

Full Length Research Paper

The effect of *Lactobacillus paracasei* on the rabbit's cholesterolemia

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Accepted 10 April, 2007

Two *Lactobacillus paracasei* (Lbp6 and Lbp9) were examined for their capacity to decrease the level of the total cholesterol, of HDL cholesterol and of LDL cholesterol in hypercholesterolemia rabbit. The obtained results indicate no modification neither of the level of total cholesterol nor of its fractions for the standard diet (T) nor of standard diet where fermented milk (FM) is added. On the other hand, they reveal significant augmentations of total cholesterol level, of HDL cholesterol and of LDL cholesterol of rabbits consuming a standard diet of cholesterol (Ch) and cholesterol with fermented milk (Ch + FM). However, the level of total cholesterol, HDL cholesterol and LDL cholesterol is weaker in rabbits that received fermented milk in addition to standard diet and cholesterol. HDL/LDL ratio, an important atherogenicity's indicator favourably. The results also show that when we stop the supplements total cholesterol level, HDL cholesterol and LDL cholesterol levels return to the initial values after 14 days. The addition of the fermented milk to rabbits' standard diet has no effect on the level of the total cholesterol and on its fractions, when these are initially normal.

Key words: *Lactobacillus paracasei*, cholesterol.

INTRODUCTION

High concentrations of blood cholesterol are associated to the development of illnesses like cardiovascular, hypercholesterolemia and lithiasis vesicular which are some of the most important mortality causes in the world (Paniangvait, 1995). Published works indicate that the reduction of excessive cholesterol level in the blood decreases the risk of these diseases (Buck and Gilliland, 1994). The hypothesis that certain fermented milk that is rich in probiotic is capable of diminishing cholesterol level is now a subject of different researches. Nutritional, dietetic and therapeutic properties have been studied in the fermented products with specific microorganisms (Dilmi-Bouras and Sadoun, 2002; Klebling et al., 2002; Oyetayo et al., 2003). The present study is devoted to examine the effect of fermented milk by two strains of *Lactobacillus paracasei* (Lbp4 and Lbp6) on the total cholesterol level, HDL cholesterol and LDL cholesterol of

hypercholesterolemia rabbits.

MATERIALS AND METHODS

Strains of bacteria used

L. paracasei C6 (Lbp6) and *L. paracasei* C9 (Lbp9) are two strains used for the fermentation of the milk (FM). The strains were obtained from Lactologia Laboratory of Navarra Public University, Spain, under frozen form in pure culture.

Rabbits

The experimentation is conducted on male rabbits (*Oryctolagus cuniculus*) of the same age (180 ± 20 days), weighing between 1400 and 1600 g at the beginning of the experimentation and coming from the same farm in Chlef. The animals are placed one by one in metallic cages of 50 cm side. They are kept in well aerated animal park, in a constant temperature of $21 \pm 1^\circ\text{C}$ with lighting of 12 h assured from 8 to 20 hours. Food and water are distributed *ad libitum* and every morning cage cleaning is done. Rabbits have slow intestinal transit and big sensitivity to nutritious cholesterol (Mayes, 1995). This characteristic may allow the extrapolation of results to man. On the other hand, working on animal will allow the best control of the type of nutrition and of addition, as well as the quantitative contribution.

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Table 1. Diet of different groups of rabbits with respect to time.

Groups	w1 and w2	w3, w4, w5, w6 and w7	w8 and w9
G1	T	T	T
G2	T	T + FM	T
G3	T	T + Ch	T
G4	T	T + Ch + FM	T

T: Standard diet: 300 g/day (lettuce, carrots, bread and water).

Ch: Cholesterol: 2 x 0.02 g/day.

FM: Fermented milk: 2 x 10 ml/day.

W: Week.

Diet's preparation "fermented milk"

The fermented milk was prepared in the laboratory as following: 120 g of skimmed instant milk (Lahda, manufactured for Edipal, Pays-Bas) was dissolved in a sufficient quantity for 1 litre of distilled sterile water. To this milk 3% of inoculum's (fermented milk with a cellular concentration of 3.6×10^8 /ml) is added. The incubation was at 37°C for 18 h. The jugs of fermented milk, of pH 5.61, are immediately taken out of steam room, rapidly cooled in the fridge at the temperature of 4°C. The purpose of this cooling is to stop the acidification and the multiplication of bacteria.

Constitution of experimental groups

In the beginning, 20 rabbits were divided into 4 groups (5 rabbits for each group). These rabbits were first fed by a standard diet (300 g/day of: lettuce, carrots, bread and water) during an adaptation period of 15 days (Table 1). Then, and during the following 6 weeks (42 days), the control group is fed by standard diet (T); whereas for the other groups, the standard diet is added either with 2 x 10 ml per day of fermented milk (FM), or with 2 x 0.02 g of standard cholesterol (reference: 139 050, Boehringer Mannheim GmbH) per day and per kg (Ch), or with cholesterol and fermented milk (FM + Ch). Finally, the rabbits were passed through two weeks of standard diet alone.

The cholesterol is added to the diet in order to raise the level of rabbit's blood cholesterol and to simulate a study on risky subjects. This cholesterol concentration that was added to rabbits' diet is relatively weak; it allows the augmentation of cholesterol without provoking an important acceleration of the development of arterial lesions (Dilmi-Bouras and Sadoun, 2002).

Faecal analysis

The faeces are recovered before the taking of the fermented milk, then each two days during and after the ingestion stop of the fermented milk. 1 g of fresh faeces is diluted in 9 ml of physiological water. After pulverizing on sterilized filter paper, 1 ml of solution is taken for the microbiological analysis.

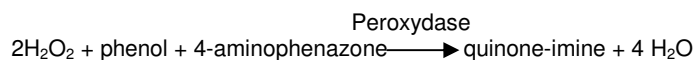
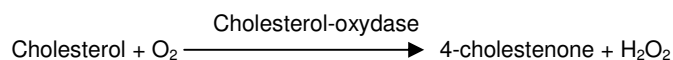
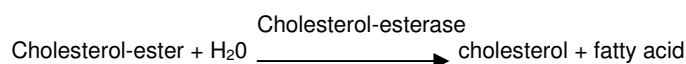
Blood analysis

The rabbits went without food 12 h before blood was taken. 2 ml was collected by puncture in the marginal vein of the ear. The blood was placed in haemolysis tubes with 1% of 5 M EDTA (antioxidant) and was allowed to stand at the laboratory's temperature until the formation of clot. After unstitching, the coagulated blood was centrifuged at 3000 rpm at 4°C for 10 min. Then, the serum was recovered for the different analysis. Control analyses were done

before the consumption of the fermented milk. After that, other analysis were conducted once a week for the total cholesterol, HDL cholesterol and LDL cholesterol.

Analysis of total cholesterol

Enzymatic colorimetric method of sera-Pak test is used: US patent 291.121 and Foteigh extensions (Siedel et al., 1983). The principle of this method is as follows:



In the presence of peroxylase, the H_2O_2 generated reacts with the 4-aminophenazone to form a derived quinone-imine (coloured in red) where the coloration intensity, measured at 600 nm, is proportional to the concentration of the total cholesterol (Rifai et al., 1998).

Analysis of HDL cholesterol

The committee of Lipids-Lipoproteins standardization ARCOL-SFBC (French Committee of Research Coordination about Atherosclerosis and Cholesterol, French Society of Clinic Biology) has published, in 1996, recommendations for the use of the precipitation method, by sodium phosphotungstate/chlorure of magnesium, of lipoproteins containing apolipoprotein B (chylomicrons, VLDL and LDL). For this purpose, the enzymatic method Chod-PAP (reference 61 531, Bio-Mérieux S.A.), like the one described by Draeger et al. (1982), was used for the analysis of the cholesterol of lipoproteins of high density. The chylomicrons, VLDL (very low density lipoprotein) and LDL (low density lipoprotein) are precipitated by the addition of phosphotungstic acid and of ion Mg^{++} to the sample. The concentration of HDL cholesterol (high density lipoprotein), which stays floating after centrifugation, is determined by an enzymatic way.

Analysis of LDL cholesterol

The analysis of the cholesterol of low density lipoprotein is determined by the precipitation method by heparin (100.000 UI/l) and of citrate sodium (0.064 mol/l), as stabilising agent (reference 14 992,

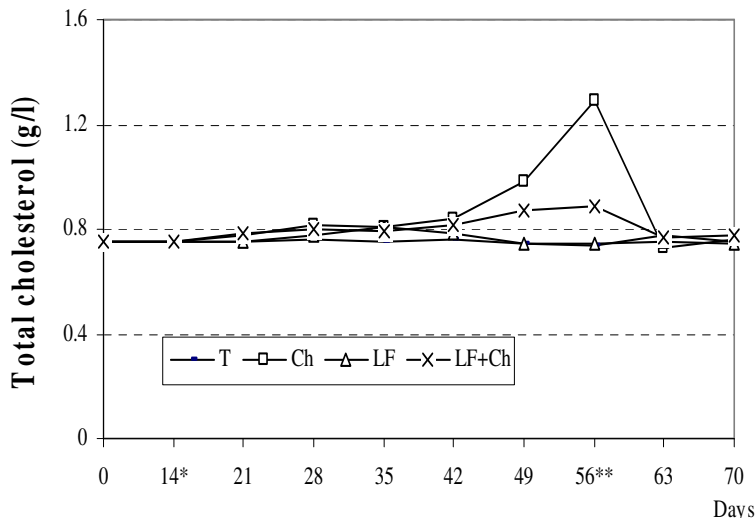


Figure 1. Evaluation of total cholesterol in the rabbits with different diets. (* -beginning of the addition; ** -stopping of the addition; T: control; Ch: cholesterol; FM: fermented milk).

diagnostica-Merck; Wieland et al., 1983). LDL was precipitated by heparin to their isoelectric point (pH 5.12). After centrifugation, HDL and VLDL stay floating and LDL were determined by enzymatic method.

Statistic analysis of the results

The statistical analysis of results was by the ANOVA program. The average comparisons was by Newman-Keuls' test and probability (P).

RESULTS AND DISCUSSION

Viability of Lbp6 and Lbp9

The number ($> 10^8$ cells/g) and the survival duration of Lbp6 and Lbp9 in the digestive tube of rabbits, during the additional diet of fermented milk are sufficient for the analyses. Lbp6 and Lbp9 are durable and observable during the consumption of the fermented milk.

Evaluation of cholesterol

The experimentation was conducted on hypercholesterolemic rabbits, in order to well define the potential activity of *L. paracasei* C6 and *L. paracasei* C9 of fermented milk on the reduction of the total cholesterol, HDL cholesterol and LDL cholesterol. High concentration of standard cholesterol (>0.05 g) provokes leg paralysis and the appearance of other symptoms in the rabbit. A study by Jacotot (1992) and confirmed by Dilmi-Bouras and Sadoun (2002) shows that the addition of 0.05 to 0.2 g/100 g to rabbits' diet causes an increase in cholesterolemia and an acceleration of arterial lesion's development.

The FM and cholesterol were consumed at 100% because they were orally administrated to rabbits by the help of tube-fed. However, there are always lettuce fragments, carrots and bread. These quantities are not important and represent $10 \pm 3\%$ of the standard diet. The results in Figure 1 show that standard diet alone (T) or with added fermented milk (FM) have not modified the level of the total cholesterol of rabbits neither during the experimentation nor after the stopping of the addition. When fermented milk and cholesterol were added to the standard diet (FM + Ch), the level of the total cholesterol reduces in comparison to the standard diet alone. After the stopping of the addition of the diet of cholesterol, the level of rabbit's cholesterol blood diminished in order to achieve its initial value, identical to the control, in 7 to 14 days.

From the found results, we can conclude that *L. paracasei* C6 (Lbp6) and *L. paracasei* C9 (Lbp9) of fermented milk (FM) diminish the excessive level of total cholesterol blood of rabbits appreciably. Similar results were found in rat (Bertazzoni et al., 2004) and *in vitro* with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Dilmi-Bouras, 2006). Also, hypocholesterolemia action of *Lactobacillus casei* was observed by Oyetayo et al. (2003) and Brashears and Gilliland (1997). However, Klebling et al. (2002) and Desreumaux (2003) did not observe any significant reduction of the level of total cholesterol in men. De Roos and Katan (2000) concluded that the hypocholesterolemia action of probiotics is not completely elucidated. Generally, the lactic bacteria present a large capacity for the degradation of different hydrates of carbon and similar compounds. They also present a large capacity of adaptation to different conditions and consequently can change their metabolic ways (Torre-Hernandez, 2000). Recent results show that

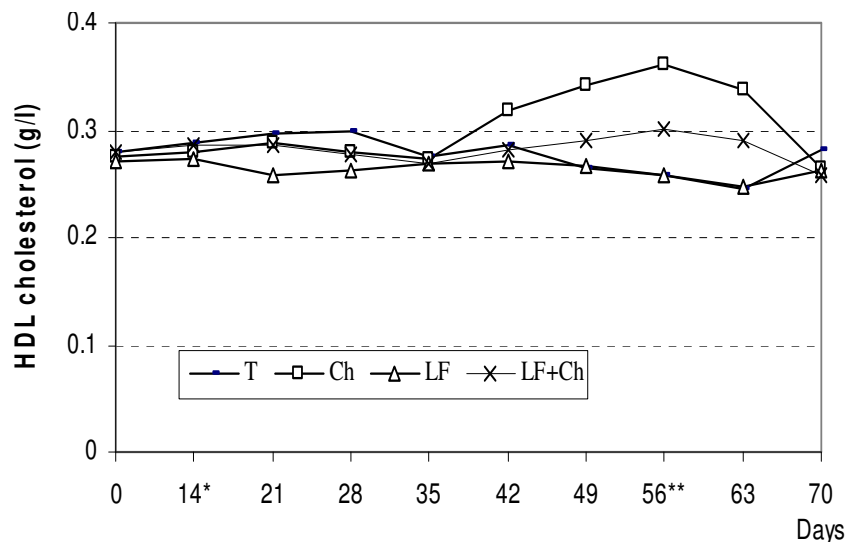


Figure 2. Evaluation of HDL cholesterol in the rabbits with different diets. (*- beginning of the addition; **- stopping of the addition; T: control, Ch: cholesterol; FM: fermented milk).

L. casei is capable of growing in acidic pH while keeping its probiotics properties (Oozeer et al., 2002; Bertazzoni et al., 2004).

This variation in results is essentially due to the fermented strains used; for there is a considerable variation in the assimilation of cholesterol, not only between the different species studied but also between strains of the same kind. According to Haggerty et al. (1984) different bacteria have different capacity to produce substances that can react on the cholesterolemia as orotic acid or hydroxy-methyl-glutarate.

On the other hand, we observed that in presence of normal blood cholesterol level, hypocholesterolemia action of fermented milk (FM) is not significant ($P > 0.01$) and cholesterol level of rabbits did not change during the whole period of experimentation. This phenomenon is may be due to endogen regulation of cholesterol. In physiological conditions, the contribution of hepatic cholesterol to different tissues is sufficient so that the endogen synthesis in these tissues could be inhibited (Agerback et al., 1995). Also, the arrival of cholesterol in the cells allows the self-regulation of cellular synthesis of cholesterol and afterwards, its entrance to blood is also self-regulated (Chanu et al., 1998). It is possible that Lbp4 and Lbp6 in association (FM) assimilate the cholesterol, in spite of its normal level, but there is compensation by endogen synthesis.

Evaluation of HDL cholesterol

HDL cholesterol is the fraction of blood cholesterol transported by lipoproteins of HDL type "called good cholesterol". It has a protector effect against atherosclerosis. It

cleans the artery of all lipids deposits of bad quality and reduces the risks of coronary diseases (Faure, 2000). However, lot of epidemiological studies have established that cardiovascular risk is directly associated to the augmentation of LDL-cholesterol and the diminution of HDL-cholesterol (Egloff et al., 1999).

The purpose of this analysis is to see which cholesterol fraction is assimilated and in which proportion, by the two bacteria (Lbp6 and Lbp9) of fermented milk (FM). The standard alone diet (T) or with added fermented milk (FM) did not modified significantly ($P > 0.05$) the level of rabbits' HDL-cholesterol (Figure 2). However, for the two rabbits groups receiving cholesterol (Ch) or cholesterol and fermented milk (FM + Ch) in addition to standard diet, a significant augmentation ($P = 0.02$) of HDL-cholesterol level was observed, 0.074 g/l (25.69%) and 0.024 g/l (8.42 %), respectively. This augmentation rapidly diminishes significantly ($P < 0.008$) and reducing to the initial value 7 to 14 days after the stopping the addition.

These results show a moderate action of Lbp6 and of Lbp9 of fermented milk (FM) on HDL-cholesterol level, for the assimilated rate of the latter is always inferior to those of total cholesterol level. These results confirm the moderate action of certain lactic bacteria on the HDL-cholesterol (Dilmi-Bouras and Sadoun, 2002; Dilmi-Bouras, 2006). On the other hand, Tamaï et al. (1996) did not observe any variation in the level of HDL blood cholesterol of rats consuming cholesterol and fermented milk in addition to standard diet. However, Klebling et al. (2002) and Desreumaux (2003) have found that probiotics' adjunction to diet augments the level of HDL-cholesterol. This difference in results is probably due to the experimental subjects and/or ferment strains used.

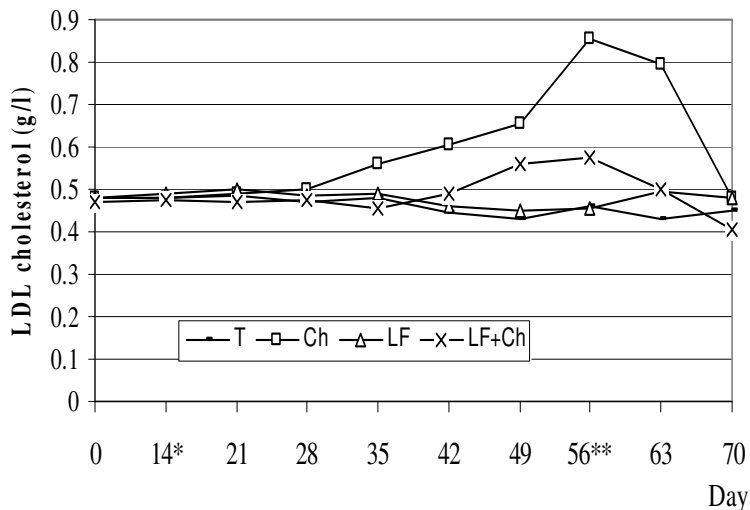


Figure 3. Evaluation of LDL cholesterol in the rabbits with different diets. (*- beginning of the addition; **- stopping of the addition; T: control; Ch: cholesterol; FM: fermented milk).

Evaluation of LDL-cholesterol

LDL-cholesterol is the fraction of blood cholesterol transported by lipoprotein of LDL type. The blood level of LDL-cholesterol "called bad cholesterol" is a risk indicator of coronary diseases more precise than the total cholesterol. This cholesterol fraction is found on artery's wall, thus participating in atherosclerosis (Faure, 2000; Perreault, 2002). Figure 3 results do not reveal any significant change ($P > 0.01$) of rabbits LDL-cholesterol receiving the standard diet only (T) or with added fermented milk (FM) during the whole experimentation. However, an important augmentation (0.363 g/l or 74.08%) of LDL-cholesterol level is observed in rabbits receiving cholesterol (Ch) besides the standard diet. This important level of LDL-cholesterol diminishes progressively reaching the initial value 14 days after stopping the addition of cholesterol. The statistical analysis of these results is very significant ($P < 0.01$).

Besides, a weaker augmentation of LDL-cholesterol level of 0.119 g/l (26.09%), until 42nd day of the addition, is observed in rabbits receiving fermented milk (FM) in addition to standard diet and cholesterol (FM + Ch). Thus, the addition of the fermented milk (FM) to standard diet and to cholesterol seems to diminish ($P < 0.05$) LDL-cholesterol level significantly in comparison to the observed levels in the rabbits which did not receive fermented milk (FM). This shows clearly that the lowering of LDL cholesterol rate is really linked to the presence of the two bacteria in the diet. These results confirm the previous work (Jaspers, 1984) showing that certain yogurt's ferments reduce, *in vitro*, the excessive level of LDL cholesterol. Similar results were found in man by Schaafsma et al. (1996) which show that the daily consumption of 125 ml of fermented milk lowers the level

of LDL cholesterol and that of total cholesterol. The action of Lbp6 and of Lbp9 of fermented milk (FM) on the reduction of level of this fraction seems interesting since the high level of LDL cholesterol is associated with atherosclerosis (Mayes, 1995; Faure, 2000; Finetin, 2001).

Conclusion

The obtained results suggest that Lbp6 and Lbp9 would have a role in the reduction of the excessive levels of the total cholesterol and of LDL cholesterol. This reduction is the result of a direct assimilation of cholesterol or the production of substances inhibiting cholesterol synthesis.

ACKNOWLEDGEMENT

Financial support was received from the University HB Chlef and from Ministry of Scientific Research (Project code: F 0201/02/05).

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