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Regulation of plasma lipid profile by *lactobacillus fermentum* (probiotic strain ME-3 DSM14241) in a randomised controlled trial of clinically healthy adults

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Abstract

Background: Previous studies showed that a probiotic-rich diet may improve the plasma lipid profile and lower the risk of cardiovascular disease (CVD). In a randomized controlled study we tested the possibility for regulation of plasma lipid profile using a kefir that contained the antioxidative probiotic strain, *Lactobacillus fermentum* ME-3 (DSM14241). The trial was performed in clinically healthy adults with borderline-high serum low-density lipoprotein-cholesterol (LDL-C) and/or high serum triglyceride (TG) levels based on guidelines from the European Cardiology Society and European Atherosclerosis Society.

Methods: One hundred sixty four participants meeting the inclusion criteria were included. Participants were randomised to receive 200 ml/day kefir, either with probiotic (PG; $n = 71$, 58 females, mean age 49.6 ± 6.5 y) or without probiotic (CG; $n = 66$, 58 females, mean age 49.9 ± 6.1 y). The probiotic contained 4×10^7 cfu/ml *L. fermentum* ME-3 (total 8×10^9 cfu/day). At 4 weeks ($n = 71$ PG and $n = 66$ CG) and at 8 weeks ($n = 43$ PG and $n = 33$ CG), we evaluated anthropologic, blood biochemical indices, and the faecal temporal persistence of the probiotic strain was assessed by real-time PCR.

Results: After 4 weeks, the lipid profiles were mostly similar between groups: only the values of oxidised LDL (ox-LDL) and TG were significantly reduced ($P < 0.001$ and $P = 0.005$, resp.). After 8 weeks, the PG group exhibited reductions in LDL-C (5 %, $P = 0.001$), ox-LDL (6 %, $P < 0.0002$), TG (17 %, $P = 0.033$). Next, the ratio of LDL-C to HDL-C was decreased only in the PG ($P = 0.013$) while in the CG it was significantly increased ($P = 0.018$). After completing the trial the changes in all above listed indices were significantly ($P < 0.05$) different between the PG and CG groups. In faeces, the prevalence of *L. fermentum* ME-3 increased after 4 and 8 weeks (both $P < 0.001$), but the counts, determined with real-time PCR, remained constant.

Conclusions: Eight weeks of consuming kefir with the antioxidative probiotic *L. fermentum* ME-3, reduced serum LDL-C, ox-LDL and TG values in clinically healthy volunteers with borderline-high lipid profile indices. Thus, *L. fermentum* ME-3 has potential to lower the risk of CVD that is tightly associated with maintenance of plasma lipid profile. This study was registered as current controlled trial http://www.controlled-trials.com/ISRCTN49744186 as ISRCTN49744186.

Keywords: Probiotic *L. fermentum* ME-3, Faecal temporal persistence, Real-time PCR, Kefir trial, Blood lipid profile, LDL-C, Ox-LDL, Triglycerides, LDL-C: HDL-C ratio

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Background

Cardiovascular disease (CVD) is the number one cause of death worldwide, and its incidence is rapidly increasing in countries with low- and mid-range economies [1, 2]. Abnormal function of the heart is related to dyslipidaemia, identified by the main marker, elevated levels of low-density lipoprotein-cholesterol (LDL-C), and the secondary marker, reduced levels of high-density cholesterol (HDL-C); these cholesterol disorders are summarized by the ratio of LDL: HDL. Numerous clinical trials have demonstrated that LDL-C can independently cause the development and progression of coronary heart disease [3]. In addition, elevated levels of triglycerides (TG) may contribute to dyslipidaemia. In a recent statement, the European Cardiology Society (ESC) and European Atherosclerosis Society (EAS) recommended that LDL-C should be used as a primary screening indicator for CVD risk estimations [4]. Thus, lowering the level of LDL-C remains the primary target of blood lipid-regulating strategies, including therapeutic dietary changes and pharmacological interventions [5].

Gut microbiota is an essential component of the complex host ecosystem involved in nutrition and health. A wide variety of host, microbiological, dietary, and environmental factors affect the metabolic relationships between gut mucosal epithelial cells and microbiota [6–9]. However, it remains unclear what role imbalanced intestinal microbiota might play in the pathophysiology of atherosclerosis, which underlies CVD. Cholesterol is obtained both through the diet and through highly-regulated biosynthesis. Serum cholesterol is predominantly eliminated from body in form of bile acids [10]. Among the normal microbiota of the gastrointestinal tract, some species and strains of lactic acid bacteria can assimilate cholesterol from consumed dietary products [11]. A meta-analysis of 13 probiotic studies indicated that a probiotic-rich diet improved the plasma lipid profile. Probiotic diets reduced the total cholesterol and LDL-C concentrations in the plasma of participants with high, borderline-high, and even normal cholesterol levels [12]. However, it is not clear how long the intervention should last to achieve a beneficial effect in healthy volunteers with borderline-high levels of LDL-C.

Lactobacillus fermentum ME-3 (DSM 14241) is a bacterial strain of human origin that was isolated from a healthy one-year-old child. *L. fermentum* ME-3 had both antimicrobial and antioxidant properties [13–16]. Various clinical studies have demonstrated several health benefits associated with consuming this strain [17–21].

In the present study, we aimed to evaluate the effects of the probiotic *L. fermentum* ME-3 on the plasma lipid profile in a randomised, double-blind, placebo-controlled, parallel-design, two-armed study (ISRCTN49744186). We included clinically healthy Estonian adults that had borderline-high levels of LDL-C and above optimum values of serum TG. We evaluated anthropologic, blood, biochemical, and faecal microbiological indices. Significant reductions in serum LDL-C and TG were the primary outcome measures for lowering the CVD risk due to borderline-high plasma lipid profiles.

Methods

Probiotic lactobacillus strain

The *L. fermentum* ME-3 strain was previously isolated from the gastrointestinal tract of healthy Estonian children [22]. The strain was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures GmbH) under the registration number, DSM 14241. The *L. fermentum* ME-3 strain used in this study was confirmed by molecular identification with polymerase chain reaction that employed an internally-transcribed spacer and 16S rRNA sequencing [23]. The strain's safety was confirmed previously in an animal model [16] and in healthy Estonian adults (registered trials ISCRNT43435738 and ISCRNT53154826).

Activity for bile hydrolysation

Enzymatic deconjugation of bile acids by the bile salt hydrolase (BSH) activity of *L. fermentum* ME-3 was tested according to Lim et al. [24]. *L. fermentum* ME-3 strains were cultured overnight on de Man, Rogosa, Sharpe (MRS; Oxoid, UK) agar in microaerobic conditions at 37 °C. For control cultures, we used three reference strains (*L. acidophilus* 821−3, *L. plantarum* 299v, and *L. plantarum* BAA793). After incubation, cultures were standardised to McFarland 3.0. For testing BSH activity, sterilised paper disks were impregnated with the cultures and placed on MRS agar plates supplemented with 4 % (w/v) tauro-deoxycholic acid sodium salt (TDCA, Sigma, USA) and 0.37 g/l $CaCl_2$. The plates were anaerobically incubated at 37 °C for 72 h, and the diameters of the precipitation zones around the disks were measured. BSH activity was then calculated by subtracting the disc diameter (DD, mm) from the inhibition zone diameter (IZD, mm), and dividing by two, as follows: BSH activity = (IZD-DD)/2. The activity of three consecutive cultures of each strain was expressed as median (CI 95 %) value.

Kefir preparation

The probiotic kefir containing *L. fermentum* ME-3 (trademark "Hellus", 2005) was developed at Tere AS in Estonia. Briefly, kefir was prepared from pasteurised (92–95 °C for 5 min) cow's milk with 2.5 % fat content; *L. fermentum* ME-3 was added as an adjunct starter. Commercial starter cultures — including kefir grains and mesophilic aromatic cultures of *Lactococcus lactis*

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subsp *cremoris, Lactococcus lactis* subsp *lactis* biovar *diacetylactis, Leuconostoc* sp, and *Lactococcus lactis* subsp *lactis* (Chr. Hansen, Denmark) — were added to the incubation tank. For production of "Hellus" kefir, freeze-dried *L. fermentum* ME-3 cultures were added to the kefir milk to a concentration of 1×10^6 colony-forming units (cfu)/ml. The kefir milk fermentation process lasted approximately 20–24 h, until achieving the required pH 4.5. After fermentation, the kefir was cooled to 8-10 °C in a heat exchanger.

The final kefir product possessed a homogenous consistency, creamy colour, shiny smooth surface, fresh and acidic taste, and mild yeast-like aroma. The average nutritional value of 100 g kefir was as follows: energy content 217 kJ/52 kcal, 3.1 g protein, 4.2 g carbohydrate, and 2.5 g fat. The content of *L. fermentum* ME-3 in the probiotic kefir was 4×10^7 cfu/ml. Similar kefir produced without the probiotic adjunct served as a control.

Subjects
This randomised, double-blind, placebo-controlled, parallel-design, two-armed study (registered trial ISRCTN49744186) was conducted in South Estonia, according to the guidelines laid down in the Declaration of Helsinki 1996–2000. The study was approved by the Ethics Committee of the University of Tartu (protocol number 210/T-3; 18.01.2011). All participants signed the written informed consent at enrolment and were given the option to withdraw from the study at any time.

Subject inclusion criteria were a desire to participate; age 35–65 years; blood lipid fractions: total cholesterol >5.2 mmol/l, LDL-C >3.4 mmol/l, and/or TG >1.7 mmol/l, in addition, subjects had to be willing to maintain a stable diet and physical activity level. Exclusion criteria included a history of gastrointestinal disease; food allergy or acute infection; use of any antimicrobial agent within the preceding 2 months; use of any regular concomitant medication within the preceding 2 weeks, including non-steroidal anti-inflammatory drugs and antioxidant products; pregnancy or breastfeeding; any serious organ or systemic disease; high blood pressure (>140/95 mmHg); eating disorder; extensive exercise; genetic hyperlipidaemia; drug or alcohol abuse; active weight loss >5 kg in the prior 3 months; participation in other studies within the last 30 days or during the study; and not wishing to participate. Also, subjects were excluded when they had used any concomitant treatment within the preceding 2 months that could influence evaluation of the efficacy and tolerability of the investigational study product, including lipid-lowering drugs (e.g., statins, bile acid sequestering agents, cholesterol absorption inhibitors, nicotinic acid) or dietary supplements (e.g., omega-3 fatty acids, calcium, oat fibre, niacin, green tea extract, plant sterols, soy protein, psyllium seed husk, or probiotics/prebiotics).

Participants were randomly assigned to receive either probiotic or placebo using a computer-generated randomisation list prepared by the independent statistician. The 164 eligible subjects were randomised to receive either probiotic treatment (PG; *n* = 83) or control kefir (CG; *n* = 81; Fig. 1).
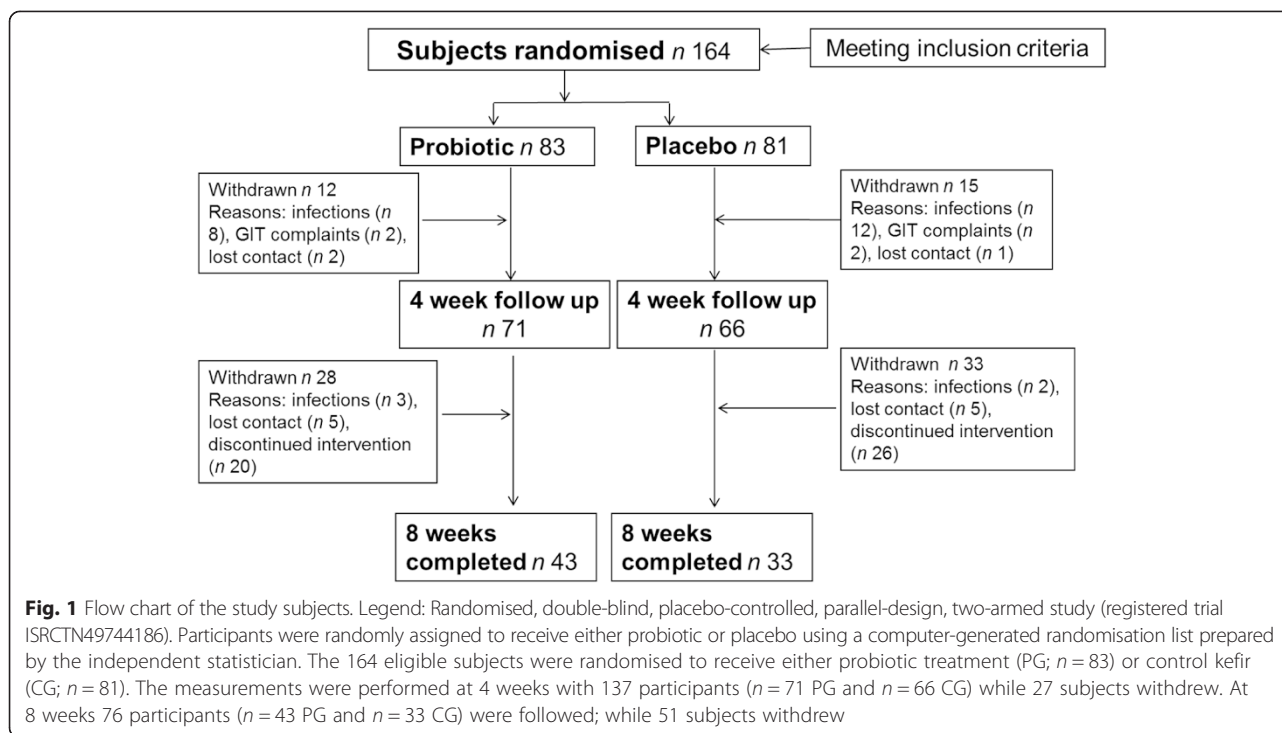
The sample size was calculated for a two-armed intervention investigation that included 4 weeks of probiotic kefir consumption. We assumed a predicted change of 0.2 U in the LDL-C level between the probiotic and control groups during the treatment period. The planned sample size of 83 subjects provided sufficient power (0.90) to detect a significance level of $P < 0.05$ with the Student's *t*-test.

Study protocol
Each group consumed 200 ml/day of kefir without (CG) or with (PG) the probiotic *L. fermentum* ME-3. The total amount of *L. fermentum* ME-3 consumed was 8×10^9 cfu/day. The trial lasted for 8 weeks. The weekly food diary was not performed but the appearance of gastrointestinal complaints was registered (Fig. 1). Measurements were carried out at the start of the study (baseline values) with 164 participants that met the inclusion criteria. We conducted measurements at 4 weeks with 137 participants (*n* = 71 PG and *n* = 66 CG) while 27 subjects withdrew (*n* = 12 PG and *n* = 15 CG) due to respiratory infections, intestinal complaints, and loss of contact. At 8 weeks 76 participants (*n* = 43 PG and *n* = 33 CG) were followed; while 51 subjects withdrew due to the infections, loss of contact and motivation (Fig. 1).

Clinical investigations
Subjects in both groups habitually consumed a Western-type diet, typically rich in potatoes, vegetables, meat, eggs, but also characterised with a high content of fibre (black bread) and several dairy products. The trial code was broken after 8 weeks. At each measurement timepoint (baseline, 4 and 8 weeks), the subjects underwent a clinical examination. Plasma samples were collected after an overnight fast and abstinence from any medications, tobacco, alcohol, tea, and coffee. Each participant was evaluated for anthropometric indices. Body mass index (BMI) was calculated as the weight (kg) divided by squared height (m^2) [25]. A sphygmomanometer was used to measure blood pressure, after subjects rested for 5 min in the sitting position. After the blood pressure measurement, blood samples were collected. Markers for determining cardiovascular health were selected based on suggestions by the NDA of the European Food Safety Authority [26]. At baseline, the two groups did not significantly differ in the selected anthropometric,

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Fig. 1 Flow chart of the study subjects. Legend: Randomised, double-blind, placebo-controlled, parallel-design, two-armed study (registered trial ISRCTN49744186). Participants were randomly assigned to receive either probiotic or placebo using a computer-generated randomisation list prepared by the independent statistician. The 164 eligible subjects were randomised to receive either probiotic treatment (PG; $n = 83$) or control kefir (CG; $n = 81$). The measurements were performed at 4 weeks with 137 participants ($n = 71$ PG and $n = 66$ CG) while 27 subjects withdrew. At 8 weeks 76 participants ($n = 43$ PG and $n = 33$ CG) were followed; while 51 subjects withdrew

clinical, and biochemical indices (Table 1). Similarly, at the end of the trial (8 weeks), the PG comprised 36 women and 7 men, with mean BMI 26.5 kg/m^2, and mean age 50.4 y; the CG comprised 30 women and 3 men, with mean BMI 27.1 kg/m^2, mean age 50.8 y. The two groups showed no significant differences. All volunteers were considered clinically healthy according to the tested baseline and reference values (Nordic Reference Interval Project; NORIP). Significant reductions in serum LDL-C and TG were considered the primary outcome measures for lowering the CVD risk score.

The obtained faecal samples were stored in a domestic refrigerator at 4 °C for no more than 2 h before transportation to the laboratory. There, the samples were frozen at −80 °C until analyses for determining the *L. fermentum* ME-3 prevalence and counts.

Measurement of oxidized LDL

Oxidized LDL (oxLDL) was measured with an ELISA kit (Cat. No. 10-1143-01, Mercodia AB, Uppsala, Sweden) [17]. This solid-phase, two-site enzyme immunoassay was based on the direct sandwich technique, in which two monoclonal antibodies were directed against separate antigenic determinants on the oxidized apolipoprotein B molecule [27]. During incubation, oxLDL in the sample reacted with the anti-oxLDL antibodies bound to the microtitration wells. After washing, a peroxidase-conjugated anti-human apolipoprotein B antibody recognised the oxLDL bound to the solid phase. After a second incubation and washing, the bound conjugate was

detected in a reaction with 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped by adding acid, and the colour intensity (U/l) was measured spectrophotometrically at 450 nm.

Metabolic indices (plasma glucose, total cholesterol, LDL-C, HDL-C, and TG) were analysed with standard laboratory methods and certified assays in the clinical laboratory of the Tartu University Clinics, Estonia. As references, we used indices for routine laboratory tests proposed by the Nordic Reference Interval Project (NORIP, http://www.tandfonline.com/doi/pdf/10.1080/00365510410006324#.Vhpmx27-VCE).

Molecular assessment of L. fermentum ME-3

Bacterial DNA was extracted from faecal samples with a QIAamp DNA stool mini kit (QIAgen, Hilden, Germany) with some modifications. Faeces (0.22 g) was suspended in 200 μl TE buffer (10 mM Tris, 10 mM EDTA pH 8, 20 mg/ml lysozyme, and 200 U/ml mutanolysin), and incubated for 1 h at 37 °C. To these samples, we added 0.3 g of 0.1-mm zirconia/silica beads and 1.4 ml ASL solution from the stool mini kit. The tubes were then agitated for 3 min at 5000 rpm in a mini-bead beater (Biospec Products Inc., USA). The protocol was completed as described by the manufacturer (QIAgen, Germany).

To establish a quantitative assay, we created plasmids that contained the PCR-amplified region of target bacteria with the pGEM-T vector system (Promega, Madison, WI). The PCR amplicon derived from *L. fermentum* ME-3 was inserted into the plasmid vector, and the recombinant

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Table 1 Clinical data of study participants

Indices		Probiotic group (PG)	Control group (CG)	PG vs CG
		(*n* = 71)	(*n* = 66)	*P*-values
Sex (female/male)		58/13	58/8	0.443
Age (y)	Mean (SD)	49.6 (6.5)	49.9 (6.1)	0.821
	Median (percentile 25–75 %)	50 (45–54)	49 (46–54)	
Body mass index (kg/m^2)	Mean (SD)	25.9 (3.8)	26.7 (4.1)	0.277
	Median (percentile 25–75 %)	26.6 (23–29)	26.2 (24–29)	
Systolic blood pressure (mmHg)	Mean (SD)	117.2 (13.8)	119.6 (11.2)	0.271
	Median (percentile 25–75 %)	116 (106–129)	119 (113–126)	
Diastolic blood pressure (mmHg)	Mean (SD)	77.7 (8.9)	81.1 (8.4)	0.022
	Median (percentile 25–75 %)	77.5 (72–83)	81.8 (75–85)	
Fasting glucose (mmol/l)	Mean (SD)	4.8 (0.5)	4.6 (0.6)	0.218
	Median (percentile 25–75 %)	4.8 (4.4–5.1)	4.6 (4.4–4.9)	
HbA1c (%)	Mean (SD)	5.8 (0.3)	5.7 (0.3)	0.467
	Median (percentile 25–75 %)	5.8 (5.6–6.0)	5.7 (5.5–6.0)	
Total cholesterol (mmol/l)	Mean (SD)	5.9 (0·7)	6.0 (0.8)	0.584
	Median (percentile 25–75 %)	5.8 (5.4–6.2)	5.9 (5.3–6.5)	
LDL cholesterol (mmol/l)	Mean (SD)	3.8 (0.7)	3.7 (0.8)	0.441
	Median (percentile 25–75 %)	3.8 (3.3–4.3)	3.7 (3.2–4.1)	
HDL cholesterol (mmol/l)	Mean (SD)	1.7 (0.5)	1.8 (0.5)	0.109
	Median (percentile 25–75 %)	1.5 (1.3–2.1)	1.8 (1.5–2.1)	
LDL-C:HDL-C ratio	Mean (SD)	2.4 (0.9)	2.2 (0.8)	0.110
	Median (percentile 25–75 %)	2.3 (1.8–3.1)	2.1 (1.7–2.6)	
Triglyceride (mmol/l)	Mean (SD)	1.2 (0.7)	1.1 (0.5)	0.706
	Median (percentile 25–75 %)	1.0 (0.7–1.5)	0.9 (0.8–1.2)	

Data represent the mean ± standard deviation and median (CI 25 % and 75 %). For continuous variables, the differences between groups were tested with the Student's *t*-test, and gender distributions were compared with Fisher's exact test. The only statistical difference was found in the level of diastolic blood pressure (NORIP, http://www.tandfonline.com/doi/pdf/10.1080/00365510410006324#.Vhpmx27-VCE)

vector was transformed into chemically competent *E. coli*. Plasmids were purified with a MaxiPrep kit (Qiagen), and then, serially diluted, and finally, measured in a spectrophotometric analysis (Quibit™, Invitrogen) [28]. The target DNA was quantified by comparing with serial, 10-fold dilutions (10^1 to 10^5 plasmid copies) of previously quantified plasmid standards. Comparisons between the plasmid standards and samples were run in triplicate.

Real-time PCR was performed with the ABI PRISM 7500 HT Sequence Detection System (Applied Biosystems, USA) equipped with optical grade 96-well plates. For detection of *L. fermentum* ME-3, the reaction mixture (25 μl) for the TaqMan assay contained 2× TaqMan Universal PCR Master Mix (PE Applied Biosystems, USA), 25 pmol primers (ME31 forward: 3′-CTTTTTAC CGCCAAAAGCAG-5′; ME-3 reverse: 3′-AGCCCTT GTCGGTGATATTG-5′), 10 pmol of TaqMan probe (5′-FAM-TTGACACAGACTCGAGCAGT-Tamra-3′), and 200 ng of extracted DNA. The thermocycling

program included an initial cycle of 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 1 min. Data was analysed with Sequence Detection Software, version 1.6.3 (Applied Biosystem, USA). *L. fermentum* ME-3 quantities were expressed as the $\log_{10}$ number of gene copies/g faeces.

Statistical analysis

Statistical analyses were performed with the R program, v. 3·0·2 (A Language and Environment, http://www.r-project.org) and GraphPad Prism, version 4·00 for Windows (GraphPad Software, San Diego, CA). All data were expressed as the mean and standard deviation (SD; Tables 1 and 2) or as the medians with two-sided confidence intervals for counts of BHS and *L. fermentum* ME-3. Sex and temporal colonisation with probiotic bacteria were compared between groups with Fisher's exact test (Table 1, Fig. 3). Baseline and intervention data (BL 1–4 and BL 2–8 weeks) were compared with

Table 2 The impact of consumption of probiotic or control kefir for 4 and 8 weeks on biochemical indices of blood sera in healthy subjects

Indices	Probiotic group (PG)						Control group (CG)						Extent of changes between PG and CG	
	Mean (SD)		p=	Mean (SD)		p=	Mean (SD)		p=	Mean (SD)		p=	p=	
	Baseline 1	4 weeks	Baseline 1 vs 4 weeks	Baseline 2	8 weeks	Baseline 2 vs 8 weeks	Baseline 1	4 weeks	Baseline 1 vs 4 weeks	Baseline 2	8 weeks	Baseline 2 vs 8 weeks	Baseline 1 to 4 weeks	Baseline 2 to 8 weeks
	n 71	*n* 71		*n* 43	n 43		*n* 66	*n* 66		*n* 33	*n* 33			
Total cholesterol (mmol/L)	5.9 (0.7)	6.0 (0.8)	0.259	5.9 (0.9)	5.7 (0.8)	0.257	6.0 (0.8)	6.1 (0.9)	0.364	6.2 (1.0)	6.2 (0.9)	0.031	0.798	0.019
HDL cholesterol (mmol/L)	1.7 (0.5)	1.7 (0.5)	0.943	1.7 (0.4)	1.6 (0.4)	0.229	1.8 (0.5)	1.8 (0.5)	0.001	1.8 (0.5)	1.8 (0.5)	0.385	0.025	0.551
LDL cholesterol (mmol/L)	3.8 (0.7)	3.8 (0.7)	0.083	3.8 (0.7)	3.6 (0.7)	0.001	3.7 (0.8)	3.8 (0.8)	0.822	4.0 (0.8)	4.0 (0.7)	0.736	0.298	0.037
LDL-C/HDL-C	2.5 (0.9)	2.4 (0.9)	0.303	2.5 (0.8)	2.4 (0.8)	0.013	2.2 (0.8)	2.3 (0.8)	0.001	2.3 (0.7)	2.4 (0.7)	0.018	0.014	0.004
Oxidized LDL (U/L)	53.7 (15.1)	50.8 (14.0)	˂0.001	52.7 (14.1)	50.4 (14.4)	˂0.001	50.5 (12.5)	49.6 (12.3)	0.164	51.0 (11.2)	50.8 (11.6)	0.630	0.103	0.0002
Triglycerides (mmol/L)	1.2 (0.7)	1.0 (0.5)	0.005	1.0 (0.4)	1.0 (0.6)	0.033	1.1 (0.5)	1.0 (0.4)	0.165	1.0 (0.4)	1.1 (0.3)	0.507	0.244	0.044
Glucose (mmol/L)	4.8 (0.5)	4.8 (0.5)	0.294	4.8 (0.4)	4.9 (0.6)	0.649	4.6 (0.6)	4.7 (0.6)	0.279	4.7 (0.6)	4.7 (0.6)	0.472	0.834	0.964
HbA1c (%)	5.8 (0.3)	5.5 (0.4)	≤0.001	5.4 (0.2)	5.5 (0.2)	≤0.001	5.7 (0.3)	5.6 (0.3)	≤0.001	5.4 (0.2)	5.4 (0.2)	≤0.001	0.220	0.719

Data represent the mean (standard deviation. BL 1 data were comprised of $n = 71$ volunteers of PG group and $n = 66$ volunteers of CG who completed the trial at 4 week; BL 2 data comprised of $n = 43$ volunteers (PG) and $n = 33$ (CG) completing the trial at 8 weeks. Baseline 1 and 2 and intervention data (4 weeks and 8 weeks, resp) were compared with a paired *t*-test within groups, and extent of changes between PG and CG was compaired with an unpaired *t*-test

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a paired *t*-test or with a Wilcoxon signed-rank test, according to the data distribution (Table 2). The change (mean ± SD) of individual biochemical indices from baseline of particular participants to 4 and 8 weeks in the probiotic and control groups was assessed with the paired t-test (Fig. 2). The extent of changes between probiotic and control groups (the PG and CG groups) was compared with the t-test. Differences were considered statistically significant when *P* was <0.05 (Table 2).

Results

Bile salt hydrolysis activity

The bile salt hydrolysis activity (BSH) of *L. fermentum* ME-3 and *L. plantarum* BAA793 was equal: in both the median (CI 95 %) was 1.5 (1.5). Still it was somewhat higher than those of the other two control strains, *L. acidophilus* 821–3 and *L. plantarum* 299v with 1.0 (1.0) and 0.5 (048–1.48), respectively.

Changes in body mass index, blood pressure and lipid fractions

Consumption of probiotic kefir for 4 or 8 weeks did not influence the BMI nor blood pressure values. No BMI or blood pressure changes were observed in either the PG or CG (data not shown).

After 4 weeks, the lipid profiles were mostly similar between groups. Still, the TG level in the PG was reduced ($P = 0.005$), and this effect was sustained at 8 weeks of probiotic kefir consumption (17 % reduction, $P = 0.033$). The ox-LDL levels also decreased with probiotic intervention. In the PG, the ox-LDL decreased by 5 % at 4 weeks ($P < 0.001$) and by 6 % ($P < 0.001$) at 8 weeks, but no change was observed in the CG.

After 8 weeks of intervention, the PG group exhibited significant reduction in LDL-C (5 %, $P = 0.001$). Thus, after this period the differences between the PG and CG groups in the LDL-C, ox-LDL and TG indices ($P = 0.037$; 0.0002; 0.044; Fig. 2) could be well demonstrated.

Additionally we found that, in the CG, the total cholesterol level was increased from 6.0 mmol/l at baseline to 6.2 mmol/l after 8 weeks of kefir consumption ($P = 0.031$), which trend was not detected in the PG. To characterise additional clinically important changes in the lipid profile (also known as the atherogenic index), we calculated the ratio of LDL-C to HDL-C (Table 2). After 8 weeks, this ratio was decreased only in the PG ($P = 0.013$) while in the CG it was significantly increased ($P = 0.018$). As a result, the extent of changes between groups in total cholesterol and LDL-C/HDL-C ratio occurred significant (P values 0.014 and 0.004, respectively) which showed a beneficial effect from the probiotic kefir intervention absent with ordinary kefir.
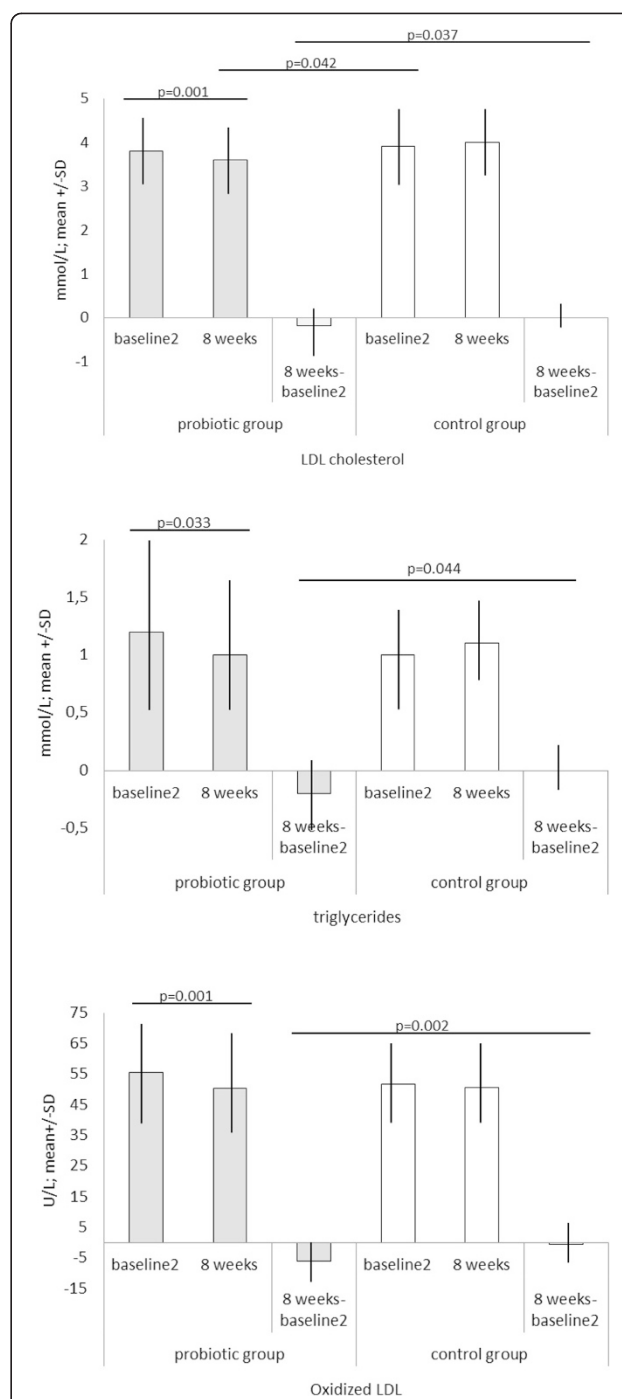
Fig. 2 The reported changes, from the adjusted baseline 2 to 8 weeks, for individuals in the probiotic and control groups. Legend: Changes (mean ± SD) are shown for (LDL serum LDL-C, (TG) serum TG, and (oxLDL) serum oxLDL (paired t-test); the extent of changes was compared between probiotic and control groups (unpaired *t*-test)

Glycated haemoglobin (HbA1c)

Glycated haemoglobin levels were reduced after 4 and 8 weeks in both the PG and CG. However, no significant

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shifts were observed in the corresponding glucose values (Table 2).

Molecular detection of L. fermentum ME-3 with real-time PCR

The prevalence and counts of probiotic bacteria were evaluated in the collected faecal samples with real-time PCR and specific *L. fermentum* ME-3 primers. At baseline, *L. fermentum* ME-3 was found in samples from 10 individuals. The PG exhibited significant increases in the prevalence of *L. fermentum* ME-3. Only 8 % of subjects harboured *L. fermentum* ME-3 at baseline, but the prevalence rose to 62 % at 4 weeks and 56 % at 8 weeks (both $P < 0.001$) (Fig. 3). These results suggested that more than half the volunteers in the PG exhibited temporal colonization by *L. fermentum* ME-3.

The median counts did not change over time; at baseline, the median (CI 95 %) was 3.31 (2.20–4.95) $\log_{10}$ gene copies/g faeces, and at 4 and 8 weeks, the median counts were 4.83 (4.47–4.40) and 4.40 (4.11–4.67) $\log_{10}$ gene copies/g faeces, respectively.

Discussion

The present randomised, double-blind, placebo-controlled, parallel-design, two-armed study (ISRCTN 49744186) aimed to evaluate whether consumption of a functional dairy food product could cause reductions in borderline-high serum LDL-C and serum TG values in Estonian adults. The main finding of our study was the positive primary outcome: the significant reductions in LDL-C (5 %) and TG (17 %) levels in the volunteers that consumed probiotic kefir with *L. fermentum* ME-3 for 8 weeks. Present results show that daily consumption of kefir with antioxidative BSH-active *L. fermentum* ME-3 (DSM14241) over longer period of time is efficacious for regulating the lipid profile in borderline

Fig. 3 Prevalence (%) of *L. fermentum* ME-3 observed among faecal samples obtained from subjects in the probiotic and control groups. Legend: The presence of *L. fermentum* ME-3 was determined with real-time PCR and *L. fermentum* ME-3-specific primers. The baseline values represent the numbers of BL1 both for PG and CG

hyper-cholesterolaemic subjects. This finding supports the numerous clinical trials [3, 4] having demonstrated that LDL-C reduction is the main factor in prevention of the development of coronary heart disease. Earlier studies have demonstrated that some lactic acid bacteria, such as *L. plantarum* 299v and *L. acidophilus* L1, could lower LDL-C levels [29, 30], and that *L. reuteri* NCIMB 30242 after different periods of consumption (from 4 to 6 weeks) reduced both LDL-C and total cholesterol levels of adults [31]. In children with dyslipidemia a formulation of three strains of bifidobacteria caused up to 4 % reduction of TG and LDL-C values after 3 months consumption [32]. In contrast, other probiotic preparations, such as VSL#3 comprising different probiotic strains of *Lactobacillus* and *Bifidobacterium* species reduced the TG levels in critically ill patients, but did not influence the LDL-C [33]. In the present investigation, the gut microbial ecosystem was the target of the probiotic *Lactobacillus* sp., and the kefir served as a probiotic carrier. The antioxidative and anti-atherogenic effects of *L. fermentum* ME-3 were previously described in numerous *in vitro* experiments, animal experiments, and in human trials, with different dairy products, including goat milk, cheese, and yoghurt [14, 16–19].

The positive effects of lactobacilli on various markers of lipid metabolism in the human body are likely due to the sum of multiple different mechanisms [34]. Pereira et al. [35] demonstrated that short-chain fatty acid concentrations, specifically the molar proportion of propionate and/or bile salt deconjugation, comprised the major mechanism involved in the purported cholesterol-lowering properties of *L. fermentum.* Lee et al. (2010) revealed that several regulatory proteins mediated serum cholesterol reduction by a probiotic strain [36]. Lactic acid bacteria, including *L. fermentum* strains, could assimilate cholesterol, and thus, lower serum cholesterol levels [37]. However, the effect of *Lactobacillus* spp. strains on levels of serum cholesterol and cholesterol fractions is strain-specific, and depends on the origin, properties and formulation of a certain strain [38–40]. In the present study, the human origin strain, *L. fermentum* ME-3, exhibited considerable BHS activity. Moreover, we found that the reduction in LDL-C level was consistent with previous data that showed that *L. fermentum* ME-3 reduced the serum ox-LDL levels [17–19].

Another potential mechanism involved in the cholesterol-lowering properties of *L. fermentum* is related to the observation that *L. fermentum* ME-3 showed strong glycosyl-hydrolase activities. These activities included alpha-galactosidase, beta-galactosidase, alpha-glycosidase, beta-glycosidase, and beta-glycuronidase [41]. The concerted actions of these enzymes derived from *L. fermentum* caused the release of a number of bioactive
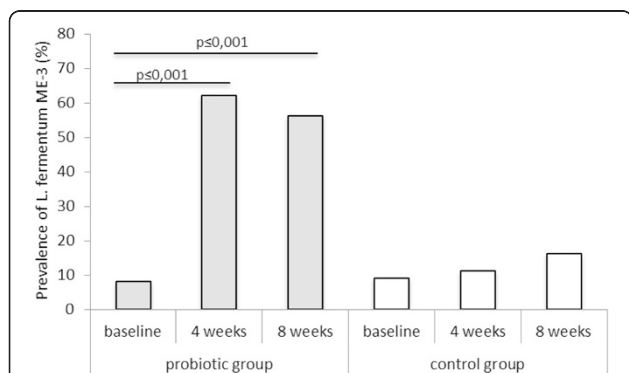
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compounds from various large complexes formed from foodstuffs; some of these bioactive compounds could affect the blood lipid profile [37]. For example, some aglycones derived from food-borne glycosides had positive effects on the blood lipid profile by increasing hepatic fatty acid oxidation and inhibiting cholesterol synthesis [42–44].

Additionally, it is widely known that the high postprandial response of serum TG is related to an elevated insulin response and an elevated hepatic synthesis of TG and cholesterol. We recently showed that consumption of *L. fermentum* ME-3 kefir after a standard breakfast led to a significantly reduced postprandial TG response [18]. This result may also contribute to the positive effects of *L. fermentum* ME-3 on the blood lipid profile, when it is consumed long-term, like that established in the present study. It was remarkable that *L. fermentum* ME-3 consumption reduced the TG more rapidly (4 weeks *vs.* 8 weeks) than it impacted the LDL-C levels.

In the present study, we tested healthy volunteers with borderline-high levels of lipid fractions at baseline. This design enabled us to document the efficacy of *L. fermentum* ME-3 consumption. Other authors have also highlighted a need to select groups with borderline values of biomarkers from the general population [12]. The present study recruitment design was quite similar to that used in testing efficacy in individuals with diseases, where the proved diagnosis served as the basis for consecutive patient selection. In our study, all randomly selected individuals of both intervention groups were healthy and agreed to maintain a standard Western-type diet rich in potatoes, vegetables, meat, eggs, one also characterized by a high content of grain fibre and dairy products. A limitation of this study is that there is no evaluation of the dietary intake in terms of its possible implications. Variations in diet over weeks may be responsible for some of the changes observed in lipid profile between probiotic and ordinary kefir. However, none of the routine clinical indices such as body mass index or blood pressure changed either in the probiotic or control groups. Thus the work presented here suggests the special regulation of the host lipid metabolism with a probiotic strain *Lactobacillus fermentum* ME-3. The main limitation of this study was the high number of drop-outs. Remarkably, only two persons in the PG and two in the CG complained of gastrointestinal symptoms, the other drop-out persons were affected mainly with an outbreak of respiratory infections or discontinued the intervention for loss of motivation to follow the prescriptions during the long study. However, we observed significant changes in the primary outcome measure LDL-C.

Previous studies have shown particularly good faecal recovery of *L. fermentum* ME-3 after consumption of probiotic yoghurt and cheese [17, 45]. The probiotic kefir tolerates the technology involved in preparation, and it remains viable throughout its shelf life [18]. However, the probiotic strain was detected in only 62 % of faecal samples after 4 weeks and 56 % after 8 weeks. These results may be due to the relatively low dose consumed (8×10^9 cfu/day). Saxelin et al. (1995) reported that a dose above 10^{10} cfu/day was necessary for even brief persistence of a probiotic strain [46]. Nevertheless, the *L. fermentum* ME-3 counts in positive faecal samples were quite high (up to 10^6 copy numbers/g) and the beneficial shifts in lipid profiles confirmed the presence of *L. fermentum* ME-3 in the gut, seemingly in the higher parts, where metabolites are absorbed. A recent clinical trial tested 3 weeks of consuming probiotic cheese with 2×10^{10} cfu/day of *L. plantarum* Tensia. Similarly, faecal recovery was detected in only 64 % of patients on a hypocaloric diet, although compliance was high, due to strict control by a hospital nurse [47]. Moreover, the faecal counts of *L. plantarum* Tensia did not exceed 10^3 gene copies/g, which was substantially lower than with *L. fermentum* ME-3. Our results suggested that the intervention should last at least 8 weeks, based on the expected primary outcome, despite the equivalent prevalence of *L. fermentum* ME-3 temporal colonization after 4 and 8 weeks. Interestingly, the prevalence of *L. fermentum* as species was reported to be 21 % in Estonian adults [48]. In the present study, we found that 9 % of subjects at baseline were persistently colonized with *L. fermentum* ME-3 strain.

In Estonia, *L. fermentum* ME-3 has been marketed for the past 10 years in dairy products. This may have facilitated its symbiosis with the hosts. In future investigations, it would be interesting to assess how a sustainable colonization of the probiotic strain is influenced by baseline data of different indigenous lactobacilli to regulate the health indices of the general population.

It was recently shown that total cholesterol does not rank very high in either its long-term impact or as a lipid marker, in terms of its clinical validity, response to changes in therapy, and long-term signal-to-noise ratio [2] Thus, our findings that the probiotic *L. fermentum* ME-3 had a beneficial effect on LDL-C, ox-LDL, the LDL-C to HDL-C ratio, and TG levels indicated that the blood lipid anti-atherogenic profile had improved, which translated as a reduced risk of CVD.

Conclusion

Eight weeks of consuming kefir with the antioxidative probiotic *L. fermentum* ME-3, reduced serum LDL-C, ox-LDL and TG values in clinically healthy volunteers with borderline-high lipid profile indices. Thus, *L. fermentum* ME-3 has the potential to lower the risk of

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CVD that is tightly associated with maintenance of plasma lipid profile.

Authors' contributions

MM, ES, and MZ participated in the design and coordination of the study; JS applied the molecular methods; PH investigated the patients and collected the clinical data; TK, KZ, and PH performed the clinical laboratory tests and microbiological assays; MM, JS, PH, ES, TK, and MZ participated in writing the manuscript. All authors read and approved the final manuscript.

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