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Antioxidative probiotic fermented goats' milk decreases oxidative stress-mediated atherogenicity in human subjects

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The increasing interest in a healthy diet is stimulating innovative development of novel scientific products in the food industry. The viable lactic acid bacteria in fermented milk products, such as yoghurt, have been associated with increased lactose tolerance, a well-balanced intestinal microflora, antimicrobial activity, stimulation of the immune system and antitumoural, anticholesterolaemic and antioxidative properties in human subjects. Recently, we have studied a human *Lactobacillus* spp. strain that possesses antioxidative activity. The aim of the present pilot study was to develop goats' milk fermented with the human antioxidative lactobacilli strain, *Lactobacillus fermentum* ME-3, and to test the effect of the fermented probiotic goats' milk on oxidative stress markers (including markers for atherosclerosis) in human blood and urine and on the gut microflora. Twenty-one healthy subjects were assigned to two treatment groups: goats' milk group and fermented goats' milk group (150 g/d) for a period of 21 d. Consumption of fermented goats' milk improved anti-atherogenicity in healthy subjects: it prolonged resistance of the lipoprotein fraction to oxidation, lowered levels of peroxidized lipoproteins, oxidized LDL, 8-isoprostanes and glutathione redox ratio, and enhanced total antioxidative activity. The consumption of fermented goats' milk also altered both the prevalence and proportion of lactic acid bacteria species in the gut microflora of the subjects. We conclude that the goats' milk fermented with our special antioxidative lactobacilli strain *Lactobacillus fermentum* ME-3 exhibits anti-atherogenic effects.

Fermented goats' milk: Antioxidative activity: Oxidized LDL: Glutathione redox ratio: Atherosclerosis

Human microflora that inhabit the gastrointestinal tract (GIT) are part of an extremely complex and well-balanced ecosystem, where GIT micro-organisms interact not only with each other, but also with their host cells (Falk *et al.* 1998). Recently it has been shown that the regulation of GIT-associated lymphoid tissue may occur by action of probiotics, so it is possible that lactic acid bacteria (LAB) as beneficial organisms interact positively with the intestinal cells of the host (Isolauri *et al.* 2000; Kailasapathy & Chin, 2000). Viable LAB (probiotics) of human origin help to restore normal intestinal microbial functions, alleviating disease symptoms in patients with GIT infection, stimulating the immune system, expressing anti-cancerogenic and anti-atherogenic effects (de Roos & Katan, 2000; Lin & Chang, 2000; McFarland, 2000; Isolauri, 2001).

The antioxidative effect of LAB has been reported only recently (Kaizu *et al.* 1993; Lin & Yen, 1999; Lin & Chang, 2000; Kullisaar *et al.* 2002). At the same time it has been established that a wide variety of reactive oxygen species are continuously produced in the human body and in food (De Zwart *et al.* 1999; Demple *et al.* 1999). Damage caused by reactive oxygen species plays a substantial role in the pathogenesis of cancer, cardiovascular diseases, allergies and atherosclerosis (Agerholm-Larsen *et al.* 2000).

In a previous study, we have reported that a *Lactobacillus fermentum* strain, deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ 14241, assigned as ME-3), possessed substantial antimicrobial and antioxidative activity, expressed manganese superoxide dismutase, eliminated hydroxyl radicals and contained reduced glutathione, a potent cellular antioxidant (Kullisaar *et al.* 2002). The antioxidative activity expressed by some *Lactobacillus* strains used as food components and probiotics may have a substantial impact on human welfare (Lin & Chang, 2000; Oxman *et al.* 2000). To assess such possibilities, we have developed probiotic fermented goats' milk (GM), based on fresh GM and fermented with a human antioxidative strain *L. fermentum* ME-3. The aim of our present study was to test the effect of the probiotic fermented GM on oxidative stress markers (including markers for atherosclerosis) of human blood and urine and on the lactic acid microflora of the gut.

Abbreviations: CFU, colony-forming units; EPI, epi-prostaglandin $F_{2\alpha}$; GIT, gastrointestinal tract; GM, goats' milk; LAB, lactic acid bacteria; LPF, lipoprotein fraction; TAA, total antioxidant activity; TAS, total antioxidant status.

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Materials and methods

Subjects

The healthy volunteers were chosen according to self-assessment as healthy and some of them had taken part in earlier trials. The study participants were five men and sixteen women, mean age 50 (range 35–65) years. Inclusion and exclusion criteria in the pilot study were: age >35 years, no drugs of any kind, no vitamin supplementations, no other yoghurts, no special diet. Twenty-one subjects took part in this trial: during the 3-week study, participants came every day to the Department of Microbiology, University of Tartu, where sixteen subjects consumed 150 g GM fermented with the antioxidative lactobacilli strains/d (study group) and five subjects consumed 150 g fresh GM/d (control group). Thus, the dose of probiotic lactobacilli for a subject was 3×10^{11} colony forming units (CFU) per d. At the end of the pilot study all consumers confirmed that the fermented GM had a good taste and they consumed it with pleasure, but the GM consumers did not like it very much, because of the particular taste and smell.

Origin of microbial strains and product development

Combining the probiotic *Lactobacillus* strain with some other lactobacilli of different origin, we developed the fermented probiotic GM. All lactobacilli strains belonged to the culture collection of the Department of Microbiology, Tartu University. The lactobacilli strains selected for the present study had been isolated from the human GIT (Sepp *et al.* 1997; Mikelsaar *et al.* 2002). Three selected lactobacilli strains (*L. fermentum* ME-3, *L. buchneri* S-15, *L. plantarum* LB-4) fermented GM successfully and provided a yoghurt-like consistency and a satisfactory taste. *L. fermentum* ME-3 originated from a healthy 1 year-old Estonian child and was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as 14241, *L. buchneri* S-15 originated from 1–2-year-old healthy Swedish infants and *L. plantarum* LB-4 originated from cheese whey.

L. fermentum ME-3 was included as a probiotic strain with high-grade antioxidative properties. We established initially that the other strains did not have principal antioxidativity (measured by using two different methods for total antioxidative activity (TAA)) (Kullisaar *et al.* 2002). Some obligatively heterofermentative lactobacilli species (*L. buchneri*, *L. brevis*) have shown potent enzymatic activity towards GM short-chain fatty acids (A Vafopoulou-Mastrojiannaki and E Litopoulou-Tzanteaki, unpublished results). *L. buchneri* strain S1-5 reduced the specific taste of GM. *L. plantarum* LB-4 was included as a strong producer of exopolysaccharides, which gives the fermented milk a cream-like consistency and recommended acidity.

Each LAB strain was incubated for 48 h in de Man, Rogosa and Sharpe medium (CM 361; Oxoid Ltd, Basingstoke, Hants., UK) at 37°C for 48 h in microaerobic conditions. The fresh GM was inoculated with a mixture of probiotic strains (20 ml/l) and incubated at 37°C for 24 h. To get the proportional mixture every strain was incubated for 48 h in de Man, Rogosa and Sharpe medium (CM 361; Oxoid Ltd) at 37°C for 48 h in microaerobic conditions. The product, ready to use, was cooled and stored at 4°C.

Specimen collection and microbial analyses of faeces

The faecal samples were collected at the beginning (day 0) and at the end of the trial (day 21) and kept at −80°C before analysis. Serial dilutions of the weighed faecal samples were prepared with phosphate buffer (pH 7·2) and 0·05 ml of each dilution was plated onto de Man, Rogosa and Sharpe agar medium (Mikelsaar *et al.* 1972). The plates were incubated at 37°C for 4 d microaerobically in a 10 % CO_2 environment (CO_2 thermostat IG 150; Jouan, Saint-Herblain, France). Representative colonies were selected on the basis of colony morphology, cells microscopy and Gram staining.

The counts of lactobacilli were given as log CFU/g faeces. In addition, the relative amounts of the particular microbes were expressed as a proportion (%) of the total count. The *Lactobacillus* spp. isolates were identified according to the absence of catalase activity, production of gas from glucose, growth temperature at 15°C and fermentation of sorbitol and tagatose (Lenzner *et al.* 1984; Mikelsaar *et al.* 2002). Vancomycin resistance differentiated Vancomycin-sensitive cocci, *L. acidophilus* group and *L. salivarius* from facultative and obligatively heterofermentative lactobacilli. Tests (Lenzner & Lenzner, 1982) to verify hydrolysis of arginine and production of lysozyme were also carried out. *L. fermentum* was identified according to gas production, no growth at 15°C, arginine hydrolysis and lysozyme activity.

Experimental protocol

Blood was sampled from the antecubital vein before and after consumption of GM or fermented GM. Blood (serum or plasma) was analysed for TAA, total antioxidant status (TAS), glutathione redox ratio (oxidized glutathione:reduced glutathione), oxidation resistance of blood lipoprotein fraction (LPF) (lag time of LDL + VLDL fraction), baseline value of diene conjugates of LPF, and the oxidized LDL level, and urine was analysed for 8-isoprostanes (8-epi-prostaglandin $F_{2\alpha}$ (EPI).

Total antioxidative activity and status

TAA of serum was assessed by using the linolenic acid test. This test evaluates the ability of sample to inhibit linolenic acid (L 2376; Sigma, St Louis, MO, USA) peroxidation (Pähkla *et al.* 1998). The standard of linolenic acid (10 mg/l ethanol (960 ml/l)) was diluted in isotonic saline (9 g NaCl/l; 8 ml standard/l). SDS (0·1 g/l) (lauryl sulphate L-5750; Sigma) was added to 0·4 ml linolenic acid, diluted in isotonic saline (9 g NaCl/l) and the sample. The incubation was started by adding 100 m $FeSO_4$ (Final Fe concentration 0·2 mM; F-7002, Sigma) and the mixture was incubated at 37°C for 60 min. Then the reaction was interrupted by adding 0·035 ml butylated hydroxytoluene (B-1378; Sigma) and the mixture was treated with 0·5 ml acetate buffer (pH 3·5), consisting of glacial acetic acid and sodium acetate trihydrate (A-6283 and S-8625

respectively; Sigma), and heated with freshly prepared thiobarbituric acid solution (10 ml/l) (T-5500; Sigma) at 80°C for 40 min. After cooling, the mixture was acidified by adding 0·5 ml cold 5 M–HCl, extracted with 1·7 ml cold 1-butanol (BT-105; Sigma) and centrifuged at 3000 ***g*** for 10 min and the thiobarbituric acid reactivity (μM malondialdehyde equivalents) of the butanol fraction was measured spectrophotometrically at 534 nm. The TAA of sample was expressed as inhibition by the sample of linolenic acid standard peroxidation as follows: $(1 - (A_{534}$ (sample)/A_{534} (linolenic acid as control)) × 100, where A is absorbance. The higher value (%) of TAA indicates a higher TAA of the sample. Peroxidation of linolenic acid standard in the isotonic saline (9 g NaCl/l, without serum) served as a control.

To measure TAS of serum, we used a commercially available kit (TAS; Randox Laboratories Ltd, Ardmore, UK). This method is based on the inhibition of the absorbance of the ferrylmyoglobin radicals of 2,2′ -azinobis-ethylbenzothiazoline 6-sulfonate generated by activation of metmyoglobin peroxidase with H_2O_2. The suppression of the absorbance of 2,2′-azinobis-ethylbenzothiazoline 6-sulfonate radicals by sample depends on TAS of the sample under investigation (Rice-Evans & Miller, 1994). The assay procedure was as follows. To 1 ml chromogen (metmyoglobin) solution was added 0·02 ml blood serum (blank was ultrapure water) and standard (6-hydroxy-2, 5,7,8-tetramethylchroman), mixed well and initial absorbance was read. Then 0·2 ml substrate (H_2O_2 in stabilized form) was added, mixed, incubated at 37°C and the absorbance at 600 nm was read after exactly 3 min. The TAS values are expressed as Trolox units (mmol/l). Trolox is water soluble vitamin E (2·5 mM) it was prepared by dissolving 0·15641 g Trolox in 250 ml PBS.

Reduced and oxidized glutathione and glutathione redox status

Total glutathione and oxidized glutathione were measured by using the method described earlier (Griffith, 1980). The samples were deproteinated with metaphosphoric acid (100 ml/l) (M 5046; Sigma). An equal volume of metaphosphoric acid was added to the sample and mixed vigorously. The mixture was allowed to stand at room temperature for 5 min and centrifuged at 3000 ***g*** for 5 min. The supernatant fraction was carefully collected and stored at −20°C if the assay was not performed immediately. For measurement of the glutathione content, to 0·1 ml sample was added 0·005 ml 4 M-triethanolamine (TEAM reagent T1377; Sigma) in water and mixed immediately. Then the sample was divided into two portions. For assay of oxidized glutathione, reduced glutathione was derivatized by adding 0·1 ml 2-vinylpyridine (13,229-2; Sigma-Aldrich, Steinheim, Germany) in 1 mM-ethanol to a portion of the sample, mixing on a vortex mixer and keeping at room temperature for 1 h. To determine the content of oxidized glutathione, 0·1 ml derivatized sample was added to 0·2 M-sodium phosphate buffer (pH 7·5) containing 0·01 M-EDTA, 0·5 U glutathione reductase (G-4751; Sigma) and 0·3 mM-NADPH (N-7505; Sigma) was added and mixed immediately. The enzymatic reaction was initiated by addition of 0·1 ml 1 mM-5,5′-dithio-bis-2-nitrobenzoic acid (D-8130; Sigma) in 0·2 M-sodium phosphate buffer (pH 7·5) containing 0·01 M-EDTA (Griffith, 1980). The change in optical density was measured spectrophotometrically at 412 nm after 10 min. The glutathione content was calculated on the basis of a standard curve generated with known concentration of glutathione. The amount of reduced glutathione was calculated as the difference between the total glutathione and oxidized glutathione (total glutathione − oxidized glutathione = reduced glutathione). The glutathione content was expressed as μg/ml sample or as glutathione redox ratio (oxidized glutathione:reduced glutathione).

Isolation and oxidation of lipoprotein fraction

Blood samples were collected by venepuncture into tubes containing EDTA and plasma was obtained by centrifugation at 1500 ***g*** for 15 min. The LPF (non-HDL fraction) was precipitated from 2 ml twice-diluted plasma by adding 0·2 ml precipitation reagent (dextran sulfate (20 g/l)–Mg Cl_2 (2 M, pH 7·0) (1:1, v/v)); vortexing for 1 min and centrifuging at 1500 ***g*** for 10 min (Zhang *et al.* 1994). In order to remove EDTA from the LPF, the pellet was suspended in 2 ml PBS (9 ml/l) and reprecipitated by adding 0·1 ml precipitation reagent, vortexed and centrifuged. The precipitated LPF was dissolved in 2 ml PBS (40 ml/l) and this solution was used immediately. The protein content in LPF was assayed by using the method of Lowry *et al.* (1951). The protein concentration of EDTA-free LPF was adjusted to 2 mg protein/ml. The resistance of LPF to Cu-catalysed oxidation (lagphase of LPF) was estimated according to the method described earlier (Esterbauer *et al.* 1989; Zhang *et al.* 1994) with some modifications. Briefly, the oxidation was initiated by the addition of a freshly prepared aqueous solution of $CuSO_4.5H_2O$ (final concentration 45 μM) to the LPF (2 mg protein/ml) and the oxidation of this fraction was evaluated by continuously monitoring the formation of conjugated dienes at a maximum absorbance of 234 nm at different intervals of incubation at 37°C. The kinetics of the diene formation (the increase of the absorbance *v.* time) can be divided into three phases: lag phase (during which the diene absorption increases only weakly); propagation phase (rapid increase of the diene absorption); decomposition phase. The resistance to oxidation is defined as the length of lag phase. The lag phase (lag time) was calculated from the interval between the intercept of the tangent of the slope of the curve with time-scale axis. LDL baseline of diene conjugation was evaluated as arbitrary units of absorbance of conjugated dienes at 234 nm.

Oxidized LDL level

Oxidized LDL level was measured by using a Mercodia Oxidized LDL ELISA kit (catalogue no. 10-1143-01; Mercodia AB, Uppsala, Sweden). Mercodia Oxidized LDL ELISA is a solid-phase two-site enzyme immunoassay, based on direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein

B molecule. During the incubation and simple washing step that removes non-reactive plasma components, a peroxide-conjugated anti-apolipoprotein B antibody recognizes the oxidized LDL bound to the solid phase. After a second incubation and simple washing step that removes unbound enzyme-labelled antibody, the bound conjugate is detected by reaction with 3,3′,5,5′-tetramethylbenzidine. Adding acid stopped the reaction and the microtitration strips were read spectrophotometrically at 450 nm.

The content of 8-isoprostanes in urine

This assay is a competitive ELISA for determining levels of 8-EPI in biological samples (BIOXYTECH 8-Isoprostane Assay, catalogue no. 21019; Cutter Circle, Portland, OR, USA). Briefly, 8-EPI in the samples or standards competes for binding (to the antibody coated on the plate) with 8-EPI conjugated to horseradish (*Amoracia rusticana*) peroxidase. The peroxidase activity results in colour development when the substrate is added. The intensity of the colour is proportional to the amount of 8-EPI–horseradish peroxidase bound and inversely proportional to the amount of 8-EPI in the samples or standards.

Statistical analysis

Calculations were performed using commercially available statistical software packages (Statistics for Windows, Stat Soft Inc.; Graph Pad PRISM, version 2.0; GraphPad Software Inc., San Diego, CA, USA) and software R, version 1.6.0 for Windows (The R Project, 2002). The values are given as means and standard deviations. Statistically significant differences inside the two groups were determined by using Student's *t* test. In all analyses $P<0{\cdot}05$ was considered statistically significant. The differences between GM and fermented GM groups (an effect of fermentation) were determined by using regression analysis.

The relative amounts of the probiotic strains colonizing the GIT of subjects in the study groups were expressed as a proportion of the total count (%), using the Bioquant statistical program (Mändar *et al.* 1992), which gives output data for every micro-organism as an absolute count (log CFU/g) and their percentage in the total count with its normal values. Mann–Whitney rank sum tests and Fisher exact tests were used to compare the prevalence, counts and proportions of lactobacilli strains in faecal samples.

The Ethical Committee of Tartu University approved the study according to the Helsinki-II declaration. All subjects gave written consent before the experimental procedure and all had been carefully informed.

Results

The fresh GM used contained four different unidentified species of cocci (total counts 10^9 CFU/ml) and *L. plantarum* (3×10^4 CFU/ml). After fermentation the increase in counts of lactic acid-producing microflora of the product was seen. The counts of lactococci and *L. plantarum* increased nearly 15-fold (cocci 10^{10}, *L. plantarum* 10^6 CFU/ml). The counts of probiotic *L. fermentum* ME-3 reached 3×10^9 CFU/ml product, and the counts of the other starter cultures were somewhat lower (*L. plantarum* LB-4 reached 3×10^8 and *L. buchneri* S-15 up to 4×10^8). The product, ready to use, was cooled and stored at 4°C.

Prolonged consumption (21 d) of fermented probiotic GM altered the counts of lactobacilli after treatment with fermented GM (6·0 (SD 2·3) *v.* 7·6 (SD 0·9) CFU/g, $P=0{\cdot}025$). In addition, the prevalence and proportion of LAB species also changed in the study group compared with their microflora before the trial. *L. fermentum* appeared in all individuals (*n* 16) in the study group ($P<0{\cdot}001$), while in four subjects it was found before the trial. In the control group *L. fermentum* appeared only in one person. In addition, some new species, such as *L. acidophilus* and *L. salivarius*, were isolated from the GIT of the fermented GM consumers (Table 1). The proportional amount of *L. fermentum* and *L. plantarum* was significantly increased $P<0{\cdot}01$ and $P<0{\cdot}05$ respectively (Table 1). The indices of *L. casei*, *L. brevis* and *Thermobacteria* did not change in both groups.

Total antioxidative activity and total antioxidant status tests

Consumption of GM or fermented GM for 21 d enhanced TAA and TAS in both groups. There was an additional increase of TAA and TAS in the fermented GM group, but it was not statistically significant (Table 2).

Reduced and oxidized glutathione and glutathione redox ratio

The 3 weeks consumption of GM or fermented GM lowered the glutathione redox ratio (oxidized glutathione:reduced glutathione). This decrease was statistically significant ($P<0{\cdot}008$) in both groups. Fermented GM had no statistically significant additional effect (Table 2).

The lag time and baseline value of diene conjugates level of the lipoprotein fraction and level of oxidized LDL

Consumption of fermented GM for 3 weeks increased the lag time statistically significantly ($P<0{\cdot}003$); however, the additional effect of fermented GM remained statistically non-significant (Table 2). The baseline value of diene conjugates level of LPF (non-HDL fraction) was decreased only in the fermented GM group and the effect of fermentation was statistically significant ($P<0{\cdot}003$) (Table 2). The amount of oxidized LDL decreased only in the fermented GM group and this effect of fermentation was also statistically significant ($P<0{\cdot}05$) (Table 2).

The content of 8-isoprostanes (8-epi-prostaglandin $F_{2\alpha}$) in urine

Only the consumption of fermented GM lowered the 8-EPI levels in urine ($P<0{\cdot}005$). In the GM group the level of 8-EPI increased, but it was not a statistically significant elevation (Table 2).

Table 1. Changes in faecal microflora of the goats' milk and the fermented goats' milk groups during the 21 d trial‡

Lactobacillus species		Colonized subjects(*n*)	Total counts of lactobacilli (median, CFU/g)	Relative amount of lactobacilli species in total count (%)	
				Range	Median
Fermented goats' milk group (*n* 16)					
L. fermentum	Before	4	1×10^7	0·7–5·77	0
	After	16†††	2×10^7	0·5–49·9	6·10***
L. plantarum	Before	5	1×10^6	0·005–28·5	0
	After	13†	4×10^6	0·08–7·69	1·96*
L. buchneri	Before	–	–	–	–
	After	2×10^7	1	8·5	0
L. acidophilus	Before	–	–	–	–
	After	3	1×10^6	0·5–2·31	0***
L. salivarius	Before	–	–	–	–
	After	1	1×10^8	3·85	0
Thermobacterium spp.	Before	3	2×10^7	1·39–17·3	0
	After	2	5×10^7	0·017–3·82	0
L. casei	Before	8	6×10^5	0·06–39·0	0·05
	After	4	2×10^6	0·67–16·0	0
L. brevis	Before	2	3×10^6	0·07–0·28	0
	After	3	3×10^6	0·08–0·45	0
Goat milks' group (*n* 5)					
L. fermentum	Before	–	–	–	–
	After	1	1×10^8	32·9	0
L. plantarum	Before	1×10^6	2	0·026–2·74	0·01
	After	–	–	–	–
L. buchneri	Before	–	–	–	–
	After	–	–	–	–
L. acidophilus	Before	–	–	–	–
	After	1	8×10^3	2·44	0
L. salivarius	Before	–	–	–	–
	After	–	–	–	–
Thermobacterium spp.	Before	1	5×10^7	21·7	0
	After	1	1×10^6	30·49	0
L. casei	Before	2	3×10^6	1·37–50	0·68
	After	3	1×10^5	0·5–6·0	0·58
L. brevis	Before	–	–	–	–
	After	1	2×10^6	0·66	0

CFU, colony-forming units; –, Below the detection level (< 10 CFU/g).
Median values were significantly different from those before the treatment (Mann-Whitney rank sum test): *$P<0·05$, ***$P<0·001$.
Values were significantly different from those before treatment (Fisher exact test): †$P<0·05$, †††$P<0·001$.
‡ For details of subjects, treatments and procedures, see p. 450.

Discussion

The intestinal bacterial flora have a close relationship with the well-being of the host. In particular, bacteria that produce harmful substances directly damage the GIT, and some of such substances are absorbed causing disorders in various organs, inducing atherosclerosis, hypertension, carcinogenesis, liver diseases, autoimmune diseases and depressed immunity. These observations imply that improving composition of intestinal microflora could have great impact on the health of man.

In a previous study we have reported that our *L. fermentum* strain (assigned ME-3) has substantial antioxidant activity (Kullisaar *et al.* 2002). Based on GM, we have developed a fermented GM product with antioxidative lactobacilli strain *L. fermentum* ME-3 and carried out a pilot study concerning the antioxidative and antiatherogenic effects of this fermented GM.

The first finding of our present study was that the fermented GM consumers showed substantial gastrointestinal persistence of *L. fermentum* ME-3. Thus, we propose that in fermented GM consumers *L. fermentum* ME-3 survived the passage through the GIT. This is supported by the fact that before the trial, only four of sixteen subjects were colonized with *L. fermentum*. After the trial all sixteen members of the probiotics fermented GM group were colonized with this species, and in much higher quantities than before (Table 1), according to the bacteriological methods used. Concerning the faecal *Lactobacillus* sp., large individual variations have been described (Mikelsaar *et al.* 1998). The smaller set of *Lactobacillus* sp. in GM drinkers could be explained by the rather small number of subjects. Gastrointestinal appearance of *L. fermentum* in one of the subjects in the GM group (Table 1) can be considered as insignificant and random because our pilot trial did not prescribe severely restrictive special diets. Thus, we propose that during fermented GM consumption a clear persistence of *L. fermentum* ME-3 takes place (the nucleic acid-based typing of the re-isolated strain might be performed in the future).

Persistence of *L. fermentum* ME-3 can make the interaction between the cells of the host and *L. fermentum*

Table 2. Effect of goats' milk and fermented goats' milk on systemic (total antioxidant activity, total antioxidant status, isoprostanes) and cellular (glutathione redox ratio) oxidative stress markers and plasma lipoproteins †
(Mean values and standard deviations)

	Goats' milk (n 5)				Fermented goats' milk (n 16)					
	Before‖		After		Before‖		After			
Group…	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Additive effect of fermentation‡	Data for healthy population§
Total antioxidant activity (%)	41	6	45***	5	38	3	45***	3	Increase ($P>0{\cdot}05$)	35–45
Total antioxidant status (mmol/l)	0·86	0·11	1·06**	0·04	0·82	0·14	1·14***	0·08	Increase ($P>0{\cdot}05$)	0·65–1·34
Redox ratio¶	0·14	0·03	0·11**	0·02	0·15	0·01	0·11**	0·04**	Decrease ($P>0{\cdot}05$)	$<0{\cdot}2$
Lag time of lipoprotein fraction (min)	44	7	47	12	42	8	46**	9	Increase ($P>0{\cdot}05$)	>30
Baseline value of diene conjugates of lipoprotein fraction (arbitrary units)	0·33	0·11	0·33	0·11	0·27	0·06	0·23***	0·06	Decrease ($P<0{\cdot}05$)	$<0{\cdot}33$
Oxidized LDL (U/l)	97	3	114*	14	98	12	81*	8	Decrease ($P<0{\cdot}05$)	26–126
8-Isoprostanes (ng/l)	4·54	0·85	5·23	0·31	5·54	0·48	5·03**	0·56	Decrease ($P<0{\cdot}05$)	

Mean values were significantly different from those before treatment: *$P<0{\cdot}05$, **$P<0{\cdot}008$, ***$P<0{\cdot}003$.
† For details of subjects, treatments and procedures, see p. 450.
‡ Regression analysis.
§ Our reference values.
‖ There was no significant difference between the values before treatment for the two treatment groups, except for 8-isoprostanes ($P<0{\cdot}05$).
¶ Oxidized glutathione:reduced glutathione.

ME-3 or its metabolites possible. Our next findings show that such interaction has an anti-atherogenic response. Only the consumption of fermented GM lowered statistically significantly the conjugated diene level in plasma LPF ($P<0{\cdot}003$), diminished the level of oxidized LDL ($P<0{\cdot}05$) and suppressed production of 8-EPI ($P<0{\cdot}008$) (Fig. 1 and Table 2). It is known that abnormal modification of plasma lipoproteins ultimately results in severe oxidation of lipoproteins (oxidized LDL). The latter plays a crucial role in the pathogenesis of atherosclerosis, being highly atherogenic. It directly damages the endothelial cells, disturbs recruitment of monocytes, facilitates conversion of monocytes to macrophages and macrophages to foam cells and eventually to fatty streak (Roberts & Cooper, 2001). As all observed effects of fermented GM on plasma lipoproteins have an anti-atherogenic character (decreased amount of oxidized LDL in the subjects' blood, lowered conjugated diene level of LPF and increased oxidation resistance of LPF), we can state that our fermented GM has the anti-atherogenic effect.

It is well known that antioxidants can increase oxidation resistance of serum lipoproteins (Nyyssönen *et al.* 1994; Terahara *et al.* 2000). Among commercial strains *Lactobacillus* GG has a preventive activity against the cholesterol-induced peroxidation damages in the plasma lipoproteins in rats (Broccali *et al.* 2000) and a GAUSIDO culture (*Enterococcus faecium* and *Streptococcus thermophilus*) decreased the baseline of LDL-cholesterol in human plasma (Agerholm-Larsen *et al.* 2000).

Anti-atherogenicity of our fermented GM may be expressed by interplay of different factors. It is known that some lactobacilli enable production of antioxidants in the human GIT (Lin & Chang, 2000; Ljungh *et al.* 2002). Most LAB try to eliminate excess of oxygen radicals and H_2O_2 by superoxide dismutase or by glutathione (Archibald & Fridovich, 1981; de Vos, 1996). Our strain *L. fermentum* ME-3 possessed a notable level of reduced glutathione (Kullisaar *et al.* 2002), whereas the presence of some other thiol compounds in ME-3, able to scavenge reactive oxygen species and to maintain the needed cellular redox status, cannot be excluded. As the intestinal environment is highly prone to development of remarkable oxidative stress, it is possible that the active interaction

Fig. 1. The oxidation resistance (lag time) and maximal oxidation level of lipoprotein fraction (non-HDL fraction) before (■) and after (•) consumption of fermented goats' milk (one typical experiment). For details of treatments and procedures, see p. 450.

between our antioxidative probiotic and mucosal cells helps to maintain physiological redox status in the intestinal mucosa cells of the host. Like the gut-associated lymphoid tissue (Isolauri, 2001), the antioxidative network may also be affected by intestinal events in human subjects. Thus, putative secretion of anti-atherogenic compounds generated by probiotics might be expressed at the level of other body cells and lipoprotein particles. Our present results confirmed that the consumption of fermented GM substantially reduced the 8-EPI level in urine. Isoprostanes have been known to be good indices of body total oxidative stress-based atherogenicity and 8-EPI is probably the most valid direct measure of oxidative stress *in vivo* (Morrow *et al.* 1995; Patrono & Fitzgerald, 1997; O'Brien *et al.* 2000).

Statistically significant elevation of TAA and decrease in isoprostanes and redox ratio confirms the improvement of both systemic and cellular antioxidativity of plasma. Some effects seem to be partially specific for GM, as both fermented GM and GM elevated the values of TAA and TAS and decreased the redox ratio. However, the elevation of these indices was expressed more in the fermented GM group and these variables refer to improved antioxidative status of subjects. GM contains casein (specially type S_1), short-chain fatty acids (greater proportion of capric, caprylic and caproic acids), several kinds of minerals (especially Ca, P, Co and Mo) and vitamins (especially inositol, vitamin A and niacin) (E Alichanidis and A Polychoniadou, unpublished results), biomolecules, which can be incorporated into human plasma and contribute to plasma antioxidative capacity (Nakagawa *et al.* 2000), and/or bioactive antimicrobial peptides (Epland & Vogel, 1999; Hancock & Diamond, 2000), which may have also some antioxidative properties. Although it is very difficult to compare the action of LAB with the other antioxidants, having different characteristics, different intestinal absorption and different metabolism (Terahara *et al.* 2000), our present results show that *L. fermentum* ME-3 may have an effect as a promising anti-atherogenic probiotic. Certainly, systemic investigations are now needed to establish molecular aspects and mechanisms (including anti-atherogenic) underlying the positive effect of our probiotic.

In summary, the present pilot study has confirmed that our antioxidative probiotic *L. fermentum* ME-3 is able to survive in the GIT and exhibits antioxidative and anti-atherogenic effects in human subjects.

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