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

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Running Head: Hyperlipidemia-associated fecal microbiota

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Abstract

Hyperlipidemia starts at a pediatric age and represents an unquestionable risk factor for cardiovascular disease. Modulation of the intestinal microbial ecosystem (IME), in principle, can ameliorate lipid profiles. In this study, we characterized the IME of children and adolescents with primary hyperlipidemia by analyzing fecal samples through 16S rRNA gene profiling (n=15) and short chain fatty acid (SCFA) quantification (n=32). The same analyses were also carried out on age-matched normolipidemic controls (n=15). Moreover, we evaluated the modulatory effect of regular hazelnut intake (approximately 0.43 g of hazelnuts with skin per kg of body weight) on the IME of 15 children and adolescents with hyperlipidemia for eight weeks. We found alterations of numerous operational taxonomic units (OTUs) potentially associated with SCFA-producing bacteria and reductions in the fecal levels of acetate, butyrate and propionate in hyperlipidemic subjects. Furthermore, we observed that an eight-week hazelnut intervention may induce limited changes in fecal microbiota composition but can significantly modulate the fecal levels of predominant intestinal SCFAs, such as acetate. Finally, correlation analyses indicated that changes in lipidemic parameters are linked to modifications of the abundance of specific bacterial taxa, such as the families Lachnospiraceae and Ruminococcaceae and the genera Akkermansia, Bacteroides, Roseburia, and Faecalibacterium. This study suggests that children and adolescents with primary hyperlipidemia possess an altered IME. The promising results presented here support the need for future dietary interventions aimed at positively modulating the IME of hyperlipidemic subjects.

Key words: intestinal microbiota, 16S rRNA gene profiling, short chain fatty acids, hazelnuts, primary hyperlipidemia, children.
**Background**

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

Dietary interventions appear to be a promising strategy for managing premature hyperlipidemia. For instance, the intake of a fiber-containing food supplement was shown to reduce total cholesterol, LDL-C levels, and apolipoprotein B in hypercholesterolemic children (Guardamagna et al. 2013). In addition, daily consumption of yogurt supplemented with phytosterols modulated lipid profiles by reducing LDL-C (Guardamagna et al. 2011a). In another trial, a probiotic formulation resulted in decreased LDL-C and improvements in triglyceridemia and HDL cholesterol (HDL-C) levels (Guardamagna et al. 2014). Mechanisms that support the potential efficacy of the abovementioned interventions include the suppression of liver cholesterol synthesis (Guardamagna et al. 2011b), reduction of intestinal cholesterol absorption (Guardamagna et al. 2011a), and production of conjugated linoleic acid in the gut (Guardamagna et al. 2014).

In principle, modulation of the gut microbiota could represent an additional strategy for the amelioration of lipid profiles. In fact, expanding scientific evidence indicates that the gut microbiota mediates pathophysiological mechanisms that alter lipid metabolism and other related metabolic traits (Ghazalpour et al. 2016). Particularly, the intestinal microbiota has been recognized as a metabolically active endocrine organ of the human superorganism that can be a therapeutic target for hyperlipidemia and associated cardiometabolic diseases (Brahe et al. 2016; Ghazalpour et al. 2016). In fact, numerous metabolites produced by intestinal bacteria, such as trimethylamine/trimethylamine-N-oxide, secondary bile acids, catecholamines, and short chain fatty acids (SCFAs), are not simply absorbed and metabolized by human cells for energy needs but also...
act as hormone-like factors that impact numerous aspects of host physiology (Boulange et al. 2016; Brown and Hazen 2015). Particularly, SCFAs, including acetate, butyrate, propionate and valerate, in addition to being used an energy source by the colonic epithelium (in particular, butyrate), are sensed by dedicated receptors on numerous diverse cell populations around the human body, resulting in the regulation of energy metabolism, insulin sensitivity, and immune responses (Brown et al. 2003; Brown and Hazen 2015; De Vadder et al. 2014; Gao et al. 2009; Kimura et al. 2013; Saemann et al. 2000).

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

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

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

Participants. The volunteers considered in the present study were derived from a subgroup of children and adolescents with primary hyperlipidemia; they were aged between 7 and 17 years old and were participants in a national project aimed at characterizing this pediatric population (Deon et al. 2017b) and investigating the effect of regular intake of hazelnuts with skin for eight weeks on several markers related to lipid metabolism and oxidative stress (Deon et al. 2017a; Deon et al. 2017b). The trial was registered at ISRCTN.com (identifier no. ISRCTN12261900). Subjects participating in the intervention study were recruited among patients receiving care at the Department of Health Science and Pediatrics at the University of Turin (Italy) after a screening for eligibility. In addition, 15 age-matched normolipidemic volunteers were recruited in the same area and time frame as the controls for microbial ecology analysis of fecal samples. The age and sex of the participants are shown in Table S1.

The recruited hyperlipidemic subjects were asked to collect a stool sample before and after the intervention. Thirty-two stool samples were collected for analysis at baseline, but only 15 samples were available after eight weeks of hazelnut with skin (HZN+S) consumption. The effect of dietary intervention on the fecal microbiota composition was analyzed in 15 subjects, whereas the levels of SCFAs were evaluated in the whole group of children and adolescents with primary hyperlipidemia who provided stool samples at baseline.

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

**Experimental design.** All enrolled patients were following nutritional recommendations suggested for pediatric hyperlipidemia based on the *cardiovascular health integrated lifestyle diet* (CHILD-1) (Expert Panel on Integrated Guidelines for Cardiovascular et al. 2011). Nutritional recommendations were as follows: 55% of daily energy intake from carbohydrates, 15% from proteins, and 30% from fats (saturated fat 7-10%). Additional recommendations were that dietary cholesterol should be lower than 100 mg/1000 kcal (and no more than 300 mg/day) and soluble fiber intake should be 10-25 g/day.
Dietary intervention consisted of an 8-week intake of HZN+S. Subjects were provided with pre-
weighed vacuum-packed portions of Italian roasted Corylus avellana L. (cultivar ‘Tonda Gentile
delle Langhe’ from Piedmont, Italy). The quantity of hazelnuts per packet was calculated by
considering doses generally advised for adults and were adjusted to the children’s body weight
(approximately 0.43 g kg\(^{-1}\) body weight up to a maximum of 30 g, which is the recommended daily
dose for an adult). All participants were encouraged to maintain the same dietary pattern and
lifestyle habits throughout the 8-week intervention study. Subjects were required to exclude the
intake of other nuts, dried fruits, probiotic or prebiotic foods or supplements from one month before
the beginning of the study until the end of the experiment. Traditional yogurt was allowed. To
check compliance to dietary recommendations, subjects and their families were asked to fill in
weekly food diaries as previously reported (Deon et al. 2017a).

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

The fecal samples were collected from each participant in a sterile plastic pot within 24 h before
visits at baseline and following the HZN+S intervention. A single fecal sample was also collected
from 15 age-matched controls. According to the recommendations for “storage conditions of
intestinal microbiota matter in metagenomic analysis” (Cardona et al. 2012), participants were asked
to deliver the fecal sample to the laboratory within 24 h. Upon delivery, stool samples were stored at
-80°C until DNA extraction. Subjects were asked to return any uneaten HZN+S packages at the visit.
Compliance was assessed by weighing returned packages and checking weekly food diaries.
Bacterial profiling of fecal microbiota. The bacterial community structure of fecal samples was determined by 16S ribosomal RNA gene profiling with Illumina MiSeq System at the Center for life – Nanoscience, Istituto Italiano di Tecnologia (Roma, Italy). Briefly, metagenomic DNA was extracted from 200 ± 10 mg of stool within 30 days from delivery by means of a PowerFecal DNA Isolation Kit (Mo Bio Laboratories, Cabru s.a.s., Biassono, Italy) according to the manufacturer’s specifications. A DNA fragment encompassing the V3 and V4 regions of the 16S rRNA gene was amplified from metagenomic DNA with the primer pair selected by Klindworth et al. in (Klindworth et al. 2013). The sequencing runs were multiplexed and barcode sequences were used to discriminate the samples. 16S rRNA gene sequence reads were analyzed through the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0 using the last version of Greengenes (gg_13_5) as reference taxonomic database. We used a reference picking to cluster and assign the taxonomy to the reads. We add this information in the section Materials and Methods as suggested. Analysis of the α- and β-diversities were performed using QIIME. For α-diversity inverse Simpson, Chao1 and Shannon indexes were calculated. Concerning β-diversity, the UniFrac algorithm was used to obtain the phylogenetic information needed to generate the dissimilarity matrix. Bacterial abundances in each fecal sample were determined at the operational taxonomic unit (OTU) level. Sequence reads from 16S rRNA gene profiling have been deposited in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under accession code PRJEB10296.

Quantification of fecal short chain fatty acids (SCFAs). SCFAs were quantified in the fecal samples collected from 32 subjects, including the 15 who completed the intervention trial. SCFA quantifications were performed as previously described (Gargari et al. 2016). In brief, stools (200 ± 10 mg) were extracted in 10 ml of 0.001% HCOOH by vortexing for 1 min. The supernatant was then recovered through centrifugation at 1000 x g for 2 min at 4 °C. All extracts were stored at -20 °C until UPLC-HR-MS analysis, which was carried out on an Acquity UPLC separation module.
(Waters, Milford, MA, USA) coupled with an Exactive Orbitrap MS with an HESI-II probe for electrospray ionization (Thermo Scientific, San Jose, CA, USA). The ion source and interface conditions and other detailed information were as specified in (Gargari *et al.* 2016). The eluents were 0.001% HCOOH in MilliQ-treated water (solvent A) and CH$_3$OH:CH$_3$CN (1:1, v/v, solvent B); UPLC elution gradient: 0% B for 4 min, 0-15% B in 6 min, 15–20% B in 5 min, 20% for 13 min, and then return to initial conditions in 1 min. The UPLC eluate was analyzed in full-scan MS in the range 50–130 m/z. The resolution was set at 50 K, the AGC target was 1E6, and the maximum ion injection time was 100 ms. The MS data were processed using Xcalibur software (Thermo Scientific). Five-point external calibration curves prepared with analytical grade SCFAs (Sigma-Aldrich, Milan, Italy) were adopted to quantify acetate, butyrate, isobutyrate, isovalerate, lactate, propionate, pyruvate, succinate, and valerate in fecal samples. SCFA concentrations were expressed in millimoles per kilogram of wet feces.

**Statistics.** Statistical analyses were performed using R statistical software (version 3.1.2). A 0 (zero) was given to any taxon that was not detected in a specific sample to enable comparisons. All statistical tests were performed considering three study populations: (i) hyperlipidemic subjects before HZN+S intake, (ii) hyperlipidemic subjects after HZN+S intake and (iii) normolipidemic subjects as controls. Differences in the microbiota composition of these populations were determined using a Wald test following DESeq2 (paired between the comparison of hyperlipidemic subject populations) read count normalization. After DESeq2 normalization, only OTUs with a number of reads higher than 5 in at least one quarter of the samples were considered for statistical analysis. Significant differences between groups of samples in β-diversity were assessed through the non-parametric statistical test ANOSIM (analysis of similarities). Differences in SCFA concentrations were evaluated by analyzing the data with a non-parametric Wilcoxon-Mann-Whitney test using paired data (when allowed).
The correlation analyses were carried out using Kendall and Spearman formulas with changes over the treatment in bacterial taxa abundances (DESeq2-normalized data), lipidemic profile data and fecal SCFA concentrations in hyperlipidemic subjects.

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

RESULTS

**16S rRNA gene profiling and SCFA quantification revealed significant differences in the fecal microbial ecosystem between hyperlipidemic and control subjects.** A total of 13,830,110 filtered high-quality sequence reads was generated (average of 153,668 ± 60,148 paired reads per sample). We identified a total of 109 bacterial families (100 in hyperlipidemic samples and 89 in controls) and 269 bacterial genera (244 in hyperlipidemic subjects and 193 in controls). We found a minimum number of 39 families and 69 genera and a maximum of 63 families and 110 genera per fecal sample in hyperlipidemic subjects; a minimum of 44 families and 80 genera and a maximum of 64 families and 113 genera were found in controls. Only approximately 15% of families ($n=16$) and 10% of genera ($n=26$) were detected in all 45 analyzed fecal samples; the same 16 families and 26 genera were the only ones detected in all 30 hyperlipidemic fecal samples, whereas 30 families and 46 genera were found in all 15 control samples. These data suggest that bacterial composition varied more among hyperlipidemic samples than among control fecal samples, which was also evidenced by the analysis of $\beta$-diversity (Fig. 1).

The analysis of $\beta$-diversity performed with the unweighted UniFrac algorithm evidenced a significant segregation of controls from hyperlipidemic samples ($p<0.05$ and number of permutations=99 according to ANOSIM test), which was principally led by the abundance of *Faecalibacterium* spp. and two unidentified Clostridiales genera (Fig. 1B). Also, the intrasample ($\alpha$)-diversity analyzed through the Chao1 index evidenced a difference between hyperlipidemic and
control samples (Fig. 2). Specifically, Chao1, which estimates the abundance of OTUs in a single sample, was significantly higher in the control samples (p<0.001 according to unpaired Mann-Whitney U test) than in the hyperlipidemic samples; nonetheless, we did not find any difference between the groups when we analyzed α-diversity with the inverse Simpson index, which estimates biodiversity also considering OTU evenness (Fig. 2B). In addition, microbiota diversity analyses failed to reveal age-related differences in the IME of volunteers (Fig. S1).

Overall, the taxonomic composition of the fecal microbiota of both hyperlipidemic and control subjects was characterized by a dominance of Firmicutes bacteria (mean relative abundance of 67% in hyperlipidemic samples and 68% in controls; Fig. S2); particularly, three Clostridiales families, i.e., Lachnospiraceae, Ruminococcaceae, and an undefined family of the order, constituted more than 50% of all bacteria in both hyperlipidemic and control subjects (Fig. S2). In addition, the most abundant non-Firmicutes families were Bacteroidaceae (phylum Bacteroidetes, mean relative abundance of 17% in hyperlipidemic samples and 16% in controls) and Bifidobacteriaceae (phylum Actinobacteria, mean relative abundance of 7% in hyperlipidemic samples and 6% in controls; Fig. S2).

To infer taxonomic signatures distinguishing the fecal microbiota structure of the 15 hyperlipidemic participants and the 15 aged-matched normolipidemic controls, we performed a comparative analysis at the OTU level through the DESeq negative binomial distribution method. We found 229 OTUs whose abundance was significantly different between the two groups of subjects (Fig. S3 and Fig. 3): 193 OTUs were increased in the controls, whereas only 36 OTUs were increased in the hyperlipidemic samples (Fig. S3). Most of the OTUs belonged to the phylum Firmicutes and particularly to the families Ruminococcaceae and Lachnospiraceae; notably, controls were enriched in OTUs belonging to well-recognized butyrate producing bacteria, such as the genus *Roseburia* and the species *Faecalibacterium prausnitzii*. We also observed a significant reduction in an OTU ascribed to the species *Akkermansia muciniphila* in the hyperlipidemic samples. In addition, 23 OTUs belonging to the phylum Bacteroidetes were significantly enriched...
in controls compared to hyperlipidemic samples, whereas only 3 *Bacteroides* OTUs were reduced (Fig. S3).

Finally, the IME of enrolled children and adolescents was characterized through the quantification of 8 SCFAs and lactate in fecal samples by UPLC-HR-MS (Table S2). Specifically, we analyzed the data from the fecal samples of 15 hyperlipidemic subjects undertaking the hazelnut intervention together with 17 additional fecal samples from hyperlipidemic subjects at baseline. We found that the fecal levels of several SCFAs were significantly different between hyperlipidemic and control subjects; specifically, hyperlipidemia was associated with significantly lower concentrations of acetate (p<0.001 according to unpaired Mann-Whitney U test), butyrate (p<0.01) and propionate (p<0.01), whereas lactate (p<0.05), isobutyrate (p<0.01) and pyruvate (p<0.001) were significantly increased compared to control subjects (Fig. 4). Notably, the fecal levels of acetate (p<0.001), propionate (p<0.001) and butyrate (as a trend, p=0.085) were significantly lower in hyperlipidemic samples when we also included data collected in a previous study on 25 healthy adult volunteers in the analysis (Gargari *et al.* 2016) (Fig. S4); these volunteers were considered to be an additional reference for healthy/physiological fecal SCFA levels, knowing that the human gut microbiota acquires an adult-like structure by the third year of life (Lozupone *et al.* 2013). The levels of valerate, isovalerate and succinate were not significantly different between the groups.

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

**Hazelnut intake induced limited changes in bacterial abundances but modulated SCFA levels in feces.** The α- and β-diversity of fecal samples were not significantly affected by the eight-week hazelnut intervention (Fig. 1 and Fig. 2). Furthermore, we did not find any OTU that was modified with an FDR adjusted p value (padJ) lower than the significance limit of 0.05 (Fig. S5). Only an
undefined Clostridiales OTU, which was reduced after the intervention, displayed a padJ value lower than 0.1 (Fig. S5).

Subsequently, we quantified the fecal level of 8 SCFAs and lactate after hazelnut consumption in 14 hyperlipidemic subjects that completed the intervention (Fig. 4). We found that acetate increased significantly over the intervention (p<0.05 according to paired Wilcoxon test). An increasing trend (p=0.079) was also observed for propionate.

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

**The modification of several taxa of the fecal microbiota correlates with changes in the host’s hyperlipidemia biomarkers.** We performed correlation analyses between the variations observed in the abundance of bacterial taxa and changes in the lipid profile of hyperlipidemic subjects. Specifically, we considered the serum levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), non-HDL cholesterol, and triglycerides (TG). We found that lipidemic parameters changed over the intervention and were associated with variations in 144 OTUs, 19 of which were significantly different between dyslipidemic and control samples (Fig. 5). We found an inverse correlation between the changes in TC and the variation of several Ruminococcaceae OTUs, particularly *Faecalibacterium prausnitzii*. The changes in HDL-C inversely correlated with fluctuations in the abundance of several Clostridiales and *Collinsella* OTUs; on the contrary, a positive correlation for HDL-C was observed with variations in a smaller number of Clostridiales and mostly Lachnospiraceae, including an OTU ascribed to the genus *Roseburia*. Only 15 OTUs were found to be correlated with LDL-C changes; 13 of them (7 positively and 6 negatively correlated) were ascribed to the order Clostridiales. Notably, triglyceridemia was inversely correlated with the change of 22 OTUs ascribed to the genus *Bacteroides*, 3 Lachnospiraceae OTUs, 3 OTUs ascribed to the species *F. prausnitzii*, and 2
Akkermansia muciniphila OTUs. In contrast, triglyceridemia modifications were positively correlated with changes of 5 Coprococcus OTUs.

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

DISCUSSION

In this study, we show for the first time that young individuals with inherited hyperlipidemia may possess a dysbiotic gut bacterial ecosystem. We came to this result by comparing the IME of hyperlipidemic children and adolescents (ages ranging from 7 to 17 years old, mean age of 11) with the IME of age-matched normolipidemic controls (ages ranging from 5 to 17 years old, mean age of 11). Although the age range was considered quite wide, spanning from early school-aged childhood to late adolescence, we did not observe age-related differences in the IME of volunteers (Fig. S1), which is in accordance with the scientific literature demonstrating that the intestinal microbiota of children reaches an adult state at approximately 3 years of age (Lozupone et al. 2013; Matamoros et al. 2013; Rodriguez et al. 2015).

Notably, diet can play a leading role in shaping the gut microbiota (Amato et al. 2015; De Filippo et al. 2010); therefore, possible differences in eating behaviors between hyperlipidemic and normolipidemic counterparts could have been contributed to IME differences. However, we calculated through food diaries and food frequency questionnaires that macronutrient contributions to the overall diet were comparable between the two groups of subjects (data not shown). Particularly, fiber intake was at the lower range of CHILD-1 recommendations as previously published (Deon et al. 2017a). Moreover, children maintained their dietary behavior, even if a slight
increase in energy and fat intake (by approximately 100 kcal) was registered due to the hazelnut intervention.

In general, the microbiota composition of subjects investigated here is in accordance with the literature, which shows that Firmicutes bacteria constitute between 50 and 80% of the taxa in the core intestinal microbiota of healthy adults, including Lachnospiraceae and Ruminococcaceae as the most abundant and metabolically active taxa (Jalanka-Tuovinen et al. 2011; Peris-Bondia et al. 2011; Tap et al. 2009). Moreover, the fecal microbiota structure of all subjects included in this study is in accordance with a study that evidenced a similar dominance of Firmicutes in children following an urban Western lifestyle compared to children with a rural diet (De Filippo et al. 2010).

Nonetheless, the results of the present study also evidenced that the fecal microbiota of hyperlipidemic subjects is characterized by the alteration of numerous operational taxonomic units (OTUs), many of which belong to the Clostridiales order, whereas several Bacteroidetes OTUs were found to be significantly reduced. Hyperlipidemia is a predisposing factor and an intrinsic feature of several diseases, such as obesity, in which a similar alteration of the microbiota has been observed. In fact, a number of studies have shown that obesity associated with dysbiosis enhances energy extraction from food and increases low-grade inflammation and is characterized by an increase of bacteria from the phylum Firmicutes (mainly Clostridiales) and a specific decrease in Bacteroidetes (Armougom et al. 2009; Baothman et al. 2016; Ley et al. 2006; Santacruz et al. 2010). In addition, an enhanced Firmicutes/Bacteroidetes ratio has been associated with a high-protein, high-fat Western diet (Amato et al. 2015) and distinguished European from African children aged 1–6 years old (De Filippo et al. 2010).

Data on type 2 diabetes, which can be a hyperlipidemia-associated disease, indicated only a moderate degree of gut microbial dysbiosis and instead reported a ‘functional dysbiosis’ in which a decrease in the intestinal level of butyrate was observed (Tilg and Moschen 2014). The loss of butyrate producing bacteria and decreased butyrate levels in the gut have also been often reported in intestinal inflammatory conditions (Sokol et al. 2008; Van Immerseel et al. 2010). Accordingly, in
our study, compared to normolipidemic controls, we found in hyperlipidemic subjects a significantly reduced abundance of OTUs ascribed to well-recognized butyrate producing bacteria, such as *Faecalibacterium prausnitzii* and *Roseburia* spp. (Louis and Flint 2009), together with a significantly lower concentration of fecal butyrate.

Butyrate is mostly produced by Clostridiales bacteria, whereas acetate and propionate are principally derived from the primary metabolism of members of the phylum Bacteroidetes (Macfarlane and Macfarlane 2003). In accordance with the observed lack of several Bacteroidetes OTUs, we found that the fecal levels of acetate and propionate were significantly decreased in hyperlipidemic subjects. Acetate and propionate produced by the gut microbiota are rapidly absorbed and reach the liver via the portal circulation, where they are used as an energy source (Canfora *et al.* 2015; den Besten *et al.* 2013) and participate in lipogenesis and gluconeogenesis, respectively (Canfora *et al.* 2015). There is experimental evidence suggesting that acetate and propionate may regulate cholesterol metabolism by decreasing the activity of hepatic 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS) and reductase (HMGCR) (den Besten *et al.* 2013); in addition, acetate may increase cholesterol 7-α-hydroxylase (CYP7A1) (Fushimi *et al.* 2006; Rodwell *et al.* 1976). Notably, HMGCS and HMGCR are involved in the initial steps of cholesterol biosynthesis (Rodwell *et al.* 1976), whereas CYP7A1 participates in cholesterol-bile acid conversion (den Besten *et al.* 2013). It can be then argued that reduction of the intestinal levels of acetate and propionate in hyperlipidemic subjects may contribute to their altered cholesterol metabolism. Interestingly, in our study, following the dietary intervention with hazelnut, we observed a significant increase in acetate and a trend to rise of propionate levels with an assumed potential benefit for individuals with hyperlipidemia. At the same time, as previously reported (Deon *et al.* 2017a), hazelnut consumption significantly affected serum lipid profile (i.e., time effect reduction of LDL-C and non HDL-C and increase of HDL-C/LDL-C) and erythrocyte phospholipid fatty acid composition (i.e., increased levels of MUFAs, oleic acid and MUFAs/SFAs ratio).
In addition to the lower abundance of predominant intestinal SCFAs (acetate, butyrate and propionate, representing up to 95% of the SCFAs present in the colon), we found in hyperlipidemic fecal samples increased levels of lactate, isobutyrate and pyruvate. Lactate is produced by several members of the intestinal microbiota, such as lactic acid bacteria, bifidobacteria and enterobacterial species (e.g., *E. coli*). However, under normal physiological conditions, lactate does not accumulate in the colon since it is consumed by other intestinal microorganisms. Particularly, lactate is converted to butyrate by several gut commensals, such as *Eubacterium hallii*, *Anaerostipes caccae* and *Roseburia intestinalis* (Bourriaud *et al.* 2005; Duncan *et al.* 2004; Flint *et al.* 2015; Van den Abbeele *et al.* 2013), which are all members of the order Clostridiales. Therefore, an accumulation of lactate can be plausibly considered to be a metabolic signature of dysbiosis; accordingly, the shifts of bacterial metabolism from short chain fatty acid to lactate production and the resulting intraluminal pooling of lactate have been associated with pathological conditions (Bustos *et al.* 1994; Huda-Faujan *et al.* 2010; Vernia *et al.* 1988). Reportedly, lactate is present at low concentrations (<3 mmol l\(^{-1}\)) or is not detected in the feces of healthy individuals (Duncan *et al.* 2007; Vernia *et al.* 1988), whereas concentrations up to 100 mmol l\(^{-1}\) have been reported in gut disorders (Hove *et al.* 1994; Vernia *et al.* 1988). Therefore, considering that in our study lactate exceeded the level of 1 mmol per kg of feces in only 5 out of 48 analyzed samples, the actual physiological significance of fecal lactate as detected here in hyperlipidemic subjects is questionable.

Isobutyrate, which is produced in the gut by the degradation of amino acids, such as valine (Zarling and Ruchim 1987), has been found to be correlated with behavior changes induced by prebiotics in mice (Burokas *et al.* 2017), whereas pyruvate, which can derive from bacterial autolysis or exfoliated apical enterocytes, has been associated with inflammatory bowel disease (Huda-Faujan *et al.* 2010). However, the actual importance of the modification of these organic acids in the human gut is unclear, and the literature is too limited yet to allow a complete interpretation of our results.
Although the hazelnut intervention significantly modified the fecal levels of SCFAs, the abundances of bacterial taxa in the fecal microbiota were only mildly affected. The observed modifications of SCFA levels that occurred after hazelnut consumption may be plausibly explained by the intake of fiber derived from the overall diet, including the contribution of hazelnuts. However, the elaboration of dietary intake did not support such a hypothesis since fiber intake did not change following the intervention (i.e., approximately 10 g per day as a mean in this target population) (Deon et al. 2017a). Moreover, despite the fact that energy intake did not change following the regular consumption of hazelnuts, it should be underlined that increased intake of total fat (+5%) and monounsaturated fatty acids (+4.7%) was observed (Deon et al. 2017a). Finally, children also had an increased intake of phytosterols, tocopherols and polyphenols following hazelnut consumption. Consequently, in light of the recognized activity of phenolic compounds as potential modulators of the microbiota (Valdes et al. 2015), the contribution of these bioactives on bacterial fermentation in the gut cannot be excluded.

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

**Conclusions**

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

Last few years, several *in vitro* and *in vivo* studies have demonstrated the ability of nut intake to modulate the abundance of specific microbial taxa of the gut microbiota and change the intestinal concentration of SCFAs (Burns *et al.* 2016; Liu *et al.* 2016; Mandalari *et al.* 2010; Schlormann *et al.* 2016; Ukhanova *et al.* 2014). Nonetheless, this research was mostly focused on almonds, and to
the best of our knowledge, only one study investigated hazelnuts, showing an increase in butyrate through *in vitro* fermentation of a human fecal sample (Schlormann *et al.* 2016).

In conclusion, our study is the first human trial investigating the potential role of hazelnuts as an IME modulator, and it specifically suggests that a dietary intervention with hazelnuts as part of a controlled diet for hyperlipidemia could be an effective and practical strategy to manage this condition.

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Legends

Fig. 1. Inter-sample ecological diversity based on 16S rRNA gene profiling data of fecal samples from hyperlipidemic subjects participating in the hazelnut intervention trial and normolipidemic controls. Principal coordinates analysis of weighted (A) and unweighted (B) UniFrac distances based on OTU abundances. The first two coordinates (PC1 and PC2) are displayed with the percentage of variance explained in brackets. In panel B, a bi-plot is represented showing five of the genera that better describe the diversity among samples. The control group is significantly different in unweighted UniFrac according to ANOSIM test (p<0.05; number of permutations=99).
Fig. 2. Intrasample ecological diversity based on 16S rRNA gene profiling data of fecal samples from hyperlipidemic subjects participating in the hazelnut intervention trial and normolipidemic controls. A, rarefaction curves obtained using Chao1 index fixing a maximum of 50,000 reads per sample. B, α-diversity analysis based on Chao1 and inverse Simpson indexes. T0, samples before the hazelnut intervention; T1, samples after the hazelnut intervention. ***, P<0.001 according to Mann-Whitney U (unpaired) test.

Fig. 2
Fig. 3. Taxonomic units in fecal samples distinguishing hyperlipidemic from control subjects. Heat map based on the normalized abundance of OTUs (horizontal axis) for an individual fecal sample (vertical axis). The figure includes only the OTUs that were significantly different between hyperlipidemic and control samples through the DESeq2 negative binomial distribution method. Lachn., Lachnospiraceae; R., Ruminococcaceae.
Fig. 4. Fecal levels of short chain fatty acids and lactate in hyperlipidemic and control subjects. *add, hyperlipidemic subjects not included in the intervention trial; T0, samples before the hazelnut intervention; T1, samples after the hazelnut intervention. *u.d.l., under detection limit. *, p < 0.05; **, p<0.01, ***, p<0.001 according to Mann-Whitney U (unpaired) test. #, p<0.05 according to Wilcoxon (paired) test.

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