools,

primarily next-generation sequencing (NGS), enables to examine the entire genomic content of the microbial community, including those organisms that are uncultivable (8).

The most popular approach consists in targeting the bacterial 16S ribosomal RNA (rRNA) gene since this gene is present in all bacteria and archaea and contains nine highly variable regions (V1–V9), which allows species to be easily distinguished (4). The sequencing of 16S rRNA gene amplicons is a cost-effective method, able to sufficient resolution (9). However, amplicon sequencing typically only resolves the taxonomic composition of the gut microbiome, without providing direct evidence of the biological functions associated with the gut microbial community (9). Thus, recently developed computational approaches, such as implemented in "Phylogenetic Investigation of Communities by Reconstruction of Unobserved States" (PICRUSt) (10), were successfully employed to infer the community's functional potential by bridging 16S rRNA gene information with reference genomes (9).

Microbiota's composition and function differ according to different locations in the human body (3) and to several characteristics of the host, including age, sex, race, and lifestyle factors (11).

1.2 Microbiota in the gastrointestinal tract

The GM is the community of microorganisms colonizing the gastrointestinal tract, including bacteria, viruses, archaea, protozoans, and fungi (12). The number of microorganisms inhabiting the gastrointestinal (GI) tract has been estimated to exceed 10^{14}, which encompasses ~10 times more bacterial cells than the number of human cells and over 100 times the amount of genomic content as the human genome. This complex ecosystem is composed of approximately 300 to 500 bacterial species (13,14).

The concentration of microbiota increases steadily along with the GI, with small numbers in the stomach, but very high concentrations in the colon (Figure 1.1). In fact, the stomach and proximal duodenum are exceptionally inhospitable, and very few bacteria are resistant to the acidic condition found in these districts (15).

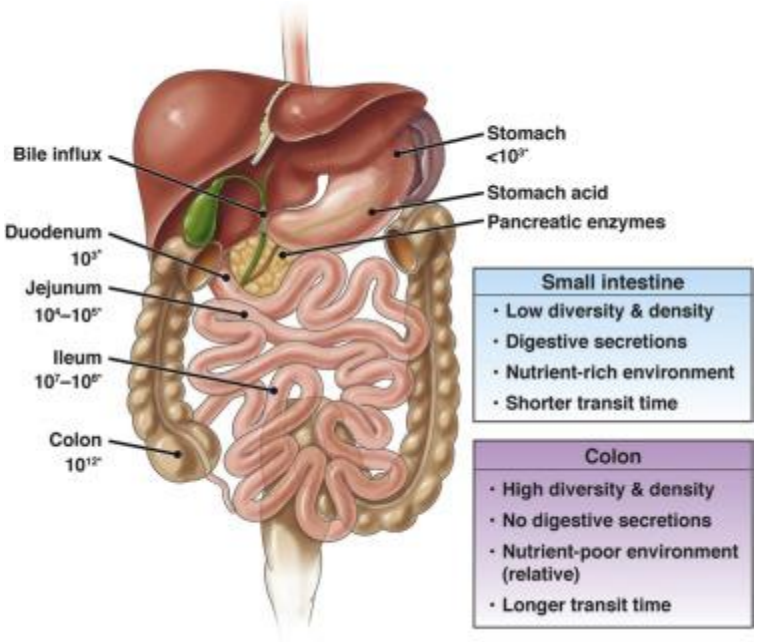

Figure 1.1. Overview of the bacterial colony density through the digestive tract and distinguishing characteristics of the small intestine and the colon (16).

1.2.1 The stomach

In the stomach, gastric acid secretion leads to drastic swings in pH from as low as 2 to neutral, depending on mealtimes and dietary composition (17). The low acid conditions of the stomach provide an important barrier to some pathogens ingested with food and water. For example, *Vibrio cholera* is acid sensitive, necessitating the ingestion of large numbers of viable cells to cause disease (17). However, other microorganisms can tolerate the acidic environment and survive passage through the stomach (e.g. *Salmonella* spp. and *Shigella* spp.). *Helicobacter pylori* is probably the most famous of gastric residents because of its associations with peptic ulcers and gastric cancer (18). Since the discovery of *H.pylori*, a large number of acid-resistant bacterial strains were detected in the stomach, including *Streptococcus*, *Neisseria,* and *Lactobacillus* (19).

In the study of Bik and collaborators, characterizing the bacterial microbiota in the human stomach of healthy subjects, 1056 non-*H. pylori* clones, 127 phylotypes, and five dominant genera (*Streptococcus*, *Prevotella*, *Rothia*, *Fusobacterium,* and *Veillonella*) were identified (20). Similarly, another study investigating the difference in microbial communities among the GI tract identified 262 phylotypes in three *H.pylori*-negative stomach samples. However, most of the prominent phylotypes (e.g. *Streptococcus*, *Actinomyces*, *Prevotella,* and *Gemella*) were also abundant in the throat, suggesting that they may represent swallowed microorganisms from upstream microbiota (21).

In general, the most common bacteria in the stomach are streptococci, followed by lactobacilli (22). At the phyla level, members of Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria, Bacteroidetes, and Gemmatimonadetes have been identified (23).

Several innate defenses (low pH, migrating motor complex and the entero-salivary circulation of nitrate), as well as external factors (diet, *H. pylori* infection, proton pump inhibitors, antibiotics and stomach diseases), have been shown to influence significantly the microbiota composition in the

stomach (23). In addition, an altered composition has been associated with stomach diseases. For example, a significantly higher abundance of the Firmicutes phylum and the *Streptococcus* genus (within Firmicutes) was observed in *H.pylori*-negative patients with antral gastritis (24). Furthermore, it has been shown that gastric cancer patients presented a diversified composition of gastric microbiota, exemplified by the reduction of *Porphyromonas*, *Neisseria*, *TM7* group, *Prevotella pallens*, *Streptococcus sinensis*, and the simultaneous enrichment of *Lactobacillus coleohominis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Lachnospiraceae* (25).

1.2.2 The duodenum

A bacterial density of 10^3 CFU per mL is found in the duodenum (5). In a recent work characterizing the GM from stomach to colon using culturomics and next-generation sequencing, the number of reads in the stomach and the duodenum was significantly lower than in the left colon, thus indicating a lower bacterial abundance in the duodenum. The microbiota of the stomach and the duodenum presented also a lower biodiversity than the ileum, the right, and the left colon. In addition, they also observed a decreased abundance of aero-intolerant species in the stomach and the duodenum than in the ileum, right colon and left colon. At the same time, some taxonomic levels were identified only in the upper gastrointestinal tract, such as *Spirochaetes*, *Deinococcus-Thermus, Tenericutes*, *Acidobacteria*, *Aquificae,* and *Negibacteria* (26). The work of Seekatz et al., analyzing the GM of the stomach, the duodenum, the proximal/mid/distal jejunum, and stool of healthy volunteers, confirmed the low diversity in sites of the upper GI tract, compared with the lower tracts. Furthermore, higher levels of Bacteroidetes species (within the genus *Prevotella*) were detected in the stomach and duodenum, compared to stool. When considering the change of the identified microbial patterns in the duodenum over time in the same subjects, they observed that the relative abundance of *Streptococcus*, *Prevotella*, and *Pasteurellaceae* ssp. fluctuated in all individuals and these fluctuations were associated with pH

(27). Alteration of the duodenum composition has been associated with obesity and celiac disease (28,29).

1.2.3 The jejunum

The jejunum presents a bacterial colony density between 10^4-10^5 CFU per mL (16). This tract is the primary site for the absorption of nutrient-derived carbohydrates, amino acids, small peptides, and vitamins. Because of the required invasive procedures for sample collection and the rapidly fluctuating environmental conditions, the characterization of the microbiota in this area is challenging and less is known about its composition, compared with other GI tracts.

In the work of Sundin and collaborators, jejunum samples from 20 subjects were collected and the microbiota was analyzed by NGS: the jejunal lumen contained a distinctive bacterial population consisting primarily of facultative anaerobes and oxygen-tolerant obligate anaerobes, similar to those found in the oral cavity. However, Enterobacteriaceae were found to be more frequent in the jejunum than in the oral microbiota. Furthermore, no evidence for retrograde movement of the most abundant colonic microbes to the jejunum was found (30).

1.2.4 The ileum

Bacterial population increase from approximately $10^{4–5}$ CFU per mL in the duodenum to $10^{7–8}$ CFU per mL in the distal ileum, where transit slows. Besides, the proportion of gram-positive to gram-negative, as well as facultative anaerobic and strict anaerobic species increases from proximal to distal segments of the small intestine and colon (16). As already discussed for the jejunum, the human small bowel microbiota has been poorly characterized. In a recent study analyzing the ileal bacterial microbiota in 27 elderly Caucasian subjects, the microbiota of the distal part of the human ileum was oral-like and strikingly different from the colonic microbiota. The most abundant phylum in the distal ileum was

Firmicutes, followed by Actinobacteria, and the microbiota was dominated by the facultative anaerobic genera *Streptococcus*, *Actinomyces*, *Gemella*, *Granulicatella*, and *Rothia* (31).

1.2.5 The colon

In the colon, bacterial density reaches 10^{12} CFU per mL (5). Most human gut microorganisms are strictly anaerobic and they belong to the phyla Bacteroidetes, Firmicutes, and Proteobacteria. Other gut bacteria with the minor absolute percentage in the healthy gut (usually below 1%) mostly belong to the phyla Actinobacteria, Verrumicrobia, Acidobacteria, or Fusobacteria (32). At the family level, the colon is characterized by the presence of Bacteroidaceae, Prevotellaceae, and Rikenellaceae (all within the phylum Bacteroidetes), Lachnospiraceae and Ruminococcaceae (both within the phylum Firmicutes) (33).

Interindividual variation within the adult GM is very high. In 2011, Arumugam et al. combined metagenomes from Danish, French, Italian and Spanish, Japanese and American individuals (39 in total) in order to investigate the phylogenetic composition of the GM: they identified three enterotypes by the variation in the levels of one of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) (34). However, none of several measured host properties in the work of Arumagam et al., namely nationality, gender, age or body mass index (BMI), significantly correlates with the different enterotypes.

During the last years, the presence of a specific enterotype in the gut has been further investigated and it has been demonstrated that it is influenced by many factors, including diet, lifestyle factors, and the use of antibiotics (35). However, the concept of enterotype is currently under discussion. In fact, since its discovery, many studies have shown that samples from different enterotypes do not separate into distinct clusters, and, when looking at the relative abundance of *Bacteroides* and *Prevotella*, a

continuous gradient rather than a gap is observed when comparing the samples. Therefore, doubts about the presence of discrete enterotypes have been recently emerged (35).

Despite the high interindividual variation of the GM, it has been established that there is a high overall temporal stability of the microbial community within an individual, in terms of both composition and microbial diversity (4,36). Each individual holds a unique GM profile that plays many specific functions in host nutrient metabolism, modulation of the central nervous system, maintenance of structural integrity of the gut mucosal barrier, immunomodulation, and protection against pathogens (32,37).

1.3 The gut microbiota across age

The development of the microbiota is generally believed to begin from birth (4). Considering the role of the placenta in keeping the fetus sterile, the presence of any bacterium in the uterus is traditionally considered as a potential danger to the fetus (38). In fact, intrauterine growth has been identified as one of the causes of preterm birth (39). However, recent studies on healthy newborns have shown the presence of bacteria in placenta tissue, umbilical cord blood, amniotic fluid, and fetal membranes (38).

A recent study characterized the microbiota of meconium and fecal samples, obtained during the first 3 weeks of life from 14 donors, by using culture and molecular techniques: Bacilli and other Firmicutes were the main bacteria groups detected in meconium while Proteobacteria dominated in the fecal samples. Furthermore, culture technique showed that *Staphylococcus* predominated in meconium and that *Enterococcus,* together with Gram-negative bacteria such as *Escherichia coli*, *Escherichia fergusonii*, *Klebsiella pneumoniae,* and *Serratia marcescens* were more abundant in fecal samples. Taken together, these findings indicated that the meconium had a specific microbiota that differed from that of feces after the first week of life (40).

The composition of the human GM changes dramatically during the first few years of life, with an increase of gut diversity over time. Different factors influence the development of the intestinal microbiota in infants, including the delivery mode, the feeding method (breastfeeding vs. formula), the use of probiotics and antibiotics, and the introduction of complex dietary substrates during weaning (41).

1.3.1 Mode of childbirth delivery

Upon delivery, the neonate is exposed to a wide array of microbes from a variety of sources, including maternal bacteria. Many studies have suggested that delivery mode shapes the microbiota's establishment and, subsequently, its role in child health (42,43).

During a vaginal delivery, the passage through the birth canal affords the neonate a microbiota similar to the mother's vagina, while caesarian-delivered neonates' microbiota resembles the mother's skin and environmental microbes (43). More specifically, the microbiota of vaginal-born infants is dominated by *Bifidobacterium*, *Lactobacillus* and *Bacteroides species* (44), while caesarian-delivered infants harbored bacterial communities similar to those found on the skin surface, dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. (43).

It is not clear whether the diversity and colonization pattern of the GM associated with the caesarian delivery persist during childhood. Results of a recent systematic review showed that specific microbial trends were still present during the first three months of life, but disappeared after 6 months (45). On the other hand, in the work of Salminen et al., bifidobacterial levels in the stool of caesarian delivered children were comparable to those of vaginally delivered children at seven years of age, while the number of Clostridia was significantly higher in vaginally born children (42). Another study observed that infants born by cesarean delivery, compared with infants born by vaginal delivery, had bacterial communities with significantly lower abundances of *Escherichia–Shigella* and absence of *Bacteroides* (46). Previous research has suggested that some species of *Bacteroides* influence the immune systems of their hosts and help to quell inflammation (47), so it can be inferred that the absence of this genus may affect the immune function.

Moreover, the variation in microbial patterns and diversity in the first phase of life, associated with the type of childbirth delivery, can influence the normal physiology and the predisposition to diseases, considering the recognized role of the GM in shaping the immune response (43,48).

1.3.2 Breastfeeding

Human milk contains many hundreds to thousands distinct bioactive molecules that protect against infection and inflammation and contribute to immune system maturation, organ development, and healthy microbial colonization (49).

Over the first 3 years of life, the relatively simple and dynamic GM start to evolve toward an adult state more complex and more stable (50).

Breastfeeding has been shown to influence microbiota composition directly, by providing the substrates for bacterial proliferation and sources of bacterial contamination, and indirectly, by modulating the morphology, cell composition and physiology of the intestinal mucosa, and the pancreatic function (51). In particular, oligosaccharides, of which human milk is a rich source, are considered to be natural prebiotics and can actively promote the growth of specific microbial species, such as bifidobacteria in the infant intestinal microbiota (52). At the same time, human milk contains immunoglobulins (Ig), which are important components that protect the neonatal gut against pathogenic bacteria (53), and cytokines with anti-inflammatory and immunosuppressive properties (54).

Results of a meta-analysis of seven studies comparing the microbiota of exclusively breastfed (EBF) infants and non-EBF infants, showed that the relative abundance of both Bacteroidetes and Firmicutes was increased in non-EBF infants. More specifically, the genera *Bacteroides*, *Eubacterium*, and *Veillonella* were increased in non-EBF vs. EBF infants. In addition, the relative abundance of predicted pathways related to carbohydrate metabolism was increased in non-EBF infants, which also presented a

decrease in crucial pathways related to lipid metabolism/homeostasis, free radical detoxification, and metabolism of cofactors and vitamins (55).

In summary, the mode of delivery together with breastfeeding have an influence on bacterial colonization and growth in early life and consequently in the development of infant immunity. In fact, gut microbes exert fundamental roles in infant development and the maturation of the immune system (56). For this reason, it has been proposed that the risk of metabolic, inflammatory, and neurodegenerative diseases can be potentially predicted by the characterization of the microbiota of the early stages of life (57).

1.3.3 Introduction of solid food

With the introduction of solid foods, the diversity of the intestinal microbiota increases with Actinobacteria and Proteobacteria becoming the dominant components of the infant microbiota (57). This transition of the intestinal microbiota usually takes 3–5 years. In this phase, a modification toward a more adult microbiota takes place (58).

In particular, the level of the dominant Bifidobacteria, which are abundant in breastfed infants, decreases with the addition of solid foods. At the same time, ingestion of table foods has been associated with an increase in the abundance of Bacteroidetes, increase in fecal short-chain fatty acid (SCFA) levels, enrichment of genes associated with carbohydrate utilization, vitamin biosynthesis, and xenobiotic degradation, and a more stable community composition, all of which are characteristics of the adult microbiota (59). At the genus level, an increase in *Clostridium*, *Roseburia*, *Bacteroides*, *Bilophila*, and *Anaerostipes* has been observed (58).

1.3.4 The gut microbiota in later life

During childhood (age 1-7 years) a gradual decrease in bacterial diversity and functional complexity has been observed, together with a higher degree of interpersonal variation (60). In particular, pre-school and school children present an increase in Actinobacteria, Bacteroidetes, Bacilli, and Ruminococcaceae and a decrease in Methanobacteriales, compared with adults individuals (60–62).

The composition and diversity of the GM also change during adolescence, and the trajectory of these changes is affected by environmental factors (63).

Findings from a recent work comparing the GM composition between adolescents and adults revealed a statistically significant higher abundance of the genera *Bifidobacterium* and *Clostridium* among adolescents (64).

The stabilization of the microbiota is reached around the age of 18, but it can be influenced by various factors such as eating habits and possible pathologies (65).

Beyond pathological situations or drastic changes in lifestyle, the GM remains almost stable throughout life, until old age, when a reduction in the number and diversity of microbial species has been observed. In particular, a reduced concentration of *Bifidobacterium* and an increase in Enterobacteriaceae has been associated with the elderly. In this phase of life, a greater frequency of *Clostridium* (C.) *difficile, C. perfringens,* and *Escherichia coli* has also been reported (66).

1.4 Role of the gut microbiota in human health

The GM is essential for intestinal development, homeostasis and protection against pathogens, maturation of the host's innate and adaptive immune responses, and metabolic processes, including digestion of polysaccharides and fibers not digested by the host, with a trophic effect on the intestinal epithelium, biosynthesis of vitamins, xenobiotic degradations, and metabolism of secondary bile acids (67,68).

1.4.1 Metabolic functions

Fermentation of the carbohydrates that escaped proximal digestion and indigestible fibers by colonic organisms, such as *Bacteroides*, *Roseburia*, *Bifidobacterium*, *Fecalibacterium*, and Enterobacteria, results in the synthesis of SCFA (68). The indigestible dietary fibers include glycans, such as cellulose, pectin, and amylose. These polymers of monosaccharide units can be metabolized by intestinal bacteria thanks to the presence of glycoside hydrolases (69).

The ratio of SCFA concentrations in the colonic lumen is about 60% acetate, 25% propionate, and 15% butyrate (70). Butyrate can provide energy to colonocytes and then prevent autophagy (71). Recently, the expression “butyrate paradox” (72) have been used to indicate the duplex action of butyrate in the colon, which has shown to stimulate the physiological pattern of proliferation in the basal crypt in the colon, reducing at the same time the number and the size of aberrant crypt focus, which are the earliest detectable neoplastic lesions (73). Other functions of the butyrate in the colon include its influence in ion transport, the anti-inflammatory action, primarily via inhibition of nuclear factor κB (NF-κB), the reinforcing of the colonic defense barrier, the regulation of colonic motility, and the activation of immune cells through specific G-protein-coupled receptors (GPRs) for SCFAs, namely GPR41 (or FFA3) and GPR43 (or FFA2) (70).

Propionate is almost quantitatively sequestrated in the liver where it may act as a gluconeogenic substrate (74). An high propionate/acetate ratio has been associated with a lower level of inflammation in vitro (75).

Some acetate is converted to butyrate by lumenal bacteria; however, most of the acetate remains available to peripheral tissues and it can be used for lipogenesis in adipose tissue or it can be oxidized by muscles (76).

A recent work has shown that SCFAs can inhibit lipolysis in human adipocytes, while branched SCFAs (BSCFAs), e.g., isobutyric and isovaleric acid, which are generated by fermentation of branched amino acids, have effects on both adipocyte lipid and glucose metabolism, and have shown anti-obesity proprieties in humans and animal models (77).

Several amino acid transporters presented on the bacterial cell allow the entrance of amino acids from the intestinal lumen into bacteria, where they can be converted into small signaling molecules and antimicrobial peptides (bacteriocins). Other important amino acid transformations include the conversion of L-histidine to histamine by the bacterial enzyme histamine decarboxylase, and the conversion of glutamate to γ-aminobutyric acid (GABA) by glutamate decarboxylases (68).

It is well known that the GM can synthesize certain vitamins, notably vitamin K, and B group vitamins including biotin, cobalamin, folates, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine (78). Recent advance in metagenomics allowed to obtain a comprehensive overview of vitamins biosynthesis pathways. In particular, it has been shown that among 256 common gut bacteria, about half of their genomes contained genes encoding the synthesis of B vitamins. Due to the impact of the GM in B-vitamins production, changes in its composition can severely affect our dietary B-vitamin requirements (79). Similarly, another work focusing on vitamin K found genes encoding vitamin K biosynthesis pathways in the genomes of 118 out of 254 gut bacteria (80).

Bile acids are amphipathic molecules synthesized in the liver from cholesterol, which is stored in the gallbladder and released into the small intestine after food intake. While the majority of bile acids are actively absorbed in the distal ileum and recycled back to the liver, a small fraction (1–5%; 200–800 mg daily in humans) escapes this enterohepatic circulation and enters the colon (78). Gut bacteria are involved in the production of secondary bile acids, due to the modification of primary bile acids by bacterial enzymes in the intestine. The predominant secondary bile acids produced by microbial activities in humans are the deoxycholic acid (DCA; 3α,12α-dihydroxy-5β-cholan-24-oic acid) and the lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid) (81). Bacterial transformations that allow the production of these acids include deconjugation, 7α-dehydroxylation, and 7α dehydrogenation (82). Secondary bile acids can be absorbed and returned to the liver for re-conjugation before re-entering the enterohepatic circulation or undergo further bacterial processing (83). Many metabolites of secondary bile acids can be found in human feces (84). The excretion of secondary bile acids by feces stimulates a new synthesis of primary bile acids from cholesterol and has been shown to improve lipid profile in animal models (85).

The metabolism of GM also includes tryptophan metabolism. Tryptophan can be produced by the microbial flora by the degradation of dietary proteins and can be converted into several catabolites including indole, tryptamine, indoleethanol, indolepropionic acid, indolelactic acid, indoleacetic acid, skatole, indolealdehyde, and indoleacrylic acid. These catabolites have a large impact on host physiology through different mechanisms, such as the regulation of mucosal homeostasis and the release of glucagon-like peptide 1 (GLP-1) by enteroendocrine cells. Furthermore, these catabolites can modulate the innate and adaptive immune responses and the release of 5-hydroxytryptamine (5-HT, serotonin) by enterochromaffin cells. 5-HT stimulates gastrointestinal motility by acting on enteric nervous system neurons (86).

Gut microbes are not only able to metabolize many classes of dietary compounds, including complex polysaccharides, lipids, proteins, and phytochemicals, but are also able to transform industrial chemicals, pollutants, and pharmaceuticals. In fact, the compounds that are not absorbed in the small intestine can arrive in the large intestine, where they may be transformed by the GM. At the same time, compounds administered via other routes (e.g., intravenous injection) can also reach gut microbes through biliary excretion. The products of gut microbial metabolism can be then absorbed by the host or interact locally with the epithelial cells in the GI tract. Finally, these microbial metabolites are excreted in feces or filtered by the kidneys and eliminated in the urine (87).

1.4.2 Immune function

As previously stated, GM is essential for the maturation of the immune system. Gut microbes allow the immune system to discriminate between commensal bacteria and pathogens. In particular, this function is achieved thanks to the activity of Toll-like receptors (TLRs), that are expressed in the membranes of epithelial and lymphoid cells (88).

The role of the intestinal bacteria in the development of the immune system has been firstly demonstrated in animal models: germ-free mice are characterized by atrophy of Peyer's patches with few germinal centers, immature mesenteric lymph nodes, few isolated lymphoid follicles, decreased levels of antimicrobial peptides and IgA and an overall reduced number of lymphocytes B, lymphocytes T and dendritic cells. In addition, germ-free mice also present a decrease in RegIIIγ and Angiogenin-4 (89). As a consequence, germ-free animals are more susceptible to infections by certain bacterial, viral, and parasitic pathogens (90).

At the same time, colonization of germ-free animals with bacteria has shown to trigger IgA production. It is interesting to note that this increased IgA production persists even after the disappearance of

bacteria from the gut, suggesting that IgA-secreting B cells are imprinted by microbial exposure, rather than requiring ongoing stimulation (91).

The regulatory T cells are one of the key factors in the establishment of immunological tolerance. In particular, Foxp3+ regulatory T (Treg) cells are responsible for both peripheral and mucosal homeostasis and can arise as differentiated cells in the thymus and the GI tract (92). Findings from an animal model suggest that the peripheral induction of Foxp3+ T reg cells can take place in the gut-associated lymphoid tissue (GALT) (93). A proportion of induced Treg cells in the colonic tissue is specific for antigens derived from the commensal microbiota (94). Furthermore, the induction of Treg cells is proposed as one of the mechanisms of action of probiotics—defined bacteria that are known to confer a health benefit to the host (92).

In general, SCFAs produced by the GM have anti-inflammatory effects on immune cells. For instance, SCFAs reduce expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-6 and IL-12 by macrophages and dendritic cells (95).

In particular, the receptor GPR109A expressed in colon epithelial has been shown to be activated by butyrate produced by gut bacteria. In fact, this receptor was found to be decreased in germ-free mice (96). The activation of GPR109A by butyrate suppresses inflammation and the process of carcinogenesis in the colon by promoting anti-inflammatory properties in macrophages and dendritic cells, which induce the differentiation of Treg cells that produce IL10 (96). Furthermore, dendritic cells exposed to butyrate express the immunosuppressive enzymes indoleamine 2,3-dioxygenase 1 and aldehyde dehydrogenase 1A2 (Aldh1A2) and suppress the conversion of naïve T cells into pro-inflammatory IFN-γ-producing cells (97).

Another recognized role of the GM is the protection against foreign pathogens. Commensal bacteria can prevent pathogen infection by altering host environmental conditions or by competitive exclusion

(98). For example, *Bacteroides thetaiotaomicron* holds a sialidase activity and can reduce the gut levels of sialic acid, a terminal sugar of some mucosal glycans. This sugar can be used as a source of nutriment by two bacteria pathogens, namely *Salmonella typhimurium* and *Clostridium difficile,* so the presence of *B. thetaiotaomicron* in the gut can contrast the growth of these two pathogens, which conversely exhibit an impaired expansion after antibiotic exposure in mice (99,100). In addition, gut bacteria produce antimicrobial peptides and bacteriocins. Among the antimicrobial peptides, cathelicidin and defensins represent two major groups (98). *Escherichia coli* is a common producers of antimicrobial peptides (101), while bacteriocin-producing species are best represented by the family Lactobacillaceae and the genus *Bifidobacterium* (102).

1.4.3 Gut-brain axis

The gut-brain axis (GBA) consists of a bidirectional communication line between the central and the enteric nervous system. This complex network includes the central nervous system (CNS), the autonomic nervous system (ANS), the enteric nervous system (ENS) and the hypothalamic-pituitary-adrenal (HPA) axis (103).

The microbiota has the potential to influence neuronal function directly or indirectly through vitamins, neurotransmitters, and neuroactive microbial metabolites, such as SCFAs and tryptophan catabolites (104,105).

Studies on germ-free animals have shown that bacterial colonization of the gut is central for the development and maturation of both ENS and CNS. In fact, in the absence of microbial colonization, altered expression and turnover of neurotransmitters in both nervous systems and alterations of gut sensory-motor functions were observed. In addition, differences in HPA axis responses, affective, social, and eating behaviors have been observed in GF animals (103,106).

Evidence from an animal model indicates that the commensal bacteria are necessary for the normal excitability of the gut sensory neurons, being involved in the communication between the ENS and the brain (107).

Another study in mice showed an altered HPA stress response in GF mice, which was partially corrected with oral supplementation with murine feces, containing commensal bacteria, for three weeks. However, this improvement was achieved only when considering early-life mice and was not reproduced in adult mice (108). Similarly, GF mice presented anxiety behavior in another study (109), and the alteration was reversed by introducing gut bacteria early in life.

In 2014, the work of Braniste and colleagues showed that in a model of GF mice, the lack of GM was associated with increased blood-brain barrier (BBB) permeability. This barrier controls the passage and exchange of molecules and nutrients between the circulatory system and the brain and is formed by capillary endothelial cells sealed by tight junctions, astrocytes, and pericytes. In the same work, treatment with SCFAs producing bacteria was able to produce a decrease of the BBB permeability (110).

In humans, the potential connection between the GM and brain function is suggested by the large number of studies observing an altered microbiota in subjects with neurological disorders (111), such as Alzheimer's disease, autism spectrum disorder, multiple sclerosis, Parkinson's disease, and in subjects with depression (112) or anxiety (113).

1.5 Factors shaping the composition of the gut microbiota in adult life

As previously discussed, each person has a unique microbiota, which reaches stability after adolescence and can influence the host's health status and susceptibility to a wide range of diseases (114).

The composition of the GM is affected by many factors including lifestyle factors (diet, smoking status, physical activity), disease state, medication use, in particular antibiotics use, and genetic factors (11). Among all these factors, diet plays a central role (Figure 1.2), as demonstrated by several animal and human studies (115,116).

Dysbiosis is defined as any change to the composition of resident commensal communities', relative to the community found in healthy individuals (117). This condition includes the loss of beneficial microbes, the expansion of pathobionts, and the loss of microbial diversity (118). These changes can be caused by all the factors previously mentioned (Figure 1.2). There is strong evidence that dysbiosis, being a situation of imbalance in the composition and metabolic capacity of our microbiota, increases the risk of intestinal bowel disorder (IBD), irritable bowel syndrome (IBS), diabetes, obesity, cancer, cardiovascular and central nervous system disorders (119,120).

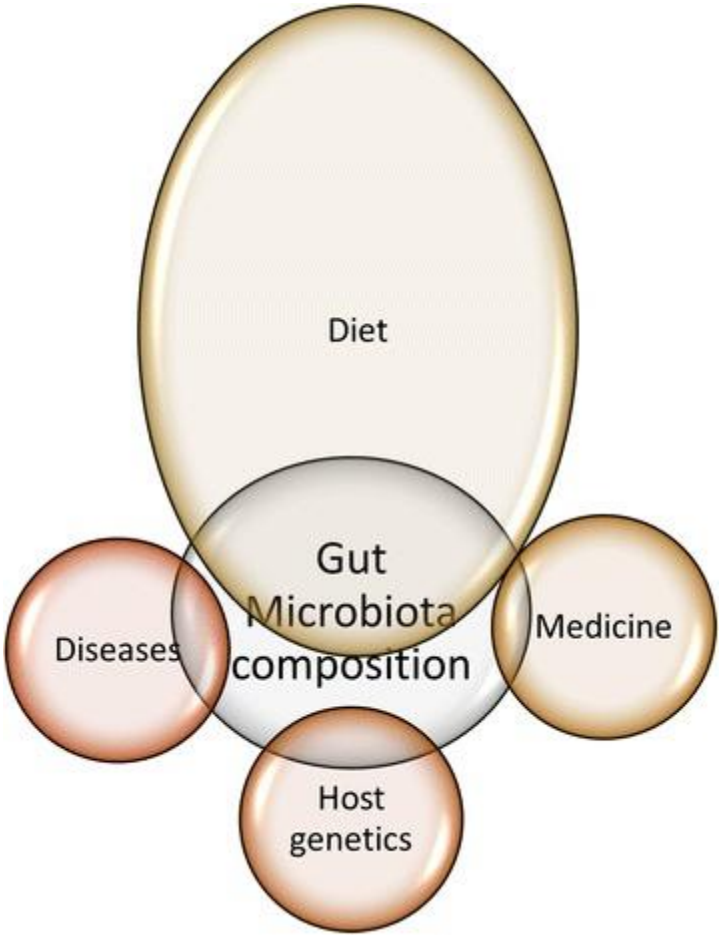

Figure 1.2. Overview of the main factors affecting gut microbiota composition, highlighting the great impact of diet (121).

1.5.1 Diet

Human studies on the association between dietary components and the GM composition have focused mainly on the intake of macronutrients (carbohydrates, fats, proteins) and their proportion in the diet (122). In addition, most intervention studies evaluating the effect of foods, nutrients, or dietary patterns on the GM were performed in animal models (123).

Non-digestible dietary carbohydrates are fermented by the gut bacteria in the colon (124). The main categories are resistant starches (RS) and non-starch polysaccharides (NSPs); however, also a small portion of oligosaccharides and monosaccharides reach the colon and can be metabolized by the gut

flora (125). As previously mentioned, complex indigestible dietary carbohydrates can be converted into SCFAs, with 10% of our daily energy requirement coming from colonic fermentation (126).

A recent observational study investigated the association between dietary patterns and the GM in 27 healthy volunteers: a high polysaccharides intake was positively associated with Actinobacteria, *Coprococcus*, *Bifidobacterium*, and *Roseburia*, and negatively associated with Proteobacteria. When considering the carbohydrate intake in general, a positive association with *Roseburia, Bifidobacterium,* and *Lactobacillus* was found (127).

In the study of Walker et al., 14 men were provided successively with a control diet, a diet high in RS or NSPs, and a reduced carbohydrate weight-loss (WL) diet, over 10 weeks. The RS diet increased the relative abundance of *Ruminococcus bromii* and *Eubacterium rectale,* while the WL diet was associated with a decrease in *Eubacterium rectale* and *Collinsella aerofaciens*. Interestingly, the NSPs did not show a large impact on the gut microbial profiles (128). In line with these findings, in the study of Abell et al., 46 healthy adults were randomized for testing the effect of two different diets (rich in NSPs only or rich in NSPs and RS) on the relative abundance of *R.bromii:* only the diet rich in both NSPs and RS was able to increase its abundance, while the effect of NSPs alone was not significant (129).

In addition, the crossover study of Martínez and colleagues, testing the effect of different types of RS starch on the GM in 10 healthy volunteers, showed that different types of RS starch presented functional differences in their effect on human fecal microbiota composition, suggesting that the chemical structure of RS determines its accessibility by different groups of colonic bacteria (130).

At the same time, the effect of low-carbohydrate diets was evaluated in recent human studies, in which subjects with a range of different diseases, including metabolic and autoimmune disease, were enrolled. In general, findings on the effect of Ketogenic diet (characterized by a very low intake of

carbohydrates) on the GM remain controversial, with some studies showing a negative effect (a decreased in alpha diversity and microbial richness) and other showing an increase in beneficial bacteria (*Akkermansia muciniphila* and Lactobacilli) (131).

Taken together, these studies demonstrate that the proportion of carbohydrates intake in the diet modulates the GM composition, and intervention strategies based on polysaccharides supplementation in humans can alter the microbial community, though the inter-subject variations in the response to these interventions should be taken into account.

A recent systematic review focused on the impact of dietary fat intake on the human GM: in total, six randomized controlled trials (RCT) and nine cross-sectional observational studies were included in the review. While observational studies indicated an association between the composition of intestinal microbiota and different amounts and types of dietary fat, RCTs showed a more modest effect. However, a high intake of fat and saturated fatty acids (SFA) was associated with a decrease in microbiota richness and diversity, while diets high in monounsaturated fatty acids (MUFA) were associated with a decreased of the total bacterial number. On the other hand, a diet rich in dietary polyunsaturated fatty acids (PUFA) did not affect richness and diversity (132).

A further RCT published in 2019, investigated the effect of three diets, differing by the percentage of fat content, in affecting the GM composition of 217 healthy volunteers: the low-fat diet was associated with increased abundance of *Blautia* and *Faecalibacterium*, whereas the high-fat diet was associated with increased *Alistipes*, *Bacteroides* and decreased *Faecalibacterium*. In general, the low-fat diet was associated with beneficial effects on GM composition. In addition to the changes in microbial patterns, the concentration of total SCFAs was significantly decreased in the high-fat diet group, in comparison with the other groups (low-fat diet and moderate-fat diet). On the other hand, in agreement with the

findings of the previously discussed systematic review (132), bacterial diversity was significantly increased in the low-fat diet group, compared to the high-fat diet group (133).

Another systematic review published in 2019 assessed the evidence for the effect of dietary fats intake on metabolic syndrome (MetS) occurrence and reversion. Thirty articles (14 observational and 16 clinical trials) were included in the review. Most of the observational studies found beneficial associations between MUFA and PUFA (including n-3 and n-6 subtypes) intake and MetS components. In addition, clinical trials also supported the benefits of MUFA- or PUFA-enriched diets (including low-fat diets) in reducing MetS (134).

Although the effect of dietary fats intake on specific microbial patterns requires further investigation, sufficient evidence from human studies suggests an impact of dietary fats intake in modulating GM profiles and/or diversity and its potential in influencing metabolic outcomes.

Regarding protein intake, most of the dietary proteins are digested in the small intestine. However, excessive protein intake can increase their arrival in the colon, where bacterial enzymes (proteases and peptidases) can hydrolyze the proteins into smaller peptides and amino acids (135). The main products of this degradation are SCFAs and ammonia. More specifically, about 30% of the substrate is converted to the major SCFAs (acetate, propionate, and butyrate) and the major BCFAs (isobutyrate, 2-methylbutyrate, and isovalerate). Other bacterial fermentation products include p-cresol, phenylpropionate (from tyrosine), phenylacetate (from phenylalanine), and indole (from tryptophan) (136). The activity of bacterial proteases and peptidases elevates intestinal pH and an alkaline pH favors pathogen proliferation and can affect gut health. Furthermore, an increased fermentation of proteins in the colon was associated with the development of enteric diseases (135).

In addition to the capacity of gut bacteria to ferment proteins in the colon, different components of protein food sources can be further metabolized by bacterial activity. For example, L-carnitine and

phosphatidylcholine, present in red meats and eggs, can be metabolized to trimethylamine and trimethylamine oxide (TMAO) (137). In humans, circulating TMAO levels are reported to be associated with cardiovascular and neurological disorders (138).

1.5.2 Prebiotics

The term “prebiotic” was used for the first time in 1995, when it was defined by Glenn Gibson and Marcel Roberfroid as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (139). The authors of the work referred to non-digestible oligosaccharides and fructooligosaccharides, which can significantly modulate the colonic microbiota by increasing the number of specific bacteria and thus changing the composition of the microbiota. In particular, these oligosaccharides stimulate the growth of endogenous bifidobacteria. In addition, prebiotics can modulate lipid metabolism, most likely via fermentation products (140).

In 2004, the first definition of prebiotics was updated as follows ‘‘selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the GI microflora that confers benefits upon host wellbeing and health’’(141). This second concept extended the potential benefits of prebiotics to all the GI tract and highlighted that the changes in the gut are connected with the general health of the host.

Finally, prebiotics have been recently defined as “a non-digestible compound that, through its metabolization by microorganisms in the gut, modulates the composition and/or activity of the GM, thus conferring a beneficial physiologic effect on the host”(142). This recent definition differs from the previous ones for the absence of requirements of selectivity or the specificity of prebiotics, the extension of the term metabolism (more general than fermentation), and the causality role of prebiotics

in modulating the GM. Furthermore, Bindels et al., considering that compounds that are supposed to reach the large intestine require specific characteristics that do not apply to other body sites, underlined the rationale to reserve the term “prebiotic” to nutritional strategies that target the GM specifically (142).

A prebiotic should answer to three criteria:

- are resistant to gastric acidity and hydrolysis by mammalian enzymes and GI absorption;
- can be fermented by intestinal microflora;
- selectively stimulates the growth and/or activity of intestinal bacteria associated with health and wellbeing (141).

There are many types of prebiotics. The majority of them are a subset of carbohydrate groups and are mostly oligosaccharide carbohydrates (143). Prebiotics are naturally presented in several dietary foods but in low amounts. For this reason, they are mostly produced artificially. The capacity to digest specific prebiotics differs from different bacterial species. For example, *Bifidobacterium* spp. can ferment starch and fructans. It is well known that prebiotics is able to increase the abundance of strains of *Bifidobacterium*, which produce lactate and acetate (144). In particular, a large number of studies conducted on humans volunteers demonstrated that the oral administration of different types of saccharides (galacto-oligosaccharide, fructo-oligosaccharide and oligofructose, inuline, lactulose) was able to increase the levels of *Bifidobacterium* (145). Some studies also observed an increase in *Lactobacillu*s, or a decrease in Clostridia and Enterobacteria (145).

Prebiotics not only have protective effects on the gastrointestinal system but also on other parts of the body, such as the central nervous system, immune system, and cardiovascular system. Other prebiotic effects include defense against pathogens, mineral absorption, bowel function, metabolic effects (such as the regulation of cholesterol levels and lipogenesis) and satiety (146).

1.5.3 Probiotics

In 2001, an Expert Consultation of international scientists working on behalf of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) provided the first definition of probiotics: "live microorganisms which when administered in adequate amounts confer a health benefit on the host"(147).

Species of *Lactobacillus* and *Bifidobacterium* are most commonly used as probiotics, but the yeast *Saccharomyces cerevisiae* and some *E. coli* and *Bacillus* species are also employed. Recently, *Roseburia* spp., *Akkermansia* spp., *Propionibacterium* spp. and *Faecalibacterium* spp. have been also tested in human and animal models (146).

Probiotics can exert a beneficial effect on human health through a wide range of mechanisms, including:

- the block of pathogens entry into the epithelial cell by providing a physical barrier (148) or by inducing the host's production of β-defensin and IgA (149);
- the fortification of the intestinal barrier by maintaining tight junctions and inducing mucin production (148,149);
- the stimulation of epithelial cell proliferation (149);
- the immunomodulation through the mediation of anti-inflammatory cytokine secretion (148,149);
- the effect on gut motility and nociception through regulation of pain receptor expression and secretion of neurotransmitters (149).

Various probiotic species have been reported to prevent many degenerative diseases, including obesity, diabetes, cancer, cardiovascular disease, malignancy, liver diseases, and IBD (46). Unfortunately,

research elucidating mechanisms of probiotics has often relied on in vitro or animal studies, not always translated to humans (146). In addition, there are substantial differences among probiotic strains, so the observed effects of a probiotic depend on the specific formula that has been used.

However, some probiotics have confirmed their potential benefits on human diseases, including *Clostridium difficile* infection, IBS, sepsis, diarrhea, and colitis (148,150–153).

1.5.4 Smoking status

A considerable number of studies observed an altered GM in smokers, including both changes in the microbial diversity and composition (154). The alterations found in humans can be explained by different mechanisms that have been proposed in animal studies.

In a mice model, the chronic exposure to cigarette smoke was associated with the increase in immune cell numbers and differentiation, along with important changes in immune cell composition. Moreover, increased apoptosis in the follicle-associated epithelium was observed (155). Another study confirmed the induced changes in the immune function in the gut and underlined the alteration of epithelial mucus profiles, associated with a dysfunction of the mucus barrier (156). In addition, an *in vitro* experiment demonstrated that nicotine holds antibacterial proprieties, being able to reduce the count of the commensal bacteria used in the model (157). Other indirect effects can be connected with changes in the gut, including the delay of the gastric empty and pH changes in the duodenum (158,159).

A recent review summarizing the impact of smoking on GM reported an increase in Proteobacteria and Bacteroidetes phyla, as well as in the genera *Clostridium*, *Bacteroides* and *Prevotella* in smokers. In particular, facultative or obligatory anaerobic genera were most abundant in the smoking environment. On the other hand, Actinobacteria and Firmicutes phyla, as well as the genera *Bifidobacterium* and *Lactococcus*, were decreased in smokers (154). In this work, some alterations were in line with those

typically observed in other diseases, including obesity and IBD. In this perspective, the further work of Huang et al., published in 2019, revised the current literature on the association between GM and lifestyle factors, focusing on healthy subjects, in order to exclude potential bias connected with the presence of diseases. However, they did not find human studies that recruited only healthy smokers (160).

It is well known that smoking is associated with an increased risk of some gastrointestinal diseases (Crohn's disease and IBD) and both these conditions have been associated with alteration in the GM. However, the causal relationship between microbiota alterations and disease progress remains enigmatic (160). Follow-up studies are currently required in order to elucidate the complex link between smoking, GM composition and diversity, and the development of diseases.

1.5.5 Antibiotics and other medications

One of the roles of the intestinal microbiota on human health is connected with its capacity to provide a defense against foreign pathogens. For this reason, the disruption of the microbiota with oral antibiotics can facilitate the emergence of several enteric pathogens (161).

The study of Ng et al. on antibiotics treated mice, revealed that the oral administration of an antibiotic (streptomycin) induces an increase in free sialic acid and consequent growth of opportunistic pathogens, namely mutants of both *Salmonella typhimurium* and *Clostridium difficile* (99). Of course, the potential growth of pathogens it is not the only negative effect of antibiotics in the gut: a general decrease of bacterial diversity and shifts of microbial patterns have been observed in human and animal models (162). These changes can affect host health by many different mechanisms, including the loss of bacterial ligands, important for the immune function. More specifically, a number of studies have shown that the depletion of the microbiota, as a consequence of antibiotic treatment, results in reduced

TLR signaling (163). The impact on the immune system was also demonstrated by the decreased production of antimicrobial peptides in the gut, reduced expression of REG3, reduced systemic IgG, disrupted mucus layer, and reduced activity of macrophages (163). In addition, different studies showed that antibiotics treated mice presented a decrease in SCFAs in feces, which was sometimes persistent weeks after the cessation of the treatment (164).

When considering the impact of selected groups of antibiotics on the normal intestinal microbiota, ciprofloxacin, and cephalosporins shows a strong suppression on Enterobacteria, while clindamycin exerts a strong suppression on anaerobes. In addition, the antibiotics vancomycin has an impact on aerobic Gram-positive cocci, though the association was found in both directions (165).

Some of the microbial changes caused by antibiotics administration can be persistent: one study verified the effect of a 7-days administration of clindamycin treatment on the fecal microbiota in 4 volunteers that were followed up to 2 years: the effects of antibiotics administration were still persistent for up to 2 years post-treatment (165).

Maier et al. in 2018 analyzed the effect of 1197 pharmaceutical compounds (including 835 human-targeted drugs) on commensal bacteria, using an *in vitro* model. They observed an anticommensal activity in 203 (24%) human-targeted drugs. Most drugs were effective against smaller subsets of strains with the exception of 40 drugs that affected at least 10 strains. In particular, species responded to drugs included *Roseburia intestinalis*, *Eubacterium rectale* (both butyrate- producers) and *Bacteroides vulgatus* (propionate-producer) (166). These findings were recently confirmed in a recent population- based study, analyzing 1883 fecal samples and looking to association between the metagenome and the using of 41 drug categories: 19 of 41 drugs were found to be associated with microbial features, including proton-pump inhibitors, metformin, antibiotics, and laxatives (167).

1.5.6 Genetic factors

Goodrich et al. in 2014 compared the GM across 1,081 fecal samples from 977 individuals, obtained from the TwinsUK population, including 416 twin-pairs. When considering the microbiota's diversity, they observed that microbiota was more similar overall within individuals (resampled) than between unrelated individuals and was also more similar within twin pairs, compared to unrelated individuals. In addition, they found a greater similarity of Lachnospiraceae and Ruminococcaceae families among monozygotic twins compared with dizygotic twins. When calculating the heritability of each taxonomic level, they discover that the family Christensenellaceae is the most highly heritable taxon. In general, almost 10% of all the identified taxa presented a certain grade of hereditability (>0.2) (168). This is of great interest considering that the family Christensenellaceae was associated with the lean phenotype in many population-based studies (169).

The association between host genome and intestinal microbial composition was evaluated also by Turpin et al. in 2016, using data from a cohort of 1561 healthy participants. After correction for false discovery rate, heritability analysis of the microbiota showed that genetic factors contributed to abundance for 20 of 249 total bacterial taxa. In this work, the order Methanobacteriales, the family Methanobacteriaceae, and the genus *Methanobrevibacter* presented the highest level of hereditability (170). Similarly to the family Christensenellaceae, also mthanobacteria have been associated with leanness in several studies (169).

In another Genome-wide association studies (GWAS), significant associations between host genetic variation and microbiome composition were observed in 10 of the 15 body sites tested. When looking to the host genes that have been previously associated with the microbiome, they discovered that these genes were involved in pathways associated with obesity and IBD (171).

Similarly, another GWAS study with 1514 participants showed several significant associations between microbial levels and single-nucleotide polymorphisms (SNPs). Among the most significant association with SNPs there were the family Methanobacteriaceae, the genus *Blautia* and the species *Dialister invisus* and *Bacteroides xylanivorans*. Authors reported that some of these taxa have been associated with obesity and, in addition, they were able to observe an interesting association between the abundance of *Lactococcus* bacteria and a SNP associated with body fat distribution (rs2294239) (172). In the same study, the gut microbiome was also linked with SNPs previously associated with IBD and asthma (172).

2. Research activities

My research topic during the course of the three years of Ph.D. related to the interface between diet, GM, and host health in the human model. My primary research goal was to investigate the association between GM and obesity, as well as the impact of weight-loss interventions in modulating its composition. The aims, methodology, and results of this research project are described in detail in the following chapters (3-7). In this chapter, I provide a summary of other related research activities.

Starting from the first year, I collaborated on a project aimed to investigate the composition and structure of the fecal microbiota in a cohort of Italian patients with Parkinson's disease (PD) compared to healthy subjects (HCs), with a focus on the impact of therapeutic drugs on microbiota modification. Direct analysis of the fecal metabolome was also implemented to understand the functional contribution of the microbial community and to identify potential metabolic alterations associated with the microbiota in PD patients. Clinical (i.e. phenotype, duration of disease) and lifestyle factors (i.e. smoking status, diet, physical activity) were considered as potential confounding factors, whose impact was evaluated at the multivariate analysis. My contribution to the project comprises the formal analysis, in terms of the performing laboratory experiments and statistical analysis.

Parkinson's disease is a neurodegenerative disorder characterized by the accumulation of intracellular aggregates of misfolded alpha-synuclein along the cerebral axis. Several studies report the association between intestinal dysbiosis and Parkinson's disease, although a cause-effect relationship remains to be established. The preliminary results of the research project were firstly presented at the Italian Society of Microbiology (SIM), 46° National congress (2018 Sept 26-29, Palermo, IT) (173), and the overall results have been included in two scientific papers, one recently published (174) and one currently in submission (175).

Firstly, the GM composition of 64 PD and 51 HCs was determined using a next-generation sequencing (NGS) and real metagenomics shape approach: the most significant changes within the PD group highlighted a reduction in bacterial taxa, which are linked to anti-inflammatory/neuroprotective effects, particularly in the Lachnospiraceae family and key members, such as *Butyrivibrio, Pseudobutyrivibrio, Coprococcus*, and *Blautia*. Some of the included confounding factors (sex, age, BMI, smoking status, coffee consumption) influenced the microbiota community, although the statistically significant differences in the composition of the intestinal microbiota in the PD group compared to the HC group was maintained at various taxonomic levels when adjusting for these factors at the multivariate analysis. The direct evaluation of fecal metabolites revealed changes in several classes of metabolites; in particular in the content of lipids (linoleic acid, oleic acid, succinic acid, and sebacic acid), vitamins, (pantothenic acid and nicotinic acid), amino acids (isoleucine, leucine, phenylalanine, glutamic acid, and pyroglutamic acid) and other organic compounds (cadaverine, ethanolamine, and hydroxy propionic acid). Interestingly, most modified metabolites strongly correlated with the abundance of members belonging to the Lachnospiraceae family, suggesting that these gut bacteria correlate with altered metabolism rates in PD. The findings of this study indicate that Parkinson's disease is associated with gut dysregulation that involves a synergistic relationship between gut microbes and several bacterial metabolites favoring altered homeostasis (174).

Secondly, taking into account that the effect of Levodopa (LD) has been poorly assessed and those of LD-carbidopa intestinal gel (LCIG) have not been evaluated so far, the aim of a further study belonging to the same research project was to identify the effect of LD and, in particular, LCIG on GM and metabolome. Faecal DNA samples from 107 patients were analyzed by NGS, with PD patients being classified in different groups: patients treated with LCIG (LCIG-Group) (n= 38) and on LD (LD-Group) (n= 46), and 23 patients without antiparkinsonian medicaments (Naïve-Group). Faecal metabolic extracts were evaluated by Gas Chromatography Mass Spectrometry (GC-MS). Findings

from this study, included in a research paper currently under review, suggest that LD and mostly LCIG might significantly influence the microbiota composition and host/bacteria metabolism acting as stressors in precipitating a specific inflammatory intestinal microenvironment, potentially related to the PD disease state and progression (175).

In addition, since the first year, I have been involved in another project focused on the analysis of the intestinal microbiota of Sardinian centenarians, and the functional implications on health status. Although it is widely recognized that life expectancy is influenced by a combination of genetic environmental and lifestyle factors, it is still unknown whether specific characteristics of these factors and their combination may facilitate the emergence of extreme longevity. Sardinia has been classified among the five "Blue Zones", distributed in the five continents of the world, with the highest frequency of long-lived. In particular, it is distinguished by the high frequency of the male gender, unlike the other areas (176).

The ongoing research project aims to determine the presence of a microbiological signature peculiar to the state of longevity, evaluating the role of the GM and its possible implications on health status. The secondary objectives include the identification of key factors associated with longevity, with a focus on diet. In order to achieve this aim, anamnestic, and anthropometric data are being collected, together with the results of the following clinical assessments: performance in activities of daily living (Activities Daily Living test) (177); cognitive impairment (Mini-Mental State Examination test); adherence to the Mediterranean diet (Mediterranean Diet Score test) (178); physical activity evaluation (Physical Activity Scale for the Elderly test) (179); nutritional status (Mini Nutritional Assessment test) (180). It has been proposed that the Mediterranean diet (MD) can lead to an average lifespan, probably thanks to its anti-inflammatory properties which would determine a decrease in chronic diseases associated with aging. This is the first study to collect data on MD adherence in Sardinian centenarians

(≥ 100 years), establishing the correlation with the composition of the GM. Another distinguishing feature of the study is that the control group consists not only of a group of adults (29-65 years), in common with the study design of other Italian studies on GM in centenarians (181,182), but also of a group of nonagenarians (90-99 years) recruited in the same geographical area, in order to clarify whether the distinctive characteristics of the GM of centenarians can emerge even by making a comparison with a population very close in age. Ninety-two participants have been recruited so far (17 centenarians, 29 nonagenarians, 46 controls), whose samples have been sequenced by NGS. My contribution to the project includes data curation, methodology, formal analysis and visualization.

As expected, many microbial alterations were in common with centenarians and nonagenarians, both of which showed signatures typical of old age, such as a depletion in Bacteroidetes and corresponding taxa, in particular *Bacteroides* spp., and a depletion in *Faecalibacterium prausnitzii*, *Akkermansia muciniphila* and *Bifidobacterium* spp. Moreover, centenarians compared with nonagenarians presented an increased abundance of *Cloacibacillus* (within Synergistetes), *Bacteroides intestinalis*, and *Phascolarctobacterium faecis* (within Firmicutes), and a decreased abundance of Lactobacillaceae, *Lactobacillus* and *Bifidobacterium angolatum*. Interestingly, many correlations between the content of microbial taxa and clinical data were found in centenarians, including negative correlations between Firmicutes taxa and the Activities Daily Living score; positive correlations between Firmicutes taxa and alcohol consumption; positive correlations between *Cloacibacillus* and Verrucomicrobia taxa and adherence to MD; negative correlations between Proteobacteria taxa and Mini-Mental State Examination score and Physical Activity Scala for Elderly score. These results have been included in a research paper, currently in preparation (183).

During the second year, I took part in a systematic review (184) carried out by the International Agency for Research on Cancer, in which I was hosted as Ph.D. student for eight months. The microbiome has

been hypothesized to play a role in cancer development. Taking into account the diversity of published data, the aim of the review was to provide an overview of available epidemiologic evidence linking the microbiome with cancer. A systematic review was conducted using a tailored search strategy in Medline and EMBASE databases to identify and summarize the current epidemiologic literature on the relationship between the microbiome and different cancer outcomes (published until December 2019). Concerning my contribution, I participated to the consensus discussions regarding the final exclusion criteria and to the drafting of the systematic review .

In total, 124 eligible articles were identified. Fifty studies reported differences in the gut microbiome between patients with colorectal cancer and various control groups. The most consistent findings were for *Fusobacterium*, *Porphyromonas*, and *Peptostreptococcus* being significantly enriched in fecal and mucosal samples from patients with colorectal cancer. For the oral microbiome, significantly increased and decreased abundance was reported for *Fusobacterium* and *Streptococcus*, respectively, in patients with oral cancer compared with controls. Findings of the review suggest that, although strong evidence was available for certain taxa of the gut microbiome and colorectal cancer risk, and for the oral microbiome and oral cancer risk, for most of the microbiome taxa/indicators the evidence was still too weak to draw firm conclusions in relation to their role in cancer. Therefore, future prospective studies with prediagnostic specimen collection using standardized methods, consistent laboratory methodology and bio-informatics, and inclusion of quality control samples are required to establish causal links (184).

The research project related to the association between GM and obesity is described in detail in the following chapters. Also in the case of this project, sample and data collection started during the first year. My contribution to the project consists of data curation, methodology, formal analysis and visualization, and scientific publications writing. The preliminary results have been presented at the

Society of Obesity (SIO), IX National Congress (2018 Oct 11-13, Milan, IT) (185). Findings from this project have been included in two publications, one recently published (186) and one submitted (187).

3. The association between obesity and gut microbiota

The worldwide prevalence of obesity nearly tripled between 1975 and 2016. In 2016, more than 1.9 billion adults aged 18 years and over were overweight. Of these over 650 million adults were obese (188). In the same year, more than a third of the Italian population was overweight (35.3%) and one person out of ten was obese (9.8%) (189).

Overweight is defined as a BMI (weight in kilograms/height^2 in meters) of 25 to 29.9 kg/m^2, obesity as a BMI of >30 kg/m^2. Increased BMI has been associated with an increased risk of all-cause mortality (190,191) and specific causes of mortality including cancer, cardiovascular, and respiratory deaths (191).

Obesity is a chronic disease characterized by a multifactorial etiology including genetic, behavioral, environmental, physiological, social, and cultural factors that result in a positive energy balance that promotes excessive fat deposition (192).

Several scientific evidences recognize that the metabolic activity of the GM can play an important role in the pathogenesis of obesity. Different mechanisms through which GM can promote the deposition of fat have been hypothesized, including the suppression of the fasting-induced adipose factor and the reduction of AMP-activated protein kinase, with an increase of lipoprotein lipase activity, the extraction of energy from fiber, the changes in intestinal permeability and the consequent increase in LPS, and the metabolism of bile acids (191,193,194). Some of these mechanisms are further described in the following sections.

3.1 Gut microbiota and appetite and satiety control

The appetite and satiety control systems are placed at the hypothalamic level, where information from peripheral signals and other brain areas is integrated. This transmission aims to keep body weight constant, so this means that the body tries to maintain the energy balance, i.e. the energy input must be equal to the energy output more or less the reserves. The incoming energy derives from the digestion and absorption of nutrients, the outgoing energy is the sum of basal metabolism, physical activity, and thermogenesis induced by the diet (195).

The signals that reach the hypothalamus allow both short-term regulation (hormones, information from the gastrointestinal tract, blood glucose, levels of physical activity), and long-term regulation (amount of body reserves). The hypothalamic arcuate nucleus contains the NPY and peptide linked to agouti (AgRP) neurons. Leptin and insulin exhibit an inhibitory action toward these neurons, while ghrelin activates them. Y1 and Y5 are NPY receptors, and MC3R and MC4R (agouti inhibits them) are agouti receptors. Overall this population of neurons activates the lateral hypothalamus (appetite center) and has an inhibitory action at the level of the paraventricular nucleus and the medial hypothalamus (196).

On the other hand, in the arcuate nucleus there is another population of neurons, which produce POMC and cocaine and amphetamine-regulated transcript (Cart), whose action determined the satiety feeling. POMC undergoes post-transcriptional changes, from which alpha MSH is obtained, which binds to the MC4R neurons (the same for which agouti is an inhibitor). The activation of MC4R neurons has a stimulating effect at the medial hypothalamus and paraventricular nucleus levels, and an inhibitory effect at the lateral hypothalamus level (196). Leptin activates these neurons. Other hormones with anorexigenic action are incretins, pancreatic polypeptide (PP), cholecystokinin (CCK) and peptide YY3−36 (PYY) (197).

The vagus nerve extends into the lamina propria of the intestinal villi and terminates at the basolateral cell membrane of enteroendocrine cells (EECs), which expresses receptors for gut hormones such as ghrelin, leptin, CCK, glucagon-like peptide1 (GLP1), and PYY, thus leading to receptors activation and subsequent neuronal stimulation (198). After food ingestion, vagal fibers converge in the nucleus tractus solitarius and the signals are integrated into the hypothalamus (199).

There is a constant communication between the gut-brain axis and the EECs, the enteric nervous system (ENS), and the vagus nerve. Emerging evidence suggests the GM may influence weight-gain through several inter-dependent pathways including controlling appetite/satiety (197).

In fact, different studies have shown that the GM can regulate the production of hormones implicated in the satiety and appetite control (200,201)**.** For example, in the study of Breton et al. intestinal infusion of *Escherichia coli* proteins in mice increased the plasmatic levels of PYY and induced a reduction of the meal size. In line with these findings, the increased presence of *E. coli* correlated inversely with BMI in obese patients (202). Another human study, focusing on the association between the GM and the plasmatic levels of ghrelin and leptin in rats, found a positive correlation between *Bifidobacterium* and *Lactobacillus* and leptin plasmatic levels, which were negatively correlated with the genera *Clostridium*, *Bacteroides* and *Prevotella*. Authors also noticed that the plasmatic levels of ghrelin negatively correlated with *Bifidobacterium*, *Lactobacillus,* and *Blautia coccoides* and positively correlated with *Bacteroides* and *Prevotella* (203). Interestingly, the taxa that positively correlated with leptin and negatively correlated with ghrelin have been negatively associated with obesity in several clinical trials (193). Furthermore, in rodents, increased production of acetate by an altered GM was associated with the activation of the parasympathetic nervous system and increased glucose-stimulated insulin and ghrelin secretion, hyperphagia, and obesity (74).

Together with the possible modulation of ghrelin and leptin release, other potential mechanisms linking the regulation of appetite/satiety control and the composition of the GM are the following:

- the digestion of nutrients and fiber produces a wide range of metabolites, including ATP, lactate, and SCFA. These metabolites can stimulate EECs in the gut, promoting the release of PPY and GLP-1 and potentially regulating the enteric neurons;
- upon bacterial lysis, bioactive compounds such as Lipopolysaccharides (LPS), 5-hydroxytryptamine and indole, are released. Their action on EECs and enterochromaffin cells can active the enteric nervous system, regulating intestinal mobility and permeability;
- it has been demonstrated that some bacterial proteins mimic host hormones in animal models. More specifically, the caseinolytic peptidase B protein homolog, found in *E.coli*, has a mimetic action of αMSH, though the activation of MCR receptors by this protein remains to be clarified;
- the LPS can mediate the production of endocannabinoids in the gut, which were found to be elevated in the hypothalamus of obese rodents and are known for their orexigenic effects;
- ATP produced by bacterial metabolism in the gut can bind receptors in NPY/AgPR neurons, situated in the hypothalamic arcuate nucleus (Figure 3.1) (204).

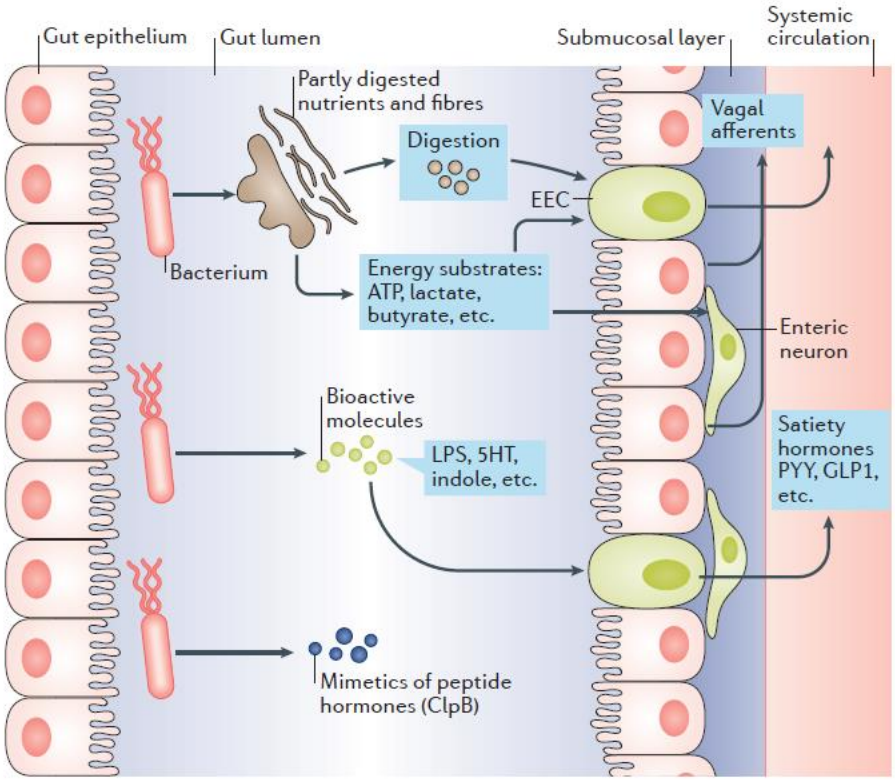

Figure 3.1. Bacterial mechanisms of regulation of satiety hormones production in the gut, These mechanisms, which can explain the association with obesity and intestinal dysbiosis (204), include:the change in intestinal permeability induced by LPS and other bioactive compounds, the increased production of endocannabinoids mediated by LPS, and the impact on the release of ghrelin and leptin.

3.2 Energy harvesting and SCFAs

A recent meta-analysis, investigating the presence and amount of SCFAs in stool samples of obese subjects, focused on the significant increase in acetate, propionate, and butyrate, compared with lean individuals. However, these changes in SCFAs profiles were not associated with the composition of the GM at the phylum level (205).

The type and amount of SCFAs and gases produced in the gut depend on multiple factors including diet, gut microbial community composition, and colonic transit time (206). In general, SCFAs represent

an additional source of energy: their energy contribution is estimated to account for ~10% of the human caloric requirements (207).

Increased energy harvesting associated with GM is a possible explanation for obesity (208).

In 2006, Ley et al. characterized the GM in obese individuals, proving for the first time the reduced abundance of taxa belonging to the Bacteroidetes phylum and the proportional increase in members of the Firmicutes phylum in this population (209). This pattern, which was confirmed in many clinical trials in the following years (193), is associated with a higher presence of enzymes for complex carbohydrate degradation and fermentation, leading to increased energy harvesting from the diet (210).

Some authors have proposed that it is not the Firmicutes/Bacteroidetes ratio to have a role in the pathogenesis of obesity but the altered proportions of Actinobacteria in obese individuals (211,212). Another link with energy production is connected with the higher presence of H_2-oxidizing methanogenic Archaea (213). It is supposed that these microorganisms oxidize H_2 produced by H_2-producing bacteria from the Prevotellaceae family (phylum Bacteroidetes), and that the H_2-utilization accelerates fermentation of polysaccharides by Prevotellaceae, and consequently results in the more considerable energy uptake by obese individuals (212).

The elucidation of the relationship between obesity and SCFAs is made complex by their demonstrated beneficial effects in human health (214). In line with these findings, a recent RCT enrolling obese men demonstrated that the administration of SCFAs was able to increase fat oxidation, energy expenditure, and PYY levels (215).

As previously discussed, in healthy individuals SCFAs are involved in the activation of GPRs (GPR41 and GPR43) in the gut, which leads to increased production of hormones involved in the satiety controls, such as GLP-1 or peptide YY (216). In addition, it has been shown that SCFAs increase the AMPK activity in liver and muscle tissue, due to a direct increase of the AMP/ATP ratio (217). This

leads to increased expression of peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α, which controls the transcriptional activity of several transcription factors involved in the regulation of cholesterol, lipid, and glucose metabolism. As a consequence, increased AMPK is connected with higher fatty acid oxidation in the liver and muscle tissues and inhibition of *de novo* fatty acid synthesis in the liver. Furthermore, SCFAs increase thermogenesis and fatty acid oxidation in the brown adipose tissue and to decrease fat accumulation in the white adipose tissue. The effect of SCFAs in white adipocytes is mediated by activation of GPR41 receptors and the consequent production of leptin (217).

It is unclear whether the beneficial effect of SCFAs is somehow compromised in obese subjects, or whether the effect is simply not strong enough to compensate for incorrect lifestyle habits or genetic predisposition (217). It has been proposed that in the case of the obese condition, the binding of SCFAs to GPRs might be attenuated, leading to increased intestinal energy harvesting and hepatic lipogenesis (205).

It is important to specify that the production of SCFAs is not the only responsible for the increase in energy harvesting. In fact, experiments on GF mice showed that the colonization with gut bacteria doubles the density of small intestinal villi capillaries. The angiogenesis, mediated by the action on Paneth cells, determines an increased absorption of monosaccharides from the gut into the portal blood (218).

3.3 Gut microbiota and increase in gut permeability

The intestinal epithelium, in the physiologic context, forms a barrier, which prevents the permeation of pro-inflammatory molecules, such as pathogens, toxins, and antigens, from the luminal environment into the mucosal tissues and circulatory system. Epithelial tight junctions (TJs) maintain the intestinal barrier, allowing at the same time the permeability of ions, nutrients, and water (219). Alteration in the intestinal barrier has been associated with a wide range of diseases, including IBD and obesity (220).

Guo et al. demonstrated that bacterial LPS induces an increase in intestinal permeability by increasing the enterocyte membrane TLR-4 expression and the TLR-4–dependent increase in CD14 membrane expression (221). In addition, in mice, a high-diet was associated with increased LPS release and consequent increase in intestinal permeability, secondary to reduced expression of TJ proteins (occludin and zonula occludens-1) (222).

Therefore, intestinal dysbiosis caused by an incorrect diet or other factors can affect intestinal permeability, leading to increased plasma levels of LPS. This condition can cause chronic low-grade inflammation, triggering metabolic disorders characterized by nonalcoholic fatty liver disease, insulin resistance, the onset of type 2 diabetes, and ultimately, obesity (223,224). In the further section, the mechanisms involved in the development of inflammation by LPS are described.

3.4 Interaction with the immune system and inflammation

In 2007, Cani et al. demonstrated for the first time that LPS from Gram-negative bacteria can trigger an inflammatory process by binding to the CD14/TLR-4 complex at the surface of innate immune cells. In this work on mice model, a 4-week high-fat diet increased the proportion of LPS-containing gut bacteria, and consequently, the plasma LPS concentration. The increased levels of LPS in the plasma were associated with increased fasted glycemia and insulinemia, and with adipose tissue weight gain (223). In fact, CD14- mice are resistant to the inflammatory state caused by a high-fat diet (224), and mice lacking TLR4 are substantially protected from the effect of systemic lipid infusion on metabolism (225).

In addition to the stimulation of CD14 and TLR-4 associated with a dysbiotic microbiota, other mechanisms related to the immune function have been proposed. For example, mice deficient in TLR-5 exhibit hyperphagia and features of metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased adiposity, associated with intestinal dysbiosis (226).

Another receptor activated by gut bacteria compounds and implicated in metabolic disorders is the TLR2: in a mice model, the administration of LPS in TLR2 mice induced higher levels of ALT and TNF-alpha levels and reduced levels of IL-6 (a cytokine with protective effects for the liver) in serum, compared with TLR2-deficient mice. In this study, LPS-induced NF-Kb was associated with liver injury. Consequently, the activation of TLR2 can be associated with systematic and liver inflammation (212,227).

Similarly, TLR9- and MyD88-deficient mice showed less insulin resistance than controls, after administration of a diet deficient in choline. In this model, TLR9 signaling activated Kupffer cells, with the production of IL-1beta, and leading to steatosis, inflammation, and fibrosis in the liver (228).

Furthermore, MyD88 deletion in mice protect against diet-induced obesity, by increasing the energy expenditure, the improved glucose homeostasis, the reduction of hepatic steatosis, and the decrease of body fat mass by 30% (229).

Taken together, these findings suggest that the activation of the immune system by the GM can contribute to inflammation and metabolic disease (212).

3.5 The gut microbiota and the fasting-induced adipose factor

Another potential mechanism linking obesity and GM is represented by the inhibition of the Fasting Induced Adipose factor (FIAF/angiopoietin-like protein 4/peroxisome proliferator-activated receptor-gamma angiopoietin-related protein) (230). FIAF is an essential regulator of lipid metabolism and adiposity. It is physically associated with lipoproteins and can inhibit lipoprotein lipase (LPL). Due to this action, FIAF overexpression causes a reduction in adipose tissue weight, by stimulating fatty acid oxidation. On the other hand, its inhibition mediated by GM produces a decrease in fatty acid oxidation and promotes fat storage (208).

3.6 The gut microbiota-liver axis

In 2004, Bäckhed et al. demonstrated that the colonization of germ-free mice with normal microbiota produced a 60% increase in body fat content and insulin resistance within 14 days, despite reduced food intake (230). In this work, many potential mechanisms for explaining how GM can increase body weight have been proposed. When looking at the effects in the liver, they found:

- increase in triglyceride content in the liver, accompanied by elevation of the expression of enzymes involved in fatty-acids metabolism and increased levels of monosaccharides in the liver (derived from gut fermentation);
- increase in Sterol response element-binding protein 1 (SREBP-1) and Carbohydrate response element-binding protein (ChREBP), two transcription factors that mediate hepatocyte lipogenic responses to insulin and glucose (231).

Nonalcoholic fatty liver disease (NAFLD) is a condition characterized by deposition of fat within the liver cells, in the absence of alcohol consumption (232). This condition is particularly common among obese individuals and associated with sedentary behaviors and high-fat diets (233). NAFLD includes a spectrum of diseases that range from simple steatosis (pure NAFLD) to nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (234).

Intestinal dysbiosis can contribute to the development of NAFLD and its progression through various mechanisms, including the carbohydrate fermentation with SCFAs production and the increase in the *de novo* lipogenesis; modulation of the endocannabinoid system; modulation of choline metabolism; modulation of bile acid homeostasis; endogenous ethanol formation; and increase in LPS release (208).

The development of NAFLD is intimately connected with the function of other organs. In the adipose tissue, a condition of insulin resistance can determine the increased production of free fatty acids (FFA), which are found elevated in the plasma of obese subjects (235). FFA in the liver can be used for

the synthesis of triglycerides. In addition, their accumulation predisposes to lipotoxicity that promotes NASH development. Lipotoxicity is characterized by a dysregulation of the lipid environment and/or intracellular lipid composition, with the accumulation of harmful lipids (234). Another connection between pathogenesis of NAFLD and the adipose tissue is the stimulation of the endocannabinoid system in adipocytes (that can be influenced by the diet), which causes inhibition in adiponectin secretion. The GM can modulate the release of adiponectin (236), and its decreased levels in plasma are associated with insulin resistance and metabolic syndrome (237).

Enzymes produced by the GM are able to convert dietary choline (found in dairy products, meat, fish, wheat, soybeans, and beans) into toxic metabolites, namely dimethylamine and trimethylamine, which are subsequently converted in the liver into trimethylamine oxide (TMAO). TMAO contributes to the inflammation of hepatocytes and the progression of NAFLD into NASH (233).

Furthermore, in the liver the bile acids are responsible for the activation of the Farnesoid X receptor (FXR) receptors. FXR plays a crucial role in the regulation of bile acids synthesis, secretion, and transport, being an important enterohepatic regulator of bile acids homeostasis (238). The decreased activity of FXR receptors in the liver was associated with the development of NAFLD and with obesity, and many FXR agonists have been developed as therapeutic targets for metabolic diseases (239). The GM modulates obesity and associated metabolic phenotypes in part through its action on the FXR signal (240).

Moreover, patients with nonalcoholic steatohepatitis (NASH) were found to have significantly higher levels of blood ethanol than healthy controls (241). It has been demonstrated that among gut bacteria, some of them can produce ethanol (233), which can be used as a substrate for triglycerides synthesis. In fact, its oxidation produces acetate and acetaldehyde. Acetate is the substrate for fatty acids synthesis, while acetaldehyde can induce the formation of reactive oxygen species (ROS) (233). Thus, ethanol

production is linked to increased oxidative stress, leading to activation of the inflammatory cascade, which ultimately causes fatty liver and cirrhosis (242).

Regarding the increased levels of LPS in the plasma (endotoxemia), this condition causes the activation of Kupffer cells and hepatic stellate cells, with a consequent activation of the TLRs 9 and 4 and the tumor necrosis factor-alpha (TNF-α). The final effect is an increased production of pro-inflammatory cytokines by liver macrophages, causing inflammation of hepatocytes, which is closely connected with the progression of NAFLD (242).

3.7 The gut microbiota-adipose tissue axis and the role of endocannabinoids

In addition to the effect of the GM on adipose tissue mediated by FIAF inhibition, which promotes fat storage, another mechanism linking obesity, gut dysbiosis, and the adipose tissue is connected with the action of LPS. In fact, LPS is able to stimulate an immune response, leading to inflammation and immune cells penetration (201). More specifically, LPS is involved in the transition of macrophages from the M2 to the M1 phenotype and may activate caspase-4/5/11 (243). In humans, it has been demonstrated that increased LPS release is associated with impaired lipid handling and function in adipose tissue as well as with increased inflammation (244). In addition to these findings, in an *in vitro* model, short-term exposure to LPS impaired the adipogenic capacity and was able to induce premature senescence of adipocyte cells (245).

The endocannabinoid system (ECS) is a complex cell-signaling system, comprising of cannabinoid (CB) receptors, which are present in the brain and peripheral tissue, including the liver and the adipose tissue (233). In obese individuals, increased plasma LPS and endocannabinoid levels and increased ECS tone in the adipose tissue have been reported (246). The study of Muccioli et al. on a mice model demonstrated the capacity of the GM in modulating the expression of endocannabinoid receptors in the gut (i.e. CB1) and proved that the expression of these receptors influences the gut permeability and the

LPS plasma levels. The authors proposed that the endocannabinoid system, modulated by the GM, can be implied in the reduction of gut permeability, thus enforcing the LPS-induced inhibitory effect on adipocyte differentiation and lipogenesis (246). In line with these findings, in a recent animal study, the blocking of CB1 (using a CB1 antagonist) was able to improve diet-induced obesity and to reduce inflammation in the adipose tissue (247).

Figure 3.2 summarizes all the potential mechanisms linking GM with the pathogenesis and development of obesity (208).

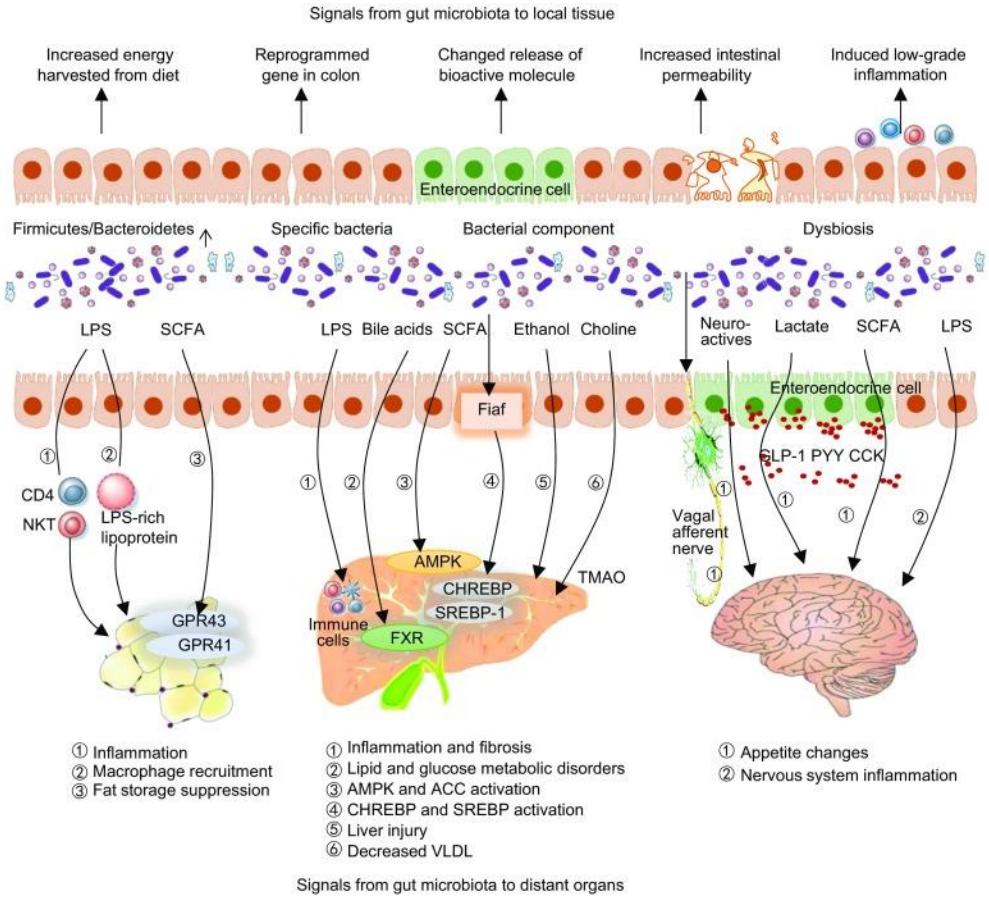

Figure 3.2. Impact of gut microbiota on obesity development and progression (208). Bacterial LPS can cause an alteration of the intestinal permeability, leading to increased concentrations in the plasma. LPS can activate toll-like receptors 4 in the immune cells and the target organs, causing inflammation and neuroinflammation. In the adipose tissue, LPS suppresses adipogenesis. At the same time, the inhibition of FIAF by gut bacteria cause a reduced activity of lipoprotein lipase (LPL), thus decreasing fatty acids oxidation. In addition, SCFAs bind GP43 and GPR41 receptors, inhibiting lipolysis, and encouraging adipocyte differentiation. In the liver, the inhibition of FIAF with decreased activity of LPL determines the activation of *de novo lipogenesis* and the increase in triglycerides contents. Furthermore, intestinal dysbiosis can decrease the activity of FXR receptors in the liver, an important regulator of bile metabolism. The inflammation caused by LPS, ethanol production, and choline metabolism can cause the development of NAFLD. SCFAs, but also lactate, ethanol, and neuropeptides produced by the gut, can activate the production of gut hormones from enteroendocrine cells, or being directly involved in the control of satiety and appetite mechanism in the brain.

4. Aims and methodology

Obesity is a non-communicable chronic disease, associated with increased risk of diabetes, tumors and cardiovascular diseases. In this complex scenario, the gut microbiota can cause weight gain or can influence the response to the therapy of obesity. However, there are only a few studies examining the association between GM composition and obesity in the Italian adult population. In addition, elaborating effective strategies aimed at obtaining weight loss while simultaneously restoring the gut microbial composition represents a still open challenge. In fact, if beneficial modulation of GM can facilitate both the improvement of metabolic outcomes and weight-control in the long term, on the other hand negative impacts on the already compromised gut microbial balance can have a deleterious effect on the colon. This consideration could identify a potential cause of the higher relapse rates registered after drastic diets.

This thesis aims to:

- determine the compositional and functional profiles of the GM of a cohort of overweight/obese Italian patients (OB) with various degrees of visceral obesity, who did not follow any type of dietary program for at least 12 months;
- identify specific microbial features in the study population and its association with anthropometrical and clinical parameters;
- investigate the potential role of a nutritional intervention based on moderate caloric restriction (duration = 3 months) in modulating the GM composition and diversity.

4.1 Patients and control recruitment

Institutional review boards and human subject committees at participating institutions approved the study (Prot.PG/2020/2973). All subjects gave written informed consent in accordance with the Declaration of Helsinki. We enrolled 46 obese/overweight patients (OB) and 46 healthy normal-weight controls (NW). The OB patients were prospectively enrolled at the Obesity clinic of the AOU Cagliari hospital (Cagliari, Italy), coordinated by Prof. Fernanda Velluzzi. The healthy controls were enrolled as volunteers during the same period of patient recruitments. Subjects of both sexes aged at least 18 years were included. The inclusion criteria for the OB group were a BMI≥25 and being "diet-free". We defined as "diet-free" patients that did not follow any specific diet within the 12 months prior to recruitment, in order to define the GM during their usual dietary habits. Exclusion criteria were the following: therapy with antibiotics, proton pump inhibitors, or metformin in the last 3 months; the use of prebiotics, probiotics, or dietary supplements in the last 3 months; the presence of intestinal bowel disease (IBD); the history of cancer; the diagnosis of psychiatric disorders. A group of 46 healthy normal-weight subjects (NW), matched for gender and age, was recruited as control at baseline. As well as OB patients, NW subjects were "diet-free", and did not report bodyweight changes in the last 2 years.

4.2 Samples collection

Stool samples from each subject were collected at outpatient facilities and delivered to the laboratory within 3 hours. Fresh samples were stored at -80°C until further processing. A group of OB patients (N=23) underwent a NI aimed at weight loss. For these patients, the samples were collected both at baseline, before the starting of the NI, and after three months of NI.

4.3 Anthropometric measurements

All the anthropometric measurements were collected on the same day as sample collection. The height expressed in cm was measured with a stadiometer. The body weight, in kilograms, was measured with an impedance scale (TANITA BC-420), also used for the analysis of the body composition. The impedance analysis was performed at room temperature, with patients on fast (for at least 2 hours) and without having performed moderate-intense physical activities in the 24 hours before the test. The following parameters of body composition were extracted and collected for the present study: bodyweight (BW) expressed in Kg, BMI, fat mass (FM) expressed in Kg and percentage, muscle mass (MM) expressed in Kg, basal Metabolic Rate (BMR). The BMI was calculated by the ratio of weight in kilograms and height in meters squared.

4.4 Nutritional assessment

The nutritional anamnesis was performed before starting the sample collection, in order to select DF patients. A weighted 3-day food intake record (3d-FR, including 2 days of the week and 1 weekend day) was collected on the same day of sample collection. The analysis was performed with the Winfood ® software (version 3.0) and the following parameters were obtained: average daily caloric intake expressed in Kcal, daily percentage of macronutrients intake (carbohydrates, lipids, and proteins), daily percentage of saturated lipids intake (on the total lipids intake), and daily intake of fiber expressed in grams. Overall dietary habits were evaluated through the Mediterranean Diet Score (MedDietScore, range 0–55), a composite index of eleven food items that assesses adherence to the Mediterranean diet (MD), where higher scores indicate higher compliance (178).

4.5 Physical activity assessment

The level of physical activity (PA) was assessed on the same day of sample collection by using the International Physical Activity Questionnaire short-form (IPAQ-SF). It consists of seven questions to

capture the average daily time spent sitting, walking, and engaging in moderate and vigorous PA over the last seven days (248).

4.6 Nutritional intervention

Patients followed a prescribed diet, with a daily caloric intake equivalent to their BMR (±10%), as detected by the impedance analysis, and in any case below 1400 Kcal for women and 1600 Kcal for men. The diet was a 7-day meal plan (three meals and two snacks), with the indications of the food and their weighted intake expressed in grams and had a balanced composition in macronutrients (carbohydrates 55%, lipids 25%, protein 20%; fiber ≥25grams/day), as recommended by the Reference intake levels of nutrients and energy for the Italian population (LARN) guidelines (249). Vegetables, fruit, cereal, fish, and white meat typical of the Mediterranean style were inserted into the diet. Patients received the recommendation to use extra-virgin olive oil as a seasoning and to increase the consumption of whole grains, avoiding added sugars and industrial foods. In particular, the following nutritional intakes were included in the diet: 5 portions per day of fruits and vegetables; 3 portions per week of poultry; limited intake of red meat (maximum 3-4 times per month); 2 portions per week of eggs; 2 portions per week of dairy products; 4 portions per week of fish; 3 portions per week of pulses.

4.7 Characterization of the gut microbiota by 16s rRNA gene sequencing

The characterization of the GM was performed at the Microbiological Laboratory of the Department of Biomedical Sciences of the AOU of Cagliari, coordinated by Prof. Aldo Manzin, while the metagenomic analysis, consisting of sequencing the genomic material on the Illumina platform MiSeq, was performed at the Technology Park of Sardinia (CRS4 center, Pula).

The overall method used for the analysis includes the following phases:

- sampling and extraction phase of total genomic DNA (human and bacterial);
- amplification phase and quantitative analysis with Real-Time PCR;
- sequencing phase.

4.7.1 Extraction of total genomic DNA

The QIAamp DNA stool kit (Qiagen, Hilden, Germany) was used for extracting DNA from stool samples.

The overall procedure consists of two main steps:

- lysis and separation of impurities from stool samples in InhibitEX Buffer;
- purification of DNA on QIAamp Mini spin columns.

In the first step of the protocol, stool samples are lysed in InhibitEX Buffer. During lysis, DNA-degrading substances and PCR inhibitors present in the stool samples are separated from the DNA by the InhibitEX buffer. The sample matrix is pelleted by centrifugation and the DNA, present in the supernatant, is purified on QIAamp Mini spin columns.

The second step involves the digestion of proteins, the binding of DNA to the QIAamp silica membrane, and the washing of impurities.

Proteins are digested and degraded under denaturing conditions during a 70°C incubation with proteinase K. Buffering conditions are then adjusted to allow optimal binding of DNA to the QIAamp membrane, and the sample is loaded onto the QIAamp spin column. DNA is adsorbed onto the QIAamp silica membrane during a brief centrifugation step. Optimized salt concentrations and pH conditions in the lysate ensure that the remains of digested proteins and other impurities, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp membrane. DNA bound to the QIAamp membrane is washed in two centrifugation steps. Optimized wash conditions using two wash buffers ensure the complete removal of any residual impurities, without affecting the DNA binding. Purified, concentrated DNA is eluted from the QIAamp Mini spin column in a low-salt buffer. Eluted DNA is collected in standard 1.5 ml microcentrifuge tubes.

The overall procedure is reported below:

- weigh 180-200 mg of sample into a 2 ml tube and place the tube on ice;
- add 1.4 ml of ASL buffer on each stool sample. Vortex continuously for 30 seconds or until the stool sample is accurately homogenized;
- heat the suspension for 5 minutes at 70 ° C;
- vortex for 15 seconds and centrifuge at the maximum speed for 30 seconds;
- pipette 1.2 mL of the supernatant into a new 2 ml tube and discard the pellet;
- add 1 mL of Inhibitex Tablet on each sample and vortex for 1 minute or until the Buffer is completely suspended. Incubate the suspension for 30 seconds at room temperature for allowing the adhesion of the inhibitors to the Inhibitex matrix;
- centrifuge at the maximum speed for 3 minutes for allowing the binding of the inhibitors to the Inhibitex matrix;
- pipette all the supernatant into a new 1.5 ml tube and discard the pellet;
- centrifuge the sample at the maximum speed for 3 minutes;

- pipette 15 μL of proteinase K into a new 1.5 ml tube;
- add 200 μL of the supernatant from the previous step into the 1.5 ml tube containing proteinase K;
- add 200 μL of Buffer AL and vortex for 15 seconds;
- incubate at 70 ° C for 10 minutes;
- add 200 ml of ethanol (96-100%) to the lysate and vortex;
- label the cap of a new QIAamp column placed in a 2 ml collection tube;
- carefully transfer the lysate from the previous step to the QIAamp spin column. Close the cap and centrifuge at maximum speed for 1 minute. Place the QIAamp spin column on a new 2 ml collection tube, and discard the tube containing the filtrate;
- carefully open the QIAamp spin column and add 500 μL of Buffer AW1. Close the cap and centrifuge at maximum speed for 1 minute. Add the QIAamp spin column to a new 2 mL collection tube, and discard the collection tube containing the filtrate;
- carefully open the QIAamp spin column and add 500 μL of Buffer AW2. Close the cap and centrifuge at maximum speed for 3 minutes. Discard the collection tube containing the filtrate;
- place the QIAamp spin column on a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at maximum speed for 1 minute;
- place the QIAamp spin column on a new 1.5 mL labeled tube. Carefully open the QIAamp spin column and pipette 200 μL of buffer AE. Close the lid and incubate for 1 minute at room temperature, then centrifuge at full speed for 1 minute to elute the DNA.

4.7.2 Amplification phase and quantitative analysis with Real-time PCR

The concentration of bacterial DNA extracted from the fecal samples was determined by Real-Time PCR. Primers were complementary upstream and downstream of the V3 and V4 16S rRNA gene regions.

The DNA used for the creation of the standard curve was extracted from pure colonies of *Escherichia coli* incubated at 37 ° C for 24 hours on Agar Mc Conkey culture medium. For this purpose, the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used. The overall procedure is reported below:

- suspend 4-5 bacterial colonies in 180 μL of ATL Buffer;
- add 20 μL of proteinase K;
- vortex and incubate at 56 ° C until a homogeneous lysate is obtained;
- add 200 μL of Buffer;
- incubate at 70 ° C for 10 minutes;
- briefly centrifuge in order to remove any drops formed on the tube cap;
- transfer the suspension to a QIAamp column inserted in a 2 mL collection tube and centrifuge at 8000 rpm for 1 minute;
- place the column inside a new collection tube and add 500 μL of Buffer AWI;
- centrifuge for 1 minute;
- add 500 μL of Buffer AW2;
- centrifuge at 14000 rpm for 3 minutes;
- place the column in a new microcentrifuge tube, discarding the collection tube containing the filtrate;
- add 200 μL of Buffer AE;

- incubate at room temperature for 5 minutes;
- centrifuge at 8000 rpm for 1 minute.

Real-Time PCR is based on the use of a fluorescent molecule, SYBR Green I (Roche Applied Science, Mannheim, Germany), intercalant of DNA that emits a light signal detected in real-time at each cycle of amplification. The PCR phases are those of a traditional PCR: in fact, during the annealing phase, the primers hybridize to the strand of the target DNA and form a double-stranded DNA in which the SYBR Green intercalates.

In the extension phase, SYBR Green continues to be inserted among the neo-synthesis strands by increasing the fluorescent signal, which is measured at 530 nm when, at the end of this growth, it reaches its maximum value, so the maximum amount of SYBR Green I is intercalated.

The DNA extracted from a pure colony of *E. coli* was quantified with the spectrophotometer NanoDrop 1000 (NanoDrop, Wilmington, DE, USA), and the detected concentration was 43.2 ng/μL. For the construction of the first point of the standard curve, the DNA solution at known concentration was diluted by a factor of 6, obtaining a starting solution for creating a curve of 7.2 ng/μL. This solution was then diluted in four successive dilutions by a factor of 10, in order to obtain decreasing concentrations up to the value of 7.2 ng/μL.

Each of the five DNA dilutions of known concentration was processed in duplicate with Real-Time PCR for the creation of the standard curve. For each of the 5 points of the standard curve, the following reagents were inserted:

- 10 μL of SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA);
- 0.5 μL of Primer Forward (10 μM);
- 0.5 μL of Primer Reverse (10 μM);
- 8 μL of DEPC water;
- 1 μL of DNA (final reaction volume of 20 μL).

The DNA at unknown concentration extracted from the faecal samples of the subjects was processed by inserting in reaction 5 μL of DNA, 10 μL of SYBR Green Master Mix, 0.5 μL of Primer Forward and 0.5 μL of Primer Reverse and 4 μL of water DEPC. Finally, a negative control was added.
The primers used for amplification with Real-Time PCR are complementary upstream and downstream of the V3 and V4 regions of the 16S rRNA and are characterized by the following sequence:

16S F2 (Forward Primer):

5'-CCTACGGGNGGCWGCAG-3'

16S R2 (Reverse Primer):

5'-GACTACHVGGGTATCTAATCC-3'

Quantitative PCR was performed using the CFX96 Touch Real-Time System (Bio-Rad, Hercules, CA, USA) tool and Bio-Rad CFX software manager version 3.1, using the following program:
a phase of activation of the DNA polymerase at 98 ° C for 3 minutes, followed by 32 cycles characterized by the repetition of a DNA denaturation phase at 98 ° C, lasting 15 seconds, and an annealing-extension phase at 59 ° C for 50 seconds.

4.7.3 Sequencing phase

The NGS MiSeq (Illumina inc., San Diego, CA, USA) platform (Figure 4.1) follows an operational workflow that includes the template preparation, the cluster generation, the sequencing and imaging, and the bioinformatics data analysis.

Figure 4.1. MiSeq (Illumina) Sequencer.

The 16S library workflow consists of six phases, which are shown in Figure 4.2.

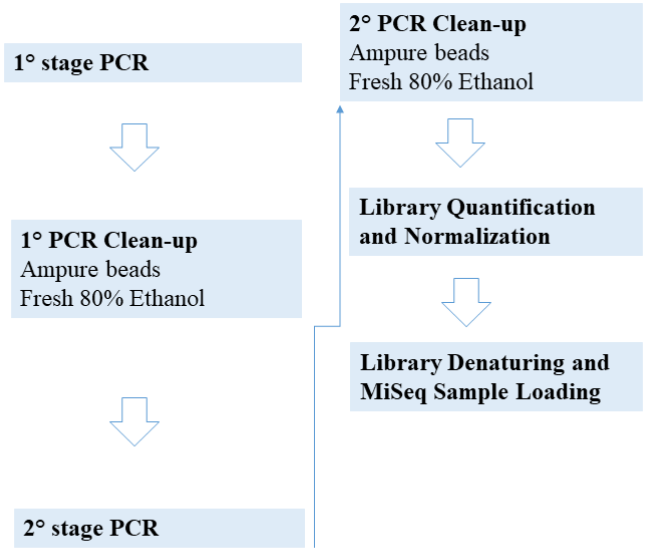

Figure 4.2. 16S Library Preparation Workflow.

Barcoded amplicon libraries for the analysis on the Illumina MiSeq platform were generated using degenerate primers targeting the V3 and V4 hypervariable region of the bacterial 16 S rRNA gene and Nextera XT index kit (Illumina inc., San Diego, CA, USA).

Template preparation

Metagenomics studies are commonly performed by analyzing the ribosomal RNA 16S region of prokaryotes. The first step of the template preparation phase is to amplify the V3 and V4 16S rRNA gene region, in order to obtain millions of identical copies (clones) of the molecules that compose the library. The forward and reverse primers are designed with overhang adapters (Figure 4.3), which are oligonucleotides of known sequence. The overhang adapters are used to insert the Illumina Index and the adapters (used in the subsequent phases of the template preparation).

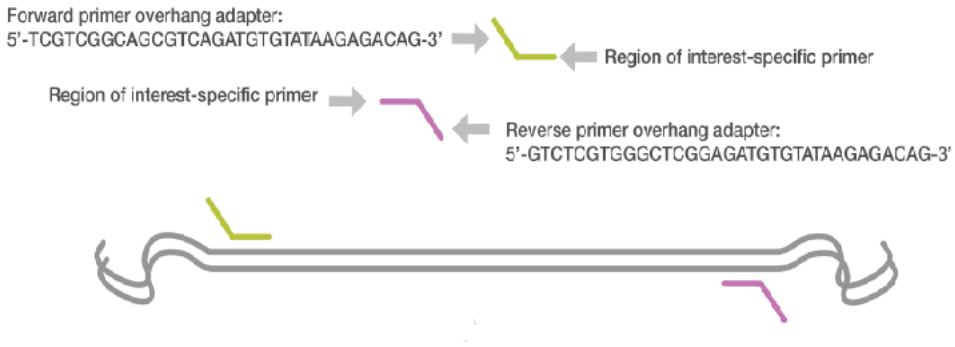

Figure 4.3. Template obtained by the insertion of the region of interest-specific primers with overhang adapters.

PCR reaction was prepared using the following reagents for each sample:

- 5 µL di PCR *Buffer* 10X;
- 1.5 µL di $MgSO_4$ 50 mM;
- 1 µL di dNTPs 10 mM;
- 1 µL di Forward Primer 10 µM;
- 1 µL di Reverse Primer 10 µM;
- 0,2 µL di Taq DNA Polymerase 5U/µL;
- 12,5 ng of DNA.

A final volume of 50 µL was obtained adding DEPC water.

DNA amplification was performed by a 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) using the following program:

• 95°C for 3 minutes;

• 25 cycles of:

— 95°C for 30 seconds

— 55°C for 30 seconds

— 72°C for 30 seconds;

• 72°C for 5 minutes;

• hold at 4°C.

Results of amplified DNA products (460 bp) ran on agarose gel 1%, using the intercalant SYBR *Safe*. The agarose gel was prepared at a concentration of 0,5 % agarose (1 g of agarose in 50 ml of TAE, Tris-Acetyl-EDTA 1X), using SYBR Safe ™ SYBR Green I as a stain.

SYBR Safe ™ SYBR Green I is a cyanine. It binds to DNA, allowing us to visualize the DNA under ultraviolet (UV) light. The DNA-dye complex absorbs blue light (max = 497 nm) and emits green light (max = 520 nm). In this way, it is possible to observe DNA migrations, which form a typical band being trapped on the meshes of electrophoretic gel. The migration is made possible by applying an electrical potential to the base of the tray in which the gel and TAE 1X buffer are inserted. The samples are loaded onto 10 µl of DNA and 2µL of 6X DNA Loadyng Dye (Thermo Scientific, USA). In order to verify the size in bp of the sample bands, a marker of molecular weight (ladder FastRuler® Middle Range DNA) has been added to the agarose gel. The marker separates in the gel, forming bands of known dimensions.

Preparation of 1% agarose gel

The following steps were performed for the agarose gel preparation:

- measure 1 g of agarose;
- mix agarose powder with 100 mL 1xTAE in a microwavable flask;
- microwave for 1-3 minutes until the agarose is completely dissolved;
- let agarose solution cool down to about 50 °C (about when you can comfortably keep your hand on the flask);
- add 12µL Sybr Green in a falcon containing 40mL of agarose solution;
- slowly pour the agarose into a gel tray with the good comb in place, push away any bubbles using a pipette tip;
- wait until the gel has completely solidified;
- once solidified, place the agarose gel into the gel box (electrophoresis unit);
- fill gel box with 1xTAE until the gel is covered.

Samples were loaded using 10 µL of DNA and 2µL of Loading buffer. Loading buffer serves two purposes: 1) it provides a visible dye that helps with gel loading and allows to observe how far the DNA has migrated; 2) it contains a high percentage of glycerol that increases the density of DNA sample causing it to settle to the bottom of the gel well, instead of diffusing in the buffer.

The molecular weight ladder was loaded into the first lane of the gel guide (2 µL loading buffer; 7 µL marker/ladder; 3 µL buffer 1xTAE). Samples were loaded into the additional wells of the gel. Gel ran at 100 V for 30 minutes. At this point, electrodes were disconnected from the power source, and the gel was removed from the gel box. DNA bands were visualized by transilluminator, using the ladder as a guide.

Purification of PCR products (PCR clean-up 1)

PCR products have been purified by using AMPure XP beads (Beckman Coulter). This step allows to separate the amplified material from the primers and primer's dimers present in the reaction. The overall procedure is described below:

- centrifuge the Amplicon PCR plate at 1000 x g at 20 ° C for one minute;
- add 20 μL of AMPURE XP beads and incubate at room temperature for 5 minutes;
- place the multiwell plate containing the samples on a magnetic stand for 2 minutes;
- remove and discard the supernatant;
- wash the beads with 200 μL of 80% ethanol, incubate at room temperature for 30 seconds and remove the supernatant (2 times);
- allow the beads to air-dry for 10 minutes;
- remove the Amplicon PCR plate from magnetic stand and add 52.5 μL of 10 mM Tris at pH 8.5;
- gently pipette mix up and down ten times;
- incubate at room temperature for 2 minutes;
- place the Amplicon PCR plate on a magnetic stand for 2 minutes;
- transfer 50 μL of supernatant of each sample in a new multiwell.

Index PCR

The second PCR step was performed in order to index the different samples and insert the sequencing adapters. In this phase, index sequences act as a *barcode*. This procedure allows us to obtain a unique identification of each sample in the pooling process.

The function of the sequencing adapters is to bind to complementary oligonucleotides present on the surface of the sequencing flow cell. Sequencing adapters and dual index barcodes were added by using the Nextera XT Index Kit (Illumina inc., San Diego, CA, USA).

In particular, pairs of indices, each with eight bases, were used: Nextera XT Index 1 (i7), adjacent to the P7 adapter sequence, and Nextera XT Index 2 (i5) adjacent to the P5 adapter sequence. The Nextera XT Index Kit for 96 samples uses 12 different Index 1 and 8 different Index 2 (Table 4.1). The combination of the different types of Index 1 and 2 made it possible to index all our samples (in duplicate).

Table 4.1. Index Primer 1 and Index Primer 2 and their sequences

Index 1 (i7)	Sequence	Index 2 (i5)	Sequence
N701	TAAGGCGA	S501	TAGATCGC
N702	CGTACTAG	S502	CTCTCTAT
N703	AGGCAGAA	S503	TATCCTCT
N704	TCCTGAGC	S504	AGAGTAGA
N705	GGACTCCT	S505	GTAAGGAG
N706	TAGGCATG	S506	ACTGCATA
N707	CTCTCTAC	S507	AAGGAGTA
N708	CAGAGAGG	S508	CTAAGCCT
N709	GCTACGCT		
N710	CGAGGCTG		
N711	AAGAGGCA		
N712	GTAGAGGA		

The dual indices were placed in a TruSeq Index Plate Fixture according to the following procedure: Index Primers 2 (with a white cap) were arranged vertically, aligned with rows A through H. Index Primer 2 tubes (orange cap) were arranged horizontally and aligned with columns 1 through 12 (Figure 4.4).

Then the PCR reaction was prepared according to the following protocol: 5 μL of DNA purified with AMPure XP beads, 5 μL of Nextera XT Index Primer 1, 5 μL Nextera XT of Nextera XT Index Primer

2, 25 µL 2x KAPA HiFi HotStart ReadyMix and 10 µL of DEPC water. The 96-well plate was then centrifuged at 1000 x g at 20 ° C for 1 minute.

Afterward, the PCR reaction was performed by using a 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) according to the following amplification profile:

• 8 cycles of:

— 95°C for 30 seconds

— 55°C for 30 seconds

— 72°C for 30 seconds;

• 72°C for 5 minutes;

• hold at 4°C.

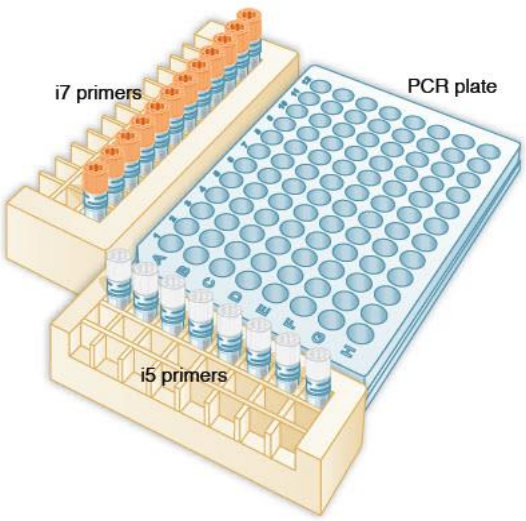

Figure 4.4. TruSeq Index Plate Fixture showing Index 2 primers (white caps) and Index 1 primers (orange caps).

Purification of PCR products (PCR clean-up 2)

In a similar way to PCR clean-up 1, the PCR products were purified from any contaminants present in the reaction. The protocol below differs from the previous one from the number of reagents used. The overall procedure is reported below:

- centrifuge the Index PCR plate at 280 x g at 20 ° C for one minute;
- add 56 μL of AMPURE XP beads to each sample and incubated at room temperature for 5 minutes;
- place the multi-well containing the samples in the magnetic stand for 2 minutes and remove the supernatant;
- wash each sample with 200μL of 80% ethanol and remove the supernatant (two times);
- allow the bead to dry-air for 10 minutes;
- incubate at room temperature for 2 minutes;
- remove the Amplicon PCR plate from magnetic stand and add 52.5 μL of 10 mM Tris at pH 8.5;
- gently pipette mix up and down ten times;
- incubate at room temperature for 2 minutes;
- place the Amplicon PCR plate on a magnetic stand for 2 minutes;
- transfer 25 μL of supernatant into the wells of a new multi-well.

At this point, the samples were diluted 1:50 and analyzed with the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), in order to estimate the size of the resulting libraries.

Library Quantification, Normalization, and Pooling

The concentration of each library was calculated by Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA).

The following formula was used for calculating DNA concentration in nM:

(concentration DNA library in ng/μL) × 10^6/(660 g/mol × average library size)

Genomic libraries were diluited using 10 mM Tris pH 8.5 to 4nM. Finally, 5 μL of diluted DNA were aliquoted from each library, and the aliquots of each library (with unique indices) were mixed.

Library Denaturing

Before proceeding with cluster generation and sequencing, the denaturing of the libraries was performed: 5 μL of the library were suspended with 5 μL of 0.2N NaOH. After having homogenized the solution by using a vortex, the samples were centrifuged at 20 ° C for one minute.

Subsequently, the samples were incubated for 5 minutes at room temperature, in order to allow the denaturing of the DNA.

At the end of this step, the denatured libraries were diluted with 990 μL of HT1 hybridization buffer, obtaining a final concentration at 20 pM. The sample was further diluted to 8 pM by resuspending 240 μL of the library with 360 μL of HT1 hybridization buffer.

Denaturation and Dilution of PhiX Control

In order to evaluate the correct execution of sequencing, a positive PhiX v3 control was used. The first step of the control preparation phase is its dilution: 3 μL of 10 mM Tris (pH 8.5) were added to 2 μL of the PhiX library (10 nM), in order to obtain a final concentration of 4 nM.

At this point, 5 μL of 0.2N NaOH were added to the 5 μL of the positive control. After homogenizing the solution by using a vortex, the sample was incubated at 96 ° C for 5 minutes, with the aim to obtain the heat denaturation of the control libraries.

Then, 990 μL of frozen Hybridization Buffer HT1 were added to the tube containing the PhiX library, to reach a final concentration of 20 pM.

Subsequently, 240 µL of the denatured library of the PhiX v3 control were resuspended in 360 µL of HT1 hybridization buffer, to reach the final concentration of 8 pM, equivalent to that of the samples library.

Finally, the two just denatured and diluted libraries were combined into a single tube containing: 30 µL of PhiX library and 570 µL of the genomic library to be sequenced.

Immediately before loading the library onto the MiSeq platform, the final denaturation was carried out with heat: the solution containing the libraries was incubated at 96 ° C for 2 minutes; at the end of the incubation the tube was inverted 1-2 times for mixing and immediately placed in ice for 5 minutes. The prepared library was loaded onto the MiSeq reagent cartridge, containing the reagents. The disposable cartridge provided by the Miseq Reagent Kit v3 was used, which consists of several sealed wells pre-filled with the reagents necessary for sequencing.

Samples loading

When the reagent cartridge was completely defrosted and ready for use, libraries were loaded in the cartridge: the seal that covers the tank was drilled by using the tip of a clean pipette and 600 µL of the library were pipetted in the appropriate gallery.

Cluster generation

The templates were placed in the Flow-cell Plate (Figure 4.5) of the Illumina system and two different oligonucleotides were immobilized on this surface.

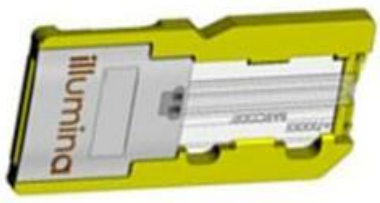

Figure 4.5. Flow-cell Plate Illumina.

In the plate, the template adapters, and their complementary oligonucleotides hybridize. After the immobilization of the *templates*, the extension process begins, in which the DNA polymerase synthesizes the filaments complementary to the *templates*. The molecules obtained are denatured and the original template filament is washed away from the system.

The newly synthesized strand, which remains bound to the plate, flexes until it binds with the other extremity to its complementary oligonucleotide on the plate, assuming a "bridge" shape "(Figure 4.6).

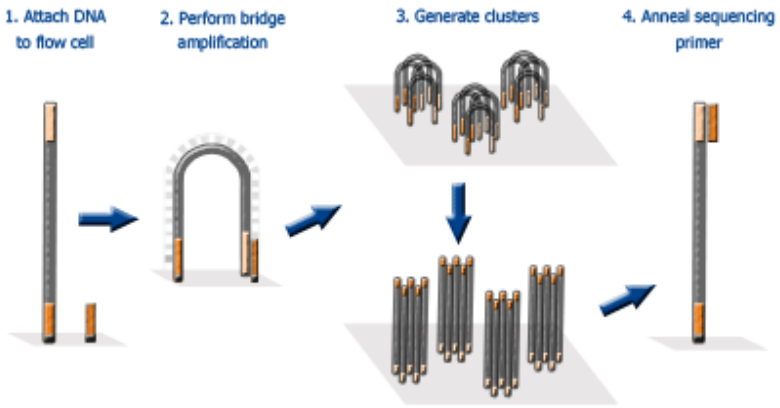

Figure 4.6. Template binding and cluster formation.

The fragments bound to the bridge are then amplified; a subsequent denaturation frees the fragments from one extremity which bends again to bind to the adjacent complementary oligonucleotide of the plate.

The operation is repeated cyclically and the immobilization-synthesis-denaturation steps continue until a cluster of thousands of fragments is obtained, bound with an extremity to the substrate, and collected in a very limited space. The process is called "*bridge*-PCR".

Sequencing

Sequencing was performed using the Illumina MiSeq platform. The sequencing primer is aligned to the fragments of each cluster, allowing the sequencing reaction to start.

Each sequencing cycle involves a DNA polymerase and deoxynucleotide (dNTP) reversible terminators labeled with four different fluorescent molecules, one for each DNA base.

The reversible terminators have the characteristic of being able to be chemically reported to the original nucleotide structure. After each incorporation, a laser excites the fluorescent marker of the terminator dNTP, generating a light emission that allows the identification of the base. The fluorescent molecule is then removed and the terminator is chemically transformed, reactivating the synthesis that allows the sequencing of the next base.

The *Illumina MiSeq* platform can produce up to 15 gigabases of data in one run, 25 million *"reads*" with a length of 2 * 300 bp each, ensuring a high degree of accuracy and reliability.

The sequencing data obtained from MiSeq were analyzed using MiSeq Reporter or on a network server using BaseSpace 16S Metagenomics App (Illumina inc., San Diego, CA, USA). Operational taxonomic units (OTUs) were taxonomically classified with an accurate GreenGenes database and the analysis of computer data was performed using the QIIME 1.8 "Quantitative Insights Into Microbial Ecology" program.

To obtain greater comparisons between the different samples the Shannon-Wiener diversity index (H ') was calculated as:

In which: $$H' = -\sum_{i=1}^{S} p_i \log_2 p_i$$

pi = frequency of the i-th estimated taxon as ni / N

N = the total number of individuals in the sample

ni = abundance of the i-th sample taxon

This index takes into account the total number of OTUs (Operational Taxonomic Units) present and their relative prevalence within each sample.

Sequences containing ambiguous or low-quality bases were filtered out using QIIME filter53.

Remaining sequences were assigned to each sample according to the unique barcodes.

4.8 Data processing and statistical analysis

Alpha diversity was generated with the script alpha_rarefaction.py in QIIME in order to obtain the Shannon index. Alpha diversities were compared by using the Mann-Whitney's test. Beta diversity was generated in R-vegan, using Bray-Curtis distance. Non-Metric Multidimensional Scaling (NMDS) was conducted on community distance matrix obtained in vegan and NMDS plots were built using the ggplot2 package. The statistical significance of beta diversity among the two groups was determined with Permutational Multivariate Analysis of Variance (PERMANOVA) (R-vegan, function adonis). For the comparison of beta diversity before and after NI, the strata argument was specified in the adonis function (R software v. 3.5.2).

For the analysis of the taxonomic levels, only bacteria present in at least 25% of our samples, and with a relative abundance, ≥0.1% in cases and/or controls were considered. The statistical significance of the difference in the Firmicutes/Bacteroidetes ratio between OB and NW was calculated by using the Mann-Whitney test. MaAsLin algorithm was used to perform a multivariate analysis comparing OB and NW at baseline, adjusted for sex, age, and smoking status. In addition, Linear discriminant analysis Effect Size (LEfSE) was employed to identify distinguishing taxa between the two groups at multiple levels and to visualize the results using taxonomic bar charts. LEfSE algorithm and MaAsLin algorithm were performed on Galaxy computational tool v. 1.0.1. (http://huttenhower.sph.harvard.edu/galaxy/) (250,251).

The comparison between GM profiles before and after the intervention was performed by using the Wilcoxon test for paired data. For the analysis of the taxonomic levels, only bacteria present in at least 25% of our samples and with a relative abundance ≥0.1% before or after intervention were considered.

All the p-values (p) were adjusted for false discovery rate (FDR) and q-values (q) < 0.05 were considered as statistically significant.

The association between the relative abundance of significant taxonomic levels and clinical parameters was evaluated by calculating the Spearman's correlation (R software v. 3.5.2). $p < 0.05$ were considered as statistically significant.

Anthropometric measurements and nutritional data before and after NI were compared using a t-test for paired data (R software v. 3.5.2). $p < 0.05$ were considered as statistically significant.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, v. 1.1.4) (10) was performed on Galaxy computational tool to infer metagenome composition in the samples. QIIME pipeline was used for OTUs picking from data generated on the Illumina platform. After the OTUs normalization by copy number, metabolic pathways were predicted and classified by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database orthologs, at hierarchical level 3. OTUs contributing to the abundance of functional categories in each sample have been detected by the function "metagenome contribution by higher category", using KEGG's classification type. The differences between obese/overweight patients and normal-weight subjects were analyzed for all metabolism pathways that presented mean differences of at least 0.1% between the two groups, using the Statistical Analysis of Metagenomic Profiles (STAMP) software (252). The statistical significance was tested using Welch's test, with a Storey FDR correction (FDR cutoff <0.05). The statistical significance of differences in metabolism pathways after NI was analyzed for all metabolism pathways that presented mean differences (the mean after NI minus the mean before NI) of at least 0.1% after the intervention, using Wilcoxon test for paired data (R software v.3.5.2) with Benjamini and Hochberg correction (FDR cutoff <0.05).

5. Results

5.1 Characteristics of the study population

Table 5.1 shows the anthropometric and clinical characteristics of OB at baseline (N=46), compared with NW (N= 46).

Overall, the analysis of clinical data showed that the OB group diverged significantly from healthy controls in terms of BMI, waist circumference and various lifestyle factors. As regards lifestyle data, the nutritional anamnesis highlighted an excessive consumption of sugary drinks, sweets, industrial food, and a low consumption of fruit, vegetables, and pulses, as well as the absence of whole grains in OB patients. In line with these findings, the analysis of the food diaries pointed out an excess of simple carbohydrates and saturated fatty acids and lack of fibers in the same subjects; on the other hand control subjects presented, as expected, a healthier diet, in terms of caloric intake, macronutrients quality and fiber intake, which were more in line with LARN guidelines (249). Noteworthy, OB patients presented an excessive intake of saturated lipids, significantly greater than that of NW and, contrariwise, a low daily intake of fiber, significantly lower than that of NW. MedDietScore highlighted good compliance to MD in NW, while it was low in OB, although this difference was not statistically significant. The level of physical activity was low in the group of patients and significantly reduced compared to NW, who, in contrast, showed a medium-high level.

Table 5.1. Clinical characteristics of study participants

	NW	**OB**	***p***
N	46	46	
Age (M ± SD)	49 ±11	50 ±12	0.345
Female (n, %)	40, 87.0	40, 87.0	1.000
Anthropometric data			
BMI (M ± SD)	21.6 ± 2.1	36.0 ± 6.0	**9.9×10^{-5}**
Waist circumference (cm) (M ± SD)	73.7 ± 5.7	111 ± 15	**7×10^{-6}**
Overweight (N, %)		5, 11	
Obesity Class I (N; %)		17, 37	

Obesity Class II (N; %)		17, 37	
Obesity Class III (N, %)		7, 15	
Lifestyle factors			
Smoking status Yes (n, %)	8, 17.8	12, 26	0.339
Daily caloric intake kcal (M ± SD)	1467.8 ± 162.3	1810 ± 627	**1.563×10^{-8}**
Daily carbohydrates intake % (M ± SD)	50.9 ± 3	50 ± 7	**8×10^{-6}**
Daily lipids intake % (M ± SD)	27.2 ± 4.3	33 ± 6	**0.006**
Daily saturated lipids intake/total lipids intake % (M± SD)	27.8 ± 4.3	38 ± 7	**0.022**
Daily proteins intake grams (M ± SD)	62.3 ± 8.8	78 ± 43	**0.001**
Daily fiber intake grams (M ± SD)	20.0 ± 3.2	15 ± 8	**0.018**
MedDietScore (M ± SD)	33.4 ± 4.1	28 ± 5	0.106
IPAQ METs/week (M ± SD)	2604.8 ± 135.4	662 ± 28	**1×10^{-6}**
Dyslipidemia			
Yes (n, %)	0	12, 26	
Alteration in glucose metabolism*			
Yes (n, %)	0	32, 70	
Hypertension			
Yes, %	0	11, 24	

*impaired Fasting Plasma Glucose (FPG) between 100–125 mg/dl (6.9 mmol/l), impaired glucose tolerance (IGT) if 2 h post-OGTT plasma glucose was 140–199 mg/dl (7.8-11.0 mmol/l), T2DM if FPG was ≥ 126 mg/dl (≥ 7 mmol/l) on two days apart, or if 2h post-OGTT plasma glucose was ≥ 200 mg/dl (≥ 11.1 mmol/l). The statistical significance was evaluated by *t* test for independent samples for continuous variables and by Pearson's chi-squared test for categorical variables. Bold values denote statistical significance ($p < 0.05$). NW = healthy normal-weight controls, OB = overweight and obese patients.

At the time of the second sample collection (after 3 months of NI), the 23 patients (F/M=20/3) included in the intervention presented decreased body weight, waist circumference, and fat mass. In addition, the analysis of the 3-d FR, reporting the nutritional intake in the 3-days before the second sample collection, showed an important decrease in the caloric intake compared with baseline, indicating a good adherence to the diet. The anthropometric measurements and the nutritional intake before and after intervention are shown in Table 5.2.

In addition, the analysis of the 3-d FR, reporting the nutritional intake in the 3-days before the second sample collection, showed a decrease in the caloric intake compared with baseline, and a lower, although not significant, intake of saturated lipids, along with a higher intake of fiber, without variation in carbohydrates intake, indicating a good adherence to the diet.

Table 5.2. Anthropometric measurements and nutritional intake before (T0) and after (T3) nutritional intervention (N=23).

Clinical parameter	T0	T3	*p*
Weight (Kg), M (SD)	89.5 (19.3)	82.8 (17.0)	**0.015**
Waist circumference (cm), M (SD)	108 (14)	102 (16)	**0.040**
Body mass index, M (SD)	35.2 (4.3)	33.6 (4.5)	**0.001**
Fat mass (Kg)	37.8 (10.2)	32.7 (8.2)	**0.0002**
Muscle mass (Kg)	47.2 (14.0)	47.6 (9.8)	0.493
Daily caloric intake (Kcal), M (SD)	1779 (534)	1341 (298)	**0.007**
Carbohydrates intake (%), M (SD)	50 (6)	50 (8)	0.578
Lipids intake (%), M (SD)	33 (6)	29 (9)	0.196
Saturated lipids intake/Total lipids intake (%), M (SD)	39 (5)	35 (8)	0.139
Daily proteins intake (grams/day), M (SD)	73 (23)	64 (13)	0.384
Daily fibers intake (grams/day), M(SD)	14 (6)	17 (6)	0.234

Statistical significance was evaluated by using a t-test for paired data (R software v3.5.2). Bold values denote statistical significance ($p < 0.05$).

5.2 Gut microbiota composition and diversity

After applying filters based on the frequency and relative abundance of each taxonomic level (at least 25% of our samples and with a relative abundance ≥0.1% in cases and/or controls), 201 OTUs were identified (8 phyla, 37 families, 63 genera, 93 species). The median (range) of each OTU is reported in Table 5.3.

Table 5.3. Median and range of the relative abundance of each taxonomic level in overweight and obese patients (OB) and normal-weight controls (NW)

	OB		NW	
Taxonomic profile	**Median**	**Range (min, max)**	**Median**	**Range (min, max)**
Actinobacteria	2.12	(0.51, 22.23)	1.86	(0.14, 14.45)
Bacteroidetes	29.69	(2.94, 74.72)	40.59	(7.18, 83.43)
Chloroflexi	0.05	(0.02, 0.59)	0.08	(0.01, 0.67)
Cyanobacteria	0.16	(0.08, 1.97)	0.40	(0.05, 3.95)
Firmicutes	54.00	(15.39, 87.86)	47.10	(13.85, 67.75)
Proteobacteria	3.61	(1.36, 23.48)	3.18	(1.31, 30.61)
Synergistetes	0.06	(0.01, 0.25)	0.03	(0.00, 2.33)
Verrucomicrobia	0.13	(0.01, 36.7)	0.24	(0.00, 25.08)
Alcaligenaceae	0.13	(0.01, 6.49)	0.39	(0.00, 2.62)
Anaerobrancaceae	0.03	(0.00, 0.96)	0.06	(0.00, 2.17)
Bacteroidaceae	16.13	(1.05, 57.72)	25.53	(4.52, 66.51)
Bifidobacteriaceae	1.30	(0.09, 20.90)	1.05	(0.01, 13.86)
Caldilineaceae	0.05	(0.00, 0.58)	0.08	(0.01, 0.65)
Clostridiaceae	4.85	(1.47, 11.65)	4.55	(0.74, 20.62)
Comamonadaceae	0.03	(0.00, 0.93)	0.04	(0.00, 0.71)
Coprobacillaceae	0.05	(0.00, 1.61)	0.02	(0.00, 4.70)
Coriobacteriaceae	0.47	(0.06, 8.94)	0.22	(0.02, 3.52)
Desulfohalobiaceae	0.05	(0.00, 0.63)	0.07	(0.00, 1.27)
Desulfovibrionaceae	0.16	(0.01, 9.93)	0.27	(0.00, 2.61)
Enterobacteriaceae	0.71	(0.04, 21.37)	0.89	(0.03, 12.87)
Erysipelotrichaceae	0.21	(0.02, 3.85)	0.25	(0.01, 7.40)
Eubacteriaceae	0.09	(0.02, 0.36)	0.07	(0.02, 0.33)
Flavobacteriaceae	1.61	(0.04, 7.65)	3.89	(0.18, 21.96)
Gemellaceae	0.08	(0.02, 0.48)	0.05	(0.01, 0.17)
Lachnospiraceae	15.14	(3.35, 79.95)	10.39	(0.00, 26.07)
Lactobacillaceae	0.11	(0.01, 4.06)	0.08	(0.00, 1.09)
Microbacteriaceae	0.10	(0.04, 4.52)	0.10	(0.00, 6.01)
Nostocaceae	0.08	(0.03, 1.79)	0.13	(0.02, 3.61)
Odoribacteraceae	0.12	(0.00, 0.68)	0.28	(0.00, 3.18)
Paenibacillaceae	0.05	(0.01, 0.43)	0.03	(0.01, 0.36)
Paraprevotellaceae	0.08	(0.00, 14.47)	0.05	(0.00, 3.03)
Pasteurellaceae	0.02	(0.00, 2.19)	0.02	(0.00, 1.86)
Peptococcaceae	0.17	(0.04, 0.95)	0.18	(0.02, 1.57)
Porphyromonadaceae	1.16	(0.08, 6.86)	2.61	(0.09, 14.87)
Prevotellaceae	0.16	(0.01, 24.45)	0.04	(0.00, 39.80)
Rikenellaceae	0.02	(0.00, 4.27)	0.00	(0.00, 0.50)
Ruminococcaceae	15.24	(1.33, 52.40)	16.75	(4.23, 48.19)
Sphingobacteriaceae	0.38	(0.02, 3.45)	1.12	(0.15, 9.52)

Table 5.3. Median and range of the relative abundance of each taxonomic level in overweight and obese patients (OB) and normal-weight controls (NW)

	OB		NW	
Taxonomic profile	**Median**	**Range (min, max)**	**Median**	**Range (min, max)**
Streptococcaceae	0.25	(0.06, 10.21)	0.10	(0.02. 1.48)
Streptomycetaceae	0.09	(0.04, 1.31)	0.07	(0.02, 0.86)
Succinivibrionaceae	0.00	(0.00, 0.26)	0.00	(0.00, 23.55)
Thermicanaceae	0.03	(0.00, 3.45)	0.01	(0.00, 0.32)
Thiotrichaceae	0.03	(0.00, 0,31)	0.06	(0.01, 0.78)
Veillonellaceae	4.23	(0.27, 38.39)	2.61	(0.08, 15.71)
Verrucomicrobiaceae	0.12	(0.00, 36.50)	0.24	(0.00, 25.00)
Acetobacterium	0.09	(0.02, 0.36)	0.07	(0.02, 0.30)
Acidaminococcus	0.02	(0.00, 4.77)	0.00	(0.00, 9.34)
Akkermansia	0.06	(0.00, 31.54)	0.20	(0.00, 22.30)
Alkaliphilus	0.32	(0.04, 3.86)	0.43	(0.04, 6.37)
Anaerobranca	0.03	(0.00, 0.96)	0.06	(0.00, 2.17)
Anaerofilum	0.08	(0.00, 0.74)	0.12	(0.01, 1.35)
Anaerostipes	0.09	(0.00, 1.41)	0.08	(0.00, 2.65)
Bacteroides	16.13	(1.05, 57.72)	25.53	(4.52, 66.51)
Bifidobacterium	1.29	(0.08, 20.75)	1.04	(0.01, 13.84)
Bilophila	0.06	(0.00, 1.93)	0.12	(0.00, 1.06)
Blautia	6.76	(1.29, 18.49)	4.80	(0.00, 15.90)
Butyricimonas	0.04	(0.00, 0.59)	0.06	(0.00, 2.52)
Caldilinea	0.05	(0.00, 0.58)	0.08	(0.01, 0.650)
Caloramator	0.03	(0.01, 1.95)	0.07	(0.01, 14.09)
Candidatus Blochmannia	0.02	(0.01, 0.10)	0.03	(0.00, 2.87)
Catenibacterium	0.00	(0.00, 1.61)	0.00	(0.00, 4.67)
Clostridium	2.46	(0.74, 7.69)	2.17	(0.46, 10.65)
Collinsella	0.17	(0.00, 7.08)	0.08	(0.00, 2.14)
Coprococcus	0.30	(0.02, 4.44)	0.30	(0.04, 2.83)
Desulfonauticus	0.05	(0.00, 0.62)	0.07	(0.00, 1.27)
Desulfotomaculum	0.05	(0.00, 0.47)	0.07	(0.00, 1.50)
Desulfovibrio	0.08	(0.01, 9.54)	0.04	(0.00, 2.49)
Dialister	0.00	(0.00, 17.15)	0.01	(0.00, 15.22)
Dorea	0.27	(0.00, 5.27)	0.16	(0.00, 5.30)
Dysgonomonas	0.06	(0.00, 3.58)	0.04	(0.00, 12.40)
Enterobacter	0.07	(0.00, 2.67)	0.01	(0.00, 0.49)
Erysipelothrix	0.12	(0.02, 1.72)	0.16	(0.01, 7.34)
Escherichia	0.20	(0.00, 11.14)	0.04	(0.00, 1.80)
Eubacterium	0.02	(0.00, 2.12)	0.01	(0.00, 0.37)
Faecalibacterium	8.68	(0.42, 45.17)	6.10	(0.68, 37.40)
Flavobacterium	1.49	(0.02, 7.55)	3.42	(0.00, 21.82)
Gemella	0.08	(0.02, 0.48)	0.05	(0.00, 0.17)
Kitasatospora	0.08	(0.02, 1.22)	0.05	(0.01, 0.82)
Klebsiella	0.04	(0.00, 0.34)	0.00	(0.00, 10.64)
Lachnobacterium	0.01	(0.00, 2.97)	0.01	(0.00, 3.19)
Lachnospira	0.41	(0.04, 3.55)	0.47	(0.01, 4.68)
Lactobacillus	0.10	(0.01, 3.88)	0.08	(0.02, 1.02)
Luteibacter	0.00	(0.00, 0.04)	0.00	(0.00, 0.02)
Megamonas	0.00	(0.00, 13.29)	0.00	(0.00, 0.11)
Megasphaera	0.03	(0.00, 25.59)	0.01	(0.00, 0.24)
Mitsuokella	0.00	(0.00, 3.89)	0.00	(0.00, 0.20)
Natronincola	0.43	(0.11, 1.94)	0.28	(0.07, 1.35)
Negativicoccus	0.06	(0.00, 4.84)	0.01	(0.00, 0.27)
Oscillospira	1.58	(0.15, 18.28)	3.16	(0.00, 20.02)
Parabacteroides	0.88	(0.00, 6.29)	1.92	(0.08, 14.76)
Paraprevotella	0.06	(0.00, 14.47)	0.04	(0.00, 1.63)

Table 5.3. Median and range of the relative abundance of each taxonomic level in overweight and obese patients (OB) and normal-weight controls (NW)

	OB		NW	
Taxonomic profile	**Median**	**Range (min, max)**	**Median**	**Range (min, max)**
Pedobacter	0.13	(0.00, 2.82)	0.44	(0.03, 3.89)
Peptoniphilus	0.07	(0.03, 0.75)	0.07	(0.00, 0.59)
Phascolarctobacterium	0.33	(0.03, 27.00)	0.97	(0.00, 7.47)
Prevotella	0.16	(0.01, 24.45)	0.04	(0.00, 39.80)
Pseudobutyrivibrio	0.31	(0.01, 5.04)	0.23	(0.00, 1.90)
Rikenella	0.02	(0.00, 4.27)	0.09	(0.00, 1.38)
Roseburia	1.75	(0.00, 52.25)	1.00	(0.00, 14.23)
Ruminococcus	3.50	(0.41, 13.09)	3.53	(0.00, 25.46)
Sedimentibacter	0.07	(0.01, 0.32)	0.07	(0.01, 0.94)
Serratia	0.09	(0.00, 5.70)	0.02	(0.00, 0.24)
Slackia	0.12	(0.03, 1.73)	0.06	(0.01, 1.18)
Sphingobacterium	0.09	(0.00, 0.54)	0.18	(0.00, 2.25)
Streptococcus	0.23	(0.05, 9.99)	0.10	(0.02, 1.45)
Sutterella	0.12	(0.01, 6.48)	0.39	(0.00, 2.61)
Thermicanus	0.03	(0.00, 3.45)	0.01	(0.00, 0.32)
Thiothrix	0.03	(0.00, 0.31)	0.06	(0.01, 0.78)
Veillonella	0.10	(0.01, 4.02)	0.03	(0.01, 1.79)
Acidaminococcus fermentans	0.00	(0.00, 2.95)	0.00	(0.00, 0.16)
Acidaminococcus intestini	0.01	(0.00, 1.90)	0.00	(0.00, 2.34)
Akkermansia muciniphila	0.06	(0.00, 31.54)	0.20	(0.00, 22.29)
Alkaliphilus crotonatoxidans	0.08	(0.00, 3.71)	0.22	(0.01, 6.31)
Alkaliphilus peptidifermentans	0.04	(0.00, 1.16)	0.02	(0.00, 4.40)
Anaerobranca zavarzinii	0.03	(0.00, 0.96)	0.06	(0.00, 2.17)
Anaerofilum pentosovorans	0.04	(0.00, 0.61)	0.06	(0.01, 1.23)
Bacteroides caccae	0.12	(0.00, 2.64)	0.43	(0.00, 4.51)
Bacteroides cellulosilyticus	0.03	(0.00, 0.79)	0.10	(0.00, 4.24)
Bacteroides coprocola	0.03	(0.00, 12.88)	0.01	(0.00, 11.67)
Bacteroides coprophilus	0.00	(0.00, 2.62)	0.00	(0.00, 3.51)
Bacteroides denticanum	0.10	(0.00, 3.91)	0.07	(0.01, 4.51)
Bacteroides dorei	0.31	(0.00, 18.13)	0.99	(0.00, 13.89)
Bacteroides eggerthii	0.00	(0.00, 13.40)	0.00	(0.00, 2.92)
Bacteroides fragilis	0.00	(0.00, 0.48)	0.00	(0.00, 4.28)
Bacteroides intestinalis	0.00	(0.00, 1.69)	0.00	(0.00, 3.61)
Bacteroides massiliensis	0.00	(0.00, 2.08)	0.00	(0.00, 1.57)
Bacteroides ovatus	0.13	(0.00, 7.41)	0.27	(0.00, 14.55)
Bacteroides paurosaccharolyticus	0.11	(0.00, 0.79)	0.13	(0.00, 0.70)
Bacteroides plebeius	0.00	(0.00, 8.26)	0.00	(0.00, 13.97)
Bacteroides rodentium	0.91	(0.043, 6.07)	2.29	(0.39, 13.66)
Bacteroides salanitronis	0.00	(0.00, 7.60)	0.00	(0.00, 1.48)
Bacteroides salyersiae	0.00	(0.00, 0.95)	0.00	(0.00, 9.30)
Bacteroides sartorii	0.16	(0.01, 4.03)	0.21	(0.02, 0.71)
Bacteroides stercorirosoris	0.27	(0.04, 2.02)	0.39	(0.11, 2.44)
Bacteroides stercoris	0.13	(0.00, 11.69)	0.08	(0.00, 20.16)
Bacteroides thetaiotaomicron	0.15	(0.00, 3.16)	0.30	(0.00, 6.49)
Bacteroides uniformis	0.80	(0.00, 6.21)	2.21	(0.31, 16.21)
Bacteroides vulgatus	0.68	(0.02, 20.48)	1.08	(0.04, 27.46)
Bacteroides xylanisolvens	0.64	(0.05, 6.14)	0.88	(0.14, 16.24)
Bifidobacterium adolescentis	0.05	(0.00, 7.55)	0.03	(0.00, 3.05)
Bifidobacterium catenulatum	0.01	(0.00, 7.30)	0.00	(0.00, 1.59)
Bifidobacterium choerinum	0.02	(0.00, 0.77)	0.02	(0.00, 0.85)
Bifidobacterium kashiwanohense	0.02	(0.00, 2.57)	0.00	(0.00, 0.250)
Bifidobacterium longum	0.12	(0.00, 7.76)	0.18	(0.00, 8.62)
Bifidobacterium scardovii	0.05	(0.00, 2.38)	0.06	(0.00, 1.64)

Table 5.3. Median and range of the relative abundance of each taxonomic level in overweight and obese patients (OB) and normal-weight controls (NW)

	OB		NW	
Taxonomic profile	**Median**	**Range (min, max)**	**Median**	**Range (min, max)**
Bifidobacterium stercoris	0.20	(0.07, 5.58)	0.11	(0.00, 2.40)
Bilophila wadsworthia	0.05	(0.00, 1.92)	0.12	(0.00, 1.06)
Blautia coccoides	1.39	(0.25, 5.70)	1.01	(0.22, 5.07)
Blautia hansenii	0.29	(0.02, 1.36)	0.24	(0.02, 0.94)
Blautia obeum	0.01	(0.00, 0.51)	0.04	(0.00, 0.96)
Blautia wexlerae	0.32	(0.02, 5.90)	0.30	(0.04, 2.33)
Caldilinea tarbellica	0.05	(0.00, 0.58)	0.08	(0.01, 0.65)
Caloramator mitchellensis	0.02	(0.00, 1.93)	0.05	(0.00, 13.63)
Candidatus Blochmannia rufipes	0.00	(0.00, 0.00)	0.00	(0.00, 2.86)
Citrobacter freundii	0.00	(0.00, 4.98)	0.00	(0.00, 0.04)
Clostridium alkalicellulosi	0.31	(0.00, 2.55)	0.29	(0.03, 3.44)
Clostridium frigoris	0.07	(0.00, 2.23)	0.03	(0.00, 1.49)
Clostridium thermosuccinogenes	0.04	(0.00, 0.43)	0.05	(0.01, 0.48)
Collinsella aerofaciens	0.13	(0.00, 6.28)	0.05	(0.00, 1.82)
Coprococcus eutactus	0.00	(0.00, 2.46)	0.00	(0.00, 1.12)
Desulfonauticus autotrophicus	0.05	(0.00, 0.62)	0.07	(0.00, 1.27)
Desulfotomaculum indicum	0.05	(0.00, 0.47)	0.07	(0.00, 1.50)
Desulfovibrio fairfieldensis	0.01	(0.00, 8.00)	0.01	(0.00, 2.00)
Desulfovibrio piger	0.02	(0.00,1.04)	0.00	(0.00, 0.56)
Dialister invisus	0.00	(0.00, 17.14)	0.00	(0.00, 15.22)
Dorea formicigenerans	0.05	(0.00, 2.53)	0.04	(0.00, 0.70)
Dysgonomonas wimpennyi	0.06	(0.00, 3.58)	0.03	(0.00, 12.19)
Erysipelothrix inopinata	0.01	(0.00, 0.44)	0.04	(0.00, 3.35)
Erysipelothrix muris	0.08	(0.01, 1.72)	0.09	(0.01, 2.55)
Escherichia albertii	0.17	(0.00, 8.59)	0.03	(0.00, 1.78)
Eubacterium biforme	0.00	(0.00, 1.95)	0.00	(0.00, 0.36)
Faecalibacterium prausnitzii	1.41	(0.00, 6.71)	0.85	(0.00, 9.77)
Klebsiella variicola	0.01	(0.00, 0.04)	0.00	(0.00, 4.46)
Lachnospira pectinoschiza	0.26	(0.04, 2.25)	0.37	(0.01, 1.99)
Megamonas funiformis	0.00	(0.00, 4.86)	0.00	(0.00, 0.07)
Megasphaera hominis	0.02	(0.00, 8.38)	0.01	(0.00, 0.07)
Negativicoccus succinicivorans	0.06	(0.00, 4.84)	0.01	(0.00, 0.27)
Oscillospira eae	0.43	(0.03, 4.22)	0.74	(0.08, 7.12)
Oscillospira guilliermondii	0.05	(0.00, 0.44)	0.09	(0.03, 0.55)
Parabacteroides distasonis	0.24	(0.00, 3.39)	0.59	(0.00, 10.73)
Parabacteroides goldsteinii	0.03	(0.00, 0.33)	0.05	(0.00, 3.81)
Parabacteroides johnsonii	0.04	(0.00, 4.23)	0.03	(0.00, 2.81)
Parabacteroides merdae	0.28	(0.00, 2.74)	0.49	(0.00, 4.70)
Paraprevotella clara	0.03	(0.00, 3.46)	0.03	(0.00, 1.42)
Pedobacter kwangyangensis	0.01	(0.00, 1.08)	0.03	(0.00, 2.14)
Phascolarctobacterium faecium	0.03	(0.01, 9.51)	0.01	(0.00, 3.98)
Phascolarctobacterium succinatutens	0.01	(0.01, 5.72)	0.12	(0.00, 2.75)
Prevotella copri	0.00	(0.00, 24.28)	0.00	(0.00, 26.17)
Prevotella shahii	0.01	(0.00, 1.86)	0.00	(0.00, 1.28)
Pseudobutyrivibrio xylanivorans	0.31	(0.01, 5.04)	0.23	(0.00, 1.90)
Rikenella microfusus	0.02	(0.00, 4.27)	0.09	(0.00, 1.38)
Roseburia faecis	0.55	(0.00, 6.19)	0.45	(0.00, 2.82)
Ruminococcus albus	0.00	(0.00, 1.61)	0.06	(0.00, 1.97)
Ruminococcus bromii	0.11	(0.00, 1.99)	0.30	(0.00, 6.69)
Ruminococcus callidus	0.02	(0.00, 0.97)	0.05	(0.00, 2.26)
Ruminococcus gnavus	0.32	(0.00, 9.82)	0.10	(0.01, 1.04)
Ruminococcus torques	0.03	(0.00, 2.23)	0.03	(0.00, 1.71)
Sphingobacterium bambusae	0.02	(0.00, 0.28)	0.02	(0.00, 2.22)

Table 5.3. Median and range of the relative abundance of each taxonomic level in overweight and obese patients (OB) and normal-weight controls (NW)

	OB		NW	
Taxonomic profile	**Median**	**Range (min, max)**	**Median**	**Range (min, max)**
Sphingobacterium shayense	0.04	(0.00, 0.44)	0.09	(0.00, 1.23)
Streptococcus vestibularis	0.05	(0.00, 4.17)	0.02	(0.00, 0.61)
Sutterella wadsworthensis	0.01	(0.00, 5.90)	0.00	(0.00, 1.23)
Veillonella atypica	0.01	(0.00, 1.06)	0.01	(0.00, 0.57)

Medians were expressed as percentage (0-100), NW = normal-weight healthy controls, OB = overweight and obese patients.

5.2.1 Characterization of the gut microbiota of overweight and obese patients at baseline

The Firmicutes/Bacteroidetes ratio was significantly higher in OB (p = 0.007;OB: Median = 1.77, range=0.25-21.90; NW: Median=1.27, range=0.17- 9.24).

The following significant results were found when performing the MaAsLin algorithm adjusted for sex, age, and smoking status (Table 5.4): the phylum Bacteroidetes was decreased in OB (q= 0.024). The following Bacteroidetes families were also decreased in OB: Flavorabacteriaceae, Porphiromonadaceae, Shingobacteriaceae (q= 0.004;0.011;0.000, respectively), while the following Firmicutes families were increased: Lachnospiraceae, Gemellaceae, Paenibacillaceae, Streptococcaceae, Thermicanaceae (q= 0.034, 0.002;0.016;0.008;0.020, respectively). The family Odoribacteriaceae within the phylum Firmicutes was depleted (q=0.019). At the genus level, *Flavobacterium*, *Rikenella*, *Pedobacter*, *Shingobacterium*, and *Parabacteroides*, within the phylum Bacteroidetes, were decreased (q= 0.018;0.004;2.92 x 10^{-9};0.028;0.015, respectively) and *Catenibacterium*, *Gemella*, *Megamonas*, *Mitsuokella*, *Streptococcus*, *Thermicanus*, *Magasphaera*, and *Veillonella*, within the phylum Firmicutes, were increased (q= 0.005;0.001;0.000;1.98 x 10^{-08};0.008;0.020;1.01 x 10^{-8};0.003). In addition, the genera *Enterobacter*, *Escherichia*, *Klebsiella*, and *Serratia* within the phylum Proteobacteria were increased (q=0.002;0.018;0.001;0.004, respectively), while the genus *Sutterella* within the same phylum was decreased (q=0.046). In total, 6 Bacteroidetes species were decreased (*B. uniformis*, *B. rodentium*, *R. microfusus*, *P. kwangyangensis*, *P. distasonis*,

A. intestini; q between 0.000 and 0.041); 4 Firmicutes species were increased (*E. biforme*, *R. gnavus*, *M. funiformis*, *M. hominis*, *V. atypica*, q between 0.000 and 0.041); *O. eae* within the phylum Firmicutes was decreased (*q*=0.021); 2 Proteobacteria species were increased (*D. piger*, *E. albertii*; q=0.011 and 0.020, respectively) and *C. blochmannia rufipes*, within the phylum Proteobacteria was decreased (q= 3.49 x 10^{-8}).

Table 5.4. Statistically significant differences in the relative abundance of bacterial taxa between overweight and obese patients (OB) and normal-weight controls (NW)

Phylum	**Family**	**Genus**	**Species**	***q***	**Coefficient**	↓/↑
Bacteroidetes				0.0249	-0.1425	↓
Bacteroidetes	Bacteroidaceae	*Bacteroides*	*B. rodentium*	0.0197	-0.0390	↓
Bacteroidetes	Bacteroidaceae	*Bacteroides*	*B. uniformis*	0.0004	-0.0731	↓
Bacteroidetes	Flavobacteriaceae			0.0042	-0.0617	↓
Bacteroidetes	Flavobacteriaceae	*Flavobacterium*		0.0183	-0.0537	↓
Bacteroidetes	Porphyromonadaceae			0.0107	-0.0442	↓
Bacteroidetes	Rikenellaceae	*Rikenella*		0.0037	-0.0128	↓
Bacteroidetes	Rikenellaceae	*Rikenella*	*R. microfusus*	0.0037	-0.0128	↓
Bacteroidetes	Sphingobacteriaceae			0.0001	-0.0368	↓
Bacteroidetes	Sphingobacteriaceae	*Pedobacter*		2.92 x 10^{-9}	-0.0292	↓
Bacteroidetes	Sphingobacteriaceae	*Pedobacter*	*P. kwangyangensis*	0.0076	-0.0063	↓
Bacteroidetes	Sphingobacteriaceae	*Sphingobacterium*		0.0281	-0.0100	↓
Bacteroidetes	Tannerellaceae	*Parabacteroides*		0.0152	-0.0373	↓
Bacteroidetes	Tannerellaceae	*Parabacteroides*	*P. distasonis*	0.0411	-0.0229	↓
Firmicutes				0.0212	0.1176	↑
Firmicutes	Acidaminococcaceae	*Acidaminococcus*	*A. intestini*	0.0004	0.0034	↑
Firmicutes	Eubacteriaceae	*Eubacterium*	*E. biforme*	0.0016	0.0026	↑
Firmicutes	Gemellaceae			0.0016	0.0065	↑
Firmicutes	Gemellaceae	*Gemella*		0.0008	0.0075	↑
Firmicutes	Lachnospiraceae			0.0343	0.0604	↑
Firmicutes	Odoribacteraceae			0.0186	-0.0157	↓
Firmicutes	Paenibacillaceae			0.0156	0.0092	↑
Firmicutes	Ruminococcaceae	*Oscillospira*	*O. eae*	0.0211	-0.0225	↓
Firmicutes	Ruminococcaceae	*Ruminococcus*	*R. gnavus*	0.0156	0.0163	↑
Firmicutes	Selenomonadaceae	*Megamonas*		0.0004	0.0023	↑
Firmicutes	Selenomonadaceae	*Megamonas*	*M. funiformis*	0.0004	0.0023	↑

Table 5.4. Statistically significant differences in the relative abundance of bacterial taxa between overweight and obese patients (OB) and normal-weight controls (NW)

Phylum	Family	Genus	Species	*q*	Coefficient	↓/↑
Firmicutes	Selenomonadaceae	*Mitsuokella*		1.98 x 10^{-8}	0.0036	↑
Firmicutes	Streptococcaceae			0.0075	0.0106	↑
Firmicutes	Streptococcaceae	*Streptococcus*		0.0082	0.0102	↑
Firmicutes	Thermicanaceae			0.0196	0.0067	↑
Firmicutes	Thermicanaceae	*Thermicanus*		0.0196	0.0067	↑
Firmicutes	Veillonellaceae	*Megasphaera*		1.01 x 10^{-8}	0.0054	↑
Firmicutes	Veillonellaceae	*Megasphaera*	*M. hominis*	0.0001	0.0050	↑
Firmicutes	Veillonellaceae	*Veillonella*		0.0025	0.0088	↑
Firmicutes	Veillonellaceae	*Veillonella*	*V. atypica*	0.0410	0.0035	↑
Proteobacteria	Betaproteobacteria	*Alcaligenaceae*		0.0473	-0.0213	↓
Proteobacteria	Desulfovibrionaceae	*Desulfovibrio*	*D. piger*	0.0111	0.0062	↑
Proteobacteria	Enterobacteriaceae	*Candidatus Blochmannia*	*C. B. rufipes*	3.49 x 10^{-8}	-0.0498	↓
Proteobacteria	Enterobacteriaceae	*Enterobacter*		0.0024	0.0146	↑
Proteobacteria	Enterobacteriaceae	*Escherichia*		0.0183	0.0221	↑
Proteobacteria	Enterobacteriaceae	*Escherichia*	*E. albertii*	0.0197	0.01954	↑
Proteobacteria	Enterobacteriaceae	*Klebsiella*		0.0006	0.01168	↑
Proteobacteria	Sutterellaceae	*Sutterella*		0.0462	-0.0215	↓
Proteobacteria	Yersiniaceae	*Serratia*		0.0035	0.0114	↑

A multivariate association with linear models (MaAsLin) was used to perform a multivariate analysis on GM composition between OB and NW (adjusted for sex, age and smoking status) on R software v.3.5.2. NW = normal-weight healthy controls, OB = overweight and obese patients, q-values: p-values adjusted for Benjamini and Hochberg false discovery rate (FDR) correction test for multiple comparisons (FDR<0.05), Coefficient = median difference between OB and NW (median relative abundance in OB minus median relative abundance in NW), ↓ = significantly reduced in OB, ↑ = significantly increased in OB. q-value equal to or less than 0.05 was considered statistically significant.

The Linear Discriminant Analysis Effect Size (LEfSe) was additionally performed on statistically significant bacterial taxa obtained from the multivariate analysis and confirmed after the False Discovery Rate (FDR) adjustment, in order to consider not only the statistical significance but also the biological consistency. Results were ranked by their Linear Discriminant Analysis (LDA) score (Figure 5.1): the Bacteroidetes phylum and its members Flavobacteriaceae, *Flavobacterium,* and *Bacteroides* spp. were identified as the main biomarkers in NW, whereas in the OB group the strongest associations

were related to Firmicutes phylum and its taxa Lachnospiraceae and *Megasphaera*, and to *Escherichia* and *E. albertii* (belonging to the Proteobacteria phylum).

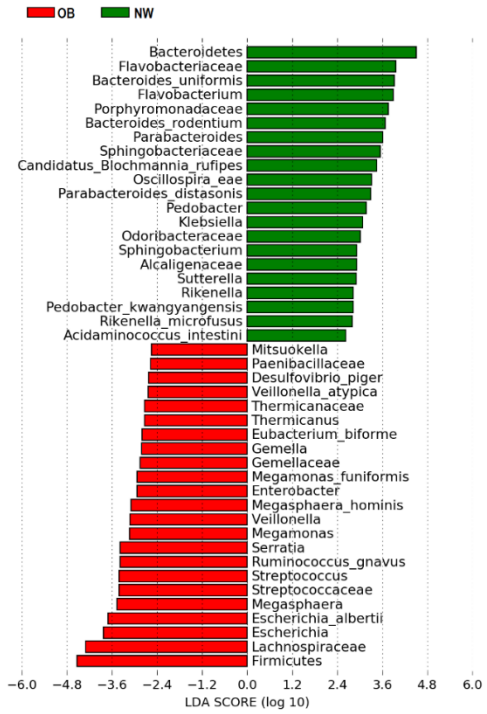

Figure 5.1. Linear Discriminant Analysis Effect Size (LEfSe) of microbial taxa between overweight and obese patients (OB) and normal-weight controls (NW). Results are ranked by the Linear Discriminant Analysis value (LDA score): bacteria in red were more abundant in OB, while bacteria in green were more abundant in NW.

5.2.2 Correlation between significant taxa and clinical parameters in OB and NW

The taxa associated with OB were positively correlated with the fat mass (*Megamonas*) and negatively correlated with the level of physical activity (*Megamonas*, *M. funiformis*, *Megasphaera*, *M. hominis*) and with the muscle mass (Thermicanaceae, *Thermicanus*, *D. piger*). On the other hand, the taxa less abundant in OB compared to NW were negatively correlated with fat mass (Flavobacteriaceae, *Flavobacterium* Porphyromonadaceae, *P. kwangyangensis*), waist circumference (Flavobacteriaceae, *Flavobacterium, P. kwangyangensis*) and BMI (Flavobacteriaceae, *Pedobacter*); while, were positively correlated with BMI (Odoribacteraceae) and MedDietScore (Flavobacteriaceae, *Flavobacterium*,

Sphingobacteriaceae, *Pedobacter*). The taxa associated to NW were negatively correlated with age (*Sphingobacterium*), while, *Flavobacterium* positively correlated with daily protein intake and the level of physical activity. The taxa less abundant in NW compared to OB were negatively correlated with the level of physical activity (*E. biforme*), BMI (*Megasphaera, M. hominis*) and age (*Candidatus B. rufipes*).

Table 5.5. Spearman's correlation between significant taxa and clinical parameters in overweight and obese patients

Phylum	Family	Genus	species	Variable	R	*p*
Bacteroidetes	Flavobacteriaceae			BMI	-0.399	0.006
				FM (%)	-0.365	0.013
				MedDietScore	0.307	0.038
				WC (cm)	-0.322	0.029
		Flavobacterium		FM (%)	-0.367	0.012
				MedDietScore	0.298	0.045
				WC (cm)	-0.325	0.028
	Odoribacteraceae			Age	0.345	0.019
	Odoribacteraceae			BMI	0.019	0.017
	Porphyromonadaceae			FM (%)	-0.292	0.049
	Rikenellaceae	*Rikenella*		Age	0.371	0.011
	Rikenellaceae	*Rikenella*	*R. microfusus*	Age	0.371	0.011
	Sphingobacteriaceae			MedDietScore	0.332	0.024
		Pedobacter		BMI	-0.297	0.045
				MedDietScore	0.308	0.037
		Pedobacter	*P. kwangyangensis*	BMI	-0.470	0.001
				FM (Kg)	-0.406	0.005
				MedDietScore	0.312	0.035
				WC (cm)	-0.414	0.004
	Tannerellaceae	*Parabacteroides*		Proteins (g)	0.311	0.045
Firmicutes	Selenomonadaceae	*Megamonas*		IPAQ (METs/weeks)	-0.342	0.020
		Megamonas		FM (%)	0.352	0.017
			M. funiformis	IPAQ (METs/weeks)	-0.304	0.045
	Thermicanaceae			MM (Kg)	-0.325	0.027
		Thermicanus		MM (Kg)	-0.325	0.027
	Veillonellaceae	*Megasphaera*		IPAQ (METs/weeks)	-0.342	0.020
			M. hominis	IPAQ (METs/weeks)	-0.365	0.013
Proteobacteria	Desulfovibrionaceae	*Desulfovibrio*	*D. piger*	MM (Kg)	-0.374	0.010

Spearman's correlations were calculated in R software (v.3.5.2). Only significant bacterial taxa identified in the multivariate analysis were correlated to clinical parameters. FM (%) = Fat Mass expressed in percentage, FM (kg) = kilograms of fat mass, IPAQ (METs/weeks) = number of METs for week, MM (kg)= Muscle Mass expressed in kg, WC= Waist circumference, BMI = Body Mass Index, MedDietScore = Mediterranean Diet Score, OB = overweight and obese patients. R = r coefficient. *p*-value equal to or less than 0.05 was considered statistically significant.

Table 5.6. Spearman's correlation between significant taxa and clinical parameters in normal-weight controls

Phylum	Family	Genus	species	Variable	R	*p*
Bacteoidetes	Flavobacteriaceae	*Flavobacterium*		Proteins (g)	0.305	0.039
				IPAQ (METs/weeks)	0.304	0.040

Table 5.6. Spearman's correlation between significant taxa and clinical parameters in normal-weight controls

Phylum	Family	Genus	species	Variable	R	*p*
		Sphingobacterium		Age	-0.362	0.013
Firmicutes	Eubacteriaceae	*Eubacterium*	*E. biforme*	Proteins (g)	0.299	0.043
				IPAQ (METs/weeks)	-0.397	0.006
	Veillonellaceae	*Megasphaera*		BMI	-0.364	0.013
	Veillonellaceae	*Megasphaera*	*M. hominis*	BMI	-0.319	0.031
	Enterobacteriaceae	*Candidatus Blochmannia*	*C. B. rufipes*	Age	-0.438	0.002

Spearman's correlations were calculated in R software (v.3.5.2). Only significant bacterial taxa identified in the multivariate analysis were correlated to clinical parameters, IPAQ (METs/weeks) = number of METs for week, BMI = Body Mass Index, NW= normal-weight healthy controls, R= r coefficient. *p*-value equal to or less than 0.05 was considered statistically significant.

5.2.3 Comparison of the same patients before and after the NI (N=23)

After 3 months of NI (T3), a reduction of the ratio Firmicutes/Bacteroidetes (T0:Median= 2.96, range= 0.25-18.96; T3:Median= 1.64, range= 13.70-0.30) was found. However, this reduction was not significant (p=0.128).

When considering FDR adjustment, a total of 24 significant results (one at the phylum level, 3 at the family level, 7 at the genus level, 13 at the species levels) were observed.

After NI, the relative abundance of the phylum Proteobacteria increased (q=0.001). One family within the phylum Bacteroidetes was enriched (Shingobacteriaceae, q=0.003), while 2 families within the phylum Firmicutes were depleted (Ruminococcaceae, q=0.000, and Veillonellaceae, q=0.004). At the genus level, the relative abundance of 6 Firmicutes genera changed significantly (increase in *Catenibacterium* and *Veillonella*, q=0.049 and 0.004 respectively; and decrease in *Megamonas*, *Roseburia*, *Ruminococcus*, q= 0.011,0.046,0.026,0.001 and 0.012, respectively). At the species level, there was an increase in 4 Bacteroidetes species (*B. cellulosilyticus*, *B. uniformis*, *P. stercorea*, *S.shayense*, q between 0.001 and 0.039) and a decrease in 6 Firmicutes species (*M. funiformis*, *R.faecis*, *R. albus*, *R. callidus*, *P. xylanivorans*, *S.vestibularis*, *q* between 0.001 and 0.038). An increased relative abundance of *C.eutactus*, *S.hydroxybenzoicus* and *V.montpellierensis* (all within the phylum

Firmicutes, q= 0.005,0.042 and 0.001, respectively) was also observed. All the significant results are shown in Table 5.7. and plotted in Figure 5.2.

Table 5.7. Significant changes in the bacterial relative abundance after nutritional intervention in overweight and obese patients.

Phylum	**Family**	***Genus***	***Species***	**Median (IQR) at T0**	**Median (IQR) at T3**	**Prevalent direction of change (N)**	***p***	**q**
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*B. bifidum*	0 (0.002)	0.006 (0.073)	↑ (15)	0.043	0.196
Bacteroidetes	Bacteroidaceae	*Bacteroides*	*B. cellulosilyticus*	0.019 (0.050)	0.036 (0.238)	↑ (20)	0.006	**0.039**
			B. rodentium	0.706 (0.643)	1.431 (2.603)	↑ (16)	0.012	0.059
			B. stercorirosoris	0.226 (0.199)	0.289 (0.400)	↑ (16)	0.016	0.091
			B. uniformis	0.803 (0.970)	1.967 (2.808)	↑ (17)	0.005	**0.036**
	Tannerellaceae	*Parabacteroides*		0.742 (0.689)	1.485 (1.646)	↑ (16)	0.016	0.093
			P. distasonis	0.211 (0.367)	0.309 (0.613)	↑ (17)	0.019	0.097
	Prevotellaceae	*Prevotella*	*P. stercorea*	0 (0)	0 (0.001)	≡ (14), ↑ (8)	3.09×10^{-5}	**0.001**
	Sphingobacteriaceae			0.302 (0.389)	0.442 (0.454)	↑ (13)	1.62×10^{-4}	**0.003**
	Sphingobacteriaceae	*Sphingobacterium*		0.088 (0.119)	0.115 (0.308)	↑ (12)	1.26×10^{-3}	**0.011**
			S. shayense	0.039 (0.042)	0.055 (0.083)	↑ (16)	1.62×10^{-4}	**0.003**
Chloroflexi	Caldilineaceae	*Caldilinea*		0.042 (0.097)	0.063 (0.096)	↑ (16)	0.045	0.196
			C. tarbellica	0.042 (0.097)	0.063 (0.096)	↑ (14)	0.045	0.196
Firmicutes	Acidaminococcaceae	*Acidaminococcus*	*A. fermentans*	0.003 (0.009)	0.018 (0.025)	↑ (14)	0.036	0.099
	Erysipelotrichaceae	*Catenibacterium*		0.002 (0.113)	0.005 (0.366)	↑ (19)	0.007	**0.049**

Table 5.7. Significant changes in the bacterial relative abundance after nutritional intervention in overweight and obese patients.

Phylum	Family	*Genus*	*Species*	Median (IQR) at T0	Median (IQR) at T3	Prevalent direction of change (N)	*p*	q
	Lachnospiraceae			15.329 (10.160)	11.358 (13.241)	↓ (14)	0.042	0.194
	Lachnospiraceae	*Coprococcus*	*C. eutactus*	0.004 (0.089)	0.018 (0.399)	↑ (20)	0.001	**0.005**
		Pseudobutyrivibrio	*P. xylanivorans*	0.410 (0.665)	0.216 (0.402)	↓ (17)	5.23×10^{-5}	**0.001**
		Roseburia		1.904 (2.995)	1.379 (2.298)	↓ (19)	0.004	**0.026**
		Roseburia	*R. faecis*	0.489 (0.594)	0.264 (0.360)	↓ (16)	2.70×10^{-5}	**0.001**
Firmicutes	Selenomonadaceae	*Megamonas*		0 (0.002)	0 (0)	≡ (14), ↓ (9)	0.007	**0.046**
		Megamonas	*M. funiformis*	0 (0.002)	0 (0)	≡ (13), ↓ (10)	0.005	**0.038**
	Ruminococcaceae			15.395 (23.822)	13.491 (14.593)	↓ (12)	2.62×10^{-4}	**0.003**
	Ruminococcaceae	*Oscillospira*	*O. eae*	0.440 (0.632)	0.568 (0.825)	↑ (14)	0.048	0.196
		Ruminococcus		3.561 (4.819)	2.284 (2.619)	↓ (12)	2.70×10^{-5}	**0.001**
			R. albus	0.001 (0.102)	0.005 (0.045)	↓ (13)	0.003	**0.012**
			R. bromii	0.056 (0.252)	0.154 (0.491)	↑ (12)	0.039	0.186
			R. callidus	0.011 (0.116)	0.013 (0.074)	↓ (12)	0.001	**0.008**
			R. gnavus	0.312 (0.607)	0.215 (0.433)	↓ (15)	0.042	0.196
	unclassified Tissierellia	*Sedimentibacter*	*S. hydroxybenzoicus*	0.075 (0.078)	0.073 (0.082)	↓ (13)	3.33×10^{-4}	**0.004**
	Streptococcaceae			0.218 (0.329)	0.114 (0.136)	↓ (17)	0.009	0.051
		Streptococcus		0.215 (0.332)	0.114 (0.134)	↓ (16)	0.015	0.073

Table 5.7. Significant changes in the bacterial relative abundance after nutritional intervention in overweight and obese patients.

Phylum	Family	*Genus*	*Species*	Median (IQR) at T0	Median (IQR) at T3	Prevalent direction of change (N)	*p*	q
Proteobacteria		*Streptococcus*	*S. vestibularis*	0.038 (0.060)	0.014 (0.029)	↓ (20)	3.09×10^{-5}	**0.001**
	Veillonellaceae			3.338 (6.828)	3.492 (6.288)	↓ (12)	2.95×10^{-4}	**0.004**
	Veillonellaceae	*Veillonella*		0.059 (0.123)	0.150 (0.185)	↑ (17)	3.73×10^{-4}	**0.004**
			V. montpellierensis	0.024 (0.047)	0.038 (0.048)	↑ (15)	1.27×10^{-4}	**0.001**
	Sutterellaceae	*Sutterella*		3.492 (3.670)	3.502 (5.981)	↓ (12)	2.70×10^{-5}	**0.001**
				0.108 (0.187)	0.204 (0.420)	↓ (12)	0.001	**0.012**
			S. wadsworthensis	0.002 (0.033)	0.003 (0.029)	↑ (10)	0.018	0.093

Median = median values of the bacterial relative abundance before (T0) and after (T3) nutritional intervention (NI) (bacterial relative abundance is expressed as percentage from 0 to 100); IQR = interquartile range calculated as the difference between upper and lower quartiles; N = number of patients that reported the most prevalent direction of change in the bacterial relative abundance (↓ = reduced after NI, ↑ = increased after NI, ≡ = no different after NI). Results were obtained by the Wilcoxon test for paired data performed on R software (v. 3.5.2). q = p adjusted for Benjamini and Hochberg's false discovery rate (FDR) correction test for multiple comparisons (FDR < 0.05). ↓ = significantly reduced after the NI, ↑ = significantly increased after the NI. Bold values denote statistical significance ($q \leq 0.05$).

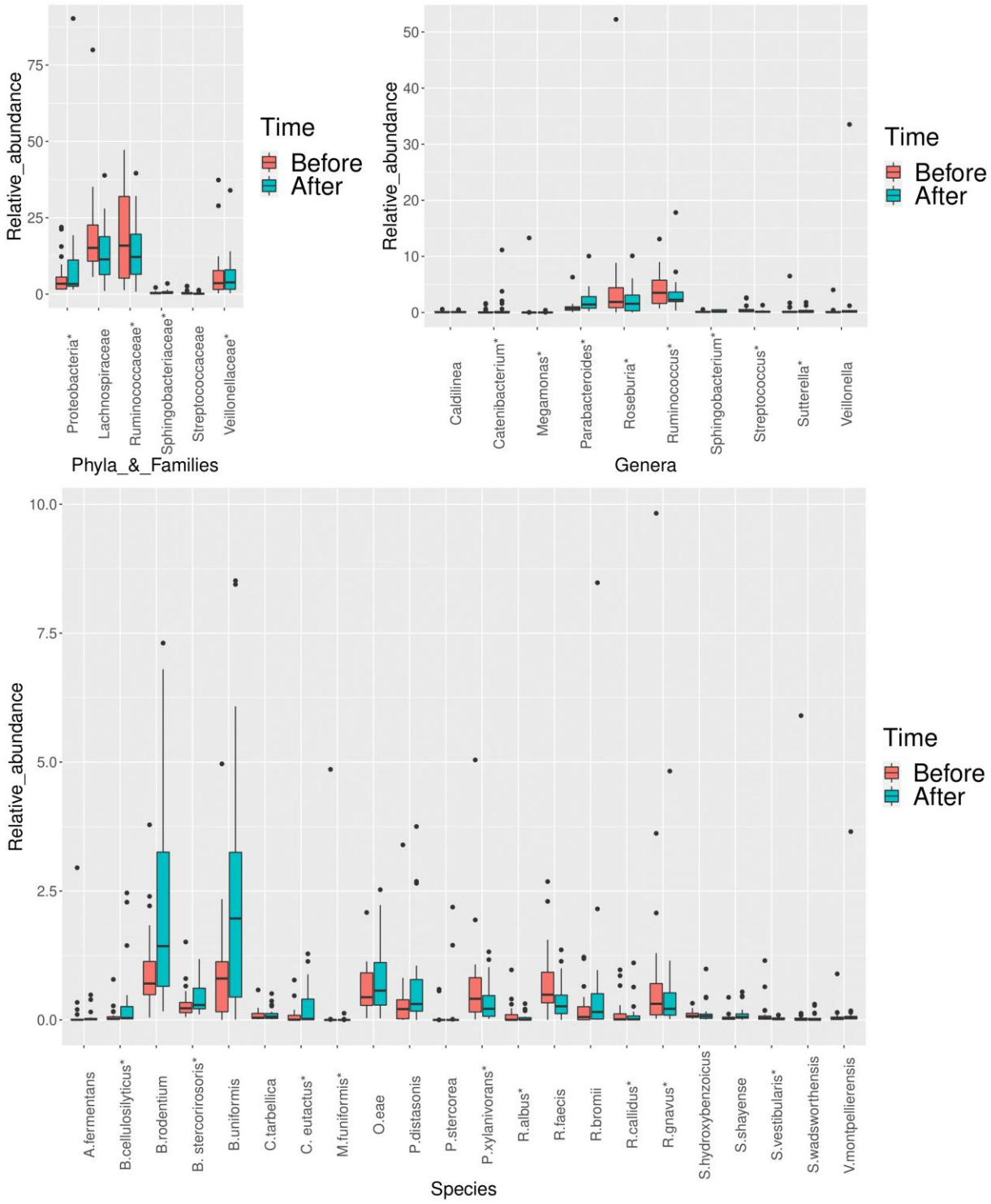
Relative_abundance
75
50
25
0
Proteobacteria*
Lachnospiraceae
Ruminococcaceae*
Sphingobacteriaceae*
Streptococcaceae
Veillonellaceae*
Phyla_&_Families
Time
Before
After
Relative_abundance
50
40
30
20
10
0
Caldilinea
Catenibacterium*
Megamonas*
Parabacteroides*
Roseburia*
Ruminococcus*
Sphingobacterium*
Streptococcus*
Sutterella*
Veillonella
Genera
Time
Before
After
Relative_abundance
10.0
7.5
5.0
2.5
0.0
A.fermentans
B.cellulosilyticus*
B.rodentium
B. stercorirosoris*
B.uniformis
C.tarbellica
C. eutactus*
M.funiformis*
O.eae
P.distasonis
P.stercorea
P.xylanivorans*
R.albus*
R.faecis
R.bromii
R.calidus*
R.gnavus*
S.hydroxybenzoicus
S.shayense
S.vestibularis*
S.wadsworthensis
V.montpelliérensis
Species
Time
Before
After

Figure 5.2. Statistically significant differences in bacterial relative abundance in overweight and obese patients after nutritional intervention, at the phylum, family, genus, and species levels, respectively. The significance level was obtained by performing the Wilcoxon test for paired data. p-values were adjusted for False Discovery Rate (FDR) (FDR < 0.05). A change in twenty-four taxa was found when considering the FDR adjustment (as indicated by the asterisk). In the box plots, the boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. Points above and below the whiskers indicate outliers outside the 10th and 90th percentiles. Each group is identified by colors, as indicated on the right side of the figure (before intervention = pink, after intervention = light blue). Every sample is represented by a black dot.

5.2.4 Alpha diversity (Shannon index)

The bacterial community of OB and NW presented a similar Shannon index (OB: Median= 2.40, range= 1.17-3.10; NW: Median=2.39, range= 1.84-2.68; p =0.833). After 3 months of NI, the 23 patients presented an increased Shannon index, though the difference was not significant (T0:Median= 2.31, range= 1.56-4.18; T3: Median= 2.32, range= 1.58-4.28; p=0.065).

5.2.5 Beta diversity (Bray-Curtis index)

The Non-Metric Multidimensional Scaling (NMDS) plot generated using Bray-Curtis distance, as previously described, showed a separation between the GM communities of OB and NW (Figure 5.3). In fact, PERMANOVA analysis, adjusted for sex, age and smoking status, indicated a significant difference between the two groups (Degrees of freedom=1, Sum of squares=0.5492, Mean of squares=5.0297, F=0.0533, p=0.002).

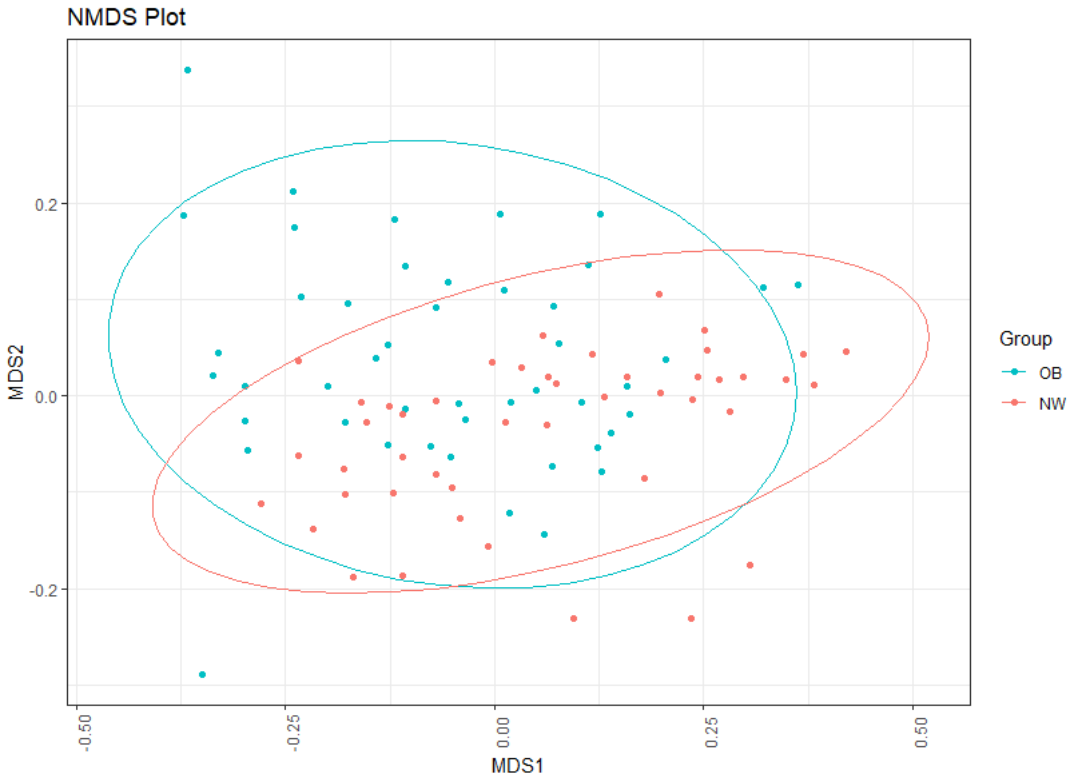

Figure 5.3. Bacterial beta diversity in overweight and obese patients (OB) and normal-weight controls (NW). The Figure shows a three-dimensional scatter plot, generated using Non-Metric Multidimensional Scaling (NMDS) conducted on community distance matrix obtained in vegan, with the Bray-Curtis distance, representing the distance of microbial communities among OB (light blue spheres) and NW (pink spheres). Each group is identified by colors as indicated on the right side of the figure. The p-value was calculated using the PERMANOVA method adjusted for sex, age, and smoking status (R software, v3.5.2, package vegan) to compare beta diversities between each category. The two groups were significantly different in terms of Beta diversity (p-value= 0.002).

5.3 Predicted functional metagenome

Comparative prediction analysis of the functional metagenome (PICRUSt) of the GM was performed, as previously described. When comparing OB and NW, a total of 6 different metabolic pathways (mean the difference between the two groups ≥ 0.1%) were identified (Figure 5.4), with 5 being more expressed and 1 being less expressed in OB (q between 2.84E-04 and 6.68E-03). One metabolic pathway related to the metabolism of cofactors and vitamins (porphyrin and chlorophyll metabolism) and 3 metabolic pathways involved in membrane transport (ABC transporter, phosphotransferase system), transporters, and transcription pathways were more abundant in OB. On the other hand, 1

metabolic pathway related to glycan biosynthesis metabolism (glycan degradation) was enriched in NW.

After NI, a significant difference in 7 metabolic pathways (q between 0.022 and 0.043) was found: 1 pathway related to membrane transport (ABC transporters), 1 pathway associated with transporters, 2 pathways related to cell motility ("flagellar assembly", "bacterial motility proteins"), and 1 pathway associated with sporulation decreased. On the other hand, the pathways "lipopolysaccharides biosynthesis proteins" and "membrane and intracellular structural molecules" increased (Figure 5.5).

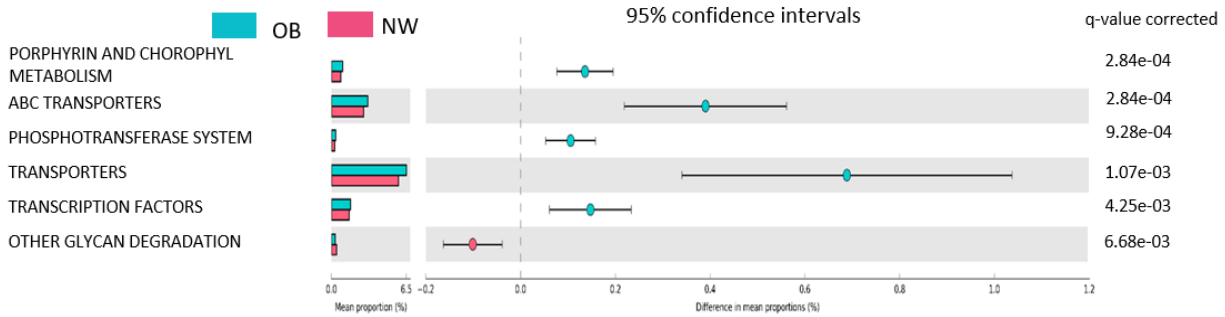

Figure 5.4. Comparative prediction of the functional metagenome of the gut bacterial microbiota between overweight and obese patients (OB) and normal-weight controls (NW). The figure shows a graphic representation of the significant predicted metabolic pathways using PICRUSt by analysis of the corresponding OTU table generated by QIIME for the bacterial communities. Six metabolic pathways differed significantly between OB and NW. Pathways that were more abundant in OB are on the positive side (light blue circle with 95% CI). Pathways that were more abundant in NW are on the negative side (light blue circle with 95% CI). The q-values represent the Storey FDR-corrected p-value (Welch's test). Mean proportions are shown in stacks for OB (light blue) and NW (pink). The difference in mean proportions indicates the mean proportion in OB minus the mean proportion in NW.

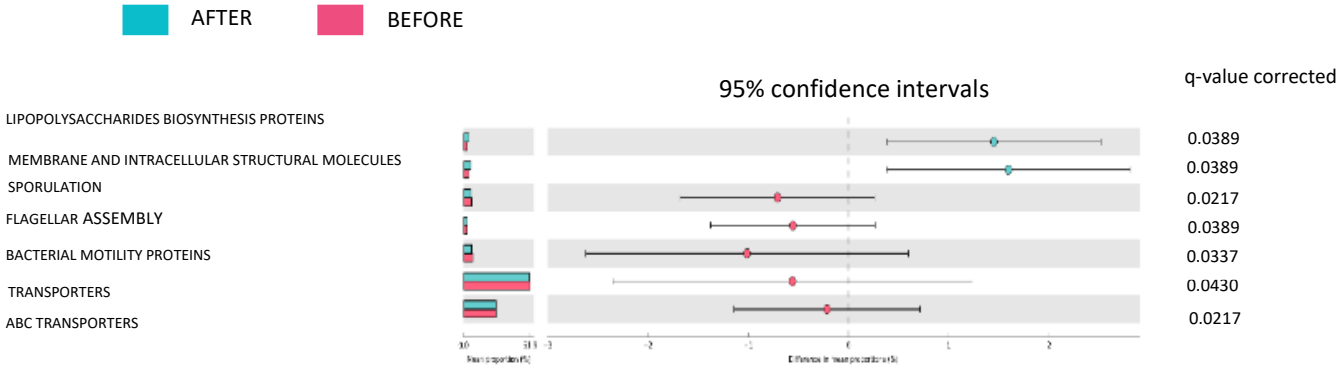

Figure 5.5. Comparative prediction of the functional metagenome of the gut bacterial microbiota before and after nutritional intervention in overweight and obese patients. The figure shows a graphic representation of the significant predicted metabolic pathways using PICRUSt by analysis of the corresponding OTU table generated by QIIME for the bacterial communities. Seven metabolic pathways were significantly different after the NI. Pathways that were more abundant after NI are on the positive side (light blue circle with 95% CI). Pathways that were less abundant after NI are on the negative side (pink circle with 95% CI). The q-values represent the Benjamini Hochberg's FDR-corrected p-value (Wilcoxon test for paired data). Mean proportions are shown in stacks (before NI=pink, after NI=light blue). The difference in mean proportions indicates the mean proportion after NI minus the mean proportion before NI.

6. Discussion

The present thesis characterized the GM of 46 overweight and obese patients (OB), recruited in an Obesity clinic in Cagliari (Sardinia, Italy). Normal-weight healthy individuals were used as controls. Cases and controls were matched for age, sex, and smoking status. The sample size consisted mainly of women, no-smokers, with a mean age of 49-50 years old.

The work also evaluated the impact of a weight-loss intervention that lasted 3 months in modulating GM composition and diversity (N=23). The intervention was based on a prescribed diet based on the Mediterranean model, with a caloric intake equivalent to the BMR of the patients (moderately hypocaloric diet) and a balanced distribution of macronutrients.

6.1 Gut microbiota associated with obesity and comparison with literature

The difference in microbiota composition between obese and normal-weight subjects has been largely analyzed so far, even if data remain controversial. Three different meta-analyses did not show significant results when looking at the relative abundances of Firmicutes or Bacteroidetes (or their ratio) and the alpha diversity level (253–255).

Regarding alpha diversity, no significant results where obtained when comparing OB with NW. In contrast, a recent metagenomics meta-analysis considering a total of 1473 patients spanning seven diseases showed a significant decrease in taxonomic richness in obese patients (256). However, the analysis of beta diversity showed significant separation between NW and OB in the present work. This is partly in line with the literature, though results remain inconsistent (255).

Here, obesity patients presented a higher relative abundance of Firmicutes and a lower relative abundance of Bacteroidetes, and the Firmicutes/Bacteroidetes ratio was more than twice that observed

in controls. It is important to underline that, although in meta-analyses the bioinformatics procedures are the same for all the included studies, there may be still differences in samples storing, DNA extraction and sequencing phase performed in different laboratories, or differences among the recruited populations. This variability between studies can partly explain the lack of significant results in meta-analyses.

In the present work, the trend regarding these two phyla was evident also when considering the lower taxonomic levels. Some of the identified patterns associated with being obese/overweight agree with those of previous studies.

In the study of Peters et al. two families within the phylum Firmicutes (Lactobacillaceae and Streptococcaceae) were more abundant among obese patients (257). Here, the same trend was observed, but only the increase in Streptococaccaeae was confirmed at the multivariate analysis. On the other hand, the family Christensenellaceae, within Firmicutes, was associated with the lean phenotype in many population-based studies (169). However, this taxonomic level was not significant in the present work.

In agreement with the present findings, the butyrate-producing family Lachnospiraceae was found to be associated with obesity in another clinical trial, including 3 patients with morbid obesity (258), and in animal models (259).

Regarding the genera within the phylum Firmicutes, the genus *Megasphaera*, in the current analysis increased in obese patients, was associated with obesity also in a meta-analysis of 5 studies (255). This genus, belonging to the family Veillonellaceae, can utilize lactate to produce propionate (260). In addition, members of the genus *Megasphaera*, can produce different SCFAs (propionate, acetate, and butyrate) (261). Similarly, an increase in the anaerobic *Veillonella* genus, which includes SCFAs-producing bacteria (262), was found in this study. Furthermore, the genus *Oscillospira* (within the

phylum Firmicutes), decreased in OB at baseline, has been associated with low-BMI/leanness in several studies (263,264). This genus metabolizes glucoronate, a sugar found on the cell surface and in the extracellular matrix of most human tissues (265). The degradation of host glucoronate by *Oscillospira* causes an energy expenditure for the host that may explain its association with leanness.

A recent study by Candela et al. characterizing the GM in 21 overweight or obese adults with type II diabetes, showed a lower relative abundance of *Lachnospira*, which increased in our subjects. On the other hand, they found an increase in *Streptococcus*, in agreement with this study, and a decrease in the SCFAs-producers *Roseburia* and *Faecalibacterium* (266). In the present study, *Roseburia* was decreased, in agreement with Candela et al., but *Faecalibacterium* was not significant. This genus and its species (*F. praunitzii*) have been negatively associated with obesity in many clinical trials (255). In fact, *F. praunitzii*, not significant in the present work, is a butyrate producer with anti-inflammatory effects (267).

The recent work of Del Chierico et al. identified an increase in two Firmicutes species in 20 adults with severe obesity compared with 12 normal-weight controls (268). Here, an increase in Firmicutes species was noted, though the identified species were different than those of the study of Del Chierico et al.

Regarding the gut microbial patterns within the phylum Bacteroidetes, a depletion in three families was found in the present study (Flavobacteriaceae, Porphyromonadaceae, Sphingobacteriaceae). Interestingly, a reduction in Flavobacteria members was observed in obese patients when analyzing the salivary microbiome (269). At the same time, Porhyromonadaceae decreased after antibiotic treatment (270) and after a high-fat diet (271) in animal models, showing how the abundance of this family can be influenced by lifestyle factors. One clinical trial found an increase in Prevotellaceae in obese patients (258), but this taxonomic level was not significant in the present study. In mice, a 4-weeks intervention based on the increase in physical exercise determined the increase in the relative

abundance of the family Sphingobacteriaceae (271). The association between physical activity and an increased abundance of Sphingobacteriaceae might explain the reduction of this taxon in obese patients.

The genus *Parabacteroides* (within Bacteroidetes), here decreased in cases, was negatively associated with BMI in two population studies (258,272), in agreement with the present study. Furthermore, Wang et al. recently reported the beneficial effect of Parabacteroides sp (*P. distasonis*) in modulating body weight in obese mice, via secondary bile acid-activated FXR signaling in the liver, and succinate-activated intestinal gluconeogenesis (273). Different results were obtained on the association between overweight/obesity (266,274) and the genus *Prevotella*, which was not significant in the present study.

In addition, two Bacteroidetes species (*B. uniformis*, *P.distasonis*), here less abundant in cases, were depleted in obese patients also in another study characterizing the GM of 28 adults, whose 13 obese (275). The depletion of *B. uniformis* in obese subjects was confirmed also in a recent study with a larger sample size (72 obese patients and 79 controls) (276). Remarkably, the administration of *B. uniformis* strains improved metabolic and immune dysfunction associated with intestinal dysbiosis in obese mice (277).

The increased Firmicutes/Bacteroidetes ratio and the microbial patterns within these two phyla can be associated with increased hydrolysis of non-digestible polysaccharides, and with increased nutrients absorption (278), resulting in an increase in calories production. In addition, the increased relative abundance of Firmicutes has been found to raise the number of lipid droplets and the export of fatty acids to the liver in animal models (279,280).

In the present work, the genera *Enterobacter*, *Escherichia*, *Klebsiella* and *Serratia* within the Enterobacteriaceae family (Proteobacteria phylum) were increased in obese patients. The increase of Enterobacteriaceae is in line with other human studies on obesity (257,275,281). This family represents

an important source of LPS, which can affect the intestinal permeability leading to an increased concentration of LPS in the plasma ("endotoxemia"), associated with the low-grade chronic inflammation typical of obese subjects (282). The increase of taxonomic levels within Enterobacteriaceae is in line with the study of Candela et al, previously mentioned (266).

The genus *Bifidobacterium*, within the phylum Actinobacteria, was decreased in obese patients in the study of Million et al. (283). In an animal model, the administration of a *Bifidobacterium* spp. showed anti-obesity effects in rats fed with a high-fat diet (284). Due to its beneficial proprieties, *Bifidobacterium* has been proposed as probiotics for the treatment of obesity (285). However, in the present work, its abundance was not different between cases and controls and no significant results were found within the phylum Actinobacteria after multivariate analysis.

Many clinical trials observed a negative association between the species *Akkermansia muciniphila* (within the phylum Verrucomicrobia) and obesity/overweight (258,276,286,287). Firstly, the group of Cani et al. demonstrated that obesity was negatively associated with the abundance of *A. muciniphila* in mice, and that its administration was able to improve metabolic profiles (288). Recently, a proof of concept study showed that the supplementation with *A. municiphila* in obese individuals was associated with decreased body weight, inflammation, and insulin resistance (289). However, this taxonomic level was not significant in this work.

Lately, an association between obesity and H_2-oxidizing methanogenic Archaea was proposed (213), due to their capacity to oxidize H_2 (produced by H_2-producing bacteria), leading to accelerated fermentation of polysaccharides and consequently higher energy uptake by obese individuals (212). However, no significant results on methanogenic Archea were found in the present work.

Few studies evaluated the association between body composition in obese/overweight subjects and gut microbial patterns. In fact, BMI is often the only anthropometric parameter available, especially in

population studies. We were able to associate some of the identified microbial patterns in obese/overweight individuals with clinical and anthropometrical characteristics. A total of 5 Bacteroidetes taxa (Coriobacteriaceae, Flavobacteriaceae, Porphiromonadaceae, *Flavobacterium, P. Kwangyangensis*) were negatively correlated with fat-mass (FM) and waist circumference (WC). *Oscillospira*, decreased in cases at baseline, was also negatively associated with FM, while other Firmicutes taxa, increased in cases at baseline, were positively correlated with FM. Interestingly, we also observed negative correlations between Firmicutes taxa and the muscle mass and/or the physical activity level (*Megasphaera, M. hominini*s, Thermicanaceae, and *Thermicanus*).

Our findings are only partially in line with those of previous Italian works. On one hand, Del Chierico et al. have recently associated both *Parabacteroides* and *Parabacteroides distasonis* with NW, in agreement with our study (268). On the other hand, the recent study by Campisciano et al. found an equal ratio Firmicutes/Bacteroidetes in OB compared with NW, and an increase in Bacteroides, both in contrast with our findings (274).

6.2 Impact of the nutritional intervention and comparison with literature

Considering the modifiable nature of the GM, and the driven role of the diet in determining its composition (121), the effect of many weight-loss interventions on GM composition was investigated in the last decade.

The present work compared the GM of 23 patients after 3 months of NI aimed to lose weight. At the time of the second sample collection, the patients presented decreased body weight, waist circumference, and fat-mass. Furthermore, the NI showed a positive effect on the modulation of bacterial taxa, especially in those belonging to the phyla Firmicutes and Bacteroidetes. In detail, paired analysis showed a total of 24 significant results (one at the phylum level, 3 at the family level, 7 at the genus level, 13 at the species levels) after FDR adjustment.

Regarding the gut microbial alterations within the phylum Bacteroidetes after NI, an increased abundance in the family Sphingobacteriaceae and its species *S. shayense*, and a raise in *B. uniformis* were observed. Both these three taxa were identified as being negatively associated with obesity at baseline. Remarkably, the administration of *B. uniformis* strains improved metabolic and immune dysfunction associated with intestinal dysbiosis in obese mice (277). We also observed a depletion of Firmicutes taxa known for being associated with obesity (i.e. Veillonellaceae, *Megamonas*, and *M. funiformis)* that express propionate production pathways (290,291).

Walker et al. investigated the impact of 3 different diets on modulating the GM composition of 14 obese men. Volunteers were provided successively with a control diet, diets high in resistant starch (RS) or non-starch polysaccharides (NSPs), and a reduced carbohydrate weight loss (WL) diet, over the course of ten weeks (292). The findings of Walker et al. suggested that the supplementation with RS can balance the reduction of the Lachnospiraceae family and/or its members, caused by the WL diet. Interestingly, this reduction was also found after different types of hypocaloric diets (high protein, fiber-rich, or with prebiotics supplementation) in other studies (266,293,294).

Similarly, in the present work a decrease in Lachnospiraceae members (*Roseburia*, *Roseburia faecis*, *Pseudobutyrivibrio xylanivorans*) was found, although an increase in *Coprococcus eutactus*, within the same family, was also observed. Members of this family can hydrolyze starch and other sugars to produce butyrate and other Short-Chain Fatty Acids (SCFAs) and play a central role in the mechanisms of bacterial cross-feeding (295). The genus *Roseburia* in particular is among the most involved in the control of gut inflammatory processes, atherosclerosis, and maturation of the immune system (296). However, it should be noted that despite the well-known benefits provided by the members of this family, Lachnospiraceae was positively associated with metabolic diseases in humans and animal models (168,258,297,298). At the same time, higher SCFAs production (acetate, propionate, and butyrate) was

associated with gut dysbiosis and obesity in a recent study with a large sample size (299), and the finding was confirmed in a random effect meta-analysis published last year (300).

In the present work, the changes in GM composition after the NI suggest a decrease in SCFAs producing bacteria (Lachnospiraceae and Veillonellaceae, *Ruminoccocus* spp., and *Megamonas*). It is unclear whether the beneficial effect of SCFAs is somehow compromised in obese subjects, or the effect is simply not strong enough to compensate for an incorrect lifestyle and/or genetic predisposition (217). Noteworthy, an increase in *O. eae*, within *Oscillospira*, was observed in the present study after the NI, although it was not confirmed after FDR adjustment.

Cancello et al. evaluated the efficacy of a short-term dietary intervention on the GM of elderly Italian women with obesity: after 15 days of hospitalization following a hypocaloric Mediterranean diet, they noticed a decrease in pro-inflammatory bacteria, along with a moderate weight loss and improved metabolic function. In line with the present work, the diet provided a daily energy deficit equal to 250 kcal. The study showed the efficacy of a balanced diet with moderate caloric restriction, even of short duration, in improving gut health, reversing the GM dysbiosis found at baseline (301).

One recent study, including 20 obese patients, investigated the effect of two different diets. More specifically, participants were randomized into two groups: one received a Mediterranean diet (MD) for 1 year, and one received a control diet (LFHCC), with a lower intake of fats and monounsaturated fatty acids, for the same time. The LFHCC diet increased *Prevotella* and *F. prausnitzii*, and decreased *Roseburia*. On the other hand, MD decreased the abundance of *Prevotella* and increased the abundance of *Roseburia*, *Oscillospira*, and *Parabacteroides distasonis*. In addition, MD increased the levels of Proteobacteria, in line with our study. The authors proposed a protective effect of both diets on the development of type II diabetes (302). In line with the LFHCC diet in this study, an increase in *Prevotella* bacteria (*P. stercorea*) and a decrease in *Roseburia*, both confirmed after FDR adjustment,

were found in the present work. Noteworthy, *Oscillospira* spp and *P. distasonis*, increased after MD in the study of Haro et al., were increased also in the present study.

In the study of Candela et al. the GM of the patients was analyzed after 3 weeks of a hypocaloric fiber-rich diet (Ma-Pi 2), using a control group who received a diet based on nutritional guidelines, with a lower intake of carbohydrates and fiber (CTR). Both diets were effective in counteracting the decrease of *Bacteroides*, *Dorea,* and *Faecalibacterium* and in increasing *Akkermansia* abundance values (these taxa were not significant in this study). On the other hand, both diets supported the reduction of *Ruminococcus*, in line with the present study, while only the CTR diet restored normal levels of *Lachnospira* and *Roseburia* and only the Ma-Pi 2 diet group recovered the levels of *Oscillospira*, *Collinsella*, and *Streptococcus* (266). In the present study, *Roseburia* and *Streptococcus* decreased after the diet, in contrast with the observations of Candela et al.

Russell et al. experimented with a weight-loss dietary approach, based on a 7-days of a low-carbohydrates and high- proteins (HPLC) diet, in 17 obese men. The HPLC diet decreased the abundance of *Roseburia* and *Eubacterium rectale*, and the proportion of butyrate in stools. In addition, the diet reduced the concentrations of fiber-derived metabolites in feces. For this reason, the authors concluded that the HPLC diet showed a detrimental effect on colon health (303).

In 2016, Pataky et al. analyzed the impact of 3-weeks of a hypocaloric hyperproteic diet on the GM of 15 overweight/obese patients with NAFLD. In contrast with the study of Russell et al., they found an increase in butyrate producers, such as *Faecalibacterium* and *Butyricicoccus,* though a reduction of *Lachnospira* was also observed (294).

A recent systematic review on the effect of nutritional intervention on GM showed that restrictive diets (very low energy or low-carb) decrease the microbiota abundance, and generally reduce the butyrate

producers *Lactobacillus* spp. and *Bifidobacterium* spp., within the phylum Firmicutes (304), which were not significantly altered after the NI in the present work.

In the study of Louis et al., 16 obese patients underwent a 52-week weight-loss program comprising a very low-calorie diet, exercise, and behavioral therapy, and were followed up for 2 years. Despite the initial impact of the intervention on gut microbial profiles, they noticed a trend to return to the initial situation both at the taxonomical and functional level after one year, except for the increase in *Akkermansia* abundance, which remained stable over two years. Authors noticed that participants who succeeded in losing their weight consistently over the two years had at baseline a microbiota enriched in *Alistipes* and *Pseudoflavonifractor* (both within the phylum Bacteroidetes). On the other hand, patients not persistent with the weight-loss, had a GM enriched in *Prevotella*, *Megamonas* and *Phascolarctobacterium* (305). In particular, *Megamonas* (within Firmicutes) was associated with obesity in the present and in other studies, but its additional association with weight-regain represents a novel finding. Interestingly, a decrease in *Megamonas* was observed in this work after the intervention.

In addition, also in another intervention study with several time points, the microbial changes obtained by the program were not stable over time. In that study, 37 patients underwent a weight-loss program divided into 3 steps (with a gradual increase in the caloric intake), which lasted 15 weeks in total. The participants were followed up for 36 months: almost all genera which showed significant changes at 6 weeks compared to baseline (*Streptococcu*s; *Pseudoflavonifractor*; *Roseburia;* Lachnospiraceae incertae sedis; *Eggerthella* and *Veillonella),* returned to their initial level after 15 weeks. At the end of the study, only *Collinsella* (within the phylum Actinobacteria) still exhibited a stable lower abundance, compared to baseline (306).

The findings of the previously discussed studies prove the current need to develop intervention strategies that allow not only the restoring of the intestinal balance after weight-loss but also the maintenance of this achievement in the long-term.

In this regard, in a recent work Grembi et al. recruited subjects from a randomized trial of 609 adults undergoing a 12-months weight-loss program, based on a low-carb or low-fat diet. A discovery cohort of 66 subjects (32 randomized to the low-carb diet and 34 to the low-fat diet) was considered for the analysis of the GM. In particular, the study focused on the association between microbial patterns at baseline and the successful 12-months weight loss. Multiple stool samples were collected before the starting of the diet and 10 weeks after the start. After that, the intervention lasted until the end of the 12 months, and participants were defined as unsuccessful (< 3% weight loss), moderately successful (3 − 10% weight loss), very successful (>10% weight loss). The same approach was followed in the validation cohort, including 56 subjects (31 on the low-carb diet and 25 on the low-fat diet). In both cohorts, the composition of the GM community, when considering both the diet regimens, did not correlate with weight-loss success. On the other hand, pre-diet daily microbiota plasticity, i.e., the amount of daily variability in an individual microbiota composition, was positively correlated with the success of the intervention. In addition, very successful participants were characterized by higher plasticity also after ten weeks, compared with unsuccessful subjects. This study was the first demonstration that the temporal variability of the microbiota is positively associated with the success in losing weight, but this result was found only for the low-fat diet. The authors hypothesized that this association was associated with the effects of the increased carbohydrates and fiber intake on the low-fat diet and with appetite suppression caused by these components of the diet (307).

When considering weight-loss strategies different than diet, bariatric surgery (BS) should be mentioned. This intervention is based on surgery on the stomach and/or intestines, aimed at losing weight. BS is an

option for obese individuals whit a BMI above 40 or a BMI between 35-40 in presence of comorbidities (298). It is currently considered as the most effective treatment for severe obesity, able to determine a dramatic and durable weight loss (308), together with remission of associated comorbidities, such as type II diabetes, and prevention of many diseases and cardiovascular events (309,310).

Bodyweight loss with BS is achieved through the reduction of nutrient digestion, alteration of food preferences, acceleration of gastric emptying, regulation of hormonal changes (e.g. GLP-1 PYY), and alterations in the metabolism of bile acids (310). It can be inferred that these mechanisms can cause changes in the GM composition. In fact, many microbial alterations have been observed after BS (311).

Several studies obtained an increased relative abundance of the phylum Proteobacteria after BS (202,258,297,312–314). Interestingly, this phylum was increased also after nutritional intervention in the current work, and after 1 year of the MD (302). However, after BS, an increase in the class Gammaproteobacteria and the pro-inflammatory genera *Escherichia*, *Klebsiella* and *Enterobacter* were also found (202,258,297,312–314), while these microbial taxa were not increased after the intervention in the present work. If~~,~~ the rise in Gammaproteobacteria can be considered as a negative impact of the BS, several studies have reported an increase in the relative abundance of the phylum Verrucomicrobia or its members (258,312,314), an increase in members of the phylum Bacteroidetes assigned to the genus *Alistipes* (202,297,314), as well as a general decrease in members of the phylum Firmicutes *(Lactobacillus* spp., *Eubacterium* spp., *Clostridium* spp., *Coprococcus comes*, *Anaerostipescaccae* and *Erysipelotrichales)* (312–314). The decrease in Firmicutes members is in line with this work. In addition, Firmicutes taxa within the genera *Veillonella* increased after BS (312) and after the NI in this study.

6.3. Functional prediction and comparison with literature

The predicted metabolic pathways indicated distinct signatures in OB and NW. More specifically, overweight and obese patients were characterized by an increase in transcription, membrane transport, and expression of vitamins cofactors.

Regarding the increase in porphyrin and chlorophyll metabolism (in the category “metabolism of cofactors and vitamins”) found in the present work, this pathway was associated with Alzheimer’s disease (315) and chronic hepatitis B (316) in humans. Furthermore, animal models demonstrated an increase in this function in presence of dysbiosis (317) and after a high-fat diet (318). Consequently, there is evidence of a relationship between this functional features and pathological conditions, and of the possibility to modulate its expression by diet. However, the link with obesity remains to be ascertained.

In line with the present study, Hou et al. observed an association between the increase in membrane transport (ABC transporters) and obesity in 87 obese children (319). ABC transporters are involved in the transport of a variety of substrates, including sugars, amino acids, glycans, cholesterol, phospholipids, peptides, proteins, toxins, antibiotics, and xenobiotics (320). Noteworthy, the alteration of ABC transporters has been associated with resistance of cancer cells to chemotherapy, cystic fibrosis, immune deficiency, and several diseases including Alzheimer’s disease (320,321). Several studies showed an increase in pathways related to amino acids utilization after BS (311), including the increase in ABC transporters (in special lysine/arginine/ornithine and histidine). In contrast, the NI evaluated in this work was associated with a decreased expression of ABC transporters.

Among the signaling pathways associated with obesity in the present work, phosphotransferase systems from the related pathway of “membrane transport” were more expressed in obese patients, in agreement with previous studies (312,319,322,323). These transporters are involved in carbohydrates

breakdown and phosphorylation, in the utilization of nitrogen and phosphorus and in the virulence of certain pathogens (324). In an animal model, the high-fat/high-sugar Western diet was associated with an enrichment of the phosphotransferase system, proving that this pathway can be modulated by diet (325).

Here, depletion of a pathway involved in glycan metabolism was observed, in line with the study of Hou et al. (319). Similarly, in the study of Del Chierico et al., biosynthesis and metabolism of glycan were enriched in NW adolescents, compared with OB adolescents (268). Interestingly, an *in vitro* model demonstrated that Firmicutes taxa possess smaller genomes and a disproportionately smaller number of glycan-degrading enzymes (326). In addition, it has been shown that colonization of germ-free mice with *Bacteroides thetaiotaomicron*, a commensal bacteria within the phylum Bacteroidetes, can increase the expression of enzymes involved in glycan synthesis (327).

On the other hand, we did not obtain significant results about lipid synthesis pathways, and results on its association remain inconsistent across studies (328,329).

In the present work, a decreased in membrane transport and cell motility was shown after the NI. In the study of Hou et al. obesity was associated with increased cell motility (319). In line with these findings, increased cell motility ("flagellar assembly") has been associated with the presence of metabolic syndrome in 16 obese adult patients and positively correlated with two sugar metabolisms (fasting blood sugar and Hemoglobin A1c levels) (305). In contrast with the present work, an increase in bacterial cell motility was observed in obese patients after BS (313).

Here, an increase in "lipopolysaccharides biosynthesis proteins" and "membrane and intracellular structural molecules" was also reported after the intervention. The lipopolysaccharides biosynthesis proteins were identified as associated with obesity in other recent studies (329,330), but the direction of the association was not consistent.

7. Conclusion and future directions

The present study extends our knowledge on the association between GM and obesity, by identifying novel taxa with potential therapeutic relevance. The findings reported in the present work demonstrated that the microbial community of diet-free obese and overweight individuals was characterized by an increase in Firmicutes and a depletion in Bacteroidetes. These changes could be associated with a higher expression of enzymes involved in carbohydrate degradation and fermentation. Although the alteration in the proportion of Firmicutes and Bacteroidetes is in line with several data in the literature, the work showed that additional and novel taxonomic levels are associated with obesity. In addition, Enterobacteriaceae members known to having an endotoxic activity were increased at baseline. The identified microbial patterns correlated with anthropometrical measurements and with the level of physical activity.

After 3 months of a weight-loss nutritional intervention, the relative abundance of many microbial patterns identified at baseline changed significantly, with the proportion of species belonging to Bacteroidetes increasing and the proportion of species belonging to Firmicutes decreasing along with weight loss. The results of the present study also highlighted the association with obesity and metabolic pathways different from that of lipids and carbohydrates. In particular, metabolic pathways related to membrane transport were enriched at baseline and decreased after the intervention. Moreover, pathways related to cell motility decreased along with weight-loss.

Considering both the compositional and the functional analysis, this work suggests the potential of a moderately hypocaloric diet in restoring the intestinal balance in obese patients.

A key strength of the present study is that all the participants belonged to a restricted geographical area (Cagliari, Sardinia, Italy), with consequent reduction in the inter-individual variability. It remains to be

ascertained whether the identified microbial changes are consistent over time and how their permanence can be influenced by lifestyle habits. These questions will be the subject of future research.

Regarding methodological issues, it should be noted that targeting 16S variable regions at the species level cannot achieve the taxonomic resolution achieved by sequencing the entire gene (around 1500 bp). This limitation should be taken into account when interpreting the results at the species level. In addition, conclusions about GM function derived from PICRUST algorithm should be considered as a support to the taxonomic information that require further validation through experimental methods.

References

1. Ursell LK, Metcalf JL, Parfrey LW, Knight R. Defining the Human Microbiome. Nutr Rev. 2012 Aug;70(Suppl 1):S38–44.

2. Ursell LK, Haiser HJ, Van Treuren W, Garg N, Reddivari L, Vanamala J, et al. The Intestinal Metabolome—an Intersection Between Microbiota and Host. Gastroenterology. 2014 May;146(6):1470–6.

3. Wang B, Yao M, Lv L, Ling Z, Li L. The Human Microbiota in Health and Disease. Engineering. 2017 Feb 1;3(1):71–82.

4. Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J. 2017 Jun 1;474(11):1823–36.

5. Sekirov I, Russell SL, Antunes LCM, Finlay BB. Gut Microbiota in Health and Disease. Physiol Rev. 2010;90:46.

6. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep. 2006 Jul;7(7):688–93.

7. Conrad R, Vlassov A. The Human Microbiota: Composition, Functions, and Therapeutic Potential. Med Sci Rev. 2015 Oct 1;2:92–103.

8. Panek M, Paljetak HČ, Barešić A, Perić M, Matijašić M, Lojkić I, et al. Methodology challenges in studying human gut microbiota – effects of collection, storage, DNA extraction and next generation sequencing technologies. Sci Rep. 2018 Mar 23;8(1):1–13.

9. Ji B, Nielsen J. From next-generation sequencing to systematic modeling of the gut microbiome. Front Genet [Internet]. 2015 [cited 2019 Nov 27];6. Available from: https://www.frontiersin.org/articles/10.3389/fgene.2015.00219/full

10. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol. 2013 Sep;31(9):814–21.

11. Hasan N, Yang H. Factors affecting the composition of the gut microbiota, and its modulation. PeerJ [Internet]. 2019 Aug 16 [cited 2019 Nov 27];7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6699480/

12. Ding R, Goh W-R, Wu R, Yue X, Luo X, Khine WWT, et al. Revisit gut microbiota and its impact on human health and disease. J Food Drug Anal. 2019 Jul 1;27(3):623–31.

13. Quigley EMM. Gut Bacteria in Health and Disease. Gastroenterol Hepatol. 2013 Sep;9(9):560–9.

14. Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. Genome Med [Internet]. 2016 Apr 27 [cited 2020 Mar 10];8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4848870/

15. Dieterich W, Schink M, Zopf Y. Microbiota in the Gastrointestinal Tract. Med Sci [Internet]. 2018 Dec 14 [cited 2019 Nov 27];6(4). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6313343/

16. Kastl AJ, Terry NA, Wu GD, Albenberg LG. The Structure and Function of the Human Small Intestinal Microbiota: Current Understanding and Future Directions. Cell Mol Gastroenterol Hepatol. 2020 Jan 1;9(1):33–45.

17. Tuohy K, Scott K. The Microbiota of the Human Gastrointestinal Tract: A Molecular View. In: Diet-Microbe Interactions in the Gut: Effects on Human Health and Disease. 2015. p. 1–15.

18. Wroblewski LE, Peek RM, Wilson KT. Helicobacter pylori and Gastric Cancer: Factors That Modulate Disease Risk. Clin Microbiol Rev. 2010 Oct;23(4):713–39.

19. Nardone G, Compare D. The human gastric microbiota: Is it time to rethink the pathogenesis of stomach diseases? United Eur Gastroenterol J. 2015 Jun;3(3):255–60.

20. Bik EM, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F, et al. Molecular analysis of the bacterial microbiota in the human stomach. Proc Natl Acad Sci U S A. 2006 Jan 17;103(3):732–7.

21. Andersson AF, Lindberg M, Jakobsson H, Bäckhed F, Nyrén P, Engstrand L. Comparative Analysis of Human Gut Microbiota by Barcoded Pyrosequencing. PLOS ONE. 2008 Jul 30;3(7):e2836.

22. Engstrand L, Lindberg M. Helicobacter pylori and the gastric microbiota. Best Pract Res Clin Gastroenterol. 2013 Feb;27(1):39–45.

23. Wu WM, Yang YS, Peng LH. Microbiota in the stomach: new insights. J Dig Dis. 2014 Feb;15(2):54–61.

24. Li X-X, Wong GL-H, To K-F, Wong VW-S, Lai LH, Chow DK-L, et al. Bacterial microbiota profiling in gastritis without Helicobacter pylori infection or non-steroidal anti-inflammatory drug use. PloS One. 2009 Nov 24;4(11):e7985.

25. Meng C, Bai C, Brown TD, Hood LE, Tian Q. Human Gut Microbiota and Gastrointestinal Cancer. Genomics Proteomics Bioinformatics. 2018 Feb;16(1):33–49.

26. Mailhe M, Ricaboni D, Vitton V, Gonzalez J-M, Bachar D, Dubourg G, et al. Repertoire of the gut microbiota from stomach to colon using culturomics and next-generation sequencing. BMC Microbiol [Internet]. 2018 Oct 24 [cited 2019 Nov 27];18. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6201554/

27. Seekatz AM, Schnizlein MK, Koenigsknecht MJ, Baker JR, Hasler WL, Bleske BE, et al. Spatial and Temporal Analysis of the Stomach and Small-Intestinal Microbiota in Fasted Healthy Humans. mSphere [Internet]. 2019 Apr 24 [cited 2019 Nov 27];4(2). Available from: https://msphere.asm.org/content/4/2/e00126-19

28. Wacklin P, Laurikka P, Lindfors K, Collin P, Salmi T, Lähdeaho M-L, et al. Altered duodenal microbiota composition in celiac disease patients suffering from persistent symptoms on a long-term gluten-free diet. Am J Gastroenterol. 2014 Dec;109(12):1933–41.

29. Angelakis E, Armougom F, Carrière F, Bachar D, Laugier R, Lagier J-C, et al. A Metagenomic Investigation of the Duodenal Microbiota Reveals Links with Obesity. PLOS ONE. 2015 Sep 10;10(9):e0137784.

30. Sundin O, Mendoza-Ladd A, Zeng M, Diaz-Arevalo D, Morales E, Fagan B, et al. The human jejunum has an endogenous microbiota that differs from those in the oral cavity and colon. BMC Microbiol. 2017 Dec 1;17.

31. Villmones HC, Haug ES, Ulvestad E, Grude N, Stenstad T, Halland A, et al. Species Level Description of the Human Ileal Bacterial Microbiota. Sci Rep. 2018 Mar 16;8(1):1–9.

32. Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, et al. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms. 2019 Jan 10;7(1).

33. Donaldson GP, Lee SM, Mazmanian SK. Gut biogeography of the bacterial microbiota. Nat Rev Microbiol. 2016 Jan;14(1):20–32.

34. Arumugam M, Raes J, Pelletier E, Paslier DL, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature. 2011 May;473(7346):174–80.

35. Cheng M, Ning K. Stereotypes About Enterotype: the Old and New Ideas. Genomics Proteomics Bioinformatics. 2019 Feb 1;17(1):4–12.

36. Guinane CM, Cotter PD. Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. Ther Adv Gastroenterol. 2013 Jul;6(4):295–308.

37. Amon P, Sanderson I. What is the microbiome? Arch Dis Child - Educ Pract. 2017 Oct 1;102(5):257–60.

38. Rodríguez JM, Murphy K, Stanton C, Ross RP, Kober OI, Juge N, et al. The composition of the gut microbiota throughout life, with an emphasis on early life. Microb Ecol Health Dis [Internet]. 2015 Feb 2 [cited 2019 Nov 28];26. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4315782/

39. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet Lond Engl. 2008 Jan 5;371(9606):75–84.

40. Moles L, Gómez M, Heilig H, Bustos G, Fuentes S, de Vos W, et al. Bacterial Diversity in Meconium of Preterm Neonates and Evolution of Their Fecal Microbiota during the First Month of Life. PLoS ONE [Internet]. 2013 Jun 28 [cited 2019 Nov 28];8(6). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3695978/

41. McBurney MI, Davis C, Fraser CM, Schneeman BO, Huttenhower C, Verbeke K, et al. Establishing What Constitutes a Healthy Human Gut Microbiome: State of the Science, Regulatory Considerations, and Future Directions. J Nutr. 2019 Nov 1;149(11):1882–95.

42. Salminen S, Gibson GR, McCartney AL, Isolauri E. Influence of mode of delivery on gut microbiota composition in seven year old children. Gut. 2004 Sep 1;53(9):1388–9.

43. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A. 2010 Jun 29;107(26):11971–5.

44. Martin R, Makino H, Cetinyurek Yavuz A, Ben-Amor K, Roelofs M, Ishikawa E, et al. Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota. PloS One. 2016;11(6):e0158498.

45. Rutayisire E, Huang K, Liu Y, Tao F. The mode of delivery affects the diversity and colonization pattern of the gut microbiota during the first year of infants' life: a systematic review. BMC Gastroenterol. 2016 Jul 30;16(1):86.

46. Azad MB, Konya T, Maughan H, Guttman DS, Field CJ, Chari RS, et al. Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. CMAJ Can Med Assoc J. 2013 Mar 19;185(5):385–94.

47. Callaway E. C-section babies are missing key microbes. Nature [Internet]. 2019 Sep 18 [cited 2019 Nov 28]; Available from: https://www.nature.com/articles/d41586-019-02807-x

48. Magne F, Puchi Silva A, Carvajal B, Gotteland M. The Elevated Rate of Cesarean Section and Its Contribution to Non-Communicable Chronic Diseases in Latin America: The Growing Involvement of the Microbiota. Front Pediatr [Internet]. 2017 Sep 4 [cited 2019 Nov 28];5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5591430/

49. Ballard O, Morrow AL. Human Milk Composition: Nutrients and Bioactive Factors. Pediatr Clin North Am. 2013 Feb;60(1):49–74.

50. van den Elsen LWJ, Garssen J, Burcelin R, Verhasselt V. Shaping the Gut Microbiota by Breastfeeding: The Gateway to Allergy Prevention? Front Pediatr [Internet]. 2019 [cited 2019 Nov 28];7. Available from: https://www.frontiersin.org/articles/10.3389/fped.2019.00047/full#B1

51. Guaraldi F, Salvatori G. Effect of Breast and Formula Feeding on Gut Microbiota Shaping in Newborns. Front Cell Infect Microbiol [Internet]. 2012 [cited 2019 Nov 28];2. Available from: https://www.frontiersin.org/articles/10.3389/fcimb.2012.00094/full#B21

52. Ward RE, Niñonuevo M, Mills DA, Lebrilla CB, German JB. In vitro fermentability of human milk oligosaccharides by several strains of bifidobacteria. Mol Nutr Food Res. 2007;51(11):1398–405.

53. Moore RE, Townsend SD. Temporal development of the infant gut microbiome. Open Biol. 9(9):190128.

54. Dawod B, Marshall JS. Cytokines and Soluble Receptors in Breast Milk as Enhancers of Oral Tolerance Development. Front Immunol [Internet]. 2019 Jan 22 [cited 2019 Nov 28];10. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6349727/

55. Ho NT, Li F, Lee-Sarwar KA, Tun HM, Brown BP, Pannaraj PS, et al. Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations. Nat Commun [Internet]. 2018 Oct 9 [cited 2019 Nov 28];9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6177445/

56. Isolauri E. Development of healthy gut microbiota early in life. J Paediatr Child Health. 2012;48(s3):1–6.

57. Zhuang L, Chen H, Zhang S, Zhuang J, Li Q, Feng Z. Intestinal Microbiota in Early Life and Its Implications on Childhood Health. Genomics Proteomics Bioinformatics. 2019 Feb 1;17(1):13–25.

58. Davis EC, Wang M, Donovan SM. The role of early life nutrition in the establishment of gastrointestinal microbial composition and function. Gut Microbes. 2017 Mar 4;8(2):143–71.

59. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, et al. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci. 2011 Mar 15;108(Supplement 1):4578–85.

60. Derrien M, Alvarez A-S, Vos WM de. The Gut Microbiota in the First Decade of Life. Trends Microbiol. 2019 Dec 1;27(12):997–1010.

61. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 2012 Jun;486(7402):222–7.

62. Chu DM, Ma J, Prince AL, Antony KM, Seferovic MD, Aagaard KM. Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. Nat Med. 2017 Mar;23(3):314–26.

63. McVey Neufeld K-A, Luczynski P, Dinan TG, Cryan JF. Reframing the Teenage Wasteland: Adolescent Microbiota-Gut-Brain Axis. Can J Psychiatry Rev Can Psychiatr. 2016 Apr;61(4):214–21.

64. Agans R, Rigsbee L, Kenche H, Michail S, Khamis HJ, Paliy O. Distal gut microbiota of adolescent children is different from that of adults. FEMS Microbiol Ecol. 2011 Aug 1;77(2):404–12.

65. Jones L, Kumar J, Mistry A, Sankar Chittoor Mana T, Perry G, Reddy VP, et al. The Transformative Possibilities of the Microbiota and Mycobiota for Health, Disease, Aging, and Technological Innovation. Biomedicines [Internet]. 2019 Mar 28 [cited 2019 Nov 28];7(2). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6631383/

66. Nagpal R, Mainali R, Ahmadi S, Wang S, Singh R, Kavanagh K, et al. Gut microbiome and aging: Physiological and mechanistic insights. Nutr Healthy Aging. 2018 Jun 15;4(4):267–85.

67. Montalto M, D'Onofrio F, Gallo A, Cazzato A, Gasbarrini G. Intestinal microbiota and its functions. Dig Liver Dis Suppl. 2009 Jul 1;3(2):30–4.

68. Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Reddy DN. Role of the normal gut microbiota. World J Gastroenterol WJG. 2015 Aug 7;21(29):8787–803.

69. Bik EM, Ugalde JA, Cousins J, Goddard AD, Richman J, Apte ZS. Microbial biotransformations in the human distal gut. Br J Pharmacol. 2018;175(24):4404–14.

70. Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, Calignano A. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol WJG. 2011 Mar 28;17(12):1519–28.

71. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, et al. The Microbiome and Butyrate Regulate Energy Metabolism and Autophagy in the Mammalian Colon. Cell Metab. 2011 May 4;13(5):517–26.

72. Ryu SH, Kaiko GE, Stappenbeck TS. Cellular differentiation: Potential insight into butyrate paradox? Mol Cell Oncol. 2018 May 4;5(3):e1212685.

73. Wang X, Zhang A, Miao J, Sun H, Yan G, Wu F, et al. Gut microbiota as important modulator of metabolism in health and disease. RSC Adv. 2018 Dec 19;8(74):42380–9.

74. Perry RJ, Borders CB, Cline GW, Zhang X-M, Alves TC, Petersen KF, et al. Propionate Increases Hepatic Pyruvate Cycling and Anaplerosis and Alters Mitochondrial Metabolism. J Biol Chem. 2016 Jun 3;291(23):12161–70.

75. Tian X, Hellman J, Horswill AR, Crosby HA, Francis KP, Prakash A. Elevated Gut Microbiome-Derived Propionate Levels Are Associated With Reduced Sterile Lung Inflammation and Bacterial Immunity in Mice. Front Microbiol [Internet]. 2019 [cited 2019 Nov 28];10. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00159/full

76. Chambers ES, Preston T, Frost G, Morrison DJ. Role of Gut Microbiota-Generated Short-Chain Fatty Acids in Metabolic and Cardiovascular Health. Curr Nutr Rep. 2018;7(4):198–206.

77. Heimann E, Nyman M, Pålbrink A-K, Lindkvist-Petersson K, Degerman E. Branched short-chain fatty acids modulate glucose and lipid metabolism in primary adipocytes. Adipocyte. 2016 Dec;5(4):359–68.

78. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, et al. Gut microbiota functions: metabolism of nutrients and other food components. Eur J Nutr. 2018;57(1):1–24.

79. Magnúsdóttir S, Ravcheev D, de Crécy-Lagard V, Thiele I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. Front Genet [Internet]. 2015 Apr 20 [cited 2019 Nov 29];6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4403557/

80. Ravcheev DA, Thiele I. Genomic Analysis of the Human Gut Microbiome Suggests Novel Enzymes Involved in Quinone Biosynthesis. Front Microbiol [Internet]. 2016 Feb 9 [cited 2019 Nov 29];7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4746308/

81. Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. Bile Acids and the Gut Microbiome. Curr Opin Gastroenterol. 2014 May;30(3):332–8.

82. Begley M, Gahan CGM, Hill C. The interaction between bacteria and bile. FEMS Microbiol Rev. 2005;29(4):625–51.

83. Winston JA, Theriot CM. Diversification of host bile acids by members of the gut microbiota. Gut Microbes. 2019 Oct 9;0(0):1–14.

84. Gérard P. Metabolism of Cholesterol and Bile Acids by the Gut Microbiota. Pathogens. 2013 Dec 30;3(1):14–24.

85. Qi Y, Jiang C, Cheng J, Krausz KW, Li T, Ferrell JM, et al. Bile acid signaling in lipid metabolism: Metabolomic and lipidomic analysis of lipid and bile acid markers linked to anti-obesity and anti-diabetes in mice. Biochim Biophys Acta. 2015 Jan;1851(1):19–29.

86. Roager HM, Licht TR. Microbial tryptophan catabolites in health and disease. Nat Commun. 2018 Aug 17;9(1):1–10.

87. Koppel N, Rekdal VM, Balskus EP. Chemical transformation of xenobiotics by the human gut microbiota. Science [Internet]. 2018 [cited 2019 Nov 29];356(6344). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5534341/

88. Lazar V, Ditu L-M, Pircalabioru GG, Gheorghe I, Curutiu C, Holban AM, et al. Aspects of Gut Microbiota and Immune System Interactions in Infectious Diseases, Immunopathology, and Cancer. Front Immunol [Internet]. 2018 Aug 15 [cited 2019 Nov 29];9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6104162/

89. Mezouar S, Chantran Y, Michel J, Fabre A, Dubus J-C, Leone M, et al. Microbiome and the immune system: From a healthy steady-state to allergy associated disruption. Hum Microbiome J. 2018 Dec 1;10:11–20.

90. Round JL, Mazmanian SK. The gut microbiome shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009 May;9(5):313–23.

91. Slack E, Balmer ML, Fritz JH, Hapfelmeier S. Functional flexibility of intestinal IgA - broadening the fine line. Front Immunol. 2012;3:100.

92. Belkaid Y, Hand TW. Role of the Microbiota in Immunity and Inflammation. Cell. 2014 Mar 27;157(1):121–41.

93. Coombes JL, Siddiqui KRR, Arancibia-Cárcamo CV, Hall J, Sun C-M, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β– and retinoic acid–dependent mechanism. J Exp Med. 2007 Aug 6;204(8):1757–64.

94. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio C-W, Santacruz N, et al. Peripheral education of the immune system by colonic commensal microbiota. Nature. 2011 Sep 21;478(7368):250–4.

95. Kaisar MMM, Pelgrom LR, Ham AJ van der, Yazdanbakhsh M, Everts B. Butyrate Conditions Human Dendritic Cells to Prime Type 1 Regulatory T Cells via both Histone Deacetylase Inhibition and G Protein-Coupled Receptor 109A Signaling. Front Immunol [Internet]. 2017 [cited 2019 Dec 3];8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5670331/

96. Cresci GA, Thangaraju M, Mellinger JD, Liu K, Ganapathy V. Colonic gene expression in conventional and germ-free mice with a focus on the butyrate receptor GPR109A and the butyrate transporter SLC5A8. J Gastrointest Surg Off J Soc Surg Aliment Tract. 2010 Mar;14(3):449–61.

97. Gurav A, Sivaprakasam S, Bhutia YD, Boettger T, Singh N, Ganapathy V. Slc5a8, a Na+-coupled high-affinity transporter for short-chain fatty acids, is a conditional tumor suppressor in colon that protects against colitis and colon cancer under low-fiber dietary conditions. Biochem J. 2015 Jul 15;469(2):267.

98. Sivieri K, Bassan J, Peixoto G, Monti R. Gut microbiota and antimicrobial peptides. Curr Opin Food Sci. 2017 Feb 1;13:56–62.

99. Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinath S, et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature. 2013 Oct 3;502(7469):96–9.

100. Bäumler AJ, Sperandio V. Interactions between the microbiota and pathogenic bacteria in the gut. Nature. 2016 Jul 6;535(7610):85–93.

101. Sinha R, Shukla P. Antimicrobial Peptides: Recent Insights on Biotechnological Interventions and Future Perspectives. Protein Pept Lett. 2019 Feb;26(2):79–87.

102. Iacob S, Iacob DG, Luminos LM. Intestinal Microbiota as a Host Defense Mechanism to Infectious Threats. Front Microbiol [Internet]. 2019 [cited 2019 Dec 3];9. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03328/full#B33

103. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol Q Publ Hell Soc Gastroenterol. 2015 Jun;28(2):203.

104. Mohajeri MH, Brummer RJM, Rastall RA, Weersma RK, Harmsen HJM, Faas M, et al. The role of the microbiome for human health: from basic science to clinical applications. Eur J Nutr. 2018;57(Suppl 1):1–14.

105. SUDO N. Role of gut microbiota in brain function and stress-related pathology. Biosci Microbiota Food Health. 2019;38(3):75–80.

106. Mayer EA, Tillisch K, Gupta A. Gut/brain axis and the microbiota. J Clin Invest. 2015 Mar 2;125(3):926–38.

107. Neufeld KAM, Mao YK, Bienenstock J, Foster JA, Kunze WA. The microbiome is essential for normal gut intrinsic primary afferent neuron excitability in the mouse. Neurogastroenterol Motil. 2013;25(2):183-e88.

108. Sudo N. Microbiome, HPA axis and production of endocrine hormones in the gut. Adv Exp Med Biol. 2014;817:177–94.

109. Heijtz RD, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, et al. Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci. 2011 Feb 15;108(7):3047–52.

110. Braniste V, Al-Asmakh M, Kowal C, Anuar F, Abbaspour A, Tóth M, et al. The gut microbiota influences blood-brain barrier permeability in mice. Sci Transl Med. 2014 Nov 19;6(263):263ra158.

111. Cryan JF, O'Riordan KJ, Sandhu K, Peterson V, Dinan TG. The gut microbiome in neurological disorders. Lancet Neurol [Internet]. 2019 Nov 18 [cited 2019 Dec 3];0(0). Available from: https://www.thelancet.com/journals/laneur/article/PIIS1474-4422(19)30356-4/abstract

112. Naseribafrouei A, Hestad K, Avershina E, Sekelja M, Linløkken A, Wilson R, et al. Correlation between the human fecal microbiota and depression. Neurogastroenterol Motil. 2014 Aug;26(8):1155–62.

113. Yang B, Wei J, Ju P, Chen J. Effects of regulating intestinal microbiota on anxiety symptoms: A systematic review. Gen Psychiatry [Internet]. 2019 Apr 1 [cited 2019 Dec 3];32(2). Available from: https://gpsych.bmj.com/content/32/2/e100056

114. Gilbert JA. Our unique microbial identity. Genome Biol [Internet]. 2015 [cited 2019 Dec 3];16(1). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4430908/

115. Brown K, DeCoffe D, Molcan E, Gibson DL. Diet-Induced Dysbiosis of the Intestinal Microbiota and the Effects on Immunity and Disease. Nutrients. 2012 Aug 21;4(8):1095–119.

116. Zmora N, Suez J, Elinav E. You are what you eat: diet, health and the gut microbiota. Nat Rev Gastroenterol Hepatol. 2019 Jan;16(1):35–56.

117. Petersen C, Round JL. Defining dysbiosis and its influence on host immunity and disease. Cell Microbiol. 2014 Jul;16(7):1024–33.

118. Levy M, Kolodziejczyk AA, Thaiss CA, Elinav E. Dysbiosis and the immune system. Nat Rev Immunol. 2017 Apr;17(4):219–32.

119. Belizário JE, Faintuch J. Microbiome and Gut Dysbiosis. Exp Suppl 2012. 2018;109:459–76.

120. Wilkins LJ, Monga M, Miller AW. Defining Dysbiosis for a Cluster of Chronic Diseases. Sci Rep. 2019 Sep 9;9(1):1–10.

121. Baothman OA, Zamzami MA, Taher I, Abubaker J, Abu-Farha M. The role of Gut Microbiota in the development of obesity and Diabetes. Lipids Health Dis [Internet]. 2016 Jun 18 [cited 2019 Oct 30];15. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4912704/

122. Senghor B, Sokhna C, Ruimy R, Lagier J-C. Gut microbiota diversity according to dietary habits and geographical provenance. Hum Microbiome J. 2018 Apr 1;7–8:1–9.

123. Valdes AM, Walter J, Segal E, Spector TD. Role of the gut microbiota in nutrition and health. BMJ [Internet]. 2018 Jun 13 [cited 2019 Nov 27];361. Available from: https://www.bmj.com/content/361/bmj.k2179

124. Watabe J. Carbohydrate Fermentation in the Colon. J Intest Microbiol. 2005;19(3):169–77.

125. Lovegrove A, Edwards CH, De Noni I, Patel H, El SN, Grassby T, et al. Role of polysaccharides in food, digestion, and health. Crit Rev Food Sci Nutr. 2017 Jan 22;57(2):237–53.

126. Marchesi JR, Adams DH, Fava F, Hermes GDA, Hirschfield GM, Hold G, et al. The gut microbiota and host health: a new clinical frontier. Gut. 2016 Feb 1;65(2):330–9.

127. Garcia-Mantrana I, Selma-Royo M, Alcantara C, Collado MC. Shifts on Gut Microbiota Associated to Mediterranean Diet Adherence and Specific Dietary Intakes on General Adult Population. Front Microbiol [Internet]. 2018 [cited 2019 Dec 4];9. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00890/full

128. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J. 2011 Feb;5(2):220–30.

129. Abell GCJ, Cooke CM, Bennett CN, Conlon MA, McOrist AL. Phylotypes related to Ruminococcus bromii are abundant in the large bowel of humans and increase in response to a diet high in resistant starch. FEMS Microbiol Ecol. 2008 Dec;66(3):505–15.

130. Martínez-González MA, López-Fontana C, Varo JJ, Sánchez-Villegas A, Martinez JA. Validation of the Spanish version of the physical activity questionnaire used in the Nurses' Health Study and the Health Professionals' Follow-up Study. Public Health Nutr. 2005 Oct;8(7):920–7.

131. Paoli A, Mancin L, Bianco A, Thomas E, Mota JF, Piccini F. Ketogenic Diet and Microbiota: Friends or Enemies? Genes [Internet]. 2019 Jul 15 [cited 2019 Dec 6];10(7). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6678592/

132. Wolters M, Ahrens J, Romaní-Pérez M, Watkins C, Sanz Y, Benítez-Páez A, et al. Dietary fat, the gut microbiota, and metabolic health - A systematic review conducted within the MyNewGut project. Clin Nutr Edinb Scotl. 2019 Dec;38(6):2504–20.

133. Wan Y, Wang F, Yuan J, Li J, Jiang D, Zhang J, et al. Effects of dietary fat on gut microbiota and faecal metabolites, and their relationship with cardiometabolic risk factors: a 6-month randomised controlled-feeding trial. Gut. 2019 Aug 1;68(8):1417–29.

134. Julibert A, Bibiloni MDM, Tur JA. Dietary fat intake and metabolic syndrome in adults: A systematic review. Nutr Metab Cardiovasc Dis NMCD. 2019 Sep;29(9):887–905.

135. Ma N, Tian Y, Wu Y, Ma X. Contributions of the Interaction Between Dietary Protein and Gut Microbiota to Intestinal Health. Curr Protein Pept Sci. 2017;18(8):795–808.

136. Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH. The influence of diet on the gut microbiota. Pharmacol Res. 2013 Mar;69(1):52–60.

137. Madsen L, Myrmel LS, Fjære E, Liaset B, Kristiansen K. Links between Dietary Protein Sources, the Gut Microbiota, and Obesity. Front Physiol [Internet]. 2017 Dec 19 [cited 2019 Dec 20];8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5742165/

138. Janeiro MH, Ramírez MJ, Milagro FI, Martínez JA, Solas M. Implication of Trimethylamine N-Oxide (TMAO) in Disease: Potential Biomarker or New Therapeutic Target. Nutrients [Internet]. 2018 Oct 1 [cited 2019 Dec 20];10(10). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6213249/

139. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr. 1995 Jun;125(6):1401–12.

140. Delzenne NM, Williams CM. Prebiotics and lipid metabolism. Curr Opin Lipidol. 2002 Feb;13(1):61–7.

141. Gibson GR, Probert HM, Loo JV, Rastall RA, Roberfroid MB. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev. 2004 Dec;17(2):259–75.

142. Bindels LB, Delzenne NM, Cani PD, Walter J. Towards a more comprehensive concept for prebiotics. Nat Rev Gastroenterol Hepatol. 2015 May;12(5):303–10.

143. Davani-Davari D, Negahdaripour M, Karimzadeh I, Seifan M, Mohkam M, Masoumi SJ, et al. Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications. Foods [Internet]. 2019 Mar 9 [cited 2020 Feb 18];8(3). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6463098/

144. Umu ÖCO, Rudi K, Diep DB. Modulation of the gut microbiota by prebiotic fibres and bacteriocins. Microb Ecol Health Dis [Internet]. 2017 Jan 1 [cited 2020 Feb 18];28(1). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5614387/

145. Conway PL. Prebiotics and human health: The state-of-the-art and future perspectives. Näringsforskning. 2001 Dec;45(1):13–21.

146. Sanders ME, Merenstein DJ, Reid G, Gibson GR, Rastall RA. Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. Nat Rev Gastroenterol Hepatol. 2019 Oct;16(10):605–16.

147. Hotel A. Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria – Joint FAO/WHO Expert Consultation. 2001 Jan 1;2014.

148. Gareau MG, Sherman PM, Walker WA. Probiotics and the gut microbiota in intestinal health and disease. Nat Rev Gastroenterol Hepatol. 2010 Sep;7(9):503–14.

149. Hemarajata P, Versalovic J. Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. Ther Adv Gastroenterol. 2013 Jan;6(1):39–51.

150. Szajewska H, Ruszczyński M, Radzikowski A. Probiotics in the prevention of antibiotic-associated diarrhea in children: a meta-analysis of randomized controlled trials. J Pediatr. 2006 Sep;149(3):367–72.

151. Barker AK, Duster M, Valentine S, Hess T, Archbald-Pannone L, Guerrant R, et al. A randomized controlled trial of probiotics for Clostridium difficile infection in adults (PICO). J Antimicrob Chemother. 2017 Nov 1;72(11):3177–80.

152. Nikolova V, Zaidi SY, Young AH, Cleare AJ, Stone JM. Gut feeling: randomized controlled trials of probiotics for the treatment of clinical depression: Systematic review and meta-analysis. Ther Adv Psychopharmacol. 2019;9:2045125319859963.

153. Milajerdi A, Mousavi SM, Sadeghi A, Salari-Moghaddam A, Parohan M, Larijani B, et al. The effect of probiotics on inflammatory biomarkers: a meta-analysis of randomized clinical trials. Eur J Nutr. 2020 Mar;59(2):633–49.

154. Savin Z, Kivity S, Yonath H, Yehuda S. Smoking and the intestinal microbiome. Arch Microbiol. 2018 Jul;200(5):677–84.

155. Verschuere S, Bracke KR, Demoor T, Plantinga M, Verbrugghe P, Ferdinande L, et al. Cigarette smoking alters epithelial apoptosis and immune composition in murine GALT. Lab Investig J Tech Methods Pathol. 2011 Jul;91(7):1056–67.

156. Allais L, Kerckhof F-M, Verschuere S, Bracke KR, De Smet R, Laukens D, et al. Chronic cigarette smoke exposure induces microbial and inflammatory shifts and mucin changes in the murine gut. Environ Microbiol. 2016;18(5):1352–63.

157. Pavia CS, Pierre A, Nowakowski J. Antimicrobial activity of nicotine against a spectrum of bacterial and fungal pathogens. J Med Microbiol. 2000 Jul;49(7):675–6.

158. Murthy SN, Dinoso VP, Clearfield HR, Chey WY. Serial pH changes in the duodenal bulb during smoking. Gastroenterology. 1978 Jul;75(1):1–4.

159. Miller G, Palmer KR, Smith B, Ferrington C, Merrick MV. Smoking delays gastric emptying of solids. Gut. 1989 Jan;30(1):50–3.

160. Huang C, Shi G. Smoking and microbiome in oral, airway, gut and some systemic diseases. J Transl Med. 2019 Jul 15;17(1):225.

161. Modi SR, Collins JJ, Relman DA. Antibiotics and the gut microbiota. J Clin Invest. 2014 Oct 1;124(10):4212–8.

162. Iizumi T, Battaglia T, Ruiz V, Perez Perez GI. Gut Microbiome and Antibiotics. Arch Med Res. 2017;48(8):727–34.

163. Zhang S, Chen D-C. Facing a new challenge: the adverse effects of antibiotics on gut microbiota and host immunity. Chin Med J (Engl). 2019 May 20;132(10):1135–8.

164. Willing BP, Russell SL, Finlay BB. Shifting the balance: antibiotic effects on host-microbiota mutualism. Nat Rev Microbiol. 2011 Apr;9(4):233–43.

165. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J. 2007 May;1(1):56–66.

166. Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. Nature. 2018 Mar 29;555(7698):623–8.

167. Vila AV, Collij V, Sanna S, Sinha T, Imhann F, Bourgonje AR, et al. Impact of commonly used drugs on the composition and metabolic function of the gut microbiota. Nat Commun. 2020 Jan 17;11(1):1–11.

168. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell. 2014 Nov 6;159(4):789–99.

169. Goodrich JK, Davenport ER, Clark AG, Ley RE. The relationship between the human genome and microbiome comes into view. Annu Rev Genet. 2017 Nov 27;51:413–33.

170. Turpin W, Espin-Garcia O, Xu W, Silverberg MS, Kevans D, Smith MI, et al. Association of host genome with intestinal microbial composition in a large healthy cohort. Nat Genet. 2016;48(11):1413–7.

171. Blekhman R, Goodrich JK, Huang K, Sun Q, Bukowski R, Bell JT, et al. Host genetic variation impacts microbiome composition across human body sites. Genome Biol [Internet]. 2015 [cited 2020 Feb 19];16(1). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4570153/

172. Bonder MJ, Kurilshikov A, Tigchelaar EF, Mujagic Z, Imhann F, Vila AV, et al. The effect of host genetics on the gut microbiome. Nat Genet. 2016 Nov;48(11):1407–12.

173. Società Italiana di Microbiologia. Abstract Book - Congresso 2018 [Internet]. SIM - Società italiana di microbiologia. [cited 2020 Oct 5]. Available from: https://www.societasim.it/congresso-2018/abstract-book/

174. Vascellari S, Palmas V, Melis M, Pisanu S, Cusano R, Uva P, et al. Gut Microbiota and Metabolome Alterations Associated with Parkinson's Disease. mSystems. 2020 Sep 15;5(5).

175. Melis M, Vascellari S, Santoru ML, Oppo V, Fabbri M, Sarchioto M, et al. Gut microbiota and metabolome distinctive features in Parkinson disease: focus on Levodopa and Levodopa Carbidopa intrajejunal gel. Revis Eur J Neurol. 2020 Sep 15;

176. Poulain M, Pes GM, Grasland C, Carru C, Ferrucci L, Baggio G, et al. Identification of a geographic area characterized by extreme longevity in the Sardinia island: the AKEA study. Exp Gerontol. 2004 Sep 1;39(9):1423–9.

177. Millán-Calenti JC, Tubío J, Pita-Fernández S, González-Abraldes I, Lorenzo T, Fernández-Arruty T, et al. Prevalence of functional disability in activities of daily living (ADL), instrumental activities of daily living

(IADL) and associated factors, as predictors of morbidity and mortality. Arch Gerontol Geriatr. 2010 May 1;50(3):306–10.

178. Panagiotakos DB, Pitsavos C, Arvaniti F, Stefanadis C. Adherence to the Mediterranean food pattern predicts the prevalence of hypertension, hypercholesterolemia, diabetes and obesity, among healthy adults; the accuracy of the MedDietScore. Prev Med. 2007 Apr;44(4):335–40.

179. Washburn RA, Smith KW, Jette AM, Janney CA. The physical activity scale for the elderly (PASE): Development and evaluation. J Clin Epidemiol. 1993 Feb 1;46(2):153–62.

180. Guigoz Y, Lauque S, Vellas BJ. Identifying the elderly at risk for malnutrition. The Mini Nutritional Assessment. Clin Geriatr Med. 2002 Nov;18(4):737–57.

181. Biagi E, Rampelli S, Turroni S, Quercia S, Candela M, Brigidi P. The gut microbiota of centenarians: Signatures of longevity in the gut microbiota profile. Mech Ageing Dev. 2017 Jul 1;165(Part B):180–4.

182. Santoro A, Ostan R, Candela M, Biagi E, Brigidi P, Capri M, et al. Gut microbiota changes in the extreme decades of human life: a focus on centenarians. Cell Mol Life Sci. 2018;75(1):129–48.

183. Palmas V, Pisanu S, Madau V, Casula E, Deledda A, Cusano R, et al. Cross-sectional study of the intestinal microbiota and its functional implications on health status in Sardinian centenarians. Prep.

184. Huybrechts I, Zouiouich S, Loobuyck A, Vandenbulcke Z, Vogtmann E, Pisanu S, et al. The Human Microbiome in Relation to Cancer Risk: A Systematic Review of Epidemiologic Studies. Cancer Epidemiol Prev Biomark [Internet]. 2020 Jul 29 [cited 2020 Aug 22]; Available from: https://cebp.aacrjournals.org/content/early/2020/08/21/1055-9965.EPI-20-0288

185. Italian Society of Obesity IX National Congress. Eat Weight Disord - Stud Anorex Bulim Obes. 2018 Oct 1;23(5):705–29.

186. Pisanu S, Palmas V, Madau V, Casula E, Deledda A, Cusano R, et al. Impact of a Moderately Hypocaloric Mediterranean Diet on the Gut Microbiota Composition of Italian Obese Patients. Nutrients. 2020 Sep 4;12(9).

187. Palmas V, Pisanu S, Madau V, Casula E, Deledda A, Cusano R, et al. Gut microbiota markers associated with obesity and overweight in Italian adults. Submitt Sci Rep.

188. WHO. Obesity and overweight [Internet]. [cited 2019 Oct 30]. Available from: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight

189. Osservatorio Nazionale 2016. Rapporto Osservasalute 2016 | Osservatorio sulla Salute [Internet]. [cited 2020 Feb 20]. Available from: https://www.osservatoriosullasalute.it/osservasalute/rapporto-osservasalute-2016

190. Aune D, Sen A, Prasad M, Norat T, Janszky I, Tonstad S, et al. BMI and all cause mortality: systematic review and non-linear dose-response meta-analysis of 230 cohort studies with 3.74 million deaths among 30.3 million participants. BMJ. 2016 04;353:i2156.

191. Bhaskaran K, dos-Santos-Silva I, Leon DA, Douglas IJ, Smeeth L. Association of BMI with overall and cause-specific mortality: a population-based cohort study of 3·6 million adults in the UK. Lancet Diabetes Endocrinol. 2018 Dec 1;6(12):944–53.

192. Romieu I, Dossus L, Barquera S, Blottière HM, Franks PW, Gunter M, et al. Energy balance and obesity: what are the main drivers? Cancer Causes Control. 2017;28(3):247–58.

193. Castaner O, Goday A, Park Y-M, Lee S-H, Magkos F, Shiow S-ATE, et al. The Gut Microbiome Profile in Obesity: A Systematic Review. Int J Endocrinol [Internet]. 2018 [cited 2018 Sep 5];2018. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5933040/

194. Davis CD. The Gut Microbiome and Its Role in Obesity. Nutr Today. 2016 Aug;51(4):167–74.

195. Hall KD, Heymsfield SB, Kemnitz JW, Klein S, Schoeller DA, Speakman JR. Energy balance and its components: implications for body weight regulation123. Am J Clin Nutr. 2012 Apr;95(4):989–94.

196. Suzuki K, Simpson KA, Minnion JS, Shillito JC, Bloom SR. The role of gut hormones and the hypothalamus in appetite regulation. Endocr J. 2010;57(5):359–72.

197. Bliss ES, Whiteside E. The Gut-Brain Axis, the Human Gut Microbiota and Their Integration in the Development of Obesity. Front Physiol [Internet]. 2018 [cited 2020 Feb 20];9. Available from: https://www.frontiersin.org/articles/10.3389/fphys.2018.00900/full

198. Dockray GJ. Enteroendocrine cell signalling via the vagus nerve. Curr Opin Pharmacol. 2013 Dec;13(6):954–8.

199. Schwartz MW, Woods SC, Porte D, Seeley RJ, Baskin DG. Central nervous system control of food intake. Nature. 2000 Apr 6;404(6778):661–71.

200. Breton J, Tennoune N, Lucas N, Francois M, Legrand R, Jacquemot J, et al. Gut Commensal E. coli Proteins Activate Host Satiety Pathways following Nutrient-Induced Bacterial Growth. Cell Metab. 2016 Feb 9;23(2):324–34.

201. Mitev K, Taleski V. Association between the Gut Microbiota and Obesity. Open Access Maced J Med Sci. 2019 Jun 30;7(12):2050–6.

202. Furet J-P, Kong L-C, Tap J, Poitou C, Basdevant A, Bouillot J-L, et al. Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers. Diabetes. 2010 Dec;59(12):3049–57.

203. Queipo-Ortuño MI, Seoane LM, Murri M, Pardo M, Gomez-Zumaquero JM, Cardona F, et al. Gut Microbiota Composition in Male Rat Models under Different Nutritional Status and Physical Activity and Its Association with Serum Leptin and Ghrelin Levels. PLoS ONE [Internet]. 2013 May 28 [cited 2020 Feb 20];8(5). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3665787/

204. Fetissov SO. Role of the gut microbiota in host appetite control: bacterial growth to animal feeding behaviour. Nat Rev Endocrinol. 2017;13(1):11–25.

205. Kim KN, Yao Y, Ju SY. Short Chain Fatty Acids and Fecal Microbiota Abundance in Humans with Obesity: A Systematic Review and Meta-Analysis. Nutrients. 2019 Oct 18;11(10).

206. Abenavoli L, Scarpellini E, Colica C, Boccuto L, Salehi B, Sharifi-Rad J, et al. Gut Microbiota and Obesity: A Role for Probiotics. Nutrients. 2019 Nov;11(11):2690.

207. Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev. 1990 Apr 1;70(2):567–90.

208. Sun L, Ma L, Ma Y, Zhang F, Zhao C, Nie Y. Insights into the role of gut microbiota in obesity: pathogenesis, mechanisms, and therapeutic perspectives. Protein Cell. 2018 May;9(5):397–403.

209. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006 Dec 21;444(7122):1022–3.

210. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006 Dec 21;444(7122):1027–31.

211. Murphy EF, Cotter PD, Healy S, Marques TM, O'Sullivan O, Fouhy F, et al. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. Gut. 2010 Dec;59(12):1635–42.

212. Kobyliak N, Virchenko O, Falalyeyeva T. Pathophysiological role of host microbiota in the development of obesity. Nutr J [Internet]. 2016 Apr 23 [cited 2020 Feb 25];15. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4841968/

213. Mathur R, Barlow GM. Obesity and the microbiome. Expert Rev Gastroenterol Hepatol. 2015 Aug 3;9(8):1087–99.

214. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. Adv Immunol. 2014;121:91–119.

215. Canfora EE, van der Beek CM, Jocken JWE, Goossens GH, Holst JJ, Olde Damink SWM, et al. Colonic infusions of short-chain fatty acid mixtures promote energy metabolism in overweight/obese men: a randomized crossover trial. Sci Rep [Internet]. 2017 May 24 [cited 2020 Feb 21];7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5443817/

216. Blaut M. Gut microbiota and energy balance: role in obesity. Proc Nutr Soc. 2015 Aug;74(3):227–34.

217. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res. 2013 Sep;54(9):2325–40.

218. Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc Natl Acad Sci U S A. 2002 Nov 26;99(24):15451–5.

219. Lee SH. Intestinal Permeability Regulation by Tight Junction: Implication on Inflammatory Bowel Diseases. Intest Res. 2015 Jan;13(1):11–8.

220. Vancamelbeke M, Vermeire S. The intestinal barrier: a fundamental role in health and disease. Expert Rev Gastroenterol Hepatol. 2017 Sep;11(9):821–34.

221. Guo S, Al-Sadi R, Said HM, Ma TY. Lipopolysaccharide Causes an Increase in Intestinal Tight Junction Permeability in Vitro and in Vivo by Inducing Enterocyte Membrane Expression and Localization of TLR-4 and CD14. Am J Pathol. 2013 Feb;182(2):375–87.

222. Sambolin-Escobales1 L, Nazario M, Torres-Aguiar R, Cruz ML, Yamamura Y, Appleyard CB, et al. A High Fat Diet Induces Decreased Levels of Colonic Tight Junctions and Inflammatory Cytokine Expression in Sprague Dawley Rats. FASEB J. 2016 Apr 1;30(1_supplement):lb698–lb698.

223. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. Diabetes. 2007 Jul 1;56(7):1761–72.

224. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes. 2008 Jun;57(6):1470–81.

225. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest. 2006 Nov;116(11):3015–25.

226. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science. 2010 Apr 9;328(5975):228–31.

227. Szabo G, Velayudham A, Romics L, Mandrekar P. Modulation of non-alcoholic steatohepatitis by pattern recognition receptors in mice: the role of toll-like receptors 2 and 4. Alcohol Clin Exp Res. 2005 Nov;29(11 Suppl):140S-145S.

228. Miura K, Kodama Y, Inokuchi S, Schnabl B, Aoyama T, Ohnishi H, et al. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. Gastroenterology. 2010 Jul;139(1):323-334.e7.

229. Everard A, Geurts L, Caesar R, Van Hul M, Matamoros S, Duparc T, et al. Intestinal epithelial MyD88 is a sensor switching host metabolism towards obesity according to nutritional status. Nat Commun. 2014 Dec 5;5:5648.

230. Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A. 2004 Nov 2;101(44):15718–23.

231. Ortega-Prieto P, Postic C. Carbohydrate Sensing Through the Transcription Factor ChREBP. Front Genet. 2019;10:472.

232. Arslan N. Obesity, fatty liver disease and intestinal microbiota. World J Gastroenterol WJG. 2014 Nov 28;20(44):16452–63.

233. JASIRWAN COM, LESMANA CRA, HASAN I, SULAIMAN AS, GANI RA. The role of gut microbiota in non-alcoholic fatty liver disease: pathways of mechanisms. Biosci Microbiota Food Health. 2019;38(3):81–8.

234. Pierantonelli I, Svegliati-Baroni G. Nonalcoholic Fatty Liver Disease: Basic Pathogenetic Mechanisms in the Progression From NAFLD to NASH. Transplantation. 2019;103(1):e1–13.

235. Boden G. Obesity and free fatty acids. Endocrinol Metab Clin North Am. 2008 Sep;37(3):635–46, viii–ix.

236. Kirpich IA, Marsano LS, McClain CJ. Gut-Liver Axis, Nutrition, and Non Alcoholic Fatty Liver Disease. Clin Biochem. 2015 Sep;48(0):923–30.

237. Matsuzawa Y, Funahashi T, Kihara S, Shimomura I. Adiponectin and metabolic syndrome. Arterioscler Thromb Vasc Biol. 2004 Jan;24(1):29–33.

238. Massafra V, Pellicciari R, Gioiello A, van Mil SWC. Progress and challenges of selective Farnesoid X Receptor modulation. Pharmacol Ther. 2018 Nov 1;191:162–77.

239. Han CY. Update on FXR Biology: Promising Therapeutic Target? Int J Mol Sci [Internet]. 2018 Jul 16 [cited 2020 Feb 24];19(7). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6073382/

240. Zhang L, Xie C, Nichols RG, Chan SHJ, Jiang C, Hao R, et al. Farnesoid X Receptor Signaling Shapes the Gut Microbiota and Controls Hepatic Lipid Metabolism. mSystems [Internet]. 2016 Oct 25 [cited 2020 Feb 24];1(5). Available from: https://msystems.asm.org/content/1/5/e00070-16

241. Zhu L, Baker SS, Gill C, Liu W, Alkhouri R, Baker RD, et al. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. Hepatol Baltim Md. 2013 Feb;57(2):601–9.

242. Campo L, Eiseler S, Apfel T, Pyrsopoulos N. Fatty Liver Disease and Gut Microbiota: A Comprehensive Update. J Clin Transl Hepatol. 2019 Mar 28;7(1):56–60.

243. Hersoug L-G, Møller P, Loft S. Role of microbiota-derived lipopolysaccharide in adipose tissue inflammation, adipocyte size and pyroptosis during obesity. Nutr Res Rev. 2018;31(2):153–63.

244. Clemente-Postigo M, Oliva-Olivera W, Coin-Aragüez L, Ramos-Molina B, Giraldez-Perez RM, Lhamyani S, et al. Metabolic endotoxemia promotes adipose dysfunction and inflammation in human obesity. Am J Physiol-Endocrinol Metab. 2018 Nov 13;316(2):E319–32.

245. Zhao M, Chen X. Effect of lipopolysaccharides on adipogenic potential and premature senescence of adipocyte progenitors. Am J Physiol - Endocrinol Metab. 2015 Aug 15;309(4):E334–44.

246. Muccioli GG, Naslain D, Bäckhed F, Reigstad CS, Lambert DM, Delzenne NM, et al. The endocannabinoid system links gut microbiota to adipogenesis. Mol Syst Biol. 2010 Jul;6:392.

247. Mehrpouya-Bahrami P, Chitrala KN, Ganewatta MS, Tang C, Murphy EA, Enos RT, et al. Blockade of CB1 cannabinoid receptor alters gut microbiota and attenuates inflammation and diet-induced obesity. Sci Rep. 2017 Nov 15;7(1):1–16.

248. Lavelle G, Noorkoiv M, Theis N, Korff T, Kilbride C, Baltzopoulos V, et al. Validity of the International Physical Activity Questionnaire Short Form (IPAQ-SF) as a measure of physical activity (PA) in young people with cerebral palsy. Physiotherapy [Internet]. 2019 Aug 22 [cited 2019 Nov 3]; Available from: http://www.sciencedirect.com/science/article/pii/S0031940618303249

249. LARN, 2014. [cited 2019 Oct 29]. Available from: https://sinu.it/tabelle-larn-2014/

250. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12(6):R60.

251. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol. 2012 Apr 16;13(9):R79.

252. Parks DH, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles. Bioinforma Oxf Engl. 2014 Nov 1;30(21):3123–4.

253. Finucane MM, Sharpton TJ, Laurent TJ, Pollard KS. A Taxonomic Signature of Obesity in the Microbiome? Getting to the Guts of the Matter. PLOS ONE. 2014 Jan 8;9(1):e84689.

254. Sze MA, Schloss PD. Looking for a Signal in the Noise: Revisiting Obesity and the Microbiome. mBio. 2016 Sep 7;7(4):e01018-16.

255. Walters WA, Xu Z, Knight R. Meta-analyses of human gut microbes associated with obesity and IBD. FEBS Lett. 2017;4223–33.

256. Armour CR, Nayfach S, Pollard KS, Sharpton TJ. A Metagenomic Meta-analysis Reveals Functional Signatures of Health and Disease in the Human Gut Microbiome. mSystems. 2019 Aug 27;4(4):e00332-18.

257. Peters B, Shapiro J, Church T, Miller G, Trinh-Shevrin C, Yuen E, et al. A taxonomic signature of obesity in a large study of American adults. Sci Rep. 2018 Dec 1;8.

258. Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, et al. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci U S A. 2009 Feb 17;106(7):2365–70.

259. Meehan CJ, Beiko RG. A Phylogenomic View of Ecological Specialization in the Lachnospiraceae, a Family of Digestive Tract-Associated Bacteria. Genome Biol Evol. 2014 Mar 12;6(3):703–13.

260. Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M, de los Reyes-Gavilán CG, Salazar N. Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. Front Microbiol [Internet]. 2016 Feb 17 [cited 2019 Nov 6];7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4756104/

261. Bhute S, Pande P, Shetty SA, Shelar R, Mane S, Kumbhare SV, et al. Molecular Characterization and Meta-Analysis of Gut Microbial Communities Illustrate Enrichment of Prevotella and Megasphaera in Indian Subjects. Front Microbiol [Internet]. 2016 [cited 2020 Apr 14];7. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2016.00660/full

262. Mashima I, Liao Y-C, Miyakawa H, Theodorea CF, Thawboon B, Thaweboon S, et al. Veillonella infantium sp. nov., an anaerobic, Gram-stain-negative coccus isolated from tongue biofilm of a Thai child. Int J Syst Evol Microbiol. 2018 Apr 1;68(4):1101–6.

263. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell. 2014 Nov 6;159(4):789–99.

264. Konikoff T, Gophna U. Oscillospira: a Central, Enigmatic Component of the Human Gut Microbiota. Trends Microbiol. 2016;24(7):523–4.

265. Tripathi CKM, Banga J, Mishra V. Microbial heparin/heparan sulphate lyases: potential and applications. Appl Microbiol Biotechnol. 2012 Apr;94(2):307–21.

266. Candela M, Biagi E, Soverini M, Consolandi C, Quercia S, Severgnini M, et al. Modulation of gut microbiota dysbioses in type 2 diabetic patients by macrobiotic Ma-Pi 2 diet. Br J Nutr. 2016 Jul 14;116(1):80–93.

267. Zhou L, Zhang M, Wang Y, Dorfman RG, Liu H, Yu T, et al. Faecalibacterium prausnitzii Produces Butyrate to Maintain Th17/Treg Balance and to Ameliorate Colorectal Colitis by Inhibiting Histone Deacetylase 1. Inflamm Bowel Dis. 2018 16;24(9):1926–40.

268. Del Chierico F, Abbatini F, Russo A, Quagliariello A, Reddel S, Capoccia D, et al. Gut Microbiota Markers in Obese Adolescent and Adult Patients: Age-Dependent Differential Patterns. Front Microbiol. 2018;9:1210.

269. Wu Y, Chi X, Zhang Q, Chen F, Deng X. Characterization of the salivary microbiome in people with obesity. PeerJ [Internet]. 2018 Mar 16 [cited 2020 Feb 28];6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5858547/

270. Clarke SF, Murphy EF, O'Sullivan O, Ross RP, O'Toole PW, Shanahan F, et al. Targeting the Microbiota to Address Diet-Induced Obesity: A Time Dependent Challenge. PLoS ONE [Internet]. 2013 Jun 7 [cited 2020 Feb 28];8(6). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3676335/

271. Kang SS, Jeraldo PR, Kurti A, Miller MEB, Cook MD, Whitlock K, et al. Diet and exercise orthogonally alter the gut microbiome and reveal independent associations with anxiety and cognition. Mol Neurodegener. 2014 Sep 13;9:36.

272. Lv Y, Qin X, Jia H, Chen S, Sun W, Wang X. The Association between Gut Microbiota Composition and Body Mass Index in Chinese Male College Students, as Analyzed by Next-generation Sequencing. Br J Nutr. 2019 Aug 9;1–17.

273. Wang K, Liao M, Zhou N, Bao L, Ma K, Zheng Z, et al. Parabacteroides distasonis Alleviates Obesity and Metabolic Dysfunctions via Production of Succinate and Secondary Bile Acids. Cell Rep. 2019 02;26(1):222-235.e5.

274. Campisciano G, Palmisano S, Cason C, Giuricin M, Silvestri M, Guerra M, et al. Gut microbiota characterisation in obese patients before and after bariatric surgery. Benef Microbes. 2018 Apr 25;9(3):367–73.

275. Verdam FJ, Fuentes S, de Jonge C, Zoetendal EG, Erbil R, Greve JW, et al. Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. Obes Silver Spring Md. 2013 Dec;21(12):E607-615.

276. Liu R, Hong J, Xu X, Feng Q, Zhang D, Gu Y, et al. Gut microbiome and serum metabolome alterations in obesity and after weight-loss intervention. Nat Med. 2017 Jul;23(7):859–68.

277. Gauffin Cano P, Santacruz A, Moya Á, Sanz Y. Bacteroides uniformis CECT 7771 Ameliorates Metabolic and Immunological Dysfunction in Mice with High-Fat-Diet Induced Obesity. PLoS ONE [Internet]. 2012 Jul 26 [cited 2019 Nov 8];7(7). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3406031/

278. Boulangé CL, Neves AL, Chilloux J, Nicholson JK, Dumas M-E. Impact of the gut microbiota on inflammation, obesity, and metabolic disease. Genome Med. 2016 Apr 20;8(1):42.

279. Chakraborti CK. New-found link between microbiota and obesity. World J Gastrointest Pathophysiol. 2015 Nov 15;6(4):110–9.

280. Semova I, Carten JD, Stombaugh J, Mackey LC, Knight R, Farber SA, et al. Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host Microbe. 2012 Sep 13;12(3):277–88.

281. Yasir M, Angelakis E, Bibi F, Azhar EI, Bachar D, Lagier J-C, et al. Comparison of the gut microbiota of people in France and Saudi Arabia. Nutr Diabetes. 2015 Apr;5(4):e153–e153.

282. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. Diabetes. 2007 Jul 1;56(7):1761–72.

283. Million M, Maraninchi M, Henry M, Armougom F, Richet H, Carrieri P, et al. Obesity-associated gut microbiota is enriched in Lactobacillus reuteri and depleted in Bifidobacterium animalis and Methanobrevibacter smithii. Int J Obes 2005. 2012 Jun;36(6):817–25.

284. An HM, Park SY, Lee DK, Kim JR, Cha MK, Lee SW, et al. Antiobesity and lipid-lowering effects of Bifidobacterium spp. in high fat diet-induced obese rats. Lipids Health Dis. 2011 Jul 12;10:116.

285. Brusaferro A, Cozzali R, Orabona C, Biscarini A, Farinelli E, Cavalli E, et al. Is It Time to Use Probiotics to Prevent or Treat Obesity? Nutrients [Internet]. 2018 Nov 1 [cited 2020 Feb 28];10(11). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6266556/

286. Brahe LK, Le Chatelier E, Prifti E, Pons N, Kennedy S, Hansen T, et al. Specific gut microbiota features and metabolic markers in postmenopausal women with obesity. Nutr Diabetes. 2015 Jun 15;5:e159.

287. Yassour M, Lim MY, Yun HS, Tickle TL, Sung J, Song Y-M, et al. Sub-clinical detection of gut microbial biomarkers of obesity and type 2 diabetes. Genome Med. 2016 Feb 17;8(1):17.

288. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et al. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A. 2013 May 28;110(22):9066–71.

289. Depommier C, Everard A, Druart C, Plovier H, Hul MV, Vieira-Silva S, et al. Supplementation with Akkermansia muciniphila in overweight and obese human volunteers: a proof-of-concept exploratory study. Nat Med. 2019 Jul;25(7):1096–103.

290. Zhang X, Shen D, Fang Z, Jie Z, Qiu X, Zhang C, et al. Human gut microbiota changes reveal the progression of glucose intolerance. PloS One. 2013;8(8):e71108.

291. Reichardt N, Duncan SH, Young P, Belenguer A, McWilliam Leitch C, Scott KP, et al. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. ISME J. 2014 Jun;8(6):1323–35.

292. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J. 2011 Feb;5(2):220–30.

293. Russell WR, Gratz SW, Duncan SH, Holtrop G, Ince J, Scobbie L, et al. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. Am J Clin Nutr. 2011 May;93(5):1062–72.

294. Pataky Z, Genton L, Spahr L, Lazarevic V, Terraz S, Gaïa N, et al. Impact of Hypocaloric Hyperproteic Diet on Gut Microbiota in Overweight or Obese Patients with Nonalcoholic Fatty Liver Disease: A Pilot Study. Dig Dis Sci. 2016;61(9):2721–31.

295. Vacca M, Celano G, Calabrese FM, Portincasa P, Gobbetti M, De Angelis M. The Controversial Role of Human Gut Lachnospiraceae. Microorganisms. 2020 Apr 15;8(4).

296. Tamanai-Shacoori Z, Smida I, Bousarghin L, Loreal O, Meuric V, Fong SB, et al. Roseburia spp.: a marker of health? Future Microbiol. 2017;12:157–70.

297. Kong L-C, Tap J, Aron-Wisnewsky J, Pelloux V, Basdevant A, Bouillot J-L, et al. Gut microbiota after gastric bypass in human obesity: increased richness and associations of bacterial genera with adipose tissue genes. Am J Clin Nutr. 2013 Jul;98(1):16–24.

298. Wolfe BM, Kvach E, Eckel RH. Treatment of Obesity: Weight Loss and Bariatric Surgery. Circ Res. 2016 May 27;118(11):1844–55.

299. de la Cuesta-Zuluaga J, Mueller NT, Álvarez-Quintero R, Velásquez-Mejía EP, Sierra JA, Corrales-Agudelo V, et al. Higher Fecal Short-Chain Fatty Acid Levels Are Associated with Gut Microbiome Dysbiosis, Obesity, Hypertension and Cardiometabolic Disease Risk Factors. Nutrients. 2018 Dec 27;11(1).

300. Kim KN, Yao Y, Ju SY. Short Chain Fatty Acids and Fecal Microbiota Abundance in Humans with Obesity: A Systematic Review and Meta-Analysis. Nutrients. 2019 Oct 18;11(10).

301. Cancello R, Turroni S, Rampelli S, Cattaldo S, Candela M, Cattani L, et al. Effect of Short-Term Dietary Intervention and Probiotic Mix Supplementation on the Gut Microbiota of Elderly Obese Women. Nutrients [Internet]. 2019 Dec 10 [cited 2020 Jun 22];11(12). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6950529/

302. Haro C, Montes-Borrego M, Rangel-Zúñiga OA, Alcalá-Díaz JF, Gómez-Delgado F, Pérez-Martínez P, et al. Two Healthy Diets Modulate Gut Microbial Community Improving Insulin Sensitivity in a Human Obese Population. J Clin Endocrinol Metab. 2016 Jan;101(1):233–42.

303. Russell WR, Gratz SW, Duncan SH, Holtrop G, Ince J, Scobbie L, et al. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. Am J Clin Nutr. 2011 May;93(5):1062–72.

304. Seganfredo FB, Blume CA, Moehlecke M, Giongo A, Casagrande DS, Spolidoro JVN, et al. Weight-loss interventions and gut microbiota changes in overweight and obese patients: a systematic review. Obes Rev Off J Int Assoc Study Obes. 2017 Aug;18(8):832–51.

305. Louis S, Tappu R-M, Damms-Machado A, Huson DH, Bischoff SC. Characterization of the Gut Microbial Community of Obese Patients Following a Weight-Loss Intervention Using Whole Metagenome Shotgun Sequencing. PloS One. 2016;11(2):e0149564.

306. Frost F, Storck LJ, Kacprowski T, Gärtner S, Rühlemann M, Bang C, et al. A structured weight loss program increases gut microbiota phylogenetic diversity and reduces levels of Collinsella in obese type 2 diabetics: A pilot study. PLoS ONE [Internet]. 2019 Jul 18 [cited 2020 Jan 14];14(7). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6638920/

307. Grembi JA, Nguyen LH, Haggerty TD, Gardner CD, Holmes SP, Parsonnet J. Gut microbiota plasticity is correlated with sustained weight loss on a low-carb or low-fat dietary intervention. Sci Rep. 2020 Jan 29;10(1):1405.

308. Pories WJ. Bariatric Surgery: Risks and Rewards. J Clin Endocrinol Metab. 2008 Nov;93(11 Suppl 1):S89–96.

309. Kuno T, Tanimoto E, Morita S, Shimada YJ. Effects of Bariatric Surgery on Cardiovascular Disease: A Concise Update of Recent Advances. Front Cardiovasc Med. 2019;6:94.

310. ULKER İ, YILDIRAN H. The effects of bariatric surgery on gut microbiota in patients with obesity: a review of the literature. Biosci Microbiota Food Health. 2019;38(1):3–9.

311. Anhê FF, Varin TV, Schertzer JD, Marette A. The Gut Microbiota as a Mediator of Metabolic Benefits after Bariatric Surgery. Can J Diabetes. 2017 Aug 1;41(4):439–47.

312. Graessler J, Qin Y, Zhong H, Zhang J, Licinio J, Wong M-L, et al. Metagenomic sequencing of the human gut microbiome before and after bariatric surgery in obese patients with type 2 diabetes: correlation with inflammatory and metabolic parameters. Pharmacogenomics J. 2013 Dec;13(6):514–22.

313. Tremaroli V, Karlsson F, Werling M, Ståhlman M, Kovatcheva-Datchary P, Olbers T, et al. Roux-en-Y Gastric Bypass and Vertical Banded Gastroplasty Induce Long-Term Changes on the Human Gut Microbiome Contributing to Fat Mass Regulation. Cell Metab. 2015 Aug 4;22(2):228–38.

314. Palleja A, Kashani A, Allin KH, Nielsen T, Zhang C, Li Y, et al. Roux-en-Y gastric bypass surgery of morbidly obese patients induces swift and persistent changes of the individual gut microbiota. Genome Med. 2016 15;8(1):67.

315. Chen J, Xie C, Zhao Y, Li Z, Xu P, Yao L. Gene expression analysis reveals the dysregulation of immune and metabolic pathways in Alzheimer's disease. Oncotarget. 2016 Oct 6;7(45):72469–74.

316. Wang J, Wang Y, Zhang X, Liu J, Zhang Q, Zhao Y, et al. Gut Microbial Dysbiosis Is Associated with Altered Hepatic Functions and Serum Metabolites in Chronic Hepatitis B Patients. Front Microbiol [Internet]. 2017 [cited 2020 Mar 3];8. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02222/full

317. Jin D, Wu S, Zhang Y-G, Lu R, Xia Y, Dong H, et al. Lack of Vitamin D Receptor Causes Dysbiosis and Changes the Functions of the Murine Intestinal Microbiome. Clin Ther. 2015 May 1;37(5):996-1009.e7.

318. Tang W, Yao X, Xia F, Yang M, Chen Z, Zhou B, et al. Modulation of the Gut Microbiota in Rats by Hugan Qingzhi Tablets during the Treatment of High-Fat-Diet-Induced Nonalcoholic Fatty Liver Disease. Oxid Med Cell Longev. 2018;2018:7261619.

319. Hou Y-P, He Q-Q, Ouyang H-M, Peng H-S, Wang Q, Li J, et al. Human Gut Microbiota Associated with Obesity in Chinese Children and Adolescents [Internet]. BioMed Research International. 2017 [cited 2019 Nov 7]. Available from: https://www.hindawi.com/journals/bmri/2017/7585989/

320. Gottesman MM, Ambudkar SV. Overview: ABC transporters and human disease. J Bioenerg Biomembr. 2001 Dec;33(6):453–8.

321. Wolf A, Bauer B, Hartz AMS. ABC Transporters and the Alzheimer's Disease Enigma. Front Psychiatry [Internet]. 2012 Jun 4 [cited 2020 Mar 3];3. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3366330/

322. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature. 2009 Jan;457(7228):480–4.

323. Kashani A, Brejnrod AD, Jin C, Kern T, Madsen AN, Holm LA, et al. Impaired glucose metabolism and altered gut microbiome despite calorie restriction of ob/ob mice. Anim Microbiome. 2019 Sep 5;1(1):11.

324. Deutscher J, Francke C, Postma PW. How Phosphotransferase System-Related Protein Phosphorylation Regulates Carbohydrate Metabolism in Bacteria. Microbiol Mol Biol Rev. 2006 Dec;70(4):939–1031.

325. Turnbaugh PJ, Backhed F, Fulton L, Gordon JI. Marked alterations in the distal gut microbiome linked to diet-induced obesity. Cell Host Microbe. 2008 Apr 17;3(4):213–23.

326. Mahowald MA, Rey FE, Seedorf H, Turnbaugh PJ, Fulton RS, Wollam A, et al. Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla. Proc Natl Acad Sci U S A. 2009 Apr 7;106(14):5859–64.

327. Hooper LV, Gordon JI. Glycans as legislators of host–microbial interactions: spanning the spectrum from symbiosis to pathogenicity. Glycobiology. 2001 Jan 1;11(2):1R-10R.

328. Yun Y, Kim H-N, Kim SE, Heo SG, Chang Y, Ryu S, et al. Comparative analysis of gut microbiota associated with body mass index in a large Korean cohort. BMC Microbiol [Internet]. 2017 Jul 4 [cited 2019 Nov 6];17. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5497371/

329. Chávez-Carbajal A, Nirmalkar K, Pérez-Lizaur A, Hernández-Quiroz F, Ramírez-del-Alto S, García-Mena J, et al. Gut Microbiota and Predicted Metabolic Pathways in a Sample of Mexican Women Affected by Obesity and Obesity Plus Metabolic Syndrome. Int J Mol Sci [Internet]. 2019 Jan 21 [cited 2019 Nov 6];20(2). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6358992/

330. Gao R, Zhu C, Li H, Yin M, Pan C, Huang L, et al. Dysbiosis Signatures of Gut Microbiota Along the Sequence from Healthy, Young Patients to Those with Overweight and Obesity. Obesity. 2018;26(2):351–61.

.