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Changes in arterial blood pressure in hypertensive rats caused by long-term intake of milk fermented by *Enterococcus faecalis* CECT 5728

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We have evaluated the changes in arterial blood pressure caused in spontaneously hypertensive rats (SHR) by long-term intake of an *Enterococcus faecalis* CECT 5728-fermented milk with significant angiotensin-converting enzyme (ACE)-inhibitory activity. After being weaned, male 3-week-old SHR were randomized into five groups. Until the 20th week of life, rats in each group were given one of the following drinking fluids: tap water (negative control 1), a fermented milk without ACE-inhibitory activity (negative control 2), captopril (100 mg/kg) (positive control), the *E. faecalis* CECT 5728-fermented milk that had significant ACE-inhibitory activity, or Ca-enriched *E. faecalis* CECT 5728-fermented milk. Animals in the different groups were then given tap water as drinking fluid from the 20th to 25th week of life. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured weekly in the rats, from the 6th to 25th week of life, by the tail-cuff method. A definite decrease in SBP and DBP could be observed in the rats treated with captopril and also in the rats that received the *E. faecalis* CECT 5728-fermented milks. The greatest antihypertensive effect was observed when the pharmacological treatment was administered. The effect of the Ca-enriched fermented milk was slightly more accentuated and more constant than the effect of the *E. faecalis* CECT 5728-fermented milk that had not been enriched in Ca. SBP and DBP increased in the treated SHR when the corresponding antihypertensive treatment was removed. Fermentation of milk with *E. faecalis* CECT 5728 may therefore be a successful strategy to produce a functional food with antihypertensive activity.

Blood pressure: Angiotensin-converting enzyme inhibition: Milk fermentation: *Enterococcus faecalis*

Hypertension is a common and usually progressive disorder, which, if not effectively treated, has a high mortality rate. The preferred antihypertensive treatments have changed progressively as better drugs have become available, and angiotensin-converting enzyme (ACE) inhibitors are widely used nowadays in the control of this disease. Through fermentation, a variety of small bioactive peptides that have ACE-inhibitory activity can be derived from milk protein hydrolysis. Some of these peptides have also been found to have antihypertensive properties in animals and human subjects. In this context, Nakamura *et al.* (1995*a*) reported the antihypertensive activity in spontaneously hypertensive rats (SHR) of both a fermented milk prepared from skimmed milk with a starter culture containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, and two biologically active peptides isolated from it (Val-Pro-Pro:VPP) and (Ile-Pro-Pro:IPP). This sour milk also reduced systolic blood pressure (SBP) and diastolic blood pressure (DBP) in hypertensive patients (Hata *et al.* 1996). Later, Sipola *et al.* (2001, 2002) demonstrated that long-term oral intake of IPP and VPP, or a sour milk product fermented by *L. helveticus* LBK-16H containing these tripeptides, attenuated the development of hypertension in young pre-hypertensive SHR. Moreover, *L. helveticus* LBK-16H-fermented milk containing bioactive tripeptides IPP and VPP in normal daily use had a blood pressure-lowering effect in hypertensive patients (Seppo *et al.* 2002, 2003).

Our research group has just demonstrated that four strains of *Enterococcus faecalis*, CECT 5727, CECT 5728, CECT 5826 and CECT 5827, are especially significant as producers of ACE-inhibitory peptides other than IPP and VPP. We have also demonstrated that the fermented milks produced using these selected *E. faecalis* strains possess acute antihypertensive effect in SHR after a single oral administration. Nevertheless, they did not modify the arterial blood pressure of the Wistar–Kyoto rats that were used as normotensive controls of the SHR (Muguerza *et al.* 2005).

The administration of Ca is paradoxically associated with a decrease in arterial blood pressure, and different studies carried out by our research group have also demonstrated that Ca-enriched diets can control hypertension in rats (López-Miranda *et al.* 1998; Civantos *et al.* 1999; Civantos Calzada & Aleixandre de Artiñano, 2003; Civantos & Aleixandre, 2004). Dietary Ca supplements also have beneficial effects in hypertensive patients (for reviews see Aleixandre & Puerro, 1993; Aleixandre *et al.* 1993; Pryer *et al.* 1995; Allender *et al.* 1996; Resnick, 1999).

Abbreviations: ACE, angiotensin-converting enzyme; DBP, diastolic blood pressure; IPP, Ile-Pro-Pro; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; VPP, Val-Pro-Pro.

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The aim of the present study was to evaluate the blood pressure-lowering effect caused in SHR by long-term intake of both an *E. faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity and a similar *E. faecalis* CECT 5728-fermented milk with a high Ca content. Different parameters of these products (ACE-inhibitory activity, proteolysis degree reflecting functional peptide content, Ca content) were controlled and determined throughout the experimental period. To facilitate the interpretation of our results, we also measured the animals' weight gain and consumption of solid freely accessible feed and drinking fluid.

Methods

Fermented milks

The strain used in the present study to produce fermented milk with ACE-inhibitory activity was *E. faecalis* CECT 5728, which belongs to the Grupo Leche Pascual S.A. culture collection. This micro-organism was maintained as stock culture in agar slants of M17 (Biokar Diagnostics, Beauvois, France), stored at 4°C in aerobic conditions and sub-cultured periodically.

Pre-cultures of *E. faecalis* CECT 5728 were prepared from commercial homogenized ultra heat-treated skimmed milk fortified with two commercial milk protein concentrates: 1·8 % (w/w) Belka DL 403 (Bel Industries, Paris, France) and 1·8 % (w/w) MTM E70 (Meggle, Wasserburg, Germany). The mix was heated to 95°C for 15 min, cooled to 42°C and inoculated with a loop of the stock culture to yield an initial bacterial concentration of 10^5–10^7 colony-forming units per ml. Incubation was performed at 42°C overnight. This pre-culture was used to inoculate a milk formulation base to obtain a stock of fermented milk with high ACE-inhibitory activity. This stock was prepared with sweetened milk (10 % sugar) standardized to a fat level of 1·5 % and supplemented with 1·8 % (w/w) MTM E70. It was pasteurized to 95°C for 15 min, quickly cooled to 4°C and stored before use. For inoculation, this milk base was warmed to 42°C and the pre-culture was added to a final dose of 3 % (w/w). Fermentation was carried out during 24 h at 42°C. The process was stopped by pasteurization of the fermented milk at 75°C for 1 min. Then it was cooled to 4°C and stored until being used to prepare the fermented milks used in the experimental trial. Aliquots of the stock were taken to measure its ACE-inhibitory activity.

For the experimental groups that were to be treated with *E. faecalis* CECT 5728-fermented milk in the present study, the fermented milk was always prepared from stock fermented milk normalized to an ACE-inhibitory activity of about 3000 IU/ml. We attained this ACE-inhibitory activity by blending the stock with variable quantities of a sweetened aromatized commercial yoghurt drink (Yosport®; Grupo Leche Pascual, Aranda de Duero, Spain) depending on the initial ACE-inhibitory activity of the stock. In one of these experimental groups, the standardized fermented milk was enriched with Ca (≈2 g/l). Only sweetened aromatized commercial yoghurt drink was used to prepare the fermented milk assigned for the negative control group in this study. Finally, all the fermented milk mixes were pasteurized at 75°C for 1 min, rapidly cooled to 4°C and kept until use.

The *o*-phthaldialdehyde method was used to measure proteolysis in the fermented milks (Church *et al.* 1983). A standard calibration curve was prepared with increasing concentrations of casein peptone hydrolysate. Results were expressed as mg casein peptone hydrolysate per ml sample. Unfermented milk was used as blank and subtracted from the value for each sample.

In order to measure the ACE-inhibitory activity, aliquots of fermented milks were pasteurized, vigorously stirred and centrifuged at 20 000 ***g*** for 10 min to obtain the whey fraction. The collected supernatants were filtered through a Whatman no. 40 filter, analysed by semi-preparative reverse-phase HPLC and used to determine the ACE-inhibitory activity by spectrophotometric assay according to the method of Cushman & Cheung (1971). The ACE-inhibitory activity was expressed as inhibition units according to the definition given by Nakamura *et al.* (1995*b*).

A standard HPLC method for quantifying inorganic cations was used to determine the Ca content in the whey fraction of the fermented milks. The chromatographic system (Hewlett-Packard Co., Palo Alto, CA, USA) consisted of a Hewlett-Packard 1050 pump, a Metrohm 690 UV–visible detector of ionic conductivity (Metrohm Ltd, Herisau, Switzerland) and a Waters 717 plus injector (Waters, Milford, MA, USA). Samples were warmed to 60°C under continuous stirring and cooled to room temperature. Aliquots (5 ml) were transferred to porcelain dishes, dried at 90°C overnight and incinerated at 550°C for 90 min. Ashes were dissolved in aqueous nitric acid solution (25 %) and the solvent evaporated at 100°C before incineration at 550°C for 90 min. Then, the pellets were re-dissolved in the same nitric acid solution, and the solution filtered through a Whatman no. 40 filter and diluted 1:1000 (v/v) with distilled water. Diluted samples were filtered through a 0·45 μm syringe filter. Finally, the processed samples were analysed in an HPLC column (Metrohm Ltd) of 150 mm × 4·0 mm, with a 7 μm particle stationary phase. The chromatographic conditions were as follows: column temperature, 25°C; flow, 1·0 ml/min; injection volume, 20 μl; mobile phase, 4 mmol tartaric acid/l and 1 mmol 2,6-pyridinedicarboxylic acid/l.

Table 1 shows the exact characteristics and nutritional details of the different fermented milks used in this study.

Experimental procedure in rats

After being weaned at 3 weeks, male SHR (Charles River Laboratories España S.A., Barcelona, Spain) were caged in groups of five at a temperature of 23°C with 12 h light/12 h dark cycles. They were in turn randomized with *ad libitum* intake into five groups of animals. Until they were 20 weeks old, the rats in these groups received drinking fluid as follows: tap water (negative control 1), a fermented milk without ACE-inhibitory activity (negative control 2), captopril (positive control, 100 mg/kg; Sigma, St. Louis, MO, USA), *E. faecalis* CECT 5728-fermented milk that had significant ACE-inhibitory activity (about 3000 UI/ml), or Ca-enriched *E. faecalis* CECT 5728-fermented milk that also had about 3000 UI ACE-inhibitory activity per ml. Thereafter, from the 20th to 25th week of life, animals in the different groups received tap water as drinking fluid. During the experimental period, the SHR of the five established groups were fed on a solid standard diet for rats (A04; Panlab, Barcelona, Spain).

SBP and DBP were measured weekly in the rats, from the 6th to 25th week of life, by the tail-cuff method (Buñag, 1973). Before measurement, the rats were kept at 37°C for 10 min to make the pulsations of the tail artery detectable. The original technique for measuring arterial blood pressure using the tail-cuff method provides only SBP values, but the equipment used in the present study, LE 5001 (Letica, Hospitalet, Barcelona,

Table 1. Characteristics of the different fermented milks

(Mean values and their standard error for a minimum of six determinations)

	Negative control		CECT 5728		Ca-CECT 5728	
	Mean	SEM	Mean	SEM	Mean	SEM
Energy (kJ/100 ml)	335·1	0·08	335·1	0·08	335·1	0·08
Protein (g/100 ml)	3·75	0·02	3·75	0·02	3·75	0·02
Fat (g/100 ml)	1·35	0·02	1·35	0·02	1·35	0·02
Carbohydrate (g/100 ml)	13·14	0·02	13·14	0·02	13·14	0·02
ACE-inhibitory activity (IU/ml)	153	53	2951[a]	364	2951[a]	364
Proteolysis degree (mg peptide/ml)	0·74	0·06	1·82[a]	0·10	1·82[a]	0·10
Calcium content (g/l)	1·57	0·02	1·57	0·02	2·03[a,b]	0·02

Negative control, milk fermented by *Lactobacillus delbrueckii* and *Streptococcus thermophilus* without ACE-inhibitory activity; CECT 5728, *Enterococcus faecalis* CECT 572-fermented milk; Ca-CECT 5728, Ca-enriched *E. faecalis* CECT 5728 fermented-milk; ACE, angiotensin-converting enzyme

[a]$P<0·05$ *v.* negative control; [b]$P<0·05$ *v.* CECT 5728.

Spain), has a high-sensitivity pulse transducer coupled with an accurate microprocessor program, and allows us to distinguish between SBP and DBP. The arterial blood pressure measurements were performed at the same time of day (between 09.00 and 13.00 hours) in order to avoid any influence of the circadian cycle. Moreover, the researchers who measured the arterial blood pressure in the animals of the different groups did not know the exact drinking fluid that had been administered to each of these groups.

We took weekly measurements of rat weight up to the 25th week of life in the different groups. The consumption of drinking fluids and freely accessible feed was also estimated weekly in the animals of the different groups throughout the experimental period.

All of the experiments were performed as authorized for scientific research (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food).

Statistical analysis

The results are expressed as means with their standard error for a minimum of eight rats and were analysed by one-way ANOVA. Differences between the groups were assessed by the Bonferroni test. We considered the differences between the means to be significant when $P<0·05$.

Results

The SHR of the negative control groups showed a gradual increase in SBP and DBP from weaning that reached maximal values at 11 weeks of life. From this age the arterial blood pressure of the rats remained constantly high and their SBP and DBP values were similar between weeks 11 and 25. These variables were slightly lower in the SHR of the negative control 2 group that drank the fermented milk without ACE-inhibitory activity than in the SHR of the negative control 1 group that drank tap water. A definite decrease in SBP and DBP could be observed in the rats of the positive control group treated with captopril from the 6th to the 20th week of life. In these animals, both SBP and DBP values remained constant during this period. The *E. faecalis* CECT 5728-fermented milks also decreased SBP and DBP in the SHR. The antihypertensive effect of these products was less accentuated than the effect of the pharmacological treatment, but could be clearly appreciated from the 13th to the 20th week of life. The effect of the Ca-enriched fermented milk was more accentuated than the effect of the *E. faecalis* CECT 5728-fermented milk that had not been enriched in Ca. An increase in SBP and DBP was observed in the SHR that had been treated with the *E. faecalis* CECT 5728-fermented milks when these rats were given tap water as drinking fluid at the 20th week of life. SBP and DBP were in fact similar in these SHR and in the SHR of the negative control groups when the animals were 25 weeks old. SBP and DBP also increased somewhat in the SHR that had received captopril when we took away the pharmacological treatment. However, despite this, these variables were lower in the 20- to 25-week-old SHR that received the drug than in the SHR of the same age in the other groups (see Figs 1 and 2). Considering the results presented earlier, we believe that comparisons of the SBP and DBP values in the 11-, 20- and 25-week-old SHR of the different groups permit us to establish the effect of the products administered in the animals. Table 2 shows these values and these comparisons.

The body weight of the SHR increased progressively in all groups. The increase was less accentuated in the SHR of the positive control 1 group treated with captopril than in the other groups. On the contrary, the rats treated with the fermented milks exhibited a significantly increased weight gain compared with the animals that drank tap water. The increase was less accentuated in the SHR treated with the Ca-enriched *E. faecalis* CECT 5728-fermented milk, and these animals showed slightly lower body weight values than the SHR of the groups treated with the other fermented milks (see Fig. 3). The comparisons of the body weight values in the 11-, 20- and 25-week-old SHR of the different groups are shown in Table 2.

Between the 5th and 20th weeks of life, we observed a considerable dry food intake and a scarce liquid food consumption in the SHR of the negative control 1 group and in the group treated with captopril. By contrast, dry food intake decreased and liquid diet consumption increased in the SHR that drank the different fermented milks during this period. The increase in liquid consumption was more noticeable in the SHR treated with the fermented milk without ACE-inhibitory activity. From the 20th to the 25th week of life, the consumption of solid food and drinking fluid was very similar in all groups. It should be remembered that during this period all of the animals drank tap water as had the animals included in the negative control 1 group from the beginning of the experiment (see Figs 4 and 5).

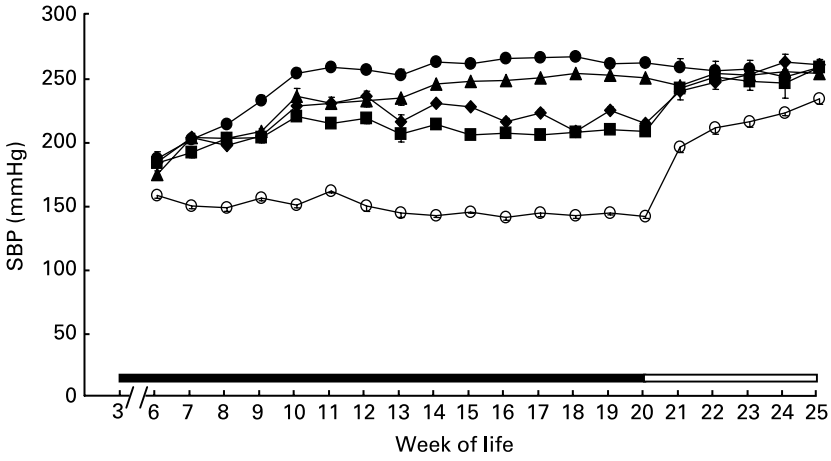

Fig. 1. Systolic blood pressure (SBP) of spontaneously hypertensive rats. The animals drank different fluids from weaning until the 20th week of life (treatment period indicated by a solid bar): tap water (•), fermented milk without angiotensin-converting enzyme (ACE)-inhibitory activity (▲), captopril 100 mg/kg (○), *Enterococcus faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity (◆), or Ca-enriched *E. faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity (■). All rats drank tap water from the 20th until the 25th week of life (follow-up period indicated by an open bar). Values are means with their standard error shown by vertical bars for a minimum of eight rats.

Comparisons of the values of dry food intake and liquid food consumption in the 11-, 20- and 25-week-old SHR of the different groups are also shown in Table 2.

Discussion

Before starting the present study, we knew that milk fermented by *E. faecalis*, and some peptides with *in vitro* ACE-inhibitory activity isolated from this milk, decreased arterial blood pressure in SHR when acutely administered by intragastric intubation. Hypertension is a chronic pathology that requires chronic treatment, and the long-term administration of functional products without side-effects is an attractive possibility to be considered in treating this pathology. For these reasons, we believed it advisable to carry out the present study in which we investigated the changes in arterial blood pressure in SHR caused by long-term oral intake of a milk fermented by one *E. faecalis strain* (*E. faecalis* CECT 5728) characterized as a producer of ACE-inhibitory peptides. Since we had also demonstrated with previous studies that Ca-enriched diets could control hypertension in SHR (Civantos & Aleixandre, 2004), we also believed it interesting to evaluate the effect on blood pressure of long-term oral intake of a Ca-enriched *E. faecalis* CECT 5728-fermented milk in these animals.

As can be seen in Figs 1 and 2, the SHR that drank tap water throughout the experimental period showed a gradual increase in SBP and DBP, which reached maximum values at about 11 weeks of life. The other groups of rats also showed an accentuated increase in arterial blood pressure from weaning until the 12th week of life, after which point arterial blood pressure reached stable levels in all groups. It was not surprising to observe lesser values of SBP and DBP during the experimental period

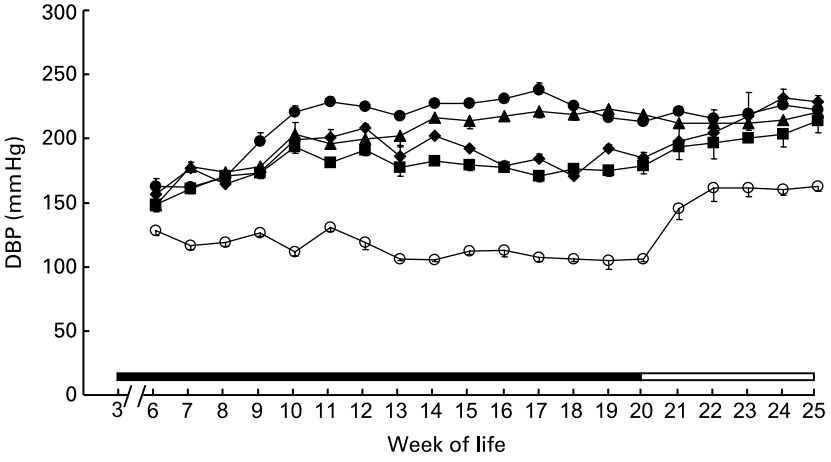

Fig. 2. Diastolic blood pressure (DBP) of spontaneously hypertensive rats. The animals drank different fluids from weaning until the 20th week of life (treatment period indicated by a solid bar): tap water (•), fermented milk without angiotensin-converting enzyme (ACE)-inhibitory activity (▲), captopril 100 mg/kg (○), *Enterococcus faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity (◆), or Ca-enriched *E. faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity (■). All rats drank tap water from the 20th until the 25th week of life (follow-up period indicated by an open bar). Values are means with their standard error shown by vertical bars for a minimum of eight rats.

Table 2. Values of the studied variables in the 11-, 20- and 25-week-old spontaneously hypertensive rats of the different groups
(Mean values and their standard error for a minimum of eight determinations)

	Tap water		Negative control		Positive control		CECT 5728		Ca-CECT 5728	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
11 weeks old										
SBP (mmHg)	258·1	3·03	229·32[a]	2·30	160·70[a,b]	1·51	229·66[a,c]	4·90	214·01[a,b,c,d]	3·25
DBP (mmHg)	228·27	3·72	196·06[a]	4·07	130·53[a,b]	2·86	201·00[a,c]	6·19	181·07[a,c,d]	3·25
Body weight (g)	282·3	4·5	302·3	3·8	264·5[b]	4·7	312·5[a,c]	6·5	315·0[a,c]	10·1
Liquid consumption (ml/d per rat)	29·02	2·70	80·71	14·14	35·97	3·69	59·54	1·02	67·28	31·13
Solid consumption (g/d per rat)	20·10	0·64	6·66[a,c]	1·22	20·25	0·28	4·51[a,c]	0·03	3·14[a,c]	1·46
20 weeks old										
SBP (mmHg)	261·13	2·69	250·00	2·77	140·53[a,b]	1·52	213·89[a,b,c]	2·71	207·33[a,b,c]	2·00
DBP (mmHg)	213·13	1·91	218·50	2·14	106·06[a,b]	2·17	184·83[a,b,c]	4·03	178·80[a,b,c]	6·08
Body weight (g)	353·5	5·2	377·2	6·8	326·6[b]	7·5	386·5[a,c]	7·4	407·0[a,c]	10·5
Liquid consumption (ml/d per rat)	28·83	0·05	71·21[a]	1·84	39·39[b]	0·30	58·64[a]	1·99	73·67[a,c]	6·06
Solid consumption (g/d per rat)	19·46	0·83	8·11[a]	0·09	18·60[b]	0·87	5·98[a,c]	1·23	6·59[a,c]	1·21
25 weeks old										
SBP (mmHg)	258·10	5·00	253·30	4·17	233·14[a]	4·33	260·00[c]	4·04	257·94[c]	1·49
DBP (mmHg)	222·60	2·25	220·60	1·63	162·40[a,b]	3·62	228·40[c]	5·10	214·00[c]	0·50
Body weight (g)	366·4	4·4	389·3	12·3	350·8	14·8	378·2	16·4	366·1	6·7
Liquid consumption (ml/d per rat)	36·28	2·29	33·93	2·21	31·07	3·50	37·98	8·35	45·95	2·21
Solid consumption (g/d per rat)	20·37	0·78	20·45	0·94	19·57	0·13	20·82	0·17	20·58	0·07

Negative control, fermented milk without angiotensin-converting enzyme (ACE)-inhibitory activity; positive control, captopril (100 mg/kg); CECT 5728, *Enterococcus faecalis* CECT 5728-fermented milk; Ca-CECT 5728, Ca-enriched *E. faecalis* CECT 5728-fermented milk; SBP, systolic blood pressure; DBP, diastolic blood pressure.
[a]$P<0·05$ *v.* tap water; [b]$P<0·05$ *v.* negative control; [c]$P<0·05$ *v.* positive control; [d]$P<0·05$ *v.* CECT 5728.

in the group of SHR that were treated with captopril, because this drug is a prototype ACE inhibitor with clinical use in hypertensive patients. The groups of SHR treated with the *E. faecalis* CECT 5728-fermented milk, and with the Ca-enriched *E. faecalis* CECT 5728-fermented milk, also showed a clear decrease in arterial blood pressure that was probably related to the antihypertensive peptides produced when milk is fermented by this bacterium. The data obtained when we evaluated the ACE-inhibitory activity and the proteolysis degree of these milks (shown in Table 1) clearly guarantee that biologically active peptide fragments are implicated in their effects on arterial blood pressure. In fact, our research group has already isolated some peptides from the *E. faecalis* CECT 5728-fermented milk (mainly the peptidic sequence Leu-His-Leu-Pro-Leu-Pro) that are ACE inhibitors and have an acute antihypertensive effect in SHR. These data have been included in a patent presented by Muguerza *et al.* (2003).

As mentioned earlier, Sipola *et al.* (2001) demonstrated that long-term oral administration of IPP and VPP, two tripeptides isolated by Nakamura *et al.* (1995*a*) from a milk fermented by *L. helveticus* and *S. cerevisiae*, and also the chronic intake of a sour milk containing these tripeptides, attenuated the development of hypertension in young SHR. As in the present study, Sipola *et al.* measured the SBP of the SHR by the tail-cuff method

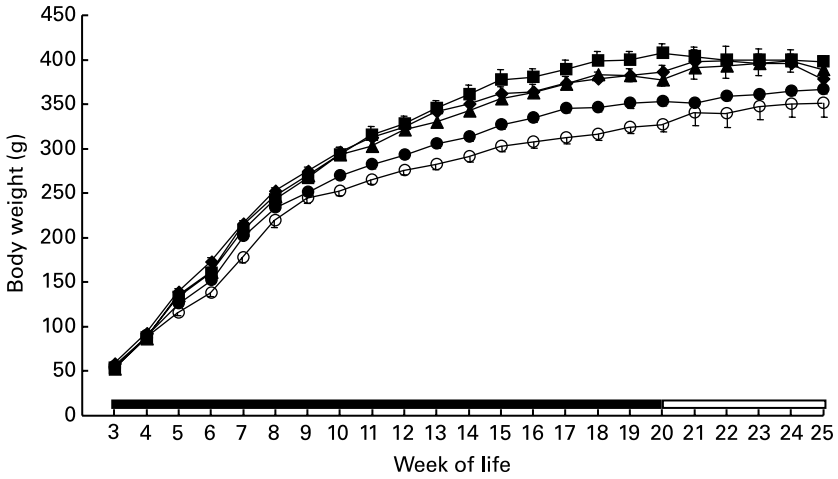

Fig. 3. Body weight of spontaneously hypertensive rats. The animals drank different fluids from weaning until the 20th week of life (treatment period indicated by a solid bar): tap water (•), fermented milk without angiotensin-converting enzyme (ACE)-inhibitory activity (▲), captopril 100 mg/kg (○), *Enterococcus faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity (◆), or Ca-enriched *E. faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity (■). All rats drank tap water from the 20th until the 25th week of life (follow-up period indicated by an open bar). Values are means with their standard error shown by vertical bars for a minimum of eight rats.

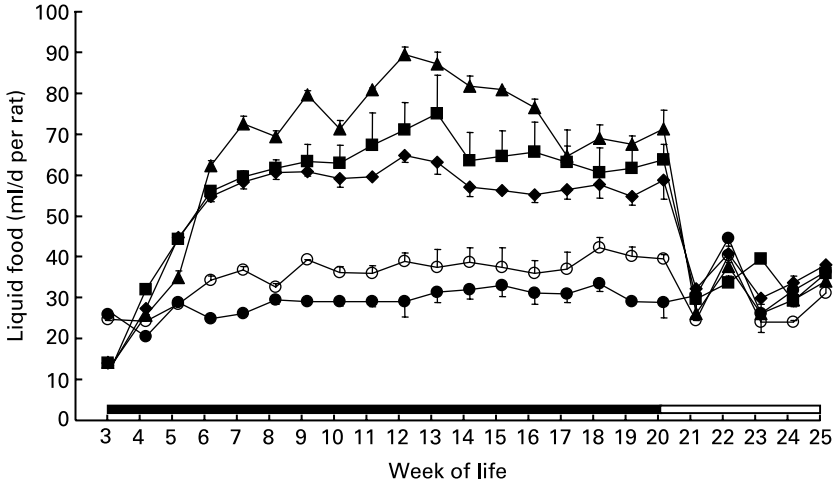

Fig. 4. Liquid food consumption of spontaneously hypertensive rats. The animals drank different fluids from weaning until the 20th week of life (treatment period indicated by a solid bar): tap water (•), fermented milk without angiotensin-converting enzyme (ACE)-inhibitory activity (▲), captopril 100 mg/kg (○), *Enterococcus faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity (◆), or Ca-enriched *E. faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity (■). All rats drank tap water from the 20th until the 25th week of life (follow-up period indicated by an open bar). Values are means with their standard error shown by vertical bars for a minimum of eight rats.

from the 6th week of life, but they did not report data on the DBP of these animals. These researchers concluded that one possible mechanism underlying the antihypertensive effect of the sour milk containing VPP and IPP was the ACE-inhibitory activity of these sequences. Most of the peptide fragments with ACE-inhibitory activity that have been isolated from hydrolysates of food materials, among them the peptidic sequences VPP and IPP, and also the sequence Leu-His-Leu-Pro-Leu-Pro isolated from the *E. faecalis* CECT 5728-fermented milk, have a proline group in the carboxyl terminus.

In the present study we have also shown that SBP and DBP were slightly lower in the SHR that drank the fermented milk without ACE-inhibitory activity (milk fermented by *Lactobacillus delbrueckii* and *Streptococcus thermophilus*) than in the SHR that drank tap water. Ashar & Chand (2004) very recently demonstrated that *L. delbrueckii* also has proteolytic ability to generate ACE-inhibitory peptides during milk fermentation, but the antihypertensive effect of these peptides has still not been demonstrated. Thus, we cannot discard that some functional antihypertensive peptides may also be produced in the fermentation process when bacteria different from *E. faecalis* ferment the milk. In addition, as can be seen in Figs 1 and 2, in the group of rats treated with the Ca-enriched milk the antihypertensive effect was particularly constant, and the development of hypertension was somewhat more extensively attenuated than in the group of rats treated with the *E. faecalis* CECT 5728-fermented milk not enriched in Ca. The increase in Ca content seems therefore to improve the antihypertensive properties of the *E. faecalis* CECT 5728-fermented milk. Sipola *et al.* (2001) also concluded that the

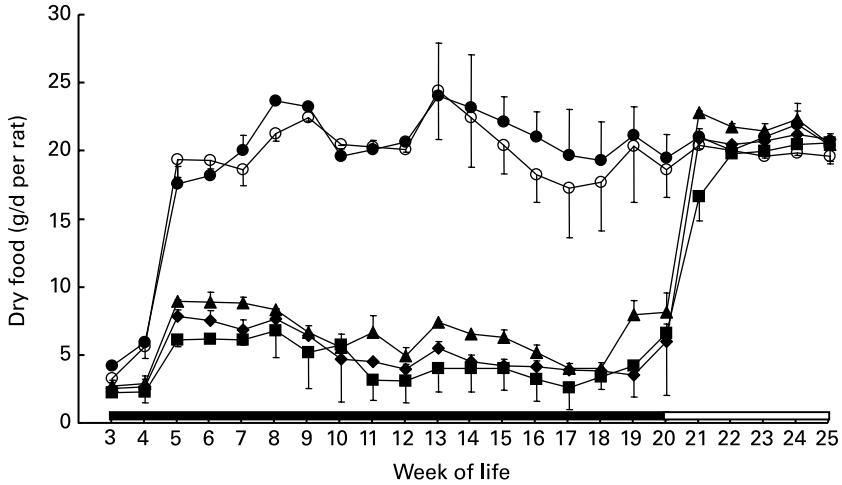

Fig. 5. Dry food intake of spontaneously hypertensive rats. The animals drank different fluids from weaning until the 20th week of life (treatment period indicated by a solid bar): tap water (•), fermented milk without angiotensin-converting enzyme (ACE)-inhibitory activity (▲), captopril 100 mg/kg (○), *Enterococcus faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity (◆), or Ca-enriched *E. faecalis* CECT 5728-fermented milk with significant ACE-inhibitory activity (■). All rats drank tap water from the 20th until the 25th week of life (follow-up period indicated by an open bar). Values are means with their standard error shown by vertical bars for a minimum of eight rats.

role of Ca in the antihypertensive effect of the milk fermented by *L. helveticus* and *S. cerevisiae* cannot be ruled out.

After withdrawal of the treatments, SBP and DBP in the animals that had drunk the *E. faecalis* CECT 5728-fermented milks rose gradually, and approximately 2 weeks later these variables reached similar levels to those obtained in the control groups. This also demonstrates that functional antihypertensive peptides are present in these products. Sipola *et al.* (2001) also described a gradual rise in the SBP of SHR after withdrawal of the treatments used in their study (the fermented milk containing VPP and IPP and the treatment with these two tripeptides). In the present study, it was also logical to observe an increase in arterial blood pressure of the rats that had been treated with captopril after the withdrawal of this treatment. Since this drug is a potent ACE inhibitor, the reversion of its effect was, nevertheless, less noticeable than the reversion of the effect of the *E. faecalis* CECT 5728-fermented milks.

We have also studied the influence of the different treatments on weight gain and food consumption of the SHR, and it is evident that the nutritional value of the fermented milks consumed by the rats was adequate as they improved animal growth (Fig. 3). This effect is independent of the ACE-inhibitory activity because, on the one hand, the group of rats treated with captopril had similar body weight gain to those that drank tap water and, on the other, the increase in body weight was also clear in the group of SHR that drank the milk fermented by bacteria other than *E. faecalis*. Moreover, since liquid consumption increased in the groups of SHR that were drinking the fermented milks, we can assume that these products are pleasant to ingest. The increase in liquid consumption is logically accompanied by a decrease in dry food consumption; Fig. 4 shows this decrease in the groups of SHR that drank the fermented milks.

The experimental model of SHR has usually been used to carry out initial studies to evaluate the antihypertensive effect of functional products and bioactive peptides derived from food proteins (Karaki *et al.* 1990; Saito *et al.* 1994; Yamamoto *et al.* 1994, 1999; Kuwabara *et al.* 1995; Fujita & Yoshikawa, 1999; Nurminen *et al.* 2000; Shin *et al.* 2001; Sipola *et al.* 2001; Wu & Ding, 2001). The results that we have obtained in this rat strain with the *E. faecalis* CECT 5728-fermented milk suggest the possibility of using this milk in the non-pharmacological treatment of hypertension. In fact, all of the mentioned data demonstrate that milk fermentation with *E. faecalis* CECT 5728 may be a successful strategy to produce a functional food with antihypertensive activity. Nevertheless, we have to bear in mind that some differences exist in bowel structure and function, and also in microflora, between rodents and man. It is therefore evident that before using the milk fermented by *E. faecalis* CECT 5728 in human subjects, it would be necessary to carry out clinical studies to demonstrate their efficiency and to guarantee their safe use in hypertensive patients. The present results also suggest that the increase in Ca content may improve the antihypertensive properties of the *E. faecalis* CECT 5728-fermented milk. This should also be considered in the future to elaborate a functional food with the *E. faecalis* CECT 5728-fermented milk.

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