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ORIGINAL COMMUNICATION

Long-term consumption of fermented dairy products over 6 months increases HDL cholesterol

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Objective: Assessment of the hypocholesterolaemic effect of yoghurt supplemented with *Lactobacillus acidophilus* 145 and *Bifidobacterium longum* 913 in women.
Design: The cross-over study consisted of three periods (7 weeks each): first period, control yoghurt for all 29 women; second period, probiotic yoghurt for 18 women, control yoghurt for 11 women; third period, the reverse of that in the second period.
Setting: Department of Nutritional Physiology, Institute of Nutritional Science, Friedrich Schiller University, Jena.
Subjects: Twenty-nine healthy women, aged 19–56 y. Fifteen of these were normocholesterolaemic and 14 women were hypercholesterolaemic.
Intervention: Yoghurt (300 g) daily containing 3.5% fat and starter cultures of *Streptococcus thermophilus* and *L. lactis*. Probiotic yoghurt was the control yoghurt enriched with *L. acidophilus* 145, *B. longum* 913 and 1% oligofructose (synbiotic).
Results: The mean serum concentration of total cholesterol and the LDL cholesterol was not influenced by the synbiotic ($P > 0.05$). The HDL concentration increased significantly by 0.3 mmol/l ($P = 0.002$). The ratio of LDL/HDL cholesterol decreased from 3.24 to 2.48 ($P = 0.001$).
Conclusions: The long-term daily consumption of 300 g yoghurt over a period of 21 weeks (control and synbiotic) increased the serum concentration of HDL cholesterol and lead to the desired improvement of the LDL/HDL cholesterol ratio.
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Keywords: probiotic yoghurt; *Lactobacillus acidophilus* 145; *Bifidobacterium longum* 913; blood lipids; cholesterol; SCFA

Introduction

Oral consumption of probiotics has been associated with the prevention, alleviation or cure of diverse intestinal disorders such as lactose intolerance, viral and bacterial diarrhoea, constipation, inflammatory bowel disease and food allergy (McNaught & MacFie, 2001). *Lactobacillus acidophilus*, *L. casei*, *Bifidobacterium bifidum* and *B. longum* have been used as probiotics in humans (Playne, 1994).

The contribution of probiotic bacteria in yoghurt to the improvement of intestinal microflora has been widely recognized. For effectiveness, these bacteria should overcome the adverse effects of the low pH of yoghurt, antagonistic action of other fermenting flora, the hostile gastrointestinal environment and competition with gut microflora (Kailasapathy & Rybka, 1997).

Cholesterol-lowering as a potential beneficial effect of probiotic yoghurt has raised much interest. Several *L. acidophilus* strains of human origin are able to remove cholesterol from culture media during growth in the presence of bile. The mechanism of action is probably that the bacteria deconjugate bile acids which, when deconjugated, coprecipitate with cholesterol at a pH < 5.5 (Brashears *et al*, 1998). The liver will compensate for the loss of bile acids by converting cholesterol into new bile acids. This conversion might lower serum cholesterol levels (Buck & Gilliland 1994; Gilliland *et al*, 1985; Lankaputhra & Shah, 1995).

Anderson and Gilliland (1999) hypothesized that every 1% reduction in serum cholesterol concentration is associated with an estimated 2–3% reduction in risk of coronary heart disease. A regular intake of fermented milk containing an appropriate strain of *L. acidophilus* has the potential to reduce risk of coronary heart disease by 6–10% (Anderson & Gilliland, 1999).

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The recent introduction of the concept of prebiotics has directed attention towards the possibility that alterations in gut microflora induced by the fermentation of non-digestible components of the diet may also have the potential to influence systemic lipid metabolism (Taylor & Williams, 1998). Prebiotics enhance the growth of intestinal bacteria presumed to promote health (Jacobasch *et al*, 1999). This possibility has been strengthened by the observation that, in animals, dietary oligofructosaccharides cause suppression of hepatic triacylglycerides and VLDL cholesterol synthesis, resulting in marked reduction in serum triacylglycerides and, to a lesser extent, serum cholesterol levels (Bengmark, 2000; Berg, 1998; Topping *et al*, 1993).

A synbiotic is a mixture of a probiotic and a prebiotic that beneficially affects the host by improving the survival and establishment of life microbial dietary supplement in the gastrointestinal tract (Kailasapathy & Chin, 2000). Consuming synbiotics (for instance, probiotic bacteria and oligofructose in yoghurt), the expected benefits could be the improved survival of probiotics via a more efficient implantation of colonic microbiota. The prebiotic should also stimulate the growth of both the exogenous (probiotic) and endogenous bacteria, such as bifidobacteria (Kailasapathy & Chin, 2000).

However, the results of different studies regarding the hypocholesterolaemic effects of pro- and prebiotics are still contradictory. These results range from significant decrease of serum cholesterol (Taylor & Williams, 1998; Anderson & Gilliland, 1999; Agerholm-Larsen *et al*, 2000) via constant values (Trapp *et al*, 1993; De Roos *et al*, 1999), to an increase of cholesterol levels (McNamara *et al*, 1989; Mikes *et al*, 1995).

The aim of our study was to investigate whether the intake of *L. acidophilus* 145 and *B. longum* 913 in combination with oligofructose as prebiotic in yoghurt lowers the blood lipids of women. The effect of the prebiotic was to be estimated by analysing the content of the short-chain fatty acids in fresh faeces samples.

Subjects and methods

Subjects

Twenty-nine women, aged between 19 and 56 y, took part in the study. The volunteers had to be free of coronary heart disease and diabetes and were not allowed to use pharmaceuticals known to affect the blood lipid mechanism.

The volunteers were divided into two subgroups of normo- and hypercholesterolaemic subjects. Women with a serum total cholesterol concentration higher than 6.5 mmol/l belong to the hypercholesterolaemic group (Table 1). The other women represented the normocholesterolaemic group. The value of 6.5 mmol/l (250 mg/dl) is commonly used as the cut-off value for hypercholesterolaemia. The concentration of HDL cholesterol did not differ between the normo- and the hypercholesterolaemic groups, whereas the concentration of LDL cholesterol in the hypercholesterolaemic group was significantly higher.

The study was approved by the Ethical Committee of the Medical Faculty of the Friedrich Schiller University of Jena. Informed consent was obtained from all volunteers.

Study design

At the beginning of the study each woman had to record her individual intake of food and drinks over a 7 day period to estimate the individual nutrient and energy intake. From this diet protocol the individual intake of nutrients was estimated using PC-software PRODI 4.0.

After a 6 week adaptation period, in which the women integrated 300 g of control yoghurt into their daily diet, a 9 day collection period followed (Figure 1). In this 9 day period of defined diet, only prescribed and weighed foods were allowed to be eaten. The first 2 days of this period were used for transition from the individual diet to the nutrient-defined diet. From day 3 to 9, 24 h urine and faeces were collected in their entirety. Faeces were collected in plastic pots and immediately frozen at -20°C. One faecal sample was prepared for each subject by pooling all daily individual stools. During each collection period identical defined menus were offered to guarantee the comparability (see *'Diets'*, below).

Upon finishing the first period of the study, the women were randomly allocated to receive either control or probiotic yoghurt during the next 6 weeks (Figure 1). The start of the second period for the control and probiotic groups was staggered by 9 days. Due to variation in menstrual cycles and the consequences for sample collection, the groups consisted of 11 and 18 women, respectively. After the second period of

Table 1 The baseline values of all volunteers and of the both subgroups (given as mean ± s.d.)

	All volunteers ($\bar{x}$ ± s.d.; range)	*Hypercholesterolaemic group (n = 14)*	*Normocholesterolaemic group (n = 15)*
Age (y)	34 ± 9 (19–56)	37 ± 12	31 ± 6
Height (m)	1.66 ± 0.05 (1.58–1.80)	1.66 ± 0.04	1.66 ± 0.06
Body mass (kg)	65 ± 11 (50–95)	67 ± 9	64 ± 13
Body mass index (kg/m^2)	23.7 ± 3.1 (17.3–29.4)	24.2 ± 2.9	23.3 ± 3.4
Total cholesterol (mmol/l)	6.6 ± 1.5 (4.2–12.7)	7.6 ± 1.6	5.7 ± 0.6
HDL cholesterol (mmol/l)	1.3 ± 0.2 (1.0–1.6)	1.3 ± 0.2	1.2 ± 0.2
LDL cholesterol (mmol/l)	4.9 ± 1.4 (3.0–10.8)	5.7 ± 1.6	4.1 ± 0.4
Triacylglycerides (mmol/l)	1.1 ± 0.5 (0.4–2.4)	1.3 ± 0.6	0.9 ± 0.4

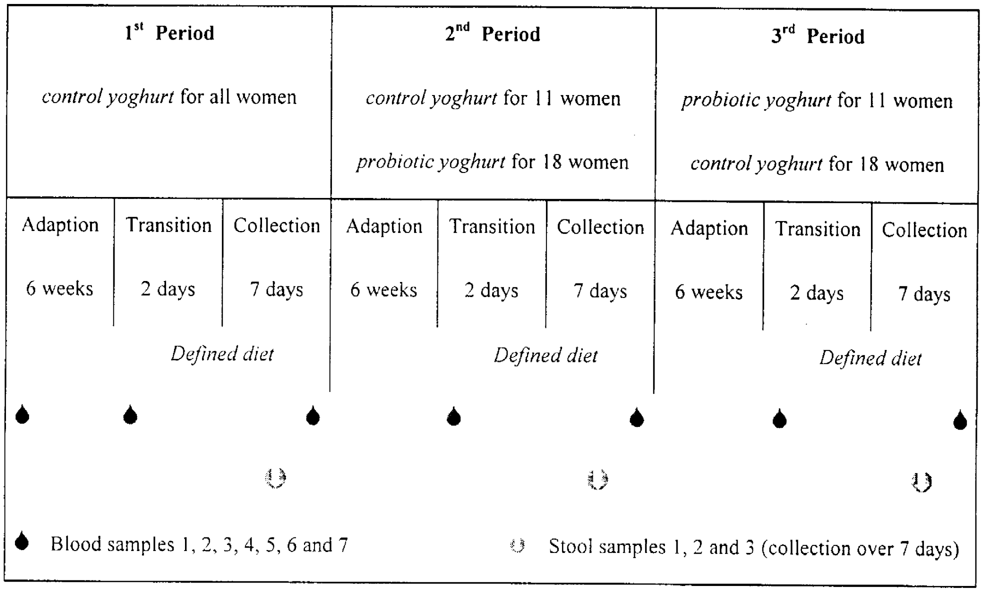

Figure 1 Cross-over design and periods of defined diet divided into adaptation, transition and collection days.

defined diet, the women consuming control yoghurt in the second period of the study had to consume probiotic yoghurt in the third period and vice versa.

Venous blood samples were taken after overnight fasting at the beginning of the study (blood sample 1), at the beginning of each defined-diet period (blood samples 2, 4 and 6), and at the end of each collection period (blood samples 3, 5 and 7, Figure 1).

Diets

The diets consumed during the days of defined nutrient intake were prepared in the kitchen of the institute and weighed accurately to the nearest gram. All foods were bought in batches at the beginning of the study, prepared, weighed out into portions and stored frozen until required. All meals were pre-packed for home consumption. No other foods were permitted. The volunteers were allowed to choose their drinks; they recorded the amount of individually consumed drinks and saved an aliquot of 10 ml in a tube for analysis.

Yoghurts

The control yoghurt was a normal dairy product containing 3.5% fat and starter cultures of *Streptococcus thermophilus* and *L. lactis*. A part of each batch of the control yoghurt was enriched with *L. acidophilus* 145 and *B. longum* 913 (Wisby GmbH & Co.KG, Germany) and 1% of oligofructose to produce a synbiotic. The yoghurt was produced once a week (Molkerei Schwarza) and immediately consumed. Microbiological analyses of the probiotic product confirmed the presence of 10^6-10^8 CFU of *L. acidophilus* 145 and at least 10^5 CFU of *B. longum* 913 per gram yoghurt. The counts of *L. acidophilus* did not decrease during the one-week storage period, whereas the count of *B. longum* decreased to 10^3 CFU/g. The strain of *L. acidophilus* 145 was selected by virtue of its level of resistance to stomach acid and bile acids (Wisby GmbH & Co. KG, personal communication). The contents of water, fat, protein and energy were comparable in both dairy products.

Analytical techniques

Blood samples were centrifuged for 20 min at 3000 U/min. The resulting serum was stored at $-80°C$ until analysis. Total cholesterol, HDL cholesterol and triacylglycerides were measured after an enzymatic preparation on a Beckman autoanalyser (CX 7 Synchro) using Beckman test kits. LDL cholesterol was computed according to Friedewald's formula.

Antibodies against oxidized LDL were analysed using enzyme-immunoassays (Biomedica Gesellschaft mbH). The colouring of the sample proportionally to the concentration of the antibody against oxidized LDL was measured with an ELISA-reader.

The individual urine samples were used to confirm the correct intake of defined foods in the collection period by analysing the nitrogen balance. Short-chain fatty acids in fresh faeces were analysed to observe an effect of the prebiotic (1% oligofructose). After the extraction of faeces with ice-cold water, the GC-measurements (Shimadzu GC 17A) were done using a 15 m-FFAP-column and a temperature programme (start temperature 130°C, increase 35°C/min and final temperature 170°C).

Statistical analyses
The results of the study were evaluated by analysis of variance to compare changes during the consumption of probiotic yoghurt or control yoghurt and also to compare normo- and hypercholesterolaemic women within and across these groupings. Statistical differentiation was performed with the Statistical Package for the Social Sciences (SPSS/PC+, version 10.0; SPSS Inc., Chicago, IL, USA). Data were tested for significance of differences between periods with Sidak adjustment for GLM-repeated measurements. For each comparison a value of $P<0.05$ was considered. Data are presented as the mean±s.d.

In the analysis of the results all subjects were initially dealt with as one large group, and were only assigned to one of two smaller groups on the basis of the results, namely into a normocholesterolaemic or hypercholesterolaemic subgroup.

Results

Blood lipids
A significant increase of 0.06 mmol/l ($P=0.005$) in total cholesterol of hypercholesterolaemic women after the consumption of probiotic yoghurt was detected (Table 2). Whereas the mean LDL cholesterol concentration of all volunteers was unchanged by the type of yoghurt ($P>0.05$), the HDL cholesterol concentration increased significantly by 0.32 mmol/l ($P=0.002$) after consumption of the probiotic yoghurt. This effect was observed in normocholesterolaemic, as well as in hypercholesterolaemic women (Table 2). Thus, the ratio of LDL to HDL cholesterol decreased significantly ($P=0.001$). The ratio of the normocholesterolaemic women decreased by about 1 unit ($P=0.02$). The concentration of triacylglycerides was unchanged after the consumption of probiotic yoghurt in normocholesterolaemic, as well as in hypercholesterolaemic women ($P>0.05$; Table 2).

Independent of the division of the women into the normocholesterolaemic or hypercholesterolaemic group and independent of the kind of yoghurt, the HDL cholesterol increased significantly by 0.4 mmol/l (38%, $P<0.01$) during 21-week long-term consumption. The concentration of triacylglycerides in all volunteers ($n=29$) did not significantly change during the study period ($P>0.05$).

Antibodies against oxidized LDL
The mean titre of antibodies against oxidized LDL was sometimes smaller than the standard deviation. The titres of antibodies varied between 42 and 1666 U/ml in the 29 women. The mean values were higher after the intake of probiotic yoghurt (238 *vs* 356 U/ml), but the influence was not statistically significant ($P>0.05$).

Short-chain fatty acids in faeces
Total short-chain fatty acids (SCFA) and the distribution of acetate, propionate and butyrate were similar in volunteers consuming control or probiotic yoghurt (Table 3). The pH value of the fresh faeces was significantly ($P=0.01$) affected by the treatment, being lower (−0.2) in the mean of all volunteers consuming synbiotic (Table 3).

Discussion

The results did not show a cholesterol-lowering effect of the *L. acidophilus* 145 and *B. longum* 913, but there was a significant increase of the HDL cholesterol. The reason for this could be a relatively high intake of milk fat (10.5 g/day), due to the consumption of 300 g full-fat yoghurt per day. The most important result is an improvement of the ratio of LDL/HDL cholesterol, an index of artherogenicity (Gordon *et al*, 1989). A decrease of this ratio has often been found as a consequence of decreased LDL-cholesterol (Eichholzer &

Table 2 Values of serum lipids (mmol/l) in the normocholesterolaemic and hypercholesterolaemic subgroups and of all volunteers (mean values±s.d.)

	Group	*Control yoghurt*	*Probiotic yoghurt*	P
Total cholesterol	Normocholesterolaemic	5.15±1.07	5.10±0.59	0.833
	Hypercholesterolaemic	6.03±1.11	6.62±1.07	0.005
	All	5.32±1.28	5.83±1.14	0.110
LDL-cholesterol	Normocholesterolaemic	3.52±0.73	3.19±0.59	0.066
	Hypercholesterolaemic	4.24±1.23	4.49±1.18	0.219
	All	3.87±1.05	3.82±1.12	0.690
HDL-cholesterol	Normocholesterolaemic	1.25±0.52	1.55±0.22	0.039
	Hypercholesterolaemic	1.32±0.31	1.66±0.36	0.024
	All	1.28±0.43	1.60±0.30	0.002
LDL/HDL	Normocholesterolaemic	3.07±1.01	2.11±0.51	0.020
	Hypercholesterolaemic	3.43±1.20	2.87±1.07	0.089
	All	3.24±1.10	2.48±0.90	0.001
Triacylglycerides	Normocholesterolaemic	0.83±0.39	0.80±0.24	0.715
	Hypercholesterolaemic	1.02±0.42	1.03±0.32	0.789
	All	0.92±0.41	0.91±0.30	0.865

Table 3 Distribution of short-chain fatty acids (mol%), the total concentration of SCFA (mmol/g) and the pH value of fresh faeces in the normocholesterolaemic and hypercholesterolaemic subgroups and of all volunteers (mean values ± s.d.)

	Group	*Control yoghurt*	*Probiotic yoghurt*	*P*
Acetic acid	Normocholesterolaemic	60.3 ± 5.9	59.3 ± 4.7	0.494
	Hypercholesterolaemic	62.4 ± 6.5	63.5 ± 6.4	0.688
	All	61.4 ± 6.2	61.3 ± 5.9	0.977
Propionic acid	Normocholesterolaemic	16.5 ± 3.3	17.8 ± 3.0	0.261
	Hypercholesterolaemic	15.0 ± 3.1	15.6 ± 3.3	0.597
	All	15.7 ± 3.3	16.8 ± 3.3	0.228
Butyric acid	Normocholesterolaemic	16.1 ± 5.4	16.5 ± 4.5	0.717
	Hypercholesterolaemic	14.0 ± 5.2	14.0 ± 5.1	0.978
	All	15.1 ± 5.3	15.3 ± 4.8	0.841
Total SCFA (mmol/g)	Normocholesterolaemic	0.09 ± 0.02	0.08 ± 0.03	0.785
	Hypercholesterolaemic	0.09 ± 0.04	0.07 ± 0.02	0.126
	All	0.09 ± 0.03	0.08 ± 0.03	0.144
pH of faeces	Normocholesterolaemic	6.86 ± 0.49	6.73 ± 0.38	0.181
	Hypercholesterolaemic	7.09 ± 0.55	6.82 ± 0.31	0.030
	All	6.97 ± 0.52	6.77 ± 0.34	0.010

Stahelin, 1993; Khedkar *et al*, 1993; Chae, 1995; Taylor & Williams, 1998; Anderson & Gilliland, 1999; Agerholm-Larsen *et al*, 2000). The ability of certain strains to assimilate cholesterol in the presence of bile acids suggests a possible association between gut microflora and cholesterol absorption. The daily intake of 375 g *L. acidophilus*-yoghurt and tablets with lyophilized *L. acidophilus* caused a decrease of total cholesterol in male volunteers by 4.4 and 5.3%, respectively (Lin *et al*, 1989; Schaafsma *et al*, 1998). Schaafsma *et al*, (1998) demonstrated a special LDL lowering influence of adding 2.5% oligofructose to this yoghurt. Almost all of these effects were attributed to either *L. acidophilus*, the fructose-oligosaccharides or to a combination of these factors.

In contrast to the results of Agerbaek *et al* (1995) and Bertolami *et al* (1999), Mikes *et al* (1995) detected a significant increase of the serum concentration of total and the LDL cholesterol. Over 6 weeks, the 12 volunteers were given a daily oral dose of *E. faecium*. The mean levels of serum cholesterol and LDL cholesterol showed a biphasic effect— an elevation followed by a sharp decrease (on the 14th day after the last dose of *E. faecium*). The HDL cholesterol showed the opposite trend, with levels being continually elevated in this study.

In the present study, the LDL/HDL ratio decreased due to the continuous increase of the HDL cholesterol. We could not find comparative studies with such results. The reason for the increasing HDL cholesterol levels may be the fatty acid distribution of the milk fat and the sphingolipids contained:

1. Samuelson *et al* (2001) concluded from their study with 94 15-y-old children, that milk fat contains components which counterbalance the expected positive relationship between saturated fat intake and serum cholesterol.
2. An inverse association between intake of milk products and LDL/HDL ratio was also found by Smedman *et al* (1999). The authors suggest that the relatively high amounts of saturated fatty acids (C12:0 and C14:0) in milk fat are responsible for the increase of HDL cholesterol (Temme *et al*, 1996).
3. In an experiment with low-fat diets in pigs, Allan *et al* (2001) found that a supplementation with milk fat, coconut oil or olive oil significantly increased HDL cholesterol.

Studies with test animals have shown that feeding sphingolipids reduces serum LDL cholesterol and elevates HDL cholesterol (Vesper *et al*, 1999). Short-term (Imaizumi *et al*, 1992) and long-term (Kobayashi *et al*, 1997) feeding experiments with rats indicated that sphingolipids reduce total plasma cholesterol. Plasma cholesterol was 30% lower in rats fed semi-purified diets supplemented with a mixture of sphingomyelin and glycosphingolipids. Full-fat milk contained 120 mg sphingolipids per litre (Vesper *et al*, 1999). The daily intake of the 300 g full-fat yoghurt increased the sphingolipid intake by 36 mg. The total daily intake was calculated with 318 mg sphingolipids (related to sphingomyelin, Vesper *et al*, 1999). The intake of the yoghurt increased the sphingolipid intake by 11%. Last but not least, an exchange of carbohydrates by lipids (integration of 10.5 g milk fat in the diet) increases HDL cholesterol too. These may be the reasons for the continuous increase of the HDL-cholesterol levels during our long-term study (21 weeks).

The yoghurt used in our study contained 1% oligofructose, but there were no changes in the concentration of short-chain fatty acids in the stool. In subjects consuming high amounts of soluble dietary fibre (eg pectin, oat bran), the molar concentrations of propionate and butyrate were increased (Topping *et al*, 1993; Fleming *et al*, 1992). Because of the similar concentrations of the acetate, propionate and butyrate in the faeces of subjects consuming control or probiotic yoghurt, it can be concluded that the concentration of 1% oligofructose was insufficient to influence the faecal concentrations of short-chain fatty acids.

Several authors have suggested that gut adaptation could modify the beneficial effects of fibre-rich foods or of oligofructose (Rao *et al*, 1994; Le Blay *et al*, 1999). In long-term experiments with rats, the oligofructose-induced increase of intestinal lactic acid-producing bacteria was lost, but the butyrogenic properties of oligofructose were maintained (Le Blay *et al*, 1999).

The reason for the lower pH of the faeces in the verum group (Table 3) could not be determined. Some dietary components (eg various lipids) can influence the pH level of the faeces independent of the concentration of the short-chain fatty acids (Topping *et al*, 1993). In the last sections of the colon of pigs there was no correlation between total short-chain fatty acids and pH value. Topping *et al* (1993) also found no relationship between the individual fatty acids and the pH value.

The faecal water of volunteers participating in the study and consuming the probiotic yoghurt induced fewer DNA strand breaks in HT29 clone 19a cells (comet-assay) in comparison to the control group (Oberreuther *et al*, 1998). The lower pH value could have importance for this modulation of genotoxicity in human colon cells. Bacteria-induced enzymatic activities of glucuronidase, azoreductase and nitroreductase are involved in processes converting procarcinogens into carcinogens. A synbiotic mixture consisting of *L. acidophilus* 74-2 and oligofructose reduced the activity of β-glucuronidase *in vitro* (Gmeiner *et al*, 2000).

Conclusion

The results of this study demonstrate that the ingestion of 300 g yoghurt fermented by traditional starter cultures and supplemented with *L. acidophilus* and *B. longum* did not lower the total and LDL cholesterol in 29 healthy women. The increase of the HDL cholesterol may be an effect of the long-term consumption of yoghurt (21 weeks) and lead to an improvement of the LDL/HDL ratio. There was no influence of the combination of probiotics with oligofructose on the concentration and distribution of short-chain fatty acids in the stool.

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