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Corresponding Author:	Chiara Devirgiliis Council for Agricultural Research and Economics (CREA) Rome, ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Council for Agricultural Research and Economics (CREA)
Corresponding Author's Secondary Institution:	
First Author:	Paola Zinno
First Author Secondary Information:	
Order of Authors:	Paola Zinno
	Vincenzo Motta
	Barbara Guantario
	Fausta Natella
	Marianna Roselli
	Cristiano Bello
	Raffaella Comitato
	Domenico Carminati
	Flavio Tidona
	Aurora Meucci
	Paola Aiello
	Giuditta Perozzi
	Fabio Virgili
	Paolo Trevisi
	Raffaella Canali
	Chiara Devirgiliis
Order of Authors Secondary Information:	
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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

	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. 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. 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. 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.
Suggested Reviewers:	Thomas Thymann Kobenhavns Universitet ttn@nexs.ku.dk Expertise in animal and human nutrition
	Jordi Estellè Institut National de la Recherche Agronomique jordi.estelle@inra.fr Expertise in characterization of complex microbial communities
	Maria De Angelis Universita degli Studi di Bari Aldo Moro maria.deangelis@uniba.it Expertise in food microbiology
	Carlotta De Filippo Italian National Research Council carlotta.defilippo@ibba.cnr.it Expertise in diet-microbiota interactions

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

Paola Zinno[1a], Vincenzo Motta[2a], Barbara Guantario[1], Fausta Natella[1], Marianna Roselli[1], Cristiano Bello[1], Raffaella Comitato[1], Domenico Carminati[3], Flavio Tidona[3], Aurora Meucci[3], Paola Aiello[1], Giuditta Perozzi[1], Fabio Virgili[1], Paolo Trevisi[2], Raffaella Canali[1]* and Chiara Devirgiliis[1]*

[1]*Research Centre for Food and Nutrition, CREA (Council for Agricultural Research and Economics), Via Ardeatina 546, 00178 Rome, Italy*

[2]*Department of Agricultural and Food Sciences (DISTAL), University of Bologna, Viale Fanin 46, 40127 Bologna, Italy*

[3]*Research Centre for Animal Production and Aquaculture, CREA (Council for Agricultural Research and Economics), Via A. Lombardo 11, 26900 Lodi, Italy*

[a]The authors contributed equally to the work

*Corresponding authors:

Chiara Devirgiliis, Research Centre for Food and Nutrition, CREA, Via Ardeatina 546, 00178 Rome, Italy; e-mail: chiara.devirgiliis@crea.gov.it; phone n.: +390651494647.

ORCID: 0000-0002-7114-7726

Raffaella Canali, Research Centre for Food and Nutrition, CREA, Via Ardeatina 546, 00178 Rome, Italy; e-mail: raffaella.canali@crea.gov.it; phone n.: +390651494497.

ORCID: 0000-0003-0012-4831

Keywords

Fermented food, folate, homocysteine, microbiome

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 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.

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.

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.

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.

Introduction

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

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

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

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

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

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

Materials and Methods

Bacterial strains and growth conditions

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

Fermented milk preparation and analyses

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

Experimental design, animals and diets

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

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

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

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

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

Plasmatic Hcy determination

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

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

Hepatic SAM measurement

Hepatic SAM measurement was performed according to [35,36] with some modifications. Because of the instability of SAM in tissue, sample preparation and SAM analysis were performed the same day of the excision. Liver was homogenised and deproteinised in ice cold 0.4 M perchloric acid. The homogenate was centrifuged 10 min at 7000g and the supernatant was mixed with 1µM of [$^{2}H_3$]-SAM (Biochemical Research 2000 S.r.l., Rome, Italy) used as internal standard. The solution was applied to an OASIS solid-phase-extraction (SPE) column (Waters, Milford, MA, USA), which was conditioned with methanol and 10 mM SDS pH 2.1 (50:50, v/v). The column was rinsed with H_2O and Methanol 15% (50:50, v/v) and sample eluted with methanol.

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

Ltd, Cheshire, UK) eluted at flow rate of 0.25 ml min^{-1}. Mobile phase A was H2O containing 0.1% formic acid while mobile phase B was acetonitrile containing 0.1% formic acid. Elution was carried out using a gradient commencing at 100% B for 10 min, then changing to 10% B in 17 min and finally returning to 100% B. The flow from the chromatograph was injected directly into the ESI source. The API 3200 ES source was tuned by infusing SAM (1 µg ml^{-1}) into the source at a flow rate of 10 ml min^{-1}. The MS operated with an electrospray voltage at 4500V and with source temperature of 550°C. Nitrogen was used as ion spray (GS1), drying (GS2) and curtain gas at 60, 60 and 20 arbitrary units, respectively. The declustering potential (DP), collision energy (CE) and entrance potential (EP) were 30, 25 and 6, respectively. SAM and [$^2H_3$]-SAM were detected with MRM transition of 399/250 and 402/250, respectively. Data acquisition and processing were performed using Analyst software 1.5.1. The quantification was performed by using a calibration curve of standards.

DNA extraction from faecal samples

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

NGS analysis

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

Bioinformatics

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

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

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

Statistical analysis on microbiota data

The effect of treatments on alpha diversity (Shannon index) was evaluated by one-way ANOVA, followed by *post-hoc* Tukey-Kramer test in R package "TDK". Differences with P values < 0.05 were considered significant, and the Benjamini & Hochberg correction was applied for multiple comparison (Padj). Normal distribution and homogeneity of variance were assessed with Shapiro-Wilk and Levene's tests, respectively, prior to the analysis. The effect of treatment on Bray-Curtis distance matrix (beta diversity) was evaluated by nonparametric MANOVA with the *adonis* function in R package "vegan", followed by pairwise *adonis*. Differences with P values < 0.05 were considered significant, and the Benjamini & Hochberg correction was applied for multiple comparison in pairwise *adonis* (Padj). The adonis procedure consists in a permutational multivariate analysis of variance (PERMANOVA), which is a hypothesis test on the partitioning of variation in multivariate datasets. The datasets are represented through distance matrices based on dissimilarity measures (Bray-Curtis in our case) and the response to one or more factors are assessed in an analysis of variance design with a permutational algorithm [42]. The differentially abundant genera were identified by linear discriminant analysis (LDA) effect size (LEfSe), an algorithm designed for biomarker discovery that identifies the significantly enriched taxa in different experimental groups [43], implemented in Calypso (version 8.20) web-application [44] using a threshold of 2 on the logarithmic (log_{10}) LDA score for discriminative genera. Briefly, the LEfSe procedure uses in the first step the factorial Kruskal-Wallis sum-rank test to detect taxa with significant ($P < 0.05$) differential abundance with respect to the class of interest; then the biological consistency is assessed using a set of pairwise tests among groups using the Wilcoxon rank-sum test ($P < 0.05$); finally the LDA is used to estimate the effect size of each differentially abundant taxon.

Statistical univariate analysis

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

Results

Nutritional and microbiological features of dairy matrices

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

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

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

Growth parameters

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

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

Supplementation with milk-based food matrices reverts hyperhomocysteinemia

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

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

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

Milk-based food matrices affect faecal microbiota composition

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

LEfSe analysis also indicated that the genera *Eubacterium*, *Coprobacillus*, *Clostridium* and *Erysipelotrichaceae* characterise the FD group, suggesting a possible link between these bacterial groups and hyperhomocysteinemia. It is interesting to note that altered levels of *Erysipelotrichi* have been previously reported to be associated to a choline-deficient diet [61,62]. However, more studies are needed to further investigate the role of specific gut microbes in folic acid deficiency and high plasma Hcy levels as well as the effect of long term treatment with functional fermented products on the prevention of cardiovascular and neurological diseases. 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 in our laboratories.

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Conflict of interests

The authors declare that they have no conflict of interests.

Availability of data and materials

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

Consent for publication

All authors read and approved the final manuscript.

Figure captions

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

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

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

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

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

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

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

Figure 1

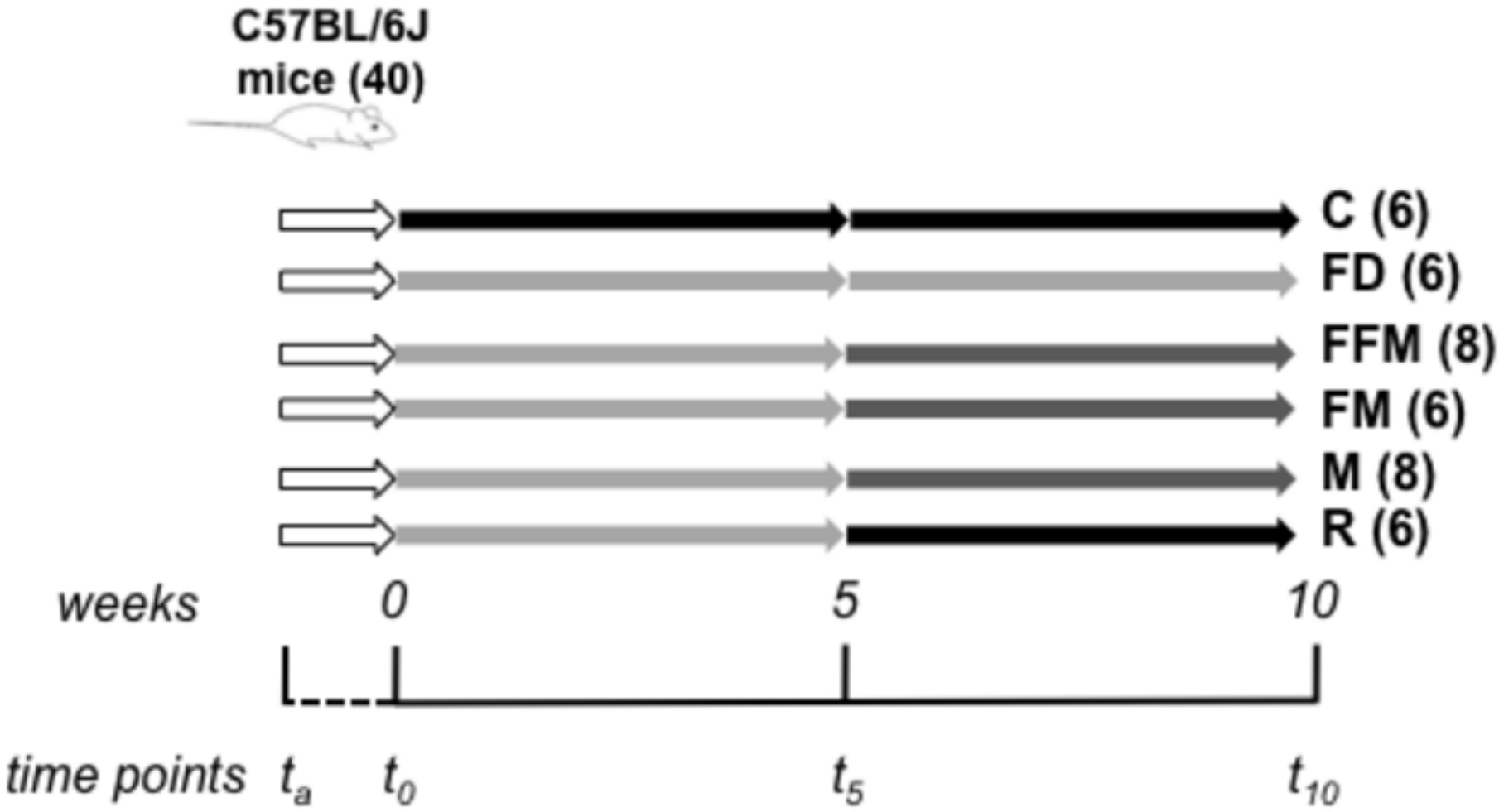

Figure 2

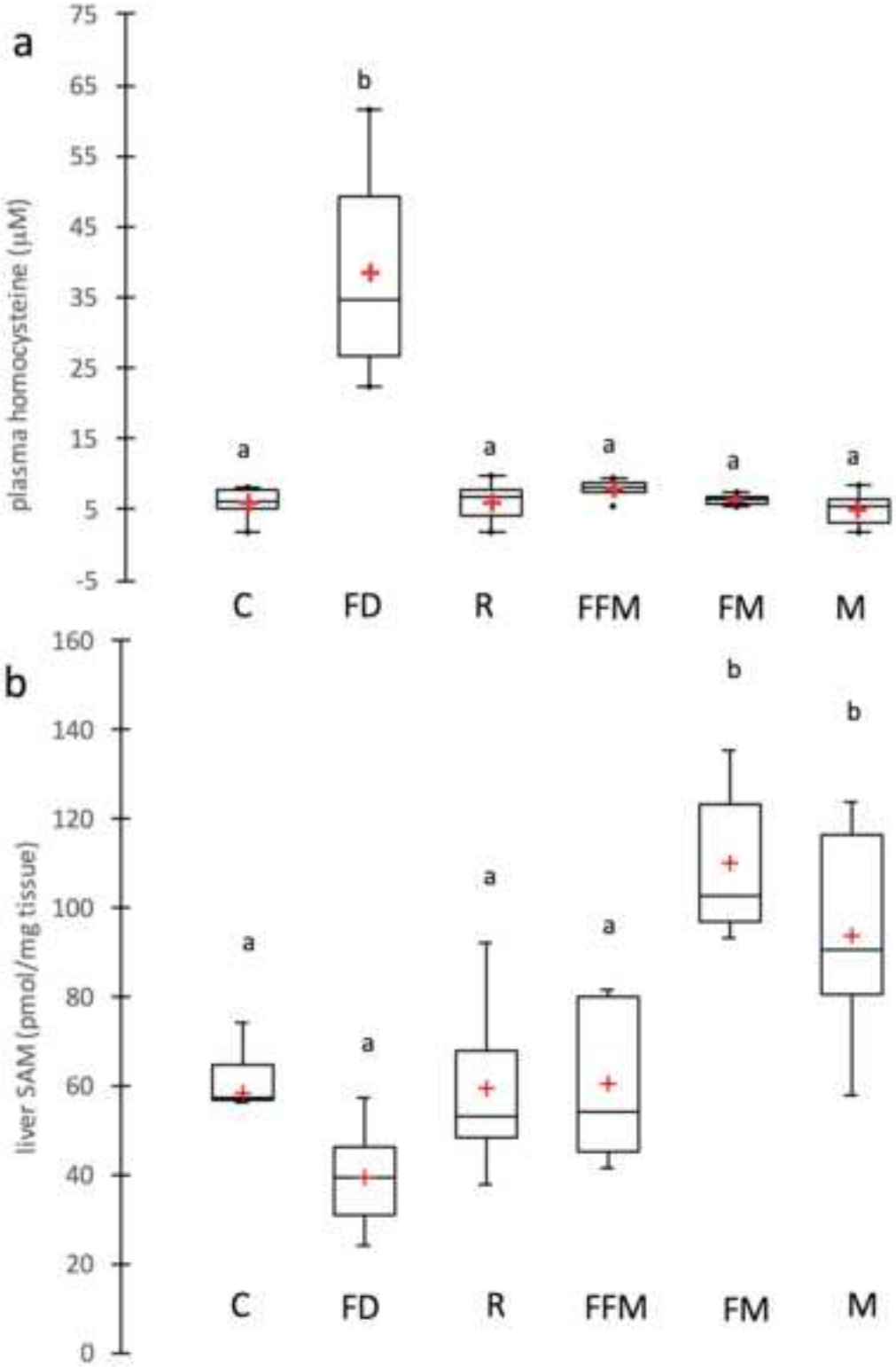

Figure 3

Click here to access/download;Figure;Fig3.tiff

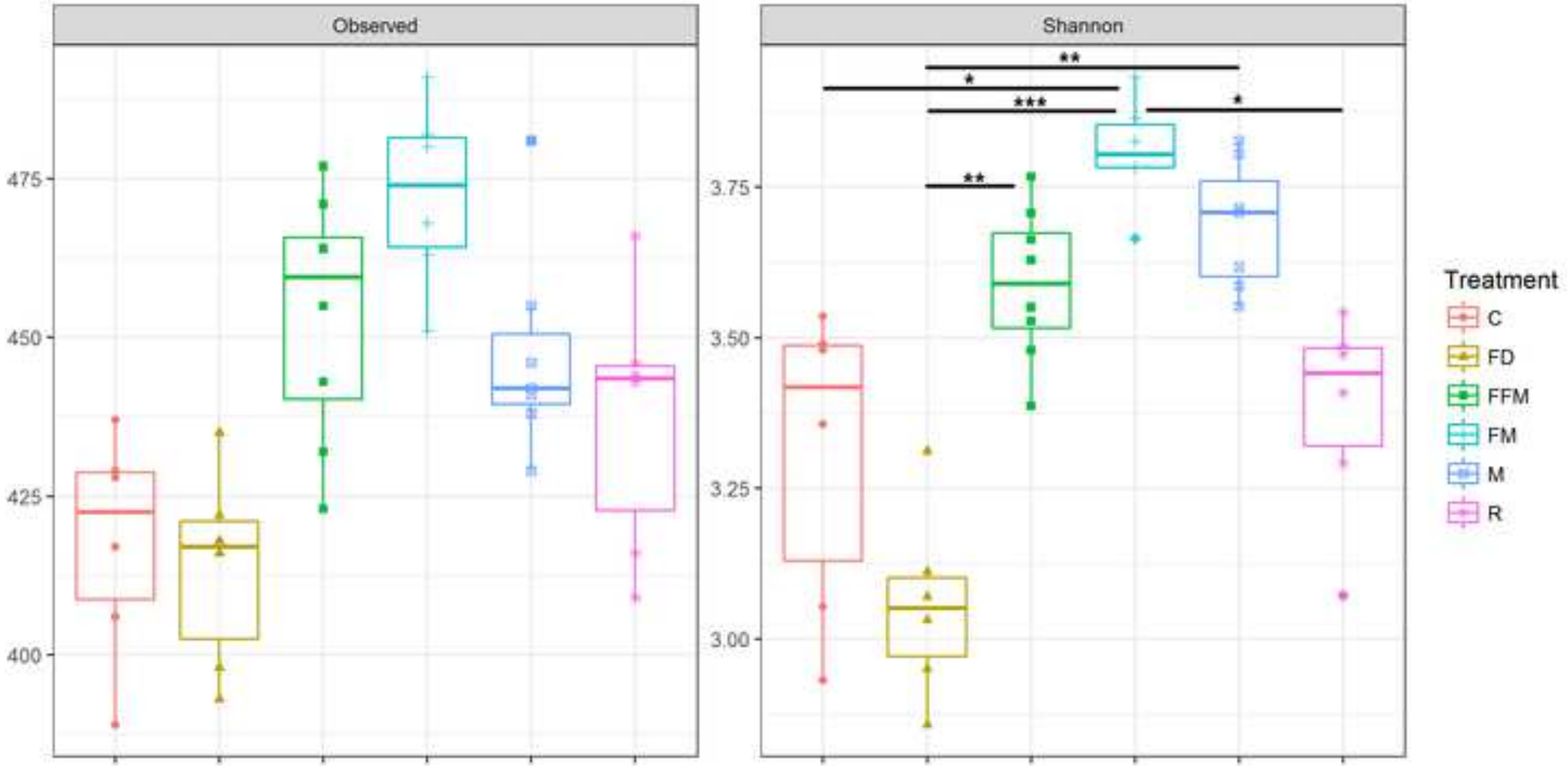

Figure 4

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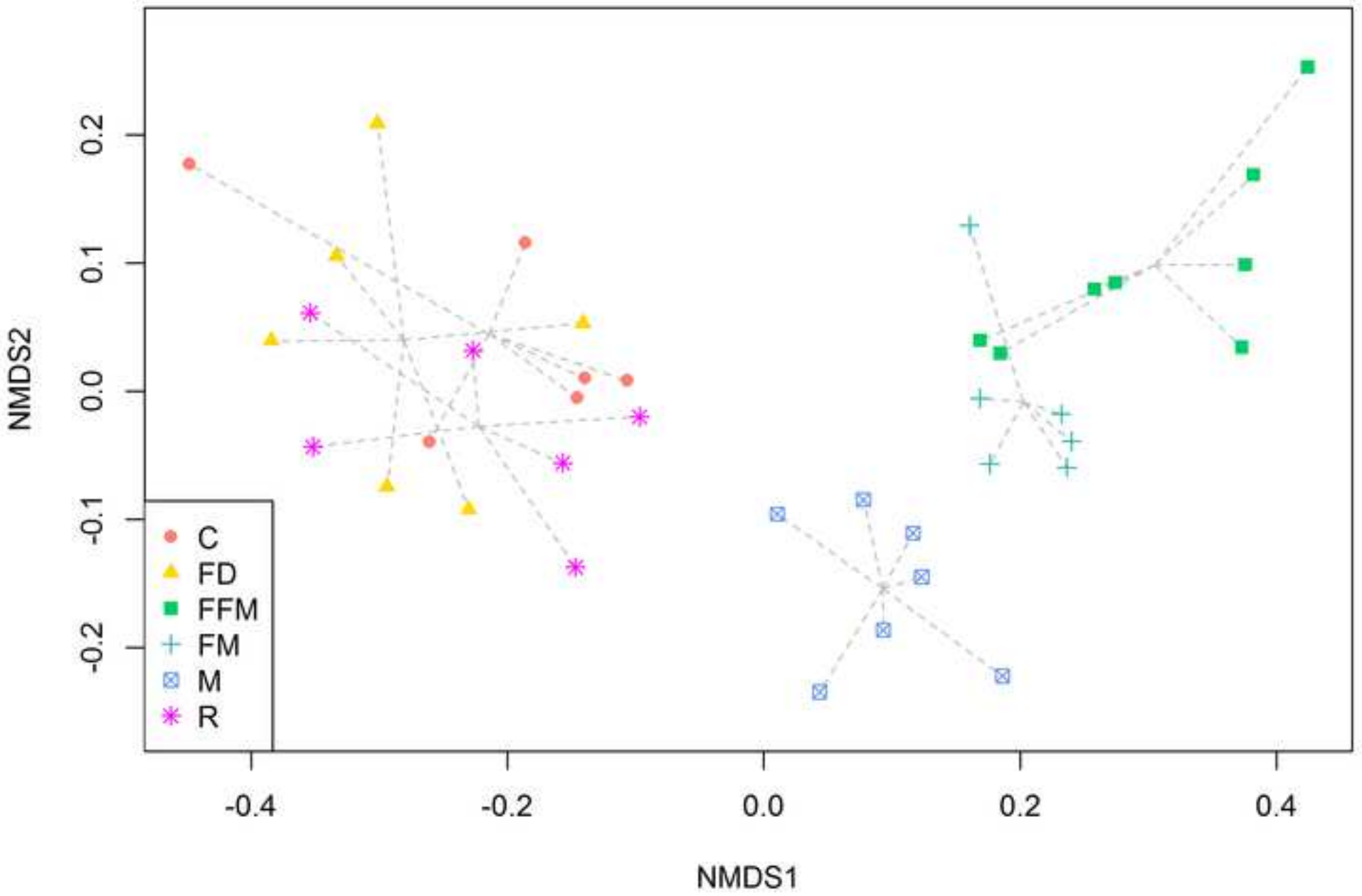

Figure 5

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Figure 6

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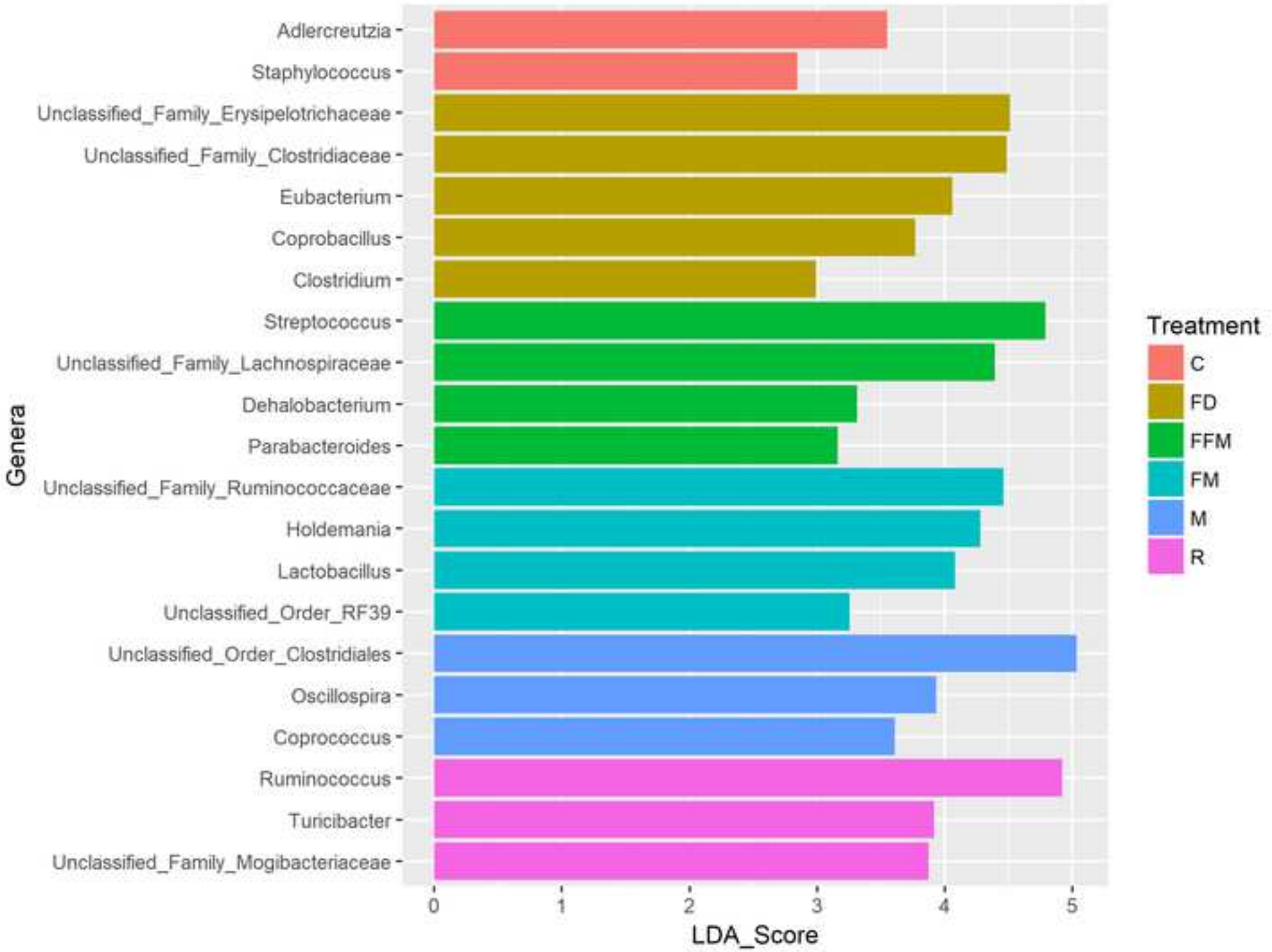

Table 1. Diet compositions

Ingredient	*Control Diet*[a]	*Folate Deficient Diet*	*Supplemented Diet*
		(g/Kg)	
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

[a]AIN-93M, modified by substitution of casein with casein-like aminoacid mix
[b]FFM, 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
[c]Folic acid amount in Vitamin mix was 2 mg/kg diet

Table 2. Fermented and control milk composition

	Bacterial strain	Bacterial titer (log_{10} CFU/ml)	pH	Lactose (g/100g)	Glucose (g/100g)	Galactose (g/100g)	Citric acid (g/100g)	Lactic acid (g/100g)	Folate (ng/g)
FFM	*S. thermophilus* 563 *L. delbrueckii* 1021	9.10 ±0.01[a] 8.48 ±0.03[b]	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]
FM	*L. helveticus* 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

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^{a,b}	4.0 ± 0.3^{a}	4.2 ± 0.4^{a,b}	4.3 ± 0.2^{a,b}	4.3 ± 0.2^{b}	4.1 ± 0.2^{a,b}

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

Table S1

Table S2

Figure S1

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Electronic Supplementary Material

FigS1.tif

Supplementary file 1

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Electronic Supplementary Material

Supplementary file 1new.xlsx