

*Full Length Research Paper*

# The role of some probiotic lactic acid bacteria in the reduction of cholesterol on mice

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**The potential use of Probiotics in restoring the urogenital and gastrointestinal health has received tremendous interest in the last decade, while few safety concerns are still being debated. The effect of two probiotic lactic acid bacteria strains tested for their ability to assimilation of Cholesterol *In Vivo* (in previous work; *In Vitro*) and their effect on feeding mice on general health indicators, hematological parameters and their ability to reduce the cholesterol level in blood serum; the results showed that no adverse effect on the hematological parameters and the probiotic strains (*Lactobacillus acidophilus* P106, *Lactobacillus acidophilus* P110) effect on reduce the level of cholesterol in the blood serum especially *Lactobacillus acidophilus* P106.**

**Keywords:** Lactic acid bacteria, Probiotics, *lactobacillus acidophilus*, serum cholesterol, feeding mice.

## INTRODUCTION

Probiotics, as defined by the Food and Agricultural Organization of the United Nations (FAO), are "live microorganisms administered in adequate amounts which confer a beneficial health effect on the host." The microorganisms referred to in this definition are non-pathogenic bacteria (small, single celled organisms which do not promote or cause disease), and one yeast, *Saccharomyces*. They are considered "friendly germs," due to benefits to the colon and the immune system. The word probiotic is a compound of a Latin and a Greek word; it means "favorable to life." Probiotics is also sometimes used to refer to a form of nutritional therapy based on eating probiotic foods and dietary supplements. Although probiotic supplements have also been used with farm animals. *Lactobacillus acidophilus* is a normal inhabitant of the intestine of many animals, including man. It may play an important role in maintaining a balanced intestinal flora (Lavermicocca *et al.*, 2005), and

by the production of enzymes (glucosidases) that split off various sugars from polysaccharides, so supplying energy for growth (Kim and Gilliland, 1983; Corcoran *et al.*, 2005).

Commercial interest in functional foods containing probiotic strains has consistently increased due to the awareness of the benefits for gut health and disease prevention and therapy. Research in this area is focusing on the development of new health-promoting foods as well as on selecting new cultures with an enhanced ability to colonize the human gut (Champagne and Gardner, 2005). To exert their beneficial effect after human consumption, probiotic bacteria need to survive first the manufacturing process of the carrier food and then the gastrointestinal ecosystem. The ability of probiotic strains to survive passage through the gastrointestinal tract can be mainly attributed to their acid and bile tolerance. This is an intrinsic characteristic of the strains, which can be improved by the protective action of carrier foods (Charalampopoulos *et al.*, 2003) and/or by the presence of nutrients such as metabolizable sugars (Corcoran *et al.*, 2005).

The most common foods used as vehicles for

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probiotics able to enhance the transit tolerance of bacteria are dairy products. Some strains of *Lactobacillus* and *Bifidobacterium* species can tolerate acidic stress when ingested with milk products (Hyytiä-Trees *et al.*, 1999), and the high fat content of cheeses protects probiotic populations during passage through the gastrointestinal tract (Fernández *et al.*, 2003). Current research is mainly focused on developing new probiotic nondairy foods that can contribute to the regular consumption of beneficial microorganisms. Since probiotic bacteria are only transient in the intestinal tract and do not become part of the host's gut microflora, their regular consumption is required for the maintenance of positive effects. Therefore, probiotic strains must be ingested in large quantities and on a daily basis. Procedures that may enhance the viability of probiotic populations during processing, storage, and transit through the gastrointestinal tract have recently been investigated (Champagne and Gardner, 2005). Apart from dairy products, other foods investigated as carriers for probiotic cultures include meat- and fish-based products, confectionery, table olives, soy- and cereal-based products, edible spreads, plant seed extracts, etc. (Champagne and Gardner, 2005). Cereal products have been shown to be suitable substrates for the growth of potentially probiotic lactobacilli, also due to the protective effects of soluble sugars (Charalampopoulos *et al.*, 2003).

Cholesterol acts as a risk factor in different diseases such as cardiovascular, colon cancer and hypercholesterolemia (Paniangvait *et al.*, 1995). Results in recently published works indicate that the reduction of excessive levels of cholesterol in the blood decreases the risks of these diseases. Some natural microorganisms in human intestine are beneficial in terms of lowering serum cholesterol (Fukushima *et al.*, 1999). The lactic acid bacteria (LAB), *Lactobacillus* and *Bifidobacterium* spp. in particular, have the ability to metabolize cholesterol (De Smet *et al.*, 1995). Blood cholesterol synthesis is decreased by the inhibition of HMG-CoA reductase that convert HMG-CoA to mevalonate and by organic acids in the fermented milk. Gilliland *et al.* reported that *Lactobacillus acidophilus* reduces blood cholesterol by direct breakdown of cholesterol and deconjugation of bile salt (Gilliland *et al.*, 1985). In particular, cholesterol metabolism is closely linked to the formation of bile salts,

Studying the ability of two probiotic lactic acid bacteria to improve the human health by use these strains in the feeding some mice with study their role in the hematological parameters and their ability to reduce the cholesterol level in blood serum and its number in the feces of mice during the test.

## MATERIALS AND METHODS

### Tested Microbial strains

Two isolates of *Lb. acidophilus* P106 and *Lb. acidophilus* P110 were originated from faeces of healthy breast-fed infants born in Alexandria, Egypt, aged from 3 – 6 months. These strains were used after isolation; identification and tested for their characteristic as probiotic (Gastric acid resistance, bile salt tolerance, antibacterial activity, adhesion to human mucus) (Mahrous 2006). The cultures were preserved in reconstituted skim milk in eppendorf tubes, stored at -80°C with glycerol (20%, v/v). Prior to use, strains were subcultured (1%, v/v) twice in MRS (de Man Rogosa Sharpe) broth and incubated anaerobically overnight at 37°C.

### Viability of the probiotic isolates in yoghurt

Tested probiotic strains were incubated in BBL anaerobic jar (Becton Dickinson Microbiology Systems, Sparks, MD) provided with disposable BBL gas generating pack (CO<sub>2</sub> system envelopes, Oxoid, Ltd., West Heidelberg, Victoria, Canada) for 48 h at 37° C, and then concentrated by centrifugation. The cell pellets were resuspended in 10 % skim milk at a concentration of 10<sup>8</sup>-10<sup>10</sup> CFU/ ml, the number of viable cells (colony forming units per ml, CFU / ml) and used in the manufacturing of yoghurt, according to the method of Shah, 2000.

### Animals and Conditions

Forty male ICR (CD-1) mice, approximately 4 week-old with the average initial body weight of (25.25 ± 4.50)g were obtained from Faculty of Science, Department of Zoology, Alexandria University, Alexandria, Egypt. All mice were examined for health status and acclimated to laboratory environment for 2 weeks prior to use. Temperature was maintained at 23±2°C, and relative humidity at approximately 50%, with a 12 h: 12 h light: dark photoperiod. Animals were housed in stainless-steel cages and given standard diet and water ad libitum throughout the study.

### Probiotic Feeding

Mice were randomly assigned to treatment groups according to an approximately equal mean body weight

to 4 treatment groups of 10 each. The treatments were: 1) group A, were fed by the normal yoghurt (as control); 2) group B (were fed by normal yoghurt plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal); 3) group C (were fed with combination with yoghurt starter and *Lb. acidophilus* P106 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal) and 4) group D (were fed with combination with yoghurt starter and *Lb. acidophilus* P110 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal). The experiment was carried out for four weeks (5 days/ week, 20 days) by oral gavages; dose level  $10^8$ - $10^{10}$  CFU/ ml. The administered volume of each dose was 1.0 ml/kg day, adjusted daily for recorded body weight changes during the treatment period. At the end of experiment, the mice were fasted for 12 hours before blood collection.

### Animal observations

Health status of treated mice was monitored daily throughout the experimental period. The number of animals with diarrhea was recorded daily. Mice body weight and feed consumption were recorded weekly.

Feces of each group of mice were collected daily on each of the last 5 days of the experimental period and lyophilized. 1.0 g of crushed lyophilized feces were suspended in chloroform-methanol 1:1(v/v), sonicated for 5 min, and then extracted at 60°C for 60 h. The extract was evaporated and dissolved in methanol for measurement of total bile acids (TBA) with a commercial kit (Loh *et al.*, 2002). The total cholesterol of feces was determined as described by Rudel and Morris (1973).

### Mice blood collection

After dosing (20 days), mice were anesthetized by using diethyl ether. Mice blood was obtained by cardiac puncture via aspiration through polyethylene tubing attached to a heparinized microhematocrit capillary tube which had been flamed and pulled to a fine point.

### Hematological parameters

For measuring hematocrit value, whole blood was centrifuged in 3 or 5  $\mu$ L heparinized microhematocrit capillary tubes for 90 sec in a hematocrit centrifuge. The lengths of the total sample and red cell fraction were measured on a standard ruler and the hematocrit value was calculated by dividing the length of the red cell fraction (in millimeters) by the total sample length. For the assay of hemoglobin concentration, a standard diagnostic colorimetric assay based on formation of cyanomethemoglobin via reaction of hemoglobin with potassium cyanide under alkaline conditions (Crosby *et al.*, 1954; Drabkin and Austin, 1982) was used to

determine hemoglobin concentrations in mice blood. Five  $\mu$ L of whole blood was incubated with 1.25 ml of Drabkin's solution. After at least 15 min at room temperature, absorbance at 540 nm was measured spectrophotometrically. All samples were analyzed within 4 h of blood collection. Sample hemoglobin levels were determined by interpolation from a concurrently run standard curve of human hemoglobin and fixed with 895  $\mu$ L of 2.5% paraformaldehyde. An aliquot of 50- 200  $\mu$ L aliquot of the fixed blood sample was added. Red blood cell (RBC) and white blood cell (WBC) were counted using the hemocytometer slide (Murphy, 1986). The absolute values, mean cell value (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentrations (MCHC) were calculated according to (Dacie and Lewis 1991).

### Cholesterol

The cholesterol was determined by the method of (Watson 1960). Briefly; 2.5 ml of reagent (acetic anhydride 3.5 mol/ L and acetic acid 5 mol/L) was added to 100  $\mu$ L of plasma, mixed well and incubated for 5 minutes in water bath at 20 – 25°C. Onto this mixture, 500  $\mu$ L of H<sub>2</sub>SO<sub>4</sub> was added and mixed immediately thoroughly under constant cooling and allowed to stand for 20 minutes at the same temperature. The absorbance was measured at 500 nm against blank. The concentration of cholesterol was the calculated as mg/ dL using the standard concentration.

### Statistical Analysis

Data are presented as the mean  $\pm$  standard deviation, and n represents the number of mice from the probiotics and the control. Comparisons were made by use of the student's t-test. Differences were regarded significant with *P* value less than 0.05.

## RESULTS AND DISCUSSION

### Adverse Clinical Signs

No serious adverse effects were observed for control and group B, in particularly, diarrhea and weakness compared to the other treated groups. These signs appeared in group B on day 10 of treatment and particularly progressed in the same animals throughout the second half of treatment period and disappeared in groups treated with probiotic strains. These results are in agreement with those reported previously (Murry *et al.*, 2004). Also, it has been reported previously (Lee *et al.*, 1999) that probiotics have beneficial effects on the health of the host. Kikuchi and Yajima (1992) showed that

**Table 1:** Feed consumption (g/ kg d week) of treated mice with yoghurt and probiotic microorganisms.

Week	A	B	C	D
1	390.5 ± 0.1	393.2± 0.3	408.3± 0.3	410.2 ±0.6
2	473.4 ± 0.2	459.3 ± 0.1	512.8 ± 0.1	519.1± 0.7
3	528.5 ± 0.3	520.3± 0.1*	544.3± 0.1	558.6± 0.5
4	665.7 ± 0.3	650.5± 0.1**	679.8± 0.1*	675.9± 0.8*

Data are presented as mean ± SD

All probiotic strains were added at ( $10^8$ - $10^{10}$  CFU/ ml).

\*Significantly different from control at  $P < 0.05$ .

\*\*Significantly different from control at  $P < 0.01$ .

Group A was served as control fed by the normal yoghurt ;Group B were fed by normal yoghurt plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal ;Group C were fed with combination with yoghurt starter and *Lb. acidophilus* P106 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal ;Group D were fed with combination with yoghurt starter and *Lb. acidophilus* P110 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal.

**Table 2:** Body weight and weight gain of Feeding mice with normal yoghurt and fermented milk with probiotic microorganisms and starter yoghurt.

	Weeks	A	B	C	D
<b>Body weight (g)</b>	1	26.7± 0.1	25.6±0.1	27.8± 0.5	27.6±0.1
	2	31.7 ± 0.2	30.5 ± 0.2	32.9 ± 0.2	32.5 ± 0.2
	3	37.9 ± 0.4	35.7 ± 0.3*	38.9 ± 0.3*	37.7 ± 0.3
	4	45.3 ± 0.6	40.5 ± 0.6*	46.5 ± 0.6*	45.9 ± 0.6
<b>Body weight gain (g)</b>	1-2	5 ± 0.1	4.9± 0.1	5.1 ± 0.3	4.9± 0.1
	2-3	6.2 ± 0.1	5.2 ± 0.1	6.0± 0.1	5.2± 0.1
	3-4	7.4 ± 0.2	4.8± 0.1*	7.6 ± 0.2*	8.2± 0.3
	1-4	18.6 ± 0.5	14.9± 0.5**	18.7± 0.7**	18.3± 0.2

Data are presented as mean ± SD

All probiotic strains were added at ( $10^8$ - $10^{10}$  CFU/ ml).

Group A was served as control fed by the normal yoghurt ;Group B were fed by normal yoghurt plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal ;Group C were fed with combination with yoghurt starter and *Lb. acidophilus* P106 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal ;Group D were fed with combination with yoghurt starter and *Lb. acidophilus* P110 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal.

\*Significantly different from control at  $P < 0.05$ .

\*\*Significantly different from control at  $P < 0.01$ .

probiotic microorganisms' strains are useful in the treatment of disturbed intestinal microflora and diarrheal diseases. Giving probiotics in conjunction with rehydration fluids reduced the duration of diarrhoea by around a day and reduced the risk of diarrhoea lasting four or more days by 59%. Moreover; probiotics might prevent infection because they compete with pathogenic viruses or bacteria for binding sites on epithelial cells or by producing bacteriocins.

### Feed Consumption

Mice feed consumption is presented in Table 1. It was significantly reduced in the group B at the beginning of

the treatment compared to other treated groups. No significant effects were observed in feed consumption in the remaining treated groups.

### Body and body weight gain

Mice body and organ body weight gain are presented in Table 2. Body weight gain was significantly reduced in the B group at the end of the treatment compared to the control groups. No significant effects were observed in body weights in the other two treated groups. Reduction of mice body weights and weight gain can be attributed addition of the probiotics to the diet was associated with increased feed consumption and weight gain. This

**Table 3:** Content of Total Bile Acids (TBA  $\mu\text{mol/g}$ ) and Content of Total Cholesterol (TC  $\text{mg/g}$ ) of Feeding mice with normal yoghurt and fermented milk with probiotic microorganisms and starter yoghurt.

Mice groups	Content of TBA $\mu\text{mol/g}$	Content of TC $\text{mg/g}$
A	8.68 $\pm$ 0.1	10 $\pm$ 0.2
B	12.5 $\pm$ 0.2	36.2 $\pm$
C	16.5 $\pm$ 0.1	45.8 $\pm$ 0.2
D	15.4 $\pm$ 0.1	40.9 $\pm$ 0.1

Data are presented as mean  $\pm$  SD

All probiotic strains were added at ( $10^8$ - $10^{10}$  CFU/ ml).

Group A was served as control fed by the normal yoghurt ;Group B were fed by normal yoghurt plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal ;Group C were fed with combination with yoghurt starter and *Lb. acidophilus* P106 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal ;Group D were fed with combination with yoghurt starter and *Lb. acidophilus* P110 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal.

**Table 4:** Blood analysis of mice after feeding with yoghurt and probiotic microorganisms.

Mice groups <sup>a</sup>	Hct value		Hb content		RBC		WBC	
	%	% of Control	g 100 $\text{ml}^{-1}$	% of Control	$\times 10^6$ $\text{uL}^{-1}$	% of control	$\times 10^3$ $\text{uL}^{-1}$	% of control
A	41 $\pm$ 3	100	12. 9 $\pm$ 1	100	5.6 $\pm$ 1	100	6.5 $\pm$ 2	100
B	40 $\pm$ 1	98.6	12.5 $\pm$ 2	96.9	5.5 $\pm$ 2*	98.2	6.7 $\pm$ 3**	103.1
C	41 $\pm$ 2	100	13..1 $\pm$ 1	101.6	5.6 $\pm$ 2	100	6.4 $\pm$ 2	98.5
D	41 $\pm$ 1	100	13..0 $\pm$ 1	100.7	5.6 $\pm$ 2	100	6.5 $\pm$ 1	100

Group A was served as control fed by the normal yoghurt ;Group B were fed by normal yoghurt plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal ;Group C were fed with combination with yoghurt starter and *Lb. acidophilus* P106 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal ;Group D were fed with combination with yoghurt starter and *Lb. acidophilus* P110 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal.

Hct value: Haematocrite value; Hb: Haemoglobin; RBC: red blood cells;

WBC: white blood cells

\*Significantly different from control at  $P < 0.05$

\*\*Significantly different from control at  $P < 0.01$

indicates a protective effect of these strains this result is agreement with Lee *et al.*, 1999.

### Bile acid and cholesterol concentration in feces

Lactic acid bacteria are normal components of the intestinal microflora in both humans and animals and have been associated with various health-promoting properties. One beneficial effect is a reduction in serum cholesterol levels. The effects of assigned diets on feces bile acid and cholesterol content of mice are illustrated in Table 3. The bile acid and cholesterol content of feces in mice given high-cholesterol diets (group B ,C and D) were significantly higher than those in model control mice (group A). The bile acid and cholesterol level in feces of the mice fed with yoghurt and probiotic bacteria (group C and D) were significant higher than those of experimental mice (group B) especially on group C. As deconjugated bile salts are more readily excreted in the feces than conjugated bile salts (Gilliland and Walker, 1990; DeSmet

*et al.*, 1994; De Rodas *et al.*, 1996), bacteria with BSH activity may effectively reduce serum cholesterol by enhancing the excretion of bile salts, with a consequent increase in the synthesis of bile salts from serum cholesterol; or by decreasing the solubility of cholesterol, since bile acid is essential for gastrointestinal absorption of cholesterol (Sugao and Imaizumi, 1986), and thus reducing its uptake from the gut.

### Hematological analysis

Blood parameters are presented in Table 4. There was No significant difference in the value of Haematocrite value (Hct) in the remaining treated groups compared to the control group.

Haemoglobin content, There were no significant differences in the values of Hb in the remaining treated groups.

Red blood cells count, No significant effects were observed in the treated groups compared to the control.

**Table 5:** Size of red cells and cholesterol content in blood mice after Feeding with yoghurt and probiotic microorganisms.

Mice groups <sup>a</sup>	MCV		MCH		MCHC		Cholesterol mg/dL
	Fento L (FL)	% of control	Picogram (Pg)	% of control	g/L	% of control	
A	73 ± 0.1	100	23.3 ± 1	100	328 ± 1	100	112.85 ± 7.10
B	72 ± 0.6	98.6	22.9 ± 2	98.3	325 ± 5	99	122.50 ± 5.20
C	73 ± 0.6	100	23.9 ± 5	102.6	330 ± 2	100.6	110.30 ± 2.56
D	73 ± 0.5	100	23.1 ± 2	99.1	329 ± 5	100.3	111.50 ± 5.20

Group A was served as control fed by the normal yoghurt ;Group B were fed by normal yoghurt plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal ;Group C were fed with combination with yoghurt starter and *Lb. acidophilus* P106 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal ;Group D were fed with combination with yoghurt starter and *Lb. acidophilus* P110 plus 1.0% (w/w) cholesterol and 0.2% (w/w) oxgal.

MCV: mean cell value, MCH: mean cell hemoglobin and MCHC: mean cell hemoglobin concentration.

\*Significantly different from control at  $P < 0.05$

\*\*Significantly different from control at  $P < 0.01$

The value of the WBC count in the treated groups was similar to the value of the control (Table 4). These results also are in agreement with the reported data investigated that oral injection of the probiotic strains in humans did not lead to cytokine changes beyond normal values (Gardiner *et al.*, 2002). This finding provides evidence for the safety of the probiotic cultures.

### The calculated parameters of an individual RBC characterization

The calculated parameters are presented in Table 5.

No significant differences were observed in the calculation value of Mean Cell Volume (MCV); Mean Cell Haemoglobin (MCH) and Mean Cell Haemoglobin Concentrations in any of the treated groups compared to the control.

### Cholesterol

Serum cholesterol increased in the group B compared to the other treated groups especially group C (Table 5). The use of probiotic bacteria in reducing serum cholesterol levels has attracted much attention. Lots of researchers proposed that probiotics have cholesterol reduction effects. However, the mechanism of this effect could not be explained definitely. There are two hypotheses trying to explain the mechanism. One of them is that bacteria may bind or incorporate cholesterol directly into the cell membrane. The other one is, bile salt hydrolysis enzymes deconjugate the bile salts which are more likely to be exerted resulting in increased cholesterol breakdown (Prakash and Jones 2005). Various studies have shown that some lactobacilli could lower total cholesterol and low-density lipoprotein (LDL) cholesterol. High level of serum cholesterol has been

associated with risks of coronary heart disease. (Anderson and Gilliland, 1999; Sanders, 2000; Agerholm *et al.*, 2000; Pereira and Gibson, 2002; Pereira *et al.*, 2003).

### CONCLUSIONS

Probiotics are claimed to have beneficial effects on health. However, only few well-performed studies have looked at clearly defined health effects such as serum cholesterol concentrations. Hypercholesterolemia is strongly associated with coronary heart disease and arteriosclerosis and decreasing serum cholesterol is an important treatment option. In this study we have shown that Ingestion of probiotic strains *Lb. acidophilus* P106, *Lb. acidophilus* P110 had no adverse effects on the hematological parameters. These results are in consistent with the published results indicate that LAB strains had no adverse effects on the hematological parameters.

On a final note, this study has demonstrated that the cholesterol and bile acid levels in the serum of mice fed with yoghurt fermented by *Lb. acidophilus* P106 decreased significantly, while the cholesterol and bile acid content increased in mice feces. These effects may be due in part to the deconjugation of bile salts by strains of bacteria that produce the enzyme bile salt hydrolase (BSH).

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