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

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11 Running Head: Hyperlipidemia-associated fecal microbiota

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21 # Abstract

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

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

42 ## Background

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

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

58 In principle, modulation of the gut microbiota could represent an additional strategy for the
59 amelioration of lipid profiles. In fact, expanding scientific evidence indicates that the gut microbiota
60 mediates pathophysiological mechanisms that alter lipid metabolism and other related metabolic
61 traits (Ghazalpour *et al.* 2016). Particularly, the intestinal microbiota has been recognized as a
62 metabolically active endocrine organ of the human superorganism that can be a therapeutic target
63 for hyperlipidemia and associated cardiometabolic diseases (Brahe *et al.* 2016; Ghazalpour *et al.*
64 2016). In fact, numerous metabolites produced by intestinal bacteria, such as
65 trimethylamine/trimethylamine-N-oxide, secondary bile acids, catecholamines, and short chain fatty
66 acids (SCFAs), are not simply absorbed and metabolized by human cells for energy needs but also

67 act as hormone-like factors that impact numerous aspects of host physiology (Boulange *et al.* 2016;
68 Brown and Hazen 2015). Particularly, SCFAs, including acetate, butyrate, propionate and valerate,
69 in addition to being used an energy source by the colonic epithelium (in particular, butyrate), are
70 sensed by dedicated receptors on numerous diverse cell populations around the human body,
71 resulting in the regulation of energy metabolism, insulin sensitivity, and immune responses (Brown
72 *et al.* 2003; Brown and Hazen 2015; De Vadder *et al.* 2014; Gao *et al.* 2009; Kimura *et al.* 2013;
73 Saemann *et al.* 2000).

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

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

90

91
Materials and methods

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

97 **Participants**. The volunteers considered in the present study were derived from a subgroup of
98 children and adolescents with primary hyperlipidemia; they were aged between 7 and 17 years old
99 and were participants in a national project aimed at characterizing this pediatric population (Deon *et*
100 *al.* 2017b) and investigating the effect of regular intake of hazelnuts with skin for eight weeks on
101 several markers related to lipid metabolism and oxidative stress (Deon *et al.* 2017a; Deon *et al.*
102 2017b). The trial was registered at ISRCTN.com (identifier no. ISRCTN12261900). Subjects
103 participating in the intervention study were recruited among patients receiving care at the
104 Department of Health Science and Pediatrics at the University of Turin (Italy) after a screening for
105 eligibility. In addition, 15 age-matched normolipidemic volunteers were recruited in the same area
106 and time frame as the controls for microbial ecology analysis of fecal samples. The age and sex of
107 the participants are shown in **Table S1**.

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

114 To be eligible, screened children and adolescents were required to be of normal weight with a
115 diagnosis of primary hyperlipemia, including familial hypercholesterolemia (FH), familial
116 combined hyperlipidemia (FCHL) or polygenic hypercholesterolemia (PHC), with total serum

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

134 **Experimental design**. All enrolled patients were following nutritional recommendations
135 suggested for pediatric hyperlipidemia based on the *cardiovascular health integrated lifestyle diet*
136 (CHILD-1) (Expert Panel on Integrated Guidelines for Cardiovascular *et al.* 2011). Nutritional
137 recommendations were as follows: 55% of daily energy intake from carbohydrates, 15% from
138 proteins, and 30% from fats (saturated fat 7-10%). Additional recommendations were that dietary
139 cholesterol should be lower than 100 mg/1000 kcal (and no more than 300 mg/day) and soluble
140 fiber intake should be 10-25 g/day.

141 Dietary intervention consisted of an 8-week intake of HZN+S. Subjects were provided with pre-
142 weighed vacuum-packed portions of Italian roasted *Corylus avellana* L. (cultivar 'Tonda Gentile
143 delle Langhe' from Piedmont, Italy). The quantity of hazelnuts per packet was calculated by
144 considering doses generally advised for adults and were adjusted to the children's body weight
145 (approximately 0.43 g kg^{-1} body weight up to a maximum of 30 g, which is the recommended daily
146 dose for an adult). All participants were encouraged to maintain the same dietary pattern and
147 lifestyle habits throughout the 8-week intervention study. Subjects were required to exclude the
148 intake of other nuts, dried fruits, probiotic or prebiotic foods or supplements from one month before
149 the beginning of the study until the end of the experiment. Traditional yogurt was allowed. To
150 check compliance to dietary recommendations, subjects and their families were asked to fill in
151 weekly food diaries as previously reported (Deon *et al.* 2017a).

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

159 The fecal samples were collected from each participant in a sterile plastic pot within 24 h before
160 visits at baseline and following the HZN+S intervention. A single fecal sample was also collected
161 from 15 age-matched controls. According to the recommendations for "storage conditions of
162 intestinal microbiota matter in metagenomic analysis" (Cardona *et al.* 2012), participants were asked
163 to deliver the fecal sample to the laboratory within 24 h. Upon delivery, stool samples were stored at
164 -80°C until DNA extraction. Subjects were asked to return any uneaten HZN+S packages at the visit.
165 Compliance was assessed by weighing returned packages and checking weekly food diaries.

166 **Bacterial profiling of fecal microbiota**. The bacterial community structure of fecal samples was
167 determined by 16S ribosomal RNA gene profiling with Illumina MiSeq System at the Center for
168 life – Nanoscience, Istituto Italiano di Tecnologia (Roma, Italy). Briefly, metagenomic DNA was
169 extracted from 200 ± 10 mg of stool within 30 days from delivery by means of a PowerFecal DNA
170 Isolation Kit (Mo Bio Laboratories, Cabru s.a.s., Biassono, Italy) according to the manufacturer's
171 specifications. A DNA fragment encompassing the V3 and V4 regions of the 16S rRNA gene was
172 amplified from metagenomic DNA with the primer pair selected by Klindworth et al. in
173 (Klindworth *et al.* 2013). The sequencing runs were multiplexed and barcode sequences were used
174 to discriminate the samples. 16S rRNA gene sequence reads were analyzed through the
175 bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0 using
176 the last version of Greengenes (gg_13_5) as reference taxonomic database. We used a reference
177 picking to cluster and assign the taxonomy to the reads. We add this information in the section
178 Materials and Methods as suggested. Analysis of the α- and β-diversities were performed using
179 QIIME. For α-diversity inverse Simpson, Chao1 and Shannon indexes were calculated. Concerning
180 β-diversity, the UniFrac algorithm was used to obtain the phylogenetic information needed to
181 generate the dissimilarity matrix. Bacterial abundances in each fecal sample were determined at the
182 operational taxonomic unit (OTU) level. Sequence reads from 16S rRNA gene profiling have been
183 deposited in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute
184 under accession code PRJEB10296.

185 **Quantification of fecal short chain fatty acids (SCFAs)**. SCFAs were quantified in the fecal
186 samples collected from 32 subjects, including the 15 who completed the intervention trial. SCFA
187 quantifications were performed as previously described (Gargari *et al.* 2016). In brief, stools (200 ±
188 10 mg) were extracted in 10 ml of 0.001% HCOOH by vortexing for 1 min. The supernatant was
189 then recovered through centrifugation at 1000 x *g* for 2 min at 4 °C. All extracts were stored at -20
190 °C until UPLC-HR-MS analysis, which was carried out on an Acquity UPLC separation module

191 (Waters, Milford, MA, USA) coupled with an Exactive Orbitrap MS with an HESI-II probe for
192 electrospray ionization (Thermo Scientific, San Jose, CA, USA). The ion source and interface
193 conditions and other detailed information were as specified in (Gargari *et al.* 2016). The eluents
194 were 0.001% HCOOH in MilliQ-treated water (solvent A) and CH_3OH:CH_3CN (1:1, v/v, solvent
195 B); UPLC elution gradient: 0% B for 4 min, 0-15% B in 6 min, 15–20% B in 5 min, 20% for 13
196 min, and then return to initial conditions in 1 min. The UPLC eluate was analyzed in full-scan MS
197 in the range 50–130 *m/z*. The resolution was set at 50 K, the AGC target was 1E6, and the
198 maximum ion injection time was 100 ms. The MS data were processed using Xcalibur software
199 (Thermo Scientific). Five-point external calibration curves prepared with analytical grade SCFAs
200 (Sigma-Aldrich, Milan, Italy) were adopted to quantify acetate, butyrate, isobutyrate, isovalerate,
201 lactate, propionate, pyruvate, succinate, and valerate in fecal samples. SCFA concentrations were
202 expressed in millimoles per kilogram of wet feces.

203 **Statistics**. Statistical analyses were performed using R statistical software (version 3.1.2). A 0
204 (zero) was given to any taxon that was not detected in a specific sample to enable comparisons. All
205 statistical tests were performed considering three study populations: (i) hyperlipidemic subjects
206 before HZN+S intake, (ii) hyperlipidemic subjects after HZN+S intake and (iii) normolipidemic
207 subjects as controls. Differences in the microbiota composition of these populations were
208 determined using a Wald test following DESeq2 (paired between the comparison of hyperlipidemic
209 subject populations) read count normalization. After DESeq2 normalization, only OTUs with a
210 number of reads higher than 5 in at least one quarter of the samples were considered for statistical
211 analysis. Significant differences between groups of samples in β-diversity were assessed through
212 the non-parametric statistical test ANOSIM (analysis of similarities).

213 Differences in SCFA concentrations were evaluated by analyzing the data with a non-parametric
214 Wilcoxon-Mann-Whitney test using paired data (when allowed).

215 The correlation analyses were carried out using Kendall and Spearman formulas with changes
216 over the treatment in bacterial taxa abundances (DESeq2-normalized data), lipidemic profile data
217 and fecal SCFA concentrations in hyperlipidemic subjects.

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

220

221 # RESULTS

222 **16S rRNA gene profiling and SCFA quantification revealed significant differences in the fecal**
223 **microbial ecosystem between hyperlipidemic and control subjects.** A total of 13,830,110
224 filtered high-quality sequence reads was generated (average of 153,668 ± 60,148 paired reads per
225 sample). We identified a total of 109 bacterial families (100 in hyperlipidemic samples and 89 in
226 controls) and 269 bacterial genera (244 in hyperlipidemic subjects and 193 in controls). We found a
227 minimum number of 39 families and 69 genera and a maximum of 63 families and 110 genera per
228 fecal sample in hyperlipidemic subjects; a minimum of 44 families and 80 genera and a maximum
229 of 64 families and 113 genera were found in controls. Only approximately 15% of families (n=16)
230 and 10% of genera (n=26) were detected in all 45 analyzed fecal samples; the same 16 families and
231 26 genera were the only ones detected in all 30 hyperlipidemic fecal samples, whereas 30 families
232 and 46 genera were found in all 15 control samples. These data suggest that bacterial composition
233 varied more among hyperlipidemic samples than among control fecal samples, which was also
234 evidenced by the analysis of β-diversity (**Fig. 1**).

235 The analysis of β-diversity performed with the unweighted UniFrac algorithm evidenced a
236 significant segregation of controls from hyperlipidemic samples ($p < 0.05$ and number of
237 permutations=99 according to ANOSIM test), which was principally led by the abundance of
238 *Faecalibacterium* spp. and two unidentified Clostridiales genera (Fig. 1B). Also, the intrasample
239 (α)-diversity analyzed through the Chao1 index evidenced a difference between hyperlipidemic and

240 control samples (**Fig. 2**). Specifically, Chao1, which estimates the abundance of OTUs in a single
241 sample, was significantly higher in the control samples ($p<0.001$ according to unpaired Mann-
242 Whitney U test) than in the hyperlipidemic samples; nonetheless, we did not find any difference
243 between the groups when we analyzed α-diversity with the inverse Simpson index, which estimates
244 biodiversity also considering OTU evenness (Fig. 2B). In addition, microbiota diversity analyses
245 failed to reveal age-related differences in the IME of volunteers (Fig. S1).
246 Overall, the taxonomic composition of the fecal microbiota of both hyperlipidemic and control
247 subjects was characterized by a dominance of Firmicutes bacteria (mean relative abundance of 67%
248 in hyperlipidemic samples and 68% in controls; Fig. S2); particularly, three Clostridiales families,
249 i.e., Lachnospiraceae, Ruminococcaceae, and an undefined family of the order, constituted more
250 than 50% of all bacteria in both hyperlipidemic and control subjects (Fig. S2). In addition, the most
251 abundant non-Firmicutes families were Bacteroidaceae (phylum Bacteroidetes, mean relative
252 abundance of 17% in hyperlipidemic samples and 16% in controls) and Bifidobacteriaceae (phylum
253 Actinobacteria, mean relative abundance of 7% in hyperlipidemic samples and 6% in controls; Fig.
254 S2).
255 To infer taxonomic signatures distinguishing the fecal microbiota structure of the 15
256 hyperlipidemic participants and the 15 aged-matched normolipidemic controls, we performed a
257 comparative analysis at the OTU level through the DESeq negative binomial distribution method.
258 We found 229 OTUs whose abundance was significantly different between the two groups of
259 subjects (**Fig. S3** and **Fig. 3**): 193 OTUs were increased in the controls, whereas only 36 OTUs
260 were increased in the hyperlipidemic samples (Fig. S3). Most of the OTUs belonged to the phylum
261 Firmicutes and particularly to the families Ruminococcaceae and Lachnospiraceae; notably,
262 controls were enriched in OTUs belonging to well-recognized butyrate producing bacteria, such as
263 the genus *Roseburia* and the species *Faecalibacterium prausnitzii*. We also observed a significant
264 reduction in an OTU ascribed to the species *Akkermansia muciniphila* in the hyperlipidemic
265 samples. In addition, 23 OTUs belonging to the phylum Bacteroidetes were significantly enriched

266 in controls compared to hyperlipidemic samples, whereas only 3 *Bacteroides* OTUs were reduced
267 (Fig. S3).

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

283 Overall, these data suggest that hyperlipidemia is potentially associated with gut microbiota
284 dysbiosis, which can be characterized by the alteration of numerous OTUs associated with SCFA-
285 producing bacteria and the reduction of fecal levels of acetate, butyrate and propionate.
286

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

291 undefined Clostridiales OTU, which was reduced after the intervention, displayed a padJ value
292 lower than 0.1 (Fig. S5).

293 Subsequently, we quantified the fecal level of 8 SCFAs and lactate after hazelnut consumption in
294 14 hyperlipidemic subjects that completed the intervention (Fig. 4). We found that acetate increased
295 significantly over the intervention ($p<0.05$ according to paired Wilcoxon test). An increasing trend
296 ($p=0.079$) was also observed for propionate.

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

300

301 **The modification of several taxa of the fecal microbiota correlates with changes in the host's**
302 **hyperlipidemia biomarkers.** We performed correlation analyses between the variations observed
303 in the abundance of bacterial taxa and changes in the lipid profile of hyperlipidemic subjects.
304 Specifically, we considered the serum levels of total cholesterol (TC), low-density lipoprotein
305 cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), non-HDL cholesterol, and
306 triglycerides (TG). We found that lipidemic parameters changed over the intervention and were
307 associated with variations in 144 OTUs, 19 of which were significantly different between
308 dyslipidemic and control samples (**Fig. 5**). We found an inverse correlation between the changes in
309 TC and the variation of several Ruminococcaceae OTUs, particularly *Faecalibacterium prausnitzii*.
310 The changes in HDL-C inversely correlated with fluctuations in the abundance of several
311 Clostridiales and *Collinsella* OTUs; on the contrary, a positive correlation for HDL-C was observed
312 with variations in a smaller number of Clostridiales and mostly Lachnospiraceae, including an OTU
313 ascribed to the genus *Roseburia*. Only 15 OTUs were found to be correlated with LDL-C changes;
314 13 of them (7 positively and 6 negatively correlated) were ascribed to the order Clostridiales.
315 Notably, triglyceridemia was inversely correlated with the change of 22 OTUs ascribed to the genus
316 *Bacteroides*, 3 Lachnospiraceae OTUs, 3 OTUs ascribed to the species *F. prausnitzii*, and 2

317 *Akkermansia muciniphila* OTUs. In contrast, triglyceridemia modifications were positively
318 correlated with changes of 5 *Coprococcus* OTUs.
319 Overall, these results indicate that lipid profiles of the hyperlipidemic subjects investigated here
320 are linked to modifications of the abundance of specific taxa in the intestinal microbiota, such as the
321 families Lachnospiraceae and Ruminococcaceae and the genera *Akkermansia*, *Bacteroides*,
322 *Roseburia*, and *Faecalibacterium*.
323

324 # DISCUSSION

325 In this study, we show for the first time that young individuals with inherited hyperlipidemia may
326 possess a dysbiotic gut bacterial ecosystem. We came to this result by comparing the IME of
327 hyperlipidemic children and adolescents (ages ranging from 7 to 17 years old, mean age of 11) with
328 the IME of age-matched normolipidemic controls (ages ranging from 5 to 17 years old, mean age of
329 11). Although the age range was considered quite wide, spanning from early school-aged childhood
330 to late adolescence, we did not observe age-related differences in the IME of volunteers (Fig. S1),
331 which is in accordance with the scientific literature demonstrating that the intestinal microbiota of
332 children reaches an adult state at approximately 3 years of age (Lozupone *et al.* 2013; Matamoros *et*
333 *al.* 2013; Rodriguez *et al.* 2015).
334 Notably, diet can play a leading role in shaping the gut microbiota (Amato *et al.* 2015; De
335 Filippo *et al.* 2010); therefore, possible differences in eating behaviors between hyperlipidemic and
336 normolipidemic counterparts could have been contributed to IME differences. However, we
337 calculated through food diaries and food frequency questionnaires that macronutrient contributions
338 to the overall diet were comparable between the two groups of subjects (data not shown).
339 Particularly, fiber intake was at the lower range of CHILD-1 recommendations as previously
340 published (Deon *et al.* 2017a). Moreover, children maintained their dietary behavior, even if a slight

341 increase in energy and fat intake (by approximately 100 kcal) was registered due to the hazelnut
342 intervention.
343 In general, the microbiota composition of subjects investigated here is in accordance with the
344 literature, which shows that Firmicutes bacteria constitute between 50 and 80% of the taxa in the
345 core intestinal microbiota of healthy adults, including Lachnospiraceae and Ruminococcaceae as the
346 most abundant and metabolically active taxa (Jalanka-Tuovinen *et al.* 2011; Peris-Bondia *et al.*
347 2011; Tap *et al.* 2009). Moreover, the fecal microbiota structure of all subjects included in this
348 study is in accordance with a study that evidenced a similar dominance of Firmicutes in children
349 following an urban Western lifestyle compared to children with a rural diet (De Filippo *et al.* 2010).
350 Nonetheless, the results of the present study also evidenced that the fecal microbiota of
351 hyperlipidemic subjects is characterized by the alteration of numerous operational taxonomic units
352 (OTUs), many of which belong to the Clostridiales order, whereas several Bacteroidetes OTUs
353 were found to be significantly reduced. Hyperlipidemia is a predisposing factor and an intrinsic
354 feature of several diseases, such as obesity, in which a similar alteration of the microbiota has been
355 observed. In fact, a number of studies have shown that obesity associated with dysbiosis enhances
356 energy extraction from food and increases low-grade inflammation and is characterized by an
357 increase of bacteria from the phylum Firmicutes (mainly Clostridiales) and a specific decrease in
358 Bacteroidetes (Armougom *et al.* 2009; Baothman *et al.* 2016; Ley *et al.* 2006; Santacruz *et al.*
359 2010). In addition, an enhanced Firmicutes/Bacteroidetes ratio has been associated with a high-
360 protein, high-fat Western diet (Amato *et al.* 2015) and distinguished European from African
361 children aged 1–6 years old(De Filippo *et al.* 2010).
362 Data on type 2 diabetes, which can be a hyperlipidemia-associated disease, indicated only a
363 moderate degree of gut microbial dysbiosis and instead reported a ‘functional dysbiosis’ in which a
364 decrease in the intestinal level of butyrate was observed (Tilg and Moschen 2014). The loss of
365 butyrate producing bacteria and decreased butyrate levels in the gut have also been often reported in
366 intestinal inflammatory conditions (Sokol *et al.* 2008; Van Immerseel *et al.* 2010). Accordingly, in

367 our study, compared to normolipidemic controls, we found in hyperlipidemic subjects a
368 significantly reduced abundance of OTUs ascribed to well-recognized butyrate producing bacteria,
369 such as *Faecalibacterium prausnitzii* and *Roseburia* spp. (Louis and Flint 2009), together with a
370 significantly lower concentration of fecal butyrate.

371 Butyrate is mostly produced by Clostridiales bacteria, whereas acetate and propionate are
372 principally derived from the primary metabolism of members of the phylum Bacteroidetes
373 (Macfarlane and Macfarlane 2003). In accordance with the observed lack of several Bacteroidetes
374 OTUs, we found that the fecal levels of acetate and propionate were significantly decreased in
375 hyperlipidemic subjects. Acetate and propionate produced by the gut microbiota are rapidly
376 absorbed and reach the liver *via* the portal circulation, where they are used as an energy source
377 (Canfora *et al.* 2015; den Besten *et al.* 2013) and participate in lipogenesis and gluconeogenesis,
378 respectively (Canfora *et al.* 2015). There is experimental evidence suggesting that acetate and
379 propionate may regulate cholesterol metabolism by decreasing the activity of hepatic 3-hydroxy-3-
380 methylglutaryl-CoA synthase (HMGCS) and reductase (HMGCR) (den Besten *et al.* 2013); in
381 addition, acetate may increase cholesterol 7-α-hydroxylase (CYP7A1) (Fushimi *et al.* 2006;
382 Rodwell *et al.* 1976). Notably, HMGCS and HMGCR are involved in the initial steps of cholesterol
383 biosynthesis (Rodwell *et al.* 1976), whereas CYP7A1 participates in cholesterol-bile acid
384 conversion (den Besten *et al.* 2013). It can be then argued that reduction of the intestinal levels of
385 acetate and propionate in hyperlipidemic subjects may contribute to their altered cholesterol
386 metabolism. Interestingly, in our study, following the dietary intervention with hazelnut, we
387 observed a significant increase in acetate and a trend to rise of propionate levels with an assumed
388 potential benefit for individuals with hyperlipidemia. At the same time, as previously reported
389 (Deon *et al.* 2017a), hazelnut consumption significantly affected serum lipid profile (i.e., time effect
390 reduction of LDL-C and non HDL-C and increase of HDL-C/LDL-C) and erythrocyte phospholipid
391 fatty acid composition (i.e., increased levels of MUFAs, oleic acid and MUFAs/SFAs ratio).

392 In addition to the lower abundance of predominant intestinal SCFAs (acetate, butyrate and
393 propionate, representing up to 95% of the SCFAs present in the colon), we found in hyperlipidemic
394 fecal samples increased levels of lactate, isobutyrate and pyruvate. Lactate is produced by several
395 members of the intestinal microbiota, such as lactic acid bacteria, bifidobacteria and enterobacterial
396 species (e.g., *E. coli*). However, under normal physiological conditions, lactate does not accumulate
397 in the colon since it is consumed by other intestinal microorganisms. Particularly, lactate is
398 converted to butyrate by several gut commensals, such as *Eubacterium hallii, Anaerostipes caccae*
399 and *Roseburia intestinalis* (Bourriaud *et al.* 2005; Duncan *et al.* 2004; Flint *et al.* 2015; Van den
400 Abbeele *et al.* 2013), which are all members of the order Clostridiales. Therefore, an accumulation
401 of lactate can be plausibly considered to be a metabolic signature of dysbiosis; accordingly, the
402 shifts of bacterial metabolism from short chain fatty acid to lactate production and the resulting
403 intraluminal pooling of lactate have been associated with pathological conditions (Bustos *et al.*
404 1994; Huda-Faujan *et al.* 2010; Vernia *et al.* 1988). Reportedly, lactate is present at low
405 concentrations (<3 mmol l^{-1}) or is not detected in the feces of healthy individuals (Duncan *et al.*
406 2007; Vernia *et al.* 1988), whereas concentrations up to 100 mmol l^{-1} have been reported in gut
407 disorders (Hove *et al.* 1994; Vernia *et al.* 1988). Therefore, considering that in our study lactate
408 exceeded the level of 1 mmol per kg of feces in only 5 out of 48 analyzed samples, the actual
409 physiological significance of fecal lactate as detected here in hyperlipidemic subjects is
410 questionable.

411 Isobutyrate, which is produced in the gut by the degradation of amino acids, such as valine (Zarling
412 and Ruchim 1987), has been found to be correlated with behavior changes induced by prebiotics in
413 mice (Burokas *et al.* 2017), whereas pyruvate, which can derive from bacterial autolysis or exfoliated
414 apical enterocytes, has been associated with inflammatory bowel disease (Huda-Faujan *et al.* 2010).
415 However, the actual importance of the modification of these organic acids in the human gut is unclear,
416 and the literature is too limited yet to allow a complete interpretation of our results.

417 Although the hazelnut intervention significantly modified the fecal levels of SCFAs, the
418 abundances of bacterial taxa in the fecal microbiota were only mildly affected. The observed
419 modifications of SCFA levels that occurred after hazelnut consumption may be plausibly explained
420 by the intake of fiber derived from the overall diet, including the contribution of hazelnuts.
421 However, the elaboration of dietary intake did not support such a hypothesis since fiber intake did
422 not change following the intervention (i.e., approximately 10 g per day as a mean in this target
423 population) (Deon *et al.* 2017a). Moreover, despite the fact that energy intake did not change
424 following the regular consumption of hazelnuts, it should be underlined that increased intake of
425 total fat (+5%) and monounsaturated fatty acids (+4.7%) was observed (Deon *et al.* 2017a). Finally,
426 children also had an increased intake of phytosterols, tocopherols and polyphenols following
427 hazelnut consumption. Consequently, in light of the recognized activity of phenolic compounds as
428 potential modulators of the microbiota (Valdes *et al.* 2015), the contribution of these bioactives on
429 bacterial fermentation in the gut cannot be excluded.

430 Numerous human trials in the recent years have included profiling of the intestinal microbiota
431 and identification of the expansion or depletion of specific taxa as potential markers for
432 pathological conditions or dysfunctions (Knip and Siljander 2016; Miele *et al.* 2015; Sokol *et al.*
433 2008). Nonetheless, only few studies have associated specific gut bacteria to defined
434 pathophysiological mechanisms (e.g., (Devkota *et al.* 2012)). This limited knowledge on the
435 involvement of bacteria in host physiological processes greatly limits the possibility of
436 understanding the actual biological meaning (if any) of several significant correlations that we
437 found between specific taxa of the intestinal microbiota and lipidemic profiles. However, a few
438 speculations can be reached. Interestingly, the variation of OTUs belonging to intestinal bacteria
439 with recognized anti-inflammatory properties, such as *Faecalibacterium prausnitzii* (Sokol *et al.*
440 2008) and *Akkermansia muciniphila* (Zhao *et al.* 2017), correlated inversely with changes in TG,
441 TC, LDL-C, and non-HDL cholesterol, suggesting the potential involvement of these bacteria in the

442 link between inflammation and hyperlipidemia (Feingold and Grunfeld 2000; Tall and Yvan-
443 Charvet 2015). Reportedly, *Faecalibacterium prausnitzii* is inversely associated with inflammatory
444 bowel diseases and its supplementation abolished inflammation (Sokol *et al.* 2008). Furthermore,
445 the abundance of *Akkermansia muciniphila* is lower in obesity and diabetes than in healthy controls,
446 and administration of this bacterium has been shown to reduce obesity, fat mass inflammation and
447 plasma cholesterol and triglycerides (Everard *et al.* 2013; Plovier *et al.* 2017). This last example is
448 also in accordance with the present study, where the changes in some plasma lipids were inversely
449 associated with this intestinal commensal. In addition, the change of *Bacteroides fragilis*, which is
450 another species with a reported potential anti-inflammatory role in the gut (Troy and Kasper 2010),
451 was inversely correlated with the modification of triglyceridemia. In contrast, we found a direct
452 correlation between the variations of cholesterolemia and an OTU ascribed to *Veillonella dispar*,
453 which is a potential pathobiont associated with several clinical cases of infection (Houston *et al.*
454 1997; Marchandin *et al.* 2001). *V. dispar* was also found to be enriched in colorectal carcinoma in
455 adenoma (Kasai *et al.* 2016).

456 **Conclusions**

457 The results of this study support the hypothesis that young individuals with primary hyperlipidemia
458 possess an altered (dysbiotic) intestinal microbial ecosystem, which could plausibly contribute to
459 the abnormal lipid profile of these subjects. A limitation of this study is the small sample size,
460 which may reduce the potential robustness of the obtained results. Moreover, further studies
461 focusing on the mechanisms involved in such hypothesized associations are warranted.
462 Last few years, several *in vitro* and *in vivo* studies have demonstrated the ability of nut intake to
463 modulate the abundance of specific microbial taxa of the gut microbiota and change the intestinal
464 concentration of SCFAs (Burns *et al.* 2016; Liu *et al.* 2016; Mandalari *et al.* 2010; Schlormann *et*
465 *al.* 2016; Ukhanova *et al.* 2014). Nonetheless, this research was mostly focused on almonds, and to

466 the best of our knowledge, only one study investigated hazelnuts, showing an increase in butyrate
467 through *in vitro* fermentation of a human fecal sample (Schlormann *et al.* 2016).

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

472 Acknowledgments

473 This work was financially supported by Torino University Funds and by Fondo Europeo di
474 Sviluppo Regionale - Programma Operativo Regionale 2007/2013 (FINPIEMONTE, Italy). We are
475 grateful to Ms. Claudia Bonardi, Ms. Natalja Kirika, Paola Cagliero and Mr. Carlo Casali, for their
476 technical assistance. The authors declare no conflict of interest.

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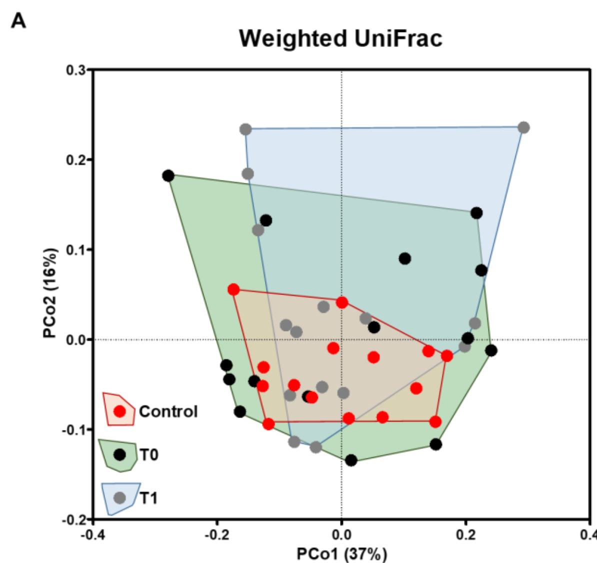
652

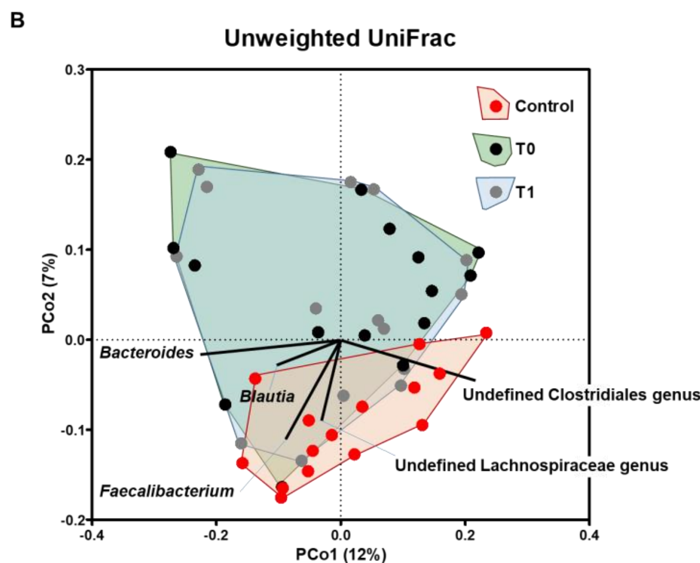
653 **Legends**

654 **Fig. 1**. **Inter-sample ecological diversity based on 16S rRNA gene profiling data of fecal**
655 **samples from hyperlipidemic subjects participating in the hazelnut intervention trial and**
656 **normolipidemic controls**. Principal coordinates analysis of weighted (A) and unweighted (B)
657 UniFrac distances based on OTU abundances. The first two coordinates (PC1 and PC2) are
658 displayed with the percentage of variance explained in brackets. In panel B, a bi-plot is represented
659 showing five of the genera that better describe the diversity among samples. The control group is
660 significantly different in unweighted UniFrac according to ANOSIM test ($p<0.05$; number of
661 permutations=99).

Fig. 1



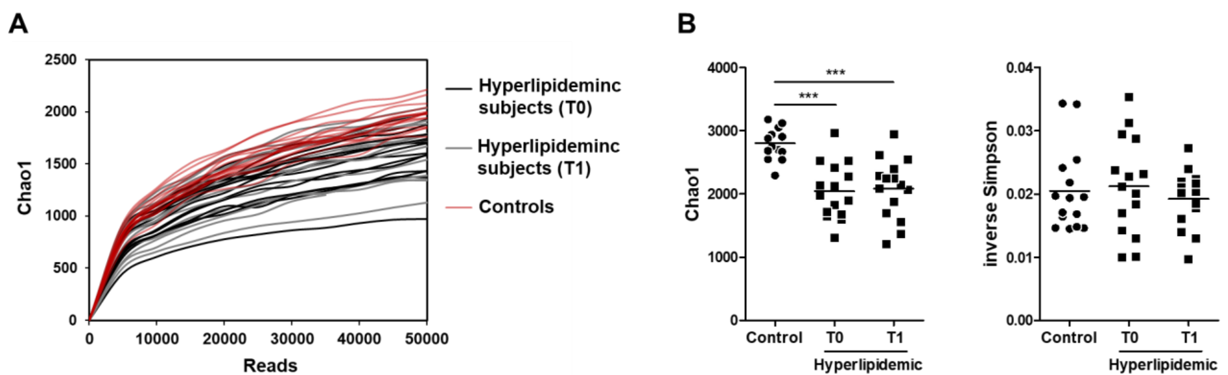
662



663

664 **Fig. 2**. **Intrasample ecological diversity based on 16S rRNA gene profiling data of fecal**
665 **samples from hyperlipidemic subjects participating in the hazelnut intervention trial and**
666 **normolipidemic controls**. **A**, rarefaction curves obtained using Chao1 index fixing a maximum of
667 50,000 reads per sample. **B**, α-diversity analysis based on Chao1 and inverse Simpson indexes. T0,
668 samples before the hazelnut intervention; T1, samples after the hazelnut intervention. ***, P<0.001
669 according to Mann-Whitney U (unpaired) test.

Fig. 2



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671

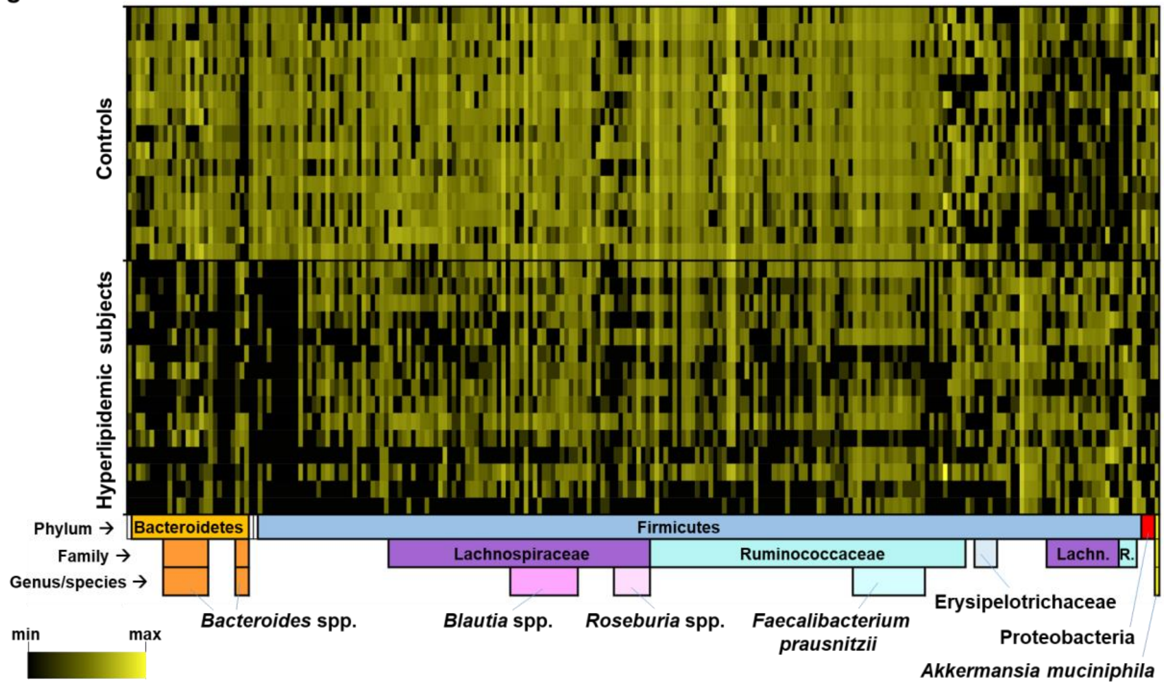
672 **Fig. 3**. **Taxonomic units in fecal samples distinguishing hyperlipidemic from control**

673 **subjects**. Heat map based on the normalized abundance of OTUs (horizontal axis) for an individual

674 fecal sample (vertical axis). The figure includes only the OTUs that were significantly different

675 between hyperlipidemic and control samples through the DESeq2 negative binomial distribution

676 method. Lachn., Lachnospiraceae; R., Ruminococcaceae.



677

678

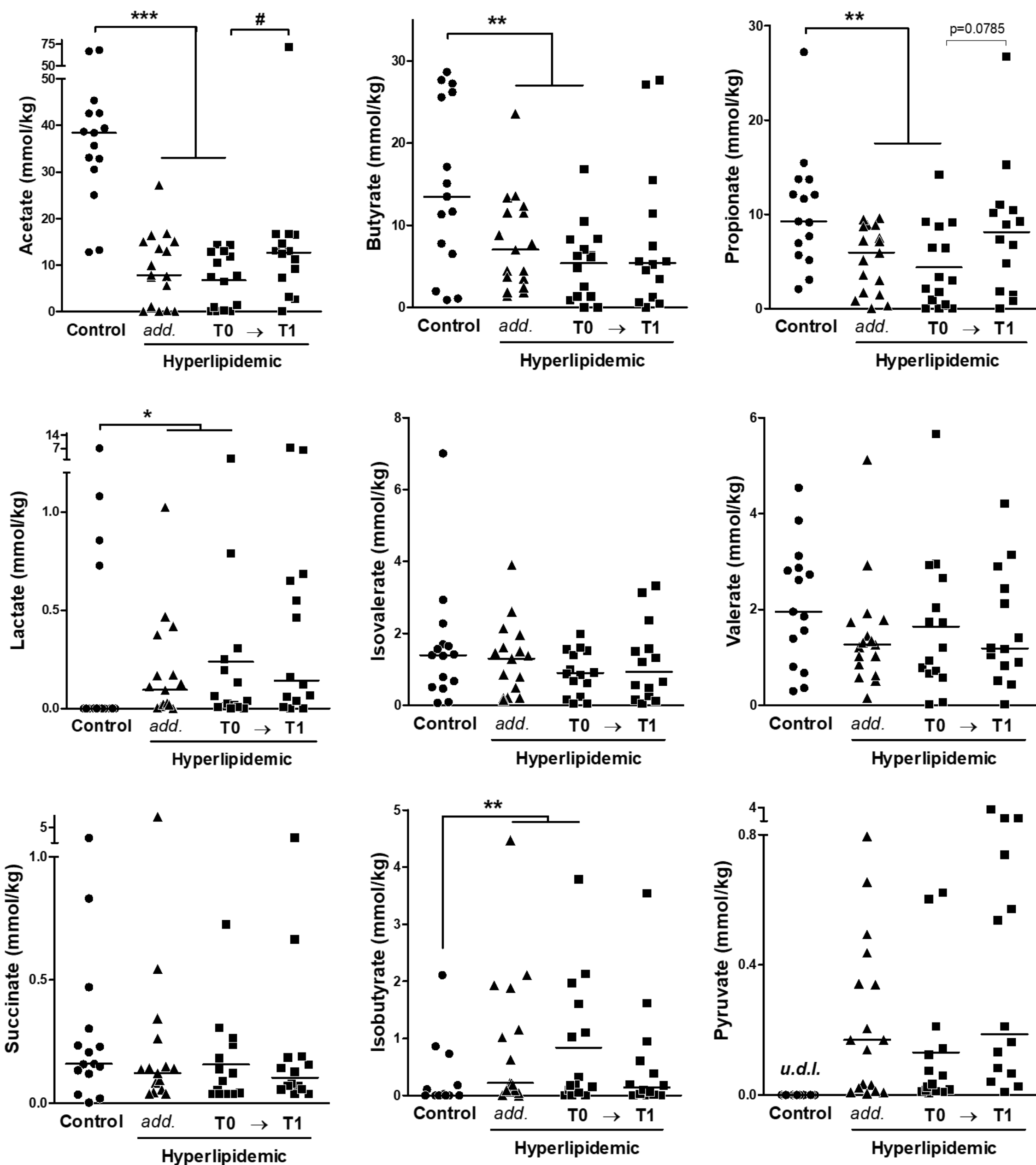
679 **Fig. 4**. **Fecal levels of short chain fatty acids and lactate in hyperlipidemic and control**

680 **subjects**. *add*, hyperlipidemic subjects not included in the intervention trial; T0, samples before the

681 hazelnut intervention; T1, samples after the hazelnut intervention. *u.d.l.*, under detection limit. *, p

682 < 0.05; **, p<0.01, ***, p<0.001 according to Mann-Whitney U (unpaired) test. #, p<0.05

683 according to Wilcoxon (paired) test.

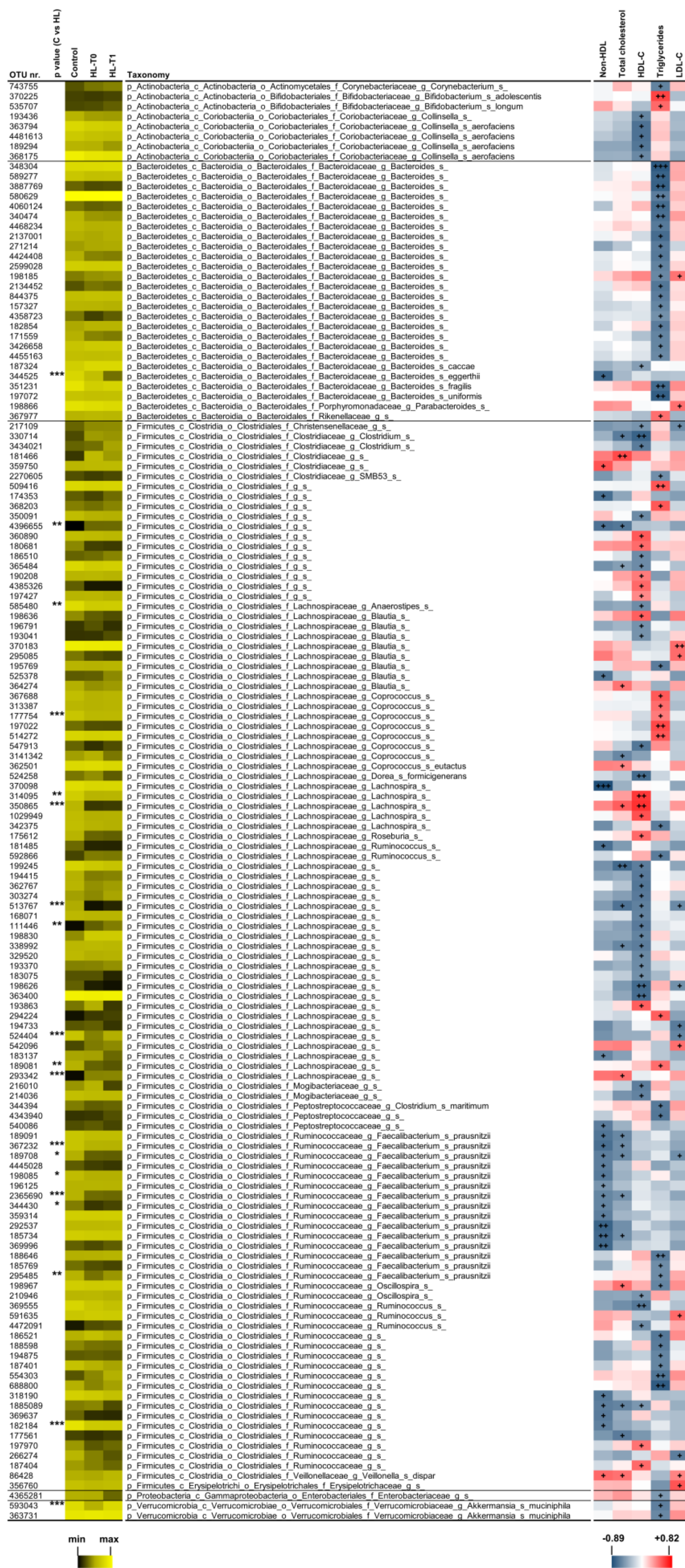


684

685 **Fig. 5**. **Correlation between lipidemic profile and changes in fecal microbiota composition**.

686 This figure only includes OTUs whose change over the hazelnut intervention significantly

687 correlated with at least one lipidemic parameter according to Kendall's Tau rank correlation. The
688 colors in the left panel represent the mean of DESeq2-normalized abundances of the OTUs in
689 control and hyperlipidemic (HL) samples before (T0) and after (T1) the intervention. Asterisks in
690 the second column indicate the FDR adjusted p values (padJ) calculated with a Wald test on
691 DESeq2-normalized data to indicate significantly different OTUs between control and HL-T0
692 samples; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. The colors in the right panel represent the R-value of
693 Spearman's correlation of the differences over the intervention trial between OTU abundance and
694 lipidemic parameters. Plus signs are according to Kendall's Tau rank correlation: +, $p<0.05$; ++,
695 $p<0.01$; +++, $p<0.001$.
696



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