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Effect of feeding aged hard cheese on blood pressure of spontaneous hypertensive rats

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Abstract:	This study investigated the long-term effect of feeding Parmigiano Reggiano (PR) cheese on blood pressure (BP) of spontaneously hypertensive rats (SHRs). Thirty male SHRs, received a daily dietary supplementation with: 0.1-0.2-0.4-0.6 g PR/rat; or captopril or water. Systolic and diastolic BP were recorded every two weeks, for 10 weeks, by a non-invasive tail-cuff apparatus. Blood samples were collected at the end of the trial to detect the presence of PR ACE-inhibitory peptides by UHPLC/ESI-MS/MS analysis. Dietary integration of PR led to a transitory reduction in BP of SHRs in the first 35 days of treatment and that decrease resulted positively associated to the different amount of PR consumed. No PR derived peptides were detected in the rats serum. The beneficial effect of hypothensive peptides may have been masked and reduced in the second part of the study by the increase in BP linked to the raise in body weight and age of SHRs.
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We would like to submit the enclosed manuscript entitled “**Effect of feeding aged hard cheese on blood pressure of spontaneous hypertensive rats**”, which we wish to be considered for publication in “International Dairy Journal”. No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was an original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part.

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Thank you and best regards.

Sincerely yours,

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Effect of feeding aged hard cheese on blood pressure of spontaneous hypertensive rats

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ABSTRACT

This study investigated the long-term effect of feeding Parmigiano Reggiano (PR) cheese on blood pressure (BP) of spontaneously hypertensive rats (SHRs). Thirty male SHRs, received a daily dietary supplementation with: 0.1-0.2-0.4-0.6 g PR/rat; or captopril or water. Systolic and diastolic BP were recorded every two weeks, for 10 weeks, by a non-invasive tail-cuff apparatus. Blood samples were collected at the end of the trial to detect the presence of PR ACE-inhibitory peptides by UHPLC/ESI-MS/MS analysis. Dietary integration of PR led to a transitory reduction in BP of SHRs in the first 35 days of treatment and that decrease resulted positively associated to the different amount of PR consumed. No PR derived peptides were detected in the rats serum. The beneficial effect of hypothensive peptides may have been masked and reduced in the second part of the study by the increase in BP linked to the raise in body weight and age of SHRs.

Key words: Parmigiano Reggiano, bioactive peptides, antihypertensive effect, SHRs, functional food

1. Introduction

The current research in the agri-food industry is oriented at characterizing and enhancing natural products rich in bioactive compounds with nutraceutical properties, able to improve consumers health and in prevention of diseases. Among animal products**,** milk and dairy foods have recently attracted growing interest other than for their high nutritional value, also as source of biologically active peptides implicated in many regulatory processes or pathways in the body (Sanchez and Vázquez, 2017), including the effectiveness in the blood pressure-lowering (Pihlanto & Korhonen, 2014; Bhat *et al.*, 2015; Beltran-Barrientos *et al*., 2016; Daliri *et al.*, 2017). Recent data highlight significant increases of hypertension, closely associated with the risk for stroke and cardiovascular disease, which represent the main causes of death in up to 30% of the adult population in developed countries (Kearney *et al.*, 2005; Danaei *et al*., 2014). Multiple and complex factors are responsible

for the pathogenesis of hypertension. These include genetics, activation of the sympathetic nervous system and renin-angiotensin-aldosterone system, endothelial disfunction, obesity, and excess dietary sodium intake (Hamrahian *et al*., 2017). Moreover, many scientific evidences (Fukuda *et al*., 2004; Pinto, 2007; Koeners *et al*., 2008) show that blood pressure increases with age and this is mostly associated with structural changes in arterial and arteriolar stiffness. Blood pressure levels are also closely related to other physiological influencing factors, such us weight. In particular, body weight and weight gain may contribute significantly to the rise in blood pressure that commonly occur with ageing (Fukuda *et al*., 2004; Yang *et al.*, 2007).

Dairy products containing bioactive peptides with potential antihypertensive effects may be considered possible candidates to develop various health-promoting functional foods in helping to prevent hypertension and cardiovascular events, also in support to pharmacological treatments, without known side effects. Most of the biopeptides investigated for their antihypertensive effect have shown to possess angiotensin-converting enzyme (**ACE**)-inhibitory activity *in vitro* (Sieber *et al.,* 2010). Angiotensin-converting enzyme is one of the key enzymes in blood pressure (**BP**) regulation. Inhibition of this enzyme leads to arteries vasodilatation and subsequent BP lowering (Tidona *et al.,* 2009). The first discovered and most extensively studied peptides showing a strong ACE-inhibitory activity *in vitro* were valyl-prolyl-proline (**VPP**, f84–86 of β-casein) and isoleucyl-prolyl-proline (**IPP**, f74–76 of β-casein as well as f108–110 of κ-casein) isolated from sour milk (Nakamura *et al.,* 1995), but other peptides, showing similar activity, have been discovered in enzymatic hydrolysates of bovine α-, β-and κ-casein (Quirós *et al.*, 2007; Stuknyte, *et al.,* 2015). Their efficacy has been demonstrated by *in vitro* assays, cell cultures and also in animal and human studies (Basiricò *et al.,* 2015; Majumder & Wu, 2015; Cicero *et al.,* 2016).

Different cheese varieties of Italian (Smacchi & Gobbetti, 1998; Bernabucci *et al.,* 2014), Spanish (Gómez-Ruiz *et al.*, 2006), Dutch (Saito *et al.,* 2000) and Swiss (Bütikofer *et al.,* 2008) origin, have been characterized for the presence of potent ACE-inhibitory peptides.

In cheeses, it has been observed that the release and the bioactivity of these naturally formed peptides depend on the variety and ripening stage of cheese. In particular, as the ripening continues, peptides can be further degraded to inactive fragments and this reduces their bioavailability and the potential antihypertensive effect *in vivo* (Pripp *et al*., 2006; Meyer *et al*., 2009; Sieber *et al*., 2010).

Gastrointestinal digestion, is expected, to further modify this pattern (Bottesini *et al.*, 2013), and indeed peptides are also likely to be formed in the gastrointestinal tract upon digestion of cheese (Parrot *et al.*, 2003).

Parmigiano Reggiano (**PR**) is an Italian Protected Designation of Origin (PDO) hard-cheese, produced in a restricted geographic area in Northern Italy according to the law that on 1955 defined the standard of this cheese. Several known biopeptides have been found in PR cheese and/or its digested products *in vitro* (Summer *et al.*, 2017). Bernabucci *et al.* (2014), in an *in vitro* study highlighted for the first time a consistent ACE-inhibitory activity of 3-kDa water-soluble extract (**WSE**) from 32-month aged PR and Grana Padano (**GP**) cheeses, attributable to the presence of ACE-inhibiting peptides naturally formed during the ripening process of cheeses. More recently, Basiricò *et al.* (2015), in an *in vitro* study revealed the presence of the potent ACE-inhibitors VPP, IPP, LHLPLP and AYFYPEL (with the respective truncated forms HLPLP and AYFYPE) in the 3-kDa ultrafiltered WSE of PR and in PR digestates. These findings might be predictive of the potential role of PR cheese in reducing BP *in vivo*. To date, nothing is known about the effects of a dietary supplementation of PR cheese on blood pressure. Therefore, we performed an *in vivo* study to investigate the potential antihypertensive effect of dietary supplementation with four different amounts of PR cheese on spontaneously hypertensive rats (**SHRs**)

2. Materials and methods

2.1. Animals and Experimental Design

All animal care and use procedures were reviewed by the Animal Care Ethics Committee of the University of Tuscia (Viterbo, Italy), approved by Italian Government Authorities, and were in accordance with European Guidelines. For the *in vivo* experiment, 30 male SHRs **(**Charles River Laboratories, Milan, Italy), 10 weeks old, were used. The SHRs were chosen for this study as they represent the best accepted animal model for biology studies on human hypertension (Bianchi *et al*., 1986).

Rats were housed in single cages under environmentally controlled conditions (23°C and 12/12 h of light/dark cycles) with an inverted light cycle (constant darkness: from 07:00 a.m. to 07:00 p.m.; constant light: from 07:00 p.m. to 07:00 a.m.) to respect the normal circadian rhythm of animals.

All rats had free access to water and standard diet (Charles River diet, Purina 5L79), provided *ad libitum*, throughout the study. Body weight (**BW**) was measured at the beginning (time 0) and at the end of the experimental period while systolic and diastolic blood pressure (**SBP** and **DBP**, respectively) were measured at time 0 and every two weeks, throughout the 10 week treatment period. After an adaptation period of three weeks to new environmental conditions, rats were subdivided into six groups (5 animals/group) balanced for body weight and BP. Each group received, in addition to the standard diet, a different daily treatment as follows: (1) 0.1 g PR cheese/rat , (2) 0.2 g PR cheese/rat, (3) 0.4 g PR cheese/rat, (4) 0.6 g PR cheese/rat, (5) captopril (50 mg/kg body weight; Sigma Aldrich, Milan, Italy) that served as positive control and (6) distilled water, as negative control. All treatments were orally administered to rats over small pieces of cookies, fat and salt free, 2 hours after turning off the light.

In this study a 12-months-age PR was chosen as this cheese cannot be sold before this period and the amounts of ACE-inhibitors, and their bioactivity, decreases as the ripening continues (Sforza *et al.*, 2012). The different doses of PR administered to rats have been chosen considering the minimum and the maximum amount of cheese that an adult man (70 kg body weight) can really

daily ingest, and with contents of fat, salt and cholesterol that do not cause side events for human health.

2.2. Blood Pressure Measurements

Systolic and diastolic blood pressure (mmHg) of rats were measured every two weeks, 6 hours after the PR, water or captopril ingestion, by using a non-invasive tail-cuff BP analyzer (BP-2000, Visitech Systems, Apex, NC, USA). To allow the optimal detection of the pulse of the tail artery, rats were kept under an infrared lamp at 36°C for 10 minutes. The SBP and DBP values were presented as the average of at least five consecutive constant measurements (Bernabucci *et al.*, 2014).

2.3. Blood collection

At the end of the experimental period, after general anesthesia, an intracardiac puncture for blood collection was performed to each rat. Blood samples were collected into vials containing heparin and immediately centrifuged. Plasma was separated and stored at -20°C until analyzed.

2.4. Biochemical Determinations

2.4.1. Reagents and solvents

Peptides H-Ile-Pro-Pro-OH, H-Val-Pro-Pro-OH, H-Leu-His-Leu-Pro-Leu-Pro-OH (99.9%) and H-His-Leu-Pro-Leu-Pro-OH (96.2%) were purchased from Bachem (Bubendorf, Switzerland). N,N-dimethylformamide was purchased from Carlo Erba (Milan, Italy). Acetonitrile (≥99.95) was purchased from Honeywell (Morris Plains, NJ, USA). Fmoc-Tyr (tBu)-Wang resin (100-200 mesh), Fmoc-Gly-Wang resin (100-200 mesh), Fmoc-Glu (OtBu)-Wang resin (100-200 mesh), Fmoc-Leu-Wang resin (100-200 mesh), Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Ala-OH and Fmoc-Phe-OH, Fmoc-Arg (pbf)-OH and HBTU were purchased from Novabiochem (Merck, Darmstadt, Germany).

Deionized water was obtained with Select water purification system (Suez water, Thame, UK). Fmoc-Pro-OH (≥99%), N, N-diisopropylethylamine (≥99.0%), piperidine (99%), triisopropylsilane (98%), DL-dithiothreitol (≥99%), trifluoroacetic acid, dichloromethane and formic acid (≥95%) were purchased from Sigma Aldrich (Saint Louis, MO, USA).

2.4.2.. Peptide Ssynthesis

Peptides RYLGY, RYLG, AYFYPEL and AYFYPE were synthesised on solid phase using an automatic peptides synthesizer with a Fmoc/*t*-butyl strategy. Peptide synthesis is a method based on building peptides on an insoluble solid support. The Wang resins used were preloaded with Fmoc-tyrosin (tBu)-OH, Fmoc-glycine-OH, Fmoc-leucine-OH and Fmoc-glutamic acid (OtBu)-OH, respectively. The synthesis was performed on a Syro I Fully Automatic peptide synthesizer (Biotage, Uppsala, Sweden) with a reaction scale of 25 µmol. Reaction steps were as follows:

a) swelling: resin was shaken with 500 µL of dichloromethane, three cycles of 10 min each;

b) deprotection of the N-term: one cycle was done with 40% v/v piperidine in dimethylformamide for 3 min, then a second cycle was done with 20% v/v piperidine in dimethylformamide for 12 min.

Resin was washed six times with dimethylformamide to remove piperidine residues;

c) activation and coupling: *in situ* activation of the ester group was performed, adding to the resin the Fmoc amino acid, HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and DIEA (N,N-Diisopropylethylamine), in the ratio 1:4.03:3.97:8. Reaction lasted 40 min;

d) reactions b and c were repeated until the end of the amino acid sequence.

The peptide was cleaved from the resin with a solution of trifluoroacetic acid, water, dithiothreitol and triisopropylsilane in the ratio 94:2.5:2.5: 1, and purified on a Sep-Pak C18 cartridge (Waters, Milford, MA, USA). Peptides were quantified with HPLC-UV (λ=214 nm) according to Kuipers and Gruppen, (2007). Reaction yields: RYLGY 29%, RYLG 50%, AYFYPEL 47%, AYFYPE 70%.

2.4.3.UHPLC/ESI-MS/MS analysis

UHPLC/ESI-MS/MS analysis stands for "Ultra High Performance Liquid Chromatography coupled to Electrospray Ionization tandem Mass Spectrometry". This is a powerful analytical technique to separate compounds in complex mixtures and to detect them with very high specificity and selectivity. MS/MS conditions for standard peptides detection were tuned by infusion (10 µL/min) of a 10 µM aqueous solution. Optimized MS/MS parameters, used for the subsequent Selection Reaction Monitoring (**SRM**), are reported in Table S1.

Separation was achieved by mean of reverse phase ultra-high-performance liquid chromatography. Aeris Peptide 1.7 µm XB-C18 column (100 Å, 150 × 2.1 mm; Phenomenex, Torrance, CA, USA) was used for the chromatographic analysis, equipped with a Security Guard ULTRA Cartridge (C18-Peptide, ID 2.1 mm; Phenomenex, Torrance, CA, USA). Chromatographic separation was run in a Dionex Ultimate 3000 UHPLC. Flow was set at 0.2 mL/min, column temperature at 35°C and sample temperature at 18°C; eluent A was water with 0.1% (v/v) of formic acid and 0.2% (v/v) of acetonitrile, eluent B was acetonitrile with 0.1% (v/v) formic acid and 0.2% of water. A gradient elution was performed, according to the following parameters: 0–7 min 100% A, 7–50 min from 100% A to 50% A, 50–52.6 min 50% A, 52.6–53 min from 50% A to 0% A, 53–58.2 min 0% A, 58.2–59 min from 0% A to 100% A, 59–72 min 100% A (total analysis time 72 min).

Detection was achieved using a triple-stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, Waltham, MA, USA) with the following parameters: solvent delay 0-7 min, acquisition 7-58.2 min, ionization type positive ions; spray voltage 3,500 V, vaporizer temperature 250°C; sheath gas pressure 22; capillary temperature 250°C. For the Selected Reaction Monitoring method, the monitored transitions are reported in Table S1. The first reported fragment was used as quantifier, the second one as qualifier. UHPLC/ESI-MS data were elaborated using Xcalibur software (Thermo Fisher Scientific, Waltham, MA, USA). Given the complexity of the

matrix (rat sera), no direct analysis of the sample was possible due to the very high counter pressure of the system. Thus, prior to injection, sample clean-up was performed by solid phase extraction (**SPE**) using Sep-Pak C18 Plus short cartridges (Waters, Milford, MA, USA) according to the manufacturer instructions. Briefly, 100 µL of sample was diluted to 2 mL with deionized water and flushed on the cartridge. Part of the interferences and salts are then removed by flushing 10 mL of solution A (water 98%, acetonitrile 2%, formic acid 0.1%). Finally, the peptide fraction is eluted with 5 mL of solution B (water 35%, acetonitrile 65%, formic acid 0.1%). Samples are dried under nitrogen flux and reconstituted with 100 µL of water acidified with 0.1% formic acid. Injection volume was 5 µL.

A calibration curve for the peptide standards was done by injecting solutions at 20, 40, 60, 80 and 100 nM. Linearity, LOD (Limit Of Detection) and LOQ (Limit Of Quantification) were calculated from these curves. The LOD and LOQ values were calculated on the peptide solutions because of the limited amount of blank plasma available. The calculation of the LOD and the LOQ on the cheese samples (which are solid) would have implied an extraction phase from the cheese, which is different from the cleanup phase of the plasma, and thus the LOD and the LOQ would have been affected by the method used. Details are reported in Table 1. Thus, a sample clean-up was performed with Sep-pak cartridges. Recovery experiments were performed by spiking the blank sera with the peptide standards to a final concentration of 50 nM. Recovery rates were: (30%) VPP, (64%) IPP, (96%) RYLGY, (80%) RYLG, (67%) AYFYPEL, (75%) AYFYPE, (75%) LHLPLP and (103%) HLPLP.

2.5. Statistical analysis

Data expressed as least-square means and standard error of the mean were analyzed by repeated measures using a general linear model procedure considering the week of sampling as the repeated effect. SBP and DBP of rats were set as dependent variables, while PR, captopril, water, and week as independent variables.

Subject (rat) was included into the model and considered as an uncorrelated random effect. Duncan's multiple range test was used to evaluate the differences and the significances were set at a value of $P < 0.05$. The analysis was carried out using the Statistica 7.0 software (Stat Soft, Inc., Tulsa, OK, USA).

3. Results

3.1. Body weight

Table 2 shows the means and SD of body weight in the different groups of SHRs, at the beginning (time 0) and at the end of the experiment. At time 0 the averages of BW were similar in all examined groups of rats (296.8 ± 1.2 g), then gradually increased untill the end of the treatment period, but no significant changes in BW gain were observed among all groups having dietary integration or water, except in rats receiving captopril (positive control) showing lower growth performance ($P < 0.05$) than other rats.

3.2. Blood pressures

No differences between the averages of systolic and diastolic pressure were found among all groups of SHRs tested at the beginning (time 0) of the study (Table 3 and 4).

Systolic pressure showed a decrease ($P < 0.05$) after 35 days from the beginning of the treatment in rats supplemented with 0.2 g/d of PR (-8 mmHg vs day 0); after 21 days in rats supplemented with 0,4 g/d of PR (-8 mmHg vs day 0), and after 21 (-8 mmHg vs day 0) and 35 days (-13 mmHg vs day 0) in rats supplemented with 0.6 g/d of PR. In the same period, dietary integration with 0.1 g/d of PR and water, did not induce significant changes in SBP.

Diastolic pressure decreased ($P < 0.05$) after 21 days of treatment in SHRs supplemented with 0.1, 0.2, 0.4 and 0.6 g/d of PR (-14, -20, -27, -31 mmHg vs day 0, respectively). Rats supplemented with 0.6 g/d of cheese maintained lower values of DBP also at 35 days (-29 mmHg

vs day 0). In the same period, dietary integration with water, did not show significant changes in DBP of SHRs.

Starting from the 35th days of PR treatment, both systolic and diastolic pressure increased ($P < 0.05$) in all groups of SHRs, reaching final values greater than that registerd at the beginning of the trial.

As expected, due to a more immediate bioavailability of the active ingredients of captopril (positive control) compared to the biopeptides present in PR, the pharmacological treatment induced a decrease ($P < 0.01$) of BP in SHRs already after 7 days (- 34 mmHg vs day 0, for SBP; - 29 mmHg vs day 0, for DBP) and BP remained low ($P < 0.01$) until the end of the experiment.

3.3. Biochemical analysis

No ACE-inhibitory peptides or other PR-derived peptides under control were detected in the sera of rats treated with the different amounts of PR (from 0.1 to 0.6 g/d of cheese/rat), neither (as expected) in the controls treated with captopril or water in blood sampled at the end of the experimental period. The LOD and LOQ values (Table 1) suggest for a very low amount of antihypertensive cheese peptides in circulating blood.

4. Discussion

This study is part of the objectives of agri-food research intended to verify the potential antihypertensive effect of hard-cheeses. In particular, our research dealt with the effect of long-term intake of PR cheese on blood pressure changes in SHRs.

Results of the present study showed that daily dietary integration with four different amounts of 12-months aged PR cheese led to a transitory reduction in BP values of SHRs. In fact, systolic and diastolic blood pressure decreased after 21 and 35 days of cheese intake and this reduction seems positively related to the amount of PR consumed.

Several potent ACE-inhibitory peptides have been found in PR cheese and/or its *in vitro* digestates (Bernabucci *et al.*, 2014, Basiricò *et al.*, 2015; Summer *et al.*, 2017). Therefore, the antihypertensive effect observed might be attributed to the activity of ACE-inhibitory peptides content in the PR given to the SHRs. After the 35th day of treatment, no significant beneficial effect of the hypotensive peptides of PR on rats pressure was observed.

Many studies report conflicting results about the real beneficial hypotensive effects *in vivo* of such biopeptides identified as ACE-inhibitors under *in vitro* conditions (Foltz *et al.*, 2010; Jäkälä & Vappaatalo, 2010). For example, acute BP lowering effect was observed in SHRs, but not in normotensive Wistar-Kyoto rats, after oral administration of food supplemented with IPP and VPP or after intravenous injection or intraperitoneal administration of ACE-inhibitory peptides from bovine lactoferrin (Nakamura *et al.*, 1995) or whey (Costa *et al.* (2005). In clinical trials, the enzymatically hydrolyzed IPP and VPP resulted hypotensive only at elevated BP and not in mild hypertensive subjects (Boelsma and Kloek, 2009) or they were able to induce a small pooled decrease in SBP and not in DBP (Engberink *et al.*, 2008). Other studies showed that IPP and VPP and other potent *in vitro* ACE-inhibitory peptides, such as α_{s1}-casein f (23–27) or the fragment 142-148 of β-lactoglobulin, did not lead to a significant change in BP of hypertensive subjects compared with placebo (FitzGerald & Meisel, 2000; van der Zander *et al.*, 2008).

Several variables may be responsible for the different BP responses *in vivo,* e.g. the different animal model used, the hypertensive state of animals or patients, the amount of peptides provided and the time and the route of administration.

But, the best approach to establish the relationship between *in vitro* and *in vivo* efficacy of biopeptides is considering their bioavailability, needed for their bioactivity *in vivo* (Vermeirssen *et al.*, 2004). Indeed, in order to exert *in vivo* biological effects, peptides must be absorbed as biologically active intact peptides until reaching the bloodstream and the target organs (Martínez-Maqueda *et al.*, 2012). This is determined by many factors linked to digestion, absorption and other processing that could make the *in vitro* digestion model not completely representative of the *in vivo*

system where biopeptides can be generated in lower amount or maybe degraded to shorter inactive fragment by proteinases and peptidases which occur during these processes (Jakala & Vappaatalo, 2010; Martínez-Maqueda, *et al.,* 2012; Gallego *et al.,* 2016). Basiricò *et al.* (2015), in an *in vitro* study, found that after digestion only a small percentage of peptides can cross intact the intestinal barrier.

Once absorbed, a further cleavage of biopeptides can occur by means of the proteases present in the plasma and the final amount of circulating intact peptides could be so low to seriously limit their bioavailability and thus their *in vivo* biological effect (Segura-Campos *et al.,* 2011). Finally, Yamada *et al.,* (2002) also reported that, after absorption, a further cause for the loss of activity by peptides is their modification in liver.

Another possible explanation for the discrepancy between *in vitro* and *in vivo* responses is that peptides to exert their biological activity (e.g. BP lowering) need to reach the blood circulation and the target organs not only in an intact form but also in a physiologically relevant concentration (Martínez Maqueda *et al.,* 2012). This is confirmed by several studies. Vermeirssen *et al.* (2002), in an *in vitro* study assessed that the peptide Ala-Leu-Pro-Met-His-Ile-Arg, derived from a tryptic digest β-lactoglobulin, resisted to gastrointestinal digestion and was absorbed intact across the Caco-2 cell monolayer (an in vitro model of human intestinal epithelium), but in too low amount to exert a hypotensive activity. Also van der Pijl *et al.* (2008), in a study on pig model, found that the synthetic tripeptides, IPP, VPP and LPP, administered intravenously or intragastrically, reached the blood circulation intact, but with a bioavailability of about 0.1% and with a half-live of absorption and elimination maximally about 5 and 15 min, respectively, suggesting that under these conditions their bioactive effect would be rather acute.

In this study, dietary supplementation with PR cheese induced only a transitory antihypertensive effect on SHRs that was no longer noticeable after week 5 and up to the end of the experimental period. This transitory antihypertensive effect is not easy to explain. A possible explaination is that in the second part of the trial the increase of BP, linked to the enhance of age

and BW of SHRs, might have masked and reduced the effects of hypothensive biopeptides. This hypothesis is also supported by the lack of detection of ACE-inhibitory peptides in the sera of SHRs at the end of the experimental period (the limits of detection for the studied peptides resulted much lower than their IC_{50}, Stuknyte *et al.*,2015) and that could explain the outcome of the study.

As previously described, daily consumption of PR induced only a transitory reduction in BP, but it is interesting to note that cheese supplementation did not even lead to an increase in the blood pressure of rats when compared with negative control, even at higher amounts of cheese given (0.6 g/d). This interesting result indicates that right cheese consumption does not represent a risk for helth.

This contradicts the belief that dairy products, as they contain saturated fats, can enhance the content of lipids in the blood and therefore can increase cardiovascular disease and mortality in populations. A recent epidemiological cohort study of individuals aged 35–70 years (enrolled for 10 years) in 18 countries, assessed the relationship between consumption of total fat and cardiovascular disease and total mortality (Dehghan. *et al*., 2017). Results showed that total fat and saturated and unsaturated fats were not significantly associated with risk of myocardial infarction or cardiovascular disease mortality. So that, global dietary guidelines that recommend to minimize the consumption of dairy products, should be reconsidered in light of these findings.

5. Conclusion

Long-term dietary supplementation with PR cheese resulted in a transitory antihypertensive effect on SHRs. This transitory effect resulted positively related to the different amount of PR ingested by hypertensive rats.

It is interesting to note that no increase in both SBP and DBP of SHRs was observed compared with negative control (water), even after ingestion of the highest amounts of cheese tested in this study, and this result would exclude the risks of PR feeding in consumers.

In conclusion, the consumption of PR cheese, although it induced a transitory reduction of BP in SHRs, should not be excluded from the diet even for hypertensive subjects. However, to confirm the potential of this cheese in BP lowering, further researches are needed to better understand the mechanisms of action involved in its biological activity.

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504 **Table S1.** MS/MS parameters used for the SRM (Selected Reactions Monitoring) analysis.

Peptide	Precursor ion (m/z)	Fragments (m/z)	Collision energy (V)	S-lens
VPP	312.2	213.0	18	79.71
		69.9	33	
IPP	326.2	213.0	17	83.19
		69.9	33	
RYLGY	671.4	274.9	43	197.70
		111.9	51	
RYLG	508.3	275.0	34	165.01
		69.9	43	
AYFYPEL	902.4	357.9	24	200.54
		234.8	46	
AYFYPE	789.3	244.8	29	177.76
		234.8	40	
LHLPLP	689.4	364.0	27	167.08
		250.9	39	
HLPLP	576.4	109.9	47	155.00
		251.0	26	

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Table 1. Calibration curves obtained for the standard peptides. R^2 is the correlation coefficient of the calibration curve. LOD and LOQ are the Limit Of Dectetion and the Limit Of Quantification of the compound, respectively.

Peptide	Line equation	R^2	LOD (nM)	LOQ (nM)
VPP	$y = 536057x - 2043266$	0.9962	8.5	25.7
IPP	$y = 525394x + 374592$	0.9977	6.6	20.0
RYLGY	$y = 97085x - 181017$	0.9999	1.5	4.6
RYLG	$y = 144622x - 662167$	0.9881	15.2	46.0
AYFYPEL	$y = 2833211x - 21179200$	0.9754	21.9	66.4
AYFYPE	$y = 1694853x + 1621413$	0.9952	9.6	29.2
LHLPLP	$y = 59484x - 198068$	0.9749	22.2	67.1
HLPLP	$y = 129742x - 54523$	0.9952	9.6	29.1

Table 2. Means and SD of body weight (g) in SHRs during the experimental period

	Treatment					
Day	0.1[1]	0.2[1]	0.4[1]	0.6[1]	Captopril[2]	Water[3]
0	294.8	298.4	296.2	297.0	296.2	298.2
	11.0	12.7	10.7	10.2	10.1	12.5
63	371.8[b]	370.0[b]	373.2[b]	368.2[b]	344.3[a]	363.4[b]
	15.3	13.3	17.7	11.2	13.8	15.2
(63 – 0)	77.0	71.6	77.0	71.2	48.1	65.2

[1]Treated groups: 0.1 g d^{-1} $head^{-1}$ of Parmigiano Reggiano (PR); 0.2 g d^{-1} $head^{-1}$ of PR; 0.4 g d^{-1} $head^{-1}$ of PR; 0.6 g d^{-1} $head^{-1}$ of PR.
[2]Positive control: 50 mg kg^{-1} body weight of captopril.
[3]Negative control.
Letters indicate differences between group within day of control: a, b =$P < 0.05$.

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531 **Table 3.** LsMean and SEM of systolic blood pressure in different groups of SHRs

Days	Treatment					
	0.1[1]	0.2[1]	0.4[1]	0.6[1]	Captopril[2]	Water[3]
0	[b] 215.0[A]	[b] 217.0[A]	[b] 214.9[A]	[b] 217.9[A]	[a] 214.7[A]	[b] 214.9[A]
	6.0	4.7	4.1	4.1	4.7	4.2
7	[b] 210.2[A]	[b] 212.4[A]	[b] 217.3[A]	[b] 212.9[A]	[b] 180.1[B]	[b] 215.3[A]
	6.7	6.2	5.5	4.3	6.2	5.9
21	[b] 210.0[B]	[b] 218.1[A]	[c] 206.3[B]	[c] 209.4[B]	[b] 174.6[C]	[b] 214.8[A]
	2.4	4.5	3.2	2.9	3.6	1.7
35	[b] 211.9[A]	[c] 209.0[A]	[b] 214.2[A]	[c] 204.9[B]	[b] 175.0[C]	[b] 211.5[A]
	2.1	3.9	3.3	4.4	3.3	2.5
49	[a] 222.9[A]	[a] 228.9[A]	[a] 228.6[A]	[a] 223.8[A]	[b] 170.6[B]	[a] 223.1[A]
	3.2	1.8	2.3	3.0	3.2	3.4
63	[a] 224.9[A]	[a] 227.8[A]	[a] 237.8[A]	[a] 226.8[A]	[b] 179.8[B]	[a] 227.4[A]
	4.1	2.8	1.8	2.4	3.6	2.4
Overal mean	214.3[A]	213.7 [A]	215.2[A]	212.1[A]	180.3[B]	215.0[A]
	1.6	1.4	1.4	1.4	1.4	1.2

532 [1]Treated groups: 0.1 g d^{-1} $head^{-1}$ of Parmigiano Reggiano (PR); 0.2 g d^{-1} $head^{-1}$ of PR; 0.4 g d^{-1} $head^{-1}$ of PR; 0.6 g d^{-1} $head^{-1}$ of PR.
533 [2]Positive control: 50 mg kg^{-1} body weight of captopril.
534 [3]Negative control.
535 Letters indicate differences between group within day of control: a, b = $P < 0.05$ within group between time; A, B < 0.05 within time between treatment.

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538 **Table 4.** LsMean and SEM of diastolic blood pressure in different treatment groups of SHRs

Days	Treatment					
	0.1[1]	0.2[1]	0.4[1]	0.6[1]	Captopril[2]	Water[3]
0	^{a} 193.5^{A} 9.2	^{b} 192.9^{A} 7.4	^{b} 196.8^{A} 7.15	^{ab} 201.3^{A} 6.3	^{a} 199.0^{A} 7.2	^{a} 197.3^{A} 6.5
7	^{a} 195.1^{A} 7.7	^{b} 193.5^{A} 7.0	^{b} 190.7^{A} 6.6	^{b} 192.5^{A} 5.3	^{b} 170.2^{B} 7.2	^{a} 196.3^{A} 7.8
21	^{b} 179.3^{B} 4.7	^{c} 172.9^{B} 12.8	^{c} 169.3^{B} 6.9	^{c} 170.0^{B} 5.5	^{c} 142.3^{C} 7.7	^{a} 192.0^{A} 7.0
35	^{a} 189.2^{A} 5.2	^{b} 191.0^{A} 4.8	^{b} 188.7^{A} 4.5	^{c} 172.9^{B} 8.4	^{c} 145.6^{C} 4.1	^{a} 194.4^{A} 5.3
49	^{a} 194.7^{A} 5.0	^{a} 203.9^{A} 1.7	^{ab} 199.6^{A} 5.0	^{a} 197.1^{A} 6.5	^{c} 141.4^{B} 4.0	^{a} 203.8^{A} 4.6
63	^{a} 201.9^{A} 5.0	^{a} 204.8^{A} 3.7	^{a} 208.1^{A} 2.3	^{a} 204.4^{A} 3.8	^{c} 161.9^{B} 4.0	^{a} 197.5^{A} 3.5
Overal means	188.6^{A} 2.1	192.3^{A} 2.1	191.6^{A} 1.9	188.8^{A} 2.1	151.9^{B} 2.0	189.2^{A} 2.1

539 [1]Treated groups: 0.1 mg d^{-1} $head^{-1}$ of Parmigiano Reggiano (PR); 0.2 mg d^{-1} $head^{-1}$ of PR; 0.4 mg d^{-1} $head^{-1}$ of PR; 0.6 mg d^{-1} $head^{-1}$ of PR.
540 [2]Positive control: 50 mg kg^{-1} body weight of captopril.
541 [3]Negative control.
542 Letters indicate differences between group within day of control: a, b = $P < 0.05$ within group between time; A, B < 0.05 within time between treatment.

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