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Supplementation with dairy matrices impacts on homocysteine levels and gut microbiota composition of hyperhomocysteinemic mice

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Supplementation with dairy matrices impacts on homocysteine levels and gut microbiota composition of hyperhomocysteinemic mice --Manuscript Draft--

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Abstract:	Purpose Several studies highlighted a correlation between folic acid deficiency and high plasma homocysteine concentration, which is considered a risk factor for multifactorial diseases. Natural folates represent an emerging alternative strategy to supplementation with synthetic folic acid, whose effects are controversial. The present work was therefore performed in hyperhomocysteinemic mice to study the impact of

	<p>supplementation with either milk, or fermented milks enriched in natural folates, produced by selected lactic acid bacteria, on plasma homocysteine levels and faecal microbiota composition.</p> <p>Methods Forty mice were divided into six groups, two of which fed control or folic acid deficient diets. The remaining four groups were fed folic acid deficient diet and then shifted to: a standard control diet or a folic acid deficient diet supplemented with folate-enriched fermented milk, fermented milk, or unfermented milk.</p> <p>Results Supplementation with dairy matrices restored homocysteine levels in folic acid-deficient mice, although impacting differently on hepatic S-adenosyl-methionine levels, and also determined specific, significant variations in faecal microbiota composition. Moreover, LEfSe analysis identified bacterial biomarker genera characterising each group of mice.</p> <p>Conclusions Taken together, our results highlighted the efficacy of natural folates. Among the three dairy matrices, folate-enriched fermented milk resulted the most effective in restoring both homocysteine and S-adenosyl-methionine levels to the control conditions. The possible relationships between the presence of bacterial biomarkers and hyperhomocysteinemia, as well as the possible implications of such microorganisms in folate metabolism, are currently under investigation.</p>
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1 **Supplementation with dairy matrices impacts on homocysteine levels and gut microbiota**
2 **composition of hyperhomocysteinemic mice**

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24

25 **Keywords**

26 Fermented food, folate, homocysteine, microbiome

27

28

29 **Abstract**

30 *Purpose* Several studies highlighted a correlation between folic acid deficiency and high plasma
31 homocysteine concentration, which is considered a risk factor for multifactorial diseases. Natural
32 folates represent an emerging alternative strategy to supplementation with synthetic folic acid,
33 whose effects are controversial. The present work was therefore performed in
34 hyperhomocysteinemic mice to study the impact of supplementation with either milk, or
35 fermented milks enriched in natural folates, produced by selected lactic acid bacteria, on plasma
36 homocysteine levels and faecal microbiota composition.

37 *Methods* Forty mice were divided into six groups, two of which fed control or folic acid deficient
38 diets. The remaining four groups were fed folic acid deficient diet and then shifted to: a standard
39 control diet or a folic acid deficient diet supplemented with folate-enriched fermented milk,
40 fermented milk, or unfermented milk.

41 *Results* Supplementation with dairy matrices restored homocysteine levels in folic acid-deficient
42 mice, although impacting differently on hepatic S-adenosyl-methionine levels, and also
43 determined specific, significant variations in faecal microbiota composition. Moreover, LEfSe
44 analysis identified bacterial biomarker genera characterising each group of mice.

45 *Conclusions* Taken together, our results highlighted the efficacy of natural folates. Among the
46 three dairy matrices, folate-enriched fermented milk resulted the most effective in restoring both
47 homocysteine and S-adenosyl-methionine levels to the control conditions. The possible
48 relationships between the presence of bacterial biomarkers and hyperhomocysteinemia, as well
49 as the possible implications of such microorganisms in folate metabolism, are currently under
50 investigation.

51

52 **Introduction**

53 Nutritional deficiencies, especially those related to B-vitamins including folic acid that lead to
54 high plasma homocysteine (Hcy) concentration [1], are considered a risk factor for various
55 diseases. Hcy is a sulphur-containing amino acid intermediate that stands at the junctions of
56 trans-methylation, re-methylation and trans-sulphuration pathways. Hcy has been associated to
57 endothelial dysfunction [2], therefore, its prompt removal is required to prevent harmful
58 accumulation. Under well-balanced dietary regimen, its clearance occurs either by re-
59 methylation to methionine or by catabolism to cysteine. Re-methylation, catalysed by methionine
60 synthase (MS), can occur via cobalamin/vitamin B12 dependent transfer of methyl groups from
61 5'-methyl tetrahydrofolate (5-MTHF), or through the betaine-homocysteine S-methyltransferase
62 (BHMT) reaction that uses betaine (a choline metabolite) as methyl donor (folate independent re-
63 methylation). In the trans-methylation pathway, methionine is condensed to ATP to yield S-
64 adenosyl-methionine (SAM), representing the primary methyl donor in biological processes. The
65 removal of methyl group from SAM during methylation reactions generates S-
66 adenosylhomocysteine (SAH), whose hydrolysis forms Hcy. Finally, catabolism of Hcy uses the
67 trans-sulphuration pathway, via vitamin B6/Cystathionine beta synthase (CBS) catalysis.

68 Moderately elevated levels of Hcy resulting from variation in gene-nutrient interactions are
69 mainly due to polymorphisms in the genes encoding for re-methylation enzymes, such as
70 methylenetetrahydrofolate reductase (MTHFR) and/or MS, combined with low B vitamin status
71 (folate, B6 or B12). Dietary pattern alone can also have an effect on Hcy levels, being
72 significantly and inversely correlated with serum folate and vitamin B12 or B6 levels. Folate
73 status is considered the most important marker of impaired Hcy methylation [3] [4,5].

74 As mentioned, hyperhomocysteinemia is associated to vascular endothelium alterations and is
75 therefore considered a risk factor for complex and multifactorial disorders including
76 cardiovascular disease and neurological dysfunction [6]. However, recent reviews and meta-
77 analyses have reported the absence of a significant effect of Hcy-lowering interventions (with
78 folic acid and/or B vitamin supplementation) on the incidence of myocardial infarction, or death
79 from any cause, or cognitive decline, with the only exception of stroke [7-11].

80 At present, the main expedient strategies to counteract hyperhomocysteinemia include the
81 supplementation or food fortification with synthetic folic acid. Conversely to naturally occurring
82 folates, mainly represented by tetrahydrofolate (THF) and its derivatives [12,13], synthetic folic
83 acid needs to be converted to the biologically active form (THF) by dihydrofolate reductase
84 (DHFR) to enter the folate cycle. The conversion of folic acid to THF is a slow process because
85 DHFR has reduced metabolic capacity that increases the risk of accumulation of unmetabolised

86 folic acid in blood, thus limiting the benefit of the use of synthetic folic acid [13,14]. Recent
87 evidence indeed suggests that the unmetabolised folic acid can be harmful, and high folic acid
88 intake was shown to exert a series of potentially toxic effects including impairment of normal
89 immune function [15,16]; cancer development and progression [17-20]; alteration of DHFR
90 enzymatic activity [14] and of liver lipid metabolism [21] as well as development of neuropathy
91 risk [22]. A growing number of studies are therefore focusing on the application of alternative
92 strategies based on the use of natural folates.

93 Several Lactic Acid Bacteria (LAB) or Bifidobacteria strains involved in dairy fermentations are
94 able to synthesise natural folates that are released into the food matrix [23,24] and are therefore
95 promising candidates to revert folate deficiency and the associated metabolic alterations [25-27]
96 [28,29]. Therefore, the aim of this work was to evaluate if supplementation with dairy matrices,
97 including fermented milk enriched in natural folates produced by suitably selected LAB strains,
98 could affect plasma Hcy levels in hyperhomocysteinemic mice. Moreover, as the majority of
99 available studies did not take into account the effect of the dietary interventions on intestinal
100 microbiota composition, or the possible colonisation capacity exerted by fermentative microbes
101 (which could provide an important additional endogenous source of folates [30]), we also
102 examined the impact of supplementations on faecal microbiota composition.

103

104 **Materials and Methods**

105 **Bacterial strains and growth conditions**

106 The LAB strains employed in this study were isolated from Italian dairy products and stored in
107 the LAB collection of CREA-ZA, Lodi (Italy). *Streptococcus thermophilus* 563 was cultivated in
108 M17 broth (Merck, Darmstadt, Germany) supplemented with 1% lactose, at 42 °C in aerobic
109 conditions. *Lactobacillus delbrueckii* subsp. *lactis* 1021 and *Lactobacillus helveticus* 989 were
110 cultivated in MRS (De Man, Rogosa, Sharpe, Merck) at 42 °C in anaerobic conditions. *L.*
111 *rhamnosus* ATCC7469 was cultivated in MRS at 37 °C in aerobic conditions.

112

113 **Fermented milk preparation and analyses**

114 Skimmed milk powder was used as starting food matrix (Oxoid, Basinstoke, England). It was
115 reconstituted with deionised water, steam sterilised, and subsequently inoculated with bacterial
116 strains, previously selected for the ability to produce folate. In particular, the folate-enriched
117 fermented milk (FFM) was obtained by co-cultivating two folate-producing strains belonging to
118 different genera, *Streptococcus thermophilus* 563 and *Lactobacillus delbrueckii* subsp. *lactis*
119 1021. To enhance folate accumulation, fermentation of FFM was carried out at 42 °C in two
120 steps: 4 h at controlled pH 6.0, obtained by addition of sterile 5 N NaOH to the culture medium,
121 followed by uncontrolled overnight fermentation (20 h). Fermented milk (FM) was produced
122 using the low-folate producer strain *Lactobacillus helveticus* 989, incubated at 42 °C for 24 h.
123 Sterile reconstituted skimmed milk lacking bacterial strains was used as unfermented milk (M).
124 After preparation, fermented milks were lyophilised and kept frozen until addition to the diets.
125 All milk supplements, including M, were subjected to the following assays: bacterial cell counts
126 (\log_{10} CFU/ml) under anaerobic conditions to evaluate the growth of *Lactobacillus* (in MRS agar,
127 42 °C, 48 h), and *S. thermophilus* strains (in M17 agar, 42 °C, 48 h); pH determination by means
128 of the Portamess 913 pH meter (Knick Elektronische, Germany); lactose, glucose, galactose,
129 citric acid and lactic acid content (g/100 g) according to the HPLC method described by [31].
130 Folate content was assayed by a microbiological assay using *L. rhamnosus* ATCC7469 as
131 indicator strain, since it is auxotrophic for folic acid [32]. Folate concentration (ng/ml) was
132 determined using folic acid (Sigma Aldrich, Italy) as standard to build the calibration curve. All
133 determinations were done in duplicate.

134

135 **Experimental design, animals and diets**

136 A total of 40, 6-weeks old C57BL/6J male mice, obtained from Charles River Laboratories
137 (Como, Italy), were kept at 23 °C with a 12 h light-dark cycle and fed *ad libitum* with a standard

138 laboratory diet (4RF21, Mucedola, Milan, Italy, www.mucedola.it). Mice had free access to food
139 and water throughout the experiments. Food intake and body weight were recorded every other
140 day and once a week, respectively. After one week of adaptation, animals were randomly divided
141 into 6 groups (6-8 mice per group), housed individually and supplemented as reported in Figure
142 1: the first 2 groups were fed control diet or folic acid deficient diet for 10 weeks (C: Control and
143 FD: Folic acid Deficient, respectively). The remaining 4 groups were fed FD diet for the first 5
144 weeks and then shifted for additional 5 weeks to: a standard control diet (R: Repleted) or a FD
145 diet supplemented with either folate-enriched fermented milk (FFM: Folate Fermented Milk), or
146 fermented milk (FM: Fermented Milk), or unfermented milk (M: Milk). The experimental
147 protocol is schematically represented in Figure 1.

148 The C diet was a modified version of the AIN-93M maintenance diet for rodents [33], where
149 casein was replaced with a casein-like aminoacid mix, and contained 0.002 g/kg folic acid, 2.5
150 g/kg choline and 3.6 g/kg methionine (Table 1). Hyperhomocysteinemia was achieved by
151 feeding mice with FD diet that was depleted of folic acid and choline (0 g/kg) to minimise the
152 content of the MS and BHMT enzyme substrates, and lowered in methionine content with
153 respect to C diet (1.8 g/kg). All purified ingredients, except the lyophilised milks, were prepared
154 and provided by Laboratori Dottori Piccioni s.r.l. (Gessate, Milan, Italy). Milk-supplemented
155 diets were appropriately balanced for protein and complex carbohydrate content (Table 1).

156 At the end of the experimental period, feces were collected and stored at -80 °C for microbiome
157 analysis. Following overnight fasting, mice were anaesthetised by intraperitoneal injection of
158 pentobarbital (10 mg/kg), blood was drawn via cardiac puncture, liver was quickly excised and
159 immediately immersed in liquid nitrogen in pieces of approximately 100 mg. Serum was
160 prepared from blood and stored at -80 °C until further analysis. All experimental procedures
161 involving animals complied with the European Guidelines for the Care and Use of Animals for
162 Research Purposes (Directive 2010/63/EU), and protocols were approved by the Ethical
163 Committee of the Food and Nutrition Research Center and by the National Health Ministry,
164 General Direction of Animal Health and Veterinary Drugs (agreement n° 268/2016). All efforts
165 were made to minimise suffering of the animals.

166 An additional, pilot group of 15 mice fed C (n = 8) and FD (n = 7) diets was employed for
167 preliminary analysis aimed at evaluating homocysteine levels after 5 weeks of depletion, as well
168 as faecal microbiota composition.

169

170 **Plasmatic Hcy determination**

171 Plasma total Hcy was measured by LC-MS/MS according to [34]. Briefly, 50 µl of plasma were
172 mixed with 25 µl of 10 µg/ml d4-Hcy (internal standard) and 50 µl of 500 mM dithiothreitol, and
173 the mixture was kept at room temperature for 15 min. Proteins were precipitated by adding 25 µl
174 of 70% trichloroacetic acid and the mixture was centrifuged for 8 min at 13.000 rpm (4 °C).
175 Supernatants were appropriately diluted, filtered through 0.20 µm syringe PVDF filters and
176 injected.

177 The analysis was performed with an HPLC system interfaced to an AB Sciex 3200 Q-Trap
178 instrument working in MRM mode. LC analyses were conducted using a system equipped with a
179 PE200 binary pump (Perkin-Elmer, USA). Samples (5 µl) were injected into a Waters XBridge
180 Phenyl column (Waters, USA) (150x2.1 mm, i.d. 3.5 µm) and eluted at a flow rate of 0.2
181 ml/min. Mobile phase A was water and mobile phase B was acetonitrile, both acidified with
182 0.1% formic acid. Elution was carried out using a gradient beginning at 30% B and changing to
183 50% B in 2 min, then remaining at 50% B for 5 min and finally to 30% B in 1 min. The column
184 was kept at 25 °C by a Peltier Column Oven Series 200 (Perkin-Elmer, USA). MS detection was
185 performed in ESI positive ion mode. Ion spray voltage was set to 4500 V, source temperature
186 was held at 600 °C, declustering potential to 35 kV, entrance potential to 9 kV and collision
187 energy to 15 kV. Transitions for Hcy and d4-Hcy were monitored at 136/90 and 140/94 m/z,
188 respectively. Data acquisition and processing were performed using Analyst software 1.5.1.

189

190 **Hepatic SAM measurement**

191 Hepatic SAM measurement was performed according to [35,36] with some modifications.
192 Because of the instability of SAM in tissue, sample preparation and SAM analysis were
193 performed the same day of the excision. Liver was homogenised and deproteinised in ice cold
194 0.4 M perchloric acid. The homogenate was centrifuged 10 min at 7000g and the supernatant
195 was mixed with 1µM of [²H₃]-SAM (Biochemical Research 2000 S.r.l., Rome, Italy) used as
196 internal standard. The solution was applied to an OASIS solid-phase-extraction (SPE) column
197 (Waters, Milford, MA, USA), which was conditioned with methanol and 10 mM SDS pH 2.1
198 (50:50, v/v). The column was rinsed with H₂O and Methanol 15% (50:50, v/v) and sample eluted
199 with methanol.

200 Quantitative on-line HPLC-ESI-MS/MS analyses were performed using HPLC system interfaced
201 to an Applied Biosystems (Foster City, CA, USA) API3200 Q-Trap instrument working with
202 triple quadrupole analyser in Multiple Reaction Monitoring (MRM) mode. LC analyses were
203 conducted using a system equipped with a 200 binary pump (Perkin-Elmer, USA). Samples were
204 injected (10 µl) into a C18 analytical column (2,1x150 mm; 3 µm bead size; Fortis Technologies

205 Ltd, Cheshire, UK) eluted at flow rate of 0.25 ml min⁻¹. Mobile phase A was H₂O containing
206 0.1% formic acid while mobile phase B was acetonitrile containing 0.1% formic acid. Elution
207 was carried out using a gradient commencing at 100% B for 10 min, then changing to 10% B in
208 17 min and finally returning to 100% B. The flow from the chromatograph was injected directly
209 into the ESI source. The API 3200 ES source was tuned by infusing SAM (1 µg ml⁻¹) into the
210 source at a flow rate of 10 ml min⁻¹. The MS operated with an electrospray voltage at 4500V and
211 with source temperature of 550°C. Nitrogen was used as ion spray (GS1), drying (GS2) and
212 curtain gas at 60, 60 and 20 arbitrary units, respectively. The declustering potential (DP),
213 collision energy (CE) and entrance potential (EP) were 30, 25 and 6, respectively. SAM and
214 [²H₃]-SAM were detected with MRM transition of 399/250 and 402/250, respectively. Data
215 acquisition and processing were performed using Analyst software 1.5.1. The quantification was
216 performed by using a calibration curve of standards.

217

218 **DNA extraction from faecal samples**

219 Total DNA was extracted from 80 mg faecal samples with the QIAamp DNA Stool Mini Kit
220 (Qiagen, Hilden, Germany) according to manufacturer's instructions. The Qiagen DNA
221 extraction method used in this work was chosen as it was listed among the most reproducible
222 kits, ensuring minimal influence on Next Generation Sequencing (NGS) data analysis [37].

223

224 **NGS analysis**

225 NGS analysis was performed on faecal DNA samples collected at the end of treatments (Figure
226 1). NGS including basic bioinformatic analysis was performed at Eurofins Genomics S.r.l.
227 sequencing laboratories (Ebersberg, Germany) by Illumina MiSeq with chemistry v3. Partial 16S
228 rRNA gene sequences were amplified using primer pairs targeting the V3-V5 region of the gene
229 (Eurofins 16S standard protocol).

230

231 **Bioinformatics**

232 One sample from treatment "M" was excluded from the analysis for insufficient yield in the
233 sequencing process (< 10,000 reads). The reads from the remaining 39 samples were imported in
234 QIIME v1.9.1 [38]. All sequences were quality checked ($Q \geq 20$) and processed applying the
235 open-reference Operational Taxonomic Units (OTU)-picking strategy with 97% sequence
236 similarity. The reads were chimera checked with chimera slayer and representative sequences
237 were taxonomy assigned against the Greengenes database V13_8 with a 90% confidence
238 threshold. Low count OTUs were removed with a threshold of 0.005% (on overall OTUs) [39].

239 The OTU table generated in QIIME was imported in R 3.3.2. The “Phyloseq” package [40] was
240 used to construct the rarefaction curves (Figure S1), which show the plateau's achievement in all
241 samples, suggesting that the sampling effort (sequencing depth) was adequate to describe the
242 variability within the microbial communities analysed.

243 The package “Phyloseq” was also used to evaluate the variability within the bacterial
244 communities (alpha diversity) calculating the Shannon Index. The dissimilarity between bacterial
245 communities (beta diversity) were evaluated through Bray-Curtis distance, using the package
246 “vegan” [41], and the distance matrix was plotted with Non Metric Multidimensional Scaling
247 (NMDS).

248

249 **Statistical analysis on microbiota data**

250 The effect of treatments on alpha diversity (Shannon index) was evaluated by one-way ANOVA,
251 followed by *post-hoc* Tukey-Kramer test in R package “TDK”. Differences with P values < 0.05
252 were considered significant, and the Benjamini & Hochberg correction was applied for multiple
253 comparison (P_{adj}). Normal distribution and homogeneity of variance were assessed with
254 Shapiro-Wilk and Levene's tests, respectively, prior to the analysis. The effect of treatment on
255 Bray-Curtis distance matrix (beta diversity) was evaluated by nonparametric MANOVA with the
256 *adonis* function in R package “vegan”, followed by pairwise *adonis*. Differences with P values <
257 0.05 were considered significant, and the Benjamini & Hochberg correction was applied for
258 multiple comparison in pairwise *adonis* (P_{adj}). The *adonis* procedure consists in a permutational
259 multivariate analysis of variance (PERMANOVA), which is a hypothesis test on the partitioning
260 of variation in multivariate datasets. The datasets are represented through distance matrices
261 based on dissimilarity measures (Bray-Curtis in our case) and the response to one or more factors
262 are assessed in an analysis of variance design with a permutational algorithm [42]. The
263 differentially abundant genera were identified by linear discriminant analysis (LDA) effect size
264 (LEfSe), an algorithm designed for biomarker discovery that identifies the significantly enriched
265 taxa in different experimental groups [43], implemented in Calypso (version 8.20) web-
266 application [44] using a threshold of 2 on the logarithmic (log₁₀) LDA score for discriminative
267 genera. Briefly, the LEfSe procedure uses in the first step the factorial Kruskal-Wallis sum-rank
268 test to detect taxa with significant (P < 0.05) differential abundance with respect to the class of
269 interest; then the biological consistency is assessed using a set of pairwise tests among groups
270 using the Wilcoxon rank-sum test (P < 0.05); finally the LDA is used to estimate the effect size
271 of each differentially abundant taxon.

272

273 **Statistical univariate analysis**

274 Values in plots and tables represent means \pm SD. Prior to analysis, normal distribution and
275 homogeneity of variance of all variables were controlled with Shapiro-Wilk and Levene's tests,
276 respectively. Statistical significance was evaluated by one-way ANOVA, followed by *post-hoc*
277 Tukey honestly significant difference (HSD) test, when normality and homogeneity of variance
278 were verified. Otherwise, Kruskal-Wallis and *post-hoc* Dunn test, and Welch one-way ANOVA
279 followed by Tamhane test were used. Differences with P values < 0.05 were considered
280 significant. Statistical univariate analysis was performed with the "Statistica" software package
281 (version 5.0; Stat Soft Inc., Tulsa, OK) and with Microsoft Office Excel 2011 upgraded with
282 XLSTAT (ver. 4 March 2014).

283

284 **Results**

285 **Nutritional and microbiological features of dairy matrices**

286 The relevant features of fermented milks (FFM and FM) and unfermented milk (M) are
287 summarised in Table 2. Sugar levels reflected the metabolic activity of bacteria present in the
288 fermented products, as compared to M: lactose, representing the main milk disaccharide, was
289 detected in higher amounts ($P < 0.01$) in M (4.35 ± 0.01 g/100 g) as compared to FFM and FM
290 (2.57 ± 0.02 and 3.37 ± 0.02 g/100 g, respectively). Lactose is the main substrate of lactic
291 fermentation, and it is converted essentially into lactic acid, which was detected only in FFM and
292 FM at 1.24 ± 0.01 and 1.29 ± 0.02 g/100 g, respectively. Lactate accumulation led to lower pH
293 values in both FFM and FM samples (i.e. pH = 3.89 and 3.77, respectively) with respect to M,
294 characterised by a pH close to neutrality (pH = 6.60) (Table 2). In order to render the food
295 matrices used for supplementation as homogeneous as possible in terms of chemical and
296 organoleptic features, M was acidified by the addition of lactic acid (0.12 g/g lyophilised milk)
297 to obtain a pH value similar to that of fermented milks.

298 The level of natural folates found in M (16.90 ± 0.31 ng/g) was similar to that observed in the FM
299 (19.40 ± 8.77 ng/g) (Table 2), whereas the FFM showed a 4-fold increase (70.33 ± 15.44 ng/g, $P <$
300 0.01), confirming the ability of the chosen bacterial strains to over-produce folates.

301 Viability of the bacterial strains in fermented milks was evaluated through plate counting. The
302 two LAB strains present in the FFM product (i.e. *S. thermophilus* 563 and *L. delbrueckii* subsp.
303 *lactis* 1021) were distinguished by colony morphologies and confirmed by microscopic
304 observation. The results obtained showed that *S. thermophilus* 563 reached a titer of 9.10 ± 0.01
305 \log_{10} CFU/ml in FFM, about one log higher than that observed for *L. delbrueckii* 1021, namely
306 8.48 ± 0.03 \log_{10} CFU/ml (Table 2). The higher titer of *S. thermophilus* 563 could be ascribed to
307 a faster growth during the first step of fermentation under pH-controlled conditions. The
308 microbial titer of the single strain *L. helveticus* 989 in FM reached a comparable level to *L.*
309 *delbrueckii* 1021 in FFM.

310

311 **Growth parameters**

312 Daily food consumption and body weight were monitored throughout the experimental period.
313 No significant differences were observed in these two parameters among the different mice
314 groups, with the exception of food intake of FM group, which resulted higher than that of FD
315 group, although with a P value of 0.049. It must be pointed out that no differences in weight as
316 well as in weight gain were observed between these two groups. Overall, these findings indicate

317 that the nutrient depletion characterising FD diet did not affect food intake, and that the milk-
318 based diets retained a good palatability with respect to C diet (Table 3).

319

320 **Supplementation with milk-based food matrices reverts hyperhomocysteinemia**

321 Preliminary analysis performed on mice fed C and FD diet showed that Hcys levels were
322 significantly increased (about 2 fold change, $P < 0.05$) after 5 weeks of FD diet with respect to C
323 (data not shown). At the end of treatments (10 weeks), supplementation with the R diet, as well
324 as with the three dairy matrices (FM, FFM and M) resulted in a significant reduction of plasma
325 Hcy levels, which reached similar values to those observed in the C group (Figure 2a). On the
326 other hand, mice fed FD diet for 10 weeks showed a significant, much more pronounced increase
327 (about 6.5 fold change) of plasma Hcy levels with respect to C group ($P < 0.0001$).

328

329 **Milk-based food matrices supplementation shows different effects on hepatic SAM** 330 **synthesis**

331 As shown in Figure 2b, nutritional deficiencies associated to FD diet induced a slight but not
332 significant reduction of liver SAM levels compared to C. Surprisingly, supplementation of mice
333 with FM and M diets caused a significant increase of SAM compared to C ($P < 0.05$), FD ($P <$
334 0.0001), FFM ($P < 0.05$) or R ($P < 0.05$) groups. On the other hand, FFM diet supplementation
335 maintained SAM levels to those of R and C diet.

336

337 **Milk-based food matrices affect faecal microbiota composition**

338 The effects of FD diet and of the different food matrices on faecal microbiota composition were
339 evaluated by assessing the relative abundance of the main taxonomic bacterial groups by NGS.
340 DNA extraction was performed from faecal samples of mice belonging to the different
341 experimental groups, collected at the end of each treatment. Preliminary analysis performed on
342 samples collected at time point t_5 (Figure 1), highlighted that microbiota composition of FD and
343 C groups resulted homogeneous at the beginning of supplementation period (ANOVA α -
344 diversity $P = 0.27$; PERMANOVA β -diversity $P = 0.23$) (data not shown). Comparison of faecal
345 bacterial communities at the end of treatments (t_{10}), indicated significant variations of microbiota
346 composition. A total of 564 OTUs were obtained from 8,331,582 quality-checked reads. The
347 distributions of the OTU counts among samples were reported in supplementary Table S1 and
348 are graphically depicted in Figure 3a.

349 Figure 3 reports the impact of diets on alpha diversity (Shannon index). The general picture
350 shows higher values of Shannon index for the treatments M, FM and FFM with respect to C, FD

351 and R, highlighting the lowest variability for the FD and the highest variability for the FM
352 bacterial communities. In particular, significant differences were observed for the contrasts FM
353 vs FD (Padj < 0.001), M vs FD (Padj < 0.01), FFM vs FD (Padj < 0.001), R vs FM (Padj < 0.05),
354 FM vs C (Padj < 0.05). Overall, these data indicate that supplementation with M, FM and FFM
355 diets is associated to an increased species diversity.

356 The analysis of beta diversity, based on Bray-Curtis dissimilarity, revealed a significant effect of
357 supplementations on bacterial community composition (Adonis, $R^2 = 0.435$ $P = 0.001$). The
358 pairwise distance matrix, plotted in two dimensions through NMDS, clearly showed the
359 clustering of samples according to treatments (Figure 4): along the first dimension, a cluster
360 formed by the bacterial communities of the C, FD and R groups significantly differed from the
361 other three clusters represented by the M, FM and FFM groups. These latter, along the second
362 dimension, significantly differed among each other (Supplementary Table S2). These findings
363 demonstrate that the three food matrices used for supplementation determined significant and
364 specific variations in microbiota composition with respect to C, FD or R diets. On the other
365 hand, FD diet did not significantly affect faecal bacterial community, which was similar to C and
366 R samples (Figure 4).

367 Taxonomical assignments of the reads at the *phylum* level showed homogeneous bacterial
368 composition within the groups, although slight inter-individual variability was observed (Figure
369 5). *Actinobacteria*, *Bacteroidetes*, *Deferribacteres*, *Firmicutes* and *Verrucomicrobia* were
370 detected as predominant bacterial phyla, with different relative proportions in each
371 supplementation group. *Firmicutes* represented the most abundant phylum in all groups, with
372 relative abundance ranging from 58 to 94% in the different samples (Figure 5). Moving through
373 the taxonomic hierarchy at the genus level, 21 different genera were identified, and in addition to
374 these, 11 unassigned genera belonging to different families or orders were reported
375 (Supplementary file 1).

376 Discriminant taxa characterising each experimental group were identified on genus aggregated
377 data by LEfSe analysis [43], allowing the detection of specific metagenomic biomarkers. Figure
378 6 shows that C samples were characterised by *Aldercreutzia* and *Staphylococcus*, while FD
379 samples were dominated by *Eubacterium*, *Coprobacillus* and *Clostridium*. Two additional
380 bacterial groups were identified at the family level, namely *Erysipelotrichaceae* and
381 *Clostridiaceae*. The R group was characterised by *Ruminococcus*, *Turicibacter* and
382 *Mogibacteriaceae*, while *Oscillospira*, *Coprococcus* and *Clostridiales* characterised M samples.
383 The groups of samples supplemented with FM and FFM milks were dominated by *Lactobacillus*,
384 *Holdemania*, *Ruminococcaceae*, *Parabacteroides*, and *Dehalobacterium*, *Streptococcus*,

385 *Lachnospiraceae*, respectively. Interestingly, one of the biomarker genera associated to FFM
386 was represented by *Streptococcus*, which was present exclusively in this group (Supplementary
387 file 1) while *Lactobacillus* was one of the specific biomarkers of FM group. Although not
388 representing a direct evidence, these findings suggest the possibility that the bacterial strains
389 employed for fermentation could be able to reach the GI tract of supplemented mice.
390

391 **Discussion**

392 Several studies highlighted a correlation between folic acid deficiency and high plasma Hcy
393 concentration, which is considered as a risk factor for various multifactorial diseases [1]. The
394 present work was performed in hyperhomocysteinemic mice with the aim of evaluating the effect
395 of the supplementation with milk or fermented milks enriched in natural folates produced by
396 suitably selected LAB, in comparison with the supplementation with synthetic folic acid. In
397 order to induce hyperhomocysteinemia, mice were fed a diet depleted in folic acid and choline,
398 and containing reduced levels of methionine, because of the metabolic interdependency of these
399 three nutrients [45,46]. High plasma levels of Hcy detected in FD mice clearly demonstrated the
400 efficacy of our experimental design to induce hyperhomocysteinemia.

401 The milk-based fermented food matrices employed for supplementation were set up to obtain
402 products enriched in natural folate content as well as high in bacterial titers. Dairy products can
403 be considered an adequate matrix to vehicle natural folate through ingestion, as folate-binding
404 proteins (FBPs) in milk improve folate stability and the bioavailability of 5-MTHF [47].
405 Together with 10-formyl-THF, 5-MTHF represents one of the major forms of folate synthesised
406 by bacteria and the most common substrate for intracellular metabolic reactions [48]. Although
407 the role of these FBPs is still unclear [49], it has been hypothesised that they can act by
408 increasing the absorption efficiency of folate introduced through the diet, protecting folate from
409 the degradation by gut microbiota [50]. While free folate is absorbed in the jejunum, the FBP-
410 linked form is mainly absorbed in the ileum, at a much slower rate than free folate, increasing the
411 absorption of folate by the host in the small intestine [51].

412 Newly isolated strains of *Lactobacillus helveticus*, *L. delbrueckii* and *Streptococcus*
413 *thermophilus* selected for their ability to produce folate were employed for the production of
414 fermented foods used in this work to supplement hyperhomocysteinemic mice. FFM supplement,
415 enriched in folate, was obtained by co-cultivation of *L. delbrueckii* 1021 and *S. thermophilus* 563
416 [32], while FM was fermented by *L. helveticus* 989 strain, which produced lower folate
417 quantities. Although several studies have shown that *Lactobacillus* species tend to consume
418 folates, the use of co-cultures of *S. thermophilus* and lactobacilli have been successfully
419 employed to produce increased levels of folate [48,52]. The amount of folate detected in FFM
420 confirmed this previous observation.

421 In our experimental design, all B vitamins involved in homocysteine metabolism were adequate
422 in content, therefore hyperhomocysteinemia represents mainly a consequence of folate
423 deficiency reduction. Surprisingly, despite their different folate content, higher in FFM with
424 respect to FM and M, the administration of the three food matrices was able to resolve
425 hyperhomocysteinemia, restoring plasma Hcy to the levels detected in C mice. However, the

426 results obtained on hepatic SAM levels suggest a different scenario. As already mentioned, SAM
427 is the methyl donor for all methylation reactions, but it is also an important regulator that,
428 depending on its concentration, drives the metabolic cycle to the trans-sulfuration or
429 remethylation pathways. In fact, when present in high concentrations, SAM allosterically binds
430 CBS, thus activating the trans-sulfuration pathway [53,54], since there is no need of additional
431 methionine synthesis. Analysis of hepatic SAM levels highlighted differences among the three
432 dairy matrices employed for supplementation of hyperhomocysteinemic mice. The high
433 concentration of SAM detected in mice supplemented with FM and M diets suggests that the
434 homocysteine recovery observed in these groups could be determined by the activation of trans-
435 sulfuration pathway rather than re-methylation pathway, according to the role of SAM as a
436 regulator of CBS activity. Indeed, our results showed that FFM exerts a different effect on SAM
437 levels with respect to FM and M diets, suggesting that the higher folate content in FFM is
438 adequate to remove the excess of homocysteine also via re-methylation pathway, as occurs
439 following C and R supplementations. It must be pointed out that the amount of natural folate
440 present in FFM is significantly lower than that in C and R diets (where it was supplied as
441 synthetic folic acid), highlighting the effectiveness of natural folates. Accordingly, other authors
442 have shown that natural folate produced by microbial fermentation is more effective than
443 synthetic folic acid, as natural folate is absorbed and transported more easily into the body
444 [51,55]. The efficacy of natural folates produced by bacteria has also been reported in a study
445 conducted on rats supplemented with engineered strains of probiotic, folate-producing
446 *Lactococcus lactis* which were able to counteract diet-induced folate deficiency by increasing
447 plasma folate concentration, as compared to synthetic folic acid [26].

448 A parallel aim of our study concerned the impact of hyperhomocysteinemia and the subsequent
449 supplementations on gut microbiota biodiversity. NGS analysis identified that faecal microbiota
450 of mice supplemented with FFM, FM and M were characterised by a higher richness of bacterial
451 species in comparison with C, FD and R groups, as revealed by Shannon index. The association
452 between microbiota biodiversity and health [56] suggests possible beneficial effect of the dairy
453 matrices employed in this study. The analysis of beta diversity, based on Bray-Curtis
454 dissimilarity, highlighted the existence of a clear effect of the supplementations on gut bacterial
455 community compositions. On the other hand, hyperhomocysteinemia was not associated with
456 significant changes in faecal microbiota composition. No differences among C, FD and R groups
457 were indeed observed, while the three dairy matrices determined specific significant variations in
458 microbiota composition in supplemented mice.

459 We took advantage of LEfSe analysis, a promising tool to identify metagenomic biomarkers
460 [43], to detect bacterial genera characterising each group. One interesting outcome of this
461 analysis was the identification of *Streptococcus* and *Lactobacillus* as specific biomarkers
462 characterising FFM and FM groups, respectively. This observation opens the possibility to
463 perform further studies aimed at evaluating the potential gut colonisation capacity of the
464 bacterial strains utilised for the fermentation processes. Indeed, an important aspect related to the
465 capability of the diet to modulate the composition of the intestinal microbiota is the contribution
466 of allochthonous bacteria, that can colonise the GI tract for a limited period of time, through the
467 consumption of fermented products or probiotic formulations. In particular, LAB and
468 bifidobacteria, representing common components of fermented food microbiota, may have
469 probiotic features, including gut colonisation capacity [57]. Vitamin-producing probiotics may
470 provide a complementary endogenous source of biomolecules that are not synthesised by
471 mammalian cells, ensuring an adequate support [58]. Several studies addressed potential
472 intervention strategies based on probiotics to counteract nutritional disorders associated to
473 cardiovascular diseases and diabetes, such as obesity, hypercholesterolemia, arterial
474 hypertension [59], considering that most of the randomised trials on vitamin and mineral
475 supplementation did not show any evident benefits on chronic disease prevention. However, it is
476 important to take into account the key role played by the food matrix in delivering health-
477 promoting bacteria, protecting them from the harsh conditions of the upper GI tract and
478 consequently enhancing their colonisation capacity. Regular consumption of fermented dairy
479 products rather than the specific intake of probiotics was shown to increase B-vitamins levels in
480 human subjects [60].

481 LEfSe analysis also indicated that the genera *Eubacterium*, *Coprobacillus*, *Clostridium* and
482 *Erysipelotrichaceae* characterise the FD group, suggesting a possible link between these
483 bacterial groups and hyperhomocysteinemia. It is interesting to note that altered levels of
484 *Erysipelotrichi* have been previously reported to be associated to a choline-deficient diet [61,62].
485 However, more studies are needed to further investigate the role of specific gut microbes in folic
486 acid deficiency and high plasma Hcy levels as well as the effect of long term treatment with
487 functional fermented products on the prevention of cardiovascular and neurological diseases.
488 The possible relationships between the presence of bacterial "biomarkers" and
489 hyperhomocysteinemia, as well as the possible implications of such microorganisms in folate
490 metabolism, are currently under investigation in our laboratories.

491

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688

689 **Conflict of interests**

690 The authors declare that they have no conflict of interests.

691

692 **Availability of data and materials**

693 Raw NGS data are available at the EMBL-EBI European Nucleotide Archive (ENA) [63], under
694 the study accession number PRJEB24434.

695

696 **Consent for publication**

697 All authors read and approved the final manuscript.

698

699 **Figure captions**

700
701 **Fig. 1** Experimental design. Arrows indicate the different diets: standard diet (white arrow);
702 control diet (black arrow); folate deficient diet (light grey arrow); supplemented folate deficient
703 diet (dark grey arrow). C: Control; FD: Folate Deficient, FFM: Folate Fermented Milk; FM:
704 Fermented Milk; M: Milk; R: Repleted. Time is expressed as weeks and it is represented by a
705 line. Dashed line refers to the adaptation period of one week, prior to the application of the
706 feeding protocol aimed at inducing hyperhomocysteinemia and including control diet. At the end
707 of the experimental period, blood was collected and faeces were sampled for gut microbiota
708 analysis. The time points are indicated as: t_a (beginning of adaptation), t_0 (beginning of
709 treatments), t_5 (shift to supplementation), t_{10} (end of treatments).

710
711 **Fig. 2.** Plasma homocysteine and liver SAM levels. Box plot representation of plasma
712 homocysteine concentrations (a), expressed as μM , and liver SAM amounts (b) expressed as
713 pmol/mg tissue, for the different experimental groups (C, FD, FFM, FM, M and R). Data were
714 analysed through ANOVA followed by *post hoc* Tukey test ($P < 0.0001$). Different letters
715 indicate significant differences.

716
717 **Fig. 3** Alpha diversity of faecal samples. Box plot of the Observed OTUs abundances and
718 Shannon index values. Samples are grouped according to the different treatments (C, FD, FFM,
719 FM, M and R). The asterisks indicate significant differences (Tukey-Kramer test): * = $P < 0.05$,
720 ** = $P < 0.01$, *** = $P < 0.001$ (P values adjusted by Benjamini & Hochberg correction).

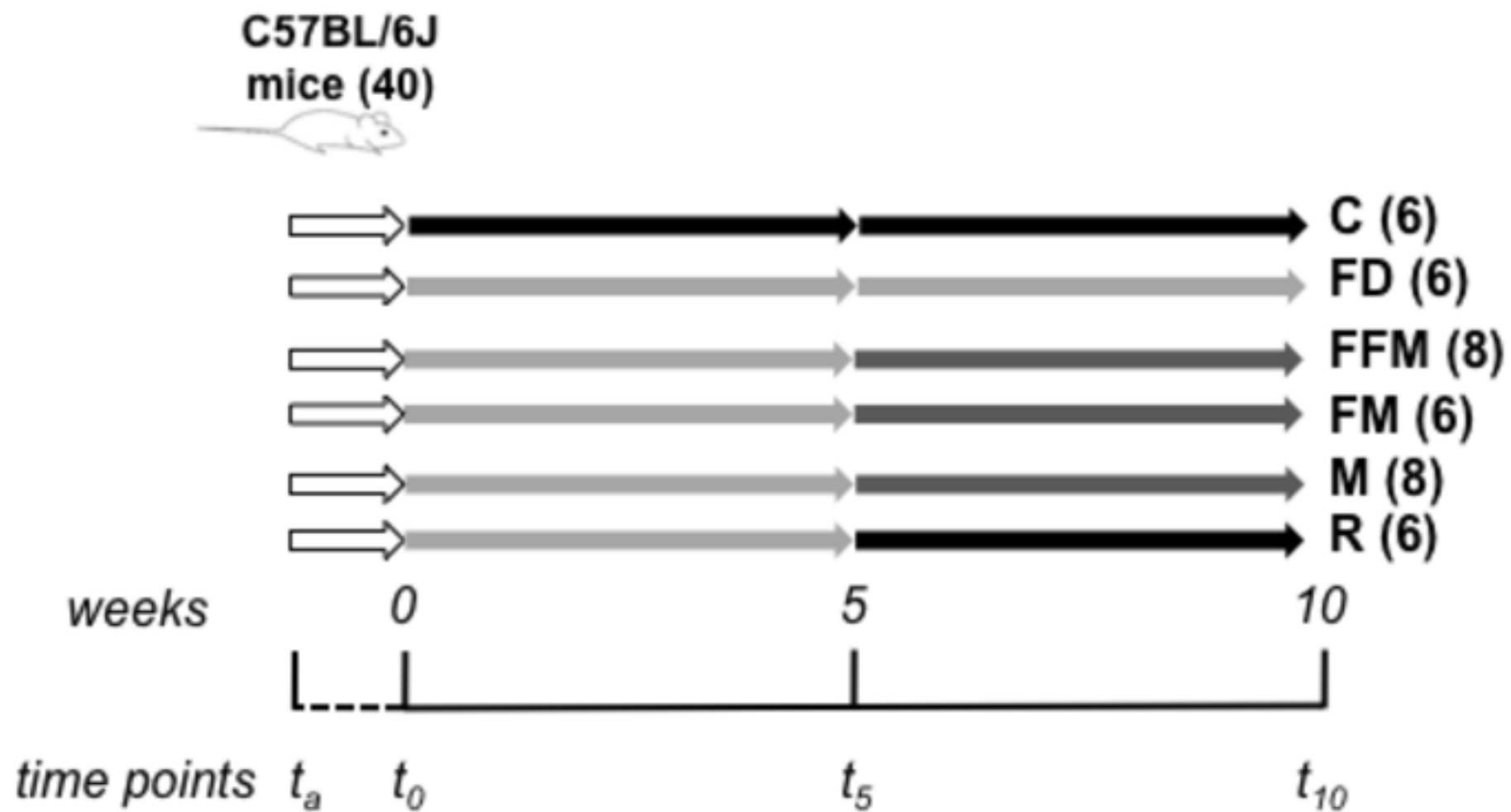
721
722 **Fig. 4** NMDS analysis of faecal samples. Non-metric multidimensional scaling plot of Bray-
723 Curtis distances. Samples are grouped according to the different treatments (C, FD, FFM, FM, M
724 and R).

725
726 **Fig. 5** Relative abundance of gut bacterial phyla obtained by NGS of faecal samples. Each bar
727 refers to a single faecal sample and depicts the proportion of OTUs per sample, expressed as
728 percentage. Colour coding of bacterial phyla is shown on the right side. “Others” includes
729 unidentified microorganisms of Bacteria kingdom and unclassified microorganisms. Samples are
730 grouped according to the different treatments (C, FD, FFM, FM, M and R).

731

732 **Fig. 6.** LEfSe analysis of faecal samples. Discriminant taxa (genus aggregated) identified by
733 linear discriminant analysis (LDA) effect size (LEfSe). Samples are grouped according to the
734 different treatments (C, FD, FFM, FM, M and R).

735
736 **Fig. S1.** Per sample rarefaction curves. Each curve shows the average number of OTUs
737 (Operational Taxonomic Units) as a function of the reads abundance subsampled at different
738 depths. Each frame and color represent the experimental treatments, the vertical gray line
739 indicates the lowest number of reads obtained on overall samples ($n = 76187$).



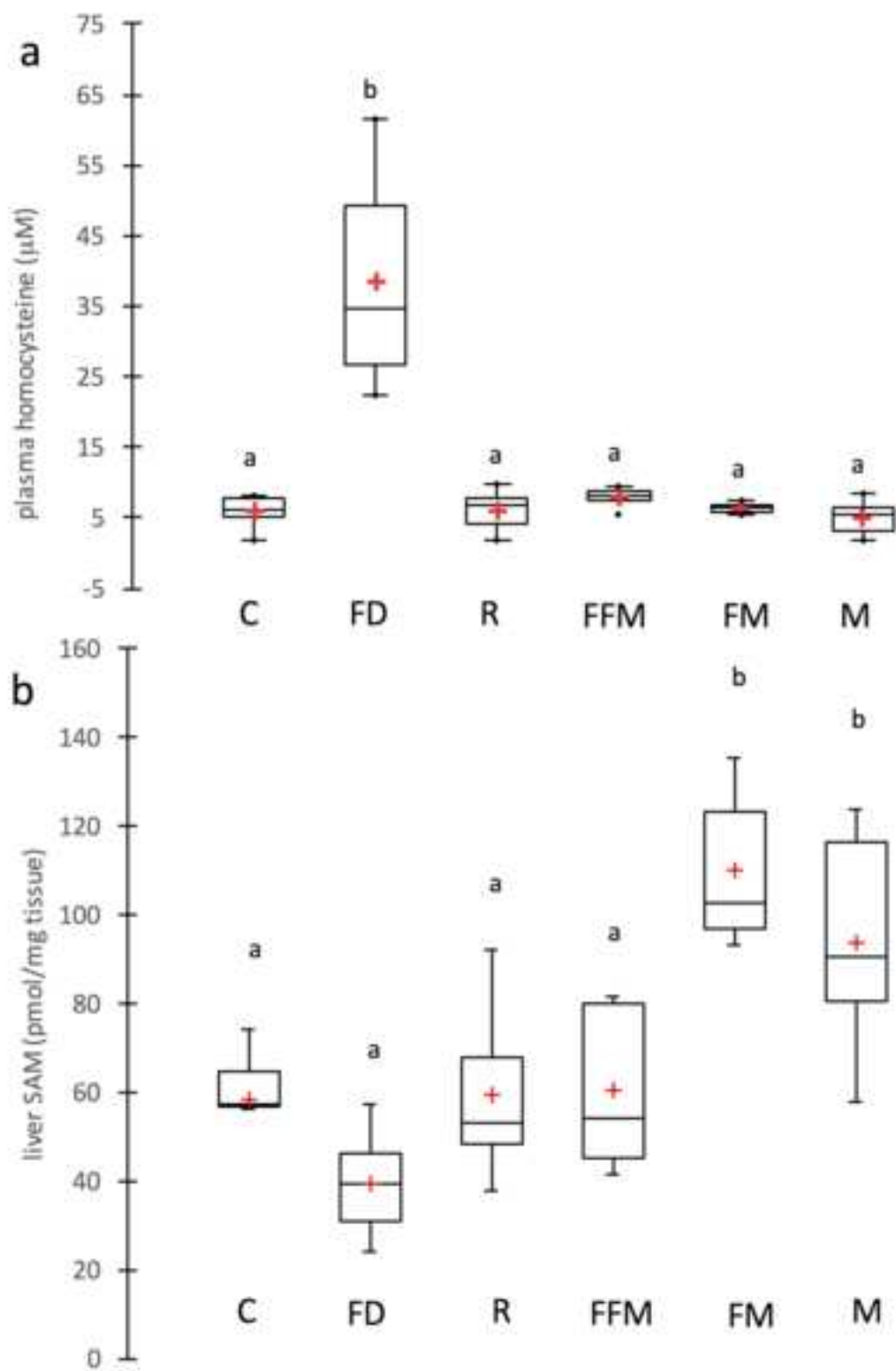


Figure 3

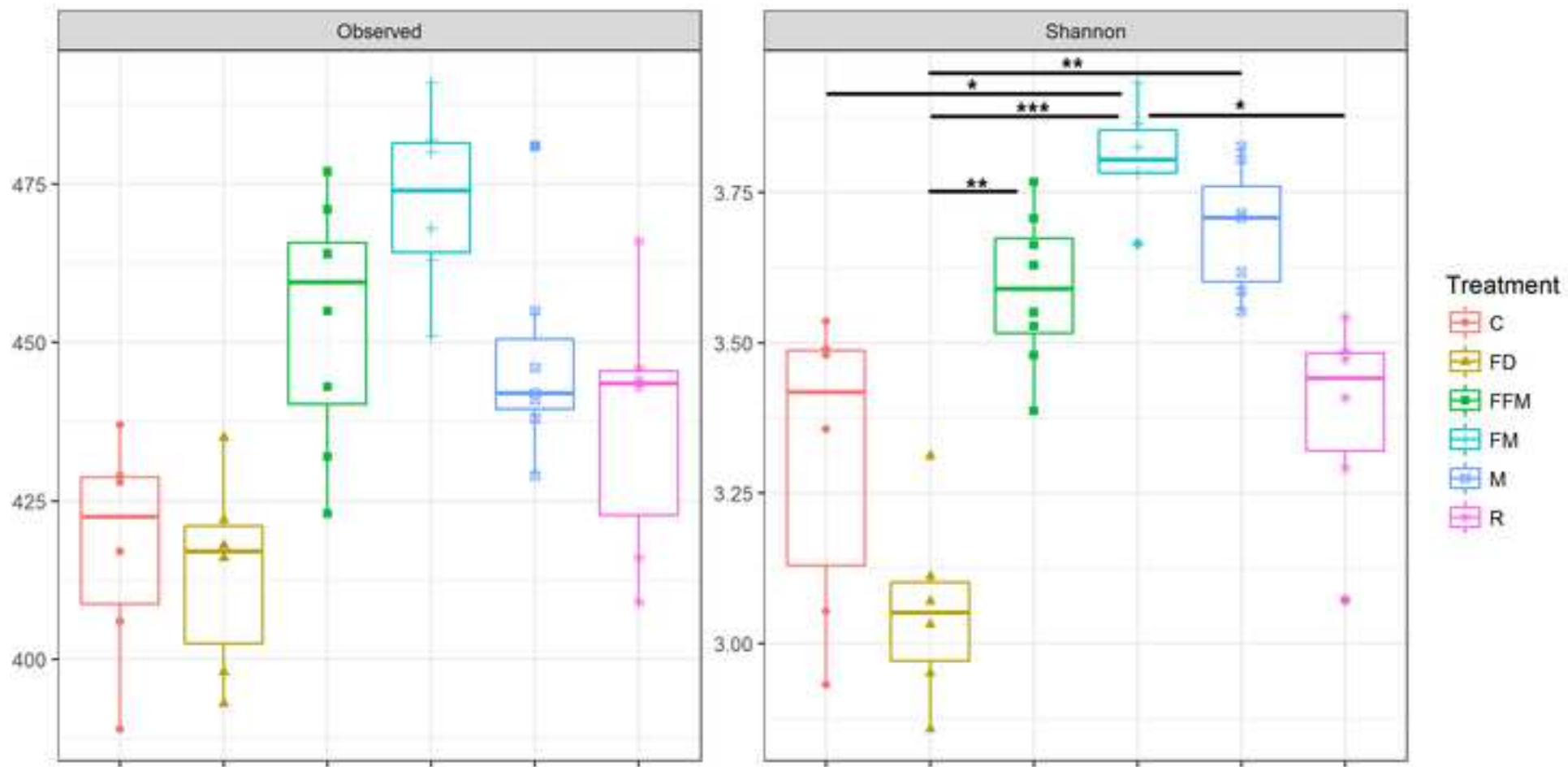


Figure 5

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Figure 6

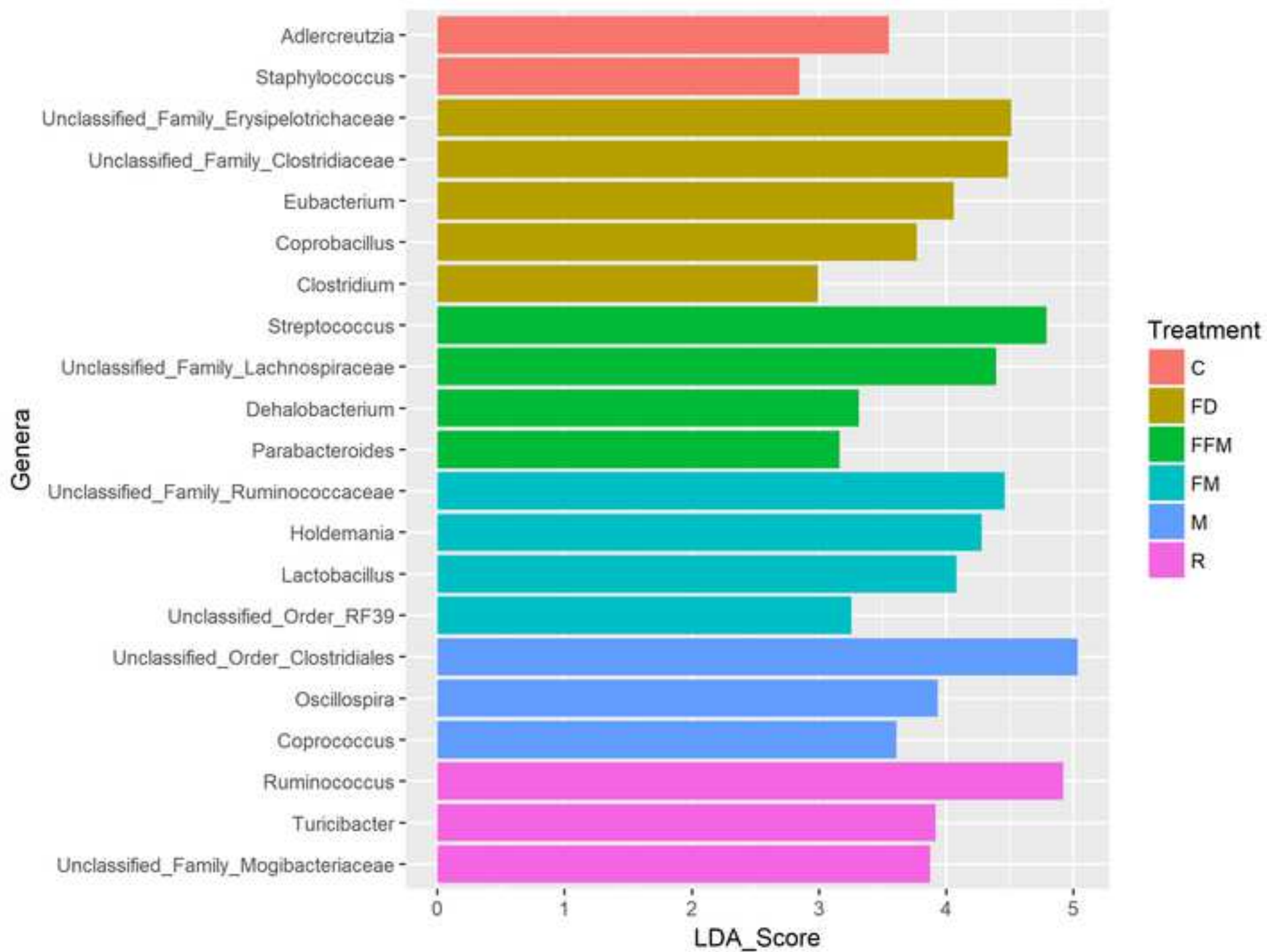


Table 1. Diet compositions

<i>Ingredient</i>	<i>Control Diet^a</i>	<i>Folate Deficient Diet</i> (g/Kg)	<i>Supplemented Diet</i>
Mais starch	467.5	468.2	189
Aminoacid mix	140	-	-
Aminoacid mix (methionine free)	-	140	-
Lyophilized milk ^b	-	-	420
Maltodextrin	155	155	155
Sucrose	100	100	100
Soya oil	40	40	40
Cellulose	50	50	50
Mineral mix	35	35	35
Vitamin mix ^c	10	-	-
Vitamin mix (folate free)	-	10	10
Choline chloride	2.5	-	-
Methionine	-	1.8	1.5
tert-Butylhydroquinone	0.008	0.008	0.008

^aAIN-93M, modified by substitution of casein with casein-like aminoacid mix

^bFFM, FM or M milk was added according to the supplementation group. Folate content in FFM, FM and M was 0.29, 0.08 and 0.07 mg/kg diet, respectively

^cFolic acid amount in Vitamin mix was 2 mg/kg diet

Table 2. Fermented and control milk composition

	Bacterial strain	Bacterial titer (log₁₀ CFU/ml)	pH	Lactose (g/100g)	Glucose (g/100g)	Galactose (g/100g)	Citric acid (g/100g)	Lactic acid (g/100g)	Folate (ng/g)
FFM	<i>S. thermophilus</i> 563	9.10 ±0.01 ^a	3.89	2.57 ±0.02 ^a	0.00 ±0.00	1.06 ±0.56 ^a	0.24 ±0.01	1.24 ±0.01 ^a	70.33 ±15.44 ^a
	<i>L. delbrueckii</i> 1021	8.48 ±0.03 ^b							
FM	<i>L. helveticus</i> 989	8.26 ±0.04 ^b	3.77	3.37 ±0.02 ^b	0.20 ±0.01	0.08 ±0.00 ^b	0.19 ±0.01	1.29 ±0.02 ^a	19.40 ±8.77 ^b
M	-	nd	6.60	4.35 ±0.01 ^c	0.00 ±0.00	0.05 ±0.00 ^b	0.22 ±0.02	0.00 ±0.01	16.90 ±0.31 ^b

Analyses were performed on fresh food matrices before freezing and lyophilisation.

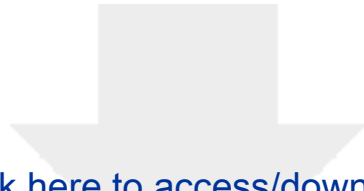
nd: not determined

Values with different superscript letters differ significantly.

Table 3. Body weight and food consumption from C, FD, R, FFM, FM or M mice.

	C	FD	R	FFM	FM	M
Body weight (g)						
Initial	19.1 ± 2.6	19.9 ± 3.0	18.6 ± 2.3	17.8 ± 2.7	17.9 ± 2.4	16.8 ± 2.0
Final	37.6 ± 3.1	37.4 ± 3.6	37.9 ± 2.6	33.4 ± 5.2	35.5 ± 4.3	37.0 ± 2.8
Gain (%)	99.7 ± 28.8	90.0 ± 17.8	105.6 ± 23.3	91.2 ± 38.8	102.1 ± 40.9	122.8 ± 23.4
Food intake (g/day)	4.1 ± 0.3 ^{ab}	4.0 ± 0.3 ^a	4.2 ± 0.4 ^{a,b}	4.3 ± 0.2 ^{ab}	4.3 ± 0.2 ^b	4.1 ± 0.2 ^{a,b}

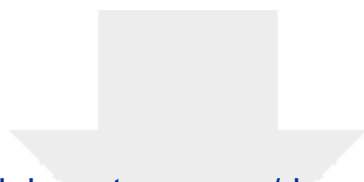
Values represent mean ± SD. Different letters indicate significant differences ($P < 0.05$).



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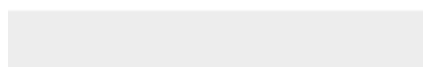
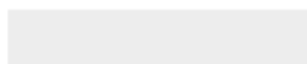
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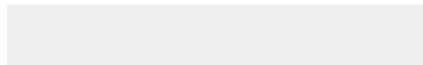
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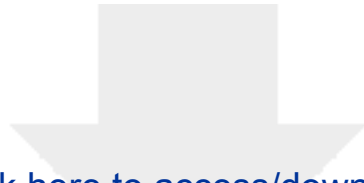




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