Food Chemistry 333 (2020) 127482

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

High protein yogurt with addition of *Lactobacillus helveticus*: Peptide profile and angiotensin-converting enzyme ACE-inhibitory activity

Flávia Giacometti Cavalheiro[a,*], Débora Parra Baptista[a], Bruno Domingues Galli[a], Fernanda Negrão[b], Marcos Nogueira Eberlin[c], Mirna Lúcia Gigante[a]

[a] Department of Food Technology, School of Food Engineering, University of Campinas, UNICAMP, 13083-862 Campinas, SP, Brazil
[b] ThoMSon Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas, UNICAMP, POB 6154, 13083-970 Campinas, SP, Brazil
[c] School of Engineering, Mackenzie Presbyterian University, 01302-907 São Paulo, SP, Brazil

ARTICLE INFO

Keywords:
Bioactive peptides
Mass spectrometry
Chemometrics

ABSTRACT

In order to evaluate differences in the peptide profile and bioactive potential in dairy products, by increasing the protein content and using proteolytic bacteria strain to enable the release of bioactive peptides, a high-protein yogurt with adjunct culture was developed. The effect of protein content, the addition of *Lactobacillus helveticus* LH-B02, and storage time were evaluated. The qualitative analysis of peptide profile was performed using a mass spectrometry approach (MALDI-ToF-MS), and the potential bioactivity evaluated by ACE inhibition activity. Protein content did not affect the peptide profile in yogurts, and the addition of *Lactobacillus helveticus* LH-B02 favored the formation of peptides recognized as bioactive, such as α_{S1}-CN f(24–32) and β-CN f(193–209). Increased protein content and adjunct culture addition increased the ACE inhibitory activity. The combination of both factors had no additional effect on the bioactive potential of yogurts.

1. Introduction

The trend of healthier food products that promote consumers' well-being has a prominent place in the market, including fermented milk and dairy products, especially yogurt. More recently, increasing interest in high-nutritional food has led to the development of high protein food products for those searching for muscle gain, decreased appetite and/or prevention of muscle atrophy, as in the case of elderly individuals (Douglas, Ortinau, Hoertel, & Leidy, 2013).

According to the Brazilian legislation, high protein content can be claimed when a food product provides at least 12 g of protein per serving of product, considering a generic 2000 kcal/day (Brasil, 2012). The use of membrane technologies, especially ultrafiltration, enabled large-scale production of caseinates, concentrates, and whey protein isolates, currently used to increase the protein content of dairy products. Calcium caseinates are the most used caseinates since they provide the technological advantage of improving the yogurt structure due to the higher number of calcium bridges (Remeuf, Mohammed, Sodini, & Tissier, 2003). Besides, calcium caseinates increase calcium intake, which might improve bone health (Thorpe, Jacobson, Layman, He, Kris-etherton, & Evans, 2008).

In addition to the nutritional importance, milk proteins, especially caseins, can be hydrolyzed, leading to the release of peptides with biological activities. The bioactive peptides are amino acid sequences that are generally inactive in the protein molecule but may exhibit activities similar to a drug or hormone, capable of modulating physiological functions when released by enzymatic action (Jin, Yu, Qi, Fangjunwang, Yan, & Zou, 2016). The most studied and proven biological activity associated with the presence of peptides in fermented dairy products is the inhibition of angiotensin-converting enzyme (ACE) activity, which is associated with a potential antihypertensive effect. ACE is part of the renin-angiotensin system, responsible for peripheral blood pressure regulation. This enzyme catalyzes the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor, and simultaneously catalyzes the degradation of bradykinin, a vasodilator peptide. Both reactions lead to the contraction of blood vessels and a consequent increase in blood pressure. Thus, through the ACE inhibitory activity, antihypertensive peptides can play an important role in the reduction of blood pressure (Nielsen, Martinussen, Flambard, Sørensen, & Otte, 2009; Otte, Lenhard, Flambard, & Sørensen, 2011).

Caseins and whey proteins are important sources of bioactive peptides, but peptides released by the hydrolysis of whey proteins have relatively lower antihypertensive activity when compared to casein-derived sequences (Fitzgerald & Murray, 2006). The release of these

* Corresponding author.
E-mail addresses: flaviagiacometti@gmail.com (F. Giacometti Cavalheiro), mirna@unicamp.br (M. Lúcia Gigante).

https://doi.org/10.1016/j.foodchem.2020.127482
Received 30 September 2019; Received in revised form 24 June 2020; Accepted 1 July 2020
Available online 06 July 2020

peptides occurs through the action of proteolytic enzymes, either during gastrointestinal digestion in the human body or during fermentation and/or ripening processes in dairy products. These enzymes may be naturally present in milk, in the human digestive tract, or associated with the presence of coagulants or lactic acid bacteria (Nielsen, Martinussen, Flambard, Sørensen, & Otte, 2009).

From the technological point of view, the production of fermented dairy products with potential biological activities, associated with the presence of bioactive peptides, requires the understanding of the process variables' effects on the peptide profile of the final product. This knowledge may allow the selection of process conditions that lead to the maximum bioactivity. These conditions include the availability of substrate, and the choice of an adjunct culture with the ability to release peptides of interest in the product. Several microorganisms have been studied for this purpose, and *Lactobacillus helveticus* strains are the most capable of producing bioactive peptides. These lactic cultures have an extremely complex proteolytic system, composed of oligopeptide transport systems through the bacterial membrane, cell wall-associated proteinases, and intracellular peptidases (Nielsen, Martinussen, Flambard, Sørensen, & Otte, 2009).

Our hypothesis for this study was that the increase of available substrate and the use of a proteolytic adjunct culture with the ability to favor the release of bioactive peptides could enable the development of a high-protein yogurt with significant antihypertensive potential. Therefore, we aimed at evaluating the effect of the protein content, the addition of *Lactobacillus helveticus* LH-B02 and the interaction of these factors on the fermentation time and ACE inhibitory activity of the yogurts. The effects of protein content, the addition of the adjunct culture and storage time on peptide profiles were also evaluated by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF-MS).

2. Materials and methods

2.1. Chemicals

All reagents used in the present study were of analytical grade. A Milli-Q water purification system (Merck Millipore, Bedford, MA, USA) was used to obtain the ultrapure water used in the experiments. Acetonitrile HPLC grade (≥99.9%), α-cyano-4-hydroxy-cinnamic acid (CHCA, 99%), trifluoroacetic acid (99%), Hipuryl-histidyl-leucine (H1635, ≥ 98%), Angiotensin Converting Enzyme from rabbit lung (A6778, ≥2.0 units/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO), and peptide calibration standard II from Bruker Daltonics (Bremen, Germany).

2.2. Preparation of lactic cultures

Mixed culture composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (YF-L812, Chr. Hansen, Hoersholm, Denmark) was activated at 45 °C for 4 h, while reactivation of *Lactobacillus helveticus* (LH-B02, Chr. Hansen, Hoersholm, Denmark) was performed at 40 °C for 18 h. Both cultures were reactivated in 10% (w/v) sterile (10 min/121 °C) reconstituted commercial skim milk (Itambé, Belo Horizonte, Brazil).

2.3. Yogurt manufacture

Commercial UHT skimmed milk (Piracanjuba, Bela Vista de Goiás, Brazil) was standardized to 10% total solids with commercial skimmed milk powder (Itambé, Belo Horizonte, Brazil) and this solution constituted the basic yogurt-making mixture, which was divided into two portions. One portion was used to manufacture control yogurts, and the second portion to produce yogurt with high protein content obtained by the addition of calcium caseinate to the base mixture, to achieve approximately 7% protein. Calcium caseinate (Fonterra, New Zealand, 92% protein) addition was performed at 60 °C under continuous mechanical stirring. Then, both control and high protein yogurt-making mixtures were heat-treated (85 °C/30 min), cooled in an ice bath and kept at 4 °C overnight to provide complete hydration of proteins before yogurt manufacturing (Needs, Capellas, Bland, Manoj, Macdougal, & Paul, 2000).

For fermentation, the mixtures (5 L for each treatment) were heated to 45 °C and subdivided into two 2.5 L portions, before inoculation with the previously activated lactic cultures. The control and high protein mixtures were inoculated with traditional lactic culture, consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (2.5% v/v), to obtain the yogurts with traditional lactic culture, or with traditional lactic culture and *Lactobacillus helveticus* (1% v/v), to obtain yogurts with adjunct culture addition. Inoculated mixtures were kept at 45 °C for fermentation. In parallel, portions of four inoculated blends were distributed into tubes (50 mL), which were kept in a water bath at 45 °C for evaluation of fermentation time. For that, every 30 min, samples were evaluated for pH. Fermentation time was considered the required period for pH mixture to reach 4.9 ± 0.05. This condition was defined according to Ribeiro, Chaves, Gebara, Infante, Grosso, and Gigante (2014) with modification and preliminary tests performed to define the ideal pH to stop the fermentation process, considering expected post-acidification related to *Lactobacillus helveticus* addition and manufacturing conditions. At the end of fermentation, yogurts were cooled in an ice bath, stored under refrigeration (4 ± 1 °C) for 24 h, shaken, distributed in 200 mL plastic cups and kept under refrigerated storage for 40 days. The complete experiment was repeated in three independent replicates, on three different days. For each process, four yogurts were manufactured with the following denomination: control yogurt, control yogurt with *Lactobacillus helveticus*, high protein yogurt, and high protein yogurt with *Lactobacillus helveticus*.

2.4. Evaluation of yogurts physicochemical and microbiological composition

After 1 day of manufacture, yogurts were evaluated for pH (digital potentiometer model DM22; Digimed, São Paulo, SP, Brazil), titratable acidity (AOAC, 2006; Method 947.05), total solids (AOAC, 2006; Method 990.19), ashes by incineration (550 °C) in a muffle furnace (AOAC, 2006; Method 945.46) and total nitrogen by the micro-kjeldahl method (AOAC, 2006; Method 991.20). Protein content was determined by multiplying total nitrogen by 6.38. Fat content was determined by the method of Mojonnier (AOAC, 2006; Method 989.05).

Total lactic acid bacteria counting was evaluated by pour-plate method and overlaid with agar MRS medium according to the methodology described by Silva, Taniwaki, Junqueira, Silveira, Nascimento, and Gomes (2013), after 1 day of storage.

2.5. Fractionation of peptides by solubility in water and ethanol 70%

Peptide extraction was performed according to the methodology described by Piraino, Upadhyay, Rossano, Riccio, Parente, and Kelly (2007) with modifications for yogurt peptide fractionation. Briefly, in order to obtain the aqueous fraction, yogurts were centrifuged for 30 min at 4 °C and 3000 *g* using an Allegra R64 centrifuge (Beckman Coulter, Indianapolis, IN, USA). Aqueous fraction (supernatant) was then filtered on glass wool and Whatman No. 113 filter paper, frozen at −80 °C and lyophilized. Duplicate aliquots of the lyophilized samples (10 mg) were dissolved in 0.5 mL of 70% ethanol, kept at room temperature for 30 min, and centrifuged at 13,000 *g* for 10 min. The supernatant, water and 70% ethanol soluble fraction, was subsequently used in MALDI-ToF-MS analysis.

2.6. Analysis of water and 70% ethanol soluble peptide fractions by MALDI-ToF-MS

Peptide profiling was performed using MALDI-ToF-MS, as described

by Galli, Baptista, Cavalheiro, Negrão, Eberlin, and Gigante (2019) and Baptista, Galli, Cavalheiro, Negrão, Eberlin, and Gigante (2018). 1 μL of each duplicate sample was applied in quintuplicate (five different spots) on a steel plate (MSP 96 polished steel target, Bruker Daltonics, Bremen, Germany). Samples were allowed to dry at room temperature followed by the application of 1 μL of the matrix solution (20 mg/mL of CHCA) dissolved in 70% acetonitrile and 0.25% trifluoroacetic acid). The instrument MALDI-ToF Autoflex III (Bruker Daltonics, Germany), equipped with SmartBeam laser, was calibrated with Peptide Calibration Standard II (Bruker Daltonics). The mass spectrometer was operated by adjusting laser power at 60–70% and voltages of ion source 1, source of ions 2, lenses, reflector and reflector 2 were 20.00, 17.77, 7.90, 21.95 and 10.03 kV, respectively. The chosen mode was a reflector of positive ions in the mass range of *m/z* 600–3500, controlled by the software FlexControl 3.3 (Bruker Daltonics), the mass suppression threshold was set at *m/z* 600 and extraction time of the pulsed ions was 30 ns. A total of 30 spectra were obtained for each yogurt and storage time considering the three independent replicates of the manufacturing processes, duplicate aliquots obtained in the peptide fractionation and five technical replicates in MALDI-ToF-MS analysis ($3 \times 2 \times 5$).

Casein-derived peptide fractions were assigned by comparing *m/z* ratios of protonated molecules and peptides previously identified in the literature, according to Piraino, Upadhyay, Rossano, Riccio, Parente, and Kelly (2007), Baptista, Galli, Cavalheiro, Negrão, Eberlin, and Gigante (2018) and Galli, Baptista, Cavalheiro, Negrão, Eberlin, and Gigante (2019). The *m/z* ratios used as reference were previously identified in fermented dairy products (Broadbent, Strickland, Weimer, Johnson, & Steele, 1998; Gagnaire, Mollé, Herrouin, & Léonil, 2001; Soeryapranata, Powers, Weller, Hill, & Siems, 2004; Ong & Shah, 2008; Kunda, Benavente, Catalá-Clariana, Giménez, Barbosa, & Sanz-Nebot, 2012; Ebner et al., 2015; Ahtesh, 2016; Jin, Yu, Qi, Fangjunwang, Yan, & Zou, 2016).

2.7. Determination of ACE inhibitory activity

Lyophilized aqueous extract obtained for analysis of peptide profile by MALDI-ToF-MS was also used to evaluate ACE inhibitory activity. The inhibitory activity was determined by the spectrophotometric method described by Cushman and Cheung (1971), with modifications proposed by Ong and Shah (2008) and further adaptations. The methodology is based on the release of hippuric acid from Hipuryl-histidyl-leucine (HHL) catalyzed by the action of ACE. For each assay, 3.8 mM HHL were diluted in 200 μL of 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl. Then 35 μL of the sample solution (15 mg lyophilized aqueous yogurt extract in 1 mL MilliQ water) were added and the tube incubated at 37 °C for 5 min. The reaction started with the addition of 20 μL of ACE solution (0.1 U/mL in borate buffer). After incubation at 37 °C for 30 min, the addition of 250 μL of 1 M HCl stopped the reaction. The hippuric acid formed in reaction was extracted by the addition of 1.5 mL of ethyl acetate followed by vortexing for 15 s and centrifugation for 10 min at 700 *g* at 20 °C. After centrifugation, 1 mL of the organic phase (ethyl acetate) was transferred to a new tube and evaporated in a water bath for 30 min at 100 °C. The remaining residue was dissolved in 1 mL of ultrapure water and absorbance of the solution was measured at 228 nm in a spectrophotometer (Orion AquaMate 8000 UV–Vis, Thermo Scientific) using ultrapure water as a blank. All samples were analyzed in triplicate. The percentage of ACE inhibition was calculated according to the following equation: ACE inhibition (%) = [1- (A-C)/(B-D)] x100, where A is absorbance with ACE, HHL and sample, B is absorbance with ACE and HHL without sample, C is absorbance with HHL and sample, and D is absorbance with HHL without ACE and sample.

2.8. Experimental design and data analysis

The effect of the treatments on yogurt composition and lactic acid bacteria counts was evaluated by Analysis of Variance (ANOVA). The effect of protein content (two levels of variation), the addition of adjunct culture (two levels of variation), as well as the interaction of these factors on fermentation time and ACE inhibitory activity, were evaluated by Analysis of Variance (ANOVA). In case of difference, the averages were compared by the Tukey's test considering a level of significance of 5%. The results were analyzed using the software Statistica 7.0.

Raw data from mass spectra were preprocessed by the software FlexAnalysis 3.4 (Bruker Daltonics) using Peptide Calibration Standard II (Bruker Daltonics) as an external standard. The most abundant signal in each spectrum was used as a reference to calculate the relative intensity of the other peptide ion peaks present in this same sample, resulting in the peak lists for each evaluated sample. The resulting .CSV files were uploaded to MetaboAnalyst 4.0 (Chong, Soufan, Li, Caraus, Li, & Bourque, 2018), an online software for statistical data analysis. Data were normalized by sum and scaled by the Pareto method. Multivariate data analysis was performed using the Partial Least Square-Discriminant Analysis (PLS-DA). PLS-DA is a supervised classification method that enables the selection of the most relevant variables for sample discrimination, according to the Variable Importance in Projection scores (VIP score $>$ 1). PLS-DA models were validated by leave-one-out cross validation using R^2 (multiple correlations) and Q^2 (cross validation) as validation metrics.

3. Results and discussion

3.1. Production and physicochemical characteristics of yogurts

The fermentation time of the mixtures was significantly affected by the increase in protein content ($p = 0.0010$) and by the addition of the adjunct culture ($p = 0.0010$), as shown in Fig. 1. The interaction between protein content and the addition of adjunct culture did not affect the fermentation time ($p = 0.3465$). Regardless of protein content, the addition of *Lactobacillus helveticus* LH-B02 reduced the fermentation time. A similar shorter fermentation time due to the addition of this bacteria was also reported by Zhou, Huo, Kwok, Li, Ma, and Mi (2019), possibly because its proteolytic action contributed to the release of amino acids that favored the development of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. In addition, *Lactobacillus helveticus* itself ferments lactose and contributes to acidification of the system (Otte, Lenhard, Flambard, & Sørensen, 2011). On the other hand, the increase in protein content by the addition of calcium caseinate increased buffering capacity of the system (Sodini, Remeuf, Haddad, & Corrieu, 2004), which resulted in a longer time to reduce pH, regardless of the type of culture used. During fermentation, casein micelles can absorb H^+ ions from the lactic acid produced by the lactic culture, releasing calcium phosphate. Thus, mixtures containing a higher amount of casein (and calcium phosphates) have better buffering capacity when compared to mixtures with lower casein content (Kindstedt, 2005). Therefore, it is observed in Fig. 1 that increasing protein content and the addition of *Lactobacillus helveticus* LH-B02 resulted in the same fermentation time as the control, that is, 180 min.

Yogurts differed significantly in all physicochemical parameters, except fat content (Table 1). *Lactobacillus helveticus* led a higher post-acidification activity, resulting in lower pH in yogurts with the addition of adjunct culture after 1 day of refrigerated storage, a similar effect also reported by Zhou, Huo, Kwok, Li, Ma, and Mi (2019).

Yogurts with calcium caseinate, which have on average 7.2 g of proteins in 100 g of product, met the requirements of the Brazilian legislation for the category of high protein content food, which is 12 g of protein per portion of the product (Brasil, 2012). Considering a 200 g portion, high protein yogurts presented approximately 14 g of protein

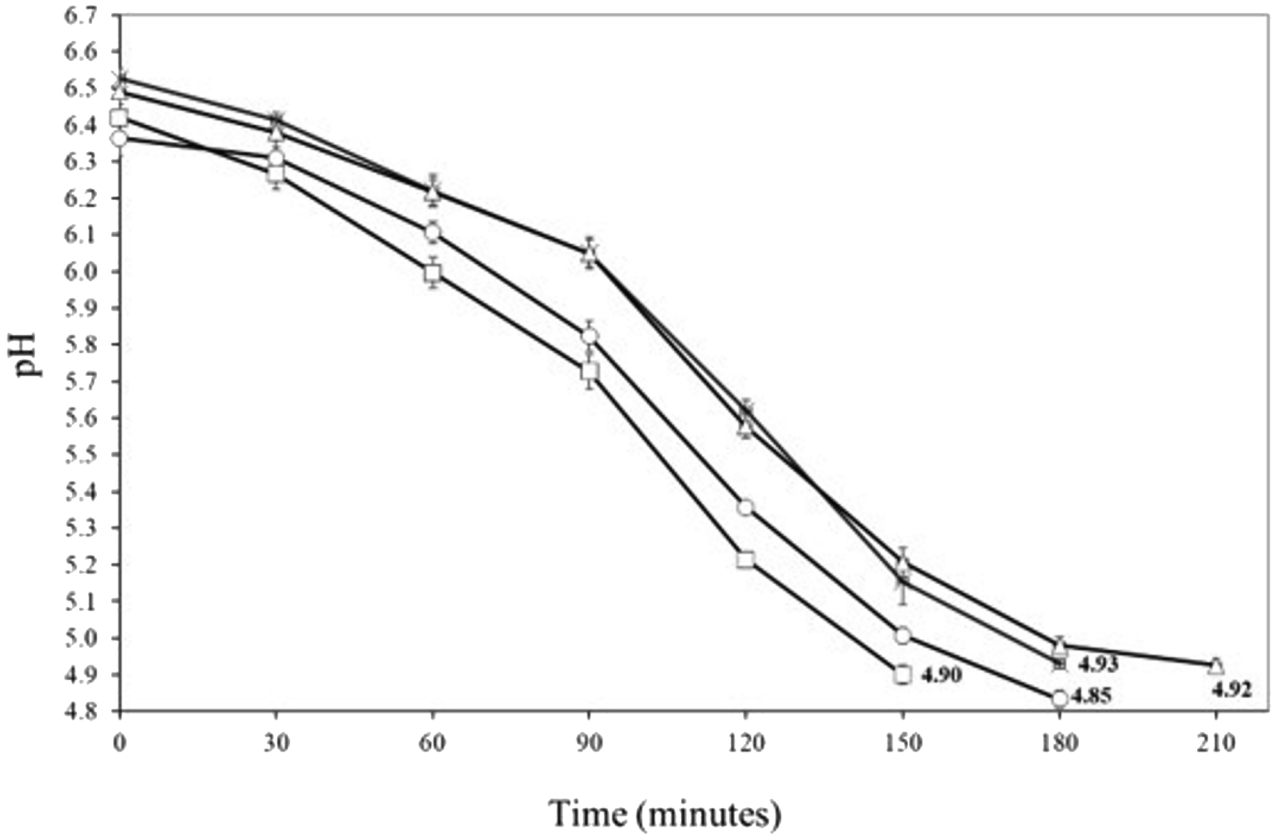

Fig. 1. pH behavior during fermentation of milk mixtures in yogurt manufacture. (×) control yogurt; (△) high protein yogurt; (○) high protein yogurt with *Lactobacillus helveticus*; (□) control yogurt with *Lactobacillus helveticus*.

per serving. All yogurts had a total lactic acid counting above 10^7 CFU/g, meeting the standards required by the Brazilian legislation (Brasil, 2007).

3.2. Peptide profile

During the 40 days of refrigerated storage, 54 peaks were detected in water and 70% ethanol soluble fraction of yogurts, using MALDI-ToF-MS analysis. Among them, 16 were derived from α_{S1}-casein, 13 from β-casein, 4 from α_{S2}-casein and 4 from κ-casein. The peptide fraction κ-CN f(44–54) (*m/z* 1252) was the most intense signal at all times for both control and high protein yogurts. On the other hand, peptides α_{S1}-CN f (24–32) (*m/z* 1053) and α_{S1}-CN f(1–16) (*m/z* 1877) were the most intense ion peaks for control yogurt with *Lactobacillus helveticus* and yogurt with high protein content and *Lactobacillus helveticus*, respectively.

Multivariate analyses were performed to compare the peptide profiles of the different yogurts and better understand the differences between the samples. The PLS-DA applied to the mass spectral data revealed that the two major principal components explained 66.9%, 66.4%, and 61.6% of the total data variance after 1, 20, and 40 days of storage, respectively (Fig. 2). These values refer to the percentage of the original data information described by the two-dimension PLS-DA score plots in Fig. 2. The addition of the adjunct culture affected the peptide profile of yogurt, evidenced by the clear separation of yogurts in two groups, with and without the addition of *Lactobacillus helveticus*, at all storage times evaluated (1, 20 and 40 days). The addition of calcium caseinate, however, did not affect the yogurts peptide profile.

During 40 days of refrigerated storage, PLS-DA analysis revealed 7 peptides as statistically significant for yogurts discrimination (VIP score > 1): κ-CN f(44–54) (*m/z* 1252), β-CN f(199–209) (*m/z* 1151), more relatively intense in control and high protein content yogurts, and

Table 1
Physicochemical composition, microbiological count, standard deviation and *p* value of yogurt after 24 h of manufacture (n = 3).

Characteristics	Control yogurt	Control yogurt with *Lactobacillus helveticus*	High protein yogurt	High protein yogurt with *Lactobacillus helveticus*	*p*-value
Physicochemical					
pH	4.60 ± 0.1^{ab}	4.34 ± 0.05^{c}	4.71 ± 0.1^{a}	4.44 ± 0.09^{bc}	0.0033
Titratable acidity (% lactic acid)	0.84 ± 0.08^{b}	1.00 ± 0.09^{ab}	1.00 ± 0.06^{ab}	1.22 ± 0.04^{a}	0.0063
Total solids (%)	9.95 ± 0.07^{b}	9.70 ± 0.13^{b}	12.78 ± 0.26^{a}	12.49 ± 0.35^{a}	< 0.0001
Protein (%)	4.53 ± 0.47^{b}	4.44 ± 0.38^{b}	7.24 ± 0.52^{a}	7.16 ± 0.25^{a}	< 0.0001
Ashes (%)	0.88 ± 0.02^{b}	0.88 ± 0.03^{b}	1.01 ± 0.04^{a}	1.00 ± 0.03^{a}	0.0004
Fat (%)	0.26 ± 0.03	0.29 ± 0.04	0.23 ± 0.09	0.29 ± 0.03	0.4428
Microbiological					
Total lactic acid bacteria count (CFU/g)	1.83 × 10^9	3.87 × 10^9	3.43 × 10^9	2.87 × 10^9	0.5880

[a, b, c] Averages with the same letter on the line do not differ significantly from each other ($p \geq 0.05$).

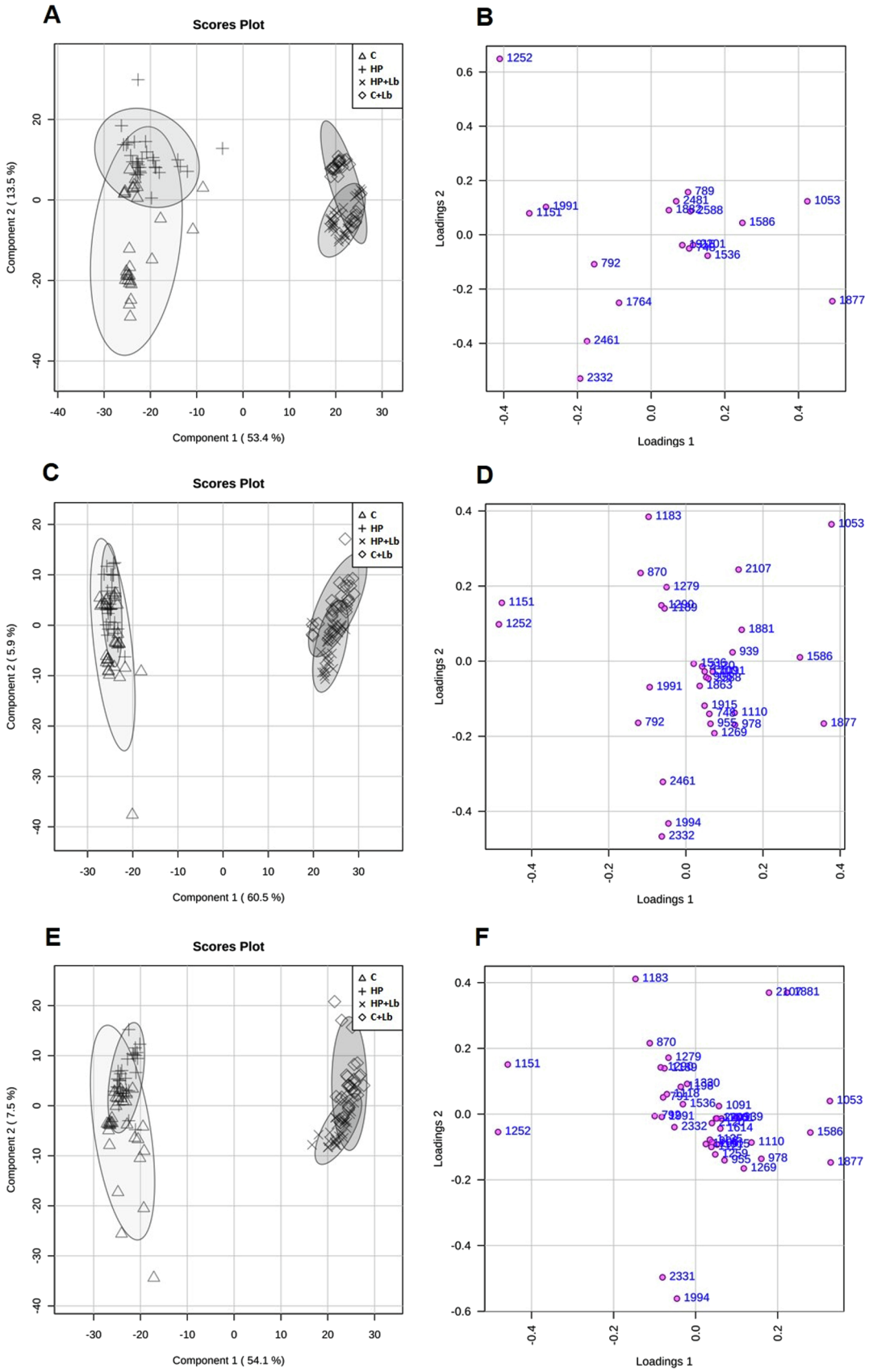

Fig. 2. Partial least square discriminant analysis (PLS-DA) of mass spectra data of peptides soluble in aqueous phase and 70% ethanol. Score plots (A, C, E) and loading plots (B, D, F) of yogurts after 1 day (A, B), 20 days (C, D) and 40 days (E, F) of storage. Numbers in Figures (△) control yogurt; (+) high protein yogurt; (×) high protein yogurt with *Lactobacillus helveticus*; (◊) control yogurt with *Lactobacillus helveticus*. The dots in the loading plots (B, D, F) represent the variables used in the chemometric analysis, expressed by their *m/z* (mass-to-charge) ratios as labels.

α_{S1}-CN f(24–32) (*m/z* 1053), α_{S1}-CN f(1–16) (*m/z* 1877), β-CN f (129–142) (*m/z* 1586), β-CN f(193–209) (*m/z* 1881), and β-CN f (191–209) (*m/z* 2107), more intense in yogurts with addition of adjunct culture (Fig. 2.E and 2.F, Fig. 2). Among them, three were recognized for their potential ACE inhibitory activity: α_{S1}-CN f(24–32) (*m/z* 1053) (Ong & Shah, 2008); β-CN f(199–209) (*m/z* 1151) (Ha, Chang, & Jo, 2015) and β-CN f(193–209) (*m/z* 1881) (Birkemo, Osullivan, Ross, & Hill, 2009). Peptide fractions β-CN f(108–113) (*m/z* 748), α_{S2}-CN f (81–89) (*m/z* 1110) and β-CN f(194–209) (*m/z* 1718), present exclusively in yogurt with adjunct culture (Table S1), are also recognized as ACE inhibitors according to Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, and Korhonen (2000), Murray and Fitzgerald (2007), and Stepaniak, Sorhaug, Jedrychowski, and Wroblewska (2001), respectively. Although they were not significant for sample classification (VIP score $<$ 1), it cannot be affirmed that they do not contribute to the yogurts' bioactive potential since it is still possible for them to present bioactivity once evaluated by *in vivo* studies.

The relative intensities of peptides β-CN f(193–209) (*m/z* 1881) and α_{S1}-CN f(24–32) (*m/z* 1053), previously described as potentially bioactive, and α_{S1}-CN f(1–16) (*m/z* 1877) were greater in yogurts with the addition of *Lactobacillus helveticus* over time. The abundance of these last two peptides in such treatments may be associated with the presence of *Lactobacillus helveticus* cell wall-associated proteinases and endopeptidases that cleave α_{S1}-CN f(1–23) fraction, leading to the release of other smaller sequences of peptides (Soeryapranata, Powers, & Ünlü, 2008). The fraction α_{S1}-CN f(1–23) is released due to the initial action of the native proteinases present in milk (cathepsins D and G), that cleave the Phe_{23}-Phe_{24} bonds of α_{S1}-casein (Considine, Geary, Kelly, & Mcsweeney, 2002), and is subsequently cleaved by aminopeptidases from *Lactobacillus helveticus*. Similarly, β-CN f(193–209) (*m/z* 1881) presence is also possibly due to the action of aminopeptidases from *Lactobacillus helveticus* LH-B02. Such enzymes use residues with hydrophobic amino acids as substrate by cleaving the Leu_{192}-Leu_{193} bond of β-casein primary sequence (Fenster, Parkin, & Steele, 1997).

On the other hand, peptides β-CN f(199–209) (*m/z* 1151), also recognized for its bioactivity, and κ-CN f(44–54) (*m/z* 1252) were more relatively intense in control and high protein content yogurts, rendering their importance to the samples separation between treatments. Cell wall-associated proteinases and peptidases might have differences regarding cleavage sites specificities among *Lactobacillus helveticus* strains. However, in general, they cleave more efficiently the β- and α_{S1}-caseins, and to a lesser extent, the α_{S2} and κ fractions (Sadat-mekmene, Genay, Atlan, Lortal, & Gagnaire, 2011). Thus, the lowest relative intensity of the peptide κ-CN f(44–54) (*m/z* 1252) in yogurts with the addition of *Lactobacillus helveticus* is possibly because it is hydrolyzed by the proteolytic system of this adjunct culture.

3.3. ACE inhibitory activity

The interaction between protein content and the type of lactic acid culture used in the yogurt manufacturing process significantly affected the ACE inhibitory activity (%) ($p < 0.05$). Fig. 3 shows that the addition of *Lactobacillus helveticus* LH-B02 in the control yogurt manufacture (approximately 4.5% protein) resulted in a significant increase in ACE inhibitory activity. However, its addition to high protein yogurt (approximately 7.2% protein) did not significantly affect the ACE-inhibitory activity. The increase in the protein content obtained by the addition of calcium caseinate itself also increased the ACE inhibitory activity of yogurts produced with traditional lactic acid culture. Thus, both the addition of *Lactobacillus helveticus* LH-B02 and the increase of protein content, through the addition of calcium caseinate, are technological strategies that can be used in yogurt processing to favor bioactive potential of this product. The association of these two strategies, however, does not represent an additional effect on ACE inhibitory activity.

The increase in ACE inhibitory activity due to the addition of *Lactobacillus helveticus* LH-B02 is associated with the modification in peptide profile resulting from the addition of this adjunct culture, which led to an increase in the relative intensity of peptides recognized as potential ACE inhibitors: α_{S1}-CN f(24–32) (*m/z* 1053) (Ong & Shah, 2008); β-CN f(193–209) (*m/z* 1881) (Birkemo, Osullivan, Ross, & Hill, 2009); β-CN f(108–113) (*m/z* 748) (Pihlanto-Leppälä, Rokka, & Korhonen, 1998); α_{S2}-CN f(81–89) (*m/z* 1110) (Murray & Fitzgerald, 2007) and β-CN f(194–209) (*m/z* 1718) (Stepaniak, Sorhaug, Jedrychowski, & Wroblewska, 2001). It is worth highlighting the importance of the amino acid specific sequence for ACE-inhibiting peptides. Peptide fractions that present in their sequence an aromatic residue at the C-terminal position are indicated as better ACE inhibitors, compared to those that do not present it (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000). The enzyme inhibition would be selective in the COOH-terminal position concerning the last two amino acids present in the sequences of inhibiting substrates, with aliphatic, basic and aromatic residues being preferred in the penultimate position and proline, aromatic and aliphatic residues in the last position. The peptide α_{S2}-CN f(81–89) (*m/z* 1110), represented by the sequence ALNEINQFY and present in yogurts with the addition of *Lactobacillus helveticus*, has two aromatic amino acids (Phenylalanine and Tyrosine) at the end of its sequence, what would result in its ACE inhibitory action. The same reasoning can be applied to peptide β-CN f(193–209) (*m/z* 1881), present in higher relative intensity in yogurts with the addition of *Lactobacillus helveticus*, is represented by the sequence YQEPVLGPVRGPFPIIV and two of its final amino acids in the sequence are aliphatic (Isoleucine and Valine) (Ong & Shah, 2008). Additionally, the higher proteolysis and the lower pH in yogurts produced with the addition of a proteolytic adjunct culture, that significantly affected the acidification behavior during the product manufacturing and storage, may have increased the calcium content in the ionic form due to the release of calcium located in the casein micelles. According to Gonzalez-Gonzalez, Tuohy, and Jauregi (2011), the ionic calcium released during milk fermentation could increase the ACE inhibitory activity, which may also explain the results obtained in the present study.

Although an increase in protein content did not show a significant modification of the peptide profile (Fig. 2), it increased the bioactive potential of yogurt (Fig. 3). This event may be related to the higher concentration of peptides inhibiting ACE activity as a result of higher substrate concentration for enzymatic activity, regardless of their diversity. This phenomenon could also be explained by the protein quality used as the substrate for ACE inhibitory peptides production (Otte, Sjalaby, Zakora, Pripp, & El-Shabrawy, 2007). With the addition of calcium caseinate, all four types of casein (α_{S1}, α_{S2}, β, and κ) had their concentrations increased in high protein yogurts, favoring the bioactivity increase in this product. In addition, the higher content of calcium ions, resulting from supplementation with calcium caseinate, may also have influenced the greater ACE inhibition in high protein yogurt (Gonzalez-Gonzalez, Tuohy, & Jauregi, 2011).

The fact that the association of the two strategies studied (calcium caseinate and adjunct culture additions) did not represent and additional effect on ACE inhibitory activity may occur because *Lactobacillus helveticus* was not able to hydrolyze such abundant protein amounts, since ACE inhibition has an exponentially relationship with the amount of proteins in the medium, and not a linear one (Nielsen, Martinussen, Flambard, Sørensen, & Otte, 2009). Thus, the addition of protein content increases the ACE inhibitory activity only up to a certain point, when the exponential curve reaches its stationary phase and further protein addition will not increase the bioactive potential. Another reasoning that would explain why ACE inhibitory activity did not statistically differ between yogurts with high protein content and high protein with the addition of *Lactobacillus helveticus* would be associated with an extensive proteolysis activity due to the adjunct culture addition, curbing ACE inhibition. This limitation occurs once ACE inhibitory activity depends on antihypertensive peptides presence, which in turn relies on the balance between the release of bioactive peptides and the

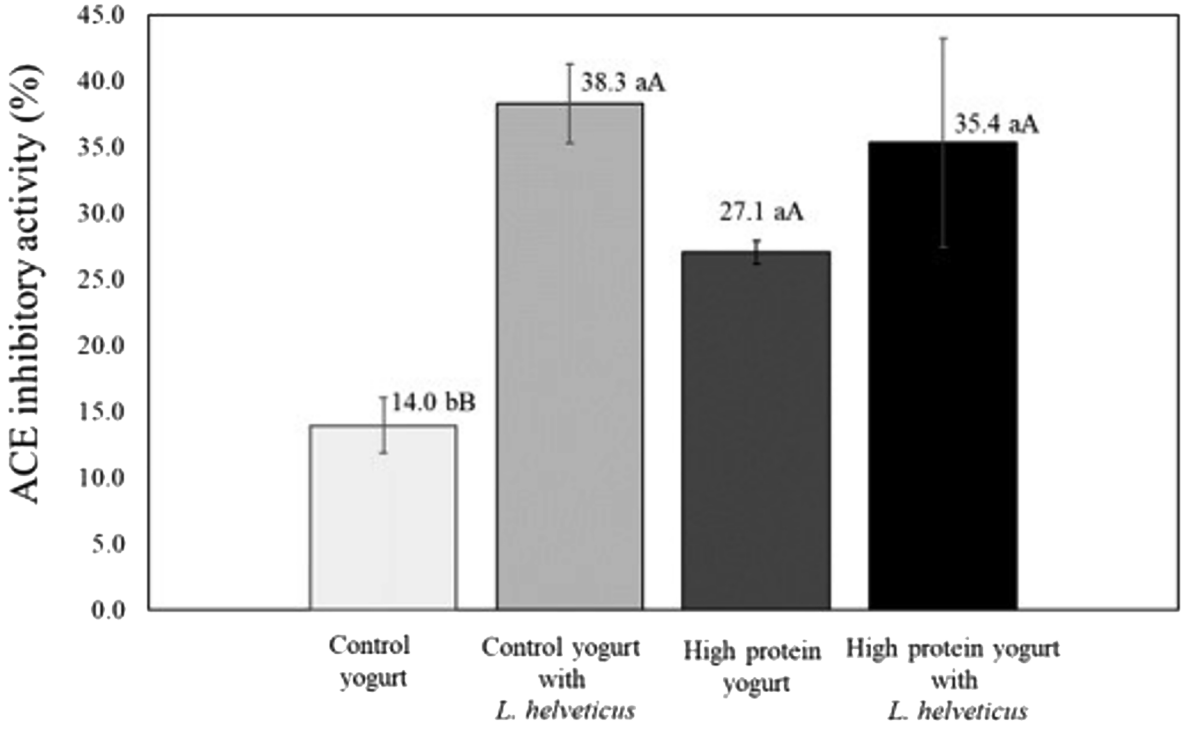

Fig. 3. Effect of interaction between protein content and lactic culture type on ACE inhibitory activity (%) (n = 3). [a,b]For each protein content, different lower-case letters show significant difference by Tukey test ($p < 0.05$). [A,B]For each type of culture, different capital letters differ significantly by Tukey test ($p < 0.05$).

cleavage of these fractions in amino acids and inactive peptides (Rutella, Tagliazucchi, & Solieri, 2016; Zhou, Huo, Kwok, Li, Ma, & Mi, 2019).

Although previous studies correlate ACE inhibition and protein increase content in fermented dairy products (Leclerc, Gauthier, Bachelard, Santure, & Roy, 2002; Akalin, Unal, & Dinkci, 2017), few are the ones that increase protein levels to the point of products being characterized as high protein and evaluate the association of this strategy with the addition of a proteolytic adjunct culture, which may explain the novelty of the data observed.

4. Conclusion

The addition of *Lactobacillus helveticus* LH-B02 favored the formation of α_{S1}-CN f(24–32) and β-CN f(193–209), considered bioactive fragments by previous studies. However, protein content did not affect the peptide profile in yogurts. Both the addition of *Lactobacillus helveticus* LH-B02 and the increase of protein content, through the addition of calcium caseinate, can be considered technological strategies that favored the bioactive potential of yogurt. However, the association of these two strategies did not represent an additional effect on ACE inhibitory activity. *In vivo* studies using animal and/or human models are needed to confirm the possible physiological effects associated with the consumption of high protein yogurt with the addition of *Lactobacillus helveticus* LH-B02.

Further studies using tandem mass spectrometry to confirm the identification of peaks in our study and the evaluation of the water and 70% ethanol insoluble fraction are also recommended.

CRediT authorship contribution statement

Flávia Giacometti Cavalheiro: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Débora Parra Baptista:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - review & editing. **Bruno Domingues Galli:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Fernanda Negrão:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Marcos Nogueira Eberlin:** Supervision, Resources. **Mirna Lúcia Gigante:** Conceptualization, Project administration, Funding acquisition, Writing - review & editing, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. The authors acknowledge the National Council for Scientific and Technological Development (CNPq) for granted scholarships.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127482.

References

Ahtesh, F. (2016, March). Anti-hypertensive (Angiotensin converting enzyme-inhibitory) peptides released from milk proteins by proteolytic microorganisms and enzymes. 306 f. Thesis (PhD) – College of Health and Biomedicine, Victoria University, Melbourne, Australia.

Akalin, A. S., Unal, U., & Dinkci, N. (2017). Angiotensin-converting enzyme inhibitory and starter culture activities in probiotic yoghurt: Effect of sodium-calcium caseinate and whey protein concentrate. *International Journal of Dairy Technology, 70*, 1–10.

AOAC International (2006). *Official methods of analysis of AOAC International.* Gaithersburg, MD, USA: AOAC International2006.

Baptista, D. B., Galli, B. D., Cavalheiro, F. G., Negrão, F., Eberlin, M. N., & Gigante, M. L. (2018). *Lactobacillus helveticus* LH-B02 favors the release of bioactive peptide during

Prato cheese ripening. *International Dairy Journal, 87*, 75–83.
Birkemo, G. A., Osullivan, O., Ross, R. P., & Hill, C. (2009). Antimicrobial activity of two peptides casecidin 15 and 17, found naturally in bovine colostrum. *Journal of Applied Microbiology, 106*, 233–240.
Brasil. 2007. Ministério da Agricultura Pecuária e Abastecimento. Instrução Normativa n° 46, de 23 de outubro de 2007, que aprova o Regulamento Técnico de Identidade e Qualidade de Leites Fermentados. Diário Oficial da União: Brasília, seção 1, p. 4, 24 de outubro.
Broadbent, J. R., Strickland, M., Weimer, B. C., Johnson, M. E., & Steele, J. L. (1998). Peptide accumulation and bitterness in cheddar cheese made using single-strain *Lactococcus lactis* starters with distinct proteinase specificities. *Journal of Dairy Science, 81*, 327–337.
Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., et al. (2018). MetaboAnalyst 4.0: Towards more transparent and integrative metabolomics analysis. *Nucleic Acids Research, 46*, 486–494.
Considine, T., Geary, S., Kelly, A. L., & Mcsweeney, P. L. H. (2002). Proteolytic specificity of cathepsin G on bovine and 3-caseins. *Food Chemistry, 76*, 59–67.
Cushman, D. W., & Cheung, H. S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology, 20*, 1637–1648.
Douglas, S. M., Ortinau, L. C., Hoertel, H. A., & Leidy, H. J. (2013). Low, moderate, or high protein yogurt snacks on appetite control and subsequent eating in healthy women. *Appetite, 60*, 117–212.
Ebner, J., Arslan, A. A., Fedorova, M., Hoffmann, C. R., Küçükçetin, A., & Pischetsrieder, M. (2015). Peptide profiling of bovine kefir reveals 236 unique peptides released from caseins during its production by starter culture or kefir grains. *Journal of Proteomics, 117*, 41–57.
Fenster, K. M., Parkin, K. L., & Steele, J. (1997). Characterization of a thiol-dependent endopeptidase from *Lactobacillus helveticus* CNRZ32. *Journal of Bacteriology, 179*, 2529–2533.
Fitzgerald, R. J., & Murray, B. A. (2006). Bioactive peptides and lactic fermentations. *International Journal of Dairy Technology, 59*, 118–125.
Gagnaire, V., Mollé, D., Herrouin, M., & Léonil, J. (2001). Peptides identified during Emmental cheese ripening: Origin and proteolytic systems involved. *Journal of Agriculture and Food Chemistry, 49*, 4402–4413.
Galli, B. D., Baptista, D. B., Cavalheiro, F. G., Negrão, F., Eberlin, M. N., & Gigante, M. L. (2019). Camembert-type cheese: Effect of heat treatment and adjunct culture *Lactobacillus rhamnosus* GG. *Food Research International, 123*, 393–402.
Gonzalez-Gonzalez, C. R., Tuohy, K. M., & Jauregi, P. (2011). Production of angiotensin-I-converting enzyme (ACE) inhibitory activity in milk fermented with probiotic strains: Effects of calcium, pH and peptides on the ACE-inhibitory activity. *International Dairy Journal, 21*, 615–622.
Ha, G. E., Chang, O. K., & Jo, S-m (2015). Identification of antihypertensive peptides derived from low molecular weight casein hydrolysates generated during fermentation by *Bifidobacterium longum* KACC 91563. *Korean Journal for Food Science of Animal Resources, 35*, 738–747.
Jin, Y., Yu, Y., Qi, Y., Fangjunwang, B., Yan, J., & Zou, H. (2016). Peptide profiling and the bioactivity character of yogurt in the simulated gastrointestinal digestion. *Journal of Proteomics, 141*, 24–46.
Kindstedt, P. (2005). American Farmstead Cheese: The complete guide to making and selling artisan cheeses. *Vermont Cheese Council,* 37–48.
Kunda, Pradeep B., Benavente, Fernando, Catalá-Clariana, Sergio, Giménez, Estela, Barbosa, José, & Sanz-Nebot, Victoria (2012). Identification of bioactive peptides in a functional yogurt by micro liquid chromatography time-of-flight mass spectrometry assisted by retention time prediction. *Journal of Chromatography A, 1229*, 121–128. https://doi.org/10.1016/j.chroma.2011.12.093.
Leclerc, P. L., Gauthier, S. F., Bachelard, H., Santure, M., & Roy, D. (2002). Antihypertensive activity of casein-enriched milk fermented by *Lactobacillus helveticus*. *International Dairy Journal, 12*, 995–1004.
Murray, B. A., & Fitzgerald, R. J. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production. *Current Pharmaceutical Design, 13*, 773.
Needs, E. C., Capellas, M., Bland, A. P., Manoj, P., Macdougal, D., & Paul, G. (2000). Comparison of heat and pressure treatments of skim milk, fortified with whey protein concentrate, for set yogurt preparation: Effects on milk proteins and gel structure. *Journal of Dairy Research, 67*, 329–348.
Nielsen, M. S., Martinussen, T., Flambard, B., Sørensen, I., & Otte, J. (2009). Peptide profiles and angiotensin-I-converting enzyme inhibitory activity of fermented milk products: Effect of bacterial strain, fermentation pH, and storage time. *International Dairy Journal, 19*, 155–165.
Ong, L., & Shah, N. P. (2008). Release and identification of angiotensin-converting enzyme-inhibitory peptides as influenced by ripening temperatures and probiotic adjuncts in Cheddar cheeses. *LWT – Food Science and Technology, 41*, 1555–1566.
Otte, J., Lenhard, T., Flambard, B., & Sørensen, I. (2011). Influence of fermentation temperature and autolysis on ACE-inhibitory activity and peptide profiles of milk fermented by selected strains of *Lactobacillus helveticus* and *Lactococcus lactis*. *International Dairy Journal, 21*, 229–238.
Otte, J., Sjalaby, S. M., Zakora, M., Pripp, A. H., & El-Shabrawy, S. A. (2007). Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: Effect of substrate, enzyme and time of hydrolysis. *International Dairy Journal, 17*, 488–503.
Pihlanto-Leppälä, A., Koskinen, P., Piilola, K., Tupasela, T., & Korhonen, H. (2000). Angiotensin I-converting enzyme inhibitory properties of whey protein digests: Concentration and characterization of active peptides. *Journal of Dairy Science Research, 67*, 53–64.
Pihlanto-Leppälä, A., Rokka, T., & Korhonen, H. (1998). Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *International Dairy Journal, 8*, 325–331.
Piraino, P., Upadhyay, V. K., Rossano, R., Riccio, P., Parente, E., Kelly, A. L., et al. (2007). Use of mass spectrometry to characterize proteolysis in cheese. *Food Chemistry, 101*, 964–972.
Remeuf, F., Mohammed, S., Sodini, I., & Tissier, J. P. (2003). Preliminary observations on the effects of milk fortification and heating on microstructure and physical properties of stirred yogurt. *International Dairy Journal, 13*, 773–782.
Ribeiro, M. C. E., Chaves, K. S., Gebara, C., Infante, F. N. S., Grosso, C. R. F., & Gigante, M. L. (2014). Effect of microencapsulation of *Lactobacillus acidophilus* LA-5 on physicochemical, sensory and microbiological characteristics of stirred probiotic yoghurt. *Food Research International, 66*, 424–431.
Rutella, G. S., Tagliazucchi, D., & Solieri, L. (2016). Survivaland bioactivities of selected probiotic lactobacilli in yogurt fermentation and cold storage: New insights for developing a bi-functional dairy food. *Food Microbiology, 60*, 54–61.
Sadat-mekmene, L., Genay, M., Atlan, D., Lortal, S., & Gagnaire, V. (2011). Original features of cell-envelope proteinases of *Lactobacillus helveticus*. *International Journal of Food Microbiology, 146*, 1–13.
Silva, N.; Taniwaki, M. H.; Junqueira, V. C. A.; Silveira, N. F. A.; Nascimento, M. S.; Gomes, R. A. R. (2013). Microbiological Examination Methods of Food and Water. A Laboratory Manual. 1. ed. Balkema: CRC Press, 2013. 456p (Chapter 14).
Sodini, I., Remeuf, F., Haddad, S., & Corrieu, G. (2004). The relative effect of milk base, starter, and process on yogurt texture: A review. *Critical Reviews in Food Science and Nutrition, 44*, 113–137.
Soeryapranata, E., Powers, J. R., & Ünlü, G. (2008). Degradation of α_{S1}-CN f1–23 by aminopeptidase N and endopeptidases E, O, O2, and O3 of *Lactobacillus helveticus* WSU19 under cheese ripening conditions. *International Dairy Journal, 18*, 178–186.
Soeryapranata, E., Powers, J. R., Weller, K. M., Hill, H. H., & Siems, W. F. (2004). Differentiation of intracellular peptidases of starter and adjunct cultures using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Lebensmittel-Wissenschaft + Technologie, 37*, 17–22.
Stepaniak, L., Sorhaug, T., Jedrychowski, L., & Wroblewska, B. (2001). Immunoreactivity and inhibition of angiotensin converting enzyme and lactococcal oligopeptidase by peptides from cheese. *Italian Journal of Food Science, 13*, 373–381.
Thorpe, M. P., Jacobson, E. H., Layman, D. K., He, X., Kris-etherton, P. M., & Evans, E. M. (2008). A diet high in protein, dairy, and calcium attenuates bone loss over twelve months of weight loss and maintenance relative to a conventional high-carbohydrate diet in adults. *Journal of Nutrition, 138*, 1096–1100.
Zhou, T., Huo, R., Kwok, L. Y., Li, C., Ma, Y., Mi, Z., et al. (2019). Effects of applying *Lactobacillus helveticus* H9 as adjunct starter culture in yogurt fermentation and storage. *Journal of Dairy Science, 102*, 223–235.
Brasil. 2012. Agência Nacional de Vigilância Sanitária. Resolução - RDC n° 54, 12 de novembro de 2012, que aprova o Regulamento Técnico sobre Informação Nutricional Complementar. Diário Oficial da União: Brasília, Distrito Federal, seção 1, p. 122, 13 de novembro.