

Effect of fermented milk containing *Lactobacillus acidophilus* and *Bifidobacterium longum* on plasma lipids of women with normal or moderately elevated cholesterol

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This study aimed to evaluate the effect of milk fermented with *Lactobacillus acidophilus* 145 and *Bifidobacterium longum* BB536 on plasma lipids in a sample of adult women. A double-blind, placebo controlled, cross-over study (two periods of four weeks each separated by a 1-week washout period) was performed in 34 women, aged between 18 and 65 years. Group A consumed 125 g fermented milk three times a day for the first 4 weeks while group B consumed regular yoghurt under the same conditions. (Groups A and B switched products for the second treatment period). Women taking the test product with a baseline total cholesterol above 190 mg/dl showed a significant reduction in LDL cholesterol. HDL cholesterol was also reduced by the test product. We conclude that the fermented milk may help to reduce LDL levels in hypercholesterolemic adult women.

Keywords: *Lactobacillus acidophilus*, *Bifidobacterium longum*, serum cholesterol, LDL, fermented milk.

The probiotic concept evolved from a theory initially proposed by Elie Metchnikoff, who suggested that the longevity of Bulgarian peasants was due to the ingestion of fermented milk containing bacteria that eliminated toxins produced by intestinal microflora (FAO/WHO, 2001; Schrezenmeier & de Vrese, 2001). Today, probiotic therapy is based on the use of probiotic bacteria to stimulate the growth of beneficial microorganisms in the gut, instead of pathogenic bacteria, so as to enhance the body's natural defence mechanisms (Isolauri, 2001; Saarela et al. 2000).

The first report of a hypocholesterolemic effect of dairy products in humans was made in the Maasai and Samburu tribes, in Africa (Mann & Spoerry, 1974; Shaper et al. 1963). These authors observed a reduction in serum cholesterol after the ingestion of large amounts (4 to 8 l/d) of milk fermented by a *Lactobacillus* strain. In recent years, clinical trials investigating the effects of fermented milk products on serum lipids have produced conflicting results. Some studies have shown significant reductions in serum total cholesterol and LDL (Schaafsma et al. 1998; Anderson & Gilliland, 1999; Ashar & Prajapati, 2000), while others have been inconclusive (de Roos et al. 1999; Agerholm-Larsen et al. 2000; de Roos & Katan, 2000;

Lewis & Burmeister, 2005). Xiao et al. (2003) showed that a fermented dairy product containing *Bifid. longum* reduced serum cholesterol in hypercholesterolemic individuals after 4 weeks of consumption. In contrast, Kiessling et al. (2002) reported that a fermented dairy product containing *Lb. acidophilus* 145, *Bifid. longum* 913 and 1% oligo-fructose (prebiotic) did not significantly affect total and LDL cholesterol concentrations in 29 healthy women.

Given the inconclusive nature of available data, the present work aimed to study the effect of fermented milk containing viable *Lb. acidophilus* and *Bifid. longum* on the serum lipid levels of normal to moderately hypercholesterolemic women.

Materials, Methods and Participants

Study design

This was a double-blind, placebo controlled, crossover study with a total duration of 9 weeks. It comprised 2 treatment periods of 4 weeks separated by a 1-week washout period. Thirty-four participants completed the study and were randomly assigned to 2 groups: group A ($n=19$) or group B ($n=15$). During the first treatment period, group A consumed 125 g test product 3 times daily (total of 375 g/day), whereas group B consumed the same

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amount of standard yoghurt (control product, containing *Streptococcus thermophilus* and *Lb. bulgaricus*). In the second period, group A consumed the standard yoghurt while group B consumed the test product. The products were taken as part of breakfast, lunch and dinner. The test product was a fermented milk containing the bacteria *Lb. acidophilus* 145 (viable counts of $1.4\text{--}2.1 \times 10^8$ CFU/g) and *Bifid. longum* BB536 (viable counts of $2.7 \times 10^7\text{--}1.0 \times 10^8$ CFU/g). Determination of viable bacteria was done by standard methods (IDF Group-E104, 1999; ISO-20128, 2006). The nutritional composition of the test and control product was similar: 4.1 g protein, 9.5 g carbohydrates (5.5 g lactose, 4.0 g sucrose), 0.1 g fat and 55 kcal per 100 g.

We tested whether the effect of fermented milk on cholesterol was dependent of initial cholesterolemia (at baseline) of the participants. Group A and B were divided into two sub-groups with total cholesterol concentrations at baseline greater or less than 190 mg/dl. In group A, there were 10 women with cholesterol values ≤ 190 mg/dl (average 161 mg/dl) and 9 women with values above 190 mg/dl (average 222 mg/dl). Group B consisted of 7 women with values ≤ 190 mg/dl and 8 women with values above 190 mg/dl (average values of 174 and 215 mg/dl respectively).

All participants were examined by a physician and a nutritionist. The physician checked their health status by means of physical examination, clinical history and routine laboratory tests (haemogram, lipid profile, hepatic function, renal function and urine analysis II). The nutritionist measured their weight, height and body composition by bioelectrical impedance (Maltron BF-906 Body Fat Analyser) at baseline and endpoint. The participants were advised to avoid consuming any fermented dairy products in the 2-week period prior to the study (Agerholm-Larsen et al. 2000). Further interviews with the nutritionist were carried out just before the start of the study and after week 4 and 9 (i.e. at the end of the first and second periods respectively). Participants were requested to keep a register of any changes in eating habits or physical activity and were encouraged to maintain them together with their body weight. Adherence to the tested products was reinforced and those who reported any missing intake of these were excluded. A 24 h recall questionnaire was applied to all participants in all interviews. Nutritional composition of diets was calculated using the software Microdiet v1.1 (2000). Blood lipids (total cholesterol, LDL, HDL and triglycerides) were analyzed by standard enzymatic techniques (Allain et al. 1974). At the end of the intervention, all patients were again weighed, measured and their body composition analyzed by bioelectrical impedance.

Participants

Participants were users of public health centres in the Madeira Autonomic Region, Portugal, who, after being

Table 1 Age and anthropometric data at baseline in both groups

Values are means \pm standard deviations

	Age (y)	BMI (Kg/m ²)	Percentage of fat (%)
Group A (n=19)	35 \pm 12	24.6 \pm 3.5	31.2 \pm 6.3
Group B (n=15)	36 \pm 10	24.9 \pm 3.4	32.3 \pm 6.4
Total (n=34)	35 \pm 11	24.8 \pm 3.4	31.7 \pm 6.3

No statistically significant differences between groups were found for any anthropometric variable at baseline

informed about the aims, design and confidentiality of the study, offered to volunteer and gave their informed consent. The study was approved by the Ethics Committee of the Regional Health Service of the Madeira Autonomic Region.

The inclusion criteria for the study were: female gender; age between 18 and 65 years; body mass index (BMI) less than 30; no coronary heart disease, diabetes, hypothyroidism or hypertension; no antidyslipidemic, antifungal, immunosuppressive or antihypertensive medication; no alcoholic habits; and no pregnancy or breastfeeding.

Forty-one people were enrolled and randomly assigned to either group A or B. Seven individuals did not complete the study because they disliked the taste of the products (n=3), could not consume the amount of product required (n=2), or got ill and chose to discontinue the treatment (respiratory tract infection medicated with antibiotic, n=2).

Statistical analysis

Lipid profiles at baseline and after the first and second treatment periods were compared by analysis of variance (ANOVA). Sample distribution normality was assessed by the One-Sample Kolmogorov Smirnov test. Comparison between groups at baseline was done using the Student's independent *t* test or the non-parametric alternative (Mann-Whitney test). Comparisons within each group between different periods were made by Student's dependent *t* test. The associations between variables were investigated using Pearson or Spearman correlation coefficients. Based a cholesterol cutoff value of 190 mg/dl (De Backer et al. 2003), a *post-hoc* analysis for the plasma lipid parameters was done within each group for patients above or below this value. The software *Statistical Package for Social Sciences* (SPSS) version 13.0 (2004) was used in this study. Differences were considered statistically significant when $P < 0.05$.

Results

The mean age, percentage body fat and BMI of groups A and B at baseline is presented in Table 1. All anthropometric and food intake variables were normally

Table 2 Average levels of blood lipids (mg/dl) at *baseline* and after each of the periods of treatment. Standard deviation values not shown for clarity purposes. Period 1 represents test product for group A and regular yoghurt for Group B. In Period 2, treatments were switched between groups

Lipids	Cholesterol (mg/dl)		LDL (mg/dl)		HDL (mg/dl)		Triacylglycerols (mg/dl)		
	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B	
Periods									
	<i>Baseline</i>	190	196	108	113	60	58	87	87
	Period 1	180	192	103	114	53	51	85	87
	Period 2	185	182	103	105	59	52	82	84
ANOVA Value- <i>P</i>		0.608		0.095		0.015		0.469	

distributed, except for ethanol intake. No statistically significant differences were found between groups A and B for any anthropometric and food intake variable at baseline ($P > 0.05$).

Study parameters

Table 2 presents the average plasma lipid levels in groups A and B before intervention (baseline), after period 1 and after period 2. Results show that only the HDL changes were statistically significant ($P = 0.015$).

Total cholesterol

We found that, in the sub-group of women with a baseline cholesterol concentration greater than 190 mg/dl, the effect of period on cholesterol average values was significant ($P = 0.008$). ANOVA was used to determine the groups and the periods in which these differences were significant. We conclude that only the individuals with more than 190 mg/dl of the group A showed significant reductions at baseline for period 1, i.e., in the period in which the test product was consumed ($P = 0.001$). No other statistically significant differences were found for total cholesterol between any group or subgroup.

LDL

The same sequence of statistical tests were used for LDL levels and it was found that the test product had a statistically significant effect in women with an initial cholesterol level above 190 mg/dl ($P = 0.014$). In group A, women with cholesterol concentrations above 190 mg/dl showed a statistically significant reduction in LDL, from 129 mg/dl (at baseline) to 109 mg/dl (at the end of period 1; $P = 0.003$). However, from period 1 to period 2 the difference in LDL concentrations (109 mg/dl to 106 mg/dl) did not reach statistical significance ($P = 0.760$). In group B, women with baseline cholesterol concentrations above 190 mg/dl showed a statistically significant reduction from period 1 to period 2 (134.5 mg/dl to 118 mg/dl). No changes were observed at the end of period. The significant differences found refer to the period in which the two groups tried the test product and represent a 16% and 12.5% decrease in groups A and B, respectively.

HDL

The differences found in HDL plasma levels were significant and occurred in both groups. In group A, a significant reduction in HDL occurred after consuming the test product ($P < 0.01$) and a significant increase occurred after consuming the control product ($P = 0.003$). In group B, a significant reduction was observed after the control product ($P = 0.001$) and no significant effect ($P = 0.773$) resulting from the test product. As for both total cholesterol and LDL, we also checked whether initial cholesterolemia of the individuals influenced HDL and we found that the results were not dependent of this factor.

Triacylglycerols

Triacylglycerols showed little variation throughout the 9 week study. ANOVA tests showed that these fluctuations did not reach statistical significance ($P = 0.949$).

Food intake and anthropometric characteristics

Analysis of energy, protein, carbohydrate, fat and ethanol intake as well as weight, height, BMI and percentage of fat intake showed no significant differences between groups A and B either at baseline, period 1 or 2 (data not shown). We found no significant association between these variables and any of the study parameters.

Discussion

This study showed that the fermented milk containing *Lb. acidophilus* 145 and *Bifid. longum* BB536, when compared with control yoghurt (*Strep. thermophilus* and *Lb. bulgaricus*), significantly reduced the LDL levels in the individuals with initial cholesterolemia above 190 mg/dl, this reduction ranging from 12.5 to 16%.

The decreases in LDL and total cholesterol values in this study are supported by previous studies on *Lb. acidophilus* and on *Bifid. longum*, in some cases with similar methodologies (Schaafsma et al. 1998; Anderson & Gilliland, 1999; Ashar & Prajapati, 2000; Xiao et al. 2003).

Schaafsma et al. (1998) showed, in a double-blind, crossover, randomized clinical trial, that fermented

milk with usual yoghurt bacteria, with *Lb. acidophilus* (DN112.053 and DN112.0969) and with addition of fructo-oligosaccharides, caused a decrease of 4.4% in total cholesterol and 5.4% in LDL, in a sample of 30 male individuals, during 3 weeks when compared with the same amount (375 g a day) of standard yoghurt (Schaafsma et al. 1998). Yet, it was not verified whether this effect was due to *Lb. acidophilus*, the fructo-oligosaccharides or both. The levels of HDL and TG remained unchanged during the study weeks.

Ashar & Prajapati (2000) evaluated the hypocholesterolemic effect of a fermented milk with *Lb. acidophilus* V3, in a parallel study of 3 weeks, and concluded that the intake of 200 ml of this test product reduced the LDL by 41% and the TC by 21% in the group of individuals with initial cholesterolemia of 2.0–2.2 g/l, an effect that can be compared with that of some statins (Ashar & Prajapati, 2000). Yet, no control by placebo or by blinding was done and all four groups of the sample had a low number of individuals (between 2 and 9). The HDL did not change in any of the groups and the TG increased in the group with total cholesterol below 2.0 g/l.

In another parallel study, but controlled by placebo and blinding, a low-fat fermented milk with yoghurt bacteria and *Bifid. longum* BL1 was compared with a standard yoghurt in 32 male individuals. After the intake of 300 ml/day of the test product for 4 weeks, it was possible to observe a reduction in TC in half of the individuals who had an initial level above 240 mg/dl (Xiao et al. 2003). In this same work, the effect of *Bifid. longum* was also tested in mice using a lyophilized powder and it was shown that TC, LDL and TG were reduced, compared with the control product.

On the other hand, there are studies that do not corroborate our results (de Roos et al. 1999; Agerholm-Larsen et al. 2000; Kiessling et al. 2002; Lewis & Burmeister, 2005). It is important to say that the different methodologies used in these studies make the comparisons difficult and inconsistent.

The mechanism by which some bacteria may reduce plasma cholesterol is a matter of debate. *In vitro* studies show that the lactic acid bacteria have the capacity to assimilate and bind to cholesterol and bile acids in the intestine and that *Lb. acidophilus* and *Bifid. longum* can incorporate cholesterol in their cell membranes. This results in the inhibition of intestinal cholesterol absorption and in the decrease of its serum levels (Dambekodi & Gilliland, 1998; Pereira & Gibson, 2002a, b; Xiao et al. 2003; Liong & Shah, 2005).

The increased excretion of bile acids due to bile salt deconjugation is another proposed mechanism (Schaafsma et al. 1998; St-Onge et al. 2000; Kimoto et al. 2002; Pereira & Gibson, 2002b; Xiao et al. 2003). Klaver and van der Meer (1993) mentioned that cholesterol coprecipitates with the deconjugated bile acids. It is known that the serum levels of cholesterol decrease whenever the intestinal reabsorption of bile acids decrease (Klaver & van

der Meer, 1993; Brashears et al. 1998; Dambekodi & Gilliland, 1998; St-Onge et al. 2000; Pereira & Gibson, 2002b; Tabas, 2002; Xiao et al. 2003).

The survival capacity of bacteria in the gastrointestinal tract, namely the tolerance to both acid and bile, seems to be crucial to the *in vivo* effect (Walker & Gilliland, 1993; Pereira & Gibson, 2002a, b; Xiao et al. 2003). This capacity has already been proved, both in animals and humans, *in vivo* and *in vitro*, for the *Lb. acidophilus* and for the *Bifid. longum* (Harrison & Peat, 1975; Buck & Gilliland, 1994; De Rodas et al. 1996; Anderson & Gilliland, 1999; Kimoto et al. 2002; Pereira & Gibson, 2002a; St-Onge et al. 2002; Xiao et al. 2003).

On the other hand, the *Strep. thermophilus* and the *Lb. bulgaricus*, the two most important microorganisms used in yoghurt production and that constituted the bacteria of the control product in our study, have a low tolerance to bile salts and to acid pH and a selective preference for sugars (Akalin et al. 1997; Agerholm-Larsen et al. 2000; Xiao et al. 2003).

In our study, a significant reduction of the HDL serum levels, not specific of the test product, was verified. Some investigations show the same effect (Danielson et al. 1989; Akalin et al. 1997; De Smet et al. 1998; Xiao et al. 2003), and this may increase the cardiovascular risk.

The results of this study show that the test product significantly reduces the LDL in the individuals with more than 190 mg/dl of TC. There is no obvious explanation for the effect to occur only in these individuals (Xiao et al. 2003). One possible explanation is the genotype of the apolipoprotein E of the participants. The influence of diet in the plasmatic lipids can be dependent on the genotype of this apoprotein (Cobb et al. 1992; Dreon et al. 1995; Dreon & Krauss, 1997; Schaafsma et al. 1998; Masson et al. 2003). According to some authors, the homozygote individuals for apo E3 are more sensitive to dietary lipids than the non-homozygote ones (van Vlijmen et al. 1994; Schaafsma et al. 1998), whereas other authors believe that the individuals with allele apo E4 are the most reactive to the diet (Lopez-Miranda et al. 1994; Weggemans et al. 2001). Presently, the effects of genetic variation are still the most controversial (Weggemans et al. 2001; Masson et al. 2003).

Results show that the product bacteria are probably responsible for these effects, but the specific action mechanisms are still to be clarified. It is speculated that the *Lb. acidophilus* and the *Bifid. longum* decrease the intestinal absorption of cholesterol both by the bile salt enzymatic deconjugation and by cholesterol assimilation and its incorporation in the cellular membrane.

The putative beneficial effects of these products upon blood lipids and cardiovascular risk may only be established with long term studies and with a more precise characterisation of the amount and type of strains used.

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