Probiotic potential of a Lactobacillus rhamnosus cheese isolate and its effect on the fecal microbiota of healthy volunteers

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Abstract

The present study describes an *in vitro* characterization of strains of lactic acid bacteria, focusing on physiological characters of probiotic interest, and a subsequent placebo-controlled, crossover administration trial, with a cohort of healthy volunteers. The strains of lactic acid bacteria were previously isolated from a fermented food (long ripened cheese) and several ones resulted to have promising probiotic characteristics. Based on comprehensive evaluation of the data obtained, one strain was chosen and supplemented in a fermented milk. The fermented milk was then used in the administration trial with the goal of assessing its effect on the composition of the intestinal microbiota, as reflected in the feces. The fermented milk, with or without probiotic, had an effect on the intestinal microbiota and significant inter-individual differences were observed in response to the intervention. A common trend was observed related to two important populations of the human gut microbiota; a reduction in the relative abundance of *Bacteroides* and increase in the abundance of *Prevotella* in subjects during treatment compared to baseline were registered.

Keywords: probiotic, fermented milk, intestinal microbiota, *Lactobacillus*

1. Introduction

Fermented foods are colonized by Lactic Acid Bacteria (LAB), not only during production but also at the moment of consumption. Increasing evidence suggests that consumption of some fermented foods can promote human health and the live microbes within them contribute to such effect (Marco et al, 2017). Delivery of high numbers of commensal microorganisms that transiently interact with the intestinal microbiota and the host may play a role in gut physiology (Plé et al., 2013). The diversity of microbes associated with fermented foods represents also a rich source of potential probiotic microorganisms. Several studies have focused on isolation and probiotic characterization of LAB from fermented foods (olives, sausages, dairy). Consequently, fermented foods can serve as vehicles for probiotic delivery (van Hylckama Vlieg et al., 2011).

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014, FAO 2001, FAO 2002). Among other beneficial health effects, modulation of the host immunity, pathogen exclusion and reinforcement of the gut barrier are most cited. Such effects may be the result of direct probiotic-host interaction or indirect, through modulation of the intestinal microbiota. Several mechanisms have been described that lead to the above described effects. Such mechanisms may be widely spread among different genera of LAB and therefore are considered core benefits, or less commonly encountered and can be considered strain-specific (Sanders et al, 2018, Hill et al, 2014).

Among probiotic intervention outcomes, the modulation of intestinal microbiota is commonly sought after as it is considered an indirect way of health maintenance or promotion (Walter et al, 2018). Advancements achieved in our understanding of the role of intestinal microbiota in human health corroborate the need to explore how

consumption of foods supplemented with probiotics influences microbiota composition and human health (Marco et al., 2017).

In a previous work, we studied a large collection of isolates, originating from a ripened cheese, focusing on certain functional properties. Most of the isolates showed a high auto-aggregation property, low hydrophobicity values and a general low survival to simulated digestion process. However, sixteen strains showed promising functional characteristics (Bautista-Gallego et al., 2014). The purpose of this study was to (i) refine the characterization of the sixteen strains, focusing on probiotic properties (adhesion to cell lines, inhibition of *Listeria monocytogenes* adhesion and invasion); (ii) to investigate the consequences of the consumption of a fermented milk supplemented with a probiotic, selected based on the characterization performed, on intestinal microbiota composition of 14 healthy volunteers.

2. Materials and methods

2.1. Bacterial isolates

Sixteen different strains, previously isolated during ripening of an Italian hard cheese, were used in this work. The strains were selected from a larger collection, based on a functional characterization that included testing for autoaggregation and hydrophobicity properties as well as survival in simulated gastrointestinal passage (Bautista-Gallego et al., 2014). They belong to *Lb. helveticus* (6), *Lb. rhamnosus* (9) and *Lb. fermentum* (1) species (Table 1). *Lb. rhamnosus* GG, a known probiotic microorganism, was used as a reference strain (Caggia et al, 2015). *Lactococcus lactis* 40FEL3 was also used in this study since it is a potent bacteriocin-producing microorganism (Dal Bello et al., 2012).

2.2. Antibiotic susceptibility

Antibiotic resistance or susceptibility were tested following the method described by Argyri et al. (2013) and Botta et al. (2014). Minimal inhibitory concentrations (MICs) of 9 antibiotics (ampicillin, AMP; gentamicin, GEN; kanamycin, KAN; streptomycin, STR; erythromycin, ERY; vancomycin, VAN; chloramphenicol, CHL; tetracycline: TET; and clindamycin, CLY) (Sigma, Milan, Italy) were determined in MRS broth. All antibiotics were added according to the breakpoint concentrations proposed by the European Food Safety Authority (EFSA, 2008). If an isolate resulted resistant to a breakpoint concentration, it was progressively tested with higher concentrations until the MIC was determined. All the assays were done in triplicate.

2.3. Cell assays

The experiments were performed using two intestinal epithelial cell lines of human origin, namely Caco-2 (ATCC HTB-37) and HT-29 (ATCC HTB-38). Caco-2 cell line was grown in an advanced Dulbecco Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Milan, Italy) whereas HT-29 was grown in McCoy's Medium Modified (Thermo Fisher Scientific). Both media were supplemented with 5% foetal calf serum (Lonza, Basel, Switzerland), L-glutamine (2 mM, Sigma), penicillin (100 U mL^{-1}, Sigma) and streptomycin (1 mg mL^{-1}, Sigma). The cell lines were routinely grown in 25 cm^2 culture flasks (Corning, New York, USA) at 37 ºC in a humidified atmosphere containing 5% CO_2 and 95% air, until confluent monolayers were obtained. The culture medium was changed routinely and once the cells reached confluence they were subpassaged. All tests were carried out in triplicate in an undifferentiated monolayer, with a suspension of 30000 cells/well (c/w) for Caco-2 and 50000 c/w in the case of HT-29.

127 *2.3.1. Adhesion assay*

128 The ability of the 16 *Lactobacillus* strains to adhere to an undifferentiated cell
129 monolayer (Caco-2 and HT-29) was studied using the protocol described by Botta et al.
130 (2014).

131 *2.3.2. Inhibition of* Listeria monocytogenes *adhesion and invasion*

132 Determination of the inhibition of *Listeria monocytogenes* adhesion and invasion
133 of a cell monolayer, by the 16 *Lactobacillus* strains, was carried out following the
134 method described by Nakamura et al. (2012) with some modifications. Briefly, 0.1 ml
135 of fresh medium (DMEM or McCoy's, depending of the cell line used) containing 7 log
136 CFU/ml of each of the *Lactobacillus* strains was added to the monolayer. After 90 min
137 of incubation at 37 ºC, the medium was removed and the monolayer washed twice with
138 Phosphate Buffered Saline (PBS, pH 7.3). Then, 0.1 ml of fresh medium containing 7
139 log CFU/ml of *L. monocytogenes* (without antibiotics) was placed in each well of a 96
140 Well TC-Treated Microplate (Corning). After 2h of incubation at 37 ºC, bacterial cells
141 that did not adhere to the monolayer were washed away with PBS. Then to quantify the
142 *L. monocytogenes* adhesion, 0.1 ml of Triton-X100 (0.25% in PBS) was added for 30
143 minutes at 37 ºC. Subsequently, *L. monocytogenes* adhered to the monolayer was
144 quantified by plating on BHI agar (Oxoid, Milan, Italy). To measure the intracellular *L.*
145 *monocytogenes*, after the 2 hours incubation and the removal of non-adhered *L.*
146 *monocytogenes*, 200 µl of fresh medium containing 50 mg/ml gentamicin (Sigma) was
147 added for 1 hour to kill extracellularly adhered bacteria (Botta et al., 2014, Corr et al.,
148 2007). The cells were then washed 3 times with PBS and lysed by the addition of 0.1 ml
149 of Triton-X100 (0.25% in PBS). Viable *L. monocytogenes* were determined by plating
150 on BHI agar (Oxoid). Inhibition of *L. monocytogenes* adhesion and invasion for each
151 *Lactobacillus* strain were referred to the control obtained only with the addition of *L.*

monocytogenes (without prior treatment with a *Lactobacillus* strain). Results were subjected to one-way ANOVA and Fisher Test was used to determine differences at $P< 0.05$, using the statistical software, Statistica 7.0 for Windows (Statsoft, Tulsa, USA).

2.4. Technological characterization

The growth/survival in skim milk (Biogenetics, Padova, Italy) of the *Lactobacillus* strains was evaluated during 7 days. Briefly, Skim Milk was inoculated firstly with 6 log CFU/ml of *Streptococcus thermophilus* (Sacco, Milan, Italy). After 6 hours at 40 ºC, the *Lactobacillus* strains were added at ~8 log CFU/ml. Cell counts were determined, at time of inoculation and after 7 days at 4 °C, on MRS agar and the survival rate of the *Lactobacillus* strains was calculated [$(CFU/mL_{t=0}/CFU/mL_{t=7})X100$]. The pH was monitored daily throughout the period of 7 days using a pH meter (Crison, Modena, Italy).

2.5. Fermented milk production and administration

Based on the previously obtained results, *Lb. rhamnosus* 14E4 was chosen to be used in the production of a probiotic fermented milk. UHT milk was heated at 42 ºC and then a *S. thermophilus* inoculum (Sacco, Milan, Italy) (≈6 log cfu/mL) was added. After 15 hours, milk was divided in two different batches. One was stored directly at 4 ºC (placebo fermented milk). The other one was inoculated with *Lb. rhamnosus* 14E4 at a final concentration of ~10 log CFU/mL and the fermented milk was incubated at 37ºC for 2 hours (probiotic fermented milk). Subsequently, the probiotic fermented milk was stored at 4 ºC until its consumption.

The fermented milk was administered to fourteen healthy volunteers (n = 14) with about equal portions of men and women aged 25–40 (30.7 ± 4.0), with BMI>18 (25.18 ±

177 5.21) who habitually followed an omnivore diet. The exclusion criteria were: acute or
178 chronic gastrointestinal diseases; eating disorders, such as anorexia, bulimia or other
179 specified feeding or eating disorder; prevalent chronic diseases, such as diabetes
180 mellitus and cancer; antibiotic treatment or surgical operations during the previous 3
181 months; pregnancy and breastfeeding.
182 Volunteers were divided in two groups; group 1 consumed the probiotic fermented
183 milk and the group 2 consumed the placebo fermented milk (Fig. 1). The probiotic or
184 placebo product was administered daily (100 mL) for 15 days. After a week of wash
185 out, intervention was inverted and the fermented milk (placebo for group 1 and
186 probiotic for group 2) was administered for another 15 days.
187 Volunteers were provided with a series of containers to collect the faeces (VWR, Milan,
188 Italy) containing 10 mL of RNA*later* (Thermo Fisher Scientific). The faecal samples
189 were collected at home and transferred to the sterile sampling containers using a
190 polypropylene spoon (3 spoons of about 15g of faecal samples) and immediately stored
191 at 4 °C. The specimens were transported to the laboratory within 12 hours of collection
192 at a refrigerated temperature and stored at -80 °C for further analysis. During the whole
193 study faeces were sampled from both groups collecting a total of 9 samples from each
194 volunteer (Fig. 1).
195
196 *2.6. RNA extraction and analysis by pyrosequencing.*
197 At each sampling point, RNA was extracted directly from faecal samples using the
198 Electtrofor-Norgen RNA purification kit (Norgen Biotek Corp. Ontario, Canada),
199 according to the manufacturer's instructions. Seven µl of TURBO-DNase (Life
200 Technologies) were added to digest the DNA in the RNA samples, with an incubation
201 of 3 h at 37°C. RNA was quantified using a NanoDrop 1000 spectrophotometer
202 (Thermo Scientific, Milano, Italy) and standardized at 500 ng/µl. Each RNA solution

was checked for the presence of residual DNA by performing PCR amplification (Ferrocino et al., 2015). When positive signals were detected, the DNase treatment was repeated.

Reverse transcription (RT) reactions were performed using an M-MLV reverse transcriptase (Promega, Milan, Italy). Five hundred ng of RNA were mixed with 1 µl of 10 µM Random Primers (Promega, Milan, Italy) and DNase- and RNase-free sterile water (Sigma) to a final volume of 10 µl and then incubated at 75 °C for 5 min. The mix was placed on ice and a mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2 mM of each dNTP, 1 µl of 200 U/µl M-MLV and 0.96 U of RNasin ribonuclease inhibitor (Promega) was transferred to the reaction tube. Reverse transcription was carried out at 42 °C for 1 hour.

Complementary DNA (cDNA) was used to study the bacterial diversity in the faecal samples. Primers Gray28F (5=-TTTGATCNTGGCTCAG) and Gray519r (5=-GTNTTACNGCGGCKGCTG), which amplify a fragment of 520 bp (Ercolini et al 2012) were used to amplify the V1-V3 region of the 16S rRNA and 454 adaptors were included in the forward primer, followed by a 10-bp sample-specific multiplex identifier (MID).

The PCR mixture contained 50 ng of template DNA, 0.4 µM of each primer, 0.50 mmol L^{-1} of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 5 µl of 10 X PCR buffer, and 2.5 U of native Taq polymerase (Thermo Fisher Scientific). The following PCR conditions were used: 94 °C for 2 min, 35 cycles of 95 °C for 20 s, 56 °C for 45 s, and 72 °C for 5 min, and a final extension at 72 °C for 7 min. PCR products were purified twice with Agencourt AMPure kit (Beckman Coulter, Milano, Italy) prior to further processing. An equimolar pool of the PCR templates was obtained prior to further processing. The amplicon pool was processed by using Titanium chemistry on a GS

228 Junior platform (454 Life Sciences, Roche, Monza, Italy) according to the
229 manufacturer's instructions.
230
231 *2.7. Bioinformatics*
232 The sequencing data obtained were analyzed by using QIIME 1.9.0 software (Caporaso
233 et al., 2010), and a pipeline previously described (Ferrocino et al., 2016). OTUs were
234 picked at 99% of similarity and representative sequences of each cluster were used to
235 assign taxonomy using the Greengenes 16S rRNA gene database (McDonald et al.,
236 2012). Alpha diversity indices were calculated by using the *diversity* function of the
237 vegan package. The Shannon-Wiener diversity index (*H'*) was further analyzed using t-
238 test, with intervention (fermented milk consumption) being the main factor. Weighted
239 UniFrac distance matrices obtained through QIIME were imported in R to obtain
240 Principal Coordinates Analysis (PCoA) plots. The OTU table obtained through QIIME
241 displays the higher taxonomy resolution that was reached by the 16S data; when the
242 taxonomy assignment was not able to reach the species level, the genus or the family
243 was displayed. OTU tables and Weighted UniFrac distance matrices were used for
244 Adonis and Anosim statistical tests in R environment (www.r-project.org) in order to
245 verify differences across subjects (volunteers) and between the samples as a function of
246 intervention (placebo and probiotic) and groups (1 and 2). OTU tables filtered for OTUs
247 presence > 3% relative abundance in at least 20 samples, were then used to build a
248 principal component analysis (PCA) as a function of the groups by using the "*made4*"
249 package of R. Kruskal–Wallis tests were used to find significant differences in
250 microbial taxa abundance according to intervention or group. A P value of 0.05 or lower
251 was considered as statistically significant. PICRUSt (Langille et al., 2013) was used to
252 predict abundances of gene families based on 16S rRNA sequences data as recently

253 described (Ferrocino et al., 2015). KEGG orthologs were then collapsed at level 3 of
254 hierarchy, and the table was imported in the "*GAGE*" Bioconductor package (Luo et al.,
255 2009) to identify inferred metabolic pathways overrepresented or underrepresented
256 between placebo and probiotic consumption.
257
258 *2.8. Nucleotide sequence accession number*
259 All the sequencing data were deposited at the Sequence Read Archive of the National
260 Center for Biotechnology Information (Sequence accession number SRP067522).
261
262 **3. Results**
263 *3.1. Probiotic characterization of* Lactobacillus *strains*
264 *3.1.1. Antibiotic resistance*
265 The results of the antibiotic resistance trials are shown in Table 1. For the antibiotics
266 ampicillin, vancomycin, gentamycin, kanamycin and streptomycin, the strains
267 belonging to 3 different species of *Lactobacillus* showed comparable levels of
268 resistance. For the antibiotics erythromycin, clindamycin, tetracycline and
269 chloramphenicol, the resistance varied among strains belonging to the same species as
270 well as between different species. *Lb. rhamnosus* 10D9 showed high resistance to all
271 antibiotics, even up to the breakpoints proposed by EFSA (2008).
272 *3.1.2. Adhesion to human cell lines and inhibition of* L. monocytogenes
273 The sixteen *Lactobacillus* strains were tested for their ability to adhere to Caco-2 and
274 HT-29 human-derived cell lines. In the case of the Caco-2 undifferentiated monolayer
275 (Fig. 2a), the adhesion capability was variable (from 0.51% to 13.65%), but ten strains
276 showed higher values than the reference probiotic *Lb. rhamnosus* GG (with five of them
277 over 10% of adhesion). The adhesion of *Lb. rhamnosus* GG was higher on the HT-29
278 cell line, reaching a value above 12 % (Fig. 2b). Also in this case, strains 2D5, 4D5,

279 5D10, 10D9, 13E7, 14E4 and 15F9 showed similar or higher percentages of adherence.
280 Globally, a better capability to adhere to the HT-29 monolayer than to Caco-2 was
281 observed.
282 Further to the adhesion to a monolayer, the strains were also tested for their ability to
283 inhibit adhesion or invasion of *L. monocytogenes* to the same two monolayers. As
284 shown in Figure 3, most strains tested, including the *Lb. rhamnosus* GG, showed limited
285 effect on adhesion of *L. monocytogenes* to Caco-2 cells. Only strains 5D10, 6E8, 13E7,
286 21E1, 10F3 and 11F9 reduced significantly the adhesion. On the other hand, 10D9
287 increased significantly the adhesion (114.81%). Twelve of the sixteen strains decreased
288 the *L. monocytogenes* invasion into Caco-2 cells. The degree of inhibition varied
289 significantly among the strains. Strain 10F3 demonstrated the highest reduction of *L.*
290 *monocytogenes* invasion, similar to the level of inhibition of the bacteriocinogenic *L.*
291 *lactis* 40FEL3 (26.08% of *L. monocytogenes* invasion in the presence of *L. lactis*).
292 Interestingly, certain *Lactobacillus* strains, including the reference *Lb. rhamnosus*, had a
293 positive effect and promoted the invasion of *L. monocytogenes* into the Caco-2 cells. In
294 the case of the inhibition of *L. monocytogenes* adhesion to HT-29 cells (Fig. 3b), some
295 strains showed a similar behaviour with respect to *Lb. rhamnosus* GG while 12D5, 6E8,
296 13E7, 14E4, 21E1, 1F4, 10F3 and 15F9 promoted a significant adhesion reduction. In
297 the case of the inhibition of the invasion, only three strains (13E7, 14E4 and 10F3) were
298 able to reduce the entry of *L. monocytogenes* into HT-29 cells better than *Lb.*
299 *rhamnosus* GG. As observed for the Caco-2 cell line, also in the case of the HT-29,
300 certain strains improved significantly the invasion of *L. monocytogenes* (21E1 and 1F8).
301 *3.2. Technological characterization of* Lactobacillus *strains*
302 In order to complement the probiotic characterization of the strains, some technological
303 parameters were also taken into consideration. More specifically strains were tested for

their ability to survive during storage of fermented milk and for their impact on the pH during storage. As shown in Table 1, the highest survival rate after 7 days was detected for strains 2D5, 4D5, 5D10, 10D9, 14E4, 21E1, 1F4, 11F9 and 15F10 (values > 100% represent growth of the strain during storage). Conversely, strains 12D5, 17D10, 6E8, 13E7, 1F8, 10F3 and 15F9 displayed the lowest survival (values < 100% represent reduced cell concentration after 7 days). Some strains showed limited acidification ability and at the end of the period reduced the pH by 0.2 units while others (21E1, 10D9, 12D5, 1F8, 1F4) sharply decreased the pH within the first day of storage, reaching final values of pH around 4.4 (initial pH was 4.88) (data not shown).

3.2. Impact of fermented milk on intestinal microbiota

RNA extracted from feces of volunteers that consumed a probiotic (fermented milk produced with the use of *S. thermophilus* and supplemented with *Lb. rhamnosus* 14E4) or placebo fermented milk (fermented milk without addition of *Lb. rhamnosus* 14E4) was used as template for the V1-V3 16S rRNA gene pyrosequencing. A total of 1,762,374 raw sequences was obtained and analyzed; 938,961 reads passed the filters applied through the QIIME split_library.py script, with an average value of 7,890 reads/sample and an average length of 452 bp. Good's estimated sample coverage (median value of 99%; Range 97-99%) indicated that satisfactory coverage was reached for all samples analyzed (Tab. S1). A high level of microbial diversity was found with an average of OTUs of 247,80 ± 84,91. A core microbiota was identified and included 9 OTUs across 90% of the samples (Fig. 4). As can be deduced from the figure, among the 9 OTUs of the core microbiota, *Bacteroides*, *Roseburia* and *Faecalibacterium* that had a median relative abundance higher than 10% also showed important variability between samples. On the other hand, OTUs with lower relative abundance (< 10%) presented limited variability between samples. Pairwise comparison of the Shannon-

329 Wiener diversity index *(H')* revealed a biological diversity ($P < 0.05$) comparing
330 samples before and after the intervention or comparing group 1 vs group 2 (data not
331 shown).
332 The fecal microbiota of each individual was clearly differentiated based on principal-
333 coordinate analysis with a Weighted UniFrac distance matrix. In addition, both Adonis
334 and Anosim statistical tests confirmed that there was a significant difference between
335 the individuals based on their microbiota ($P < 0.001$) (data not shown). Plotting the
336 Weighted UniFrac distance matrix of each subject's dataset it was possible to observe a
337 shift in the gut microbiota composition before and after intervention in 7 out of the 14
338 volunteers. Fig. S1 concerns the 7 individuals for which a shift was observed with the
339 intervention while fig. S2 concerns the 7 individuals for which such shift was not
340 evident. However this separation (of data before/after intervention) was not statistically
341 significant (ANOSIM statistical test $P>0.05$). Further, the most separated samples were
342 the ones corresponding to the subjects at time zero (Fig. S1). By comparing the initial
343 microbiota of all the subject (time 0) we did not observe a separation in terms of
344 composition across samples (ANOSIM statistical test $P > 0.05$).
345 The discrimination (ANOSIM, $P < 0.01$) on the relative abundances of OTUs associated
346 with the two groups was also taken into account (Fig. 6). The Principal Component
347 Analysis (PCA) on the OTUs table at genus level, showed a certain degree of separation
348 between subjects that used first the probiotic and then the placebo (G1) and subjects that
349 *vice versa* consumed first the placebo and then the probiotic (G2). The plot shows that
350 G1 were characterized by significantly elevated abundance of *Prevotella, Collinsella,*
351 *Faecalibacterium* and *Oscillospira* while G2 were characterized by *Sutturella,*
352 *Eubacterium* and *Odoribacter* (Fig. 6).

353 Comparing sampling during probiotic treatment against sampling at time 0 (Fig. 6A), it
354 was possible to observe an increment ($P < 0.001$) of the minor OTUs fraction (0.5-4%
355 of the total OTUs) comprising *Prevotella*, *Clostridium*, *Roseburia* and *Succinvibrio.*
356 The most remarkable differences appeared on the decrement of the abundance of
357 *Bacteroides* and *B. dorei* in samples during probiotic consumption compared to
358 baseline. Moreover, samples during placebo intervention (Fig. 6B) showed an increase
359 of *Prevotella, Ruminococcaceae* and *Sutturella* and a decrease of *Bacteroides* and
360 *Lachnospiraceae* compared to samples at baseline. Regarding the predicted
361 metagenomes, NSTI index for the samples was 0.079 ± 0.040, indicating 92% accuracy
362 for all the samples. The pathway enrichment analysis performed by GAGE of the
363 predicted metagenomes showed an enrichment of Phosphonate and phosphinate
364 metabolism (ko00440), Pentose and glucuronate interconversions (ko00040) and ABC
365 transporters (ko02010) in samples under intervention (placebo and probiotic) compared
366 to baseline (data not shown).
367
368 **Discussion**
369 In the last decades several studies report how the diet can influence the composition of
370 the human gut microbiota. Food containing LAB, such as fermented milks or cheeses
371 can modify the gut microbiota since these bacteria can easily survive and colonize the
372 gastrointestinal tract (Putignani et al. 2014). The consumption of probiotics can have
373 significant effects on human health (Larsen et al., 2010) and may change the metabolic
374 gene content in the gut (Claus et al., 2011). Short-term dietary interventions on the
375 contrary have shown to only have a minor influence on microbiota composition (Wu et
376 al. 2011; Lappi et al., 2013).

In this work we expanded the probiotic characterization of selected LAB strains with the purpose of identifying a probiotic candidate to be used in an intervention study with healthy volunteers. Antibiotic resistance was evaluated and β-Lactam antibiotic ampicillin and the broad-spectrum clindamycin were the most effective antibiotics, as observed by Botta et al. (2014). Conversely, vancomycin, kanamycin and streptomycin resulted to be less effective and higher concentrations (256 μg/ml) were required to reach inhibition. Biogenic amine production was also evaluated and only tyramine was produced by three strains at levels below 1 mg/mL (data not shown). Adhesion to Caco-2 and HT-29 was variable among the strains tested, but for several ones it was higher than the adhesion observed for *Lb. rhanmosus* GG, used as a reference probiotic strain. Variability was also observed in the capacity of the strains to limit adhesion or invasion of *L. monocytogenes* in the same two cell lines. Overall the strains did not significantly inhibit adhesion, but had an effect (mostly negative) in the invasion. Based on the results obtained, a *Lb. rhamnosus* strain was chosen and inoculated in milk fermented by *S. thermophilus* to produce a probiotic fermented milk.

The influence of the fermented milk on gut microbiota was assessed using RNA as target molecule to investigate whether the consumption could result in alterations in the microbial community structure. It was not possible to trace the *Lb. rhamnosus* or *S. thermophilus*, that were ingested with the fermented milk, in the sequencing data or by specifically targeting them by real time PCR (data not show).

The results indicate a significant inter-individual difference in response to the intervention; possibly due to the relatively small size of the cohort, such inter-individual differences may not allow for a clear effect to emerge. In fact, no clear effect on the major components of gut microbiota, similar among subjects, was found when comparing intervention and baseline samples. This is in agreement with recent DNA-

based studies evaluating the fecal microbiota before and after probiotic intervention from 12 elderly (Eloe-Fadrosh et al. 2015), 25 healthy Finnish adults (Lahti et al. 2013), seven adult female monozygotic twin pairs (Unno et al., 2015), 18 healthy Japanese adults (Kim et al., 2013) and 10 New Zealand adult subjects (Tannock et al., 2010). We found however a shift in the minor OTUs during probiotic (or placebo) intervention compared to baseline. In particular, such shift concerned an overall increase in the *Firmicutes* and a decrease in *Bacteroidetes* population as affected by the fermented milk. These results are in contrast with Unno et al. (2015) where the relative abundance of *Bacteriodetes* species increased during the intervention period and decreased during the non-ingestion period. Notwithstanding the obvious heterogeneity of the individuals, the discrepancy observed with this study may also be due to the different target molecules used, RNA instead of DNA and to the different number of subjects in the cohort (14 instead of 6). This is clearly shown from the core microbiota of this cohort, mainly represented by members belonging to *Firmicutes*. Regarding the OTUs that changed as affected by the intervention, it was possible to observe a reduction in the relative abundance of *Bacteroides* in subjects during intervention (placebo and probiotic) compared to baseline and an increase in the abundance of *Prevotella*. It has been recently reported that high *Bacteroides* levels are associated to an increased risk of weight gain and insulin resistance (Le Chatelier et al., 2013), while *Prevotella* is mostly associated with the production of short chain fatty acid (SCFA) and in details is mainly associated with the production of acetate from pyruvate via acetyl-CoA (Rey et al., 2010; Louis et al., 2014). The protective role of SCFAs against different types of disease is well documented (De Filippis et al., 2015). A recent metatranscriptomic study in a mouse model showed that the genes that exhibited the highest fold-change in expression were associated with the KEGG categories ‘carbohydrate metabolism’ and

427 ‘membrane transport’ (McNulty et al. 2015). Consistent with this, the predicted
428 metagenomes in this study showed a significant increase in abundance of genes
429 associated with sugar interconversions and genes involved in membrane transport in
430 subjects during intervention compared to baseline (data not shown).
431 The main finding from the investigation of the intestinal microbiota of subjects that
432 consumed a fermented milk, with or without a probiotic, is that changes occur for minor
433 bacterial populations. This finding is in line with previous reports that interested
434 subjects with compromised health (Ceapa et al, 2013). In probiotic interventions
435 performed on healthy adults, little or no effect on the composition of the microbiota was
436 observed (Kristensen et al, 2016). Low abundance populations, may be metabolically
437 active and interfere with metabolic networks in the large intestine, modulating intestinal
438 microbiota (Ceapa et al, 2013).
439 The strains that were explored in this study originate from a fermented food. It has been
440 proven here that a ripened cheese harbors microbes that, when ingested in large
441 quantities, have the potential to interfere and modulate the composition of the intestinal
442 microbiota. It remains to be seen what is the effect of these microbes when ingested in
443 lower concentrations and concomitantly with other, closely related and competitive
444 microbes, within a fermented food.
445
446
447 **Acknowledgements**
448 This research program has received funding from the Compagnia di San Paolo, through
449 the University of Torino (ORTO11HEXP).
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Figure legends

Figure 1. Schematic representation of the experimental trial. The figure shows the intervention for each group. Group 1 consumed first the probiotic for a period of 14 days, then there was a 1-week washout followed by placebo consumption for a period of 14 days. Group 2 consumed first the placebo for 14 days, then there was a 1 week washout followed by probiotic consumption for 14 days. The figure also shows the days of the sampling; 1 sample before the intervention, followed by three samples during the first part of the trial, 1 sample during washout, 3 more samples during the second part of the trial, followed by one last sample after the intervention (total 9 samples for each individual).

Figure 2. Adhesion profile of the lactic acid bacteria, expressed as the ratio (%) of bacteria recovered from the human cells after incubation (37 °C for 90 minutes) and the initial bacterial count of the inoculum. A) Caco-2 undifferentiated monolayer; B) HT-29 undifferentiated monolayer.

Figure 3. Adhesion and invasion inhibition of *Listeria monocytogenes* to an A) Caco-2 undifferentiated monolayer; B) HT-29 undifferentiated monolayer. Results are expressed relative to control (i.e. *Listeria monocytogenes* adhesion and invasion without the presence of a *Lactobacillus* strain, corresponding to 100% adhesion or invasion).

Figure 4. Abundance (%) of the 9 OTUs identified in 90% of the samples. Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.56 IQR from the first and third quartiles, respectively. Circles represent outliers beyond the whiskers.

643 **Figure 5.** PCA based on the OTU abundance. G1 (subjects that used first the probiotic
644 and then the placebo); G2 (subjects that vice versa consumed first the placebo and then
645 the fermented milk).
646
647 **Figure 6.** Relative abundance (percent) of the OTUs (> 0.2%) that are differentially
648 abundant ($P < 0.01$) across baseline and probiotic treated samples (Plot A), and across
649 baseline and placebo treated samples (Plot B). The red bar corresponds to the mean
650 value at time zero (baseline) for the 14 individuals. The blue bar in plot A, shows the
651 mean value for the 14 individuals during probiotic consumption (for each individual the
652 mean value of the three sampling points during probiotic consumption was used).
653 Similarly, the yellow bar in plot B, shows the mean value of the 14 individuals during
654 placebo consumption (for each individual the mean value of the three sampling points
655 during placebo consumption were considered). Refer to figure 1 for sampling points
656 during probiotic and placebo consumption.
657
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Table 1. Antibiotic MICs values (µg/mL) and survival rate in skim milk after 7 days for the 16 *Lactobacillus* strains.

Strain	Species	AMP.	VAN.	GEN.	KAN.	STR.	ERY.	CL.	TET.	CHL.	Survival rate (%)
2D5	*L. helveticus*	1	256	64	256	128	2	1	8	8	93.88
4D5		1	256	32	256	256	2	1	8	8	134.93
5D10		1	256	256	256	256	8	8	64	64	85.71
6E8		1	256	256	256	256	2	2	64	32	26.70
1F4		1	256	256	256	256	2	8	64	32	128.76
1F8		1	256	128	256	256	2	1	64	32	64.31
10D9	*L. rhamnosus*	1	256[n.r.]	256	256	256	16	16	256	256	93.81
12D5		1	256[n.r.]	128	256	256	1	1	8	8	68.86
17D10		1	256[n.r.]	128	256	128	1	1	8	8	69.97
13E7		1	256[n.r.]	128	256	256	2	1	16	8	32.56
14E4		1	256[n.r.]	256	256	256	8	1	64	32	128.76
21E1		1	256[n.r.]	128	256	256	8	1	64	32	171.67
11F9		1	256[n.r.]	256	256	256	8	8	64	256	92.08
15F9		1	256[n.r.]	128	256	256	2	2	64	32	36.22
15F10		1	256[n.r.]	256	256	256	4	2	64	32	127.27
10F3	*L. fermentum*	1	256[n.r.]	256	256	256	8	8	64	32	32.47

Note: ampicillin, AMP; gentamicin, GEN; kanamycin, KAN; streptomycin, STR; erythromycin, ERY; vancomycin, VAN; chloramphenicol, CHL; tetracycline: TET; and clindamycin, CLI.

n.r.: not required (EFSA, 2008).

Figure 1. Schematic representation of the experimental trials.

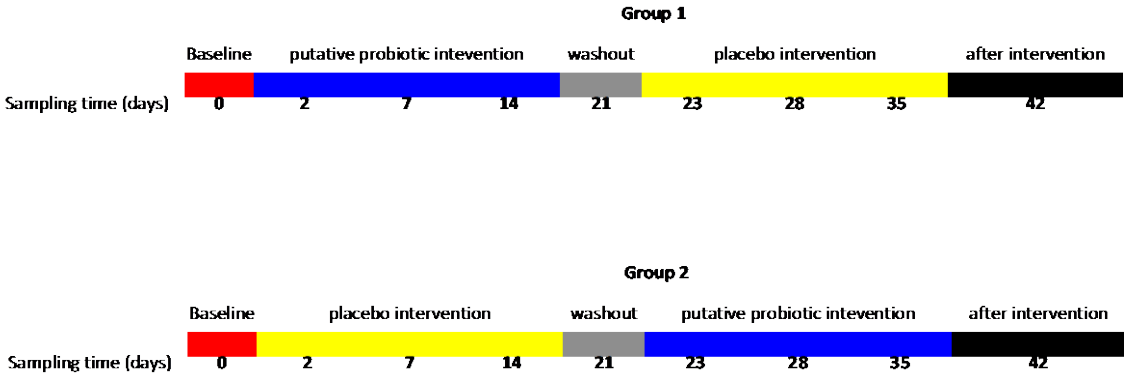
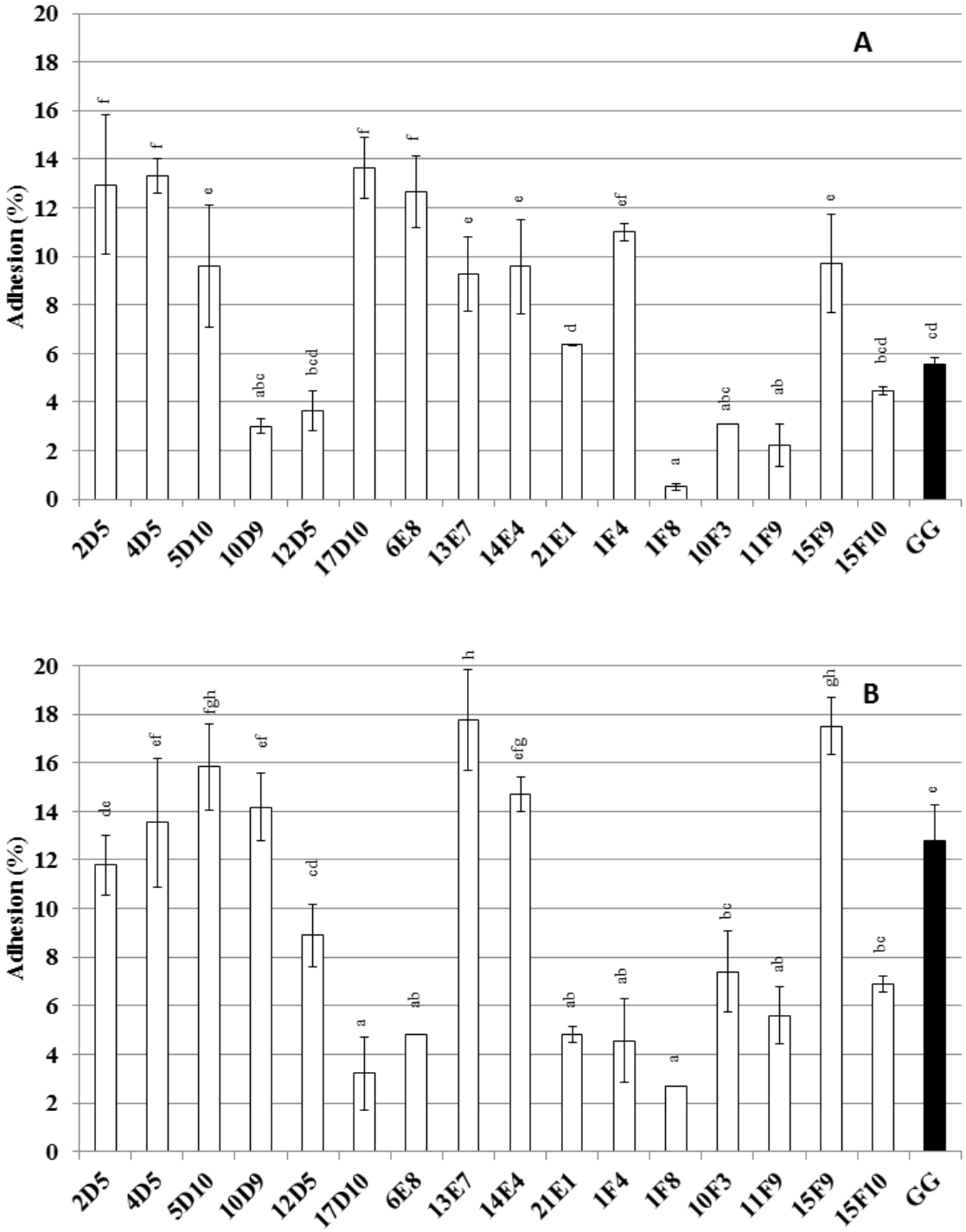


Figure 2.


A
B
Adhesion (%)
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12D5
17D10
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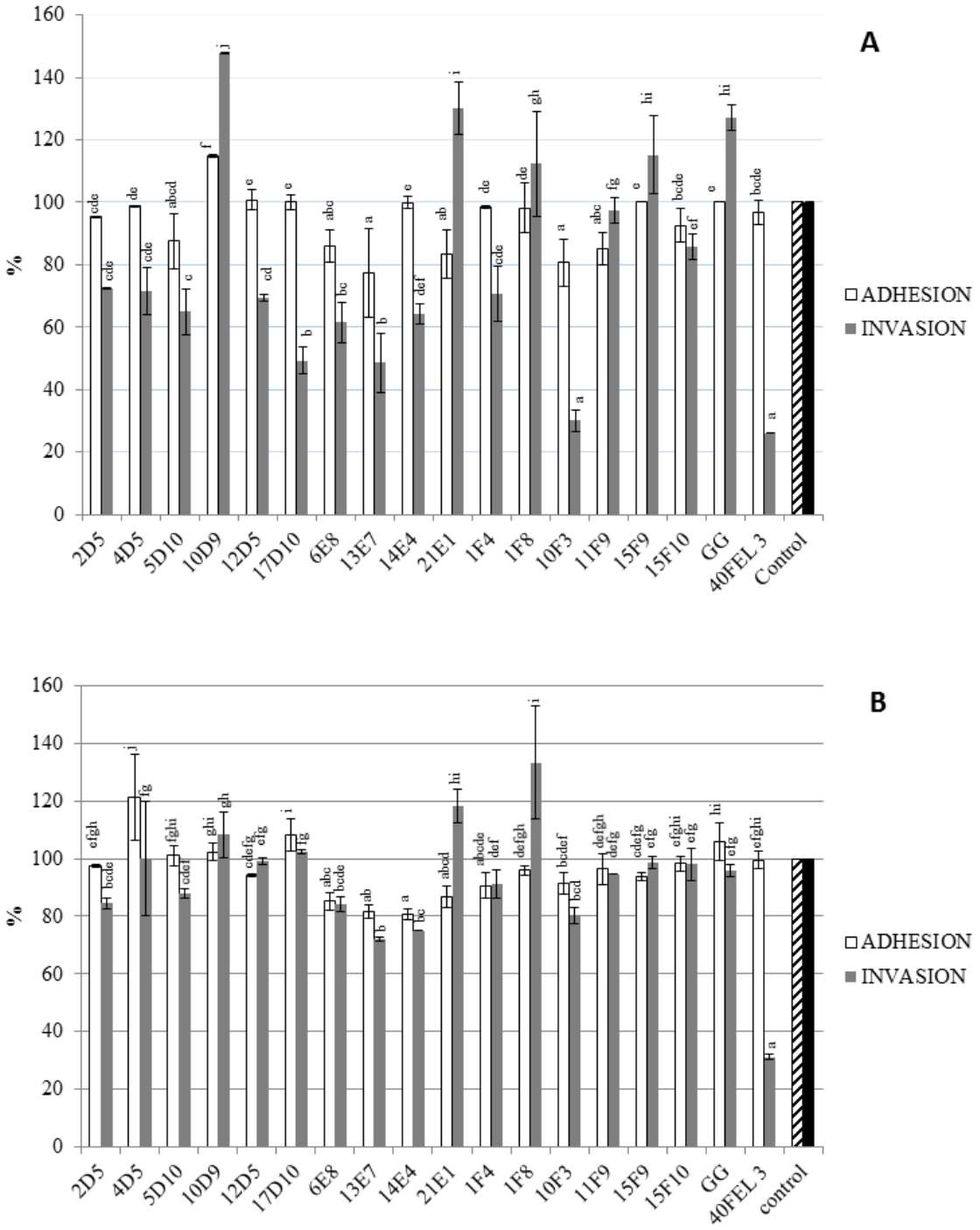
10F3
11F9
15F9
15F10
GG

Figure 3.


A
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1F4
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10F3
11F9
15F9
15F10
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40FEL 3
Control
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ADHESION
INVASION
2D5
4D5
5D10
10D9
12D5
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6E8
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control

Figure 4.

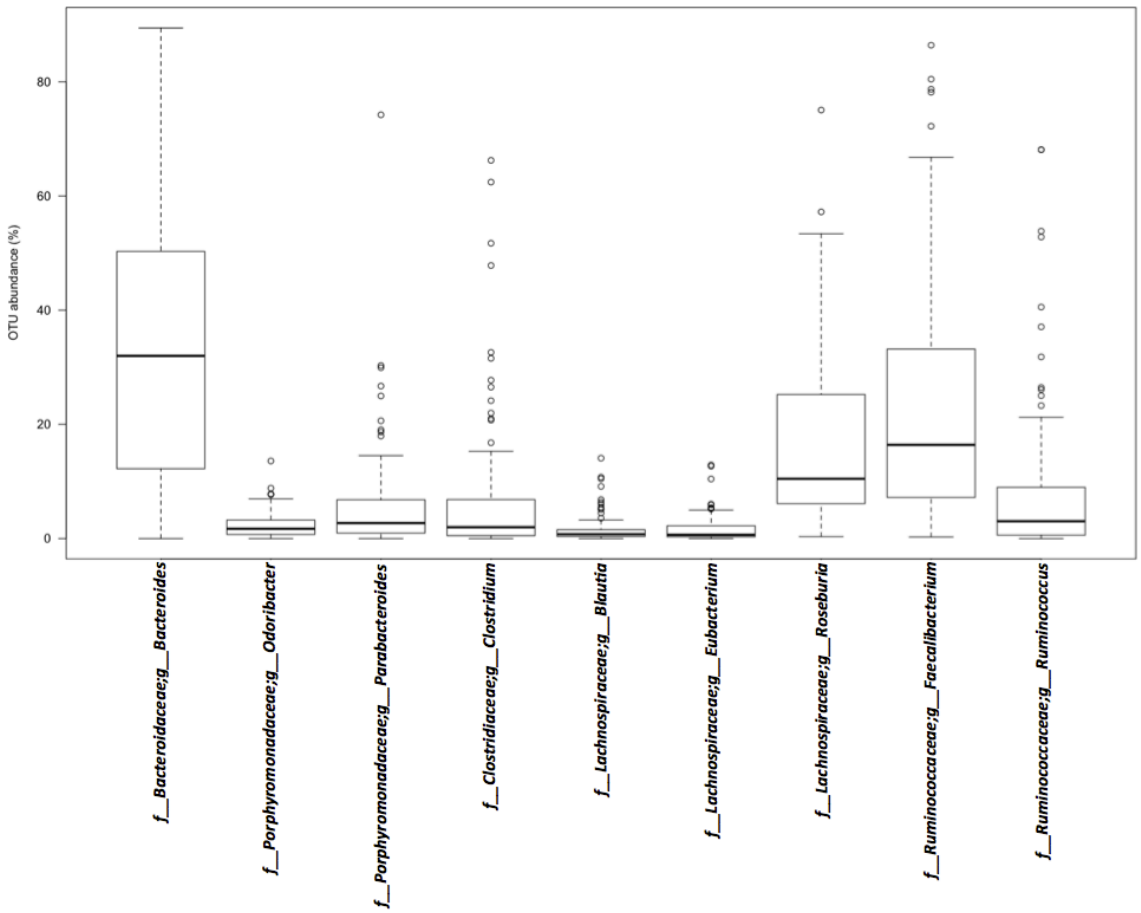


Figure 5.

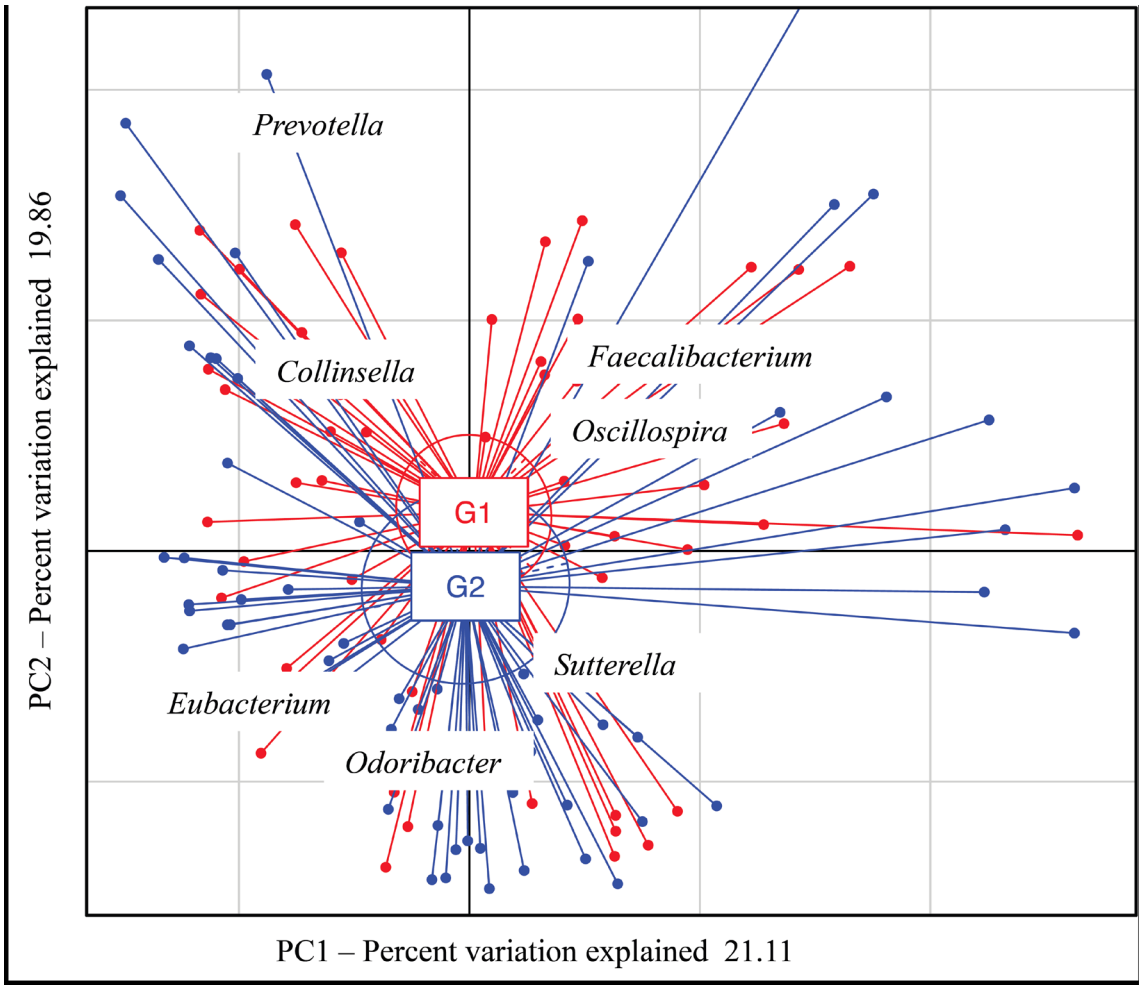
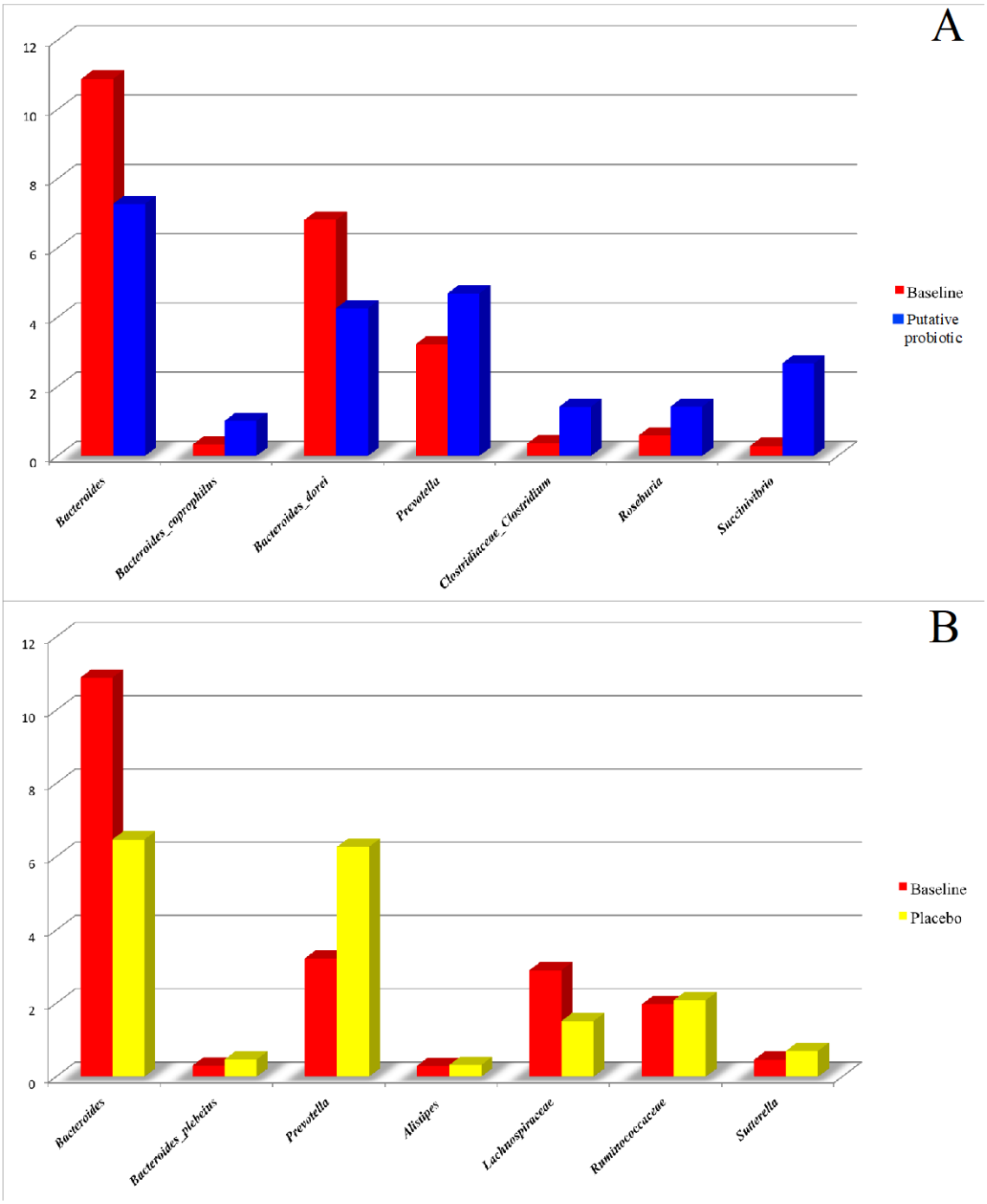
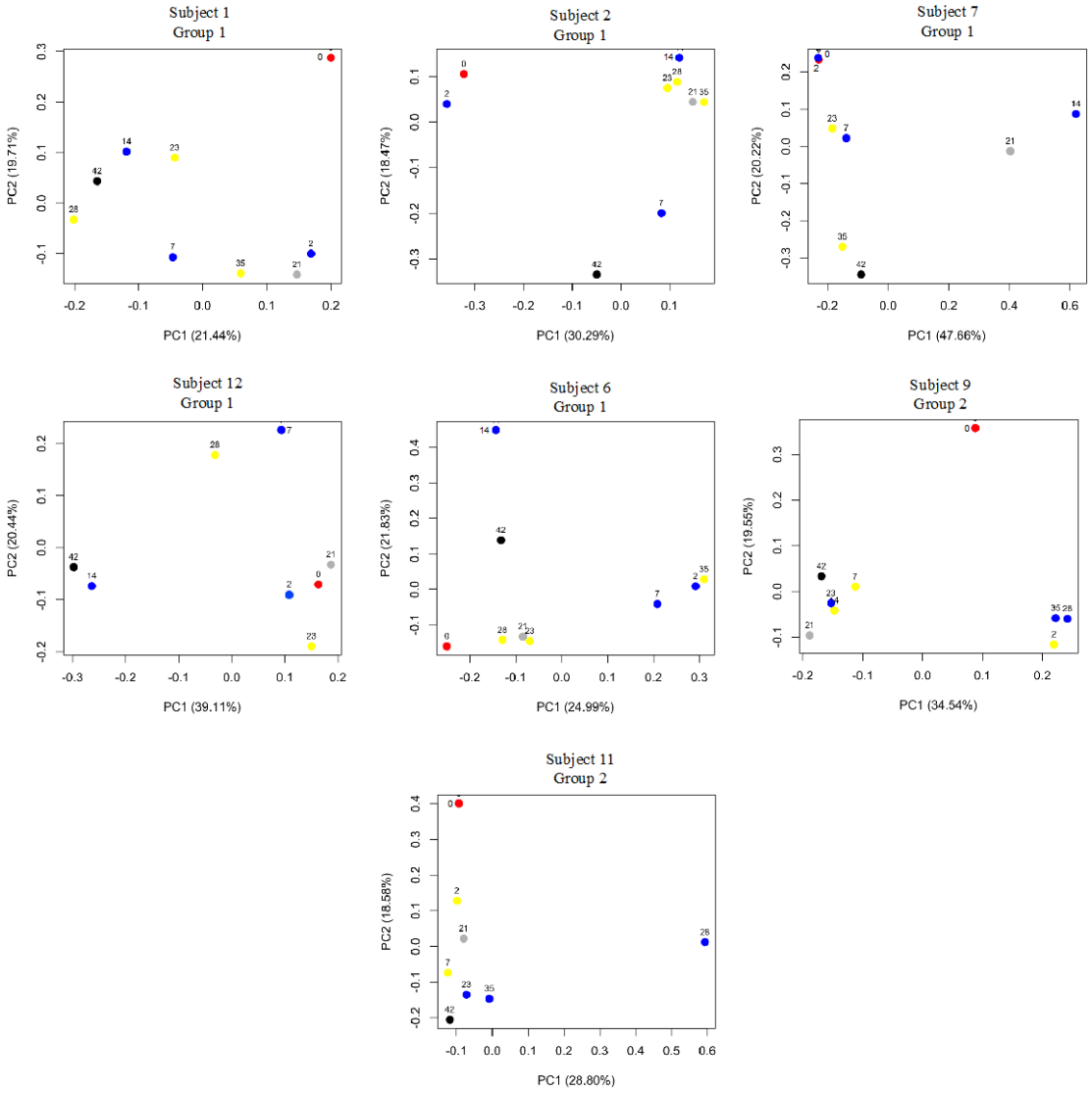

Prevotella
Collinsella
Faecalibacterium
Oscillospira
G1
G2
Sutterella
Eubacterium
Odoribacter
PC2 – Percent variation explained 19.86
PC1 – Percent variation explained 21.11

Figure 6.



Supplementary Figure S1. Principal-coordinate analysis based on Weighted UniFrac analysis of the 16S rRNA gene sequences for selected subjects. Samples from each plot

are color coded according to: baseline (red), probiotic (blue), placebo (yellow), washout (gray) and after the trial (black). Sampling day are also reported.



Supplementary Figure S2. Principal-coordinate analysis based on Weighted UniFrac analysis of the 16S rRNA gene sequences for selected subjects. Samples from each plot are color coded according to: baseline (red), probiotic (blue), placebo (yellow), washout (gray) and after the trial (black). Sampling day are also reported.