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Effect of three *Lactobacillus* strains on lipid metabolism in rats fed a high-cholesterol diet

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Probiotics are known as functional foods and have been recommended as dietary adjuncts to hypercholesterolemic subjects. The objective of the present study was to evaluate the effect of three *Lactobacillus* strains on lipid metabolism in rats fed a high-cholesterol diet. The results showed that *Lactobacillus acidophilus* CH5, *Lactobacillus casei* strain *Shirota* and *L. acidophilus* NCFM were able to deconjugate bile acids *in vitro* but the same species did not significantly alter the serum levels of cholesterol, triglycerides and bile acids of rats fed a high cholesterol diet, showing that the three probiotic species administered, although they were able to deconjugate bile acids, did not influence the lipid metabolism of the experimental animals.

Key words: Probiotic, functional food, health claim, bile salt hydrolase, lactic acid bacteria.

INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death worldwide. An estimated 25 million people will die from CVD, mainly from heart disease and stroke by 2030. Most CVD can be prevented by reducing the risk factors such as, unhealthy and high lipid diets (WHO, 2012).

There is conclusive evidences concerning the association between serum cholesterol concentrations and CVD, although, the strength of this association is still uncertain (Law et al., 1994a).

Epidemiological and experimental studies carried out between 1950 and 1990, were reviewed by Law et al. (1994b) in order to associate serum cholesterol levels and CVD. The results showed that a decrease in low density lipoprotein and total cholesterol concentration of 0.6 mmol/L (about 10%) in men is associated with a decrease in the risk of CVD of about 50% at the age of 40, 40% at 50, 30% at 60 and 20% at 70 and above. This has led to a growing search for new alternatives, especially those that may lower the cholesterol level leading to a reduction in morbidity and mortality from CVD.

The possible prophylactic and therapeutic effects of lactic acid bacteria (LAB) were studied in the early 20th century, when Metchnikoff (1908) suggested that life could be prolonged by regular consumption of fermented milks (Metchnikoff's longevity-without aging theory). However, the first study relating to intake of these bacteria leading to cholesterol reduction in humans appeared in 1974, when the authors observed a relation between high

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fermented milk consumption and reduction in serum cholesterol among the African Maasai. Besides their high-fat and cholesterol diet, these people also consumed large amounts of fermented milk. Although body weight increased, serum cholesterol decreased in the group consuming the fermented milk (Mann and Spoerry, 1974). Since then, further research has corroborated these initial observations relating fermented milk intake to cholesterolemia modulation.

Probiotics are defined by the World Health Organization (WHO) as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2006).

LAB with active bile salt hydrolase (BSH) have been suggested to lower cholesterol levels through interaction with the host bile salt metabolism (Kumar et al., 2012a). Lactobacilli with BSH activity have the advantage of surviving and colonizing the lower small intestine where the enterohepatic cycle takes place, and therefore BSH activity may be considered as an important colonization factor (Kumar et al., 2012b). The capacity of the intestinal microbiota to deconjugate bile acids through the enzyme BSH may influence cholesterol level by at least two mechanisms. Firstly, the deconjugated bile acids reduce lipid absorption, when compared with conjugated acids and may also precipitate cholesterol in the gastrointestinal tract (GIT) (Li, 2012). This may lower blood cholesterol levels due to lower cholesterol absorption in the intestine.

The second mechanism involves enterohepatic circulation. The majority of bile acids are reabsorbed in the terminal ileum. However, if they are deconjugated or dehydroxilated by the intestinal bacteria that convert the primary bile acids (cholic and chenodeoxycholic) into secondary bile acids (deoxycholic and lithocholic acids), this re-utilization will be reduced, leading to an increased bile acid excretion in the feces. To compensate for this loss, cholesterol will be used to synthetize primary bile acids in the liver and, consequently, blood cholesterol will be reduced (Begley et al., 2006). There is currently great interest in identifying the LAB strains that produce BSH enzyme so that the possible effects of bile salt deconjugation on animal cholesterolemia can be evaluated (Guo et al., 2012). The objective of the present study was to evaluate the effect of three *Lactobacillus* strains on lipid metabolism in rats fed a high-cholesterol diet.

MATERIALS AND METHODS

Culture origin and maintenance

All cultures were obtained from the stock collection of the Lactic Culture Laboratory at the Food Technology Department at the Federal University of Viçosa. The *L. acidophilus* CH5 was isolated from a commercial dairy starter culture (strain LA-5, Chr. Hansen Inc., Milwaukee, WI, USA), *L. casei* strain Shirota was isolated from the fermented milk product Yakult (Yakult Honsha Co., Ltd., Tokyo, Japan) and *L. acidophilus* NCFM was obtained originally from North Carolina State University.

The cultures were kept frozen at -80°C in sterile 12% nonfat milk solids (NFMS) and 50% glycerol solution at 20%. For complete activation, the cultures were thawed overnight under at 5°C and replicated three times in lactobacilli MRS broth (Difco Laboratories, Detroit, MI, USA) using a 1% inoculum and 20 h of incubation at 37°C and kept under refrigeration until the time of analysis.

Preparation of concentrated cultures

The *Lactobacillus* cultures were activated in 200 mL MRS broth in screw-top Erlenmeyer flasks and incubated at 37° for 20 h. After growth, the cultures were distributed in Nalgene sterilized screw-top polypropylene tubes (50 ml). They were centrifuged at 2.750 xg for 15 min, 4°C, in a Beckman GS-6R centrifuge. After discarding the supernatant, the cell concentrate from each culture was re-suspended in 50 mL sterile phosphate buffer (pH = 7.2) and centrifuged again, under the conditions previously described, and re-suspended in 10 mL sterilized NFMS thus constituting approximately 10^{10} UFC/mL cell concentrate that was plated on in MRS agar using the pour plate technique and incubated at 37°C in an anaerobic workstation (Bug box, RUSKINN, UK) for 48 h to confirm the number of viable lactobacilli cells.

BSH enzyme activity

The BSH enzyme was assessed following methodology reported by Walker and Gilliland (1993). Thirty-milliliter volumes of MRS broth containing 0.2% sodium thioglycolate (Merck, Darmstadt, Germany) and sodium taurocholate (Sigma Chemical Co., St. Louis, MO, USA) were inoculated (1%) with *Lactobacillus* cultures and incubated at 37°C under anaerobiosis (sodium thioglycolate was used as an oxygen scavenger).

One tube of each culture was removed at times of 0, 6 and 12 h in order to analyze the pH and to determine the optical density and the cholic acid deconjugate. A 1:10 dilution was made from each using sterile peptone diluent (1%), and the absorbance was measured at 620 nm using the Spectronic 20D+ spectrophotometer (Thermo Electron Scientific Instruments LLC, Madison, WI, USA) to determine the relative amounts of growth.

To measure the amount of free cholic acid liberated by each culture twenty milliliter volumes of each culture was used and adjusted to pH 7.0 with 1 N NaOH, which was adjusted to 25 mL with distilled water, and the cells were removed by centrifugation for 20 min at 8.000 xg at 1°C in a Beckman GS-6 series centrifuge (Beckman Instruments, Inc., Fullerton, CA, USA).

The supernatant (15 mL) was adjusted to pH 1 with HCl 10N and the volume completed to 24 mL with distilled water. Later, 3 mL of the solution were transferred to screw glass tubes and 9 mL ethyl acetate were added. The content of each tube was shaken and left to rest to separate the phases. Then 3 mL of the ethyl acetate phase (upper phase) were transferred to test tubes and evaporated at 60°C under continuous N_2 flow and 1 mL NaOH 0.1 N was added to each tube to dissolve the residue. Then, 6 mL H_2SO_4 16 N and 1 mL 1% furfuraldehyde were added. The tubes were shaken and heated to 65°C for 30 min, cooled to room temperature, 5 mL glacial acetic acid were added and then they were shaken. The reading was made in spectrophotometer at 660 nm and compared with a standard deconjugated cholic acid (Sigma Chemical Co., St. Louis, MO, USA) curve (1mg/mL), where the intensity of the color produced by the free cholic acid was directly proportional to its concentration in the sample. Results were expressed as micromoles of cholic acid per milliliter.

Rats and diets

Forty eight-weeks-old male adult Wistar rats were used. The ani-

Table 1. Composition of the experimental diets (g/100g).

Ingredient	Diet	
	Basal	High-cholesterol
Casein	14.00	14.00
Dextrinizedcornstarch	15.50	15.50
Sucrose	10.00	10.00
Soybeanoil	15.00	2.00
Fiber	5.00	5.00
Mineral mix	3.50	3.50
Vitaminmix	1.00	1.00
L-Cystine	0.18	0.18
Cholinebitartrate	0.25	0.25
Cornstarch	35.57	36.97
Cholesterol	-	0.60
Lard	-	11.0

mals were divided into five groups (n=8), according to their body weight, so that the mean initial body weight among the groups was the most homogenous as possible (±150 g). The animals were placed in individual cages, in a controlled temperature environment (24 ± 1° C) with 12 hour light and dark cycles (lights on 6:00 am to 6:00 pm). The animal study was conducted according to the guidelines for animal experiments, approved by the Brazilian College of Animal Experimentation.

The diets were prepared according to the standard diet for rodents AIN-93M (Rhoster Ltda, São Paulo, Brazil), as described by Reeves et al. (1993) (Table 1). One group (Basal) received the standard diet, while the other experimental groups were fed the hypercholesterolemic diet supplemented with 0.6% crystalline cholesterol (Sigma Chemical Co., St. Louis, MO, USA) as reported by Costa (1990). Afterwards, the diets were labeled, placed in plastic bags and stored under refrigeration at 5°C. Distilled water and the experimental diets were offered *ad libitum* throughout the 14 days of the experimental period.

Aliquots of 0.1 mL NFMS, with or without microorganisms, were administered orally with a micropipette once a day (6:00 to 7:00 pm) throughout the experimental period. The animals were held down until the completion of the aliquot swallow. Body weight and food intake were monitored weekly, and thus the weight gain and food efficiency ratio (FER) were calculated. At the end of the 14-day experiment, the animals were fasted for 12 h and then euthanized by carbon dioxide gas asphyxiation, followed by incision in the abdominal and thoracic cavities to collect blood by heart puncture. The blood was collected in disposable 10 mL syringes. The spleen and liver were removed under aseptic conditions, washed in 0.85% sterile saline solution, placed individually in sterile plastic bags (Whirl-Pak, Millipore sample bag - Nasco International, Inc., Fort Atkinson, WI, USA) and weighed.

Blood analysis

The rat blood samples were centrifuged at 1.500 xg f or 15 min to remove the serum. The Sigma Kit no 450-A (Sigma Chemical Co., St. Louis, MO, USA) was used for the serum dosage of the bile acids. The total cholesterol, HDL-cholesterol and triglycerides were determined by an enzymatic Kit (BioMérieux, Craponne, France). The cholesterol of the LDL + VLDL-C fractions were calculated by the difference between the total cholesterol and the HDL-C values.

Statistical analysis

A randomized block design was used for the statistical analysis of the data, that was carried out using the SAS statistical package (System for Statistical Analyses, NC, USA). The Duncan test was used to detect differences in the treatment effects (P<0.05).

RESULTS AND DISCUSSION

BSH enzyme activity

Table 2 shows that all the probiotic species tested had BSH enzyme activity, but the *L. acidophilus* CH5 and *L. casei* species presented significantly greater ability to deconjugate bile acids as compared to *L. acidophilus* NCFM (P<0.05). In a similar experiment, Liong (2006) observed that *L. acidophilus* and *L. casei* showed the greatest deconjugation capability when he studied 8 species of Lactobacilli, corroborating the results found in the present study.

Biological experiment

Table 3 shows the food intake, weight gain and food efficiency ratio of the animals that received the diets. The weight gain and FER of the animals receiving the high-cholesterol diet were greater than the animals receiving the base diet (P<0.05), probably as a consequence of their greater calorie intake, due to the greater energetic density of the high cholesterol diet. Administering probiotics to the animals receiving the high cholesterol diet did not alter the food intake, weight gain and FER among the experimental groups (P<0.05). Table 4 shows the spleen and liver weight of the experimental animals.

There was no significant difference in the liver or spleen weight index among the animals of the different treatments (P<0.05). However, assessment of the spleen of the animals receiving probiotic supplementation showed an increase of about 20%. This result shows the need to assess the bacterial translocation in this organ in experiments with probiotic intake, because the weight increase observed may show not only increase in spleen activity but also increase in translocated intestinal bacteria (Zhou et al., 2000; Machado et al., 2003). It was concluded that the species assessed presented the same profile regarding spleen weight.

Blood parameters

Table 5 shows the values for total cholesterol, HDL-C, VLDL+LDL-C, triglycerides and atherogenic index (AI = VLDL + LDL-C)/HDL-C) of the experimental animals, obtained after 14 days of administering different lactobacilli

A high fat and cholesterol diet raised the atherogenic index of the experimental animals (P<0.05), but did not

Table 2. Bile acid deconjugation of the experimental species at 0, 6 and 12 hours[a,b].

Group	Cholic acid released (µM/mL)[c]			Final pH[c]			Optical density[c]		
	0	6	12[d]	0	6	12[d]	0	6	12[d]
CH5	0.00	0.36	3.01a	6.36	5.04	4.75b	0.021	0.231	0.263a
NCFM	0.00	0.255	1.56b	6.38	4.91	4.26a	0.024	0.244	0.293a
Lc	0.00	0.00	3.00a	6.38	4.91	4.29a	0.019	0.143	0.193b

CH5: *L. acidophilus* CH5; NCFM: *L. acidophilus* NCFM; Lc: *L. casei strain Shirota*. [a]Results are expressed as mean; values are means of duplicates from two separate runs; [b]Deconjugation of taurine conjugated bile based on released of cholic acid. [c]MRS broth supplemented with 0.2% sodium taurocholate. [d]Means in the same column followed by different letters are significantly different by the Duncan test (P<0.05).

Table 3. Weight gain, food intake and food efficiency ratio (FER) of rats supplemented with different lactobacilli cultures[a].

Group	Weight gain(g/day)	Food intake(g/day)	FER (%)
Basal	3.99 ± 0.78a	18.10 ± 1.51a	22.05 ± 3.52a
HC	5.07 ± 0.76bA	18.48 ± 1.88aA	27.45 ± 4.28bA
CH5	4.88 ± 0.68A	18.10 ± 0.90A	26.94 ± 2.86A
NCFM	4.81 ± 0.91A	17.69 ± 1.23A	27.21 ± 3.89A
Lc	4.71 ± 0.46A	18.71 ± 0.40A	25.20 ± 2.26A

[a]Results are expressed as mean ± standard deviation; FER: weight gain/food intake x 100; Basal: AIN-93M diet; HC: basal diet + cholesterol + LDR; CH5: HC + *L. acidophilus* CH5; NCFM: HC + *L. acidophilus* NCFM; Lc: *L. casei strain Shirota*; Lowercase letter: Basal X HC; Uppercase letter: HC X *Lactobacillus* species; mean followed by different letters are significantly different by the Duncan test (P<0.05).

Table 4. Weight index of the organs of rats supplemented with different lactobacilli cultures[a].

Group	Weight index of the organs (g /kg body weight)	
	Liver	Spleen
Basal	49.75 ± 4.25aA	4.52 ± 1.41aA
HC	50.84 ± 4.04aA	4.26 ± 2.72aA
CH5	49.58 ± 4.10A	6.12 ± 2.05A
NCFM	52.16 ± 2.04A	6.27 ± 1.31A
Lc	50.49 ± 2.98A	6.55 ± 1.07A

[a]Results are expressed as mean ± standard deviation. Basal: AIN-93M diet; HC: basal diet + cholesterol + LDR; CH5: HC + *L. acidophilus* CH5; NCFM: HC + *L. acidophilus* NCFM; Lc: *L. casei strain Shirota.* Lowercase letter: Basal X HC; Uppercase letter: HC X *Lactobacillus* species; mean followed by different letters are significantly different by the Duncan test (P<0.05).

induce hypercholesterolemia as observed in the total cholesterol plasma concentrations (P>0.05). Although rats are resistant to developing hypercholesterolemia when submitted to a high fat and cholesterol diet (Nguyen et al., 1999; Xu et al., 2000), the results found here may have been influenced by the experimental period because rodent dietetic hypercholesterolemia induced in different time periods has shown varied responses. For example, in a similar experiment, but applied for 28 days, Costa (1990) induced hypercholesterolemia in rats using the same diet whereas Taranto et al. (2000) observed a significant increase in the cholesterol plasma concentration in mice after seven days experiment using a similar diet.

Machado et al. (2003) reported that cholesterol and cholic acid supplementation in rat diet was more efficacious for cholesterol accumulation in the liver, increasing its weight rather than the cholesterol blood level. However, this was not the case in the present study, although there was significant increase in the serum cholesterol levels in the experimental animals (P< 0.05) no increase was observed in liver weight. Administering probiotics to the experimental animals did not alter (P>0.05) the blood parameters (total cholesterol, triglycerides, HDL-C, VLDL-LDL-C and the atherogenic index) among the experimental groups.

Figure 1 shows that the concentration of serum bile acids in the animals fed the high cholesterol diet were lower than those found in the animals that received the basal diet (P<0.05). This difference may have resulted from the decrease in the intestinal reabsorption of the bile acids by the hepatic circulation in the animals fed the high cholesterol diet, corroborating a similar study by Xu et al. (2000).

Comparison of the groups that received the high-cholesterol diet showed that there was no difference between the control and the animals that received probiotics (P>0.05), which indicates that the microorganisms administered did not alter the intestinal reabsorption of these

Table 5. Total cholesterol, HDL, VLDL+LDL-C serum triglycerides (mg/dL) and atherogenic index (AI) of rats supplemented with different lactobacilli cultures[a].

Group	Total cholesterol	HDL-C	[1]VLDL±LDL-C	Triglycerides	[2]AI
Basal	97.63 ± 10.04a	48.64 ± 6.81a	48.99 ± 7.26a	140.96 ± 50.59a	1.02a
HC	106.190 ± 24.36aA	44.34 ± 5.39aA	61.85 ± 20.81aA	118.96 ± 30.17aA	1.38bA
CH5	122.20 ± 30.24A	41.23 ± 5.00A	80.97 ± 27.53A	134.73 ± 27.22A	1.96A
NCFM	121.88 ± 17.47A	44.76 ± 3.44A	77.11 ± 14.77A	154.55 ± 40.66A	1.72A
Lc	109.36 ± 10.54A	40.93 ± 6.05A	68.44 ± 7.68A	132.88 ± 20.44A	1.70A

[a]Results are expressed as mean ± standard deviation; [1]VLDL + LDL-C: cholesterol total – HDL-C; [2]AI: (VLDL + LDL-C)/HDL-C; Basal: AIN-93M diet; HC: basal diet + cholesterol + LDR; CH5: HC + *L. acidophilus* CH5; NCFM: HC + *L. acidophilus* NCFM; Lc: *L. casei strain Shirota*; Lowercase letter: Basal X HC; Uppercase letter: HC X *Lactobacillus* species; mean followed by different letters are significantly different by the Duncan test (P<0.05).

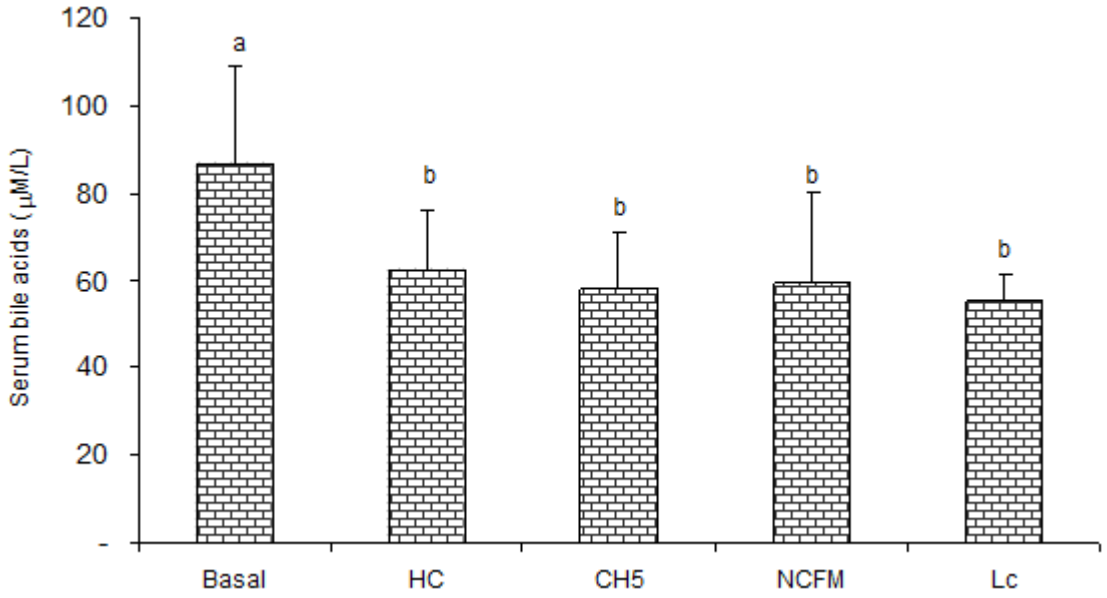

Figure 1. Serum bile acids of rats supplemented with different lactobacilli cultures. Basal: AIN-93M diet; HC: basal diet + cholesterol + LDR; CH5: HC + *L. acidophilus* CH5; NCFM: HC + *L. acidophilus* NCFM; Lc: *L. casei strain Shirota*; Lowercase letter: Basal X HC; Uppercase letter: HC X *Lactobacillus* species; mean followed by different letters are significantly different by the Duncan test (P<0.05).

acids.

In a similar experiment, Usman and Hosono (2000) studied supplementing the diet of hypercholesterolemic rats with different *Lactobacillus gasseri* strains and observed reduction in the serum bile acid concentration only in the animals that presented increased fecal excretion of these acids. The authors attributed the effects observed to the increase in the intestinal bile acid deconjugation rate promoted by the ingestion of the probiotic species. From this report, although the concentration of fecal bile acids was not assessed in the present study, it is suggested that the bile acid plasmatic concentration may have been maintained in the experimental animals because the percentage of bile acids in the animals´ feces was not altered. The ingestion of different *Lactobacillus* species in the current rat study did not seem to increase the intestinal deconjugation rate of these acids, even though the cultures did deconjugate the bile acids *in vitro*.

Guo et al. (2012) and Kumar et al. (2012), based on their *in vitro* studies, stated that the species that have BSH enzyme activities are candidates for lowering cholesterol. However, caution should be taken because in the present study the results obtained *in vitro* were not observed *in vivo*. Studies that reviewed the hypocholesterolemic effect of probiotic bacteria reported that their effect on cholesterol metabolism is not conclusive (De Roos and Katan, 2000). Recently, Li (2012) reported that more attention should be paid on the effects of probiotic on intestinal host health. The cholesterol removing capacity of probiotics as a quasi-endogenous lipid lowering mechanism is of continuing concern. Although, *in vitro* experiments and corresponding theories have become

more complete and mature, animal and human studies are still inconclusive.

Conclusions

The three species administered, although they were capable of deconjugating bile acids in the *in vitro* study, did not influence the lipid metabolism of the experimental animals. The hypolipidemic effect of probiotics demands further investigations in animal models more susceptible to develop hypercholesterolemia. Moreover, human studies would provide information on the dose necessary to promote the health benefits of probiotic bacteria in reducing the risk of non communicable diseases.

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