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Effect of Diet Therapy on Gut Microbiome in Rare Genetic Diseases

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Abstract

In the last decades, several studies have explored the human microbiota in different body niches, notably in the gastrointestinal tract. The gut microbiota has been recognized as an additional organ that co-evolves with humans, and interacts with host physiology. The gut microbiota plays a key role in the host metabolism and the extraction of energy from food.

Diet is the strongest factor shaping the gut microbiota: differences in macronutrient intake may select the growth of specific microbial taxa. Indeed, the diet provides different substrates such as undigested carbohydrates for fermentation, affecting the composition of the gut microbiota and, consequently, the production of microbial metabolites. Short chain fatty acids (SCFAs), mainly acetate, propionate and butyrate, are the major end products of the anaerobic fermentation by gut microbes.

Modifications in the proportions of the gut microbes and, consequently, the production of SCFAs have been suggested to play a role in several pathological conditions. If the diet is modifiable to stimulate the growth of beneficial microbes, it could represent a target for prevention and treatment of a possible dysbiosis.

In congenital metabolic disorders such as phenylketonuria (PKU) and glycogen storage disease (GSD), diet is considered the mainstay for the treatment of the pathology. PKU diet is based on the restriction of phenylalanine intake and the supplementation of essential amino acids and micronutrients. PKU diet is similar to a vegan alimentation, since all the animal products are excluded. GSD dietary management provides the intake of slow-release carbohydrates to prevent hypoglycaemia and limits the assumption of simple sugars.

In this research, we aimed to analyze the impact of the dietary therapy on the composition of the gut microbiota and the production of SCFAs in two congenital metabolic disorders (PKU and GSD).

PKU and GSD diseases showed an alteration of the gut microbiome. If diet represent the only treatment of the disease and is not modifiable, a personalized intervention to restore a healthy gut microbiome is to consider. Nowadays, the attention is focused on the supplementation of probiotics and prebiotics.

Riassunto

Negli ultimi venti anni, numerosi studi si sono occupati di analizzare il microbiota umano in diverse nicchie corporee, specialmente nel tratto gastrointestinale. Il microbiota intestinale è considerato un organo aggiuntivo che evolve insieme all'ospite ed influenza la fisiologia dell'ospite stesso. Inoltre, ricopre un ruolo fondamentale nel metabolismo dell'ospite e nella produzione di energia, ricavandola direttamente dal cibo introdotto.

La dieta rappresenta il fattore che più influenza il microbiota intestinale: differenze nell'assunzione di macronutrienti potrebbero selezionare la crescita di specifici taxa microbici. Infatti, la dieta fornisce substrati differenti, come carboidrati indigeriti, per la fermentazione microbica, influenzando la composizione dei microrganismi intestinali e conseguentemente la produzione dei metaboliti microbici. Gli acidi grassi a corta catena (SCFAs), principalmente acetato, propionato e butirrato, sono i maggiori metaboliti derivanti dalla fermentazione microbica intestinale.

Alterazioni nella composizione del microbiota intestinale e, conseguentemente, nella produzione di SCFAs, sembrano influenzare lo sviluppo di diverse condizioni patologiche. Se la dieta fosse modificabile in modo da poter stimolare la crescita di microrganismi benefici, tale approccio potrebbe prevenire e/o trattare una possibile disbiosi.

In malattie metaboliche ereditarie, come la fenilchetonuria (PKU) e la malattia di stoccaggio del glicogeno (GSD), la dieta rappresenta il trattamento della patologia. La dietoterapia per la PKU è basata sulla restrizione dell'apporto di fenilalanina e sulla supplementazione di amino acidi essenziali e micronutrienti. Questo trattamento dietetico è molto simile ad una dieta

vegana, perché tutti gli alimenti di origine animale sono esclusi. La terapia dietetica per i pazienti affetti da GSD prevede l'utilizzo di carboidrati a lento rilascio per prevenire l'ipoglicemia e limita l'assunzione di zuccheri semplici.
In questo progetto, l'obiettivo è stato di valutare come la dietoterapia potesse influenzare la composizione del microbiota intestinale e la produzione di SCFAs in soggetti PKU e GSD.
I nostri studi hanno dimostrato un'alterazione del microbioma intestinale nelle due patologie analizzate. In considerazione del fatto che la dieta rappresenta l'unico trattamento per entrambe le patologie e, di conseguenza, non può essere modificata, un intervento personalizzato con probiotici e prebiotici viene ipotizzato come possibile alternativa nel ristabilire un corretto equilibrio nella composizione del microbiota intestinale.

CHAPTER 1

Introduction

1.1 The microbiota: a "new entry" in the science

Over the last decades, the microbiota has received increasing importance. Despite its marginal role as "intestinal bacterial flora", discoveries on microbiota functions have underlined its potential in health and disease [1].
Since the National Institutes of Health launched the Human Microbiome Project (HMP) in 2007 [2], a plethora of studies focusing on the microbiota was published. The advancement of omics sciences has certainly accelerated the publication of human microbiome studies and the accuracy of novel findings [3].
The term microbiota was first coined by Lederberg [4] in 2001 and identifies a symbiotic, commensal and even pathogenic community of microorganisms harboured by our body, whereas the term microbiome represents the genetic material within the microbiota. Recently, Prescott underlined that the history of the microbiome did not begin in 2001 but in 1988, when it was defined by Whipps as "characteristic microbial community occupying a reasonably well-defined habitat which has distinct physiochemical properties" [5].

1.2 The gut microbiota

Humans harbour widely active microbial communities in different body niches, including the nasal-pharyngeal cavity as well as the skin, the vagina and the gastrointestinal tract. It is estimated that the human microbiota includes 10^{14} bacterial cells, exceeding 10 times the number of human cells. The microorganisms comprise bacteria, fungi, viruses, and archaea, and they have evolved with the human being, affecting health and disease [6].

Currently, the majority of research is focused on the gastrointestinal microbiota since it represents the niche with the greatest density and numbers of microorganisms. Indeed, the gut microbiota can be considered a "microbial organ" in the human body, constituting the well-recognized "superorganism". Although a significant part of the gut microorganisms has not yet been cultured, they are composed by the three domains of life: Eukarya, Archaea and Bacteria, the most represented (Figure 1, [7]).

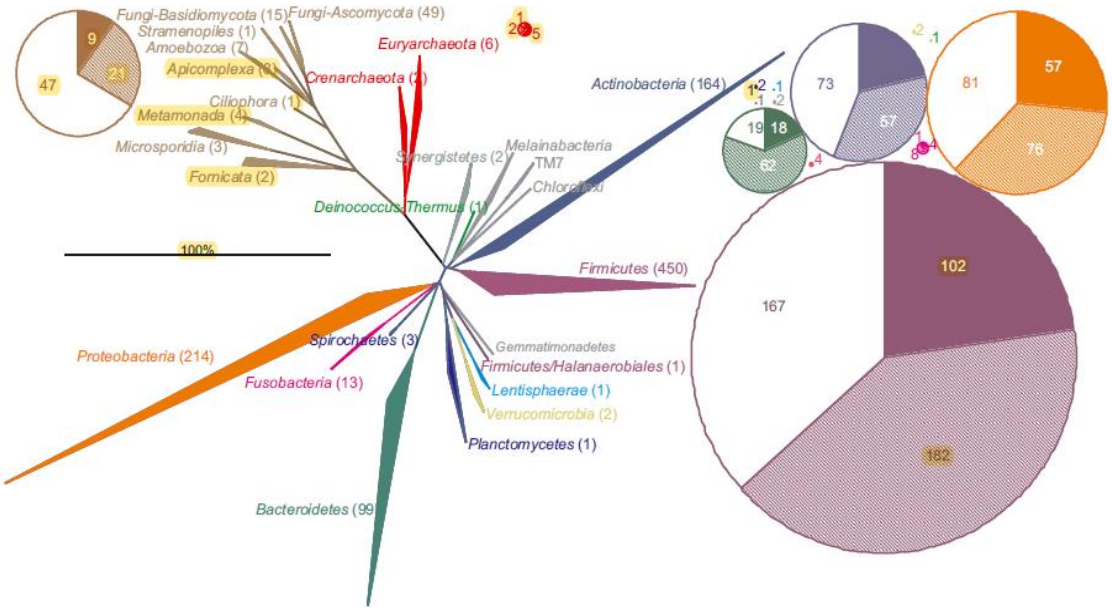

Figure 1. Phylogenetic tree of the human gut microbiota. The numbers in parentheses define the number of cultured species per phylum. The pie charts indicate distribution between species with full genome sequence (full sectors), species with partial genome sequence (semi-full sectors) and species without any genome sequence (empty sectors) explained for the three domains (Archea, Eukarya and Bacteria) [7].

The gastrointestinal tract of humans has been evaluated to harbour approximately 500-1000 different bacterial species. In addition, the dominant Archea is represented by *Methanobrevibacter smithii* [8].

Most human microorganisms in the gastrointestinal tract are strictly anaerobic and, although the gut microbiota is individually specific, it is composed mainly of two predominant phyla *Firmicutes* and *Bacteroidetes*. Minor proportions are represented by *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* [9].

In a healthy human gut, the *Firmicutes* are Gram-positive bacteria and are mainly represented by two major classes: *Bacilli* and *Clostridia*. Instead, the *Bacteroidetes* phylum is a group of Gram-negative bacteria, of which *Bacteroides* is the most representative genus [10]. *Proteobacteria* include the *Enterobacteriaceae* family, considered as pro-inflammatory bacteria, whereas the *Actinobacteria* phylum is strongly represented by *Bifidobacterium* species.

The concentration of microorganisms increases along the gastrointestinal tract, with small amounts in the stomach and very high numbers in the colon, underling that the gastrointestinal microbiota is not homogeneous [11]. The stomach is considered inhospitable for its acid condition and since very few bacteria are resistant to this environment, it harbours only 10^1 bacteria per gram content. The density and the bacterial diversity increases along the intestine, starting from a concentration of 10^3 bacteria/g and 10^4 bacteria/g in the duodenum and in the jejunum, respectively. Highest amounts are achieved in the ileum (10^7 bacteria/g) and in the colon (10^{12} bacteria/g) [9].

The microbial composition differs within the spatial sites of the gastrointestinal tract (figure 2, [12]). A study in healthy subjects reported that a biopsy of the small intestine revealed an enrichment in *Actinobacteria* and *Bacilli* class, whereas colonic samples were enriched in *Bacteroidetes* and in *Lachnospiraceae* family [9].

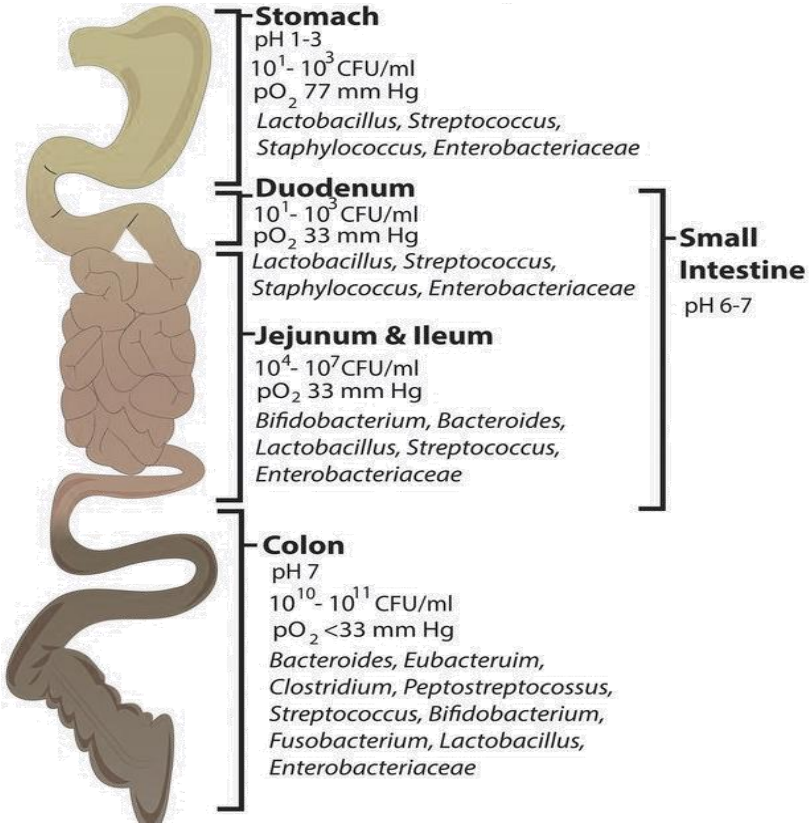

Figure 2. Microbiota composition in the different areas along the gastrointestinal tract [12].

Beyond the longitudinal heterogeneity of gut microbiota, a latitudinal variation is highlighted. The intestinal epithelium is covered by a mucus layer, considered as a physical barrier to prevent translocation of commensal and pathogen microorganisms. Only few microbes, such as *Clostridium*, *Lactobacillus*, or *Enterococcus*, are able to adhere to mucosal surfaces and utilize the mucus as nutritional source.

Since mucosa-associated microbes are in closer proximity to the host intestinal epithelium, they may more influence the immune system than luminal/fecal microbes. The latter may have a role in metabolic interactions and energy production [11]. However, most data of the gut microbiota derived from fecal samples and, to a lesser extent, mucosal biopsies. Although stool samples can

be easy to obtain, the information they give does not represent the complete image within the gut.

Gut microbiota composition is influenced by several factors, both temporal and spatial. Indeed, gut microbiome study could not be analzyed without its environmental context. Host genetics, nutrition, geographical location, and environment strongly affect the gut microbial composition. During life, the gut microbiota evolves and depends on dietary habits, life-style, and use of drugs, especially antibiotics [9].

1.3 Functions of the gut microbiota

Gut microbiota has been recognized as an additional organ that co-evolves with the human being and plays a role in host health maintenance and disease pathogenesis. Gut microbiota acts by exercising a protective, structural, and metabolic role towards the host (figure 3, [13]).

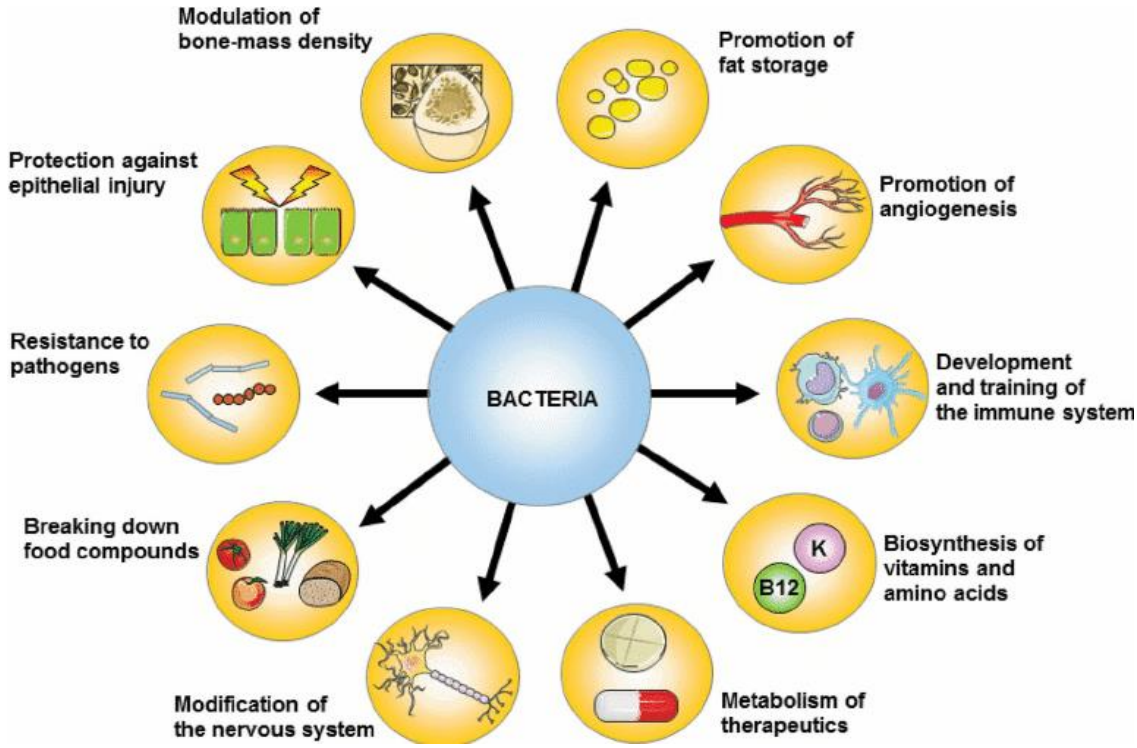

Figure 3. Main functions of bacteria in the gut [13].

1.3.1 Protective and structural functions

The gut microbiota acts as a physical barrier to hinder proliferation and growth of pathogens and is involved in maintaining intestinal integrity, by protecting against epithelial injury and promoting the enterocytes' turnover. Behind this activity, it competes against pathogens for adhesion sites and nutrients, and it produces different metabolites such as polyamines [14]. These molecules are primarily responsible for enhancing the intestinal barrier integrity by stimulating the production of junction proteins (i.e. occludin, zonula occludens 1, and E-cadherin). It has been also demonstrated their role in regulating immune response, and in the modulation of adaptive immunity [15].

1.3.2 Modulation of the immune system

Immune host homeostasis seems to be guaranteed by the dynamic and mutualistic interplay between host and gut microbiota.

The intestinal microbiota produces a huge repertoire of molecules that can trigger the host immune system. For example, gut microbes generate metabolites that bind aryl hydrocarbon receptor (AHR), a transcription factor known to play a role in xenobiotic metabolism and in regulating mucosal immune responses [14].

Moreover, it promotes a correct localization and response of Toll-like receptors (TLRs), key elements when an infection occurs. Intracellular signals of the TLRs induces the production of cytokines able to coordinate the inflammatory and immune response [10].

1.3.3 Metabolic activity of gut microbiota

The gut microbiota produces a huge repertoire of metabolites, able to interact with host cells and affect immune response and risk of disease.

Intestinal microbes synthesize vitamins, such as the K vitamin and constituents of vitamin B, especially folic acids, and produce conjugated linoleic acid (CLA). The latter is known to have several characteristics: it is antidiabetic, antiatherogenic, antiobesogenic, hypolipidemic, and possesses immunomodulatory properties [16].

The gut microbiota is also involved in modifying phenolic compounds from an inactive status to an active one. Indeed, phenolic compounds are generally inactive dietary molecules and it seems that gut microbiota allows their absorption with all positive effects associated [17].

The anaerobic fermentation of dietary components represents the principal metabolic activity of gut microbiota, leading to the production of different metabolites, principally short chain fatty acids (SCFAs). SCFAs are saturated aliphatic organic acids with one to six atoms of carbons. The main SCFAs are acetate (C2), propionate (C3), and butyrate (C4) that represent 95% of total microbial production and are found in an approximate molar ratio of 60:20:20 in the colon and feces [18].

Recently, numerous studies have underlined the importance of SCFAs in host physiology [18]. The molecular mechanisms highlighting the beneficial effects of SCFAs are subjects of numerous studies and are not completely understood. They might be involved in the prevention and treatment of metabolic syndrome, intestinal diseases and different cancers, especially colon rectal cancer [19]. In ulcerative colitis, Crohn's disease, and antibiotic-associated diarrhea (AAD), SCFAs seem to positively influence the treatment [20].

1.4 Microbial production of SCFAs

SCFAs microbial production is affected by different factors: environmental conditions, gut microbiota composition, and diet. The presence of specific bacteria in the gut and different intakes of dietary components lead to a diverse production of SCFAs (figure 4, [21]). For the SCFAs production, it is important that gut microbes work as a community and have symbiotic interactions with the host.

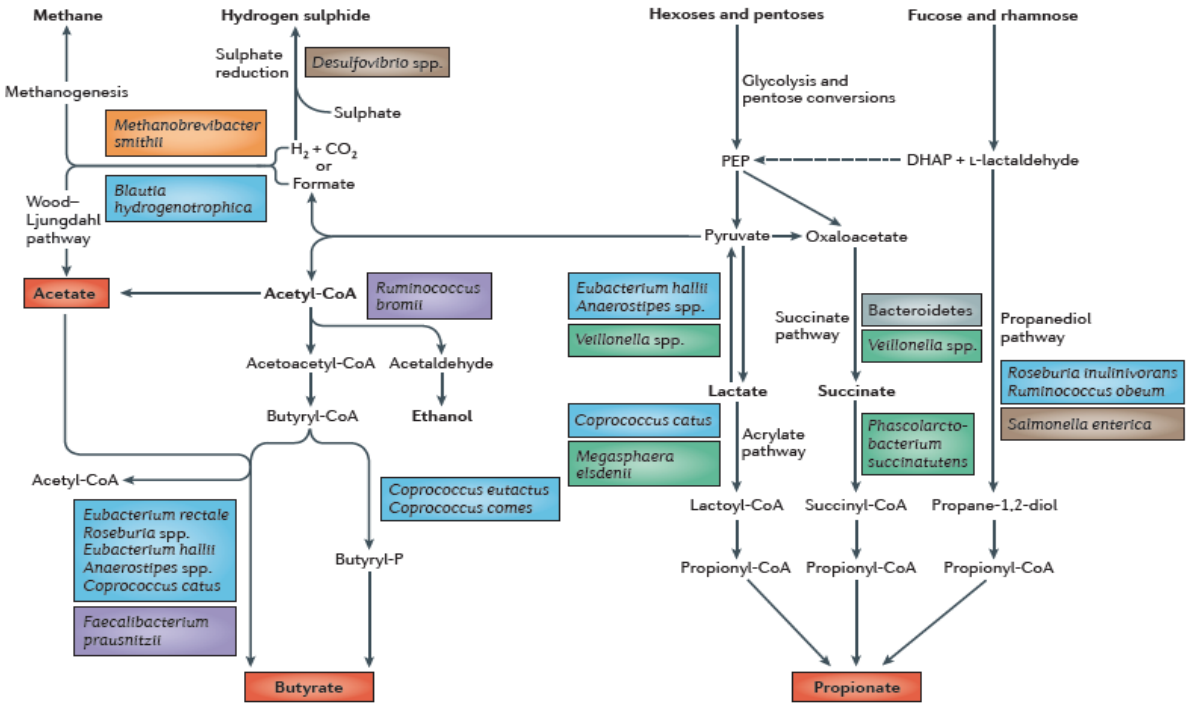

Figure 4. Pathways involved in the biosynthesis of the major metabolites that derive from carbohydrates fermentation and microbial cross-feeding [21].

Taking into account all dietary components, carbohydrates and, to a lesser extent, proteins represent substrates for fermentation in the large intestine. Saccharolytic bacteria ferment undigested carbohydrates such as dietary fibers, and resistant starches. The fermentation of these substrates results in the production of acetate, propionate and butyrate accompanied by the gases [18]. The *Bacteroidetes* phylum has acetate and propionate as its metabolic end products, while *Firmicutes* mainly produce butyrate.

Breakdown of proteins and amino acids leads to the formation of branched-chain fatty acids together with potentially toxic metabolites such as volatile sulfur compounds, phenolic compounds, and amines [18].

The gut microbiota hydrolyzes indigestible carbohydrates in oligosaccharides and monosaccharides through two major metabolic routes: glycolysis and the

pentose-phosphate pathway. The conversion of monosaccharides results in phosphoenolpyruvate (PEP), a microbial substrate essential for SCFAs production.

Most enteric bacteria, primary *Bacteroidetes*, produce acetate as the main end product. Acetate is also formed by acetogenic bacteria such as *Blautia hydrogenotrophica* from the gases H_2 and CO_2 or formate through the Wood–Ljungdahl pathway [21]. Since the production of SCFAs is the result of a mutualistic relation between bacteria, these gases are also utilized by other intestinal microbes. For instance, methanogenic bacteria produce CH_4 from both gases.

Propionate is mostly produced via succinate pathway by *Bacteroidetes* and a class of *Firmicutes*, the *Negativicutes*, such as *Phascolarctobacterium succinatutens*, *Veillonella* spp. and *Dialister* spp. [22]. Succinate is a precursor of propionate, and the conversion of succinate into propionate requires vitamin B_{12}. Some bacteria, notably *Prevotella copri* and *Ruminococcus flavefaciens*, produce succinate rather than propionate as the main end product. On the other hand, the *Negativicutes* convert succinate in propionate, and this cross-feeding may explain the low accumulation of succinate in human feces [21]. Other two pathways are known for propionate formation: the propanediol pathway and the acrylate pathway. The first one utilizes deoxyhexose sugars (fucose and rhamnose) as substrates and is present in some *Firmicutes* and *Proteobacteria*. Instead, the acrylate pathway uses lactate as substrate but is limited among colonic bacteria.

Butyrate is formed by acetyl-CoA conversion using either the butyryl-CoA:acetate CoA-transferase enzyme or, less commonly, phosphotransbutyrylase and butyrate kinase [23]. The ability to produce butyrate is mainly found in two predominant families of *Firmicutes*,

Ruminococcaceae and *Lachnospiraceae*. In particular, *Faecalibacterium prausnitzii*, species of *Ruminococcaceae*, and *Eubacterium rectale/Roseburia* spp., strains of *Lachnospiraceae*, seem to strongly contribute to the production of butyrate. In addition to butyrate, other strains of *Lachnospiraceae* produce, to a lesser extent, formate and lactate [23]. Moreover, *Eubacterium hallii* and *Anaerostipes* spp. can utilize acetate and lactate to produce butyrate and this activity may have an important role in avoiding the accumulation of lactate and guaranteeing the stability of the gut microbial ecosystem [22].

Only a few anaerobes belonging to gut microbiota possess the ability to produce both propionate and butyrate and they form them from different substrates. *Roseburia inulinivorans* forms butyrate from fructose and propionate from fucose, while *Coprococcus catus* forms butyrate from fructose and forms propionate from lactate [24].

1.4.1 Functions of SCFAs in the host

SCFAs has been recognized to exert positive effects on host metabolism, both locally and systemically (figure 5, [25]). They maintain the mucosal immunity by fortifying the intestinal epithelium and play a broad activity on host immune system through G-protein-coupled receptors (GPRs) signaling [14].

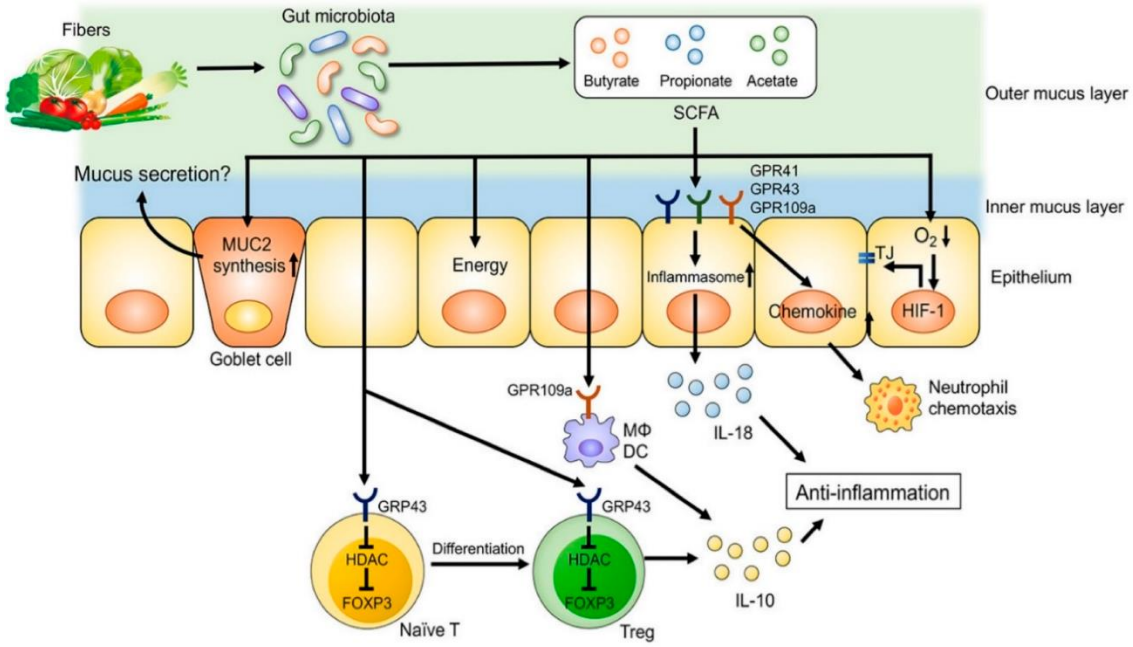

Figure 5. The role of SCFAs, produced by the gut microbiota that ferments indigestible carbohydrates, in intestinal homeostasis [25].

Acetate, propionate and butyrate are quickly absorbed from the intestinal epithelium but their destination and action differ [21]. Acetate is the most present in the peripheral blood, achieving relatively high concentrations. Propionate is mainly metabolized in the liver, whereas butyrate represents the primary source of energy for enterocytes and its presence in the systemic circulation is very low.

Since SCFAs reduce the intestinal pH, they contribute to the absorption of minerals such as sodium and magnesium, vitamins and H_2O at the colon level, where play an anti-inflammatory effect [26]. For example, butyrate inhibits the activation of transcription factor NF-kB and, consequently, the production of pro-inflammatory cytokines.

Numerous studies underlined that acetate and propionate can modulate the glycolipid metabolism by ameliorating the insulin sensitivity and inhibiting the hepatic synthesis of cholesterol, respectively [27]. Acetate serves as a

substrate for the production of long chain fatty acids, and for the synthesis of glutamine and glutamate. Instead, propionate acts as a precursor of gluconeogenesis in the liver [28].

Butyrate and, to a lesser extent, propionate inhibit the activity of histone deacetylases (HDACs) in enterocytes and immune cells. This capability promotes the hyperacetylation of histones, accompanied by some transcription factors and proteins involved in signal transduction [23]. It has been demonstrated that the hyperacetylation of histone has various consequences on gene expression. *In vitro* studies showed that butyrate at the different concentrations was able to inhibit the growth of human cells or, on the other hand, to promote the differentiation of them and induce the apoptosis of neoplastic cells [29]. Moreover, butyrate seems to increase the time of cellular division and reduce the growth rate of colon-rectal cancer cell lines.

1.5 Interplay between diet and gut microbiota

In literature, many studies indicate that diet is the strongest factor shaping the human gut microbiota and differences in macronutrient intakes can affect the growth of specific microbial taxa [30].

Diet provides diverse substrates for anaerobic fermentation by gut microbes, influencing the production and, consequently, the concentration of microbial metabolites (figure 6, [30]).

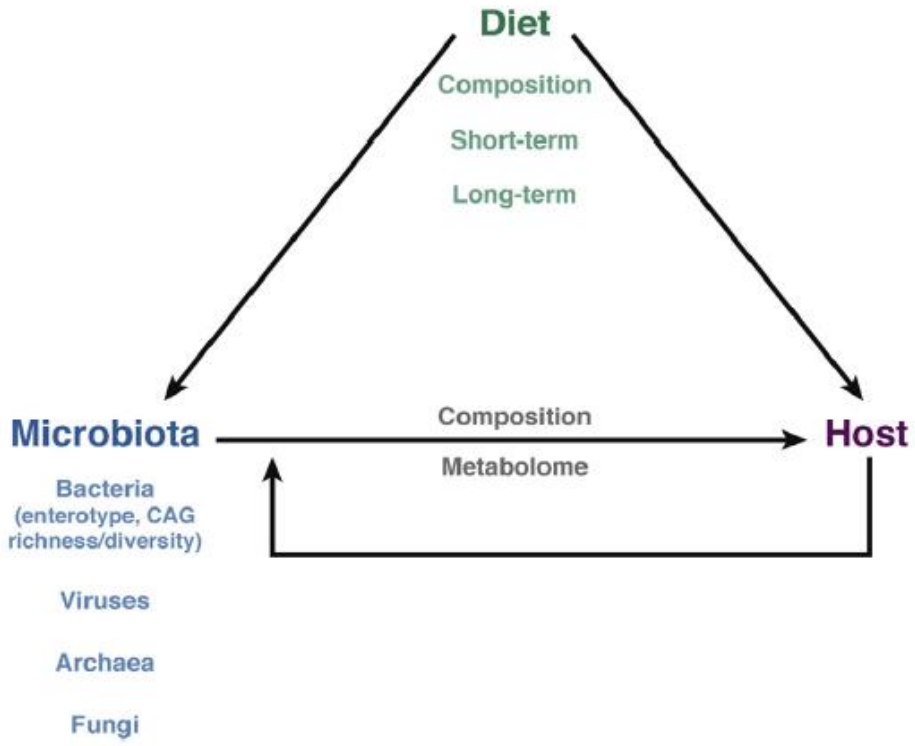

Figure 6. Interactions among diet, gut microbiota and host. Diet can influence directly the composition of gut microbes, or through the interaction with the host [30].

Short-term dietary interventions alter significantly the composition of gut microbiota in healthy subjects, and strict diets such as diet without carbohydrates seem to mostly affect the intestinal biodiversity [31].

Massive alterations in intestinal microbial population have been mainly associated with alterations in diet, affected by the consumption of dietary fiber from vegetables, fruits and cereals [30]. A landmark study of De Filippo and collaborators underlined that the gut microbiota differed significantly between children living in Europe and those living in a rural village of Burkina Faso [32]. The gut microbiota of children in Burkina Faso was characterized by greater amounts of *Prevotella*, lower abundances of *Bacteroides* and a better microbial richness compared to the gut microbiota of European children. They showed also a major production of SCFAs in Burkina Faso children than

European ones. It may speculate that the agrarian diet of Burkina Faso, rich in carbohydrates, fibers, and non-animal proteins, compared to the Western diet, with high content of animal proteins, sugars and fat, is responsible of these differences in gut microbiota composition.

Several other studies report the effects of diet on human gut microbiota. For example, a diet rich in indigestible carbohydrates and fiber has been shown to increase the production of microbial SCFAs together with the positive effects of these metabolites in the host. On the other hand, a diet rich in red meat increases the risk of the colon cancer's development [30] and impacts on the composition of the gut microbiota, by increasing the numbers of *Proteobacteria* [33].

Modifications in the composition of gut microbes and the production of SCFAs have been suggested to play a role in the development of diseases and pathological conditions [20]. Dietary interventions that aim to modify the composition of the gut microbiota might represent potential therapeutic target.

A great example of interplay between diet and gut microbiota in contributing to the pathogenesis of some diseases is certainly represented by inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis [20]. The gut microbiota in IBD patients is considered an important factor involved in the pathogenesis, and diet could restore the altered composition of gut microbes. If the diet is modifiable to stimulate the growth of beneficial microbes, it could represent a target for prevention and treatment of dysbiosis.

In congenital metabolic disorders such as phenylketonuria (PKU) and glycogen storage disease (GSD), especially in type I, diet is considered the mainstay for the treatment of the pathology [34], [35]. In these pathologies, the

diet must not be modified, therefore it is reasonable to restore the gut microbiota by other interventions, such as the use of prebiotics and probiotics.
In PKU, the diet started as soon as disease is diagnosed through the newborn screening [36]. The dietary treatment is based on restriction of phenylalanine intake and supplementation of essential amino acids and micronutrients. PKU diet is similar to a vegan diet, in which animal products are completely excluded, and provides mainly vegetables and aproteic cereals. A plant-based diet seems to promote the development of a stable microbial community: higher abundances of *Ruminococcus*, *E. rectale*, and *Roseburia* are found in vegans and vegetarians compared to onnivores [37].
However, the gut microbiota of phenylketonuric subjects is not deeply investigated, probably due to the disease's rarity. Only a Brazilian group studied the microbial community in PKU patients compared to healthy controls [38], finding a great presence of *Prevotella*, highly abundant in people who consumed a considerable amount of fiber [32].
In GSD-I, the dietary treatment aims to prevent hypoglycaemia and to minimize the second metabolic outcomes. The diet provides the intake of slow-release carbohydrates, limiting the assumption of simple sugars. The use of corn starch is peculiar and it can begin as early as six months of age, as soon as the pancreatic activity of the infants has matured [35].
Since the disease is strongly rare, studies on the gut microbiota of GSD-I and, generally, all GSD types are lacking. Only a study investigated the microbial community of different types of GSD, highlighting a strong presence of *Proteobacteria*, considered as pro-inflammatory phylum, compared to healthy controls [39].

1.6 Techniques to study the gut microbiota

Several methods have been utilized to study the composition of the gut microbiota (figure 7, [35]).

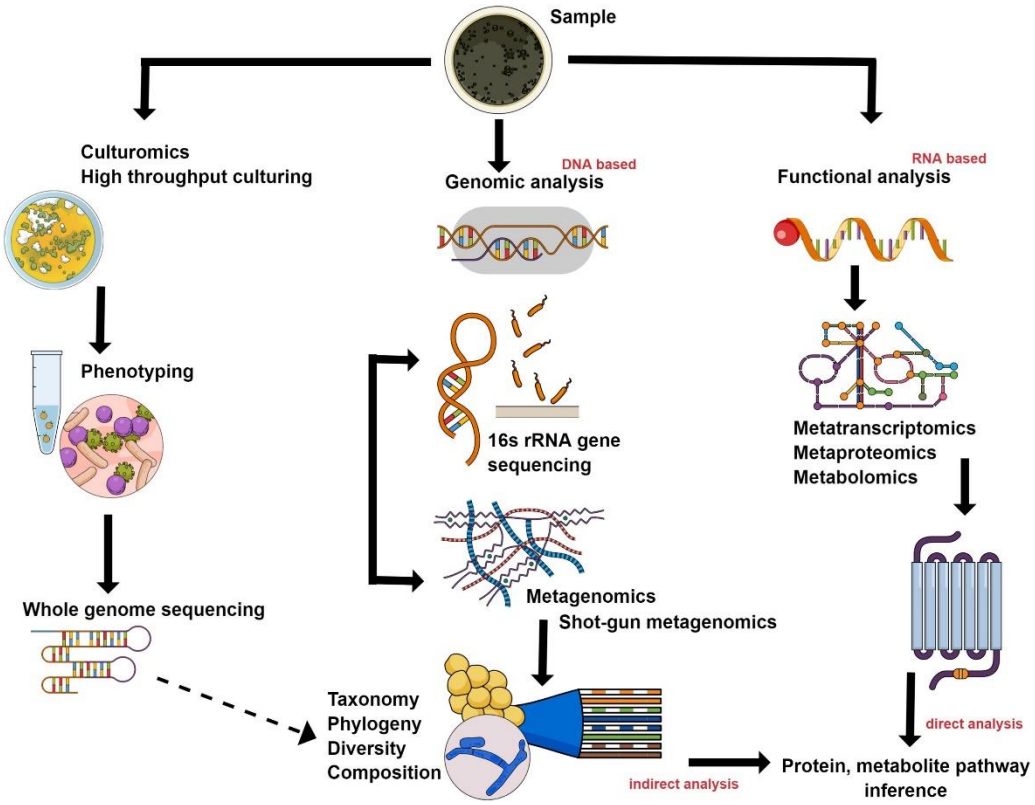

Figure 7. Techniques used to study the gut microbiota [40].

The initial researches used traditional culture techniques by exploiting morphological and biochemical characteristics of bacteria [41]. Since most bacteria in the gut are obligate anaerobes, the use of these techniques do not allow the detection of various bacteria. The culture-based techniques mainly detect aerobic microbes that easily grow *in vitro*. Hence, the usefulness of these techniques in studying the profile of the gut microbiota is strongly limited.

To overcome the limitations of the culture-based techniques, molecular approaches have been developed in the late century [41]. These techniques are based on the identification of gut bacteria through the analysis of their 16S ribosomal RNA (16S rRNA) genes.

The 16S rRNA is 1500 nucleotide long, with some differences among bacterial species. The conserved sequences are inter-spersed with hypervariable regions that show variation [41]. These hypervariable regions represent the evolutionary divergence of bacterial species and provide a useful method for phylogenetic classification. Using these methods allows the detection of bacteria that can be cultured as well as those that present some difficulties to grow.

The initial molecular techniques could exploit only major differences such as the length and nucleotide sequences. In the last 10 years, the advent of high-throughput sequencing allows a deeper analysis and is considered as the gold standard of molecular techniques.

CHAPTER 2

Aim of the Project

In literature, numerous studies have been highlighted the key role of diet in modulating the composition of the human gut microbiota. Since alterations in the gut microbial population and the production of metabolites have been associated with several pathological conditions, the project aimed to investigate the gut microbiome in inherited metabolic disorders, particularly phenylketonuria (PKU) and glycogen storage disease (GSD). In these two inherited metabolic disorders, diet represents the treatment of the pathology. Hence, the aim of this PhD thesis was to characterize a possible association between diet, gut microbiota and short chain fatty acids (SCFAs) in PKU and GSD.

The study specifically aimed to:

- Evaluate nutritional differences between the enrolled subjects
- Evaluate the effect of the diet therapy on microbial gut composition
- Evaluate the effect of the diet therapy on SCFAs production

CHAPTER 3

Materials and Methods

3.1 Subjects recruitment

3.1.1 Phenylketonuria cohort

The phenylketonuria studies included 21 children affected by classic PKU and 21 children with mild hyperphenylalaninemia (MHP) as a control group (age 4-18 years). The patients were enrolled from December 2014 to May 2016 at the Pediatric Department of San Paolo Hospital in Milan (Italy).

All PKU subjects started diet therapy as soon as the disease was diagnosed through newborn screening. To evaluate the compliance to the diet, the bloody concentration of phenylalanine (Phe) was monthly observed by the Guthrie test [36]. The compliance to the diet was under 360 μmol/L for children below 13 years, and under 600 μmol/L for subjects > 13 years.

Inclusion and exclusion criteria are shown in table 1.

Inclusion criteria	**Exclusion criteria**
Age from 4 to 18 years	Congenital malformation
Weight at birth ≥ 2,500 g	Endocrine disorders, chronic liver, and chronic or acute intestinal diseases
Gestational age 37-42 week inclusive	Treatments with antibiotic and probiotic/prebiotic assumption (including glycomacropeptide) in the previous 3 months
Diagnosis of PKU or MHP due to PAH deficiency	Patients non-compliant to the diet
Caucasian, living in Northern Italy	Subjects undergoing therapy with sapropterin dihydrochloride (synthetic copy of tetrahydrobiopterin)

Table 1. Eligibility criteria of the studies.

The studies were approved by the Ethics Committee (Comitato Etico Milano Area 1, Protocol number 2015/ST/135). The parents of enrolled children were explained of the studies protocol and signed consent form.

3.1.2 Glycogenosis cohort

The dataset of glycogenosis study included 21 subjects, 9 GSD type I patients (Ia = 4; Ib = 5), and 12 healthy controls (HC), respectively. The GSD patients were enrolled from January 2018 to June 2018 at the Pediatric Department of San Paolo Hospital (Milan, Italy). GSD patients were 27.7 ± 12.5 years old (6 males/3 females), whereas HC were 24.7 ± 7.9 (9 males/3 females). Although most GSD patients were not of pediatric age, they were followed by the Pediatric Department, a reference center for metabolic diseases.
For all the enrolled subjects, inclusion criteria were: gestational age 37-42 week inclusive, weight at birth ≥ 2,500 g, and single birth, while exclusion criteria were: treatments with antibiotics, prebiotic and/or probiotic assumption in the previous months. Specific criteria for GSD patients are shown in table 2.

Inclusion criteria	**Exclusion criteria**
Disease clinical onset during childhood	Diabetes type I/II
Diagnosis of GSD I confirmed by liver biopsy (% hepatic glycogen and glucose-6-phosphatase enzymatic activity assay)	
Dosage of deoxyglucose transport in polymorphonuclear neutrophils (only in GSD Ib patients)	
Molecular analysis of GSD	
Treatment with CS	

Table 2. Eligibility criteria for GSD patients.

The study was approved by the Ethics Committee (Comitato Etico Milano Area 1, Protocol number 2017/ST/13749). All enrolled subjects signed the consent form.

3.2 Sampling and Data Collection

From all patients and controls, we collected: anthropometric data, dietary habits, and fecal samples, stored at - 80°C until use.

3.2.1 Phenylketonuria cohort

Height, weight and *z*-score body mass index were collected. Dietary intakes were recorded by means of a three-day food diary, filled out by parents. Quantification of energy and nutrient intakes (carbohydrates, lipids, proteins, and fibers) were performed using a software (MetaDieta®, Software version 3.1, ME.TE.DA S.r.l., San Benedetto del Tronto, Italy). For each meal, the glycemic index (GI) and the glycemic load (GL) were evaluated using the Italian and International tables. If the values did not match, a mean value was used. For foods not included in these tables, GI values were calculated at the Department of Food Science, University of Parma (Italy), following the method proposed by the Food Agriculture Organization/World Health Organization and reported in Moretti et al [42]. To analyze the GI of these foods, healthy volunteers were enrolled and instructed in carrying out the method. The day before the test, the volunteers consumed a normal dinner, avoiding the foods rich in fermentable dietary fiber. After a fasting night, the volunteers consumed the foods (with 500 ml of still water) once, and 50 g of glucose as standard food three times, random during different days. Blood

samples, taken by finger-prick, were collected at 15, 30, 45, 60, 90 and 120 min after the meal's start. Whole capillary blood glucose was quantified by an automatic instrument (YSI Stat2300, YSI Inc., Yellow Springs, OH). Mean GI of each meal was measured using the following formula:

$$\mathrm{GI}_{\mathrm{meal}} = \frac{(\,_{\mathrm{i=1,..,n}}\,\mathrm{GI}_{\,\mathrm{food\ i}} \times \mathrm{grams\ of\ carbohydrates}_{\,\mathrm{food\ i}})}{\mathrm{total\ grams\ of\ carbohydrates}}$$

Daily mean GI was calculated as followed:

$$\mathrm{GI}_{\mathrm{daily}} = \frac{(\,_{\mathrm{i=1,..,n}}\,\mathrm{GI}_{\,\mathrm{meal\ i}} \times \mathrm{grams\ of\ carbohydrates}_{\,\mathrm{meal\ i}})}{\mathrm{daily\ total\ grams\ of\ carbohydrates}}$$

The GL of each meal was calculated as followed:

$$\mathrm{GL}_{\mathrm{meal}} = \,_{\mathrm{i=1,..,n}}\,\mathrm{GL}_{\,\mathrm{food\ i}}$$

3.2.2. Glycogenosis cohort

Height, weight and body mass index were calculated. The nutritional weight status was assessed following two different methods according to subjects' age. For subjects > 18 years, the WHO classification of underweight, overweight and obese was used [43], whereas for patients ≤ 18 years BMI z-scores were calculated [44].

Dietary habits were recorded through a 24-h food recall, filled out by subjects or by parents. Quantification of macronutrients was performed as we analyzed the dietary intakes of phenylketonuric patients, using the software MetaDieta®. For each patient, the use of drugs was assessed.

Besides anthropometric parameters, biochemical data of GSD patients were collected from the routine check-up. Glycemia, insulin and related values (HOMA-IR, HOMAβ, QUICKI and Tyg-Index), total cholesterol, triglycerides, uric acid, lactate and transaminases were evaluated.

3.3 Gut microbiota analysis

The study of gut microbiota was assessed firstly for the microbial DNA extraction from stool samples and, subsequently, for the application of different techniques.
All the analyses were carried out at the Clinical Microbiology Laboratory of the Department of Health Sciences, Università degli Studi di Milano (Milan, Italy).

3.3.1 Microbial DNA extraction

Stool samples were aliquoted in 200 mg, used for microbial DNA extraction and, consequently, gut microbiota analysis.
Fecal DNA extraction was performed with the Spin Stool DNA Plus Kit (Stratec Molecular, Berlin, Germany), according to the manufacturer's instructions.
Fecal samples were homogenized in a lysis buffer and incubated at 95°C for 10 minutes. After the homogenization, the samples were purified to remove PCR inhibitors and added with Proteinase K to digest proteins. After the incubation at 70°C for 10 minutes, the samples were washed with different buffers. The samples were eluted in 100 µl of elution buffer.

Concentration and purity of extracted DNA were checked through spectrophotometry using the NanoDrops (Spectrophotometer ND-1000).
A microliter of extracted DNA was read at 240 nm and the optical density (OD) was automatically converted in ng/μl to evaluate DNA concentration. Instead, the OD between 260 nm and 280 nm was useful to analyze the DNA purity and values between 1.8 and 2.0 were considered as a good purity index.

3.3.2 Gut microbiota techniques

For phenylketonuria cohort, preliminary studies on gut microbiota were carried out. Before analyzing the 16S rRNA by next generation sequencing (NGS), we investigated the gut microbial biodiversity in PKU and MHP subjects through different techniques.

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE analysis was performed by amplifying the V2-V3 region of 16S rRNA and using the following primers: HDA 1-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCACGG GGG G-ACT CCT ACG GGA GGC AGCAGT-3′) and HDA 2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′).
The PCR mix was composed by:

- 25 μl of Megamix with $MgCl_2$ (concentration = 2.5 mM)
- 2 μl of each primer (100 μM)
- 16 μl H_2O
- 5 μl of microbial DNA

The amplification cycle provided a first denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation (95°C for 30 seconds), annealing (52°C

for 30 seconds), and extension (72°C for 30 seconds). The final step was carried out at 72°C for 7 minutes.

PCR amplicons (220 bp) were checked on a 2% agarose gel and DGGE was performed. The analysis was assessed using PhorU System (Ingeny, The Haugue, Netherland) in 1X TAE (Tris-acetate-EDTA) buffer at 60°C. PCR amplicons were loaded into 8% polyacrylamide/bis (37.5:1) gels with a denaturing gradient of 40% - 60 % formamide/urea. After the electrophoretic run for 18 h at 90 V, gels were stained with SYBR Green I Nucleic Acid Stain (Roche, UK) for 30 min and visualized by UV radiation. DGGE profiles were acquired through the system Quantity One, Gel Doc (BIORAD) and analyzed with Fingerprinting II software (BIORAD), using the Pearson coefficient correlation and the UPGMA method for dendograms generation.

Absolute quantification analyses in real-time PCR (qPCR)

Absolute quantification of microorganisms was performed by real-time PCR using StepOne Plus (Applied Biosystems), and SYBR® Green (ThermoScientific, USA) as dye.

To define standard curves, control DNA was used: *Roseburia intestinalis* DSM 14610 and *Faecalibacterium prausntzii* DSM 17677 from international collection DSMZ (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH); while *Bacteroides uniformis*, *Lactobacillus reuteri* and *Bifidobacterium animalis* from Clinical Microbiology Laboratory of the Department of Health Sciences, Università degli Studi di Milano (Milan, Italy). Total DNA of each control strain was extracted by using Prepman Ultra (Applied Biosystems, Foster).

Standard curve was prepared using five serial dilutions of control DNA, and all the samples were run in triplicate.

Amplification mix was prepared with the following ingredients:

- 7.5 μl SYBR® Green
- 1.5 μl Forward Primer
- 1.5 μl Reverse Primer
- 2.5 μl H_2O

For each sample, 2 μl DNA (5 ng/μl) were added. Forward and reverse primers are shown in table 3.

	PRIMER 5′→3′	**Reference**
***Bacteroides* spp.**	fw: GAA GGT CCC CCA CAT TG rev: CAA TCG GAG TTC TTC GTG	Bartosh et al. [45]
***Bifidobacterium* spp.**	fw: CGC GTC YGG TGT GAA AG rev: CCC CAC ATC CAG CAT CCA	Delroisse et al. [46]
Faecalibacterium prausnitzii	fw: GGA GGA AGA AGG TCT TCG rev: AAT TCC GCC TAC CTC TGC ACT	Bartosh et al. [45]
***Lactobacillus* spp.**	fw: TGG AAA CAG TTG CTA ATA CCG rev: GTC CAT TGT GGA AGA TTC CC	Delroisse et al. [46]
***Roseburia* spp.**	fw: TAC TGC ATT GGA AAC TGT CG rev: CGG CAC CGA AGA GCA AT	Delroisse et al. [46]

Table 3. Forward and reverse primers of analyzed species.

The PCR conditions were the followings: 95°C for 20 seconds, 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds, and a final step of 95°C for 15 seconds and 60°C for 1 minute.

16S rRNA sequencing

To prepare a sequencing library, 25 ng of microbial extracted DNA was used for each sample. 16S rRNA gene amplicon libraries were performed with a two-step barcoding approach according to Illumina 16S Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA, USA). DNA samples were amplified using the primers provided by Nextera XT DNA Library Preparation Kit (Illumina).

In the first-step PCR, 2.5 μl of DNA was mixed with 5 μl of Amplicon PCR Reverse Primer (1 μM), 5 μl of Amplicon PCR Forward Primer (1 μM), and 12.5 μl of 2X KAPA HiFi HotStart ReadyMix.

16S rRNA gene of bacteria was amplified as follows:

- 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

To verify the size of PCR amplicon, PCR products were loaded on 2% electrophoresis gel and all the samples were ~550 bp.

Before the second index PCR, each sample was purified to eliminate free primers and primer dimer species. The clean-up included the use of AMPure XP beads, several washes with 80% ethanol and a final elution in 27.5 μl of 10 mM Tris pH 8.5.

The index PCR aimed to give each sample a specific barcode sequences at the 5′ and 3′-end of the PCR amplicon to discriminate among each other in the pooled library.

This PCR was obtained in a final volume of 50 ul with 5 µl of purified DNA, 5 µl of Nextera XT Index Primer 1 (N7xx), 5 µl Nextera XT Index Primer 2 (S5xx), 25 µl of 2x KAPA HiFi HotStart ReadyMix, and 10 µl PCR Grade Water (Dnase-Rnase free).

The amplification conditions were the following:

- 95°C for 3 minutes
- 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

Another clean-up was performed following the previous steps. Library concentration and size of PCR amplicons were measured using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) and an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA), respectively.

Once samples concentration was standardized, a 20 nM pooled library was constructed and mixed with 0.2 N fresh NaOH and hybridization buffer HT1 (Illumina) to produce the desired final concentration. A PhiX control v3 (20 nM) was prepared following the same instructions, used for the pooled library. The resulting library was mixed with the PhiX control v3 (5%, v/v) (Illumina) and 600 µl loaded on a MiSeq v2 (500 cycle) Reagent cartridge for obtaining a paired-end 2 × 250 bp sequencing. All sequencing procedures were monitored through the Illumina BaseSpace application. Demultiplexed FASTQ files were generated by Illumina MiSeq Reporter and a total of 2.5 Gbases were obtained.

Microbiota Profiling

The 16S rRNA gene paired sequences, obtained by next generation sequencing, were merged using Pandaseq (release 2.5) [47].

Reads were filtered by trimming stretches of 3 or more low-quality bases (quality < 3) and discarding the trimmed sequences whenever they were shorter than 75% of the original one.

Bioinformatic analyses were conducted using the QIIME pipeline (release 1.8.0) [48], clustering filtered reads into Operational Taxonomic Unit (OTUs) at 97% identity level and discarding singletons as possible chimeras. Taxonomic assignment was performed via the RDP classifier [49] against the Greengenes database (v 13_8).

Alpha-diversity was computed using the chao1, number of OTUs, Shannon diversity and Faith's phylogenetic diversity whole tree (PD whole tree) metrics through the QIIME pipeline; statistical evaluation among alpha-diversity indices was performed by a non-parametric Monte Carlo-based test, using 9999 random permutations. Weighted and unweighted UniFrac distances and Permanova (adonis function) in the R package vegan (version 2.0–10) [50] were used to compare the microbial community structure of enrolled subjects, both PKU and GSD. A functional prediction of the bacterial metabolic pathways was performed using PICRUSt software (version 1.0.1) and KEGG pathways database [51]. Differences in functional category profiles between breeds were assessed using Bray–Curtis distance among samples and "adonis" permutation-based test on the experimental labels.

3.3.3 Fecal metabolite measurement

Short-chain fatty acids (SCFA) were performed by gas chromatography, following a method proposed by Weaver and collaborators [52] with slight modifications.

Aliquots (200 mg) of stool samples were suspended in 1 ml of double distilled water and homogenized on a vortex mixer for 1 minute. After 30 minutes, the samples were centrifuged at 15000 rpm for 15 min. 500 µl of supernatants were mixed with 200 µl of 85% orthophosphoric acid, 200 µl of 2% (v/v) sulphuric acid and 100 µl of 2-ethyl-butyric acid (109959 Sigma) 10 mM in HCOOH 12% as internal standard.

SCFA were gently mixed for 2 minutes with 1 ml ethyl-ether/heptan (1:1 v/v) and centrifuged at 3000 rpm for 10 min. The aqueous phase was frozen at -80°C and the organic layer was collected for the analysis by a Varian 3400 CX gas liquid chromatograph equipped with a Varian 8200 CX autosampler and a HP-FFAP fused-silica capillary column (30 m, 0.53 mm i.d. with a 1-mm film).

Chromatography conditions were the following: gas carrier He with flow 15 ml/min; splitting 1:10 after 20 seconds injection; injection volume of 1 µl. Injector and detector temperatures were 110 and 260° C, respectively. The initial oven temperature was 60°C and was increased by 10°C/min to 110° C and then by 5°C/min. The temperature was held at 200°C for 5 minutes.

SCFA quantification was obtained through calibration curves of acetic, propionic, iso-butyric, butyric and iso-valeric acid in concentrations between 0.25 mM and 10 mM (10 mM 2-ethyl-butyric acid as internal standard).

In particular, SCFA concentration was expressed as mg/g feces and was obtained by applying the following formula:

$$[\mathrm{SCFA}]_{\mathrm{sample}} = \frac{[\mathrm{SCFA}]_{\mathrm{standard}}\ (\mathrm{mM}) * \mathrm{SCFA\ area}_{\mathrm{sample}} * \mathrm{PM\ SCFA}}{\mathrm{SCFA\ area}_{\mathrm{standard}}\ \mathrm{standard} * \mathrm{feces\ weight\ (mg)}}$$

For phenylketonuria cohort, fecal calprotectin quantities were measured using a commercial ELISA kit (Calprotectin ELISA kit, Immundiagnostik, Bensheim, Germany).

3.3.4 Statistical analysis

Statistical comparisons were assessed using MATLAB software (Natick, MA, USA). Comparisons between groups were performed using Student's t-test for normally distributed variables and Wilcoxon test for non-normally distributed variables.

For evaluating differences in collected data (anthropometric data, dietary habits, relative abundances of bacterial groups, and metabolites quantities), a Mann-Whitney U-test was performed.

In gut microbiota analysis, only the 25 most abundant taxa of each phylogenetic level were considered to better focus on the major players of the gut microbiota. Due to multiple testing, a Benjamini-Hochberg correction was applied, considering a FDR < 0.15 as significant. Co-abundance of microbial groups, as well as correlations between taxa and nutritional values and SCFA concentrations were performed through Spearman correlation and the associated linear regression model. P-values < 0.05 were considered as significant.

CHAPTER 4

Gut Microbiome in Phenylketonuria

4.1 Introduction

Phenylketonuria (PKU, OMIM 261600) is an autosomal recessive disorder caused by an absence or deficiency of the phenylalanine hydroxylase (PAH), an enzyme that converts phenylalanine (Phe) into tyrosine [53]. As the activity of PAH is compromised, Phe accumulates in the blood and becomes toxic to the brain. Allelic heterogeneity at PAH locus results in different metabolic phenotypes, ranging from severe and classical PKU to mild hyperphenylalaninemia.

The prevalence of PKU varies widely around the world: in Europe, the prevalence is about one case per 10000 livebirths, while in the Americas (USA and Latin America) and Asia it is lower compared to Europe [54].

If undiagnosed and untreated, the disease can result in progressive intellectual impairment together with other symptoms, such as eczematous rash, autism, seizures, and motor deficits [54]. Therefore, early diagnosis of PKU can prevent developmental problems, psychiatric symptoms and mental retardation.

Although treatment options for the management of PKU are increasing with the introductions of new possibilities, such as large neutral amino acids, or gene therapy, the restriction of dietary Phe still remains the gold standard for disease's treatment [55]. The diet usually begins immediately after the diagnosis in a neonate and it is characterized by the exclusion of foods rich in proteins (animal products, standard bread, nuts and seeds). Patients with PKU consume phe-free formula and can eat low-protein foods such as cereals and potatoes in restricted amounts.

Considering the important role of diet in shaping the gut microbial population, it is not surprising that this peculiar diet leads to shifts in gut

microbiota of patients with PKU [38]. Alterations in the gut microbiota might affect SCFA production and consequently host homeostasis.

Up to date, only a study by a Brazilian group has investigated by 16S rRNA sequencing the gut microbiota in a small cohort of PKU subjects [38].

To this end, the two following studies aimed to elucidate, by comparing the gut microbiota of phenylketonuric children with hyperphenylalaninemia, the role of a low-Phe diet as a potential responsible of microbial dysbiosis.

4.2 Manuscript 1

"Phenylketonuric diet negatively impacts on butyrate production"

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PHENYLKETONURIC DIET NEGATIVELY IMPACTS ON BUTYRATE PRODUCTION

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Abbreviation list: BMI (Body Mass Index); DGGE (denaturing gradient gel electrophoresis); GI (Glycemic Index); GL (Glycemic Load); MHP (Mild Hyperphenylalaninemia); PAH (Phenylalanine Hydroxylase); PHE (Phenylalanine); PKU (Phenylketonuria); qPCR (Real Time PC); SCFA (Short Chain Fatty Acids).

Abstract

Background and Aims: Phenylalanine (Phe) restricted diet, combined with Phe-free L-amino acid supplementation, is the mainstay of treatment for phenylketonuria (PKU). Being the diet a key factor modulating gut microbiota composition, the aim of the present paper was to compare dietary intakes, gut microbiota biodiversity and short chain fatty acids (SCFAs) production in children with PKU, on low-Phe diet, and in children with mild hyperphenylalaninemia (MHP), on unrestricted diet.

Methods: We enrolled 21 PKU and 21 MHP children matched for gender, age and body mass index z-score. Dietary intakes, including glycemic index (GI) and glycemic load (GL), and fecal microbiota analyses, by means of denaturing gradient gel electrophoresis (DGGE) and Real-time PCR were assessed. Fecal SCFAs were quantified by gas chromatographic analysis.

Results: We observed an increased carbohydrate (% of total energy), fiber and vegetables intakes (g/day) in PKU compared with MHP children ($p=0.047$), as well a higher daily GI and GL (maximum $p<0.001$). Compared with MHP, PKU showed a lower degree in microbial diversity and a decrease in fecal butyrate content ($p=0.02$). Accordingly, two of the most abundant butyrate-producing genera, *Faecalibacterium* spp. and *Roseburia* spp., were found significantly depleted in PKU children ($p=0.02$ and $p=0.03$, respectively).

Conclusion: The low-Phe diet, characterized by a higher carbohydrate intake, increases GI and GL, resulting in a different quality of substrates for microbial fermentation. Further analyses, thoroughly evaluating microbial species altered by PKU diet are needed to better investigate gut microbiota in PKU children and to eventually pave the way for pre/probiotic supplementations.

Introduction

Phenylketonuria (PKU; OMIM 261 600) is an autosomal recessive disorder of Phenylalanine (Phe) metabolism [1] caused by mutations in the phenylalanine hydroxylase (PAH) gene. Loss of PAH enzymatic activity results in an impairment of phenylalanine to tyrosine conversion, consequently increasing phenylalanine concentrations in blood (hyperphenylalaninemia, HPA) and fluids throughout the body and reaching toxic concentrations in the brain [1]. Various combinations of mutations result in a full metabolic phenotypes ranging from mild hyperphenylalaninaemia (MHP, blood Phe 120-360 μmol/L) in which treatment is not necessary, to mild, moderate and classical phenylketonuria (blood Phe levels >360 μmol/L) which require dietary management [1–3].

Despite continuing progress in the treatment, the restriction of dietary Phe throughout life remains the cornerstone of PKU management [4]. The PKU diet is mainly made up of low-protein natural foods (vegetables, fruits) and special low protein products, which are low-protein variants of some foods such as bread, pasta and biscuits. The required amount of daily protein is obtained from Phe-free protein substitutes providing essential amino acids in suitable proportions. [4,5].

The overall goals of PKU dietary management are to prevent complications associated with untreated PKU and promote normal growth and development, and adequate nutritional status [6].

In the last decade, growing evidence has suggested the involvement of gut microbiota and microbial metabolites in various physiological and pathological conditions [7]. In health, gut microbiota is mainly involved in the development and maturation of the immune system and in the regulation of several metabolic pathways. Alterations in the gut microbiota have been observed in functional and inflammatory gastrointestinal diseases, as well as in other metabolic syndromes [8]. Amongst factors that can influence the human gut microbiota composition, diet is one of the most relevant [8–10]. Thus, the low-phenylalanine diet might modify gut microbiota composition and affecting gut homeostasis of PKU subjects.

Up to date, only a Brazilian study analyzed gut microbiota in PKU children [11]. In this case-control study based on 8 PKU and 10 healthy controls, the authors demonstrated in PKU a strong reduction in both richness and evenness, and in Firmicutes population.

The aim of this paper was to compare dietary intake, gut microbiota biodiversity and short chain fatty acids production in children with PKU, on low-Phe diet, and in children with mild hyperphenylalaninemia, on unrestricted diet.

Methods

This observational case control study included 21 phenylketonuric children (age 4-18 years) gender and age (± 6 months) matched with 21 children with mild hyperphenylalaninemia, consecutively admitted to the Department of Pediatrics, San Paolo Hospital, Milan, from December 2014 to May 2016. Inclusion criteria were: gestational age 37–42 week inclusive, weight at birth ≥ 2500 g, single birth, white parents. Exclusion criteria were: congenital malformation, having endocrine disorders, chronic liver diseases, chronic or acute intestinal diseases and treatments with antibiotic and probiotic/prebiotic in the previous 3 months. PKU children non-compliant with the recommended diet were also excluded. PKU children were defined as compliant to the diet when the annual mean Phe levels, monitored monthly by the Guthrie test [12], was within the range 120–360 μmol/L for children below 13 years and was

under 600 μmol/L for patients ≥13 years. Phenylketonuric children were detected by a newborn screening test and periodically monitored in our department since diagnosis. A medical history was collected at recruitment from parents by a standardized questionnaire during a personal interview conducted by the same pediatrician that also saw the children for a general examination. Moreover, the pediatrician took anthropometric measurements of children, assisted by an experienced operator. Body weight and height were measured using a mechanical column scale (seca 711; seca GmbH & KG, Hamburg, Germany) with an integrated measuring rod (seca 220; seca GmbH & KG). The body mass index (BMI) was calculated from the ratio of weight to height squared (kg/m2). BMI z-scores were calculated and adjusted for age and gender by using WHO anthro [Available online: http://www.who.int/childgrowth/software/en/].

A fresh fecal sample was self-collected at home by each enrolled subject and stored immediately at -20° C. The collection took place in the same week as the dietary record. Subsequently, the fecal samples were transported to the laboratory and stored at -20° C until further analysis. The parents of eligible children or their legal guardian received a detailed explanation of the study, and signed a consent form. The hospital Ethics Committee approved the study protocol.

Dietary assessment

For each child, the dietary intake, including beverages, was recorded by means of a food diary filled out by parents for three consecutive days (two weekdays and one weekend day). Parents received instructions about the method for weighing and recording food. They were trained by a dietitian to weigh each food item offered to their child before consumption, to weigh leftovers and to record the weights each time [13]. Quantification and analysis of the energy intake and nutrient composition were performed with an ad hoc PC software (MètaDieta®, Me.Te.Da S.r.l., San Benedetto del Tronto, Italy). Vegetable intake quantification excluded tuber and legumes in accordance with the World Health Organization (WHO) [14]. For each food, the GI value was derived from the Italian [15] or International [16–18] tables. If there was a discrepancy between the tables [16–18] then the mean value was used. For a food not included in the Italian or International tables, the GI value was determined at the Department of food science, University of Parma (Italy), using the Food and Agriculture Organization/World Health Organization's method [19] and in accordance with the International Standards Organization guideline [20]. Then, to find out the GI of these foods healthy volunteers were recruited, after an informed consent was signed. On the day before the test the volunteers consumed a standardized dinner. Foods with known content of fermentable dietary fiber were forbidden in order to avoid any second meal effect. After fasting overnight, volunteers were tested for assessed foods (with 500 mL of still mineral water) once each, and the standardized food (50 g of glucose) three times, in random order on separate days. Blood samples were taken by finger-prick. Whole capillary blood glucose was measured by an automatic analyzer (YSI Stat2300, YSI Inc., Yellow Springs, OH). Blood samples were collected at 15, 30, 45, 60, 90 and 120 min after starting to eat. Mean GI of each meal, was calculated by the following formula [21]:

GI meal = (i=1, …, n GI food i * grams of carbohydrates food i)/ total grams of carbohydrates meal

Daily mean GI, was calculated as:

GI daily = (□i=1, …, n GI meal i * grams of carbohydrates meal i)/ daily total grams of carbohydrates

The glycemic load for each meal was calculated:

GL meal = □i=1, …, n GL food i

Bacterial DNA extraction

Total microbial DNA extraction was performed with the Spin Stool DNA Plus Kit (Stratec Molecular, Berlin, Germany) according to manufacturer instruction, using 200 mg aliquot of wet feces. Briefly, fecal sample was homogenized in a lysis buffer and incubated at 95° C for 10 minutes. The lysed sample was mixed with the matrix in InviAdsorb to remove most of the components that inhibit PCR. Proteinase K was added to the supernatant to digest and degrade proteins after incubation at 70° C. The purification of bacterial DNA was obtained by the addition of suitable buffers to remove other impurities. The filtrate was added with an elution buffer to obtain bacterial DNA in 100 µL. Microbial DNA concentration and purity was assessed by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

DGGE analysis was carried out as previously described [22], amplifying theV2-V3 region of the 16S rRNA gene using the primers: HDA1-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCACGG GGG G-ACT CCT ACG GGA GGC AGCAGT-'3) and HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA C-'3). PCR products (220 bp) were visualized on a 1.5% agarose gel and subsequently subjected to DGGE analysis. DGGE was performed using PhorU system (Ingeny, The Haugue, Netherland) in 1X TAE (Tris-acetate-EDTA) buffer at 60 °C. PCR products were loaded onto 8% polyacrylamide gels in 1 X TAE. The electrophoretic conditions were the following: 18 h at 90 V in a 40–60% denaturating agent gradient. Gels were stained in 1X TAE buffer with SYBR Green I nucleic acid stain (Roche, UK) for 30 min and visualized by UV radiation. Banding patterns of DGGE profiles were analyzed with Fingerprinting II software (Bio-Rad Laboratories), using the Pearson product moment correlation coefficient and the un-weighted-pair group method with averages (UPGMA) for the generation of dendrograms. Pearson coefficient is a measure of the degree of similarity. Two identical profiles create a similarity value of 100% whereas completely different profiles result in a similarity value of 0%.

Short Chain Fatty Acid (SCFA) measurement

SCFA concentrations were assessed in accordance with the method proposed by Weaver et al. [23] modified as previously described [24]. Briefly, 200 mg of stool were suspended in 1 mL of distilled water, homogenized on vortex mixer and, after 30 min, centrifuged (15000 rpm) for 15 min at 10°C. 500 µL of supernatant were mixed with 100 µL 85% orthophosphoric acid, 100 µL 2% (v/v), sulphuric acid and 100 µL and 2-ethyl-butyric acid 10 mM in HCOOH 12% as internal standard. SCFA were gently extracted for 1 min with 1 ml ethyl-ether/heptan (1:1 v/v) and centrifuged for 10 min at 3000 rpm. The aqueous phase was frozen and the organic layer was removed for analysis by a Varian 3400 CX (Conquer Scientific, San Diego, CA,

USA) gas liquid chromatograph equipped with a Varian 8200 CX autosampler and a HP-FFAP fused-silica capillary column (30 m, 0.53 mm i.d. with a 1-mm film). Quantification of the SCFA was obtained through calibration curves of acetic, propionic, iso-butyric, butyric and iso-valeric acid in concentrations between 2.5 mM and 10 mM (10 mM 2-ethyl-butyric acid as internal standard). Results have been expressed as mg/g of wet weight of feces.

Absolute quantification analyses in real time PCR (qPCR)

Absolute quantification by real-time PCR was performed using the following control strains: Roseburia intestinalis DSM 14610 and DSM 17677 Faecalibacterium prausntzii of international collection DSMZ (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH); Lactobacillus reuteri and Bifidobacterium animalis from Clinical Microbiology Laboratory of the Department of Health Sciences, Università degli Studi di Milano. Total microbial DNA was extracted for each control strain using Prepman Ultra (Applied Biosystems, Foster City, CA). qPCR was carried out using the StepOne Plus instrument (Life technologies) and SYBR Premix Ex Taq (Tli RNaseH Plus, Takara Bio). Standard curve was carried out for each qPCR run using five serial dilutions of control DNA. Samples and standards were run in triplicate. Specific 16S rRNA primers and qPCR conditions have been previously described [22].

Statistical analysis

Descriptive data are reported as mean and standard deviation (SD), and median and the 25th-75th centile. Normality of the distribution of continuous variables was assessed by the Kolmogorove-Smirnov test. For continuous variables, comparison between groups was performed by Student's t-test and Mann-Whitney, as appropriate. All values of $p < 0.05$ were considered to indicate statistical significance (two tailed test). The statistical package for social sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft, Redmond, WA, USA) was used for the statistical analysis.

Results

Pediatric cohort characteristics

We enrolled 21 children (10 boys/11 girls) for each group, PKU and MPH. Mean (SD) age of children was 8.69 (3.57) years. No significant difference between PKU and MHP was found for BMI z-score 0.5 (1.1) vs. 0.2 (1.0), p=0.309.

In PKU children, mean (SD) blood Phe levels at the recruitment was 262.6 (97.5) μmol/L. No PKU patient was on tetrahydrobiopterin (BH4) and large neutral amino acids (LNAA) treatments during the study.

Dietary assessment

Dietary intake of energy, macronutrients, fiber, fruit and vegetables are reported in Table 1. PKU children consumed lower proteins and higher carbohydrates (% of total energy) and fibers than MHP children ($0.001<p<0.047$). Moreover, PKU group showed a higher fruit and vegetables (overall and only vegetables) intakes than MHP ($p<0.001$).

Daily GI and GL of diet and of each meal in PKU and in MHP children are shown in Table 2. Overall, both lunch and dinner dietary GIs were higher in PKU compared with MHP children ($p<0.001$). The same results were observed for GLs ($p<0.001$).

Gut microbiota analysis

Gut microbiota biodiversity was assessed by denaturing gradient gel electrophoresis technique. DGGE gel profiles showed a high degree of individual variability in microbiota composition (Figure 1). In particular, profiles from MHP subjects revealed an enriched microbial ecosystem in comparison with PKU children. The resulting UPGMA dendrogram showed the presence of two main clusters (40.0% similarity), one represented by MHP children and the other group included PKU children.

Fecal SCFA analysis revealed a decrease in total SCFAs and butyrate production in PKU children compared with MHP children ($p=0.044$ and $p=0.026$, respectively), while no differences were observed for acetate, propionate, iso-butyrate and iso-valerate (Table 3).

Real-Time PCR was performed to quantify the abundance of *Faecalibacterium prausnitzii* and *Roseburia* spp., known butyrate-producing genera, and of *Bifidobacterium* spp. e *Lactobacillus* spp., two genera of lactate-producing bacteria. These analyses showed a depletion in *F. prausnitzii* and *Roseburia* spp. ($p = 0.02$ and $p = 0.03$, respectively) in PKU compared with MHP children (Figure 1 A-B). Moreover, PKU children showed a decrease in *Lactobacillus* spp. ($p=0.002$), while no significant differences were observed for *Bifidobacterium* spp.

Discussion

The aim of this work was to investigate the effect of a special diet on gut microbiota biodiversity and SCFA production. To this purpose, we enrolled PKU children, on a low-Phe diet, and MHP children, on an unrestricted diet. To understand key differences in the daily

nutrient intake of the two experimental groups, we performed a fine nutritional analysis that revealed differences in protein, carbohydrate and fiber intakes, although similar energy intakes were observed. As expected [5], PKU children showed a lower protein and higher carbohydrate, fiber and vegetables intakes compared with MHP children. Indeed, 85.7% of PKU children showed a protein intake (% energy) lower than 12% and 57.1% a carbohydrate intake higher than the recommended upper limit for healthy children [25]. Within the MHP group, 19.0% of children exceeded the recommended intake for both proteins and carbohydrates and only one MHP child (4.8%) had a carbohydrate intake lower than 45% of energy. Fiber and overall fruit and vegetables intake analysis demonstrated an adequate intake in a high percentage of PKU group (61.9% and 42.8%, respectively); in MHP group, only one child showed adequate intakes. It should be noted that, in planning this study, MHP children have been considered as comparison group just because they are affected by disease but are on free diet and, indeed, MHP group and healthy children were comparable for macronutrients intakes [26].

Daily glycemic index and glycemic load were higher in PKU than MHP children. This could be due to consumption of special low protein foods considering that some of them are added with glucose, dextrose or sugar as ingredients, which may increase their GI [27].

Differences in daily macronutrient intake could modulate the microbiota composition. Indeed, DGGE analysis of microbial biodiversity highlighted in PKU children a reduction in microbial richness, that is considered the first hallmark of gut dysbiosis [28].

Changes in diet could result in different substrates for microbial fermentation, leading to an enrichment or depletion of specific genera within our microbial community. Shifts in microbiota, in turn, lead to variations in microbial metabolites, mainly SCFAs [29].

Acetate, butyrate, and propionate largely derive from carbohydrates fermentation, whereas branched-chain fatty acids (BCFAs, mainly iso-butyrate and iso-valerate) from proteins and amino acids fermentation by proteolytic bacteria [30].

Total fecal SCFAs, as well as butyrate, were decreased in PKU compared with MHP children.

Butyrate is the preferred energy source for colonocytes, and participates in maintaining gut homeostasis. Several families belonging to Firmicutes have a key role in butyrate production, especially *Faecalibacterium prausnitzii* and *Eubacterium rectale/Roseburia* group [31]. Absolute quantification of key bacterial genera showed a decrease in both *F. prausnitzii* and *Roseburia* spp. as well as in Lactobacillus spp. The latters produce lactate that can be further used as substrate for other SCFAs production [32], participating in a cross-feeding mechanism.

Our results are in line with the observation by Pinheiro de Oliveira and colleagues [11] that observed a distinct microbial signature in PKU children compared with healthy children. In particular, the authors revealed in PKU children a depletion in Firmicutes, to which *Faecalibacterium prausnitzii* and *Eubacterium rectale/Roseburia* belong, and an enrichment in Bacteroidetes. According to metagenome prediction analysis, PKU microbiota presented fewer genes encoding for enzymes involved in starch and sucrose metabolism and in glycolysis/gluconeogenesis [11]. Since in the above-mentioned Brazilian study [11] a quantification of fecal SCFAs was not performed, a full comparison between the two studies was not possible.

A reduction in SCFAs production seems inconsistent with the higher fiber and vegetable (rich fiber foods) intakes that characterize PKU diets. Indeed, non-digestible fibers are fermented

in the caecum and the large intestine by gut microbiota allowing the production of SCFAs [33]. However, the quality of fibers is a key factor in determining microbial composition and its SCFA production, as different bacteria possess different metabolic pathways to break down sugars [34]. Benus and colleagues [35] investigated the effect of dietary fiber exclusion and supplementation on the intestinal microbiota and SCFA concentrations in healthy subjects. The authors observed a significant reduction in the abundance of *F. prausnitzii* and *Roseburia* spp. during both fiber-free and fiber-supplemented diets compared with normal diet, and a positive correlation between F. prausnitzii abundance and butyrate concentration. Indeed, the quality of fiber supplemented, mostly non digestible, was ineffective in promoting *Roseburia* spp. and *F. prausnitzii* proliferation [35].

In conclusion, the restricted PKU diet, characterized by a higher carbohydrate intake, including many simple sugars and non-digestible fibers, has been shown to increase glycemic index and glycemic load, resulting in a different quality of substrate for microbial fermentation.

Because phenylketonuria is good model to explore diet-induced modifications of microbiota, further studies using innovative sequencing techniques are needed to better investigate gut microbiota dysbiosis in PKU children and to eventually pave the way for pre/probiotic supplementations.

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Figure 1

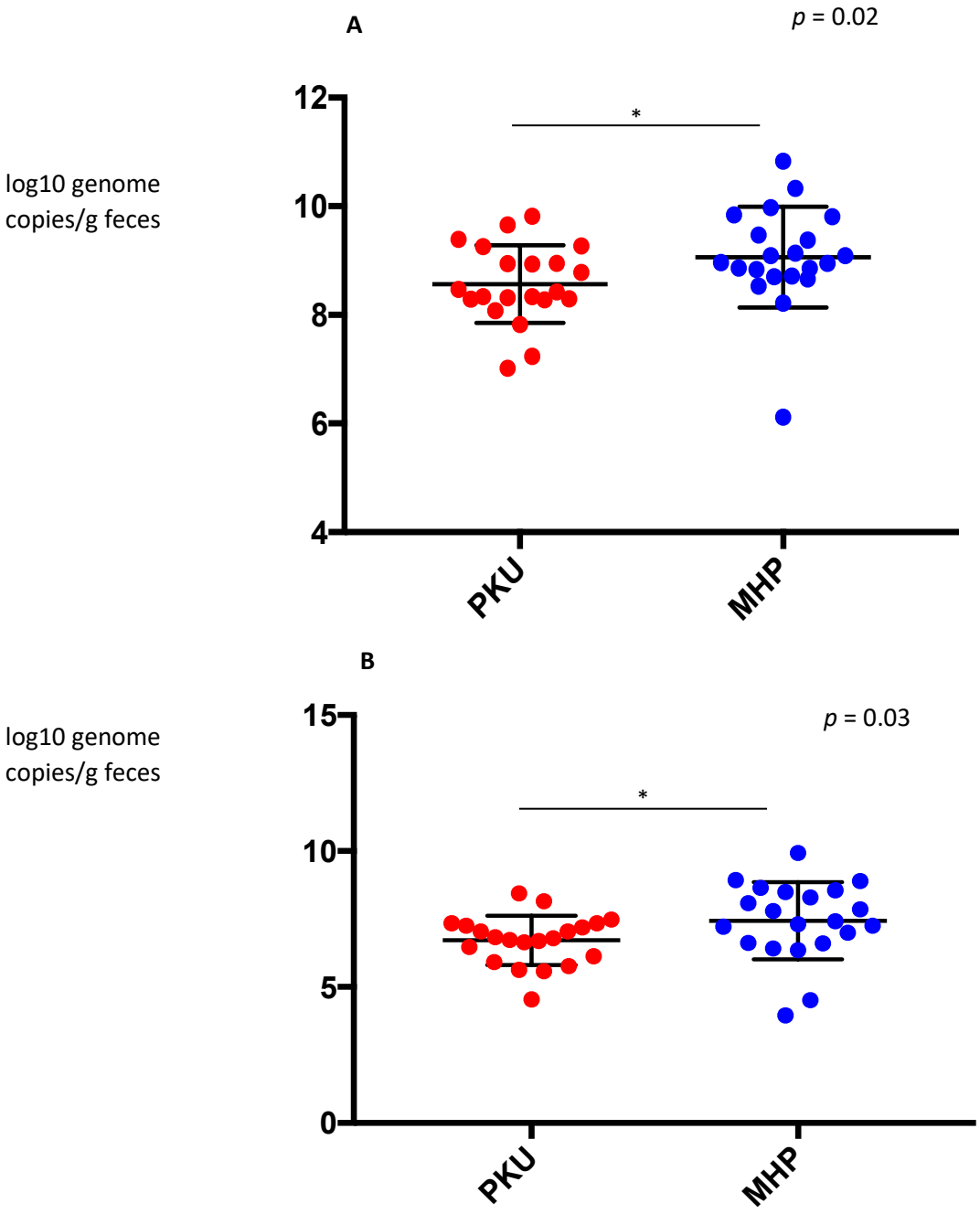

Figure 1. Microbial quantification, in phenylketonuric (PKU, red) and mild hyperphenylalaninemia (MHP, blue) children, of *Faecalibacterium prausnitzii* (A), and *Roseburia* spp (B). Abundances are expressed as log10 genome copies/g feces.

Tables

Table 1. Daily dietary intake of energy, macronutrients, fiber, fruit and vegetables in children with phenylketonuria (PKU) and with mild hyperphenylalaninemia (MHP).

	PKU children		MHP children			
Variable	**Mean (SD)**	**Median (25^{th}-75^{th} centile)**	**Mean (SD)**	**Median (25^{th}-75^{th} centile)**	***P*-value**	**Reference Values†**
Energy						
kcals	1681.3 (439.2)	1585.5 (1449.2-1942.6)	1482.3 (303.2)	1478.2 (1258.0-1623.0)	0.141	boys:1330-4020 kcals (AR)
kcals/kg weight	49.2 (10.2)	50.3 (44.9-56.4)	54.7 (20.0)	56.4 (37.1-69.5)	0.265	girls:1220-3550 kcals (AR)
Protein						
total, g	43.2 (15.1)	50.3 (32.9-52.0)	52.1 (11.1)	50.1 (41.5-61.5)	0.023*	16-50 g (AR)
% energy	10.2 (1.9)	7.5 (5.9-8.5)	14.3 (2.3)	15.7 (13.1-21.1)	<0.001*	12-15% (RI)
g/kg weight	1.3 (0.3)	1.3 (1.0-1.5)	1.9 (0.8)	1.8 (1.3-2.3)	0.011*	boys: 0.76-0.79 g/kg (AR)
						girls: 0.76-0.77 g/kg (AR)
from vegetables, g	7.3 (2.0)	7.5 (5.9-8.5)	16.8 (5.2)	15.7 (13.1-21.1)	<0.001*	
from animal sources, g	4.9 (3.2)	4.7 (2.4-7.7)	27.9 (8.4)	26.5 (22.6-32.1)	<0.001*	
from formula, g	28.7 (13.0)	25.0 (18.1-32.9)	0 (0)	0 (0)	<0.001*	
Carbohydrate						
g	254.2 (72.7)	263.2 (196.0-285.7)	207.3 (45.4)	203.8 (183.8-247.0)	0.062	
% energy	61.0 (7.0)	60.8 (55.4-65.1)	56.0 (5.9)	55.9 (52.2-61.8)	0.047*	45-60% (RI)
Fat						
g	54.9 (16.0)	55.4 (43.4-64.3)	51.8 (15.4)	48.5 (37.2-65.8)	0.409	
% energy	29.6 (6.6)	28.5 (25.3-35.7)	32.6 (4.7)	33.1 (27.6-36.5)	0.188	20-35% (RI)
Fiber						
overall. g	16.0 (9.1)	14.6 (9.2-19.7)	8.9 (2.6)	8.9 (7.5-10.1)	0.003*	
overall. g/1000 kcal	9.7 (4.9)	9.7 (5.9-11.5)	6.1 (1.8)	6.4 (4.6-7.3)	0.016*	8.40g/1000 kcals (AI)
Fruit and vegetables						
overall. g	382.6 (193.7)	351.7 (220.7-560.0)	191.8 (83.2)	208.3 (141.0-230.0)	0.001*	≥ 400 g
fruit. g	143.6 (128.5)	106.7 (45.0-199.2)	111.8 (62.1)	120.0 (76.0-153.3)	0.999	
vegetables. g	239.0 (117.7)	221.7 (158.3-312.5)	80.0 (49.0)	65.0 (42.0-93.3)	<0.001*	

AR. average requirement; RI. reference intake; AI. adequate intake.

† Energy. macronutrients and fiber [25]; amount of fruit and vegetables [26].

* Statistically significant

Table 2. Glycemic index and glycemic load of overall daily diet and meals in children with phenylketonuria (PKU) and with mild hyperphenylalaninemia (MHP).

	PKU children		**MHP children**		
Variable	**Mean (SD)**	**Median (25^{th}-75^{th} centile)**	**Mean (SD)**	**Median (25^{th}-75^{th} centile)**	***P* - value**
Glycemic index					
Overall diet	65.1 (5.2)	64.9 (62.3- 69.1)	52.8 (3.8)	53.7 (49.8- 55.3)	<0.001*
Breakfast	51.5 (19.8)	51.3 (43.8- 57.6)	47.0 (9.6)	46.5 (39.6- 53.0)	0.252
Morning snack[§]	42.2 (22.0)	38.2 (32.9- 50.2)	47.2 (24.1)	49.4 (32.3- 70.0)	0.335
Lunch	68.8 (9.3)	70.9 (63.7- 75.5)	54.1 (7.5)	54.0 (50.2- 57.1)	<0.001*
Afternoon snack	45.6 (14.7)	46.8 (39.3- 51.8)	51.7 (9.2)	53.6 (47.6- 57.5)	0.050
Dinner	69.2 (4.8)	69.6 (67.2- 71.8)	64.7 (41.3)	58.2 (44.9- 66.9)	0.001*
Glycemic load					
Overall diet	163.5 (48.6)	158.6 (130.2- 182.0)	104.1 (29.8)	92.7 (88.7- 134.4)	<0.001*
Breakfast	14.7 (8.3)	14.4 (8.4- 20.0)	15.7 (6.8)	15.0 (8.9- 19.5)	0.758
Morning snack[§]	9.9 (9.2)	6.5 (1.8- 17.0)	4.0 (4.3)	2.8 (0.2- 5.8)	0.135
Lunch	61.0 (22.5)	55.2 (44.7- 73.7)	33.5 (15.2)	27.5 (24.8- 42.6)	<0.001*
Afternoon snack	17.7 (13.0)	17.3 (8.3- 22.7)	14.9 (10.9)	12.2 (6.4- 25.5)	0.587
Dinner	62.2 (24.4)	53.8 (47.7- 84.7)	36.4 (17.5)	32.3 (25.4- 44.1)	<0.001*

[§]Four PKU children and 10 MHP children did not report any morning snack during the food diary recording.

* Statistically significant.

Table 3. Short chains fatty acids (mg/g of feces) in children with phenylketonuria (PKU) and with mild hyperphenylalaninemia (MHP).

	PKU children		**MHP children**		
Variable mg/g of feces	Mean (SD)	Median (25^{th}-75^{th} centile)	Mean (SD)	Median (25^{th}-75^{th} centile)	*P* - value
Acetate	3.0 (0.8)	3.0 (2.2-3.6)	3.6 (1.3)	3.6 (2.6-4.8)	0.161
Propionate	1.0 (0.3)	1.0 (0.8-1.2)	1.1 (0.4)	1.1 (0.8-1.4)	0.211
Iso-butyrate	0.2 (0.1)	0.1 (0.1-0.2)	0.2 (0.1)	0.2 (0.1-0.3)	0.470
Butyrate	1.0 (0.3)	1.0 (0.8-1.2)	1.3 (0.4)	1.3 (1.0-1.6)	0.026*
Iso-valerate	0.3 (0.2)	0.3 (0.2-0.4)	0.3 (0.2)	0.4 (0.2-0.5)	0.533
Total	5.5 (1.1)	5.5 (4.5-6.1)	6.5 (1.4)	6.5 (4.9-8.4)	0.044*

* Statistically significant.

4.3 Manuscript 2

"Phenylketonuria Diet Promotes Shifts in Firmicutes Populations"

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Phenylketonuria Diet Promotes Shifts in Firmicutes Populations

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Low-phenylalanine diet, the mainstay of treatment for phenylketonuria (PKU), has been shown to increase glycemic index and glycemic load, affecting the availability of substrates for microbial fermentation. Indeed, changes in the PKU gut microbiota compared with healthy controls have been previously reported. In this study we compared the gut microbial communities of children with PKU and with mild hyperphenylalaninemia (MHP, unrestricted diet). For each group, we enrolled 21 children (4–18 years old), for a total dataset of 42 subjects. We assessed dietary intake and performed gut microbiota analysis by sequencing the V3–V4 hypervariable regions of the 16S rRNA gene. Short chain fatty acids (SCFAs) were quantified by gas chromatographic analysis. While alpha-diversity analysis showed no significant differences between PKU and MHP groups, microbial community analysis highlighted a significant separation of the gut microbiota according to both unweighted ($p = 0.008$) and weighted Unifrac distances ($p = 0.033$). Major differences were seen within the Firmicutes phylum. Indeed, PKU children were depleted in *Faecalibacterium* spp. and enriched in *Blautia* spp. and *Clostridium* spp (family *Lachnospiraceae*). We found a divergent response of members of the Firmicutes phylum with respect to daily glycemic index, higher in PKU children. *Faecalibacterium prausnitzii*, unclassified *Ruminococcaceae* and, to a lesser extent *Roseburia* spp. negatively correlated with glycemic index, whereas unclassified *Lachnospiraceae* were positively associated. Indicator species analysis suggested *F. prausnitzii* be related to MHP status and *Ruminococcus bromii* to be associated with PKU. Despite PKU children having a higher vegetable and fiber intake, resembling a vegan diet, their gut microbial profile is different from the microbiota reported in the literature for individuals consuming a high-fiber/low-protein diet. Indeed, beneficial microorganisms, such as *F. prausnitzii*, considered a biomarker for a healthy status and one of the main butyrate producers, are depleted in PKU gut microbiota. We suggest that both the quality and quantity of carbohydrates ingested participate in determining the observed Firmicutes shifts on the PKU population.

Keywords: phenylketonuria, mild hyperphenylalaninemia, diet, microbiota, glycemic index, *Faecalibacterium prausnitzii*, butyrate

INTRODUCTION

Phenylketonuria (PKU; OMIM 261600) is an inherited metabolic disorder caused by a mutation in the phenylalanine hydroxylase enzyme (PAH), which converts phenylalanine (Phe) into tyrosine. As PAH activity is hampered, phenylalanine accumulates in the blood and becomes toxic to the brain (Williams et al., 2008). Allelic heterogeneity at PAH locus results in a variety of metabolic phenotypes, ranging from mild, moderate and classical PKU (blood Phe levels >360 μmol/L) to mild hyperphenylalaninemia (MHP, blood Phe levels ranging 120–360 μmol/L; Güttler and Guldberg, 1996).

Untreated PKU leads to neurodevelopmental damage and behavioral problems that are preventable by early diagnosis and dietary treatment (van Spronsen et al., 2017).

A PKU diet, started in the neonatal period and followed life-long, is characterized by low-protein natural foods (vegetables, fruits) and special low-protein products, which are low-protein variants of some foods (bread, pasta, and biscuits; Giovannini et al., 2012). Adequate protein intake is guaranteed by Phe-free amino acid mixtures (AAM) with a balanced content of amino acids and micronutrients. Despite improvements in taste, the palatability of such formulas is still less than optimal, often resulting in poor acceptance by school-aged patients. Moreover, a PKU diet has been shown to increase glycemic index and glycemic load (Moretti et al., 2017), probably due to special low-protein products frequently being enriched in sugars.

Considering the crucial role of diet in shaping the gut microbiota, i.e., the microbial community inhabiting gastrointestinal tract (Albenberg and Wu, 2014), it is not surprising that such a peculiar diet leads to microbial changes in phenylketonuric patients (Pinheiro de Oliveira et al., 2016; Verduci et al., 2018). Alterations in the gut microbiota, in turn, may influence gastrointestinal homeostasis, predispose to chronic inflammation and modulate other metabolic functions through gut-liver axis and gut-brain axis (Nieuwdorp et al., 2014).

Up to date, only a study by Pinheiro de Oliveira et al. (2016) has investigated by 16S rRNA sequencing the gut community of PKU patients. However, the small cohort (eight patients vs. ten healthy controls) and the presence of confounding factors (i.e., antibiotic treatment, age <1 year) might have mitigated the observed microbial alterations within the gut. Moreover, whether reported changes in the gut microbiota represent an effect of the disease itself or a consequence of the modified diet is still unclear.

To this end, this work aims at elucidating, by comparing the microbiota of PKU children with mild hyperphenylalaninemia, the role of the low-Phe diet as potential inducer of microbial dysbiosis.

MATERIALS AND METHODS

Subject Recruitment and Sampling

A total of 42 children (21 PKU/21 MHP) were enrolled in the study at the Pediatric Department of San Paolo Hospital in Milan, Italy (Verduci et al., 2018). Inclusion criteria were: gestational age 37–42 week inclusive, Caucasian, living in Northern Italy, birth weight ≥2,500 g, single birth, diagnosis of PKU or MHP due to PAH deficiency. Exclusion criteria were: congenital malformation, endocrine disorders, chronic liver diseases, chronic or acute intestinal diseases, treatments with antibiotic, and probiotic/prebiotic (including glycomacropeptide) in the 3 months preceding the study. All PKU subjects started the diet therapy at disease diagnosis, usually within 10 days from birth.

Blood phenylalanine concentration was monthly monitored by the Guthrie test (Guthrie and Susi, 1963).

From all subjects we collected: anthropometric data (height, weight and *z*-score body mass index), dietary habits and stool samples, stored at −80°C until use. Three-days food diaries were filled out by a parent for each enrolled subject and processed by dieticians to calculate the average amounts of energy and nutrient intake (carbohydrates, soluble and insoluble fibers, lipids, proteins) using a commercially available software (MetaDieta®, Software version 3.1, ME.TE.DA S.r.l., San Benedetto del Tronto, Italy). For each meal, the glycemic index (GI) value and glycemic load (GL) were calculated as described by Verduci et al. (2018) using the following formulas:

$GI_{meal} = (\sum_{i=1,...,n} GI_{food\,i} * \text{grams of carbohydrates}_{food\,i})/\text{total grams of carbohydrates}_{meal}$

$GI_{daily} = (\sum_{i=1,...,n} GI_{meal\,i} * \text{grams of carbohydrates}_{meal\,i})/\text{daily total grams of carbohydrates}$

$GL_{meal} = \sum_{i=1,...,n} GL_{food\,i}$.

Gut Microbiota Analysis

Fecal DNA extraction was performed using the Spin stool DNA kit (Stratec Molecular, Berlin, Germany), according to manufacturer's instructions. For each sample, 25 ng of extracted DNA was used to construct the sequencing library. The V3–V4 hypervariable regions of the bacterial 16S rRNA were amplified with a two-step barcoding approach according to the Illumina 16S Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA, USA). For library preparation, DNA samples were amplified with dual-index primers using a Nextera XT DNA Library Preparation Kit (Illumina). Library concentration and quantification were determined using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) and Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA), respectively. The libraries were pooled and sequenced with a MiSeq platform (Illumina) for 2 × 250 base paired-end reads and a total of 2.5 Gbases raw reads were obtained.

Fecal Metabolite Measurement

We performed short chain fatty acids (SCFAs) and calprotectin quantification from stool samples.

Concentrations of acetic, propionic, iso-butyric, butyric, and iso-valeric acids were assessed by gas liquid chromatography in accordance with the method proposed by Weaver et al. (1989) with slight modifications described in Borgo et al. (2017). Analyses were performed using a Varian model 3400 CX Gas-chromatograph fitted with FID detector, split/splitless injector and a SPB-1 capillary column (30 m × 0.32 mm ID, 0.25 μm film thickness; Supelco, Bellefonte, PA, USA). Results are expressed as mg/g of wet weight of feces. Quantification of the SCFAs was obtained through calibration curves of acetic, propionic,

iso-butyric, butyric, and iso-valeric acid in concentrations between 0.25 and 10 mM (10 mM 2-ethylbutyric acid as internal standard). SCFA data on the same cohort have been previously described in Verduci et al. (2018).

Fecal calprotectin concentrations were measured by a commercial ELISA kit (Calprotectin ELISA Kit, Immundiagnostik, Bensheim, Germany), according to manufacturer instructions.

Absolute Quantification of *Methanobrevibacter smithii*

Real-time PCR was carried out using a SYBRGreen chemistry (ThermoScientific, USA) and the specific primers for *Methanobrevibacter smithii* (MSfw: 5′-CCGGGTATCTAATCCGGTTC-3′ and MSrev: 5′-CTCCCAGGGTAGAGGTGAAA-3′), as previously described (Borgo et al., 2017). The following thermal cycling parameters were used for amplification of DNA: 95°C for 10 min followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. A melting curve analysis was also performed to verify amplicon specificity.

The control strain *M. smithii* DSM-861 (DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was used for the standard curve.

Microbiota Profiling

The 16S rRNA gene paired sequences obtained were merged using Pandaseq (release 2.5; Masella et al., 2012), then reads were filtered by trimming stretches of 3 or more low-quality bases (quality <3) and discarding the trimmed sequences whenever they were shorter than 75% of the original one. Bioinformatic analyses were conducted using the QIIME pipeline (release 1.8.0; Caporaso et al., 2010), clustering filtered reads into Operational Taxonomic Unit (OTUs) at 97% identity level and discarding singletons (i.e., OTUs having only 1 supporting read along the whole dataset) as possible chimeras. Taxonomic assignment was performed via the RDP classifier (Wang et al., 2007) against the Greengenes database (ftp://greengenes.microbio.me/greengenes_release/gg_13_8_otus).

Indicator species analysis was performed using the indicspecies package in R (De Cáceres and Legendre, 2009) on the QIIME-derived OTU table.

Alpha-diversity was computed using the Chao1, number of OTUs, Shannon diversity, and Faith's Phylogenetic Diversity whole tree (PD whole tree) metrics; statistical evaluation among alpha-diversity indices was performed by a non-parametric Monte Carlo-based test, using 9999 random permutations. Weighted and unweighted UniFrac distances and PERMANOVA (adonis function) in the R package vegan (version 2.0-10; Oksanen et al., 2013) were used to compare the microbial community structure of the PKU and MHP children.

Statistical Analysis

Statistical comparisons were performed using MATLAB software (Natick, MA, USA). Comparisons of the two groups were performed using Student's *t*-test for normally distributed variables and Wilcoxon test for non-normally distributed variables. For evaluating differences in relative abundances of bacterial groups, a Mann-Whitney *U*-test was performed. For each phylogenetic level, only the 25 most abundant taxa were considered, in order to focus on the major players of the gut microbiota. Due to multiple testing, a Benjamini-Hochberg correction was applied, considering a FDR < 0.15 as significant. For clarity, uncorrected *p*-values were reported in the text. Co-abundance of microbial groups, as well as correlations between taxa and nutritional values and SCFA quantities were assessed through Spearman correlation and the associated linear regression model. Unless otherwise stated, *p*-values < 0.05 were considered as significant.

RESULTS

Cohort Description

Cohort characteristics are reported in **Table 1**. At recruitment, blood Phe levels in PKU children was slightly higher than the MHP group ($p = 0.24$). Compared with MHP children, PKU children showed higher dietary intakes of carbohydrates and fibers and a significant lower consume of proteins (expressed as %). Glycemic index (GI) and glycemic load (GL), evaluated for each meal, were significantly higher in PKU children compared with MHP subjects ($p < 0.001$). Anthropometric measurements were similar, with a BMI *z*-score not significantly different in the two groups ($p = 0.31$).

TABLE 1 | Cohort characteristics and dietary habits.

	MHP ($n = 21$)	PKU ($n = 21$)	*p*-value	
Female	12	11		
Male	9	10		
Age (years)	8.0 ± 3.4	10.0 ± 3.5	0.060	
BMI z-score	0.2 ± 1.0	0.5 ± 1.1	0.310	
Blood Phe levels (mmol/L)	228.0 ± 87.1	262.6 ± 97.5	0.241	
Carbohydrate (% energy)	56.0 ± 5.9	61.0 ± 7.0	0.047	*
Fiber (overall grams)	8.9 ± 2.6	16.0 ± 9.1	0.003	**
Protein (% energy)	52.1 ± 11.1	43.2 ± 15.1	0.023	*
Lipids (% energy)	32.6 ± 4.7	29.6 ± 6.6	0.188	
Glycemic index (GI)	52.8 ± 3.8	65.1 ± 5.2	<0.001	***
Glycemic load (GL)	104.1 ± 29.8	163.5 ± 48.6	<0.001	***

Values are expressed as mean ± standard deviation; one asterisk (*) indicates p-value smaller than 0.05 ($p < 0.05$), two asterisks (**) indicate p-value smaller than 0.01 ($p < 0.01$), three asterisks(***) indicate p-value smaller than 0.001 ($p<0.001$; Mann-Whitney U-test).

Gut Microbiota Composition in PKU and MHP Children

Five samples (2 PKU and 3 MHP subjects) were excluded from the analysis due to very low raw read quantities (with an average of 189 reads compared to an average of 159,914 reads among the other samples); the final dataset for microbiota analysis, then, consisted of 37 subjects: 19 PKU and 18 MHP.

To avoid biases related to uneven sequencing depth, samples were subsampled to 50,000 reads each. After quality filtering

processes, we obtained a mean count of 49,749 ± 111 reads per sample.

Alpha-diversity analysis (data not shown) revealed no significant differences between PKU and MHP groups for any of the metrics used (number of OTUs, $p = 0.306$; chao1, $p = 0.131$; Shannon, $p = 0.894$; PD whole tree, $p = 0.31$). Beta-diversity analysis, instead, showed that the structure of the PKU fecal microbiota differed significantly from that of the MHP group according to both unweighted ($p = 0.008$) and weighted ($p = 0.032$) Unifrac distances (β-diversity, **Figure 1**).

The gut microbiota composition at the phylum and family levels is depicted in **Figure 2** and in **Table S1**. The most relatively abundant phyla in PKU and MHP subjects were Firmicutes and Bacteroidetes, the latter slightly higher in MHP subjects. Among the most relatively abundant families, we found *Veillonellaceae* to be significantly depleted in PKU children ($p = 0.002$). Although not statistically significant, *Ruminococcaceae* were enriched in MHP and *Lachnospiraceae* in PKU subjects.

At the genus level (**Figure 3**), *Faecalibacterium* ($p = 0.001$), *Ruminococcaceae (other)* ($p = 0.03$) were more relatively abundant in MHP children; although not significant, *Bacteroides* and *Prevotella* genera showed the same trend. Furthermore, we observed an increased *Prevotella/Bacteroides* ratio in MHP compared with PKU (0.14 ± 0.59; 0.02 ± 0.05, respectively).

In contrast, PKU children were characterized by a significant increase in the relative abundance of *Blautia* ($p = 0.004$), *Clostridium* (belonging to *Lachnospiraceae* family, $p = 0.002$), and *Lachnospiraceae (other)* ($p = 0.019$). Significantly altered taxa belonged to the Firmicutes phylum and are highlighted in **Table 2**.

Because of the underestimation of Archaea by 16S rRNA gene sequencing, Real-time PCR quantification of the most abundant Archaea species in human gut microbiota, *Methanobrevibacter smithii*, was performed. We did not observe significant differences between PKU and MHP subjects ($p = 0.40$).

Indicator species analysis highlighted *Akkermansia muciniphila* OTU 1045 (best BLAST hit: Accession number NR_074436.1, with 97% seq. similarity over 420 bp, e-*value* = 0.046) and *Faecalibacterium prausnitzii* OTU 3793 (accession number: NR_028961.1, with 97% seq. similarity over 420 bp, e-*value* = 0.001) to be characteristic of MHP microbiota, whereas *Ruminococcus bromii* OTU 3232 (accession number: NR_025930.1, with 97% seq. similarity over 420 bp, e-*value* = 0.018) to be associated to PKU.

Bacterial Correlation Patterns

Because cross-feeding between species is relevant in gut microbiota dynamic, we performed correlation analysis to study interactions between different members of intestinal microbiota. Several significant bacterial connections (**Table S2**), both positive and negative, have been observed. In particular, MHP indicator species *A. muciniphila* and *F. prausnitzii* showed several interactions with other members of the microbial community: *Akkermansia* was negatively related to *Unclassified Lachnospiraceae* ($R = -0.61$) and to *Blautia* spp. ($R = -0.40$), while was positively associated to *Oscillospira* ($R = 0.57$) and to *Unclassified Clostridiales* ($R = 0.34$). On the other hand, *F. prausnitzii* was only found positively related to *Ruminococcaceae* (other) genus ($R = 0.42$). The PKU indicator species, *Ruminococcus bromii*, instead, showed no correlation to other genera itself.

Correlations Between Gut Microbiota and Nutritional Values

Statistical correlation analysis between diet and microbiota showed that *Faecalibacterium* spp., significantly increased in MHP group, negatively correlated with fiber intake, both soluble and insoluble fibers ($R = -0.61$; $R = -0.37$), and with GI, GL ($R = -0.53$ and $R = -0.49$, respectively). *Ruminococcaceae* family as well as its genus *Ruminococcaceae (other)*, with higher relative abundance among MHP patients (**Figure 4**), negatively

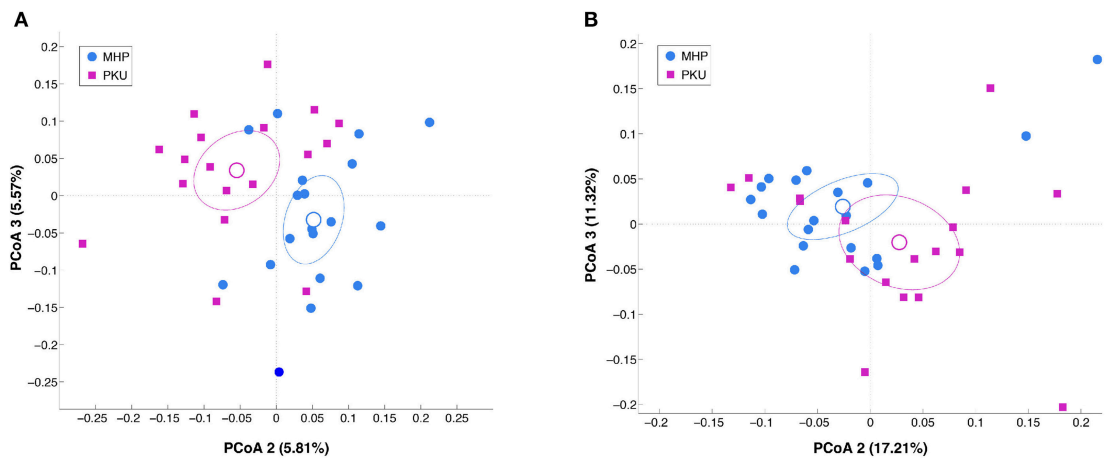

FIGURE 1 | Beta-diversity analysis in MHP (blue) and PKU (magenta). Principal coordinates analysis (PCoA) of **(A)** unweighted and **(B)** weighted Unifrac distances. Microbial communities are statistically different (*adonis* test: unweighted $p = 0.008$, $R^2 = 0.040$; weighted $p = 0.033$, $R^2 = 0.058$). Second and third principal coordinates are shown in the plot for both distances.

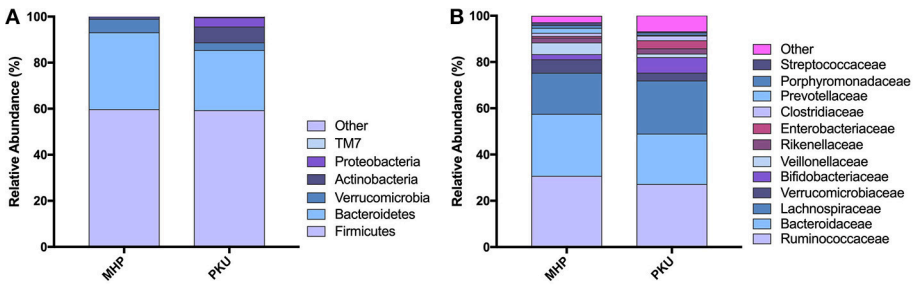

FIGURE 2 | Gut microbiota composition in MHP and PKU children. Relative abundance at **(A)** phylum and **(B)** family level. All bacterial taxa present at <1% relative abundance were grouped into the "Other" classification.

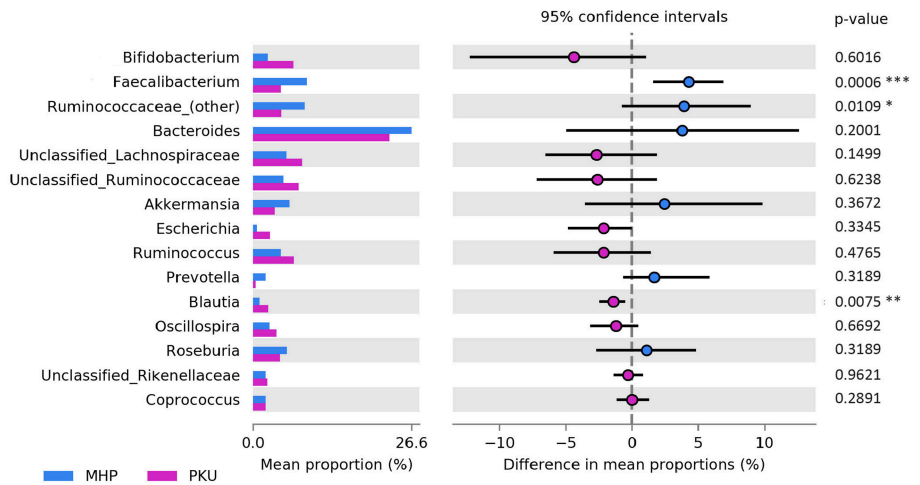

FIGURE 3 | Bacterial abundances at genus level. Genera abundances of the 15 most abundant genera are reported as mean proportion along with significant *p*-values (two-sided White's non-parametric *t*-test) and 95% of confidence interval (bootstrap method).

TABLE 2 | Genera belonging to Firmicutes phylum significantly increased or depleted in PKU children (Mann-Whitney *U*-test, *p-value* <0.05).

Genus	MHP (mean ± SD)	PKU (mean ± SD)	*p*-value	PKU
Ruminococcaceae (other)	7.07 ± 8.26	2.78 ± 4.22	0.030	↓
Lachnospiraceae (other)	0.35 ± 0.25	1.84 ± 2.52	0.019	↑
Blautia	1.07 ± 0.85	2.43 ± 2.02	0.004	↑
Faecalibacterium	8.71 ± 4.32	4.52 ± 4.14	0.001	↓
Clostridium	0.23 ± 0.29	1.92 ± 2.64	0.002	↑
Dialister	3.28 ± 3.83	0.67 ± 2.1	0.036	↓

related with GI and soluble fibers ($R = -0.40$ and $R = -0.49$, respectively), while an opposite trend was observed among the *Lachnospiraceae (other)* genus within the same nutritional values (GI $R = 0.43$; soluble fibers $R = 0.49$). *Oscillospira*, slightly higher in PKU children, was positively related to energy assumption and carbohydrates ($R = 0.43$ and $R = 0.47$, respectively); *Roseburia*, genus enriched in MHP, was found negatively related to soluble fibers ($R = -0.42$) and to lipids ($R = -0.42$). All of these correlation coefficients were statistically significant ($p < 0.05$).

SCFAs and Gut Microbiota Correlation

As reported in our previous work (Verduci et al., 2018), a decrease in total SCFAs and in particular in butyrate production was observed in PKU children compared with MHP children ($p = 0.044$ and $p = 0.026$, respectively). Although not statistically significant, acetate ($p = 0.161$) was also reduced in PKU group. Propionate and the isoforms iso-butyrate and iso-valerate, instead, were similar.

Correlation analysis with gut microbiota composition revealed several significant interactions between the relative abundance of certain taxa and fecal SCFA concentrations.

Acetate was found negatively related to *Coprococcus* ($R = -0.42$) and to *Blautia* ($R = -0.50$), genera enriched in PKU. Butyrate was found inversely correlated to *Lachnospiraceae (other)* ($R = -0.41$). Propionate was found to positively correlate to *Bacteroides* ($R = 0.49$) and inversely to *Unclassified Lachnospiraceae* ($R = -0.42$) and to *Blautia* ($R = -0.56$). The branched-chain fatty acids iso-butyrate and iso-valerate were negatively related to *Unclassified Lachnospiraceae* ($R = -0.64$, $R = -0.65$, respectively), to *Clostridium* spp. ($R = -0.45$ and $R = -0.46$) and to *Blautia* spp ($R = -0.40$, only iso-butyrate) and positively correlated to *Akkermansia* ($R = 0.47$

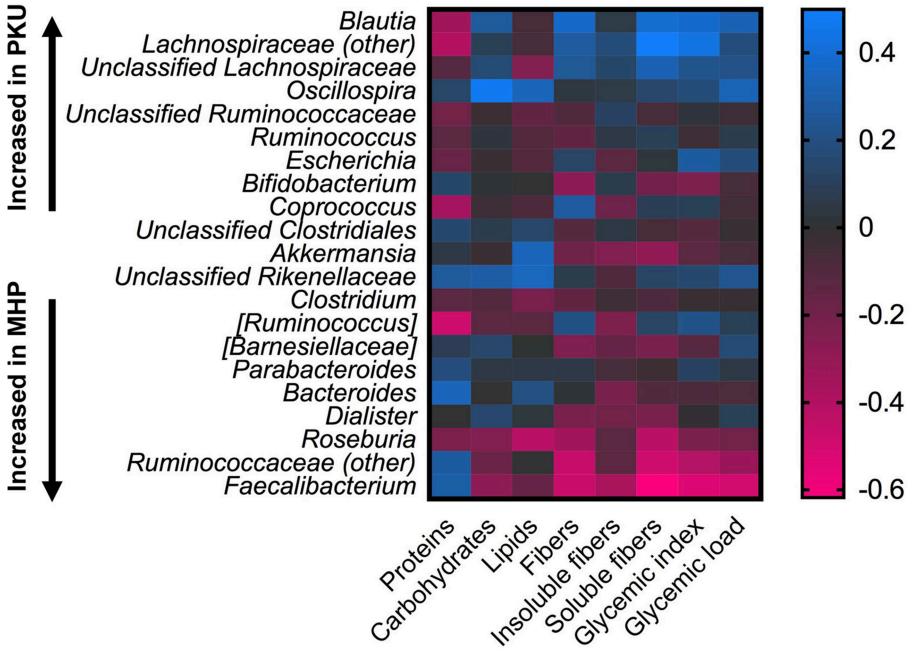

FIGURE 4 | Correlations between microbiota and dietary information. Heatmap showing Spearman's correlations between the most abundant microbial genera and nutritional values. Color intensity represents the degree of association; positive correlations are depicted in blue and negative in magenta.

and $R = 0.44$). All correlation coefficients were statistically significant ($p < 0.05$).

Fecal Calprotectin Concentrations in PKU and MHP Patients

To investigate whether PKU subjects are chronically inflamed in the gut, we quantified fecal calprotectin, a recognized biomarker for gastrointestinal diseases (Pang et al., 2014). No significant differences were recorded between PKU and MHP groups ($24.8 \pm 14.9\,\mu g/g$ and $40.6 \pm 28.3\,\mu g/g$, $p = 0.10$, respectively).

CONCLUSIONS AND DISCUSSION

The aim of this study was to investigate the impact of a low-Phe diet on microbial gut community and its possible consequences on PKU patient wellbeing. Indeed, according to European guidelines for PKU management, the recommended diet should start as early as possible, usually before the age of 10 days, to prevent neurological damage (Singh et al., 2014; van Spronsen et al., 2017). This age corresponds to a well-recognized crucial step in microbiota acquisition and maturation (Dominguez-Bello et al., 2019) on which environmental factors may have a profound impact.

To rule out a direct effect of PAH deficiency in microbiota alteration, subjects with mild hyperphenylalaninemia, under normal diet, were enrolled as control group.

Bioinformatic analyses revealed several changes in the microbial taxa inhabiting the PKU gut compared with MHP subjects, as already suggested by Pinheiro de Oliveira et al. (2016). Nevertheless, some differences were observed between their study and ours, mainly ascribable to the different enrolled control group (healthy children instead of MHP), the different age range (4 out of their 8 PKU patients were <2 years-old), the sequencing method used (Ion Torrent vs. Illumina) and the subjects' ethnicity (Brazilian vs. Italians).

In our cohort, the relative abundance of Firmicutes and Bacteroidetes was similar, with slight decrease of both phyla in the PKU group. Although not statistically significant, both *Bacteroides* and *Prevotella,* the two main genera belonging to the Bacteroidetes, were more relatively abundant in MHP children. While *Bacteroides* result is not surprising, probably related to the reduced protein intake (David et al., 2014) in Phe-free diet, *Prevotella* is usually associated with increased fiber intake (De Filippo et al., 2010), typical of the PKU diet. Similarly, the *Prevotella/Bacteroides* ratio was slightly higher in MHP group, in contrast to the common finding of a higher ratio in strict vegetarians/vegans (Wu et al., 2011; Franco-de-Moraes et al., 2017). Recently, a high *Prevotella/Bacteroides* ratio has been suggested to be associated with an improvement of glucose response, possibly preventing cardiometabolic diseases (Sandberg et al., 2018).

The most relevant shifts, however, concerned the Firmicutes phylum. Indeed, the PKU gut microbiota was enriched in *Blautia* spp. and *Clostridium* spp. and depleted in *Faecalibacterium*

spp., as anticipated in our previous work by absolute real-time PCR quantification (Verduci et al., 2018). As discussed, it was reasonable to expect that the higher fiber intake in PKU patients would have increased *Faecalibacterium* spp. proliferation, but the relative abundance of this genus showed an opposite trend. However, as described by Benus et al. (2010), a fiber-supplemented diet does not necessarily increase *F. prausnitzii* and *Ruminococcus* populations compared with a normal balanced diet. Moreover, the quality of fibers (Verduci et al., 2018) as well as the supplementation of some PKU special low protein products with inulin could also impact the abundance of these genera.

It is intriguing that the indicator species analysis showed an association between MHP phenotype and *F. prausnitzii*, a known biomarker for health status (Lopez-Siles et al., 2017). In healthy adults, this bacterial genus commonly represents more than 5% of the total gut bacterial population (Miquel et al., 2013) and is one of the major butyrate producers. Butyrate is considered the main energy source of colonocytes and displays anti-inflammatory properties in the colonic mucosa (Flint et al., 2012). *A. muciniphila* also characterized the MHP gut microbiota in the indicator species analysis. *A. muciniphila*, a mucin-degrading bacterium, is considered a potential probiotic, that is able to maintain intestinal integrity (Zhai et al., 2018). As already reported by other authors (Arumugam et al., 2011; Cani and de Vos, 2017), *A. muciniphila* is positively associated with members of the family *Ruminococcaceae*, probably sharing nutritional requirements or cross feeding phenomena.

In contrast, PKU microbial communities were characterized by *Ruminococcus bromii*, a well-known starch degrader that belongs to the non-butyrate-forming *Ruminococcaceae* (Ze et al., 2012; Kettle et al., 2015).

Overall, in agreement with the lower total fecal SCFAs content and in particular of butyrate, the PKU gut microbiota was depleted in butyrate-producing species and enriched in genera, i.e., *Blautia*, that are recognized to exert a pro-inflammatory effect on gut mucosa. Indeed, *Blautia* spp. has been demonstrated to induce cytokines secretion, like tumor necrosis factor alpha (TNF-alpha), involved in immune acute phase response (Tuovinen et al., 2013).

Moreover, about half of PKU subjects were characterized by an increase in the relative abundance of Proteobacteria, in particular *Escherichia* spp. Proteobacteria is a phylum of Gram-negative microorganisms whose membrane lipopolysaccharide is a well-known inducer of innate immune responses (Hotamisligil, 2006).

As discussed for the phyla Firmicutes and Bacteroidetes, the enrichment in Proteobacteria observed in PKU children is in contrast with the reported underrepresentation of this phylum in children with rural diet, more similar to PKU vegetarian-vegan diet than MHP, compared with children under Western-diet (De Filippo et al., 2010).

Although our study did not show any alteration in calprotectin, a recognized gut inflammation marker, previous work by our group (Moretti et al., 2017) demonstrated an increase in the triglyceride glucose index (TyG index) in PKU children compared with age- and sex-matched healthy controls. TyG index is considered a marker of low-grade inflammation and of peripheral insulin resistance (Er et al., 2016). Moretti et al. (2017) showed in PKU a positive correlation of TyG index with glycemic load, reinforcing a possible link between carbohydrate quality and metabolic disorder predisposition. Indeed, PKU children showed an increased consumption of fast-absorbing carbohydrates that escape gut microbiota fermentation (Verduci et al., 2018), resulting in higher GI and GL.

It is important to note, we also found a divergent response of the Firmicutes phylum with respect to daily GI and GL. Most members of the *Lachnospiraceae* family, with the exception of *Roseburia,* were positively correlated with both indexes (with *Blautia* as the most relevant genus), whereas members of *Ruminococcaceae* family were negatively, in particular *F. prausnitzii*.

The quality of carbohydrates ingested by PKU children might directly affect *Faecalibacterium* abundance. In accordance with our data, Fava and colleagues (Fava et al., 2013) showed that a diet enriched in carbohydrates with a high glycemic index resulted in a decreased *F. prausnitzii* abundance in subjects at-risk of developing a metabolic syndrome. A plausible explanation is that fast-absorbing carbohydrates do not represent a suitable substrate for *F. prausnitzii* growth, commonly fermenting complex carbohydrates. On the other hand, short chain carbohydrates represent a good substrate for *Blautia* spp. (Egshatyan et al., 2016), which were more relatively abundant in the PKU group. The *Blautia* genus encompasses a huge number of strains with different metabolic capabilities (Eren et al., 2015), several of them considered to be acetogens. In contrast, we found an inverse correlation with fecal acetate concentration. However, this observation is in agreement with recent findings by Org et al. (2017) that investigated the relationship between the gut microbiota composition and metabolic disorder traits. The authors also suggested a positive correlation between *Blautia* and body mass index. Indeed, PKU patients, more than MHP subjects, are at risk for excessive weight gain (Scaglioni et al., 2004; Rocha et al., 2013; Couce et al., 2018) and insulin resistance (Moretti et al., 2017; Couce et al., 2018).

The enrichment of PKU low-protein products in simple sugars, is a consequence of their poor palatability. In the last few years, the increasing awareness of the health consequences of PKU diet led to the development of new products that could implement or complement these formulas. For example, some companies have made commercially available products based on glycomacropeptide (GMP). GMP is a protein derived from cheese whey, rich in specific essential amino acids and lacking aromatic amino acids (i.e., Phe). GMP has been recently demonstrated to have prebiotic properties with beneficial effects on the gut microbiota (Sawin et al., 2015).

In addition, new formulas enriched in prolonged-release amino acids have been recently released. Such medical foods, meant to allow a physiological-like adsorption, are strongly improved in palatability thanks to the amino acids coating layer (Giarratana et al., 2018). It still remains to be evaluated whether an improvement in current free-amino acid formulas,

as well as increased attention to the management of dietary carbohydrate quality in PKU diet (with a particular focus on special low protein products), can rebalance the PKU microbial community.

ETHICS STATEMENT

The study was approved by the ethics committee (Comitato Etico Milano Area 1, Protocol number 2015/ST/135). The parents of eligible children or their legal guardian received a detailed explanation of the study and signed a consent form.

AUTHOR CONTRIBUTIONS

EB and EV designed the study. GB, FB, and CC performed experiments and data analysis, and drafted the manuscript. CC and MS performed microbiota data analysis. VR performed subject enrollment and analyzed clinical data. EB, EV, and GM performed supervision and writing—review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00101/full#supplementary-material

Raw reads are available in NCBI Short Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA447916.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.4 Discussion and Conclusion

According to the results of the studies above, a distinct alteration in the gut microbiota and the production of SCFAs was found in phenylketonuric children compared with those affected by hyperphenilalaninemia.

In our first study, we investigated the effect of low-Phe diet on gut microbiota biodiversity and SCFAs production.

We analysed dietary habits in the two enrolled groups that highlighted differences in protein, carbohydrate and fiber intakes. PKU children showed an increased glycemic index, probably due to the consumption of special low protein foods, rich in simple sugars.

Differences in daily macronutrient intake may reflect the intestinal microbial composition. Indeed, DGGE analysis showed high bacterial biodiversity between the two groups and quantitative Real-Time PCR underlined significant differences in some bacterial species, such as *Roseburia* spp. and *Faecalibacterium prausnitzii*, found depleted in PKU children.

In agreement with the depletion of these butyrate-producing species, a reduction of butyrate and, generally, total fecal SCFAs was underlined in PKU children.

The next use of the 16S rRNA gene sequencing confirmed the previous results. Major differences were found within the *Firmicutes* phylum: a depletion in *Faecalibacterium* spp. and an enrichment in *Blautia* spp. were underlined in PKU children. Interestingly, *Faecalibacterium* spp. and, to a lesser extent, *Roseburia* spp. negatively correlated with glycemic index, suggesting that the quality of ingested carbohydrates could change the gut microbial population. The PKU microbiota lacked beneficial microbes, such as *F. prausntizii*, considered as a biomarker of healthy status [56], whereas was enriched in

Blautia spp. that is recognized to exert proinflammatory effects on gut mucosa [57].

Although PKU children consumed high amounts of vegetables and fiber, their intestinal microbial profile is different from microbiota reported in literature for subjects having a high fiber/low protein intake [32].

The enrichment of PKU low-protein foods in simple sugars is made to improve their palatability. In the last years, the consequences of these products on PKU health have been clearly investigated and the development of new products is necessary. Some companies have commercialized products based on glycomacropeptide (GMP), a protein with no aromatic amino acids. GMP has been recently suggested to be a putative prebiotic with beneficial effects in restoring gut microbial alteration [58].

It is worthy to evaluate if an improvement in special low protein foods as well as a major attention to the quality of ingested carbohydrates in PKU diet may rebalance the PKU gut microbiota and consequently the production of SCFAs.

CHAPTER 5

Gut Microbiome in Glycogen Storage Disease Type I

5.1 Introduction

Glycogen storage diseases (GSD) are a group of inherited metabolic disorders due to a deficiency of one of the enzymes involved in glycogen metabolism [35]. Since glycogen is primarily stored in the liver and muscles, the disorders may affect both tissues. GSD types are grouped by the deficiency of a certain enzyme, and named with progressive numbers, as they were discovered [59]. The following study focused on GSD-I (von Gierke disease), with an overall annual prevalence of about 1 to 100000 subjects [35].

GSD-I is characterized by a defect in the glucose-6-phosphatase system, which is required for the hydrolysis of glucose-6-phosphate into glucose and inorganic phosphate [35]. This deficiency impairs free glucose availability during fasting and glucose homeostasis with consequent hypoglycemia.

The clinical onset of GSD-I usually occurs in the first year of life, with diverse symptoms such as fasting hypoglycemia, hepatomegaly, failure to thrive and growth retardation [59].

Two main subtypes of GSD-I are recognized: type Ia (GSD-Ia), responsible for 80% of cases of GSD-I, and type Ib (GSD-Ib) [35]. The two subtypes are metabolically and clinically identical but in addition patients with GSD-Ib suffer from neutropenia and neutrophil dysfunctions, and intestinal bowel disease (IBD).

Besides drug consumption such as ACE inhibitor and allopurinol, a life-long dietary treatment is the mainstay for the disease's management. The diet is based on small and frequent meals of complex carbohydrates, distributed during 24 hours, also with continuous feeding through nasogastric tube [59]. In this regimen, the use of raw corn starch is peculiar to provide a steady

intestinal release of glucose, and the reduction in sugar consumption prevents the conversion of fructose and galactose to glucose-6-phosphate.

Since the gut microbiota is strongly affected by both diet and drugs, GSD patients may present an alteration in the microbial community and a different production of SCFA.

To date, only a study has evaluated the intestinal microbial population in GSD patients, including different types of the disease such as type I, III and IX, compared with healthy controls [39].

The present research aimed to investigate and characterize the role of the peculiar diet of GSD-Ia and Ib patients on gut microbiome.

5.2 Manuscript 3

"Proteobacteria Overgrowth and Butyrate-Producing Taxa Depletion in the Gut Microbiota of GlycogenStorage Disease Type 1 Patients"

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Proteobacteria Overgrowth and Butyrate-Producing Taxa Depletion in the Gut Microbiota of Glycogen Storage Disease Type 1 Patients

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Abstract: A life-long dietary intervention can affect the substrates' availability for gut fermentation in metabolic diseases such as the glycogen-storage diseases (GSD). Besides drug consumption, the main treatment of types GSD-Ia and Ib to prevent metabolic complications is a specific diet with definite nutrient intakes. In order to evaluate how deeply this dietary treatment affects gut bacteria, we compared the gut microbiota of nine GSD-I subjects and 12 healthy controls (HC) through 16S rRNA gene sequencing; we assessed their dietary intake and nutrients, their microbial short chain fatty acids (SCFAs) via gas chromatography and their hematic values. Both alpha-diversity and phylogenetic analysis revealed a significant biodiversity reduction in the GSD group compared to the HC group, and highlighted profound differences of their gut microbiota. GSD subjects were characterized by an increase in the relative abundance of *Enterobacteriaceae* and *Veillonellaceae* families, while the beneficial genera *Faecalibacterium* and *Oscillospira* were significantly reduced. SCFA quantification revealed a significant increase of fecal acetate and propionate in GSD subjects, but with a beneficial role probably reduced due to unbalanced bacterial interactions; nutritional values correlated to bacterial genera were significantly different between experimental groups, with nearly opposite cohort trends.

Keywords: glycogen storage disease; GSD; diet; gut microbiota; short-chain fatty acids; inflammation

1. Introduction

The evidence of interplay between intestinal commensal bacteria and host physiological functions has hugely grown over the last years, shedding new light on clinical research on pathological conditions [1]. Among them, inherited metabolic disorders have been shown to be related to gut microbiota composition [2], possibly for the crucial role that diet plays both in patient treatment and in microbial metabolite production. Here, we present our study involving patients affected by glycogen storage diseases (GSD) following the vitally specific diet.

GSD are a group of hereditary metabolic disorders caused by the deficiency of one of the enzymes involved in glycogen metabolism. Glycogen is primarily stored in liver and muscle, and disorders of glycogen degradation may affect both tissues [3,4]. GSD types, grouped by the enzyme deficiency, were numbered as they were discovered, classifying them from GSD type I (von Gierke disease) to GSD type XI [5,6]. The present study focused our research on GSD-I, one of the most common types of glycogen storage diseases.

GSD-I results in a defect in the glucose-6-phosphatase system, which is required for the hydrolysis of glucose-6-phosphate into glucose and inorganic phosphate [7,8], impairing free glucose availability during fasting and glucose homeostasis with consequent hypoglycemia. The clinical onset of GSD-I usually occurs in the first year of life, during complementary feeding, with symptoms related to severe fasting hypoglycemia, hepatomegaly, failure to thrive and growth retardation. The overall annual incidence is about 1 to 100,000 subjects [9,10].

Two main subtypes of GSD-I are recognized: type Ia (GSD-Ia), due to a defect of the catalytic subunit glucose-6-phosphatase-α in the endoplasmic reticulum, and responsible for 80% of cases of GSD-I [3], and type Ib (GSD-Ib), due to a defect of the glucose-6-phosphate translocase, the transporter for the entrance of glucose-6-phosphate into the endoplasmic reticulum [9]. Patients with GSD-Ib may be clinically and metabolically identical to those with GSD-Ia (showing typical physical findings, including protuberant abdomen, truncal obesity, doll-like faces, short stature and hypotrophic muscles [6]), but in addition, most patients with GSD-Ib develop neutropenia and neutrophil dysfunction that predispose them to severe infections and to inflammatory bowel disease (IBD) [11,12]. Although the development of IBD is associated to GSD-Ib, few cases of IBD were recently reported in GSD-Ia [13,14]. Dietary treatment is the cornerstone of GSD-I therapy, and it starts at diagnosis and is life-long. This regimen is characterized by small frequent meals high in complex carbohydrates (preferably with high fiber content) distributed over 24 h [15], including the night, and/or continuous feeding through nasogastric tube [16]. Thus, over the total amount of daily energy intake, the carbohydrate consumption is 60–70%, while 10–15% of calories are derived from proteins and the remaining calories from fat [17,18]. Raw cornstarch is typically introduced between 6 months and 1 year of age [15], since its slow digestion can provide a steady intestinal release of glucose, maintaining more stable glucose levels over a longer period of time [19]. The restriction in sugar consumption is also crucial in the GSD-I diet, since fructose and galactose are metabolized to glucose-6-phosphate and can further contribute to the abnormal biochemical profile; in particular, to hyperlactacidemia [17,19].

The primary aim of the dietary treatment is not only avoiding hypoglycemia, but also achieving a good metabolic control [20], minimizing the secondary metabolic derangements and reducing long-term complications. In order to prevent or treat some clinical conditions (proteinuria, osteoporosis) or biochemical abnormalities (hyperuricemia, hyperlipidemia), patients also take medications/supplementations such as an ACE inhibitor, allopurinol, fibrate, oil fish, calcium and vitamin D3 [16]. GSD-Ib patients also assume granulocyte colony-stimulating factor (G-CSF) and anti-inflammatory drugs to treat neutropenia and IBD, respectively.

Since nutritional intake is one of the most relevant factors influencing the gut microbiota's composition [21], it is reasonable to expect that such a peculiar diet, along with the daily supplementations, could impact substrates' availability for microbial fermentation, affecting the production of metabolites; in particular, short chain fatty acids (SCFAs). SCFAs, mainly represented by acetate, propionate and butyrate, are the end products of microbial fermentation in the gastrointestinal tract [22]. Their production is heavily influenced by bacterial cross-feeding interactions, in which acetate and other small molecules (i.e., lactate and succinate) act as substrates to produce butyrate and propionate, respectively [23]. SCFAs are suggested to be involved in the maintenance of the gut barrier function and in the promotion of gut homeostasis [24]. To date, there is no information about gut bacterial metabolite production and consumption regarding GSD microbiota.

The aim of our study was thus to compare dietary macronutrient intake, gut microbial biodiversity and microbial metabolite production in patients with GSD-Ia/Ib and healthy subjects, in order to better evaluate and characterize diet or disease-related microbiome differences.

2. Results

2.1. Cohort Description

Overall mean BMI values for the enrolled subjects were 26.8 ± 4.8 for GSD patients and 21.6 ± 2.9 for healthy controls (HC) ($p = 0.0176$). Within the entire dataset, 3/21 resulted obese (3/9 GSD, 0/12 HC), 4/21 overweight (3/9 GSD, 1 of which <18 years; 1/12 HC), 14/21 normal weight (3/9 GSD, 11/12 HC).

All GSD patients were taking drugs to prevent disease-related comorbidities. The reported medications/supplementations were: allopurinol (Ia = 3/4; Ib= 5/5), antihypertensive drugs (Ia = 1/4; Ib = 4/5), triglyceride lower-drugs (Ia = 1/4; Ib = 2/5), salicylates (Ia = 0/4; Ib = 2/5), granulocyte-colony stimulating factor (Ia = 0/4; Ib = 3/5) and multivitamin and calcium with vitamin D (Ia = 4/4; Ib = 5/5).

Three GSD-Ib patients were reported to be neutropenic and to have IBD.

Fasting blood samples of GSD patients were analyzed for total cholesterol, triglycerides, insulin, glucose, uric acid, liver enzymes and lactate (Supplementary Table S1). GSD patients showed slightly increased alanine aminotransferase (ALT, mean ± SD: 54.1 ± 43.44 U/L) and aspartate aminotransferase (AST, 42.5 ± 23.8 U/L) values compared to physiological levels (0–35 U/L). In particular, GSD-Ia showed higher values in both parameters (54.5 ± 28.3 U/L and 67.7 ± 47.1 U/L, respectively). GSD-Ia patients showed higher values of both total cholesterol and triglycerides (265.5 ± 152.2 mg/dL) and 422.5 ± 241.6 mg/dL, respectively), compared to normal levels (< 200 mg/dl and < 150 mg/dl, respectively). Serum lactate was also increased in GSD-Ia patients (3.8 ± 1.9 mmol/L) compared to normal levels (0.7–1.15 mmol/L).

2.2. Dietary Assessment

The daily energy intakes and the diet macronutrient compositions of enrolled subjects are reported in Table 1.

Compared to HC, GSD group showed a significantly higher daily energy ($p = 0.0468$) and carbohydrate intakes (both grams and % total energy, $p = 0.002$), but a lower lipid intake (% of total energy, $p = 0.0013$) was observed. No significant differences were observed for proteins. As expected from the dietary recommendations, sugar consumption was reduced in the GSD group ($p = 0.0013$), whereas the starch intake was higher in GSD (mean ± SD: 110.27 g ± 44.80) compared to HC (180.94 g ± 62.81) ($p = 0.004$). Total fiber intake ($p = 0.0148$) and soluble fiber intake ($p = 0.0227$) were higher in GSD patients, whereas no significant differences were detected for the insoluble fraction.

Table 1. Nutritional values of the two enrolled groups.

Nutritional Variable	HC Mean ± SD	GSD Mean ± SD	*p*-Value	
Energy Intake				
kcals	1907 ± 603	2420 ± 549	0.0468	*
Proteins				
g	74.67 ± 22.92	83.06 ± 20.56	0.3824	
% energy	16.70 ± 3.77	13.80 ± 2.20	0.0815	
Lipids				
g	77.96 ± 47.06	62.35 ± 15.47	0.7021	
% energy	36.58 ± 10.65	23.40 ± 3.26	0.0013	**
Carbohydrates				
g	216.19 ± 54.55	390.03 ± 97.78	0.0007	***
% energy	46.28 ± 9.01	60.22 ± 4.54	<0.0001	****
Sugars				
g	58.56 ± 25.44	23.75 ± 9.11	0.0013	**
% energy	11.98 ± 4.74	3.56 ± 1.06	<0.0001	****
Fiber				
overall, g	15.44 ± 4.80	21.01 ± 4.37	0.0148	*
overall, g/1000 kcal	8.58 ± 2.16	9.10 ± 2.72	0.7021	
insoluble fiber, g	6.43 ± 4.44	9.59 ± 4.43	0.1285	
soluble fiber, g	2.01 ± 1.35	3.57 ± 1.23	0.0227	*

Values are expressed as means (with standard deviations). Significant differences are indicated by * (p-value < 0.05), ** (p-value < 0.01), *** (p-value < 0.001) and **** (p-value < 0.0001), Mann–Whitney test.

2.3. Microbiota Profiling

To avoid biases related to uneven sequencing depth (raw reads ranging from 56,150 reads to 350,680), samples were subsampled to 50,000 reads each by random picking. After quality filtering processes, we obtained a mean count of 40,988.261 reads per sample (total count of Operational Taxonomic Units (OTUs) for the entire dataset, average 1654 OTUs per sample).

As shown in Figure 1A, alpha-diversity showed a significant lower biodiversity within GSD subjects for each metric used (chao1, $p = 0.02$; observed species, $p = 0.02$; Shannon, $p = 0.002$; Faith's phylogenetic diversity, $p = 0.03$).

A clear difference among HC and GSD subjects was highlighted in beta-diversity as well (Figure 1B). Both unweighted and weighted Unifrac distances revealed a significant separation between groups (respectively, $p = 0.004$ and $p = 0.01$).

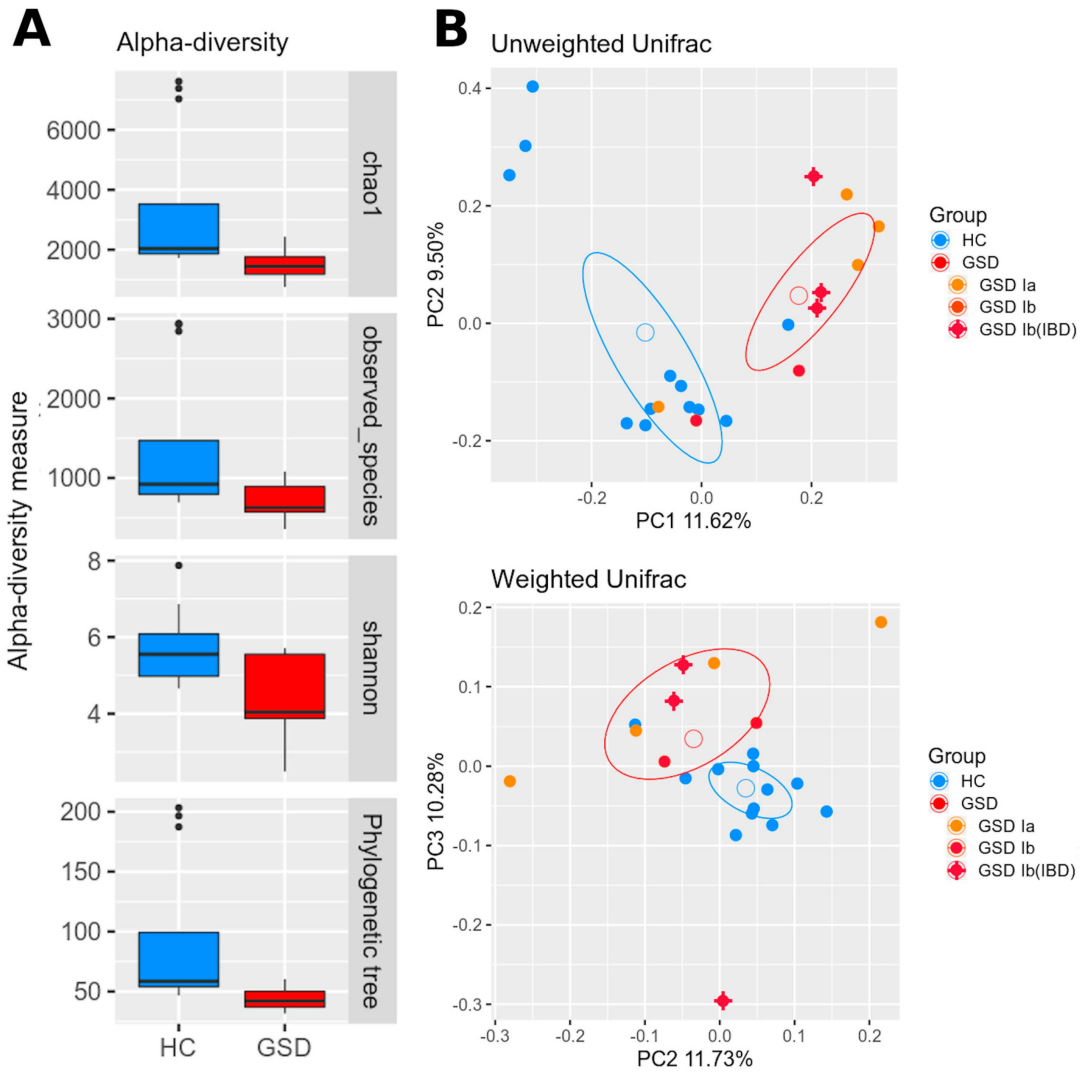

Figure 1. Biodiversity and phylogenetic analysis between cohorts. (**A**) Alpha-diversity indexes are reported for healthy control (HC) (blue) and glycogen storage disease (GSD) (red) subjects for chao1, observed species, Shannon diversity and Faith's phylogenetic metrics. Diversity among groups is statistically significant for all metrics. (**B**) Beta-diversity analysis represented by PCoA graphs of weighted and unweighted UniFrac distance between HC (blue) and GSD (red) subjects. The ellipses of mean standard error (SEM)-based data confidence are reported. Microbial communities are statistically different for both distances (adonis test: unweighted $p = 0.004$; weighted $p = 0.01$). Percentage variance accounting for the first, second and third principal components is shown along the axis. To highlight possible differences related to GSD type, a color scheme was further applied to the GSD group: GSD-Ia (orange), GSD-Ib (red), GSD-Ib with inflammatory bowel disease (IBD) (red + cross).

2.3.1. Taxonomic Characterization

We found several significant differences in taxas' relative abundances among the two groups across all phylogenetic levels.

At the phylum level (Figure 2A), differences were found in the relative abundance of Firmicutes (GSD 55.9% vs. HC 70%, although not significant) and Proteobacteria (GSD 17% vs. HC 1.4%, $p = 0.001$). Several dominant families were also significantly diverse in the two cohorts: *Ruminococcaceae* ($p = 0.002$), *Veillonellaceae* ($p = 0.030$) and *Enterobacteriaceae* ($p = 0.006$) (Figure 2B). Note, while *Ruminococcaceae* was more abundant among controls (40.3% vs. 15% GSD), both *Veillonellaceae* and *Enterobacteriaceae* were much higher among GSD patients (respectively, 13.8% and 16.3% compared to 4.7% and 1.1% among HC). As shown in Figure 2C, at the genus level GSD patients were severely and significantly depleted

in *Ruminococcus* (1.4% vs. 7.2% in HC; $p = 0.0173$), *Faecalibacterium* (7.4% vs. 19.6%; $p = 0.0209$) and *Oscillospira* (0.6% vs. 3.3%; $p = 0.0020$). In total, 1596 OTUs out of were classified as *Enterobacteriaceae* at the family level, and 792 of them were annotated as *Escherichia coli*. GSD patients were found significantly increased in *Escherichia coli* compared to HC (10% vs. 0.93%, $p = 0.0077$).

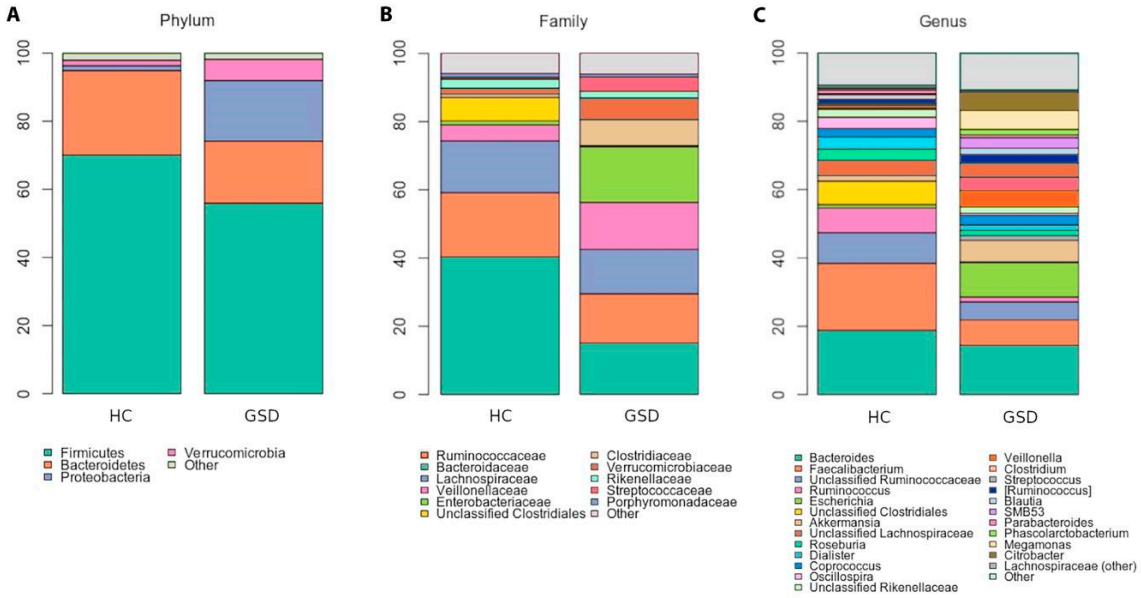

Figure 2. Taxonomic characterization. Stacked bar charts of taxonomy relative abundances at (**A**) phylum, (**B**) family and (**C**) genus levels for healthy controls (HC) and GSD patients. Only phyla, families and genera present at relative abundances >1% in at least 20% subjects (i.e.,: ≥ 4 samples) are reported. Remaining taxa are grouped in the "Other" category for each level.

All relative abundances and significant *p* values are reported in Table 2.

Table 2. Taxonomic relative abundance at the genus level.

Genus	Average Relative Abundance		*p*-Value
	HC	GSD	
Bacteroides	18.83	14.43	0.2410
Faecalibacterium	19.61	7.44	0.0209 *
Unclassified Ruminococcaceae	8.94	5.27	0.0700
Ruminococcus	7.25	1.42	0.0173 *
Escherichia	0.99	10.01	0.0077 **
Unclassified Clostridiales	6.87	0.29	0.0025 **
Akkermansia	1.63	6.26	0.2323
Unclassified Lachnospiraceae	4.48	1.41	0.0428 *
Roseburia	3.27	1.50	0.0428 *
Dialister	3.57	1.64	0.0360 *
Coprococcus	2.43	2.80	0.4138
Oscillospira	3.35	0.64	0.0020 **
Unclassified Rikenellaceae	2.35	1.87	0.1657

Table 2. *Cont.*

Genus	Average Relative Abundance		*p*-Value
	HC	GSD	
Veillonella	0.41	4.73	0.1265
Clostridium	0.76	3.94	0.4996
Streptococcus	0.51	4.16	0.1886
Blautia	1.40	1.83	0.4996
SMB53	0.29	3.05	0.0360 *
Parabacteroides	1.07	0.84	0.1658
Phascolarctobacterium	0.51	1.58	0.4946
Megamonas	0.00	5.64	0.0092 **
Citrobacter	0.07	5.33	0.3609
Bifidobacterium	0.23	0.70	0.7754
Other genera	10.68	13.41	–

The main genera in GSD patients and healthy controls, selected for <1% abundance in at least one of the two groups, are reported. All bacterial taxa present at less than 1% relative abundance were grouped into the "Other genera" classification. Significant differences are indicated by * (p-value < 0.05) and ** (p-value < 0.01).

2.3.2. Fecal Microbial Metabolites

Gas-chromatography analysis revealed an increased production of total fecal short chain fatty acid (SCFA) in GSD group ($p = 0.0159$). In particular, the concentration of acetate and propionate were higher in patients ($p = 0.031$ and $p = 0.038$, respectively), whereas the concentration of butyrate was similar in the two groups ($p = 0.8381$). No significant differences were found for the branched-chain fatty acids iso-valerate and iso-butyrate between the two groups (Supplementary Table S2).

2.3.3. Functional Prediction

At a broad functional level (level 2 KEGG), the functional analysis predicted an enrichment in genes encoding enzymes for amino acid metabolism ($p = 0.0094$); in particular, tryptophan ($p = 0.017$), glutathione ($p = 0.009$) and beta-alanine ($p = 0.0004$); and for lipid metabolism, alpha-linolenic acid especially ($p = 0.025$). Intriguingly and counterintuitively, the starch and sucrose metabolism pathways were significantly reduced in GSD subjects (respectively, $p = 0.026$).

2.4. Relationship between Microbial Population, Metabolite Content and Diet

2.4.1. Gut Microbiota and Fecal Microbial Metabolites

A correlation analysis was applied to investigate possible associations between SCFA concentration and bacterial taxa, as shown in Figure 3.

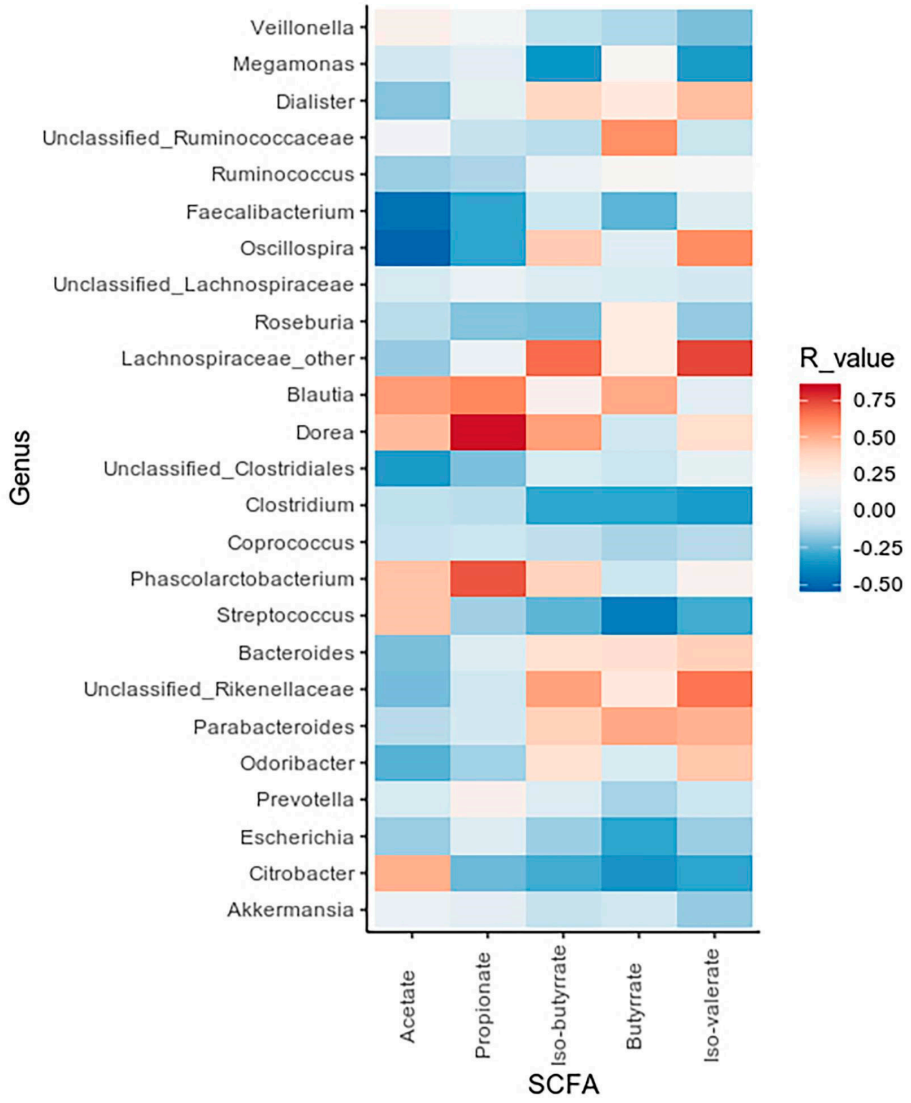

Figure 3. Correlation between SCFA values and bacterial genera. Heatmap showing Spearman's correlations between the most abundant microbial genera and SCFA concentrations. Red tiles indicate a positive correlation, blue tiles a negative one for both HC and GSD groups.

The obtained data revealed positive correlations between the *Blautia*, *Dorea* and *Phascolarctobacterium* genera, increased in GSD patients, and propionate concentration (R = 0.61, R = 0.82, R = 0.71, respectively). On the other side, we observed *Faecalibacterium* and *Oscillospira* (significantly decreased in GSD subjects) to be negatively related to acetate concentrations (R = −0.47, R = −0.51).

2.4.2. Impact of Diet on Microbial Taxa Relative Abundance

Correlations of nutritional parameters to bacteria abundances revealed several divergent relationships between the two cohorts, as shown in the Figure 4.

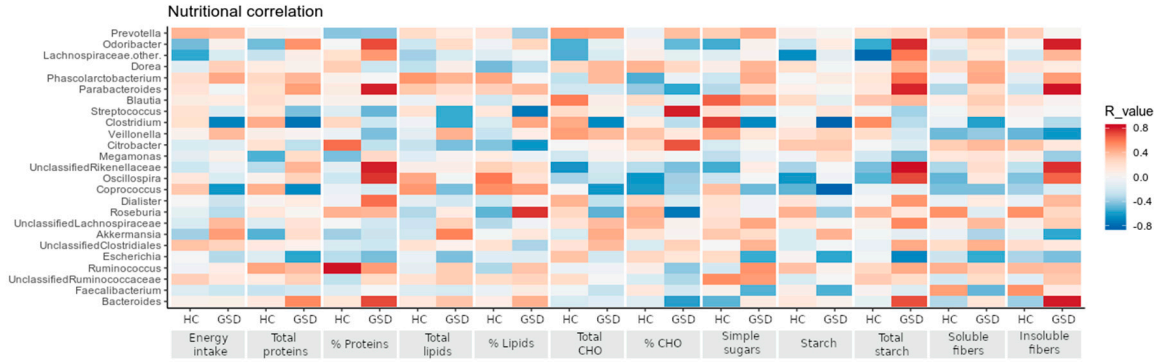

Figure 4. Correlations between nutritional and taxonomic values. Bacterial genera are reported in the same order of relative abundance as in Table 2, in correlation with the nutritional values shown in Table 1 according to Spearman's correlation. Red tiles indicate a positive correlation, blue tiles a negative one for both HC and GSD groups.

Fiber intake showed a strong positive correlation to *Odoribacter* and *Parabacteroides* genera (total fibers: R = 0.79, R = 0.78; insoluble fibers: R = 0.79, R = 0.81) only in GSD patients, whereas a milder, opposite trend characterized the HC group. In GSD patients, starch intake positively correlated with *Veillonella*, *Citrobacter* and *Akkermansia* genera (R = 0.299, R = 0.334 and R = 0.406, respectively) and negatively with *Coprococcus* and *Clostridium* genera (R = −0.826 and R = −0.823, respectively). The latter two genera, in particular, showed an opposite trend with nutritional values between the two groups (correlated positively to HC and negatively to GSD patients).

3. Discussion

Our study investigated the impact of the life-long cornstarch-rich diet characterizing the treatment of GSD patients by integrating gut microbiota, microbial metabolites and nutritional data. The identification of bacterial metabolism is crucial for the understanding of a possible microbial role in metabolic diseases. For this reason, we highlighted the importance of short chain fatty acids in gut microbiota characterization as bacteria cooperate and feed one on another's products (cross-feeding). To our knowledge, this is the first study evaluating the impact of the cornerstone diet of GSD-I on gut microbial cross-feeding and metabolites production.

During the last decade, a healthy gut microbiota has been typically characterized by members of the phyla Bacteroidetes and Firmicutes, and their genera are believed to be the main responsible bacteria for positive biodiversity in the human gut [25], as their balanced abundances and metabolite productions protect the intestinal trait, help digestion and modulate the host innate immune system [26]. In agreement with Colonetti and coworkers [27] that have analyzed the gut microbiota of different types of GSDs, we found a strong reduction in intestinal microbiota richness and diversity compared with healthy controls and a dramatic increase in the phylum Proteobacteria.

Though the GSD diet is enriched in starch and fibers, usually considered good substrates promoting beneficial microbes' growth, Proteobacteria, in particular, the *Enterobacteriaceae* family, have been suggested to exert pro-inflammatory activity both locally, at the gastrointestinal mucosa level, and systemically [28]. In turn, an inflamed gut seems to constitute a commending environment for proliferation of *Enterobacteriaceae* bacteria [29], and it is also characterized by a depletion of obligate anaerobes, typically recognized as fiber-degrading bacteria [30]. Although GSD-Ib are genetically at risk of intestinal bowel inflammation [16] and three GSD-Ib patients in our dataset were indeed affected, the enrichment in the relative abundance of *Escherichia coli* spanned both type Ia and Ib patients. This data could account for the increased abundance in genes for glutathione metabolism in GSD, since *E. coli* accumulates the tripeptide in order to protect itself from chemical and environmental stress [31]. Despite the high amount of starch in GSD diet, we predicted a reduction of the starch and sucrose metabolism genes, which could be linked to a possible intestinal imbalance caused by both

the Proteobacteria's abnormal abundance and the decrease of obligate anaerobes. Within an inflamed environment, the availability of simple sugars could be altered, and bacteria forced to exploit other nutritional sources such as amino acids. *Enterobacteriaceae*, as seen for the strain *E. coli* LF82, associated to Crohn's disease, seem to be able to catabolize dietary L-serine in order to maximize their growth [32]. On the other hand, GSD group showed increased amino-acid metabolism genes compared to HC, suggesting that *Enterobacteriaceae* may contribute to the increment of this pathway. Other taxonomic indicators of inflammatory status in GSD patients were the enrichment of the *Blautia* genus, known to stimulate cytokines secretion by host cells [33], and the significant depletion of *Oscillospira* and *Faecalibacterium* species. Data about the observed relative abundance of *Faecalibacterium* spp. and *Escherichia* spp. are in agreement to what Grabherr and colleagues [34] have observed in non-alcoholic steatohepatitis (NASH). Of note, both GSD and NASH are affected by liver damage, and the elevated ALT values found in our patients' blood samples confirms this similarity.

The depletion in *Faecalibacterium* and *Oscillospira* spp. is a hallmark for patients gut microbiota alteration, as these genera are considered as biomarkers of intestinal and host wellness. *Faecalibacterium* spp. has the ability to produce anti-inflammatory molecules [35], and also a specific protein able to block NF-κB pathway [36]. The genus *Oscillospira* has been found to be constantly reduced in inflammatory diseases as well as *Faecalibacterium* spp. with decreased abundances in Crohn's disease, both colonic and ileal [37], and in pediatric nonalcoholic steatohepatitis [38]. Moreover, several studies associated *Oscillospira* spp. to lower BMI and leanness-promoting bacteria such as *Christensenella minuta* [39,40]. Our data confirmed these observations, since we observed a higher prevalence of obesity/overweight in GSD cohort compared to HC, and a depletion of *Christensenella* spp. in the patients' gut. Colonetti and coworkers [27] did not observe changes in *Faecalibacterium* and *Oscillospira* relative abundances in their dataset, and found *Blautia* spp., enriched in our cohort, to be depleted in GSD patients. These differences could be ascribable to the multiplicity of GSDs (type Ia/Ib, III, IX vs. type Ia/Ib), the use of antibiotics before sampling (10/24 subjects vs. 0/9), the sequencing method used (Ion Torrent vs. Illumina MiSeq) and the database used for OTU processing (SILVA vs. Greengenes).

Byndloss and coworkers demonstrated, in vitro and in vivo, the existence of a vicious cycle encompassing the depletion of butyrate-producing microbes and the increase of *Enterobacteriaceae* in the gut microbiota [41]. Indeed, antibiotic-driven reduction of *Lachnospiraceae* and *Ruminococcaceae*, major butyrate producers, promotes the use of glucose instead of butyrate by colonocyte. This metabolic switch-anaerobic glycolysis-fails to suppress host-derived nitrate and oxygen production, promoting the growth of facultative anaerobes such as *Enterobacteriaceae*. On the other hand, the decrease of butyrate downregulates Tregs and epithelial PPAR-γsignaling further promotes the epithelial dysfunction.

We conducted this research considering that the bioavailability of substrates introduced with the diet drives the gut's microbial composition, and consequently, alterations in intestinal microbiota can lead to a different production of microbial metabolites.

For instance, the important role *Faecalibacterium* spp. plays in gut microbiota is directly linked to the production of butyrate, the main energy source for enterocytes with a protective role in colorectal cancer and in IBD [42]. On the other hand, the decrease of these bacteria in GSD gut microbiota did not result in a reduction of fecal butyrate concentrations, found in similar amounts in GSD and controls, whereas it could have caused the higher acetate quantities, since this fatty acid has been less used in fermentation reactions. The negative correlation we found between *Faecalibacterium* spp. and acetate concentrations leads in that direction.

Compared to HC, GSD patients were observed to have a higher concentration of total SCFAs; in particular, acetate and propionate. Those SCFAs have key-roles in gut microbial composition: for instance, acetate production is strongly regulated by the cross-feeding within the gut microbial community. Indeed, Samuel and Gordon [43] underlined that the co-colonization with *Bacteroides thetaiotaomicron*/*Methanobrevibacter smithii* increased serum acetate levels compared to *B. thetaiotaomicron* alone in gnotobiotic mice.

The higher acetate concentration can be ascribed to several intestinal bacteria found to be more abundant in GSD gut microbiota, including *Akkermansia muciniphila* and *Bifidobacterium* spp., which produce acetate by fermenting acetogenic fibers, and to a lesser extent, protein-derived peptides [44].

As well as acetate, propionate seems to exert protective and anti-inflammatory activities in IBD, ameliorating the intestinal mucosa lesion [45]. The higher propionate concentration is in agreement with the enrichment in *Veillonellaceae* family among GSD patients [46]; the genus *Veillonella* was found positively related to starch intake in patients, probably because of their peculiar diet. GSD patients were also enriched in *Megasphaera* genus, which is able to produce propionate from lactate through acrylate pathway [46].

Considering the opposite trends observed between GSD patients and HC, our results indicate that GSD patients have an ongoing alteration in gut microbial community cross-fed by increased pro-inflammatory genera and decreased beneficial bacteria. The specific dietary treatment does not seem to help the composition of gut microbial community in patients, as the anti-inflammatory genera were depleted and not sufficient to counterbalance the dysbiosis. Probiotics supplementation could offer another way to improve and ameliorate the gut microbial population in GSD patients. Carnero-Gregorio and colleagues [14] recently reported a prospective case study pointing toward this direction: by testing a probiotic mixture in a patient with GSD-Ia and Crohn-like IBD, the authors observed a reduced number of bowel episodes and an improvement the patient's quality of life. Moreover, they found a reduction in *Enterobacteriaceae* relative abundance after the probiotic treatment.

Since patients are commonly taking multiple drugs to cope with the variety of comorbidities characterizing glycogen storage diseases, we tried to evaluate their possible impacts on the microbial community. Indeed, multi-drug usage has been reported to impact microbial composition and richness, but it is difficult to assess whether the observed alterations are caused by the high number of drugs or by the disease itself, forcing the patient to take all these medications [47]. Whereas few drugs seem to have a direct effect on the microbiota, i.e., metformin or proton pump inhibitors, the association of multiple compounds is not clearly associated to the depletion or enrichment of specific taxa. All the patients but one were taking allopurinol, a common urate-reducing drug. In a rodent model of hyperuricaemia, its use was associated with an increase in the relative abundance of *Bifidobacterium* spp. [48]. In our GSD patients, we did observe a slight increase in this genus, but this observation should be confirmed in a bigger cohort.

In conclusion, we believe that our study could pave the way for further investigations of the intestinal bacterial community in GSD type Ia and Ib patients and in similar metabolic syndromes. In the frame of glycogen storage diseases, studies evaluating gut microbiota differences in large multicentric cohorts are needed to expand our results obtained by a cohort limited by the rarity of the disease, albeit homogeneous. Nevertheless, our study showed how profoundly gut microbiota can be modulated by a life-long diet. Importantly, future studies should aim at clarifying whether the observed changes are driven by nutritional parameters only or also by the disease itself.

4. Materials and Methods

4.1. Subject Recruitment and Sampling

For this study, 9 GSD type I patients (Ia = 4, Ib = 5) and 12 healthy controls (HC) were enrolled from January 2018 to June 2018. The dataset consisted of 21 subjects, gender and age matched between groups. Mean age of GSD patients was 27.7 ± 12.5 years (6 males and 3 females), while mean age of HC was 24.7 ± 7.9 years (9 males and 3 females). Despite only six subjects, three GSD and three HC, being of pediatric age, all the enrolled subjects in the study were followed by the Pediatric Department of San Paolo Hospital, reference center for metabolic diseases in Milan (Italy). For both patients and controls, inclusion criteria were: gestational age 37–42 week inclusive, birth weight ≥ 2500 g and single birth; exclusion criteria were: treatments with antibiotic and/or probiotic/prebiotic assumption during the previous 3 months.

Specific GSD inclusion criteria were: disease clinical onset during childhood and the diagnosis confirmed by liver biopsy (% hepatic glycogen and glucose-6-phosphatase enzymatic activity assay); dosage of deoxyglucose transport in polymorphonuclear neutrophils (only in patients with GSD Ib) and/or molecular analysis of GSD; to be on treatment with CS; not to have type I/II diabetes.

Stool samples, stored at −80 °C until use, were collected for each subject. Pediatricians performed anthropometric measurements (height, weight) and body mass index was calculated; the nutritional weight status was evaluated through the WHO classification of underweight, overweight and obese adult for patients >18 years [49], while for patients ≤ 18 years standard scores (z-scores) of BMI were calculated and evaluated using WHO reference standard [50].

Furthermore, a 24-h food recall was provided by patients themselves or by parents to collect dietary data. Dietary food records were processed by dieticians in order to calculate the average amounts of energy and nutrient intake (carbohydrates, soluble glucids, starch, soluble and insoluble fibers, lipids, proteins) using commercially available software (MetaDietaR, Software version 3.1, ME.TE.DA S.r.l., San Benedetto del Tronto, Italy). For each subject, the use of drugs was also evaluated.

In addition, in conjunction with the stool collection, we collected biochemical data of GSD patients from their routine check-up. The metabolic parameters evaluated were: glycemia, insulin (with HOMA-IR, HOMAβ, QUICKI and Tyg-Index calculation), total cholesterol, triglycerides, uric acid, lactate and transaminases. The HOMA-IR (homeostasis model assessment of insulin resistance) was calculated as follows: [basal blood glucose (mg/dl) × basal insulin (IU/mL)]/405; the QUICKI (quantitative insulin sensitivity index) was calculated as follows: 1/[log10 insulin (μUI/mL) + log10 basal blood glucose (mg/dL)]; HOMAβ (Homeostatic Model Assessment of β-cell function) was calculated as follows: 20 × basal insulin (μUI/mL)/[basal blood glucose (mmol/L) -3.5] [51]; the Tyg-index (triglycerides and glucose index) was calculated as follows: Log [triglycerides (mg/dL) × glycaemia (mg/dL)/2] [52].

The study was conducted at the Pediatric Department of San Paolo Hospital in Milan, with the previous approval by Ethics Committee of San Paolo Hospital in Milan (Comitato Etico Milano Area 1, Protocol number 2017/ST/13749); written informed consent was obtained from each enrolled subject.

4.2. Gut Microbial DNA Extraction and Sequencing

Fecal DNA extraction was performed using the Spin stool DNA kit (Stratec Molecular, Berlin, Germany), according to manufacturer's instructions. For each sample, 25 ng of extracted DNA was used to construct the sequencing library.

The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with a two-step barcoding approach according to the Illumina 16S Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA, USA). Briefly, DNA samples were amplified with dual-index primers using a Nextera XT DNA Library Preparation Kit (Illumina) and library concentration and quantification were determined using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) and Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA), respectively. The libraries were pooled and sequenced with a MiSeq platform (Illumina) for 2 × 250 base paired-end reads and a total of 2.5 Gbases raw reads were obtained.

4.3. Microbiota Profiling

The obtained 16S rRNA gene paired sequences were merged using Pandaseq [53] (release 2.5). Reads were filtered by trimming stretches of 3 or more low-quality bases (quality < 3) and discarding the trimmed sequences whenever they were shorter than 75% of the original one.

Bioinformatic analyses were conducted using the QIIME [54] pipeline (release 1.8.0), clustering filtered reads into Operational Taxonomic Unit (OTUs) at 97% identity level and discarding singletons as possible chimeras. Taxonomic assignment was performed via the RDP classifier [55] against the Greengenes database [56] (v 13_8).

Alpha-diversity was computed using the chao1, number of OTUs, Shannon diversity and Faith's phylogenetic diversity whole tree (PD whole tree) metrics through the QIIME pipeline; statistical evaluation among alpha-diversity indices was performed by a non-parametric Monte Carlo-based test, using 9999 random permutations. Weighted and unweighted UniFrac distances and Permanova (adonis function) in the R package vegan [57] (version 2.0–10) were used to compare the microbial community structure of GSD and HC subjects. A functional prediction of the bacterial metabolic pathways was performed using PICRUSt software [58] (version 1.0.1) and KEGG pathways database [59]. Differences in functional category profiles between breeds were assessed using Bray–Curtis distance among samples and "adonis" permutation-based test on the experimental labels.

4.4. Fecal Short Chain Fatty Acids Measurement

Fecal short chain fatty acids (SCFAs) quantification was performed by gas chromatography. Concentrations of acetic, propionic, iso-butyric, butyric and iso-valeric acids were assessed as previously described [60].

Briefly, analyses were performed using a Varian model 3400 CX Gas chromatograph fitted with FID detector, split/splitless injector and a SPB-1 capillary column (30 m × 0.32 mm ID, 0.25 µm film thickness; Supelco, Bellefonte, PA, USA). Quantification of the SCFAs was obtained through calibration curves of acetic, propionic, iso-butyric, butyric, and iso-valeric acid in concentrations between 0.25 and 10 mM (10 mM 2-ethylbutyric acid as internal standard). Results are expressed as mg/g of wet weight of feces.

4.5. Statistical Analysis

All comparisons were performed using MATLAB software (Natick, MA, USA; version 2008b).

Comparisons of the two groups were performed using Student's t-test for normally distributed variables and Wilcoxon test for non-normally distributed variables. For evaluating differences in relative abundances of bacterial groups, a Mann–Whitney U-test was performed. Due to the small number of samples, no correction methods have been applied.

Correlations between taxa and nutritional values and SCFA quantities were assessed through Spearman correlation and the associated linear regression model. p-values < 0.05 were considered as significant for each analysis.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-1989/10/4/133/s1. Table S1: Blood biochemical parameters in GSD patients. Table S2: Fecal SCFA concentrations. Raw reads are available in NCBI Short Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA614988.

Author Contributions: E.B. and E.V. conceived and designed the experiments; G.B. and E.O. performed NGS experiments and dietary assessment; M.C.C. performed short-chain fatty acids quantification; C.C. performed bioinformatics analyses; G.B., C.C. and E.B. analyzed the data; C.M. and S.P. contributed to clinical evaluation; E.B., G.B. and C.C. wrote the paper; G.M., G.B. and E.V. provided guidance in the manuscript revision. All authors have read and agreed to the published version of the manuscript

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5.3 Discussion and Conclusion

According to our study, a clear alteration of gut microbiome is present in patients affected by glycogen storage disease type I (GSD I) compared with healthy controls.

To evaluate the impact of a cornstarch-rich diet (treatment of GSD I), we anayzed gut microbiota, SCFAs and nutritional data.

Firstly, a reduction in gut microbiota richness and diversity and a strong increase in *Proteobacteria* were found in GSD I patients. Despite the GSD diet is characterized by high amount of starch and fibers, typically considered as good substrates for microbial fermentation and promotion of beneficial microorganisms' growth, GSD gut microbiota showed the characteristics of a dysbiosis.

Proteobacteria, notably the *Enterobacteriaceae* family, represent a hallmark of inflammation-associated dysbiosis and have been demonstrated to exert pro-inflammatory activity [60]. Even if GSD Ib are genetically predisposed to intestinal bowel inflammation and three GSD patients in our dataset are affected, the enrichment in *Enterobacteriaceae* and *Escherichia* spp. spanned the entire GSD group.

Other taxonomic indicators of an inflamed gut were the enrichment of the genus *Blautia* and the depletion of *Faecalibacterium* spp. and *Oscillospira* spp. [57], [61]. Indeed, the reduction of these beneficial bacteria is a hallmark for gut microbiota alteration, since they are considered as biomarkers of gut and host wellness.

SCFA quantification showed higher concentrations of acetate and propionate in GSD patients compared with healthy controls, whereas butyrate amounts were similar in the two groups. Indeed, the decrease of *Faecalibacterium* spp. did not result in a reduction of butyrate but it might be responsible for the

higher amount of acetate that is not used as microbial substrate to produce butyrate [62]. To confirm this hypothesis, a negative correlation between *Faecalibacterium* spp. and acetate was found.

The higher acetate concentration is ascribable to several gut bacteria, such as *Akkermansia muciniphila* and *Bifidobacterium* spp., found more abundant in GSD patients and able to ferment acetogenic fibers [62].

The higher quantity of propionate is in agreement with the enrichment of the genera *Veillonella* and *Megasphaera*, able to form this fatty acids through different pathways [22].

Our study indicates an ongoing alteration in gut microbiome of GSD patients. The dietary treatment does not help the composition of gut microbial population, as beneficial microbes were depleted and not sufficient to restore the dysbiosis. Probiotic supplementation may offer a different way to improve and counterbalance the gut microbiota in GSD patients.

CHAPTER 6

Conclusions and Perspectives

Alterations in the gut microbiota have been associated with the development of human pathologies, like obesity, cardiovascular diseases, as well as inflammatory gastrointestinal diseases [20].

The changes in gut microbial population are strongly affected by diet, and this reflect the microbial metabolites production [63]. In several diseases, especially inherited metabolic diseases, diet represents the cornerstone of treatment and starts as soon as the diagnosis is made. It has been demonstrated that life-long dietary managements can affect the availability of substrates for microbial fermentation.

In PKU and in GSD type I, an ongoing alteration of the gut microbiota and, consequently, a modification in the SCFAs production have been suggested [38], [39].

Indeed, according to our studies, two Brazilian studies investigated the intestinal microbial community both in PKU and in GSD, highlighting a different composition in gut microbiota of patients compared with healthy controls.

Whether controlled dietary interventions cannot be assessed since diet is the treatment, prebiotics and probiotics may represent a way to restore the gut microbial community.

Prebiotics and probiotics exert physiological functions by acting on gut microbial homeostasis [11]. Future managements for human diseases might be based on the modulation of gut microbiota with prebiotics and probiotics.

Several prospective studies should be performed to better understand the role of gut microbiota in disease's development and to determine if the gut microbiota is altered before or after the onset of the disease. An intensive and continuous use of the omics sciences (metagenomics, proteomics and metabolomics) can help in this direction [3]. Indeed, the omics should be

combined to guarantee and offer a network of correlations between gut microbiota and host.

A greater study of gut microbiota may provide new diagnostic biomarkers for human health, particularly for pathologies where there is actually no cure. Interventions on gut microbial community, able to restore the dysbiosis, may represent a possible alternative target in metabolic diseases.

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