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Verification of Hypocholesterolemic Effect of Fermented Milk on Human Subjects with Different Cholesterol Levels

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ABSTRACT. The possible hypocholesterolemic effect of acidophilus milk was evaluated on 27 human subjects having different levels of serum cholesterol, *i.e.* <2.0 (group C_1), 2.0–2.2 (C_2), 2.2–2.5 (C_3) and >2.5 g/L (C_4). The acidophilus milk was prepared by fermentation of low-fat milk with *Lactobacillus acidophilus* and was fed to each volunteer at the rate of 200 mL/d for 20 d. Blood samples from the volunteers were collected and analyzed for lipid profile twice prior to, during and after feeding, keeping a gap of 10 d between two collections. A significant decrease ($p < 0.05$) in average total cholesterol was found in the C_2 and C_3 groups, amounting to 21 and 12 %, respectively. The average LDL cholesterol decreased in C_2, C_3 and C_4 groups by 0.54, 0.26 and 0.46 g/L, respectively. In the C_2 group, the LDL/HDL and total/HDL ratio was also reduced by 1.4 and 1.3, respectively. However, in the C_1 group, the average total and LDL cholesterol level did not show any significant change but serum triacylglycerols and VLDL cholesterol showed a significant ($p < 0.05$) increase of 0.53 and 0.11 g/L, respectively. Regression analysis of the data revealed a square trend in most of the parameters over time period. Overall, the feeding had the best effect in the subjects with lipidemic status of borderline cholesterol level (2.0–2.2 g/L) group.

An elevated level of blood cholesterol is known to promote arteriosclerosis and facilitate the occurrence of myocardial infarction and stroke (Kaplan *et al.* 1988; Bierman 1991). When the sum of cholesterol synthesized and that obtained from the diet exceeds the amount required for the synthesis of membranes, bile salts and steroids; pathological accumulation of cholesterol in blood vessels can develop, resulting in obstruction of blood vessels (Lehninger 1993).

Blood cholesterol reduction through dietary modification is preferred over drugs by physicians treating hyperlipemic patients, being a natural means with no side effects. Increasing evidence suggests that selected members of the lactic acid bacteria, such as lactobacilli and bifidobacteria, and of other microorganisms (*e.g.*, enterococci) when consumed in sufficiently large amounts, exert prophylactic and therapeutic effects in humans as well as animals (Sandine *et al.* 1972; Mitsuoka 1991; Robinson 1991; Olasupo 1998; Belicová *et al.* 1999; Lauková *et al.* 1999). Use of fermented milks as a dietary means to combat hypercholesterolemia has generated some interest stemming from the observation of consistently low blood cholesterol level in Masai tribesmen in spite of large intake of diet high in saturated fat and cholesterol (Mann and Spoerry 1974). Till date, we have ample evidence that fermented milk consumption may favorably affect the lipid metabolism in animals as well as in humans (Gilliland *et al.* 1985; De Rodas *et al.* 1996; Gorbach *et al.* 1988; Hruby *et al.* 1992; Khedkar *et al.* 1993*a*). While the majority of the feeding trials on animals have yielded favorable results, contradictory results have also been reported with feeding trials on humans by some research workers (Massey 1984; Lin *et al.* 1989; McNamara *et al.* 1989).

The October 1987 report of the *National Heart, Lung and Blood Institute's National Cholesterol Reduction Program* (NCEP) defined the levels of serum cholesterol deemed to be desirable, tolerable or a serious risk factor for the development of coronary artery disease. The report classifies total cholesterol concentrations below 2.0 g/L as desirable, those between 2.0 and 2.4 g/L as borderline, and those greater than 2.4 g/L as high cholesterol concentrations that should be reduced by dietary means or appropriate therapy. A certain level of cholesterol is desired in the body. Hence, a cholesterol lowering culture may have an adverse effect in a human with already low level. It is likely that cultures may have a regulatory effect rather than lowering and that the influence may differ with persons with different levels of cholesterol. Hence, the present investigation was carried out to check the effect of feeding fermented milk prepared by a selected strain of *L. acidophilus* on lipid profile parameters in volunteers with a different level of baseline cholesterol.

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MATERIALS AND METHODS

Selection of strains. Lactobacillus acidophilus V_3, a vaginal isolate, was obtained from the culture collection of the *Dairy Microbiology Department, SMC College of Dairy Science*, Anand, and was maintained in sterilized reconstituted skimmed milk by weekly transfer. The culture was known to have the ability to deconjugate bile acids, assimilate cholesterol and an antibacterial activity against some food-borne pathogens (Ashar and Prajapati 1998). Before use in the preparation of acidophilus milk, the culture was activated by three daily transfers in reconstituted skimmed milk.

Preparation of acidophilus milk. Low-fat milk (1.6 % fat, 8.5 % SNF) of batch size of 10–20 L was heated to 120 °C for 10 min, cooled, inoculated with an active culture of *L. acidophilus* and incubated at 37 °C till setting. The curd was then broken, flavor and color were added, homogenized and packed in 200-mL aliquots in sanitized polyethylene pouches. The product was stored at 2–8 °C till use.

The experimental protocol. Human volunteers suspected to have hyperlipemia were initially screened for their blood lipid profile status. Based on two baseline readings for total serum cholesterol, the volunteers were grouped into four categories, *viz.* those having <2.0 g/L (C_1), 2.0–2.2 g/L (C_2), 2.2–2.5 g/L (C_3) and >2.5 g/L (C_4). Each group contained a certain number of volunteers, totaling 27. The personal details and medical history of all were recorded along with their visit dates for blood collection. The volunteers were given a pouch of 200 mL acidophilic milk daily for 20 consecutive days. Twelve hourly fasting blood samples were collected by venipuncture six times; two times each before, during and after feeding acidophilic milk, keeping a gap of 10 d between the two collections. Subjects suffering from hyperlipemia secondary to hypertension, diabetes, hypothyroidism, *etc.* were also allowed to take their routine medicine as prescribed by the physician. However, none of the volunteers were taking any drugs for reducing cholesterol. Collected blood samples of all subjects were analyzed for lipid profile and results were recorded and analyzed.

Analysis of serum lipid profile. Total cholesterol, HDL-cholesterol and triacylglycerols were estimated from serum with the help of commercial kits manufactured by *M/S Bayer Diagnostics India Ltd.*, Baroda (India) as per the methods suggested by them in the brochure. As far as possible, estimations were made on the day of blood collection. However, occasionally, when delay was unavoidable, the samples were stored at 2–8 °C for a maximum of 3 d prior to analysis.

Estimation of total cholesterol. According to the enzymic method given in the kit manual, daily working solution was prepared by mixing equal volumes of solution *1*, *i.e.* buffer–enzyme–chromogen, and reagent *2*, *i.e.* phenol, supplied in the kit. One mL of this working reagent was added with 10 mL of test serum. Blank as well as standard were run every time to increase the accuracy of the assay. The tubes were subsequently incubated at 37 °C in a water bath for 5 min. Thereafter, the absorbance A was read on a Hitachi 220S double-beam spectrophotometer at 505 nm against reagent blank. The cholesterol (Cho) content in g/L of the test sample was calculated as

$$\text{Cho} = A_{\text{test}}/A_{\text{std}} \times 2\,,$$

where 2 is the cholesterol content of the standard solution.

Serum triacylglycerols were estimated by an enzymic colorimetric method using 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS). Reagent *1* (containing enzymes plus chromogen) was reconstituted by addition of equal volumes of reagent *2* (buffer plus chromogen) and reagent *3* (stabilizer) in the required quantity. One mL of this reconstituted solution (working reagent) was taken in a test tube to which 10 mL of serum sample was added. Blank and standard were run during each assay. The tubes were kept at room temperature for 15 min thereafter. Finally, absorbance (A_{505}) was measured against reagent blank. The triacylglycerol (TAG) content (g/L) in test serum was calculated as

$$\text{TAG} = A_{\text{test}}/A_{\text{std}} \times 2\,.$$

HDL cholesterol in test serum was measured by the phosphotungstate method as given in the kit brochure. Precipitation step was initially carried out to remove other fractions of cholesterol from the serum. In this step, 200 mL of serum and 200 mL of precipitating reagent containing phosphotungstic acid were taken into a centrifuge tube and the contents were centrifuged for 10 min at 58–67 Hz. The clear supernatant thus obtained was treated as sample. The onward procedure was similar to the estimation of total cholesterol except that instead of 10 mL sample, 20 mL of supernatant was taken in 1 mL of working reagent. The content of HDL cholesterol (g/L) in the test sample was calculated as

$$\text{HDL} = A_{\text{test}}/A_{\text{std}}\,.$$

The other parameters were calculated (all in g/L) as

$$\text{VLDL} = \text{TAG}/5\,,$$
$$\text{LDL} = \text{Cho}_{\text{tot}} - \text{HDL} - \text{VLDL}\,.$$

Statistical analyses. The pooled as well as grouped data for lipid profile parameters obtained during the pre-feeding (−10 and 0 d), feeding (10 and 20 d) as well as post-feeding (+10 and +20 d) periods were analyzed by Randomized Block Design (RBD). Individual volunteers were taken as replicates whereas the time periods were considered as treatments. The significant differences between treatment means for the respective parameters were tested by critical difference (CD) test. The average pre-feeding (baseline) values were compared with the values obtained during subsequent feeding and post-feeding periods. Regression analysis was also done for individual groups to statistically assess the trend of lipid profile parameters. Linear equation $Y = a + bx$ and square equation $Y = a + bx + cx^2$ were applied (Snedecor and Cochran 1967). The best-fit equation was selected on the basis of significance of the value of the coefficient of determination (R^2) to explain the trend of test parameters over the test periods of time.

RESULTS AND DISCUSSION

Fresh acidophilus milk contained a viable count (on MRS agar, at 37 °C after 2 d) of 3.6/ng, 3.5 % protein, 1.6 % fat and 0.66 % titratable acidity.

The average baseline total cholesterol was 1.7 g/L in C_1, 2.1 in C_2, 2.3 in C_3 and 2.7 in C_4 groups (Table I). In other lipid profile parameters also large variations were observed. The average total cholesterol level was significantly reduced ($p < 0.05$) by 0.44 and 0.29 g/L in C_2 and C_3 groups, which included 7 and 9 subjects, respectively. When the treatment means of these two groups were tested by the least significant difference, a significant reduction was found after 20 d of stopping feeding in C_2 group while in the C_3 group it remained at par up to 20-d feeding period and subsequently reduced after a 10-d post-feeding period. In the C_1 and C_4 groups there were insignificant changes in the total cholesterol values over time.

The reduction obtained in serum total cholesterol was in line with earlier studies related to feeding trials involving human subjects (Mann and Spoerry 1974; Harrison and Peat 1975; Khedkar *et al.* 1993*a,b*; Agerbek *et al.* 1995). Working with the same strain of *L. acidophilus* V_3 Khedkar *et al.* (1993*a*) reported a significant reduction in total cholesterol (0.34 g/L) after 30 d of feeding of 200 g of acidophilic milk per d to 20 healthy human volunteers aged 50−60 years. In a similar trial involving 20 healthy male volunteers aged 40−50 years, the trend of serum cholesterol level reduction was not clearly established (Khedkar *et al.* 1993*b*).

Lin *et al.* (1989) also grouped the 354 subjects of their study based on their baseline cholesterol levels. However, they could not find a significant change in any of the lipid profile parameters in the low cholesterol (<2.0 g/L), medium cholesterol (2.0−2.2) and high cholesterol (>2.4) populations during the entire study period. One of the reasons for such insignificant result may be the low dose (2×10^6) of viable bacteria in commercially available tablet Lactinex selected for the feeding trial. The type of strain also matters.

A significant decline ($p < 0.05$) of 0.54 g/L was found in the LDL cholesterol level of the C_2 group, amounting to 41 % reduction. In other groups, there were insignificant changes in LDL cholesterol values (Table I). A gradual increase in the average serum triacylglycerols as well as VLDL cholesterol was also found in the C_1 group, while in other groups there were no significant changes. With respect to HDL cholesterol, LDL/HDL ratio and total/HDL cholesterol ratio, no significant alterations have been found in any group of volunteers at any period (Table I).

Results of regression analysis revealed that in some parameters either a linear or a square trend fitted, while in other cases no clear trend could be established (Table II). In the present analysis, equations exhibiting an R^2 value of more than 0.7 were selected to explain the trend. In the individuals having a baseline cholesterol level <2.0 g/L (C_1), a square trend of increase was observed in the average total cholesterol value. In the individuals having a baseline cholesterol level in the range of 2.0−2.2 g/L (C_2), an initial square increase in average total cholesterol values was found during feeding, followed by a sharp decline over the post feeding periods. Group C_3 showed a continuous decline.

Table I. Average lipid profile parameters

Parameter g/L	Group	Pre-feeding†	Feeding		Post-feeding		*n*	SEM	CD*	CV %
		0 d	10 d	20 d	+10 d	+20 d				
Total cholesterol	C_1	1.7	1.7	1.7	1.8	1.9	9	6.4	—*	10.9
	C_2	2.1	2.1	2.1	1.8	1.6	7	10.8	31.6	14.6
	C_3	2.3	2.2	2.1	2.0	2.0	9	7.4	21.3	10.3
	C_4	2.7	2.3	2.3	2.6	2.6	2	12.4	—*	7.1
Serum triacylglycerols	C_1	1.8	1.1	1.2	1.4	1.7	9	11.6	33.6	26.2
	C_2	1.5	1.7	1.5	1.6	1.8	7	17.2	—*	28.0
	C_3	1.9	1.7	1.8	1.6	1.9	9	17.1	—*	28.8
	C_4	1.5	1.4	1.7	1.7	1.3	2	29.5	—*	27.2
HDL cholesterol	C_1	0.46	0.43	0.47	0.36	0.43	9	4.8	—*	33.5
	C_2	0.46	0.43	0.55	0.46	0.51	7	4.7	—*	25.8
	C_3	0.46	0.41	0.49	0.42	0.43	9	2.9	—*	19.6
	C_4	0.36	0.46	0.38	0.55	0.52	2	10.4	—*	32.0
VLDL cholesterol	C_1	0.23	0.23	0.24	0.29	0.34	9	2.3	6.7	26.2
	C_2	0.31	0.34	0.30	0.31	0.36	7	3.4	—*	28.0
	C_3	0.38	0.35	0.36	0.32	0.38	9	3.4	—*	28.8
	C_4	0.30	0.27	0.34	0.35	0.27	2	5.9	—*	27.2
LDL cholesterol	C_1	0.98	1.0	1.0.2	1.1	1.1	9	7.3	—*	20.6
	C_2	1.3	1.3	1.3	1.1	0.78	7	11.7	34.2	26.9
	C_3	1.5	1.4	1.3	1.3	1.2	9	7.8	—*	17.3
	C_4	2.0	1.5	1.6	1.7	1.8	2	21.7	—*	17.7
Ratio‡ LDL/HDL	C_1	2.1	2.4	2.2	3.1	2.6	9	0.13	—*	24.7
	C_2	2.9	3.1	2.3	2.3	1.5	7	0.13	—*	22.3
	C_3	3.3	3.5	2.6	3.1	2.9	9	0.08	—*	13.9
	C_4	5.6	3.4	4.2	3.0	3.5	2	0.30	—*	22.2
Ratio‡ Cho_{tot}/HDL	C_1	3.6	4.0	3.7	4.9	4.4	9	0.13	—*	18.3
	C_2	4.5	4.8	3.9	4.0	3.2	7	0.11	—*	13.7
	C_3	5.1	5.4	4.4	4.9	4.8	9	0.07	—*	9.7
	C_4	7.4	5.0	6.1	4.7	5.0	2	0.27	—*	15.7

* — insignificant.
†Average of two baseline estimations, *i.e.* 10 d before start of feeding and on day zero.
‡The original values were transformed to square root for statistical analysis.

The average LDL/HDL cholesterol ratio showed a trend of continuous decline over the study period in the C_2 group. In this group an insignificant square trend of initial increase followed by decrease was observed for the Cho_{tot}/HDL ratio. In the C_3 group, a square trend with the average total and LDL cholesterol levels over the given test periods was detected. In the C_4 group, though neither linear nor square trend fitted with respect to total cholesterol, a square trend of decline in LDL cholesterol was found.

The results indicated that feeding of acidophilic milk was beneficial in terms of reducing cholesterol in people having borderline (2.0–2.2 g/L, C_2 group) and moderate risk (2.2–2.5 g/L, C_3 group) in relation to the occurrence of coronary heart diseases. However, when the cholesterol level was normal, it did not reduce further as in C_1. The average post-feeding value for total cholesterol of +20 d was less than the baseline in C_2, C_3 and C_4 group individuals, whereas in C_1 group it increased. Thus the test culture seems to have a cholesterol-regulating effect rather than lowering effect. In the high-risk group C_4, the reduction was insignificant. One of the limitations in this group was that the numbers of volunteers were only two.

Table II. Best fitted trend equations for lipid profile parametric values

Parameter	Group	Trend pattern	Constant a	Rate of change b_1	Rate of change b_2	Coefficient of determination R^2	Significance†
Total cholesterol	C_1	Q	172	-4.6	1.6	0.93	-
	C_2	Q	194	19.3	-5.1	0.94	-
	C_3	Q	25	-17.7	1.7	0.99	+
Triacylglycerols	C_1	Q	131	-19.8	5.6	0.98	+
VLDL cholesterol	C_1	Q	25	-3.2	1.0	0.99	+
LDL cholesterol	C_1	Q	96	2.7	0.14	0.84	-
	C_2	Q	117	19.4	-5.4	0.99	+
	C_3	Q	163	-12.6	1.0	0.91	-
	C_4	Q	240	-53.6	8.4	0.81	-
Ratio LDL/HDL	C_2	L	3.5	-0.4	-	0.83	+
Ratio Cho_{tot}/HDL	C_2	L	4.5	0.17	-0.08	0.83	-

†At 5 % level; + significant, - insignificant.

The average reduction in total cholesterol of 21 and 12 % in the C_2 and C_3 groups, respectively, during the study is very promising and reasonably comparable to that obtained by cholesterol reduction drugs. Drugs like Simvastatin and Lovastatin that inhibit cholesterol synthesis are able to reduce the total cholesterol by lowering the plasma LDL levels by 30–50 % (Brown and Goldstein 1991). However, in addition to many harmful side effects, these drugs are not available locally and their cost is also prohibitive for middle-class subjects.

The average decrease in the LDL-cholesterol level of C_2, C_3 and C_4 groups during the study is a positive indication of a hypocholesterolemic effect of *L. acidophilus*. The LDL/HDL and Cho_{tot}/HDL ratios are indicative of the risk of developing coronary heart diseases. In the C_2 group, a decline was observed in both cases, again indicating a favorable effect in persons having a borderline blood cholesterol level.

Maximum changes in the lipid profile parameters were observed during the post-feeding periods of +10 and +20 d. This indicated that the effect of feeding acidophilic milk was gradual. This may be due to a gradual establishment of culture organisms in the gut. However, this needs to be confirmed by taking more observations during the post-feeding periods. Also the feeding had a maximum effect on serum total and LDL cholesterol values, whereas the average HDL cholesterol level remained almost unchanged in all the four groups.

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