Associations between the human intestinal microbiota, *Lactobacillus rhamnosus* GG and serum lipids indicated by integrated analysis of high-throughput profiling data

Accumulating evidence indicates that the intestinal microbiota regulates our physiology and metabolism. Bacteria marketed as probiotics confer health benefits that may arise from their ability to affect the microbiota. Here high-throughput screening of the intestinal microbiota was carried out and integrated with serum lipidomic profiling data to study the impact of probiotic intervention on the intestinal ecosystem and on host, and to explore the associations between the intestinal bacteria and serum lipids. We performed a comprehensive intestinal microbiota analysis using a phylogenetic microarray before and after *Lactobacillus rhamnosus* GG intervention. While a specific increase in the *L. rhamnosus*-related bacteria was observed during the intervention, no other changes in the composition or stability of the microbiota were detected. After the intervention, lactobacilli returned to their initial levels. As previously reported, also the serum lipid profiles remained unaltered during the intervention. The most prevailing association between the gut microbiota and lipid profiles was a strong positive correlation between uncultured phylotypes of *Ruminococcus gnavus*-group and polyunsaturated serum triglycerides of dietary origin. Moreover, a positive correlation was detected between serum cholesterol and *Collinsella (Coriobacteriaceae)*. These associations identified with the spectrometric lipidome profiling were corroborated by enzymatically determined cholesterol and triglyceride levels. Actinomycetaceae correlated negatively with triglycerides of highly unsaturated fatty acids while a set of Proteobacteria showed negative correlation with ether phosphatidylcholines. Based on a high-resolution microbiota analysis, intake of *L. rhamnosus* GG did not modify the composition of the intestinal ecosystem in healthy adults, indicating that probiotics confer their health effects by other mechanisms. Our results suggest that several members of the Firmicutes, Actinobacteria and Proteobacteria may be involved in the metabolism of dietary and endogenous lipids, and provide a scientific rationale for further human studies to explore the role of intestinal microbes in host lipid metabolism.

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Introduction

Over 90% of the cells in the human body constitute microbes, the majority of them living in the lower part of the gastrointestinal (GI) tract. Hence, we are composite organisms programmed not only by the inherited and stable human genome but also by the environmentally acquired and plastic microbiome. The coding capacity of the microbiome vastly surpasses the human genome with more than three million genes (Qin *et al.* 2010). While many of the microbial functions have not yet been characterized, various mechanisms have been described by which the intestinal microbes impact our life, including the metabolism of our food, exclusion of pathogens and many signaling functions that range from modulation of the mucosal immune response to development of metabolic diseases (Holmes *et al.* 2011; Sekirov 2010). Bacterial metabolism of dietary and endogenous substances is known to generate a wide repertoire of metabolites that may have beneficial or harmful effects on the host (O'Keefe 2008; Blaut & Clavel 2007). Hence, the intestinal microbiota has a remarkable potential to influence the physiology and biology of the host (Tremaroli & Bäckhed 2012), and unlike the human genome, its gene pool can be modulated by changing the environmental conditions, such as food or drug intake, affecting the composition and function of the intestinal microbiota (Zoetendal, Rajilić-Stojanović & de Vos 2008).

Consumption of lactic acid bacteria marketed as probiotics is a common approach to maintain health (Saxelin *et al.* 2005). *Lactobacillus rhamnosus* GG is one of the most widely used probiotic bacteria that is assumed to interact with the host via binding to human mucus via its extracellular pili (Kankainen *et al.* 2009). However, the further molecular details of the probiotic signaling are not yet understood and it remains to be established whether the effect is direct, through metabolites or structural components modulating for instance the immune responses of the host or indirect, via alteration of the intestinal microbiota. From the ecological perspective, a single bacterial strain is not likely to radically alter the established intestinal community, which in adults typically consists of

45   hundreds of different species-level phylotypes that represent approximately ten bacterial phyla, vastly

46   dominated by Firmicutes, Bacteroidetes and Actinobacteria, followed by Proteobacteria, Fusobacteria

47   and Verrucomicrobia (Zoetendal, Rajilić-Stojanović & de Vos 2008). Many of the currently

48   characterized phylotypes are strict anaerobes, have not yet been cultured, and can only be recognized

49   based on molecular methods (Zoetendal, Rajilić-Stojanović & de Vos 2008). The development of

50   culture-independent molecular techniques has provided insights in the composition of the intestinal

51   microbiota before and following probiotic intake. Targeted microbiota analyses as well as the more

52   recent community-level profiling studies support the view that the effects of probiotic intake are limited

53   and only affect bacteria related to the probiotics (Satokari *et al.* 2001; Vaughan, Mollet & deVos 1999;

54   Palaria, Johnson-Kanda & O'Sullivan 2011; McNulty *et al.* 2011).

55   Animal and *in vitro* studies have shown that the intestinal microbiota can regulate host lipid

56   metabolism via numerous microbial activities (Fava 2006; Martin *et al.* 2007). The best characterized

57   mechanism is through the biotransformation of bile acids, which regulate the digestion and absorption

58   of fats, and profoundly affects the cholesterol and other lipid metabolism in the body (Gérard P. 2010;

59   Ridlon, Kang & Hylemon 2006). However, global monitoring of the serum and organ lipid profiles of

60   germ-free and conventionally raised mice suggests an even more widespread and profound influence of

61   the intestinal microbiota on host lipid metabolism, in particular on triglycerides and

62   phosphatidylcholines (Velagapudi *et al.* 2010; Orešič, Hänninen & Vidal-Puig 2008).

63   In the present study, we characterized the impact of a probiotic intervention on the composition

64   and stability of the intestinal microbiota and serum lipid profiles in healthy adults. A comprehensive

65   intestinal microbiota profiling and phylogenetic analysis was carried out with the Human Intestinal

66   Tract Chip (HITChip), a phylogenetic microarray that provides a robust and sensitive measurement

67   platform to assessing the abundance of over 1000 microbial species-like phylotypes representing the

68 majority of the known bacterial diversity of the human intestinal tract (Rajilić-Stojanović *et al.* 2009).

69 Stability of the microbiota was quantified by inter- and intra-individual correlations within and between

70 time points. The study subjects were sampled prior and after three weeks consumption of dairy milks

71 supplemented with either the probiotic *L. rhamnosus* GG or a placebo. Moreover, the microbiota

72 composition was analyzed from additional follow-up samples collected three weeks after the

73 intervention trial. With phylogenetic microarray analysis we could both deepen and expand the typical

74 bacterial analysis in probiotic trials, often limited to specific bacterial groups, such as lactic acid

75 bacteria, or to the dominant fraction of the microbiota. Previously, we have shown that the intake of *L.*

76 *rhamnosus* GG did not elicit any significant changes in the serum lipids (Kekkonen *et al.* 2008b). The

77 present study complements these results by providing longitudinal data and evidence that the probiotic

78 intervention with *L. rhamnosus* GG does not affect the overall composition or stability of the intestinal

79 microbiota. Furthermore, we have integrated the lipid profiling and global microbiota data sets to

80 investigate the overall associations between the intestinal microbes and systemic metabolites, in

81 particular serum lipids. Quantitative analyses suggest that the abundance of specific intestinal microbes

82 is correlated to that of specific lipid species. We identified mainly uncultured members of the

83 Firmicutes, Actinobacteria and Proteobacteria that seem to be involved in the absorption and

84 metabolism of the dietary and endogenous lipids, providing a scientific rationale for further human

85 studies designed to explore the role of intestinal microbes in lipid metabolism, and associations to

86 health.

87

88 **Materials and Methods**

89 *Ethics statement*

90 The trial and its protocol have been approved by the Ethics Committee of the Hospital District of

91 Helsinki and Uusimaa (Ethical protocol no HUS 3577E0/05). The subjects provided written informed

92 consent. The details of this trial have been published previously (Kekkonen *et al.* 2008a; Kekkonen *et*

93 *al.* 2008b).

94

95 *Subjects*

96 The subjects were healthy Finnish adults (n=25, 18 females, 7 males) with a mean age of 42 years

97 (23-55) and a mean BMI of 24 kg/m^2 (18-30) from Helsinki region, representing a subset of a larger

98 cohort (Kekkonen *et al.* 2008a; Kekkonen *et al.* 2008b). The subject characteristics are provided in

99 Supplementary Table S1.

100

101 *Dietary Intervention*

102 The subjects constituted two treatment groups in a randomized, double blind intervention study to

103 receive either *L. rhamnosus* GG (probiotic; n=11) or placebo (n=14). During the intervention the

104 subjects consumed daily a 250 mL milk-based fruit drink containing either *L. rhamnosus* GG (ATCC

105 53103, 6.2 x 10^7 cfu/mL) or a similar placebo drink without probiotic bacteria for three weeks. No

106 other probiotic-containing products were allowed three weeks prior or during the intervention; a list of

107 fermented foods and commercial probiotic-containing products was given to the subjects. The subjects

108 were not separately questioned about their abstinence from probiotic products, but they filled a study

109 diary recording the daily intake of the study product.

110

111 *Fecal samples and microbiota analysis with the HITChip and quantitative PCR*

112 Three fecal samples per individual were collected three weeks before, during and three weeks after the

113 intervention, as previously described (Kekkonen *et al.* 2008b). The fecal DNA extraction with modified

114 Promega method, and quantification of the probiotic counts with a strain-specific quantitative PCR

115 (qPCR) assay have been described previously (Ahlroos & Tynkkynen 2009). The phylogenetic analysis

116 of the intestinal microbiota composition with the HITChip microarray was performed as previously

117 described (Rajilić-Stojanović *et al.* 2009; Salonen *et al.* 2010). The phylogenetic HITChip microarray

118 targets the V1 and V6 hypervariable regions of the 16S rRNA gene of over 1000 bacterial phylotypes

119 that present the majority of the so far detected phylotypes of the human intestine. Phylogenetic

120 organization of the microarray probes and data preprocessing have been explained in detail elsewhere

121 (Rajilić-Stojanović *et al.* 2009; Salonen *et al.* 2010; Jalanka-Tuovinen *et al.* 2011). Quantification of

122 the relative differences in the taxon abundance between samples was obtained by summarizing the

123 probe signals to phylotype (species-like), genus and phylum levels. Genus-level taxa with ≥90%

124 sequence similarity in the 16S rRNA gene are referred to as *Species* and relatives, the latter being

125 shortened in the text as "*et rel.*" (Rajilić-Stojanović *et al.* 2009).

126 Previously, HITChip-derived microbial profiles have been shown to correlate well with those

127 obtained with fluorescence in situ hybridization (FISH) (Rajilić-Stojanović *et al.* 2009) and

128 pyrosequencing (Claesson *et al.* 2009). Samples were also used for absolute quantification of total

129 bacteria, methanogenic Archaea, *Lactobacillus* group and *Bifidobacterium* spp. with previously

130 described qPCR primers and reaction conditions (Salonen *et al.* 2010).

131

132 *Blood samples and their biochemical analyses*

133 Of the 25 study subjects used for the microbiota analysis, 22 (8 from probiotic group and 14 from

134 placebo group) were available for the parallel analysis of serum lipid profiles before and after the

135 intervention (44 samples in total). Venous blood samples from the antecubital vein were taken at

136 baseline and after the three-week intervention. The blood samples were stored at –20°C for further

137 analyses. Total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL)

138 cholesterol as well as triglyceride levels were enzymatically determined from the serum as previously

139 described (Kekkonen *et al.* 2008a).

140

Global lipid profiling: sample preparation and analysis by UPLC-MS

Extraction of lipids from the serum samples, analysis of the lipid extracts with Waters Q-Tof Premier mass spectrometer combined with an Acquity Ultra Performance LC™ (UPLC), data preprocessing and identification of the lipid molecular species is described in detail elsewhere (Kekkonen *et al.* 2008a). Lipids have been named according to Lipid Maps (http://www.lipidmaps.org) with the following abbreviations: Cer: ceramide; ChoE: cholesteryl ester; lysoPC: lysophosphatidylcholine; PA: phosphatidic acid; PG: phosphatidylglycerol; PC: phosphatidylcholine; PS: phosphatidylserine; SM: sphingomyelin; TG: triglyceride. Where the fatty acid composition could not be determined, the total number of carbons and double bonds is indicated. The first number indicates the amount of carbon atoms in the fatty acid molecule, followed by the number of double bonds. For details, see (Kekkonen *et al.* 2008a).

Statistical analyses

The effects of probiotic intervention on the intestinal microbiota were investigated with a combination of linear models, sparse principal component analysis (PCA; Shen & Huang 2008; Lê Cao, González & Dèjean 2009), unsupervised hierarchical clustering, and significance testing. The log-transformed HITChip and lipid profiling values were approximately Gaussian distributed and fulfilled the general statistical assumptions underlying the selected computational approaches. Background variables, including age, body-mass index and gender were compared in the baseline samples to exclude potentially confounding effects associated with these variables. The Wilcoxon test was used with continuous variables and the Fisher exact test for categorical variables, followed by Benjamini-Hochberg p-value correction for multiple testing. No significant differences between the background variables were observed between the treatment groups ($p > 0.05$ in all comparisons). The

164 two-group comparisons between the time points and between the treatment groups were quantified

165 based on a linear model with group-wise fixed effects and sample-specific random effects, as

166 implemented in the limma R package (Smyth 2004), to identify bacterial taxa with significant changes

167 induced by the probiotic intervention and to assess the magnitude and significance of the effects. The

168 function lmFit was used to fit the linear model, followed by significance estimation by empirical Bayes

169 as described in Smyth (2004). In addition, power calculation was carried out to assess statistical power

170 of the current study. The original data was randomly permuted, one significant 2-fold alteration was

171 inserted, and Gaussian noise was added using the same average standard deviation as in the original

172 data. Empirical power calculation with 1000 random permutations showed that 2-fold and higher

173 alterations that follow the noise levels in the original data were detected in > 99.8% of the cases with

174 sample size of 8 or more, based on the same detection criteria than in the current study, confirming the

175 high statistical power of the analysis at the present sample size. PCA is a linear dimension reduction

176 method used to compress information in the high-dimensional phylogenetic (genus-level) and lipid

177 profiles into few informative features that capture the main variation in the data and allow

178 two-dimensional visualization of sample similarities. The temporal and inter-individual similarity of

179 the microbiota and lipid profiles was assessed by average intra- and inter-individual Pearson

180 correlations (r) between and within the time points, respectively. The differences in profile similarity

181 between the probiotic and placebo groups were estimated with Wilcoxon test. The biweight

182 midcorrelation, which is more robust to outliers than the standard Pearson correlation, was used to

183 quantify correlation between the microbiota and lipid profiles across the individuals. High-throughput

184 screening studies involve considerable multiple testing and the traditional multiple testing correction

185 approaches are prohibitively conservative in this context due to their emphasis on estimating the

186 probability of a single false positive finding. Hence, we have used q-values for multiple testing

187   correction in the high-throughput screening tests that include parallel comparisons of large numbers of

188   lipids and bacterial phylotypes.

189   *Correlation analysis of the intestinal microbiota and serum lipids*

190   The biweight midcorrelation measure was used to quantify associations between the microbiota and

191   lipid profiling data sets, and between the microbiota and biochemically determined lipids. The

192   correlations between genus-level bacterial groups and lipid species were calculated together with

193   significance estimates that were corrected for multiple testing. The significantly correlated (q<0.05)

194   phylotypes and lipids were selected for further analysis. It is important to note, however, that while the

195   present sample size of 22 samples across 2 time points (before and during the probiotic intervention) is

196   sufficient for highlighting significant correlation patterns, the present experimental design cannot

197   uncover causal relationships and the observed correlations may be partly associated with diet or other

198   confounding variables that simultaneously affect both lipid and microbiota profiles. Two-way average

199   linkage hierarchical clustering of the bacterial taxa and lipids was applied to highlight and visualize

200   groups of phylotypes and lipids sharing similar correlation patterns. Analogous 'second-order'

201   correlations have previously been applied for instance in the context of gene expression studies

202   (Parmigiani *et al.* 2004; Lahti *et al.* 2012). A constant plaid model biclustering was used for systematic

203   detection of significantly correlated bacterial and lipid groups on the correlation heatmap (Lazzeroni &

204   Owen 2002). To interpret the detected biclusters, statistical enrichment (over-representation) of the

205   implicated bacteria was quantified. Moreover, enrichment analysis was carried out for lipids containing

206   even or odd number of carbon atoms, as well as the enrichment of lipids with zero, one or more double

207   bonds. The enrichment analyses were carried out with Fisher's exact test (Rivals *et al.* 2007). All

208   analyses were performed within the R statistical environment (R Development Core Team 2010).

209

210 **Results**

211 *Impact of the L. rhamnosus GG intervention in the intestinal microbiota*

212 This study characterized the impact of *L. rhamnosus* GG intervention on the stability and composition

213 of the intestinal microbiota. The subjects in the probiotic group consumed daily approximately 10^{10}

214 (10.2 $\log_{10}$) colony forming units (cfu) of *L. rhamnosus* GG. The compliance was verified with the

215 quantification of *L. rhamnosus* GG in the feces with strain-specific qPCR (Kekkonen *et al.* 2008b). The

216 average excretion of *L. rhamnosus* GG in the probiotic group was more than 1000-fold higher than that

217 in the placebo group (8.52; sd 0.73 $\log_{10}$ versus 5.20; sd 1.09 $\log_{10}$ genome copies per gram of feces,

218 respectively).

219   The stability of the microbiota during the trial was quantified by the similarity of the

220 microbiota profiles between the three time points with Pearson correlation (Table 1). The average

221 intra-individual correlations were high; 0.94-0.95 (sd 0.02-0.03). No significant difference in the

222 temporal stability of the microbiota between the probiotic and placebo groups was observed, indicating

223 that the probiotic intervention did not alter the overall microbial stability. Principal Component

224 Analysis (PCA) visualization of the relationships between the intestinal microbiota profiles

225 (Supplementary Fig. S1A) and hierarchical clustering (data not shown) further supported the

226 conclusion that there were no systematic differences in the microbiota between the intervention groups.

227 The average inter-individual microbiota correlation was 0.76-0.78 with no significant differences

228 between the probiotic and placebo groups (Table 1). The intra-individual microbiota correlations

229 (r=0.94-0.95) were notably higher than the inter-individual correlations (r=0.76-0.78), stressing the

230 subject-specificity of the microbiota.

231   Linear models were used to quantify the effects of the *L. rhamnosus* GG intervention on

232 individual taxa using genus- and phylotype-level microarray data. A specific and transient increase of

233 bacteria related to *L. rhamnosus* was detected in the probiotic group immediately after the intervention

234 (Fig. 1). There were no intervention-related effects in the other lactobacilli, Bifidobacteria or any other

235 taxa either in the probiotic or placebo group; however substantial individual variation was evident.

236   To complement the HITChip microarray analysis, we also determined the absolute counts of

237 total bacteria, methanogenic Archaea, *Lactobacillus* group and *Bifidobacterium* spp. using real-time

238 PCR. The ingestion of *L. rhamnosus GG* was reflected in the total lactobacilli that showed a highly

239 significant increase in the probiotic group after the intervention, returning to baseline levels in the

240 follow up (q<0.05 in both comparisons). No other significant changes were observed in the amount of

241 targeted microbes neither in the probiotic or placebo group. We detected substantial inter- and

242 intra-individual variation in the methanogenic Archaea but that was independent of the time point or

243 the treatment group (data not shown). These observations support the conclusion that the *L. rhamnosus*

244 GG intervention did not change the overall microbiota composition.

245

246 *High-throughput profiling of serum lipids*

247 Global profiling of the serum lipids was performed using the UPLC-MS platform to assess the serum

248 lipid profiles at the molecular species level (Kekkonen et al. 2008a). In total, 407 lipid species from 11

249 different classes were identified. The lipid profiles were dominated with triglycerides (TG; 37%),

250 phosphatidylcholines (PC; 25%) and phosphatidyletanolamines (PE; 13%) while phosphatidic acids

251 (PA), phosphatidylglycerols (PG), sphingomyelins (SM), cholesteryl esters (ChoE),

252 lysophosphatidylcholines (lysoPC), phosphatidylserines (PS), ceramides (Cer) and

253 lysophosphatidylethanolamines (lysoPE) each contributed with 7% to 0.3% to the total lipid pool.

254   The stability of the lipid profiles was quantified based on the same correlation analysis

255 approach than in the HITChip microbiota profiling analysis. No significant differences in the lipid

256   profile stability were observed between the treatment groups (average intra-individual r=0.92 and

257   r=0.93 for the probiotic and placebo groups, respectively; Table 1). In contrast to microbiota profiles,

258   the serum lipid profiles were remarkably similar, not only within the subjects but also between the

259   subjects (average inter-individual r=0.89-0.91; Table 1). No significant differences were seen between

260   the probiotic and placebo groups, or between the time points. The stability of the lipid and microbiota

261   profiles showed a weak positive correlation (r=0.27) across the subjects independent of a treatment

262   group. The global lipid profiles did not separate according to the treatment group in PCA visualization

263   (Supplementary Fig. S1B), and no statistically significant lipid alterations were detected between the

264   time points neither in the probiotic or placebo group (data not shown; Kekkonen *et al.* 2008b). The

265   fold-changes for each lipid species between the two time points followed normal distribution in both

266   intervention groups (data not shown), which further supports the conclusion that the intervention did

267   not induce systematic alterations on the lipid profiles.

268

269   *Associations between the intestinal microbiota and serum lipids*

270   In addition to studying the effects of probiotic *L. rhamnosus GG* intervention in the intestinal

271   microbiota, we investigated the co-variation of the microbiota and serum lipid profiles. As discussed

272   above, our results (Table 1, Supplementary Fig. S1) conclusively show that the probiotic intervention

273   did not affect the lipid profiles or the microbiota beyond the *L. rhamnosus* that was ingested. Hence,

274   the subjects from probiotic and placebo groups were pooled to increase the sample size and statistical

275   power in exploring the associations between the intestinal microbiota and serum lipids.

276   Heatmap visualization of the microbiota-lipid correlations across the 22 subjects from 2 time

277   points revealed intestinal bacteria and serum lipids with significantly correlated abundance patterns

278   (Fig. 2). In total, 86 bacterial group-lipid pairs with notable correlations (+/- 0.5 or higher) were

279 identified at q<0.05 significance level (Fig. 2). Among the significant correlations, 23 of the 131

280 genus-level taxa detectable by the HITChip were represented (Supplementary Table S3). Most of these

281 taxa belonged to Proteobacteria (6) and Firmicutes within Clostridium cluster XIVa (5) and Bacilli (3).

282 From the lipid side, the vast majority of the significant correlations were attributable to the most

283 dominant lipids, TGs (62%) and PCs (30%). When analyzed at the genus-level, the significant

284 correlations were strongly dominated by the positive correlations between bacteria related to

285 *Ruminococcus gnavus* and different TG species (31 of 40 significant positive correlations, average

286 r=0.61; Fig. 3A). Detailed analysis at the phylotype-level data indicated that within the *R. gnavus*

287 group, two uncultured phylotypes, uncultured human gut bacterium JW1G3 and JW1H4a, dominated

288 the correlations while the type species *R. gnavus* or related *R. torques* did not correlate significantly

289 with TG or any other lipid species. Other representatives of the *Clostridium* cluster XIVa included

290 bacteria related to *Dorea formicigenerans* that also correlated positively with four TG species (Fig. 2;

291 average r=0.58). The remaining TG correlations were negative and involved phylogenetically diverse

292 bacteria (Supplementary Table S3). Also PCs correlated significantly with numerous taxa, most of

293 which correlated negatively with ester- or ether linked PCs. Interestingly, a set of Proteobacteria

294 (*Campylobacter*, *Helicobacter* and *Moraxellaceae*) with low signal intensities had a clear negative

295 correlation with PC. Similarly, two *Lactobacillus* species (*L. rhamnosus* that was ingested during the

296 trial and *L. salivarius*) together with another group of *Bacillus*, *Enterococcus* spp., also correlated

297 negatively with PC while few Firmicutes exhibited a positive correlation with this lipid species. Among

298 the less abundant lipids (<10% of the total lipid pool), a positive correlation was detected between

299 cholesteryl ester and *Collinsella* (r=0.59; Supplementary Table S3, Fig. 4A). On the phylotype level,

300 the correlation was mainly attributable to an uncultured bacterium clone Eldhufec074 within genus

301 *Collinsella*. Another group of Actinobacteria, namely *Actinomycetaceae,* showed a significant negative

302 correlation to TG and PA.

303   Hierarchical clustering and heatmap visualization of the significantly correlated lipid-bacteria

304 pairs revealed groups of lipids and bacteria sharing similar correlation patterns. A constant plaid model

305 biclustering approach was applied to detect coherent groups of significantly correlating microbe-lipid

306 pairs (see Methods). The analysis revealed three major clusters of significantly correlated lipid-microbe

307 pairs (Supplementary Table S2). One of the observed biclusters highlights the above discussed positive

308 association between bacteria related to *R. gnavus* and TGs. Another TG cluster, containing highly

309 unsaturated long-chain fatty acids, consisted of negatively correlating bacteria related to *Megamonas*

310 *hypermegale* (family *Veillonellaceae,* Clostridium cluster IX*)* or belonging to the *Actinomycetaceae.*

311 The third cluster contained a set of Proteobacteria (*Helicobacter, Oceanospirillum and Moraxellaceae*

312 *spp.*) and bacteria related to *Eubacterium cylindroides* within the family *Erysipelotrichaceae,* which all

313 correlated negatively with ether lipids.

314   To explore the nature of the identified lipid-microbe pairs, we quantified the enrichment of

315 specific, functionally relevant lipid categories in the biclusters. In particular, we investigated the

316 enrichment of lipids with even and odd number of carbon atoms and the degree of saturation (0, 1, or

317 more double bonds). The origin of lipid can be inferred from its chemical composition: Polyunsaturated

318 fatty acids (PUFAs; more than one double bond) and lipids that have uneven number of carbon atoms

319 originating from plants, bacteria or marine organism as humans cannot synthesize them. Significant

320 enrichment of long acyl chain polyunsaturated fatty acids (two or more hydrogen bonds; $p<0.05$; Fisher

321 exact test) was detected among the TG lipids that significantly correlated with bacteria related to *R.*

322 *gnavus* (Fig. S2; Supplementary Table S2). Such a positive association suggests a role for these *R.*

323 *gnavus*-related bacteria in the absorption of dietary lipids. In support of this interpretation, TGs

324 containing fatty acids with odd number of carbons were also relatively common in this group ($p=0.1$).

325 In the other two biclusters, no significant enrichment of odd/even carbon count or saturation level was

326 detected but within the bicluster 2 (Supplementary Table S2), *Actinomycetaceae* correlated exclusively

327 with highly unsaturated PUFAs (Fig. 2).

328

329 *Associations between the intestinal microbiota and biochemically determined serum lipids*

330 The mean values (SD) for the major serum lipids were total cholesterol 5.10 (1.02), LDL cholesterol

331 3.00 (1.21), HDL cholesterol 1.50 (0.33) and TG 1.20 (0.71) mmol/L (Kekkonen *et al.* 2008a). No

332 significant changes were detected in the lipids of the probiotic or the placebo groups during the

333 intervention (Kekkonen *et al.* 2008b). Hence, we analyzed the potential associations of the

334 enzymatically determined major blood lipids with the intestinal microbiota (Table 2). A positive

335 correlation between bacteria related to *R. gnavus* and TG was observed (q<0.01; r=0.60; Fig. 3B),

336 corroborating the association identified within the global lipid analysis. In line with the spectroscopic

337 lipid analysis, representatives of Bacteroidetes and Uncultured *Clostridiales* correlated negatively and

338 other implicated Firmicutes positively with enzymatically determined TG (Table 2). Representatives of

339 Proteobacteria and Actinobacteria that correlated negatively with TG in the lipid profiling data did not

340 correlate significantly with enzymatically determined TG. Such inconsistency may partly arise from

341 methodological reasons as the enzymatic assay captures not only TG but also diacylglyceride,

342 monoacylglyceride and free glycerol, while lipid profiling captures TGs at the molecular level.

343 *Collinsella* spp. and *Eubacterium biforme et rel.* showed statistically significant (q<0.05) and

344 positive correlations to enzymatically determined total and LDL cholesterol (Fig. 4B; Supplementary

345 Table S2). No other significant correlations were identified for total or LDL cholesterol while HDL

346 cholesterol correlated significantly with numerous taxa. Eight different Firmicutes including taxa

347 related to *Ruminococcus obeum* and *D. formicigenerans* were found to correlate negatively with HDL,

348 while Uncultured *Clostridiales* I was the only taxon showing positive correlation to HDL

349 (Supplementary Table S2).

350

351 **Discussion**

352 In the present study, we analyzed the effect of probiotic intake on the stability and composition of the

353 intestinal microbiota and of the serum lipids, and the overall associations between the microbiota and

354 lipid profiles. The microbiota was analyzed using the HITChip, a phylogenetic microarray, providing

355 one of the first holistic and community-level microbiota assessments after a probiotic intervention. The

356 data published so far is largely dominated with targeted microbiota analyses that have reported a

357 generic increase of lactic acid bacteria after intake of individual lactobacilli strains (McNulty *et al.*

358 2011; Palaria, Johnson-Kanda & O'Sullivan 2011; Satokari *et al.* 2001; Savard *et al.* 2011; Vaughan,

359 Mollet & de Vos 1999; Yamano *et al.* 2006). Our community-level analysis showed that the probiotic

360 intake did not introduce any changes in the microbiota composition or stability except the specific

361 increase of *L. rhamnosus* and total lactobacilli, which likely reflected the excretion of the ingested

362 strain. Similarly, ingestion of a mixture of five different probiotic strains did not induce any significant

363 changes in the microbiota composition neither in adults nor in simplified model community in mice

364 (McNulty *et al.* 2011). Even when a combination of probiotic and prebiotic (symbiotic) food was

365 consumed, two studies based on microbiota profiling and qPCR did not identify any differences in the

366 microbiota composition between placebo and treatment besides the ingested strains (Palaria,

367 Johnson-Kanda & O'Sullivan 2011; Vitali *et al.* 2010).

368 We have shown previously using the same cohort that the ingestion of *L. rhamnosus GG* had

369 apparent effects on the host immunology (Kekkonen *et al.* 2008b). Hence, our results does not support

370 the hypothesis that probiotic bacteria would modulate the endogenous microbiota but rather points

371 towards direct signaling to host or altered gene expression of the resident microbiota as a mode of

372 probiotic action as recently proposed (McNulty *et al.* 2011).

373   Our study was carried out in healthy adults with seemingly well-established and balanced

374 microbial communities. In subjects whose intestinal ecosystem is unbalanced e.g. due to pathogen

375 overgrowth, gastrointestinal symptoms or recent intake of antibiotics, intake of probiotic bacteria may

376 modulate the microbiota. Similarly, the developing microbiota of children is potentially more

377 susceptible to environmental modulators including probiotics. Although substantial changes in infant

378 microbiota following daily intake of *L. rhamnosus GG* have indeed been reported (Cox *et al.* 2010),

379 these results must be interpreted with caution as the used phylogenetic microarray provided signals for

380 close to 50 phyla whereas the human intestinal ecosystem only contains a maximum of 10 phyla

381 (Rajilić-Stojanović, Smidt & de Vos 2007; Midgley *et al.* 2012).

382   To the best of our knowledge, this cohort is the first addressing the potential impact of

383 probiotics on global serum lipid profiles in adults. Daily intake of *L. rhamnosus GG* for three weeks

384 did not introduce any consistent alterations in the serum lipids or in the biochemically determined

385 cholesterol and triglyceride levels of the healthy, normolipidemic adults analyzed in this and previous

386 study (Kekkonen *et al.* 2008b). Probiotic bacteria have been suggested as a potential non-drug

387 treatment to lower serum cholesterol levels based on their *in vitro* described ability to deconjugate bile

388 acids and directly assimilate cholesterol. A recent meta-analysis implies that probiotic intake can lower

389 the total and LDL cholesterol (Guo *et al.* 2011), although numerous human studies have failed to

390 observe any effects on serum lipids after probiotic intake (see e.g. Sadrzadeh-Yeganeh *et al.* 2010;

391 Pereira & Gibson 2002). One explanation for the controversy is that similarly to the strain-specificity of

392 the immunomodulatory effects of probiotics (Kekkonen *et al.* 2008b), also their effects on lipid

393 metabolism are likely to vary between different strains. In atopic infants the consumption of *L.*

394 *rhamnosus GG* supplemented formulas for several months resulted in a reduced proportion of
395 α-linolenic acid and of total n-3 PUFAs, leading to increased n-6 to n-3 PUFA ratio in sera of
396 probiotic-fed infants (Kankaanpää *et al.* 2002). Decrease of n-3 fatty acids has negative health
397 implication, underlining the need for more *in vivo* research to specify the bacterial strains and target
398 population where probiotics can have beneficial effects on the host lipid metabolism.

399 Our data indicates that the variation of the intestinal microbiota is considerably higher across
400 the individuals than the variation in serum lipid profiles, highlighting the tight homeostatic control of
401 systemically circulating lipids. The intestinal microbiota is known to play an important role in the
402 regulation of systemic lipid metabolism (Martin *et al.* 2007; Velagapudi *et al.* 2010). In this study, we
403 identified several novel associations between the human intestinal commensals and serum lipids. From
404 the lipid side, TGs (62%) and PCs (30%) dominated the significant lipid-microbe correlations. TGs are
405 used for energy storage while PCs are abundant constituents of cell membranes. Both of these abundant
406 lipid classes were affected upon colonization of germ-free mice (Velagapudi *et al.* 2010). The most
407 prominent correlation was between TGs and uncultured phylotypes related to *R. gnavus* that belong to
408 family *Ruminococcaceae* in Clostridium cluster XIVa. Two characterized species within the group, *R.*
409 *gnavus* and *R. torques,* have been implicated in intestinal disorders as they appeared to be
410 overrepresented both in IBS (Kassinen *et al.* 2007; Rajilić-Stojanović *et al.* 2011) and in IBD (Joossens
411 *et al.* 2011; Prindiville, Cantrell & Wilson. 2004). The TG lipids that were positively associat*ed with R.*
412 gnavus-related phylotypes were enriched with polyunsaturated and odd-chain fatty acids. Since the
413 odd-chain fatty acids are not synthesized in the body, these positive associations suggest that the
414 implicated bacteria facilitate the absorption of polyunsaturated dietary lipids. Moreover, it cannot be
415 excluded that these bacteria are even involved in their biosynthesis as related *R. obeum* together with
416 other Firmicutes are among the intestinal bacteria capable of producing isomers of conjugated linoleic
417 acids (CLA; McIntosh *et al.* 2009).

418   Triglycerides carry different types of fatty acids, and accordingly not all TGs displayed the

419   same pattern regarding microbial correlations. For example, bacteria related to *D. formicigenerans*

420   showed positive association to saturated triglycerides with a small carbon number, i.e. including

421   palmitic acid. These fatty acids are mainly produced *de novo* in the liver (Westerbacka *et al.* 2010;

422   Kotronen *et al.* 2009). *D. formicigenerans* produces formate and acetate that are precursors of hepatic

423   lipogenesis. Recent metaproteomic work verifies that the acetate kinases involved in acetate production

424   are highly expressed in the intestinal ecosystem (Kolmeder *et al.* 2012), suggesting that several

425   intestinal bacteria have potential to regulate lipogenesis. On the other hand, *Actinomycetaceae* were

426   inversely associated with TGs containing highly unsaturated triglycerides with a high carbon number.

427   Such TGs carry physiologically important fatty acids such as C22:6 (docosahexanoic acid) and C20:4

428   (arachidonic acid). Both are important cell membrane components, the former especially in visual and

429   neural tissues, and the latter is involved in inflammatory signaling. While not much is known about the

430   intestinal *Actinomycetaceae*, both commensal and pathogenic forms of these Actinobacteria are

431   abundant in the oral cavity.

432   We identified multiple taxa correlating negatively with normal or ether linked forms of PC.

433   Dietary PC was recently identified as compound that after conversion by the intestinal microbiota

434   promotes heart disease (Wang *et al.* 2011). Unfortunately, that study did not address the identity of

435   bacteria involved in the metabolism of dietary PC, but our data suggest that different Firmicutes,

436   Proteobacteria and Fusobacteria may participate in this metabolic conversion (Fig. 2). Remarkably, the

437   presence of serum ether PCs was negatively associated with various taxa, mainly with Gram-negative

438   genera that include pathogens and commensals, such as *Helicobacter*, *Moraxellaceae* and

439   *Campylobacter*. Among the ether lipids, plasmalogens, known as endogenous antioxidants (Wallner &

440   Schmitz 2011), are the most abundant. Their negative association with Proteobacteria could indicate

441   that the implicated bacteria may induce oxidative stress in host cells, leading to depletion of

442 plasmalogens. In support of this hypothesis, lipopolysaccharide (LPS), a ubiquitous and toxic surface

443 component of Gram-negative bacteria, induces oxidative stress in mammalian cell cultures (Aly,

444 Lightfoot & El-Shemy 2010).

445   Finally, we identified positive correlation between the abundance of serum cholesterol and

446 genus *Collinsella (*Actinobacteria, family *Coriobacteriacea).* Biochemical lipid analysis indicated that

447 *Collinsella* spp. together with bacteria related to *E. biforme* correlated specifically with total cholesterol

448 and LDL but not HDL. To our knowledge, our work provides the first *in vivo* evidence about the

449 implication of *Coriobacteriacea* in human lipid metabolism. Our present findings are supported by the

450 data generated with rodent models. In a hamster model, the proportion of *Coriobacteriaceae* showed

451 high positive correlation with non-HDL plasma cholesterol levels and reacted to the intake of dietary

452 lipids (Martinez *et al.* 2009). In a mouse model, a strong positive correlation between

453 *Coriobacteriaceae* and hepatic triglycerides was identified (Claus *et al.* 2011), providing cumulative

454 indication for the involvement of *Coriobacteriaceae* in mammalian lipid metabolism. While only one

455 taxon (*Collinsella)* correlated statistically significantly with cholesterol in the lipid profiling data,

456 numerous taxa showed significant correlation to enzymatically determined HDL cholesterol. This may

457 arise from technical differences, as the lipid profiling does not quantify free cholesterol but its

458 derivative cholesteryl ester, where cholesterol is esterified to long-chain fatty acids. All taxa that were

459 associated with both HDL cholesterol and TG had opposite correlation with these lipids

460 (Supplementary Table S2), in accordance with the fact that serum TG and HDL show strong inverse

461 relationship, and their ratio is used as an atherogenic index that predicts cardiovascular risk (Jeppesen

462 *et al.* 1997). Hence, identification of bacteria that potentially affect the athreogenic index may provide

463 important clinical implications for dyslipidemic individuals.

464   While good correspondence between the HITChip and pyrosequencing studies have been

465 previously demonstrated, the main advantage of the HITChip microarray compared to the

466 pyrosequencing studies is that the microarray technology provides very standardized and cost-efficient

467 tools for deep and reproducible analysis of intestinal microbiota including phylotypes that are only

468 present in low concentrations (Claesson et al., 2009; Salonen et al. 2012). We expect that our major

469 findings, the associations between specific lipid species and bacteria related to *Collinsella* or *R. gnavus*

470 *c*ould be detected also in a standard pyrosequencing study due to the relatively high abundance of these

471 organisms. However, the associations involving less abundant taxa (Bacilli, Proteobacteria,

472 *Actinomycetaeae*) would likely have been missed with conventional sequencing depth.

473 In summary, quantitative analysis of high-throughput profiling data identified several

474 significant correlations between the intestinal microbiota and serum lipids. These results partly confirm

475 and extend previous observations in animal studies, and provide hypotheses for follow-up studies in

476 humans. It is important to note, however, that the present analysis is based on a Finnish cohort and only

477 two time points, and does not consider causal relationships between these variables. Therefore it

478 cannot be excluded that some of the observed correlations may be associated with the diet or other

479 confounding variables that simultaneously affect both lipid and microbiota profiles. However, as all

480 subjects ate their habitual diets, individual food components are not likely to cause systematic bias to

481 the observed lipid-microbe correlations, which is further supported by the observation of distinct

482 groups of correlated lipid-microbe pairs in the bicluster analysis. It can also be ruled out that the

483 intervention would have caused the correlations as control measurements from the baseline were

484 included, the ingested probiotics *L. rhamnosus* GG did not correlate significantly with any serum lipid,

485 and the drink did not otherwise alter the microbiota. Confirmation of the findings in an independent

486 cohort and a more thorough longitudinal analysis will be necessary to assess the effect of external

487 variables on the microbiota-lipid correlations and their potential causal associations. None of the

488 implicated bacterial taxa are functionally characterized, and thus the potential mechanisms of how

489 human intestinal bacteria relate to the host lipid metabolism are currently unknown. Among the

490 potential mechanisms, bacterial modification of the bile acid pool is by far the best characterized.

491 *Collinsella* spp*., R. gnavus* as well as the genus *Eubacterium* are among the intestinal bacteria capable

492 of deconjugating bile acids (Lepercq *et al.* 2004). Bile acids are cholesterol-derived detergents that play

493 a central role in the absorption of fat in the intestine but also in signaling with systemic endocrine

494 functions (Swann *et al.* 2011). It has been known for long that intestinal bacteria can chemically

495 modify bile acids but recent work suggest that the microbiota may also control their production and

496 degradation (Antunes *et al.* 2011). In mice the intestinal bacteria profoundly affect the emulsification,

497 absorption and transport of dietary fat as well as their storage and peroxidation through the metabolic

498 and signaling properties of bile acids (Martin *et al.* 2007).

499

500 **Conclusion**

501 Our data supports the concept that the overall lipid content in human serum is a composite of host and

502 microbial metabolic activity, and the intestinal commensals are implicated in the metabolism of various

503 lipid species that human body utilizes for membranes, energy storage and signaling. Considering that a

504 single gene in an intestinal bacterium could alter host fatty acid composition (Rosberg-Cody *et al.*

505 2011), we can only envisage the metabolic capacity and the functional consequences from the million

506 genes in the intestinal microbiome. As epidemiological data do not support a link between dietary

507 cholesterol and serum cholesterol levels (Lecerf & de Lorgeril 2011), the role of genetic factors in the

508 individual variability of cholesterol levels is evident. Identification of the bacteria and their

509 mechanisms that regulate host fatty acid and lipid metabolism have considerable potential for clinical

510 implications due to the profound role of these molecules for instance in cardiovascular disease and

511 regulation of inflammatory cytokine signaling (Calder 2011). Future trials involving controlled diet and

512 dyslipidemic individuals will provide further insights on the role of intestinal microbes on human lipid

513 metabolism.

514

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Table 1(on next page)

Stability of microbiota and lipid profiles in the probiotic and placebo groups

We determined the similarity (expressed as Pearson's correlation) both within and between the time points (TP) for the microbiota and lipid profiles by the average scatter r of the profiles. Lipid data is available for the first two time points (TP1 and TP2, three weeks before the intervention and during the intervention , respectively), and not available (-) for the third time point (TP3) measured three weeks after the intervention.

	Between subjects			Within subjects	
Microbiota	TP1	TP2	TP3	TP1 vs TP2	TP3 vs TP2
Probiotic	0.78	0.78	0.78	0.94	0.95
Placebo	0.76	0.77	0.77	0.94	0.95
Lipids					
Probiotic	0.90	0.89	-	0.92	-
Placebo	0.91	0.89	-	0.93	-

Table 2(on next page)

Associations between genus-level bacterial groups and enzymatically determined lipids

Associations between the relative amounts of genus-level bacterial groups as determined by the HITChip analysis and the serum lipid concentrations are quantified with a biweight correlation. Only significant positive and negative correlations are shown (q<0.05; otherwise '-'). Abbreviations: Total cholesterol (TC), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, triglyceride (TG). Correlations between genus-level bacterial groups and mass spectrometry-determined lipids are provided in Supplementary Table S3.

Phyla/Firmicute order	Genus-level taxon	TC	LDL	HDL	TG
Actinobacteria	Collinsella	0.56	0.57	-	-
Bacilli	Aneurinibacillus	-	-	-0.58	0.50
Bacteroidetes	Bacteroides plebeius et rel.	-	-	-	-0.47
Bacteroidetes	Bacteroides vulgatus et rel.	-	-	-	-0.47
Bacteroidetes	Tannerella et rel.	-	-	-	-0.45
Clostridium cluster XI	Anaerovorax odorimutans et rel.	-	-	-0.48	0.49
Clostridium cluster XIVa	Clostridium nexile et rel.	-	-	-0.45	-
Clostridium cluster XIVa	Clostridium sphenoides et rel.	-	-	-	0.46
Clostridium cluster XIVa	Dorea formicigenerans et rel.	-	-	-0.56	0.57
Clostridium cluster XIVa	Eubacterium hallii et rel.	-	-	-	0.47
Clostridium cluster XIVa	Ruminococcus gnavus et rel.	-	-	-0.46	0.60
Clostridium cluster XIVa	Ruminococcus obeum et rel.	-	-	-0.48	0.51
Clostridium cluster XV	Anaerofustis	-	-	-0.45	-
Clostridium cluster XVI	Eubacterium biforme et rel.	0.48	0.47	-	-
Clostridium cluster XVI	Eubacterium cylindroides et rel.	-	-	-0.45	-
Uncultured Clostridiales	Uncultured Clostridiales I	-	-	0.53	-0.45
Uncultured Clostridiales	Uncultured Clostridiales II	-	-	-	-0.54

Figure 1

Intervention effects on the abundance of *L. rhamnosus*.

Mean abundance of *L. rhamnosus* among the study subjects before, during and after the probiotic intervention (the time points 1-3, respectively) quantified by the HITChip hybridization signal. The error bars denote the Gaussian 95% confidence limits based on standard deviation of the mean.
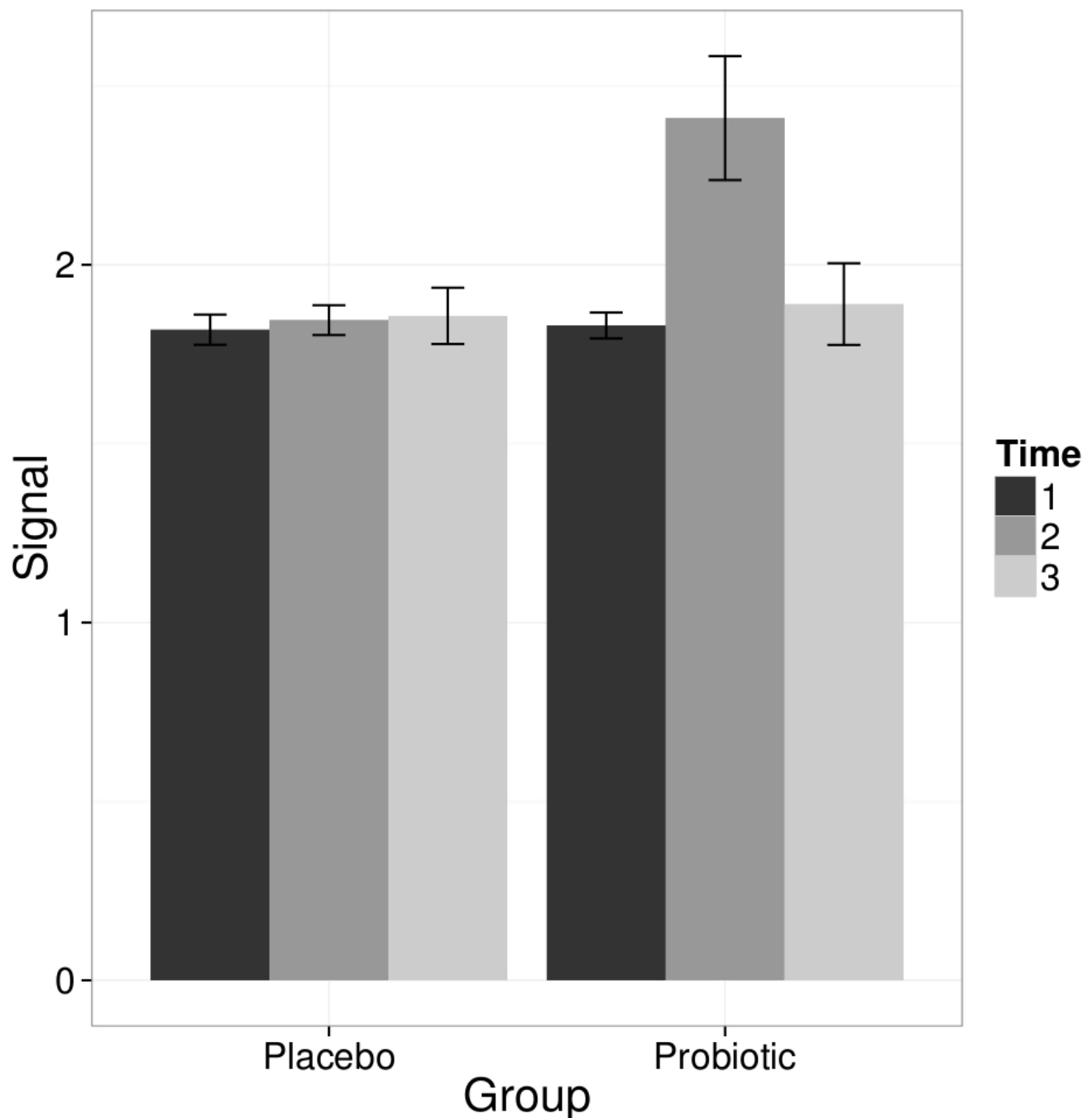
Figure 2

Correlations between intestinal genus-level phylogenetic groups and serum lipids

The correlations between the intestinal bacteria and serum lipids are indicated by colors (red: positive; blue: negative). The significant correlations (q<0.05) are indicated by '+'; only lipids and bacteria with at least one significant correlation are shown. Hierarchical clustering of the rows and columns highlights groups of significantly correlated bacteria and lipids. Lipids have been named according to Lipid Maps (http://www.lipidmaps.org) with the following abbreviations: Cer: ceramide; ChoE: cholesteryl ester; lysoPC: lysophosphatidylcholine; PA: phosphatidic acid; PG: phosphatidylglycerol; PC: phosphatidylcholine; PS: phosphatidylserine; SM: sphingomyelin; TG: triglyceride. Where the fatty acid composition could not be determined, the total number of carbons and double bonds is indicated. The first number indicates the amount of carbon atoms in the fatty acid molecule, followed by the number of double bonds. For further details, see the Methods section.
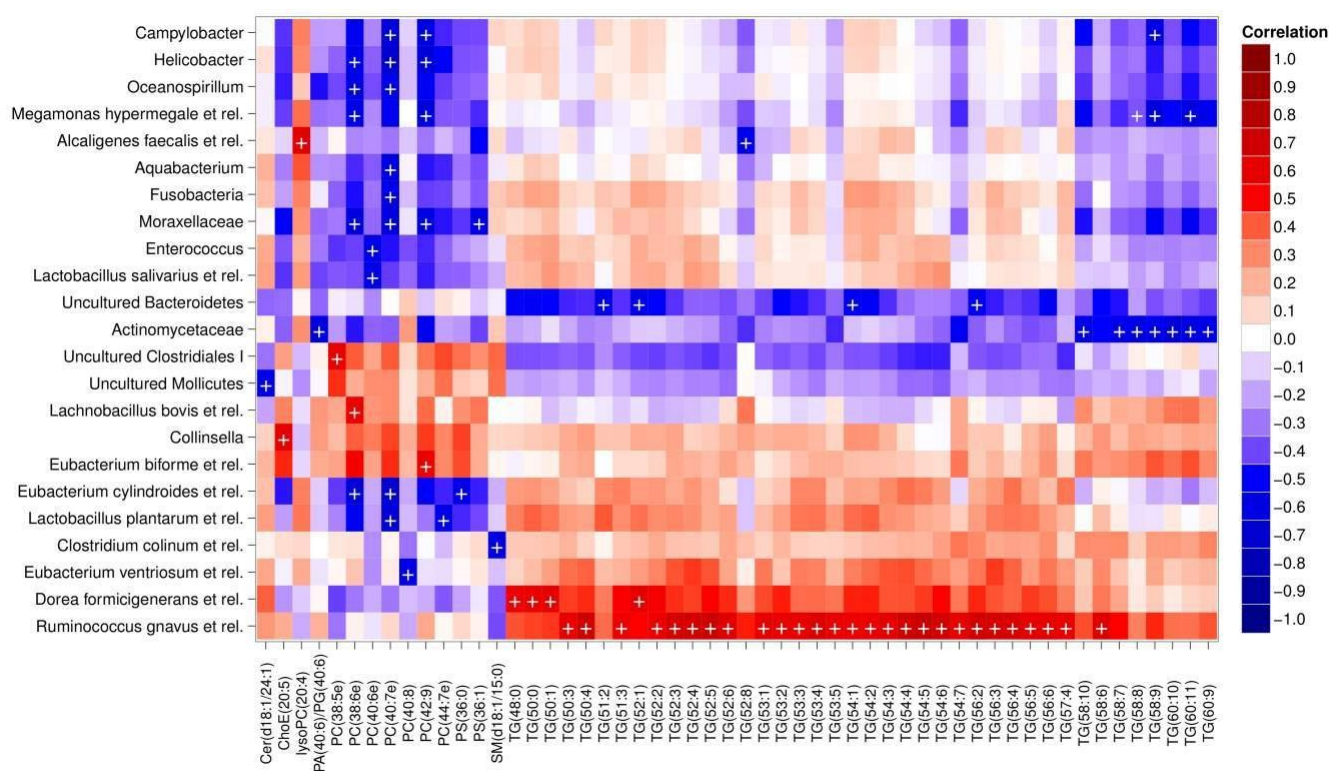
Figure 3

Association between *Ruminococcus gnavus et rel.* and serum triglyceride (TG) lipids

The relative amounts of *R. gnavus et rel.* were quantified by the HITChip analysis and the triglyceride concentration was determined based on two independent techniques: **A** the triglyceride TG(54:5) (see Fig. 2 for explanation) by mass spectrometry (r=0.61); **B** triglyceride by an enzymatic assay (r=0.60).
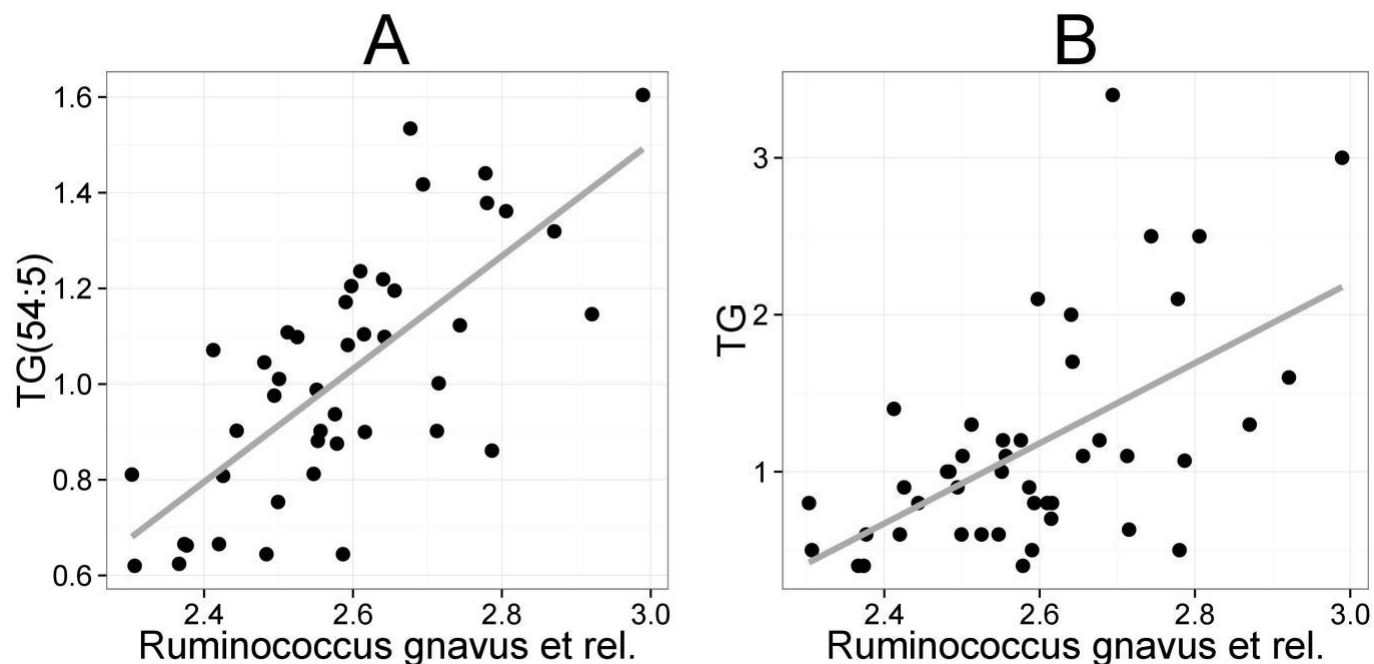
Figure 4

Association between *Collinsella spp.* and serum cholesterol

The relative amounts of *Collinsella spp.* were quantified by the HITChip analysis, and serum cholesterol levels were determined by two independent techniques: **A** Cholesterol ester ChoE(20:5) (see Fig. 2 for explanation) by mass spectrometry (r=0.59); **B** low-density lipoprotein (LDL) cholesterol by enzymatic assay (r=0.57).