Antihypertensive and hypolipidemic effect of milk fermented by specific *Lactococcus lactis* strains

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ABSTRACT

The antihypertensive and hypolipidemic effects of milk fermented by specific *Lactococcus lactis* strains in spontaneously hypertensive rats (SHR) were investigated. The SHR were fed ad libitum milk fermented by *Lc. lactis* NRRL B-50571, *Lc. lactis* NRRL B-50572, Captopril (40 mg/kg of body weight, Sigma-Aldrich Co., St. Louis, MO) or purified water for 4 wk. Results suggested that *Lc. lactis* fermented milks presented a significant blood pressure-lowering effect. No significant difference was noted among milk fermented by *Lc. lactis* NRRL B-50571 and Captopril by the second and third week of treatment. Additionally, milk fermented by *Lc. lactis* strains modified SHR lipid profiles. Milk fermented by *Lc. lactis* NRRL B-50571 and B-50572 were able to reduce plasma low-density lipoprotein cholesterol and triglyceride contents. Thus, milk fermented by *Lc. lactis* strains may be a coadjuvant in the reduction of hypertension and hyperlipidemia and may be used as a functional food for better cardiovascular health.

Key words: *Lactococcus lactis*, fermented milk, antihypertensive effect, hypolipidemic effect

INTRODUCTION

Coronary heart disease (CHD) is the single largest cause of death in developed countries (Chobanian et al., 2003). Hypertension and elevated blood cholesterol levels, particularly high low-density lipoprotein cholesterol (LDL-C), are 2 of the major modified risk factors for the development of CHD (Department of Health and Human Services, 2000).

Dairy proteins are potential ingredients in functional foods, as they may act as precursors of bioactive peptides with different physiological benefits on the human body (Korhonen, 2009). One way to increase the amount of bioactive peptides in dairy products is by milk fermentation with highly proteolytic strains of lactic acid bacteria (LAB; López-Fandiño et al., 2006) or by enzymatic hydrolysis with proteases from different sources (Tavares et al., 2011, 2012). Lactic acid bacteria present specific proteolytic systems for the generation of free AA and free peptides as a source of nitrogen required for their growth (Hugenholtz, 2008). Furthermore, peptides isoleucine-proline-proline and valine-proline-proline produced by *Lactobacillus helveticus* (LBK 16H strain) presented an antihypertensive effect in spontaneously hypertensive rats (SHR) after a long-term oral administration of the fermented milk (Sipola et al., 2002). Similarly, it has been shown that peptides released by *Enterococcus faecalis* strains from milk proteins were able to decrease arterial blood pressure in SHR (Muguerza et al., 2006; Quirós et al., 2007). However, to the best of our knowledge, the beneficial health effects of peptides in fermented milk with *Lactococcus lactis* strains have not been explored.

Lactococcus lactis is one of the most important LAB, as it generally takes the place of commercial starter cultures used in the manufacture of fermented dairy products (Odamaki et al., 2011). *Lactococcus lactis* strains are able to improve the organoleptic characteristics of dairy products, as they are responsible for the formation of volatile aroma compounds (Ayad, 2009). In fact, we were able to characterize *Lc. lactis* strains isolated from nature that produce characteristic cheese-like and yogurt-like aroma profiles in fermented milk (Gutiérrez-Méndez et al., 2008). Additionally, our research group demonstrated that *Lc. lactis* strains NRRL B-50571 and NRRL B-50572 were able to release several novel angiotensin I-converting enzyme-inhibitory peptides in fermented milks (Rodríguez-Figueroa et al., 2012b).

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Furthermore, we also demonstrated that whey fractions of these fermented milks showed an acute antihypertensive and heart rate-lowering effect in SHR after receiving a single dose (Rodríguez-Figueroa et al., 2012a).

The use of animal models to study the effect of food derived substances on CHD has been quite accepted nowadays. An SHR is characterized as presenting hyperlipidemia, hypertension, hyperinsulinemia, and type 2 diabetes (Brown et al., 2011). These characteristics made SHR one of the most useful models, besides the similarity of the pathologies mechanisms in humans (Doggrell and Brown, 1998). Therefore, several studies have been focused on the evaluation of antihypertensive and hypolipidemic effects of functional foods in SHR (Manoso et al., 2008) and human subjects (Turpeinen et al., 2009; Pal et al., 2010). However, the antihypertensive and hypolipidemic effects of long-term consumption of fermented milk with specific *Lc. lactis* strains have not been tested. Thus, the objective of this research was to evaluate the antihypertensive and hypolipidemic effects of milk fermented by specific *Lc. lactis* strains through a long-term study with SHR.

MATERIALS AND METHODS

Lc. lactis Strains

Two *Lc. lactis* strains, NRRL B-50571 and NRRL B-50572, obtained from the Dairy Laboratory collection at Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD, Hermosillo, Sonora, Mexico) were deposited at the Agricultural Research Service Culture Collection (ARS, also known as the NRRL) from the US Department of Agriculture (Peoria, IL). The strains were propagated in 10 mL of sterile lactose (5 g/L) M17 broth (Difco, Sparks, MD) and incubated at 30°C for 24 h. Fresh precultures were obtained by repeating the same procedure allowing *Lc. lactis* strains to reach 10^6 to 10^7 cfu/mL. The bacterial cell population was estimated by the pour-plate method using M17 agar containing lactose (5 g/L; Difco). To adapt bacteria to milk fermentation conditions, *Lc. lactis* working cultures

were prepared by inoculating (1%) fresh precultures in sterile (100°C, 20 min) nonfat dry milk reconstituted (10%, wt/wt) and incubated at 30°C for 12 h.

Sample Preparation

An aliquot (3%) of each working culture was obtained as described above and added to sterile skim milk to obtain the different fermented milk batches. Incubation was performed at 30°C over 48 h. Then, the fermentation process was stopped by heating at 98°C for 10 min to inactivate proteases and *Lc. lactis* strains (Guan-Wen et al., 2007); subsequently, samples were frozen at -20°C. All fermented milk samples were unfrozen and homogenized daily (model 4169, Braun, Barcelona, Spain) for 20 min before use. Total protein (method 960.52; AOAC International, 2000), calcium, magnesium, potassium (EPA 3052), total fat (method 989.04; AOAC International, 2000), and lactose (method 930.28; AOAC International, 2000) contents were evaluated (Table 1).

In Vivo Experimental Protocol

Thirty-two male SHR were obtained from Harlan Laboratories Inc. (Indianapolis, IN). The rats were randomly housed in pairs per cage at $21 \pm 2^\circ\text{C}$ with 12-h light and dark cycles, $52 \pm 6\%$ relative humidity, and with ad libitum intake of a standard diet (Teklad, Harlan Laboratories) during the experiment. Spontaneously hypertensive rats (27–28 wk old and 355 ± 24 g of BW) were randomly split into 4 groups, each group included 8 animals ($n = 8$). The first and second groups served as negative and positive controls and received purified water and Captopril (a proven hypotensive drug, 40 mg/kg of BW, Sigma-Aldrich Co., St. Louis, MO), respectively. The third and fourth groups were administered with milk fermented by *Lc. lactis* NRRL B-50571 and milk fermented by *Lc. lactis* NRRL 50572, respectively. All SHR had free access to each treatment during the 3 wk as part of the protocol. Half of the animals of each group were killed at the end of that

Table 1. Nutritional composition of milks fermented by *Lactococcus lactis* NRRL strains

Item	B-50571	B-50572	Food ¹
Energy (kcal/100 g)	62.4 $\pm$ 0.5	65.6 $\pm$ 0.3	307
Protein (g/100 g)	3.1 $\pm$ 0.2	3.3 $\pm$ 0.1	18.6
Fat (g/100 g)	ND ²	ND	6.2
Carbohydrate (g/100 g)	12.5 $\pm$ 0.3	13.1 $\pm$ 0.2	44.2
Calcium (mg/100 g)	368 $\pm$ 11.3	366 $\pm$ 3.0	1000
Potassium (mg/100 g)	439 $\pm$ 14.6	434 $\pm$ 5.2	600
Magnesium (mg/100 g)	32.2 $\pm$ 1.2	31.1 $\pm$ 0.3	200

¹Data according to Teklad Global 18% protein rodent diet, Harlan Laboratories (Indianapolis, IN).

²ND = not detected.

period. Meanwhile, the rest of the SHR only received purified water during one more week before being killed to determine a possible extended effect after cessation of the treatments. A research animal protocol was followed according to the guidelines established by the institutional ethics committee (CIOMS, 1985).

Antihypertensive Effect Measurements

The lowering blood pressure effect of milk fermented by specific *Lc. lactis* strains on SHR was monitored over time. Animals were deposited in restrainers in the warming chamber for 20 min at 32°C to detect pulsations through the caudal artery. Systolic (SBP) and diastolic (DBP) blood pressures were measured 5 times on each conscious animal before treatments and every week during the experiment. Measurements were obtained using the tail-cuff method between 9 and 12 h to eliminate circadian cycles. The noninvasive blood pressure system used in this experiment included a photoelectric sensor, an amplifier, an automatic inflation cuff, and software (model 229, IITC Life Science Inc., Woodland, CA).

Plasma Lipid Profile Evaluation

The hypolipidemic activity of milk fermented by specific *Lc. lactis* strains were also evaluated in SHR. Blood samples were collected under anesthesia by cardiac puncture in tubes with heparin (Sarstedt AG & Co., Nümbrecht, Germany). Subsequently, samples were centrifuged at $756 \times g$, 4°C for 10 min to obtain the plasma and they were frozen at -20°C for further studies. Triglycerides (TG), total cholesterol, and high-density lipoprotein cholesterol levels in plasma were determined by a commercial kit (Randox Laboratories, Kearneysville, WV), whereas LDL-C was calculated by the formula $LDL = TC/1.19 + TG/1.9 - HDL/1.1 - 38$, as reported by Ahmadi et al. (2008).

Statistical Analysis

Normality of experimental data was evaluated as a prerequisite for the analysis by one-way ANOVA. Differences between means were assessed by the Tukey-Kramer multiple-comparison test and they were considered significantly different when $P < 0.05$. Results were processed by the NCSS 2007 statistical program (NCSS Inc., Kaysville, UT). Data were presented as means $\pm$ SEM.

RESULTS AND DISCUSSION

Nutritional composition corresponding to fermented milks is presented in Table 1. Milk with *Lc. lactis*

NRRL B-50571 was not significantly different ($P > 0.05$) from milk with *Lc. lactis* NRRL B-50572. One of the most important macronutrients in fermented milk, which is related to the antihypertensive activity, may be the proteins. They eventually could be hydrolyzed by the proteolytic and peptidolytic systems of *Lc. lactis* strains to form hypotensive peptides (López-Fandiño et al., 2006). In fact, it was previously shown that the whey isolated from these fermented milk administered to SHR in a single dose was the fraction responsible for the blood pressure-lowering effect (Rodríguez-Figueroa et al., 2012a).

Conversely, the presence of calcium in dairy products has been associated to an antihypertensive effect (Jäkälä et al., 2009). However, only diets including $>2.5\%$ of calcium reduce arterial blood pressure (Civantos and Aleixandre, 2004). In this study, the calcium contents in milk fermented by *Lc. lactis* NRRL B-50571 and *Lc. lactis* NRRL B-50572 were 368 ± 11.3 and 366 ± 3.0 mg/100 g, respectively, which is equivalent to only 0.81 and 0.83% in the diet. Thus, the effect of calcium could be disregarded.

Before the experiment, SHR (27–28 wk old) SBP and DBP were 226 ± 3.2 and 180 ± 4.5 mmHg, respectively. Both *Lc. lactis* fermented milks were able to reduce blood pressure during the experiment (Figure 1 and 2). Results did not show significant differences ($P > 0.05$) between SBP measurements in the first week (Figure 1). However, by the second week, the SBP level was 20.2 mmHg lower in SHR that received milk fermented by *Lc. lactis* NRRL B-50571 than the group that received water (233.3 ± 1.8 vs. 253.5 ± 2.8 mmHg) and was not statistically different ($P > 0.05$) from those that received Captopril (223.8 ± 5.2 mmHg). In fact, by the second and third week, SHR treated with Captopril or milks fermented by *Lc. lactis* NRRL B-50571 presented a marked lowering effect on SBP. By the fourth week of treatment, SBP level was 23.3 mmHg lower in SHR that received milk fermented by *Lc. lactis* NRRL B-50571 than the group that received water (234.3 ± 1.9 vs. 257.6 ± 6.4 mmHg), and was not statistically different ($P > 0.05$) from those that received Captopril (229.5 ± 7.1 mmHg).

As observed in Figure 1, the SBP-lowering effect in SHR treated with milk fermented by *Lc. lactis* NRRL B-50571 increased with time when compared with SHR administered with water. Indeed, the maximal SBP reduction was found by the fourth week, even though animals drank only water in the last week. Thus, these results suggested an extended SBP-lowering effect after cessation of the treatment.

Diastolic blood pressure was also monitored as part of the experimental procedure. SHR treated with milk fermented by *Lc. lactis* NRRL B-50571 and B-50572

Figure 1. Systolic blood pressure (SBP) of spontaneously hypertensive rats subjected to different treatments. *Lactococcus lactis* NRRL B-50571 fermented milk (FM; ■); *L. lactis* NRRL B-50572 FM (●); Captopril (Sigma-Aldrich Co., St. Louis, MO) = positive control (▲); purified water = negative control (▼). Data are shown as mean values $\pm$ SEM ($n = 8$). Data points sharing the same letter (a–c) within a week are not significantly different ($P > 0.05$). Color version available in the online PDF.

Figure 2. Diastolic blood pressure (DBP) of spontaneously hypertensive rats subjected to different treatments. *Lactococcus lactis* NRRL B-50571 fermented milk (FM; ■); *L. lactis* NRRL B-50572 FM (●); Captopril (Sigma-Aldrich Co., St. Louis, MO) = positive control (▲); purified water = negative control (▼). Data are shown as mean values $\pm$ SEM ($n = 8$). Color version available in the online PDF.

presented a DBP-lowering effect during the experiment (Figure 2). As in SBP, the first week, DBP measurements were not significantly different ($P > 0.05$) between treatments. However, by the second week, the DBP level was 24.5 mmHg lower in SHR that received milk fermented by *L. lactis* NRRL B-50571 than the group that received water (207.2 ± 2.0 vs. 231.7 ± 3.2 mmHg) and was not statistically different ($P > 0.05$) from those that received Captopril (193.2 ± 5.3 mmHg). Furthermore, by the third experimental week, the DBP-lowering effect was not significantly different ($P > 0.05$) between SHR treated with Captopril and milk fermented by *L. lactis* NRRL B-50571 or B-50572. The most important DBP reduction, 49.8 mmHg, was observed in the fourth week of treatment in SHR that received milk fermented by *L. lactis* NRRL B-50571 against those that received water (184.3 ± 4.6 vs. 234.1 ± 2.6 mmHg).

These results were similar to those reported by others, who found that milk fermented by *Lactobacillus helveticus* LBK16H was able to reduce SBP by 21 mmHg (Sipola et al., 2002). Also, it was shown that the milk tetrapeptide α -lactorphin reduced SBP and DBP by 23 ± 4 and 17 ± 4 mmHg, respectively, after being administered to SHR (Nurminen et al., 2000). Additionally, Jauhiainen et al. (2007) demonstrated the presence of a radiolabelled analog of the milk-derived

antihypertensive peptide isoleucine-proline-proline in several rat tissues 48 h after a single oral administration. Therefore, fermented milk containing antihypertensive peptides administered for long periods may extend their bioactivity even after cessation of the treatment.

Results suggested that fermented milks were able to modify SHR lipid profiles by the third week of treatment. Spontaneously hypertensive rats that received milk fermented by *L. lactis* NRRL B-50571 presented an LDL-C of 27.1 mg/dL, which is 8.4 mg/dL lower than the LDL-C of SHR administered with water (35.5 mg/dL; Table 2). Similarly, SHR that received milk fermented by *L. lactis* NRRL B-50572 presented an LDL-C of 13.6 mg/dL, which is 21.9 mg/dL lower than the LDL-C of SHR administered with water (35.5 mg/dL; Table 2). Furthermore, LDL-C values of SHR administered with fermented milks were significantly lower ($P < 0.05$) than those for SHR administered with Captopril (Table 2).

On the other hand, high-density lipoprotein cholesterol values were not significantly different ($P > 0.05$) in SHR administered with fermented milk or water or Captopril (Table 2). According to the National Cholesterol Education Program Adult Treatment Panel III, it is a priority to reduce LDL-C as a primary target to reduce the risk of heart disease and as a secondary target to reduce the risk of metabolic syndrome (Expert

Table 2. Effects of fermented milks on serum lipids of spontaneously hypertensive rats¹

Item	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
<i>Lactococcus lactis</i> NRRL B-50572	73.7 ± 1.8	25.7 ± 1.5 ^a	26.2 ± 1.6	13.6 ± 0.8 ^a
<i>Lactococcus lactis</i> NRRL B-50571	75.2 ± 1.9	47.0 ± 3.0 ^b	25.1 ± 1.8	27.1 ± 5.2 ^{ab}
Captopril ²	76.1 ± 3.2	68.1 ± 13.1 ^c	28.6 ± 2.6	35.8 ± 3.9 ^b
Water	76.5 ± 2.1	62.0 ± 6.8 ^c	25.8 ± 2.0	35.5 ± 3.6 ^b

^{a-c}Values within a column followed by different superscript letters are significantly different ($P < 0.05$).

¹Data presented as mean ± SEM; TC = total cholesterol; TG = triglycerides; HDL-C = high-density lipoprotein-cholesterol; LDL-C = low-density lipoprotein-cholesterol.

²Sigma-Aldrich Co., St. Louis, MO.

Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001).

Spontaneously hypertensive rats that received milk fermented by *Lc. lactis* NRRL B-50571 presented a plasma TG content of 47.0 mg/dL, which is 15 mg/dL lower than the TG content of SHR administered with water (62.0 mg/dL; Table 2). Similarly, SHR that received milk fermented by *Lc. lactis* NRRL B-50572 presented a plasma TG content of 25.7 mg/dL, which is 36.3 mg/dL lower than the TG content of SHR administered with water (62.0 mg/dL; Table 2). Furthermore, TG values of SHR administered with fermented milks were significantly ($P < 0.05$) lower than those for SHR administered with Captopril (Table 2). Additionally, plasma total cholesterol content was slightly reduced in SHR that received fermented milk, although differences were not significantly different ($P > 0.05$; Table 2).

It has been reported that milk supplemented with whey protein concentrate and fermented by *Lactobacillus casei* TMC0409 and *Streptococcus thermophilus* TMC1543 decreased serum TG from 151.7 ± 13.9 to 115.7 ± 11.1 mg/dL in humans after 4 wk of treatment (Kawase et al., 2000). Even though lipid profiles of SHR treated with fermented milks were also evaluated after 1 wk treatment with purified water, no significant difference was observed among treatments ($P > 0.05$). Thus, these results suggest that no extended hypolipidemic-lowering effect exists after cessation of the treatment.

It has been reported that nongrowing and dead cells of specific strains of the genus *Lactococcus* could remove cholesterol via binding of cholesterol to the cellular surface (Kimoto-Nira et al., 2007). These authors suggested that cholesterol was bound to the bacterial cells and this was a result of the chemical and structural properties of their cell wall peptidoglycans, which contain several AA that ease the attachment of cholesterol to the cells (Kimoto-Nira et al., 2007). Therefore, as *Lc. lactis* cells were killed during the pasteurization treatment applied to fermented milk, the cholesterol lowering effect of this study may be attributed to cholesterol

removal by attachment to cells, however, this remains to be determined. However, the lowering effect on LDL-C due to the ingestion of dairy protein or peptides present in fermented milk could not be disregarded. It has been reported that obese and overweight individuals who consume whey protein for 12 wk presented an important reduction in LDL-C (Pal et al., 2010).

CONCLUSIONS

Milks fermented by specific *Lc. lactis* strains were able to reduce systolic SBP and DBP in an SHR. Moreover, the SHR lipid profile was improved by fermented milk intake for 3 wk, although the mechanism for this effect remains to be determined. The use of milk fermented by specific LAB may be considered as a coadjuvant for the improvement of cardiovascular health. To the best of our knowledge, this is the first in vivo study that showed the antihypertensive and hypolipidemic effects of continuous consumption of fermented milk with specific *Lc. lactis* strains. Thus, dairy products fermented with *Lc. lactis* strains NRRL B-50571 and NRRL B-50572 may be used as functional foods with potential benefits for cardiovascular health.

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