Hypotensive and heart rate-lowering effects in rats receiving milk fermented by specific \textit{Lactococcus lactis} strains

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Abstract
Previous studies have demonstrated that milk fermented by specific \textit{Lactococcus lactis} strains significantly inhibits the activity of angiotensin I-converting enzyme (ACE). However, the relationship between the ACE inhibitor and its \textit{in vivo} action has revealed discrepancies. Therefore, the aim of the present study was to investigate the antihypertensive and heart rate (HR)-lowering effect of milk fermented by specific \textit{L. lactis} in a murine model. Spontaneously hypertensive male rats (271 (SD 14) g) were randomised into four treatment groups that were orally administered with milk fermented by \textit{L. lactis} NRRL B-50 571 or \textit{L. lactis} NRRL B-50 572 at 35 or 50 mg protein/kg body weight (BW), respectively. Further, two more groups were fed with different solutions as controls: a saline solution as the negative control and Captopril™ (40 mg/kg BW), a proven ACE inhibitor, as the positive control. Blood pressure and HR were monitored by the tail-cuff method before the treatments and at 2, 4, 6 and 24 h post-oral administration. The results demonstrated that milk fermented by \textit{L. lactis} NRRL B-50 571 as well as by \textit{L. lactis} NRRL B-50 572 presented an important systolic and diastolic blood pressure- and HR-lowering effect. Thus, milk fermented by specific \textit{L. lactis} strains may present potential benefits in the prevention and treatment of CVD associated with hypertension in humans.

Key words: \textit{Lactococcus lactis}: Fermented milk: Antihypertensive effects: Functional food: Spontaneously hypertensive rats

Hypertension has become a serious health problem, which has been considered an important CVD risk factor, especially in developing countries\textsuperscript{(1)}. The long-term regulation of blood pressure is associated with the rennin–angiotensin system. The conversion of angiotensin I into angiotensin II, a potent vasoconstrictor octapeptide, by the angiotensin-converting enzyme (ACE, \textit{EC} 3.4.15.1) has long been known\textsuperscript{(2)}. Hence, the inhibition of this enzyme can reduce high arterial blood pressure through ACE-inhibitory compounds.

Blood pressure is monitored by systolic blood pressure (SBP) and diastolic blood pressure (DBP). Brachial SBP is overall the best predictor of future cardiovascular risk for the entire hypertensive population. However, DBP must be measured in order to calculate pulse pressure, which has become a surrogate marker of central elastic large artery stiffness and a useful predictor of cardiovascular risk in the elderly population\textsuperscript{(3,4)}. On the other hand, heart rate (HR) is an important determinant of myocardial oxygen consumption and cardiac work. Several experimental lines of research have considered HR as an important risk factor for atherosclerosis. In fact, HR reduction may represent an important strategy for the treatment of patients with a wide range of cardiovascular disorders\textsuperscript{(5)}. Therefore, SBP, DBP and HR measurements give a wide view related to cardiovascular disorders. Because of the high prevalence of hypertension and serious health consequences, lifestyle modifications, including dietary interventions, are recommended to help prevent and treat hypertension\textsuperscript{(6)}. Research with hypertensive animals\textsuperscript{(7)} and human subjects\textsuperscript{(8)} have indicated that milk peptides derived from casein and whey may have a hypotensive effect.

Milk proteins have received increased attention as potential ingredients in health-promoting functional foods. It is accepted that proteins from milk may act as precursors of biologically active peptides with different physiological effects on digestive, endocrine, cardiovascular, immune and nervous systems\textsuperscript{(9)}. Indeed, it has been reported that an effective way to increase the amount of bioactive peptides in dairy products

Abbreviations: ACE, angiotensin I-converting enzyme; BW, body weight; DBP, diastolic blood pressure; HR, heart rate; NRRL B-50 571-3, milk fermented by \textit{Lactococcus lactis} NRRL B-50 571 (35 mg protein/kg body weight); NRRL B-50 572-3, milk fermented by \textit{Lactococcus lactis} NRRL B-50 572 (35 mg protein/kg body weight); NRRL B-50 571-5, milk fermented by \textit{Lactococcus lactis} NRRL B-50 571 (50 mg protein/kg body weight); NRRL B-50 572-5, milk fermented by \textit{Lactococcus lactis} NRRL B-50 572 (50 mg protein/kg body weight); SBP, systolic blood pressure; SHR, spontaneously hypertensive rat.

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is by milk fermentation with highly proteolytic strains of lactic acid bacteria(10). Lactic acid bacteria growth in milk is dependent on specific proteolytic and peptidolytic systems for the generation of free amino acids and free peptides as a source of N(11). In fact, several studies have suggested that peptides released by Enterococcus faecalis strains from milk proteins were able to decrease arterial blood pressure in spontaneously hypertensive rats (SHR)(7,12).

L. lactis is one of the most well-studied lactic acid bacteria because of its importance as part of commercial starter cultures used in the manufacture of fermented dairy products(13). It has been reported that L. lactis strains are able to improve the organoleptic characteristics of dairy products(14). Previous studies in our laboratory have shown that specific L. lactis strains isolated from native ecosystems were able to produce remarkable aroma profiles in fermented milk(15). In addition, milk fermented by these specific strains was able to inhibit ACE activity. However, the antihypertensive effects of milk fermented by these specific L. lactis strains have not been investigated. Therefore, the objective of the present study was to evaluate the blood pressure- and HR-lowering effect of milk fermented by specific L. lactis strains in an animal model.

Materials and methods

Strains and growth conditions

L. lactis strains (NRRL B-50 571 and NRRL B-50 572) were deposited at the Agricultural Research Service Culture Collection (NRRL) of the National Center for Agricultural Utilization Research (US Department of Agriculture Peoria, IL). Strains were routinely propagated in 10 ml of sterile lactose (5 g/l) M17 broth (DIFCO) and incubated at 30°C for 24 h. Fresh cultures were obtained by repeating the same procedure. The initial starter culture of each L. lactis strain reached 10^6–10^7 colony-forming units/ml as enumerated on M17 agar supplemented with lactose (5 g/l).

Manufacturer of fermented milk samples

Organic grade A non-fat dry milk from Organic Valley® was reconstituted in purified water at 10% (w/w) and sterilised (100°C, 20 min). Every single specific L. lactis strain was inoculated with a loop in sterilised milk with an initial bacterial population of 7–8 log colony-forming units/ml as pre-cultures. The inoculated milk was incubated for 12 h at 30°C. Pre-cultures were added (3%, v/v) to the sterilised milk to obtain the different 3-litre fermented milk batches. Incubation was performed at 30°C and stopped at 48 h by pasteurisation at 75°C for 1 min to obtain the samples. Samples of specific L. lactis-fermented milk for the single-dose bioassay were obtained by centrifugation at 20000 g (J2-21 rotor; Beckman) for 10 min at 0°C. The supernatants were collected and lyophilised with a freeze-dryer (Labconco). All samples were kept at 4°C until use for further analysis. Total protein (Method 960.52 Official Method of Analysis of the Association of Official Analytical Chemists (AOAC), 1998), Ca, Mg, K (EPA 3052), total fat (Method 942.05 AOAC, 1998) and lactose (Method 930.28 AOAC, 1998) contents of the lyophilised whey were evaluated (Table 1).

Experimental protocol with spontaneously hypertensive rats

A total of forty-two male SHR (4–5 weeks old, 72 (SD 7) g body weight (BW)) were obtained from Harlan Laboratories, Inc. SHR were weaned for 8 weeks and their SBP monitored during this period. The rats were randomly housed in pairs per cage at 21 ± 2°C, 12 h light–dark cycles and 52 ± 6% relative humidity, with an ad libitum intake of a standard diet (Teklad; Harlan Laboratories, Inc.) and purified water. SHR (12–13 weeks old, 271 (SD 14) g BW) were divided into six groups of seven rats (n 7): oral administration of saline as the negative control and Captopril™ (a proven hypotensive drug, 40 mg/kg BW) as the positive control. The animals were weighed before oral administration in order to prepare the corresponding amount of lyophilised whey/kg animal weight. The lyophilised whey fractions of milk fermented by L. lactis NRRL B-50 572 or NRRL B-50 571 were dissolved in 0.8 ml of saline. The treatments were NRRL B-50 572-3 (35 mg protein/kg BW), NRRL B-50 572-5 (50 mg protein/kg BW), NRRL B-50 571-3 (35 mg protein/kg BW) and NRRL B-50 571-5 (50 mg protein/kg BW).

Conscious SHR received a single dose through a cannula between 08.30 and 09.30 hours to eliminate circadian cycles. The animals were restrained in the warming chamber for 20 min at 32°C to detect pulsations through the caudal artery. SBP, DBP as well as HR were monitored before

Table 1. Chemical composition of the whey fractions obtained from milk fermented by specific Lactococcus lactis strains

<table>
<thead>
<tr>
<th></th>
<th>NRRL B-50 571-3</th>
<th>NRRL B-50 572-3</th>
<th>NRRL B-50 571-5</th>
<th>NRRL B-50 572-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/dosage)</td>
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<td>35 (SD)</td>
<td>50 (SD)</td>
<td>50 (SD)</td>
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<tr>
<td>Fat (g/dosage)</td>
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<td>3 (SD)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lactose (mg/dosage)</td>
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<td>1310 (SD)</td>
<td>1790 (SD)</td>
<td>1870 (SD)</td>
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<tr>
<td>Ca (mg/dosage)</td>
<td>3.7 (SD)</td>
<td>3.6 (SD)</td>
<td>5.3 (SD)</td>
<td>5.23 (SD)</td>
</tr>
<tr>
<td>K (mg/dosage)</td>
<td>4.4 (SD)</td>
<td>4.3 (SD)</td>
<td>6.3 (SD)</td>
<td>6.2 (SD)</td>
</tr>
<tr>
<td>Mg (mg/dosage)</td>
<td>0.3 (SD)</td>
<td>0.3 (SD)</td>
<td>0.5 (SD)</td>
<td>0.4 (SD)</td>
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</tbody>
</table>

NRRL B-50 571-3, milk fermented by L. lactis NRRL B-50 571 (35 mg protein/kg BW); NRRL B-50 572-3, milk fermented by L. lactis NRRL B-50 572 (50 mg protein/kg BW); NRRL B-50 571-5, milk fermented by L. lactis NRRL B-50 571 (50 mg protein/kg BW); NRRL B-50 572-5, milk fermented by L. lactis NRRL B-50 572 (50 mg protein/kg BW); ND, not detected.
administration and at 2, 4, 6 and 24 h post-administration. Measurements were taken five times using the non-invasive blood pressure system using a photoelectric sensor, amplifier, automatic inflation cuff and software (Model 229; IITC). The animal experimental procedures were done following the guidelines and supervision of the Centro de Investigación en Alimentación y Desarrollo, A.C. Committee of Ethics for scientific research.

**Statistical analysis**

Data normality was evaluated as a prerequisite before one-way ANOVA was carried out. Differences among means were assessed by Fisher’s least significant difference multiple comparison test and considered significant when \( P<0.05 \). Data analyses were performed using the NCSS 2007 statistical program (NCSS Inc.).

**Results**

**Antihypertensive effects of milk fermented by specific Lactococcus lactis strains**

Fig. 1 shows that SHR became hypertensive when they were 7 weeks old. The rats presented with >150 mmHg SBP for more than 4 weeks, which is a prerequisite for being considered hypertensive\(^{(10)}\).

SBP changes are shown in Fig. 2(a). The results showed maximal SBP reductions at 6 h post-oral administration. SHR treated with the whey fractions of milk fermented by *L. lactis* NRRL B-50572-5 and *L. lactis* NRRL B-50571-3 showed a more relevant decrement in SBP (16.7 (SEM 3.5) mmHg and 17.7 (SEM 4.0) mmHg, respectively), although the treatments were not significantly different (\( P>0.05 \)).

The maximum decrease at 6 h was observed in the animals treated with Captopril\(^{(e)}\), which was significantly different from the other treatments (\( P<0.05 \)). However, the SBP measurements 24 h post-administration showed that SHR treated with the whey fraction of milk fermented by *L. lactis* NRRL B-50572-5 showed a SBP of 4.3 mmHg less than the rats that

Fig. 2. Blood pressure- and heart rate (HR)-lowering effects in spontaneously hypertensive rats (SHR) treated with milk fermented by specific *Lactococcus lactis* strains: (a) systolic blood pressure (SBP), (b) diastolic blood pressure (DBP) and (c) HR. - - - Captopril\(^{(e)}\) (positive control); - - - saline (negative control); - - , whey fraction of milk fermented by *L. lactis* NRRL B-50572-3 (35 mg protein/kg body weight (BW)); - - - , whey fraction of milk fermented by *L. lactis* NRRL B-50572-3 (35 mg protein/kg BW); - - - , whey fraction of milk fermented by *L. lactis* NRRL B-50572-5 (50 mg protein/kg BW); - - - , whey fraction of milk fermented by *L. lactis* NRRL B-50572-5 (50 mg protein/kg BW). Values are means, with their standard errors represented by vertical bars (\( n = 42 \)). NRRL B-50571-3, milk fermented by *L. lactis* NRRL B-50571 (35 mg protein/kg BW); NRRL B-50571-3, milk fermented by *L. lactis* NRRL B-50571 (35 mg protein/kg BW); NRRL B-50571-5, milk fermented by *L. lactis* NRRL B-50571 (50 mg protein/kg BW); NRRL B-50571-5, milk fermented by *L. lactis* NRRL B-50571 (50 mg protein/kg BW).

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**Fig. 1.** Development of hypertension with age. SBP (---), systolic blood pressure. Values are means, with their standard errors represented by vertical bars (\( n = 42 \)).
Table 2. Measurements of systolic blood pressure (SBP) and diastolic blood pressure (DBP) in spontaneously hypertensive rats treated with milk fermented by specific *Lactococcus lactis* strains at different times (Mean values with their standard errors)

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<thead>
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<td>2.8</td>
<td>112.48</td>
<td>1.3</td>
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<tr>
<td>Captopril</td>
<td>119.37</td>
<td>3.6</td>
<td>113.03</td>
<td>1.1</td>
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<td>NRRL B-50 571-3</td>
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<td>NRRL B-50 571-5</td>
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<td>Saline</td>
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<td>113.03</td>
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Mean values were significantly different from those of saline: *P* < 0.05; **P** < 0.01. Mean values were significantly different from those of Captopril: †† *P* < 0.05; † † † *P* < 0.01.
strains to evaluate primary hypertension are related to the ability to provide new insights into relevant mechanisms for blood pressure control in rodents and humans, and to the fact that many genes identified in animal models have been extensively studied in human subjects. Probably the most important issue of using SHR to evaluate the antihypertensive effects of specific substances is the feasibility to determine and characterise the influence of specific treatments establishing a close relationship between an antihypertensive substance and a hypotensive effect. In recent years, peptides derived from food protein substrates such as milk, egg, fish, sesame, pea, sake, rice and maize have demonstrated important antihypertensive activity.

At the beginning of the present study, we knew that specific \textit{L. lactis} strains were able to ferment milk with the ability to inhibit the activity of the ACE, which is associated with the reduction in blood pressure. In fact, these previous in vitro studies have shown the relevant capacity of \textit{L. lactis} NRRL B-50 571 and \textit{L. lactis} NRRL B-50 572 to hydrolyse milk proteins to exert antihypertensive activity. On the other hand, based on the complexity of living organisms, studies showing a clear direct correlation between \textit{in vitro} and \textit{in vivo} hypertensive activity have not been reported. Therefore, it was necessary to evaluate the antihypertensive effect of milk fermented by specific \textit{L. lactis} strains in an animal model.

The present study demonstrated the ability of specific \textit{L. lactis} strains to ferment milk with blood pressure- and HR-lowering effect \textit{in vivo}. The remarkable hypotensive effect as well as the HR reduction were observed at 6 h post-oral administration. In fact, it has been reported that there were maximal SBP and DBP reductions in SHR treated with the whey fraction of milk fermented by \textit{E. faecalis} at 4 and 6 h post-administration.

An association of specific minerals with blood pressure reduction in SHR has been reported. Therefore, it was necessary to determine the chemical composition of whey fractions from fermented milk (Table 1). Indeed, it has been reported that there was a decrement in systolic and DBP in SHR fed with an enriched Ca diet (2.5\%) during a long-term experiment. However, in the present experiment, the dose with the highest Ca concentration corresponded to less than 1\% of the SHR diet. On the other hand, the content of Mg in the SHR diet may play a role in hypertension. Sipola et al. did not report a blood pressure-lowering effect associated with Mg content in SHR after a 12-week treatment. It is important to note that the amount of Mg present in milk fermented by \textit{Lactobacillus helveticus} (0.33 mg/g) in that study was similar to that found in milk fermented by specific \textit{L. lactis} in the present study. Thus, the effect of Ca and Mg content on blood pressure reduction in SHR of the present study may not be important.

In the present study, two protein concentrations (35 or 50 mg/kg BW) were administered to SHR by a single dose. Hence, according to the results shown in Fig. 2(a), milk fermented by \textit{L. lactis} NRRL B-50 572 was dose-dependent. Nakamura et al. evaluated the capacity of milk fermented by \textit{L. helveticus} and \textit{Saccharomyces cerevisiae} to reduce SBP. SHR received 34 mg protein/kg BW of the whey fraction, resulting in a decrement of 21.5 (SEM 9.4) mmHg. Masuda et al. also used the same \textit{L. helveticus} and \textit{S. cerevisiae} strains to ferment milk by increasing the protein content to 68 mg/kg BW followed by a reduction in blood pressure to 26.4 (SEM 3.1) mmHg. These findings showed a dose-dependent relationship.

Muguerza et al. also evaluated the antihypertensive effect of milk fermented by \textit{E. faecalis} in SHR. In this case, a single dose of milk fermented by \textit{E. faecalis} CECT 5727 and CECT 5728 presented a maximal DBP reduction of 34.8 (SEM 4.5) mmHg. Chen et al. measured the antihypertensive effect of fresh low-fat milk fermented by five mixed lactic acid bacteria. The DBP value reported was 21.5 mmHg after 8 weeks of oral administration. Similar results were found in the present study for SHR treated with a single dose of milk fermented by \textit{L. lactis} NRRL B-50 572-5 (23.9 (SEM 4.4) mmHg).

On the other hand, it is important to note that a maximal SBP reduction (17.7 (SEM 4.0) mmHg) was observed in SHR treated with the lowest dose of whey fraction from milk.
fermented by *Lactis* NRRL B-50 571-3 (35 mg protein/kg BW), although this treatment was not significantly (P<0.05) different from milk fermented by *Lactis* NRRL B-50 571-5 (50 mg protein/kg BW). The dose-dependent effect for the whey fraction produced by NRRL B-50 572 but not for the whey fraction produced by NRRL B-50 571 might be related to their chemical composition. It has been reported that there is a close relationship between peptide structure and its hypertensive effect(10).

HR is a major determinant of myocardial oxygen consumption and cardiac work. Indeed, high HR has been considered as an important risk factor for atherosclerosis; therefore, its reduction may represent an important strategy for the treatment of patients with a wide range of cardiac disorders.(5). In the present study, an evident reduction in the HR of SHR was observed after the single-dose treatment (Fig. 2(c)). The whey fraction corresponding to milk fermented by *Lactis* NRRL B-50 571-3 decreased HR by 16.6 (SEM 9.2) beats/min, whereas the whey fraction of milk fermented by *Lactis* NRRL B-50 572-5 decreased HR by 16.9 (SEM 11.5) beats/min at 6 h after oral administration. On the other hand, Captopril™ decreased HR by 11.4 (SEM 5.9) beats/min. To the best of our knowledge, this is the first report on the blood pressure- and HR-lowering effect of whey fractions obtained from milk fermented by *Lactis*.

In conclusion, the present study demonstrated the relevant blood pressure- and HR-lowering effect of milk fermented by specific *Lactis* strains on SHR. Moreover, previous research in our laboratory reported the capacity of these strains to biogenerate interesting aroma profiles. Thus, whey fractions from milk fermented by specific *Lactis* strains may be used as a functional ingredient or food with important advantages in the prevention and treatment of CVD associated with hypertension. Ongoing research is being carried out to identify the sequences of the antihypertensive peptides present in the whey fraction and their mechanism of absorption.

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References


