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Gut microbiota response to consumption of milks fermented with specific strains of *Lactococcus lactis* with hypocholesterolemic effect

Miguel A. Rendon-Rosales[1], José I. Méndez-Romero[1], Adrián Hernández-Mendoza[1], Aarón F. González-Córdova[1], Miguel A. Mazorra-Manzano[1], Hugo S. García[2], Lilia M. Beltrán-Barrientos[1], María C. Estrada-Montoya[1] and Belinda Vallejo-Cordoba[1*]

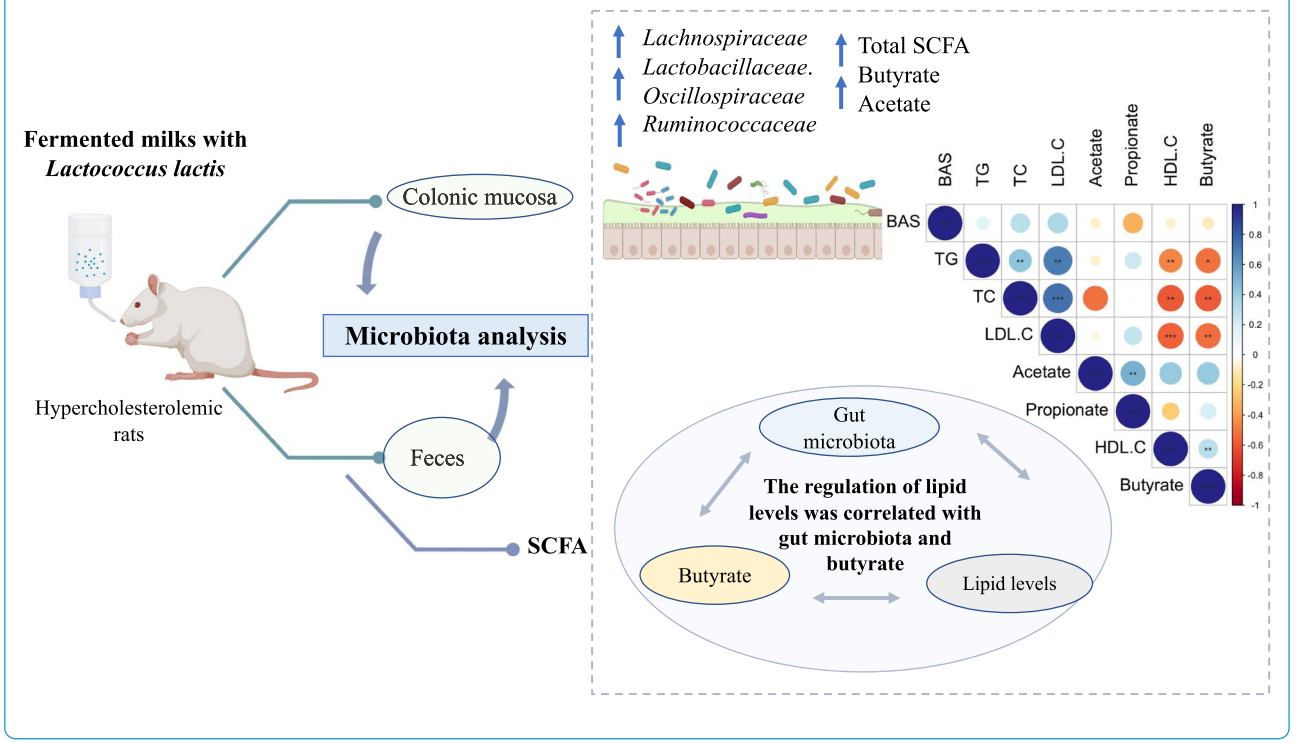
Abstract

The alteration of structure and function of gut microbiota (dysbiosis) appears to be a major factor associated with metabolic disorders such as dyslipidemia and subsequent development of cardiovascular diseases. However, the consumption of fermented milks is a promising strategy to enhance health and restore the function of gut microbiota; specifically, in individuals with intestinal dysbiosis and hypercholesterolemia. Therefore, the aim of the present study was to evaluate the potential association between gut microbiota and the hypocholesterolemic effect of fermented milks with *Lactococcus lactis* NRRL B-571 (FM-571), NRRL B-572 (FM-572) and NRRL B-600 (FM-600) in Sprague–Dawley rats. Fermented milks were administered to hypercholesterolemic Sprague–Dawley rats during seven weeks. At the end of the experimental period, fecal and colonic microbiota were characterized using 16S RNA gene sequencing. Also, the short chain fatty acids (SCFAs) content was quantified in feces. Results showed that a high-cholesterol diet (HCD) altered the bacterial community in both fecal and mucosal samples. The consumption of fermented milks, specifically FM-572 promoted changes in the structure (beta diversity) in fecal, but not in mucosal microbiota. The levels of SCFAs in feces were improved after fermented milks consumption. From all SCFAs, butyrate was negatively correlated with total cholesterol, LDL-C ($p < 0.05$) and positively correlated with HDL-C ($p < 0.05$). Furthermore, *Ruminococcaceae*, *Lactobacillaceae*, *Lachnospiraceae* and *Oscillospiraceae* families, were negatively associated with total cholesterol, LDL-C ($p < 0.05$) and positively associated with HDL-C ($p < 0.05$). The abundance of these families was increased in groups treated with fermented milks, particularly with FM-572 ($p < 0.05$). Thus, the in vivo hypocholesterolemic effect after the consumption of milks fermented with *Lactococcus lactis* strains may be related with the modulation of fecal microbiota associated with the increase of butyrate-producing bacteria. Furthermore, these associations may suggest that butyrate may influence the cholesterol metabolism, resulting in the decreasing cholesterol levels.

Keywords Hypercholesterolemia, short-chain fatty acids, Lipid metabolism, Hypocholesterolemic effect

*Correspondence:
Belinda Vallejo-Cordoba
vallejo@ciad.mx
Full list of author information is available at the end of the article

Graphical Abstract



Introduction

Cardiovascular diseases (CVDs) are the major cause of mortality worldwide. There are several risk factors associated with the development of CVD such as hypertension, obesity and dyslipidemia (James et al. 2018) In recent years, the evidence suggests the interplay between gut microbiota and the development of CVDs (Tang et al. 2017). Gut microbiota comprises around 100 trillion microorganisms, particularly bacteria, dominated by *Firmicutes* and *Bacteroidetes* phyla. Moreover, this community also includes viruses, archaea, protozoa and fungi (Rinninella et al. 2019). A healthy, balanced microbiota is important for the maintenance of homeostasis through their metabolic and immunological functions. However, the alteration of the structure and function of the microbiota affects various metabolic pathways that facilitate the development of several diseases such as CVDs (Shi 2019). The underlying mechanism has been associated with the alteration of permeability and the dysregulated production of gut-derived metabolites such as lipopolysaccharide (LPS), secondary bile acids (SBA), trimethylamine N-oxide (TMAO), uremic toxins and SCFAs (acetate, propionate and butyrate) (James et al. 2018).

Moreover, SCFAs have been widely recognized as important molecules that mediate physiological and metabolic functions between the host and gut microbiota. Although SCFAs (*e.g.* butyrate) are associated with the barrier function in the colonocytes (Peng et al. 2009; Bach et al. 2018), their function also may extend to the regulation of lipid metabolism. SCFAs may regulate cholesterol metabolism through the absorption, synthesis and excretion of endogenous or dietary cholesterol (Amiri et al. 2022). However, the alteration of the structure of microbiota, particularly the decrease of SCFAs producing bacteria, may lead to the alteration of lipid profile in bloodstream and liver. Therefore, given the importance of the function of microbiota, its modulation is an emerging potential therapeutic target to prevent or decrease the risk factors associated with the development of CVDs such as hypercholesterolemia (Vourakis et al. 2021). Since diet plays an important role in the composition of microbiota, dietary interventions with specific foods or food components (*e.g.* fiber, oligosaccharides, proteins and peptides) have exerted positive effects (Leeming et al. 2019). Fermented foods such as kefir, kimchi, kombucha and milks fermented with lactic acid bacteria (LAB), have been associated with cardioprotective properties (*e.g.* antihypertensive, hypocholesterolemic and antiobesity) (Hadjimbei et al. 2022; Melini et al. 2019).

Fermented milks have been widely recognized as functional foods with beneficial health effects. Metanalysis studies have reported that the consumption of these products can significantly reduce cardiovascular risks, mainly by their effects in the reduction of blood pressure and lipid levels such as triglycerides and LDL-C. Hence, the consumption of fermented dairy products has been recommended by the guidelines in the management of dyslipidemia (Zhang et al. 2020). Previous studies with fermented milks with specific strains of *Lactococcus lactis* showed cardioprotective properties such as antihypertensive and antithrombotic effects (Beltran-Barrientos et al. 2018; Rendón-Rosales et al. 2019; Rodriguez-Figueroa et al. 2013). Moreover, the administration of a fermented milk with *Lactococcus lactis* NRRL B-50572 decreased triglycerides and LDL-C levels in hypertensive rats (Rodriguez-Figueroa et al. 2013). Furthermore, an hypercholesterolemic model was used for evaluating the cholesterol-lowering effect of milks fermented with *Lactococcus lactis* spp. In this study, fermented milks with *Lactococcus lactis* NRRL B-50571 (FM-571), NRRL B-50572 (FM-572) and NRRL B-50600 (FM-600) showed capacity to reduce total cholesterol and LDL + VLDL and increase HDL cholesterol in plasma. Additionally, these fermented milks prevented the accumulation of lipids in liver and induced excretion of total lipids in feces (Rendón-Rosales et al. 2023). Nevertheless, the underlaying mechanisms were not investigated.

In this regard, it has been previously reported that the hypocholesterolemic effect may be associated with the participation of peptide fractions released during the fermentation process. Moreover, the water-soluble fractions derived from FM-571, FM-572 and FM-600 showed the capacity to inhibit the micellar solubility of cholesterol and bind to bile acids in vitro (Rendón-Rosales et al. 2019). Additionally, it was also suggested that gut microbiota modulation by the consumption of fermented milks may also be implicated in the regulation of cholesterol levels by the increase of SCFAs (acetate, propionate and butyrate) production. To date, it is still unknown if the hypocholesterolemic effect of these fermented milks with *Lactococcus lactis* may also be related to the modulation of gut microbiota. Therefore, the aim of this study was to characterize the structure of fecal and mucosal microbiota after the consumption of milks fermented with *Lactococcus lactis* NRRL B-50571 (FM-571), NRRL B-50572 (FM-572) or NRRL B-50600 (FM-600) with hypocholesterolemic effect in an hypercholesterolemic murine model.

Materials and methods

Preparation of fermented milks

The *Lactococcus* (*L.*) *lactis* strains previously isolated from artisanal dairy products (Gutiérrez-Méndez et al. 2010) were obtained from the culture collection of the Dairy Laboratory at the Food Research and Development Center, A.C. (CIAD, A.C. Hermosillo, Sonora, México). The fermentation of milk with *L. lactis* was performed as previously reported by Rendón-Rosales et al. (2023). Briefly, *L. lactis* NRRL B-50571, NRRL B-50572 and NRRL B-50600 were cultured in M17 broth supplemented with dextrose or lactose solution. Fresh cultures were inoculated (3% v/v) in heat-treated (110 °C/10 min) reconstituted (10% w/v) nonfat milk and incubated for 12 or 24 h. The resulting milk pre-inoculums were inoculated again in heat treated (80 °C/30 min) milk and incubated for 48 h at 30 °C to obtain the fermented milks. At the end of incubation, the fermentation process was stopped by heat treatment (75 °C/15 min) and subsequent cooling (4 °C). Fermented milks were stored at -20 °C until used in the in vivo study.

Animals and experimental design

Animal experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals and the experimental protocol was approved by the Bioethics Committee of the Research Center for Food and Development (CE/017/2019). Thirty five male Sprague–Dawley rats (six-weeks old) were obtained from the University of Sonora (Hermosillo, Sonora, México). The sample size of the experimental groups used in this study was determined according to Arifin & Azahiruddin (2017) and confirmed with Charan & Katharia (2013). Prior to the experimental period, the rats were maintained with a commercially standard diet (laboratory rodent diet, Labdiet 5001) and purified water during three weeks for acclimation in controlled conditions (20–22 °C, 50 ± 10% relative humidity and 12 h light–dark cycles). After this period, rats were randomized and divided into a control group (n = 5) which received standard diet, and the remaining five groups (n = 6) received high cholesterol diet (HCD). HCD was a mixture of standard diet (98.75%), 1% cholesterol (Sigma-Aldrich) and 0.25% cholic acid (Sigma-Aldrich). During seven weeks, the rats received diets and treatments (Table 1) ad libitum (Rendón-Rosales et al. 2023).

Sample collection for microbiota and lipid analysis

Feces were freshly collected in the last three days before euthanasia of animals. Feces were mixed and immediately store at -80 °C in sterile conical tubes for further procedures. After the experimental period, the rats were

Table 1 Diets and treatments administered to Sprague–Dawley rats during seven weeks

No	Diets	Treatment	Form of administration
1	Standard diet (SD)	Water	Daily—ad libitum
2	High-cholesterol diet (HCD)	Water	Daily—ad libitum
3	HCD	Non-fermented milk (NFM)	Daily—ad libitum
4	HCD	Fermented milk with NRRL B-50571 (FM-571)	Daily—ad libitum
5	HCD	Fermented milk with NRRL B-50572 (FM-572)	Daily—ad libitum
6	HCD	Fermented milk with NRRL B-50600 (FM-600)	Daily—ad libitum

euthanized by anesthesia administration (pentobarbital) by intraperitoneal injection (75 mg/kg of body weight). Blood samples were obtained by intracardiac puncture and collected in heparinized tubes for further plasma collection (2500 *xg*, 15 min, 4 °C). Plasma was used to determine total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG) and bile acids (BAs) with commercially available kits (Cell Biolabs, San Diego, CA and Randox Laboratories, Crumlin, U.K). The colon tissue section was removed and microbiota from colonic mucosal was collected by scraping and were kept in sterile conical tubes. All samples were immediately stored at -80 °C until bacterial genomic DNA extraction. For 16S RNA gene sequencing, either colonic mucosal or feces from two rats were pooled in equal amounts (100 mg).

Total DNA preparation

Genomic DNA was extracted from feces and colonic mucosal scrapings using the QIAamp DNA stool minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with some modifications that include the use of Tris–HCl (10 mM, pH 8.5) for DNA elution in the final step. Quality and purity of DNA was analyzed by gel electrophoresis (1% agarose gel) and spectrophotometrically using Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA). Total DNA concentration was quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA) with a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA).

Library preparation and 16S rRNA gene sequencing for fecal and mucosal microbiota analysis

To investigate the bacterial community structure, the V3-V4 region of the 16 s rRNA gene was amplified from bacterial DNA according to "16S-metagenomic sequencing library preparation guide" protocol. The first PCR amplification was performed using primers from V3-V4 region of the 16S rRNA gene that consists of forward Primer 5´-CCTACGGGNGGCWGCAG-3´ with the forward overhang 5´-TCGTCGGCAGC GTCAGATGT GTATAAGAGACAG-3´ and 16S Amplicon PCR reverse Primer 5´-GACTACHVGGGTATCTAATCC-3´ with the reverse overhang 5´-GTCTCGTGGGCTCGGAGATGT GTATAAGAGACAG. Samples were amplified in reaction volumes of 12.5 µL containing 12.5 ng of total DNA and 2×KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA). PCR was performed with the following program: initial denaturation 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. At the end of each PCR step, the products were purified using AMPure XP reagent (Beckman Coulter, Brea, CA). For library construction, purified DNA was used as a second PCR template using primers from Nextera XT index kit v2 Set A (Illumina, San Diego, CA) that contain adapters and dual-index barcodes (index 1(i7) and index 2(i5)) (Illumina, San Diego, CA) complementary to the amplicon target. The second PCR program consisted of an initial denaturation at 95 °C for 3 min, followed by 8 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and extension at 72 °C for 5 min. The resulting libraries were purified using AMPure XP reagent and assessed for quality by gel electrophoresis using a 2% agarose gel. Libraries were quantified by Qubit dsDNA HS Assay Kit and normalized to a concentration of 4 nM. The library pool was then denatured with NaOH (0.1 N), diluted to 1.4 pM and mixed with denatured PhiX (1.4 pM) to a final concentration of 1% (v/v). Libraries were loaded into a MiniSeq High Output Reagent Kit (300-cycles). Automated cluster generation and paired-end sequencing with dual reads (2×151) were performed according to the manufacturer's instructions.

Bioinformatic analysis

Data analysis was performed using the Fastq files obtained from Illumina sequencing. First, the quality reads were assessed using FastQC software v 0.11.9. Then, the reads in Fastq.gz format were analyzed using DADA2 software package pipeline, running in R software (v 3.5.3). In the first step, the analysis included the quality filtering [maxN = 0, maxEE = c (2,2), truncQ = 8, length (truncLen = 140)]. After quality evaluation, only forward sequences were used for further analysis.

Forward sequences were dereplicated and subsequent amplicon sequence variants (ASVs) were inferred. Next, chimeras were removed, and taxonomy was assigned against SILVA v138.1 reference database. ASV count, taxonomy and metadata tables were generated and subsequently utilized for analysis with the phyloseq package (McMurdie & Holmes 2013). The unidentified sequences (NA) and those assigned to eukaryotic organisms such as mitochondria were discarded and abundance filter was also applied, removing families with less than 10 counts. Next, sequencing was randomly sub-sampled with rarefaction. Microbial diversity and composition were performed using microbiome R package (v 1.0.2). Alpha diversity was determine using Gini Simpson and Shannon indexes using the Alpha Function in microbiome. The beta diversity for similarities in microbial communities between treatments was performed using Bray–Curtis. Beta diversity distance matrices were visualized with Principal Coordinate Analysis (PCoA). Analysis of compositions of microbiomes with bias correction (ANCOM-BC), with ANCOMBC package v 1.4.0, was performed to identifying the differentially abundant taxa (Lin & Peddada 2020). Venn diagrams were employed to illustrate the results of the core and specific microbiota in mucosa and feces. These diagrams were generated using the web tool https://jvenn.toulouse.inrae.fr/app/index.html.

Determination of SCFA in feces by gas chromatography

SFCAs were extracted in fecal samples according to a previously published protocol with some modifications in the chromatographic conditions (García-Villalba et al. 2012). Freeze-dried feces (50 mg) were weighed and 1.5 mL of 0.5% phosphoric acid was added and mixed for 2 min. Fecal suspensions were centrifuged at 18,000 *xg* for 5 min at 4 °C. The supernatant (1 mL) was collected, and an equal volume of ethyl acetate was added. Next, samples were mixed for 2 min and centrifuged at 18,000 *xg* for 5 min at 4 °C. The organic phase was transferred to a glass insert into an autosampler vial and spiked with hexanoic acid at a final concentration of 1000 uM. A 5-point calibration curve (200–2500 uM) was prepared with a mixture of acetic acid, propionic acid and butyric acid, and hexanoic acid as internal standard. SCFAs quantification was performed by gas chromatography (GC) with a flame ionization detector (FID). The GC system consisted of an HP 6890 (Hewlett Packard, Wilmington, DE), equipped with an automatic liquid sampler. The SCFAs were separated in a BD-FFAP capillary column at a flow of 1 mL/min using helium as the carrier gas. Samples were automatically injected in a split mode (5:1 split ratio). The oven temperature program was set with an initial temperature of 90 °C, increased to 150 °C at the rate of 10 °C/min, then to 170 °C at the rate of 5 °C/min and held for 10 min at 200 °C. Identification of the SCFAs was based on the retention time of the analytical standards of SCFAs.

Statistical analysis

All statistical tests for microbiota analysis were performed using non-parametric tests using R packages. Statistical analysis for alpha diversity was performed using the Mann–Whitney-Wilcoxon test and Kruskal–Wallis test. Analysis of similarities (ANOSIM) was used for significance calculation of beta diversity-PCoA with 999 permutations with vegan package (v1.4.0). Correlation analysis was carried out by using the Spearman coefficient via a corrplot package (v 0.92). The differential abundance was performed using ANCOM-BC followed by False Discovery Rate (FDR) for p value correction (q value) where the control (Standard diet) group was set as the reference level group. SCFAs data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey–Kramer for means comparison in GraphPad Prism version 9.51. (San Diego, CA). The significance for all statistical analysis was considered when p value was < 0.05 and asterisks in bar plots indicate the p value: $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, ns: not significant. Tendency was defined when p value was in a range of 0.05 to 0.1.

Results

Effect of a hypercholesterolemic diet and fermented milks in microbiota biodiversity

To investigate the effect of fermented milks with *L. lactis* spp. on gut microbiota in hypercholesterolemic Sprague–Dawley rats, a 16 s rRNA gene sequencing was performed to analyze the bacterial community response in colonic mucosal and fecal samples. The alpha diversity analysis detected a significant difference in Shannon ($p = 0.0004$) and Simpson ($p = 0.0027$) indices exhibiting a higher richness and diversity in colonic mucosal than in feces microbiota (Fig. 1A). PCoA based on Bray–Curtis distance showed a remarkable clustering pattern between both samples (Fig. 1B) indicating significant differences (ANOSIM analysis, $p = 0.001$) in the composition of microbiota in both samples. To investigate the impact of fermented milks consumption on microbial diversity, an independent analysis was performed for either mucosal or fecal samples for each treatment. Results showed no significant differences ($p > 0.05$) among the different treatments for both fecal and mucosal samples as shown for the alpha diversity based on the Shannon index (Fig. 2 A and B). However, the consumption of FM-572 showed a tendency to increase diversity ($p = 0.10$) when compared with the HC control (Fig. 2A) in the fecal sample.

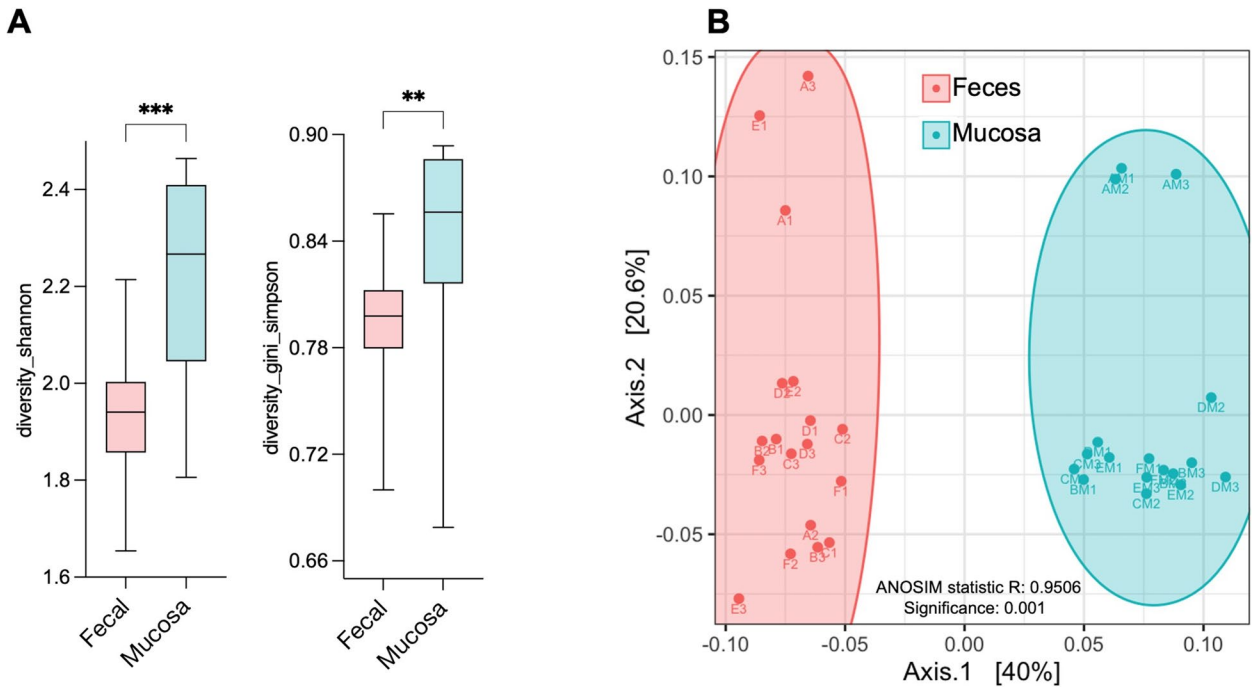


Fig. 1 Analysis of gut microbiota biodiversity comparing fecal and colonic mucosal samples **A**, Simpson, and Shannon indexes for alpha diversity in fecal and mucosal samples. **B**, PCoA of Beta diversity based in Bray–Curtis distances dissimilarity comparing fecal and mucosal samples. ANOSIM was used for statistical analysis in PCoA. For alpha diversity, the Mann–Whitney-Wilcoxon test was used for median comparison

Changes in microbiota structure were determined by Bray–Curtis dissimilarity. The Bray–Curtis analysis showed a significant separation (ANOSIM analysis, $p=0.037$) between the different treatments in feces (Fig. 2C). Specifically, there was cluster separation among the HC control and FM-571, FM-572 and the control group, suggesting that the microbiota after the consumption of fermented milks was improved since it was closer to the control group (healthy group). For mucosal microbiota, clustering was observed between the control and all hypercholesterolemic groups as depicted in PCoA (Fig. 2D). However, the consumption of fermented milks did not exert significant changes in the colonic mucosal microbiota composition ($p=0.072$).

Effect of a hypercholesterolemic diet and fermented milks on gut microbiota composition at phylum and family level

The effect of the hypercholesterolemic diet and fermented milks with *L. lactis* spp. on the relative abundance at phylum and family level were also analyzed. The predominant phyla in each group in feces and mucosal samples are shown in Fig. 3AB. *Bacteroidota* and *Firmicutes* were the dominant phyla (94%) in feces; whereas, in mucosa, *Bacteroidota*, *Firmicutes* and *Desulfobacterota* were the most abundant phyla with more than 80% of total. Moreover, *Actinobacteriota* phyla was only detected in fecal microbiota.

The high cholesterol diet increases the abundance of *Bacteroidota* and decreases *Firmicutes* phylum in feces ($p<0.05$). In mucosa, the relative abundance of both *Bacteroidota* and *Firmicutes* phylum was increased ($p<0.05$) in the hypercholesterolemic control compared to the healthy control. Moreover, the abundance of *Campylobacterota* decreased after a high cholesterol diet. Furthermore, non-fermented milk (NFM), FM-571 and FM-572 increased the abundance of *Firmicutes*, and FM-572 decreased the relative abundance of *Bacteroidota* phylum ($p<0.05$) in fecal microbiota (Supplemental Table 1). Nevertheless, no significant ($p>0.05$) changes were observed by the administration of the different fermented milks at phylum level in mucosal microbiota. A *Firmicutes* to *Bacteroidota* ratio was also determined for both samples; however, no significant changes were detected ($p>0.05$) (Fig. 3C and D). Nevertheless, the administration of FM-571 and FM-572 slightly increased *Firmicutes*/*Bacteroidota* ratio in mucosa (Fig. 3D) compared with HC control ($p=0.078$).

At family level, 29 and 24 families were identified in fecal and mucosal samples, respectively (Supplemental Fig. 1). All 24 families that were found in mucosa were also identified in feces; however, *Coriobacteriales Incertae Sedis*, *Eggerthellaceae*, *Monoglobaceae*, *Morganellaceae* and *Streptococcaceae* families were exclusive to fecal samples. Figure 4 shows the relative abundance at

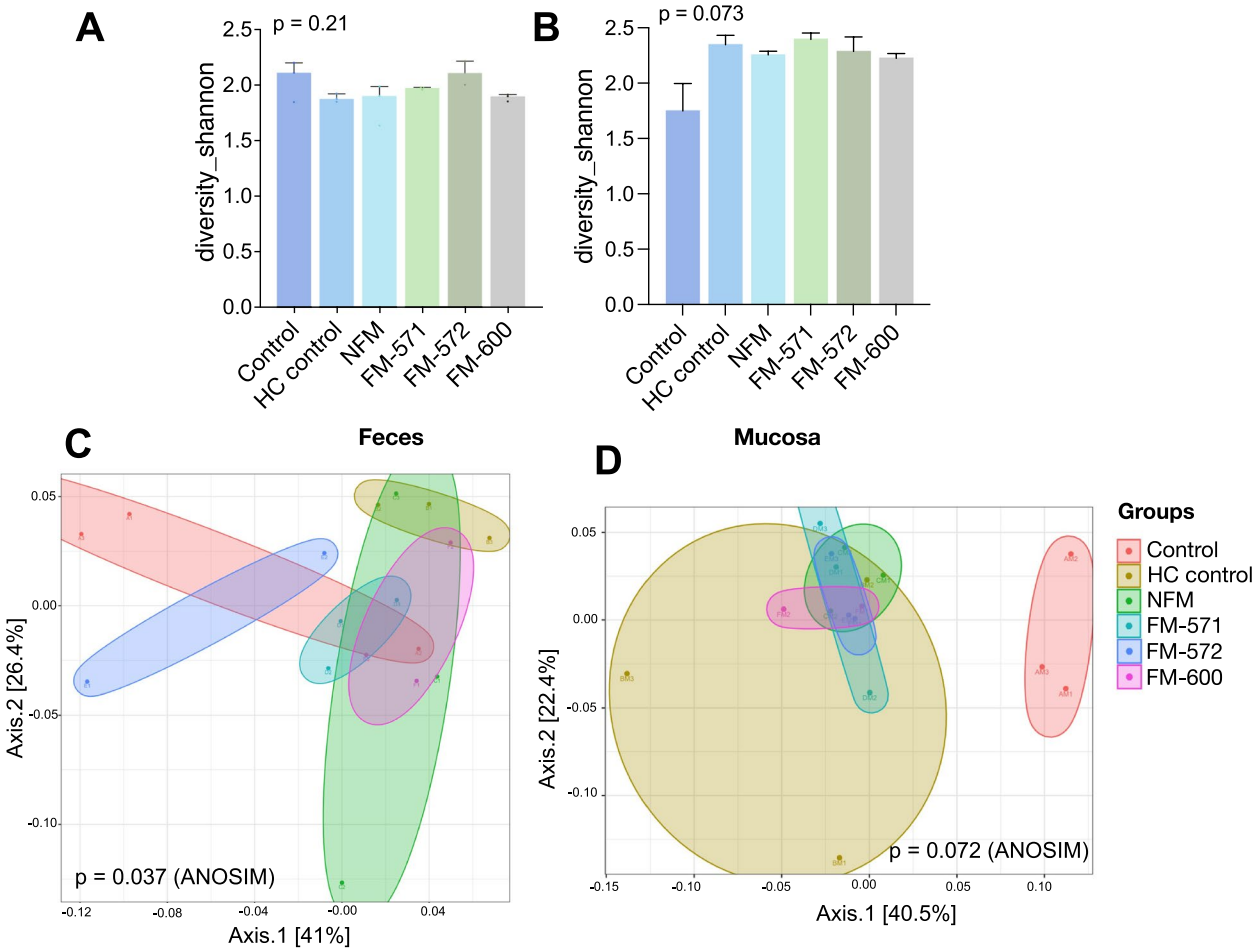


Fig. 2 Analysis of gut microbiota biodiversity in fecal and mucosal samples. **A-B** Shannon index for Alpha diversity in fecal and mucosal microbiota, respectively. **C-D** PCoA of Beta diversity based in Bray–Curtis distances dissimilarity in feces and mucosa, respectively. ANOSIM was used for statistical analysis. n.s. no statistically significant differences ($p > 0.05$) based in a non-parametric test comparison

family level in fecal and mucosal microbiota. In feces (Fig. 4A), *Muribaculaceae*, *Prevotellaceae* and *Lachnospiraceae* were the most predominant families comprising 70% of the total families. Meanwhile in mucosal microbiota (Fig. 4B), *Desulfovibrionaceae*, *Helicobacteraceae* and *Muribaculaceae* families were the representative families in mucosa with above 50% of total bacterial groups.

ANCOM-BC analysis was used for identifying the most differential abundant families among the different treatments with control group as a reference group level. The analysis revealed that in feces, *Peptococcaceae* family was increased in the hypercholesterolemic group. However, *Ruminococcaceae*, *Oscillospiraceae*, *Anaerovoracaceae* and *Rikenellaceae* were decreased in this group (Supplemental Fig. 2). In mucosa, *Eubacterium coprostanoligenes* group, *Lachnospiraceae*, *Bacteroidaceae*, *Erysipelotrichaceae*, *Tannerellaceae* families were significantly ($p < 0.05$) more abundant in the HC control compared to control group ($p < 0.05$). Whereas, *Lactobacillaceae*, *Saccharimonadaceae* and *Christensenellaceae* were differentially less abundant in the HC control group (Supplemental Fig. 3).

The consumption of fermented milks modified the relative abundance of specific taxa in fecal microbiota. In this regard, the consumption of fermented milks significantly increased the relative abundance of four specific families in fecal microbiota. In specific, *Lachnospiraceae* was increased ($p < 0.05$) in all fermented milks groups. Furthermore, FM-572 increased up to 16-fold the relative abundance of *Lactobacillaceae*. Additionally, the relative abundance of *Oscillospiraceae* and *Ruminococcaceae* families was significantly increased in FM-572 group up to two-fold ($p < 0.05$) as seen in Fig. 4 C. Contrary to this, after the consumption of fermented milks the relative abundance in mucosal microbiota did not exert an effect.

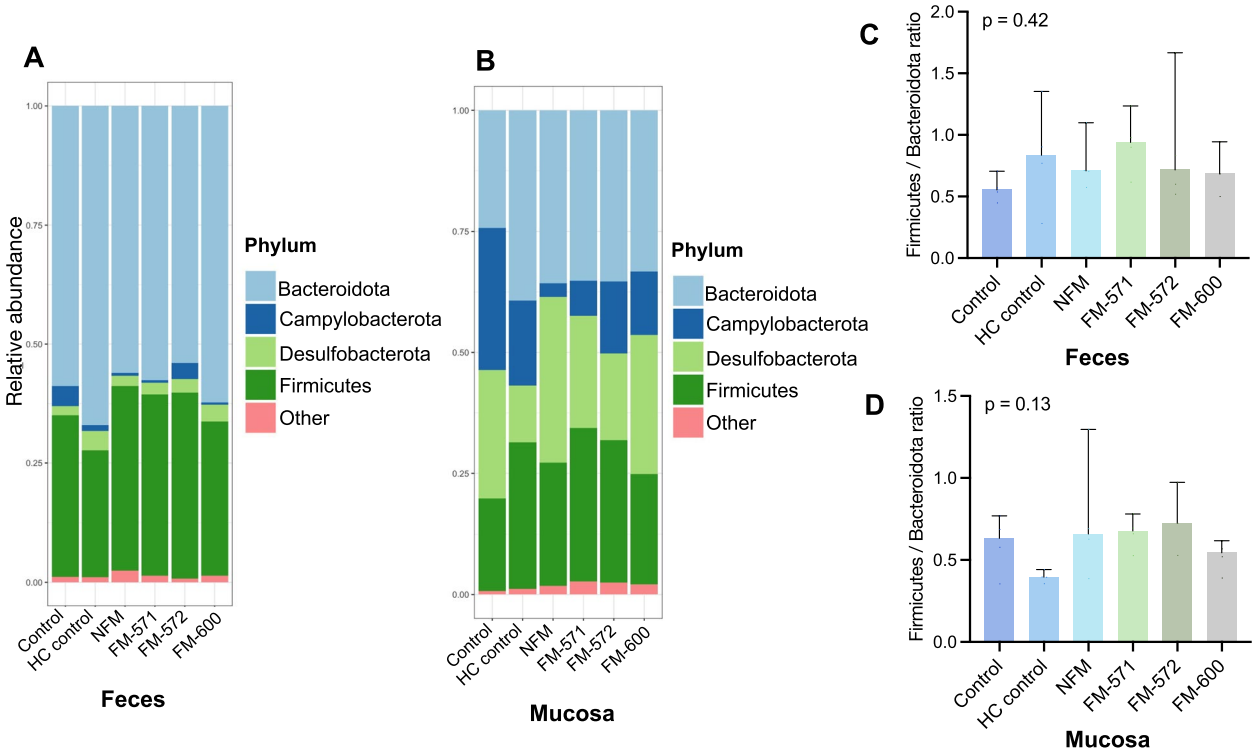


Fig. 3 Gut microbiota composition at phylum level. Relative abundance of taxa at phylum level in **A**, in feces and **B**, in mucosa. Relative *Firmicutes/Bacteroidota* ratio for **C**, fecal and **D**, mucosal samples. Bars in C and D figures represent the median with range

Effect of fermented milks in short chain fatty acids production and their association with microbiota families

To explore the effect of fermented milks on the production of SCFAs (acetate, propionate and butyrate) concentrations were determined in fecal samples at the end of the experimental period (Fig. 5A). Total SCFAs content was decreased in HC control; however, the consumption of fermented milks increased total SCFAs ($p < 0.05$), specifically in FM-572 and FM-600 groups compared with HC control and NFM. Acetate content was higher ($p < 0.05$) in FM-572 and FM-600, compared with the HC control or NFM ($p < 0.05$). Propionate content was increased in all hypercholesterolemic groups, except for NFM group, and the most significant ($p < 0.05$) increases were observed in FM-572 and FM-600. Moreover, butyrate content was notably decreased ($p < 0.05$) up to 2.5-fold in all hypercholesterolemic groups. Nevertheless, the consumption of all fermented milks, increased ($p < 0.05$) the levels of butyrate in feces compared to HC or NFM. Figure 5 shows the correlation matrix that indicates the potential association between SCFAs and the most abundant families identified in fecal and mucosal microbiota. As shown in Fig. 5B and 5C, acetate was positively ($p < 0.05$) correlated with *Lactobacillaceae* and *Oscillospiraceae* in feces and mucosa, while *Prevotellaceae* only in mucosa. Propionate was positively ($p < 0.05$) correlated with *Oscillospiraceae*, *Maribaculaceae*, *Bacteroidaceae* and *Desulfovibrionaceae*; whereas, butyrate was positively ($p < 0.05$) correlated with *Ruminococcaceae*, *Lactobacillaceae*, *Christensenellaceae*, *Eubacterium coprostanoligenes* group and *Oscillospiraceae*.

Potential association of gut microbiota with plasma lipid levels in hypercholesteremic rats

A high-cholesterol diet (1% w/w) with cholic acid (0.25% w/w) was used to induce hypercholesterolemia in Sprague–Dawley rats. This diet significantly increased TC and LDL-C, and significantly reduce HDL-C ($p < 0.05$) after seven-weeks of administration (Supplemental Fig. 4). Conversely, fermented milks with FM-572 and FM-600 significantly decreased TC and LDL-C levels compared with NFM and HC control groups ($p < 0.05$). Moreover, HDL-C was significantly increased after the administration of fermented mik with FM-600 ($p < 0.05$). The consumption of fermented milks did not influence in the TG and bile acids in plasma and fecal cholesterol ($p > 0.05$). Additionally, no significant differences were observed in food consumption of HCD in the hypercholesterolemic groups (Supplemental Fig. 5A). In addition, no significant changes were observed ($p > 0.05$) on

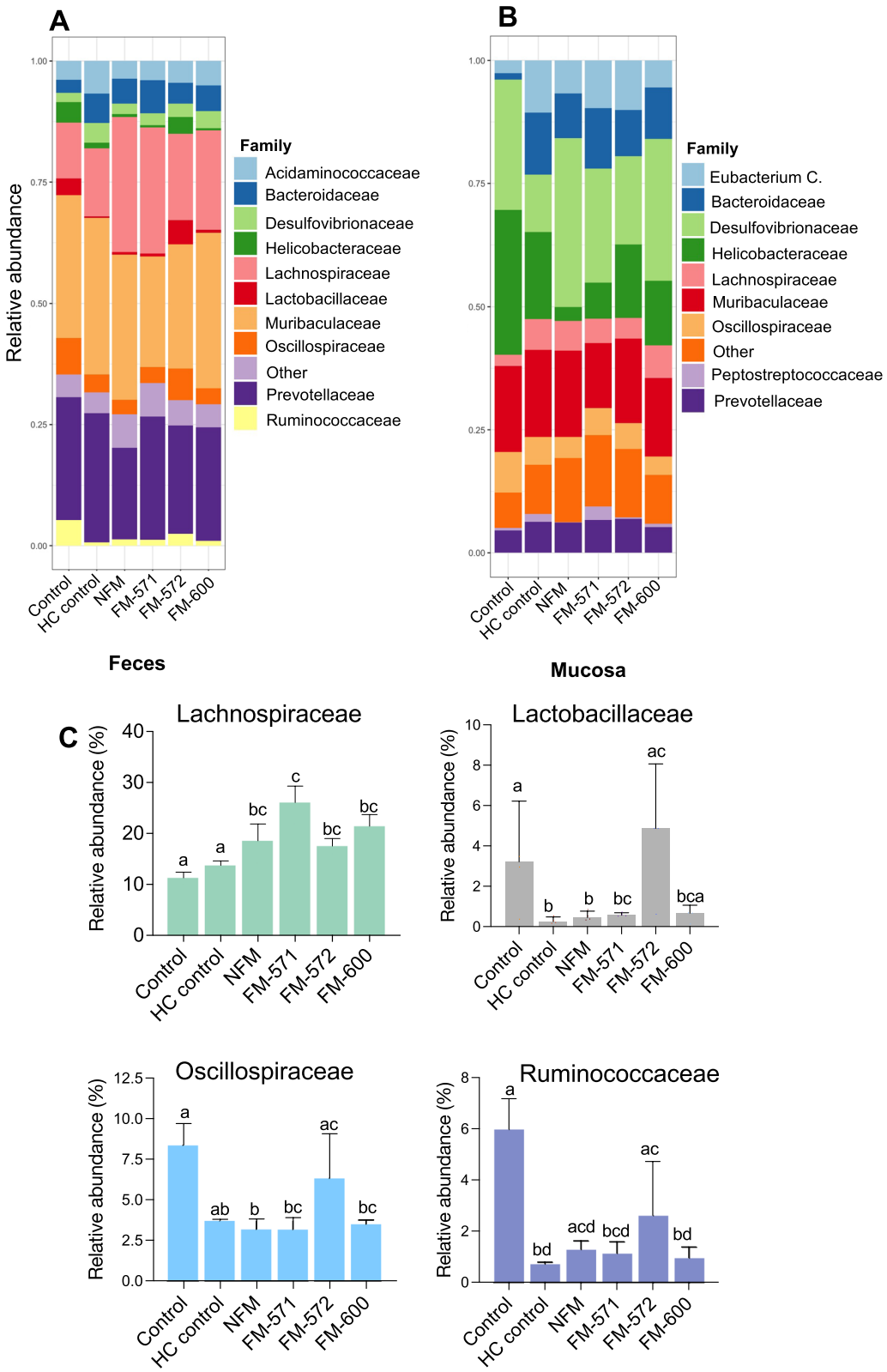


Fig. 4 Gut microbiota composition at family level. Relative abundance of taxa at family level in **A**, fecal sample and **B,** mucosal sample. **C**, Effect of consumption of fermented milks with *L. lactis* level on the relative abundance (%) of specific families. Bars represent the median with range. Different letters indicate statistical differences ($p < 0.05$) between groups

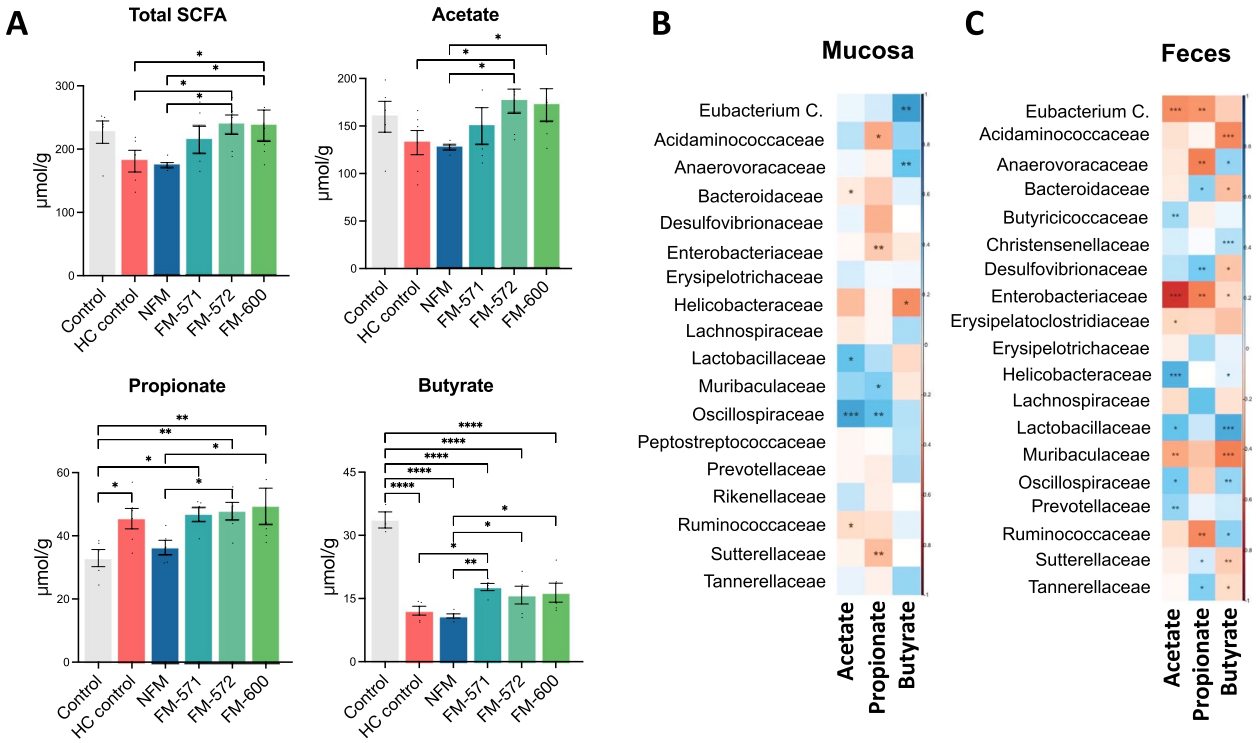


Fig. 5 **A** Concentration of short chain fatty acids (total SCFAs, acetate, propionate and butyrate) in feces of rats after fermented milks treatment. **B-C** Correlation Heatmap based on Spearman correlation coefficient between levels of SCFAs and specific families identified in mucosal and fecal samples. Asterisks indicate significative correlation (*, 0.05; **, 0.01; and ***, 0.001)

the consumption of fermented milks during the last two weeks of experimental period (Supplemental Fig. 5B).

The potential association between lipid variables (total cholesterol, LDL-C, TG, HDL-C, plasma bile acids and fecal cholesterol) and the most abundant taxa at family level in feces and mucosal samples was also explored. As shown in Fig. 6, TC, LDL-C and TG were positively associated with *Helicobacteraceae* and *Muribaculaceae* ($p < 0.05$) in both samples. However, *Oscillospiraceae* in mucosa (Fig. 6A), *Lachnospiraceae*, *Ruminococcaceae*, *Anaerovoracaceae* and *Lactobacillaceae* in feces (Fig. 6B) were negatively associated with TC. Moreover, *Eubacterium coprostanoligenes* and *Ruminococcaceae* were negatively associated with LDL-C ($p < 0.05$). Also, HDL-C levels were positively associated with *Ruminococcaceae*, *Lactobacillaceae* and *Lachnospiraceae* families in mucosa (Fig. 6A); whereas in feces, HDL-C was positively correlated with *Acidaminococcaceae* and *Desulfovibrionaceae* (Fig. 6B). Subsequently, SCFAs levels were correlated with lipid levels (Fig. 6C). From all SCFAs only butyrate was negatively correlated with plasma levels of TC, LDL-C, TG ($p < 0.05$) and positively associated with HDL-C ($p < 0.05$). These observations collectively suggest that the increased production of butyrate in groups treated with fermented milks may play an important role in the regulation of cholesterol levels and may be related to the increase of butyrate-producing bacteria.

Discussion

Over the last several decades, considerable efforts have been focused on the prevention of CVDs and their related risk factors, such as hypercholesterolemia. Indeed, hypercholesterolemia is considered the main modifiable risk factor, and their current management by clinical guidelines comprise the consumption of specific functional foods (Catapano et al. 2019; Mach et al. 2020). Nevertheless, it is important to consider that lipid metabolism may also be altered by gut microbiota, and in a dysbiosis state, it can lead atherogenesis and CVDs (Jonsson & Bäckhed 2017). It has previously been reported that FM-572 and FM-600 reduce atherogenic risk indices by the regulation of cholesterol levels (TC, non-HLDL and HDL-C) in plasma and liver in rats (Rodriguez-Figueroa et al. 2013, Rendón-Rosales et al. 2023). Nevertheless, the underlaying mechanisms is still unknown. In this study, we characterized the bacterial community of gut and feces of hypercholesterolemic Sprague–Dawley rats after the consumption of hypocholesterolemic fermented milks with *L. lactis* spp.

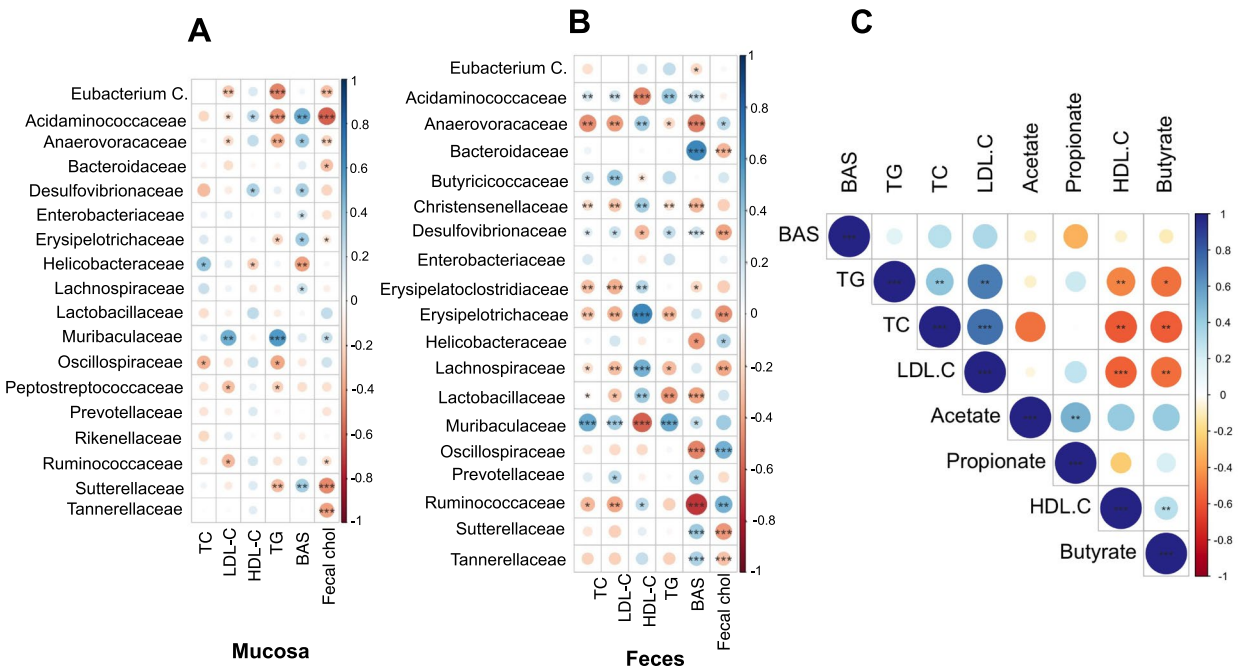


Fig. 6 Correlation heatmap based on Spearman correlation coefficient between lipid levels (TC, LDL-C, HDL-C, TG, BAs, and fecal cholesterol) and the abundant taxa identified in **A**) Mucosa and **B**) Fecal microbiota. **C**) Correlation heatmap based on Pearson coefficient between lipid levels and SCFAs. Asterisks indicate significative correlation (*, 0.05; **, 0.01; and ***, 0.001)

First, the findings showed that the consumption of formulated HCD induced alteration on plasma lipid profile, characterized by a significantly increased of LDL-C and low levels of HDL-C without changes in triglycerides and bile acids levels in plasma. Concurrently, microbiota composition was significantly altered by the consumption of a HCD resulting in a hypercholesterolemic model with an altered microbiota (dysbiosis) based in the beta diversity-analysis (ANOSIM). Our results showed that the hypercholesterolemic diet significantly increased bacteria capable to metabolize bile acids and cholesterol, such as *Bacteroidaceae* and *Eubacterium Coprostanoligenes,* and this effect may be related to the presence of these compounds in the diet (Mullish et al. 2018, Juste & Gérard 2021).

Moreover, these groups were significantly correlated with plasma bile acids and cholesterol (TC and LDL-C), suggesting their capacity to metabolize these compounds. It has been reported that *Bacteroidaceae* is positively associated with secondary bile acids and these compounds are capable to regulate cholesterol levels through the absorption, synthesis and excretion of cholesterol. The alteration of bile acid composition by gut bacteria may lead to metabolic disorders and CVDs (Mullish et al. 2018). Also, in the present study, the abundance of *Eubacterium coprostanoligenes* was significantly increased in the hypercholesterolemic groups, and this bacterial group was negatively associated with LDL-C and fecal cholesterol. It has been recently reported that this family is capable of metabolizing cholesterol producing coprostanol, which is a weakly absorbable sterol in the intestine (Juste & Gérard 2021). The significant correlations observed in the present study may suggest that *Eubacterium coprostanoligenes* group decreased dietary and endogenous cholesterol in the intestine, and thus, reducing their intestinal absorption and cholesterol in plasma. In fact, *Eubacterium coprostanoligenes* group has been proposed as an adjuvant of hypercholesterolemia treatment; nevertheless, this effect has been only been confirmed in animal models (Li et al. 1995).

Moreover, our findings revealed that the alteration of structure of gut microbiota by the hypercholesterolemic diet was also associated to a significant decrease on the abundance of beneficial bacteria, such as *Lactobacillaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Oscillospiraceae,* which have been associated to the production of SCFAs, particularly, butyrate. Interestingly, butyrate content in feces was significantly decreased in the hypercholesterolemic group. These changes were consistent with previous reports, where the abundance of these families was low in the fecal microbiota of children with high risk of stroke and primary hyperlipidemia (Gargari et al. 2018). It is noteworthy to mention that low abundance of these families

is also associated with inflammation, which may also increase the risk of the development of CVDs (Forbes et al. 2016). The decrease of abundance of these families can be explained in part by the presence of bile acids in the diet (cholic acid). Bile acids have been demonstrated to modulate gut microbiota structure by their antimicrobial activity. In fact, bile acids have been suggested to be a risk factor for development dysbiosis in metabolic diseases such as obesity (Martínez et al. 2013). The consumption of fermented milks with *L. lactis* spp. significantly reduced 20% of the cholesterol levels (TC and LDL-C) and did not decreased HDL-C levels, compared to the hypercholesterolemic rats treated with non-fermented milk. Therefore, this result reinforce the beneficial effect of fermentation process in milk increasing their nutritional and functional value.

Our findings suggest that the hypocholesterolemic effect of these fermented milks with *L. lactis* spp. may be due to gut microbiota modulation. In specific, these results revealed that the consumption of fermented milks was capable to modulate fecal microbiota but not mucosal (colonic) microbiota, according to a significantly cluster separation by Beta-diversity analysis. This result suggests that fermented milks are capable to modulate luminal microbiota which is major represented in feces (Carrol et al. 2010). It has been reported that luminal bacteria differ to mucosa-associated microbiota in their composition. Luminal bacteria is more exposed to compounds derived from digestion. Thus, the composition of luminal bacteria can be modified by dietary factors. In fact, luminal bacteria may metabolize nutrients (*e.g.,* lipids, proteins, and carbohydrates) derived from the diet, producing metabolites such as SCFAs (Wu 2020). Hence, the production of gut-derived metabolites induced by the consumption of fermented milks may exert hypocholesterolemic effect.

On the other hand, families from feces were more significantly correlated with plasma lipids and fecal SCFAs, than families from mucosal microbiota. These findings support that luminal bacteria are associated to lipid metabolism, which is consistent with previous results (Wu 2020). In particular, the abundance of *Ruminococcaceae*, *Lachnospiraceae,* and *Oscillospiraceae* in feces were increased after the consumption of fermented milks, especially with FM-572. Interestingly, these families showed a significant negative association with TC and LDL-C, and positive with HDL-C and butyrate levels in feces. These results suggest that the consumption of fermented milks may increase butyrate producing families. Altogether, our results indicate that the consumption of fermented dairy products may increase potential butyrate-producing bacteria (Veiga et al. 2014, Zhou et al. 2019).

Although the consumption of fermented milks increased the content of SCFAs in feces, only butyrate was significantly correlated with lipid levels (negatively for TC and LDL-C and positively for HDL-C). Therefore, these associations suggest that butyrate plays a beneficial role in the modulation of cholesterol metabolism. The increase of butyrate levels after the consumption of fermented products has also been observed in previous studies. These studies reported an increase of genes related to butyrate synthesis (butyryl-CoA transferase and butyrate kinase) (Berni et al. 2017). Moreover, food compounds such as exopolysaccharides (EPS) and protein/peptides present in fermented milks may be used as substrate for the fermentation in the gut (Bengoa et al. 2020, Nehal et al. 2019). Furthermore, lactate can be metabolized into butyrate in the gut by lactate-utilizing bacteria, such as *Anaerostipes* which belongs to the *Lachnospiraceae* family (Tao et al. 2016). Additionality, fermented milks may also provide SCFAs to the diet.

In a previous study, fermented milks with *Lactococcus lactis* NRRL B-50571 or NRRL B-50572 and non-fermented milk showed the presence of SCFAs (acetate and butyrate); nevertheless, only acetate was increased in fermented milks (Beltrán-Barrientos et al. 2019). The source of acetate from fermented milk may increase the SCFAs content in feces, since acetate may be used by gut commensals to produce propionate and butyrate in a growth-promoting cross-feeding process (Bengoa et al. 2020).

Although butyrate is associated with the barrier function, due to their effect in permeability (Peng et al. 2009; Bach et al. 2018), recent evidence suggests that butyrate extend its function beyond the barrier function protection. In this sense, butyrate is associated with the modulation of lipid metabolism with the consequent reduction of cholesterol levels (Amiri et al. 2022, Zhang et al. 2022). Moreover, this effect depends on multiple mechanism pathways, including the modulation of gene expression of enzymes and proteins related to synthesis and catabolism of cholesterol. In fact, the regulation of sterol regulatory element-binding protein 2 (SREBP2) and low-density lipoprotein receptor (LDLR) is also involved in this process. Butyrate via SREBP2 modulation may reduce the expression of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR) which is the rate-limiting step enzyme in the synthesis of cholesterol (Friesen & Rodwell 2004; Zhao et al. 2017). Also, butyrate can increase LDLR synthesis, which plays a role in the uptake of LDL-C by the hepatocytes, regulating the levels of cholesterol in bloodstream (Yang et al. 2020, Haghikia et al. 2022).

In the present study, propionate was not significantly associated with lipid levels; however, this SCFA

has been reported to have a cholesterol-lowering effect. This discrepancy was observed despite the fact that propionate levels were improved after fermented milks consumption. Propionate has been reported to deliver the capacity of inhibit HMGCR and suppress the expression of Niemann-Pick C1-like 1 (NPC1L1), resulting in a decrease of biosynthesis and intestinal absorption; respectively (Haghikia et al. 2022). Similarly, acetate has shown to possess an hypocholesterolemic effect by the catabolism of cholesterol through the increase of activity of cholesterol-7-α-hydroxylase enzyme reflecting in the synthesis of bile acids. It is noteworthy to highlight that butyrate also exerts an antihypertensive and anti-inflammatory effect, reduce oxidative stress, that collectively suggest a protective effect against atherosclerosis (Wang et al. 2017; Zhang et al. 2017; Amiri et al. 2022).

Moreover, results also showed that the abundance of *Lactobacillaceae* was significantly increased with the consumption of FM-572. Similar studies have reported an increase of *Lactobacillaceae* after the consumption fermented milks, which may be related to an stimulatory effect of metabolites derived from fermented milk on lactic acid bacteria in the gut. Indeed, it has been previously reported that SCFAs promotes growth of species of *Lactobacillaceae* (Berni et al. 2017). In the present study, a significant positive correlation was detected between SCFAs (acetate and butyrate) and *Lactobacillaceae*. Interestingly, species of *Lactobacillus* have been associated with metabolic health, such as the reduction of cholesterol levels, due their capacity to assimilate cholesterol (adsorption and absorption process) and bile salt hydrolase activity (Jia et al. 2023). Furthermore, other bacterial taxa, such as *Anaerovoracaceae*, *Erysipelotrichaceae*, *Erysipelatoclostridiaceae*, *Christensenellaceae* and *Oscillospiraceae* were negatively associated with cholesterol. However, it is difficult to suggest the contribution of these taxa to lipid metabolism, since there are limited reports that demonstrate their involvement.

Due the fact that the composition of fermented milks is complex and abundant in bioactive compounds, it is possible that the hypocholesterolemic effect is also mediated directly by other molecules such as bioactive peptides and exopolysaccharides, which may collectively exert a synergist effect. Further studies are needed to determine the underlying mechanisms associated with the hypocholesterolemic effect of the fermented milks with *L. lactis* spp. In summary, these fermented milks exerted an effect on the modulation of gut microbiota at mucosal and fecal level. The observed changes in specific families may contribute to a positive effect in the regulation of lipid levels in plasma. This effect may be related to the participation of SCFAs, specifically, butyrate, raising the possibility that the hypocholesterolemic effect of fermented milks is mediated by butyrate- producing bacteria.

Conclusion

The results of the present study suggest that the consumption of fermented milks with *L. lactis*, specifically with FM-572 may modulate fecal microbiota but not mucosal microbiota. These changes were consistent with the significant increase of potential butyrate-producing bacteria and the increase of fecal butyrate. In particular, there was a positive association of *Lachnospiraceae*, *Ruminococcaceae* and *Lactobacillaceae* families with cholesterol levels in plasma and fecal butyrate. Therefore, the hypocholesterolemic effect of FM-572 may be related to the increase of potential butyrate-producing bacteria. Further studies are necessary to unravel the mechanisms underlaying the hypocholesterolemic effect of milks fermented with *L. lactis*.

Abbreviations

FM-571	Fermented milk with NRRL B-50571
FM-572	Fermented milk with NRRL B-50572
FM-600	Fermented milk with NRRL B-50600
NFM	Non-fermented milk
HCD	High-cholesterol diet
L.	*Lactococcus*
SCFA	Short-chain fatty acids
TC	Total cholesterol
TG	Triglycerides
ANOSIM	Analysis of similarities
ANCOM-BC	Analysis of compositions of microbiomes with bias correction
ASVs	Amplicon sequence variants
PCoA	Principal Coordinate Analysis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43014-024-00221-z.

Additional file 1: Supplemental Figure 1. Venn-diagram illustrating the total identified families in fecal and colonic mucosal samples from rats. **Supplemental Figure 2.** Differential abundance of families identified in fecal samples expressed as Log Fold change and error standard. Differential abundance was tested using ANCOM-BC with *p*-values correction using False Discovery Rate (FDR) procedure. Standard diet (healthy) group was set as reference level group. Asterisk (*) represent the treatment where the taxa at family level was differentially abundant ($p < 0.05$). **Supplemental Figure 3.** Differential abundance of families identified in mucosal samples expressed as Log Fold change and error standard. Differential abundance was tested using ANCOM-BC with *p*-values correction using False Discovery Rate (FDR) procedure. Standard diet (healthy) group was set as reference level group. Asterisk (*) represent the treatment where the taxa at family level was differentially abundant ($p < 0.05$). **Supplemental Figure 4.** Effect of milks fermented with *L. lactis* on plasma lipid levels, total cholesterol (TC), LDL-C, HDL-C, triglycerides (TG), plasma bile acids (BAs) and fecal cholesterol. Bars represent the mean and standard deviation (SD). Different literals indicate a significant difference ($p < 0.05$) between treatments, compared with one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. n.s., no significant ($p > 0.05$). **Supplemental Figure 5.** Average daily food (**A**) and milk (**B**) consumption in the experimental period (seven weeks) by Sprague-Dawley rats. Lowercase letters indicate statistical differences ($p < 0.05$) among the experimental groups. **Supplemental Table 1.** Relative abundance of taxa at phylum level in feces and mucosa.

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Authors' contributions
Conceptualization, B.V.-C. and M.A.R.-R.; methodology, M.A.R.-R.; M.C.E.-M and J.I.M.-R.; software, M.A.R.-R. and J.I.M.-R.; validation, M.A.M.-M. and H.S.G.; investigation, M.A.R.-R.; writing-original draft preparation, M.A.R.-R.; writing-review and editing, B.V.-C., L.M.B.-B., A.H.-M., and A.F.G.-C.; project administration B.V.-C. and A.F.G.-C.; funding acquisition, B.V.-C. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
The data support the findings in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The experimental protocol was approved by the Bioethics Committee of the Research Center for Food and Development (Spanish acronym, CIAD), Hermosillo, Sonora, México (CE/017/2019).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Centro de Investigación en Alimentación y Desarrollo A.C. (CIAD, A.C.), Carretera Gustavo Enrique Astiazarán Rosas, No. 46, Col. La Victoria, 83304 Hermosillo, Sonora, México. [2]Unidad de Investigación y Desarrollo en Alimentos (UNIDA), Tecnológico Nacional de México/Instituto Tecnológico de Veracruz, M.A. de Quevedo 2779, Col. Formando Hogar, 91897 Veracruz, Veracruz, Mexico.

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