RESEARCH ARTICLE

Teodora Panayotova
Kalinka Pashova-Baltova
Zhechko Dimitrov

Authors' address:
LB Bulgaricum PLC,
14 Malashevska str.,
Sofia 1202,
Bulgaria.

Correspondence:
Teodora Panayotova
LB Bulgaricum PLC,
14 Malashevska str.,
Sofia 1202, Bulgaria.
Tel.: + 359 897296675
e-mail: t.panayotova@lbbulgaricum.bg

Article info:
Received: 1 August 2018
Accepted: 20 August 2018

Production of ACE-inhibitory peptides in milk fermented with selected lactic acid bacteria

ABSTRACT

The ability of lactic acid bacteria to release bioactive peptides is strain specific and is dependent on the dairy processing conditions. In the present study, we developed a starter for fermented milk with increased proteolytic and peptidolytic activity. The structure and concentration of bioactive peptides are strongly dependent on the strain-specific proteolytic complex. It could be expected that such peptides would be released during the process of manufacture of fermented milk. Among the possible bioactivities of released peptides is the inhibitory effect against angiotensin converting enzyme (ACE), whose increased activity could lead to an elevation of the blood pressure. Evaluation of the peptides IPP and VPP with proven ACE-inhibitory activity was performed by UPLC-MS-MS. The quantification of peptides IPP and VPP was performed by selected reaction monitoring (SRM) method using certified standards. In this study, we developed a starter containing *L. helveticus*, *L. bulgaricus*, and *S. thermophilus* strains. The strains *L. helveticus* A1 and *L. bulgaricus* J24 possess strong proteolytic complex which lead to increased content of ACE inhibitory peptides. After a pilot production of fermented yoghurt with the developed starter, the concentration of IPP and VPP was assessed by UPLC-MS-MS. The inclusion of the highly proteolytic strain *L. helveticus* A1 in the starter culture leads to increased production of bioactive peptides in the fermented milk.

Key words: lactic acid bacteria, ACE-inhibitory peptides, fermented milk

Introduction

The use of fermented milk dates back many centuries, although there is no precise record of the date when they were first made. The value of yoghurt in the human diet is determined by the nutritive value of milk from which it is made and by the chemical changes of milk constituents occurring during lactic acid fermentation. Lactic acid in yoghurt demonstrates many physiological advantages in addition to its nutritive value. During the manufacture of fermented milk, the digestibility of proteins is significantly increased, respectively due to the effect of lactic acid and the proteolytic activity of Lactic acid bacteria (LAB). Milk proteins are a good source of bioactive peptides. These peptides can be produced in two ways by milk fermentation with selected LAB and by enzymatic hydrolysis with digestive enzymes (Chobert et al., 2005). In recent years LAB have been studied for their antihypertensive potential and it is considered they can produce angiotensin-I-converting enzyme (ACE) inhibitory peptides from milk proteins during lactic acid fermentation.

Hypertension (HTN or HT), also known as high blood pressure (HBP), is a long-term medical condition in which the blood pressure in the arteries is persistently elevated. Long-term high blood pressure, however, is a major risk factor for coronary artery disease, stroke, heart failure, peripheral vascular disease, vision loss, and chronic kidney disease. Hypertension is defined as persistent systolic blood pressure (≥ 140 mmHg), and diastolic pressure (≥ 90 mmHg) (Lollo et al., 2015).

Angiotensin-converting enzyme (ACE), is a central component of the renin-angiotensin system (RAS), which controls blood pressure by regulating the volume of fluids in the body. It converts the hormone angiotensin I to the active vasoconstrictor angiotensin II. Therefore, ACE indirectly increases blood pressure by causing blood vessels to constrict. ACE-inhibitory peptides, such as casokinins and lactokinins, are released during enzymatic proteolysis of milk proteins and bacterial fermentation, therefore, fermented milk products rich in bioactive peptides represent a natural dietary approach with potential to control hypertension (FitzGerald & Meisel, 2000; FitzGerald et al., 2004).

One of the main factors in the production of ACE-inhibitory peptides in fermented milk is the type of LAB. In recent years, milk protein-based ingredients such as sodium caseinate (SCN), calcium caseinate (CaCN) and whey protein concentrate (WPC) have been preferred as they have better

http://www.jbb.uni-plovdiv.bg
DOI: 10.69085/jbb20181031

effects on yoghurt's functional and textural properties than skim milk powder (SMP) does (Alkalin et al., 2012).

The aim of this study was to detect ACE-inhibitory peptides in milk fermented with a selected LAB in media with and without protein-based ingredients.

Materials and Methods

Bacterial strain culture

The strains used in this study were *Lactobacillus helveticus* A1, *Lactobacillus delbrueckii* ssp. *bulgaricus* J24 and *Streptococcus thermophilus* TN1. Commercial DVS starter culture LBB BY 5-12 was used as a control. *Lactobacillus helveticus* A1 has been chosen as a comparative reference strain which releases the peptide Ala-Leu-Pro-Met as the main contributor to the ACE inhibition (Dimitrov et al., 2015). All bacterial strains were obtained from the Collection of LB Bulgaricum PLC (Sofia, Bulgaria). The bacterial strains were activated by 3 rounds of overnight passage at 37°C in sterile reconstituted milk at stationary conditions. The reconstituted milk was prepared by suspending 10% (wt / wt) skim milk powder in distilled water at 121°C for 6 min. After this activation bacterial strains were cultivated in 2 L bioreactors (Laboratory Bioreactors Diabench 2 Hybrid). Samples were taken at certain time intervals, depending on observed growth and acid production rates. After reaching sufficient biomass concentration bacterial strains were lyophilized at laboratory lyophilizer freeze-dryer – Zirbus 2x3x3 3526.

Media preparation and yoghurt manufacture

Fermented milks were manufactured at laboratory conditions at R&D Center of "LB Bulgaricum", Sofia, Bulgaria. There were produced 5 batches of fermented milks. For this purpose, four of samples were produced with different kind of milk-protein ingredients such as milk protein concentrate 48 (MPC 48), milk protein concentrate 52 (MPC 52), sodium caseinate (SCN), whey protein concentrate (WPC 80) and skimmed milk powder (SMP). Protein-based ingredients were delivered by P.I.C. Co. Ltd. The sixth sample was without any additives, only pasteurized cow milk. Initially, our study started with the preparation of 2 batches fermented milks, in which as a starter culture we used commercial starter culture LBB BY 5-12 with and without additives of *Lactobacillus helveticus* A1 and *Lactobacillus delbrueckii* ssp. *bulgaricus* J24. The first batch was prepared without any supplements, it contains only pasteurized cow milk. The ingredients of the second batch were pasteurized cow milk, 5% MPC 48 and 1% SMP. This media was pasteurized at 90°C for 30 min and cooled to 43°C. The first two batches were inoculated with mentioned above freeze-dried strains in an amount of 40 mg per liter. In the third batch, we used as a starter culture LBB BY 5-12 + *Lactobacillus helveticus* A1+ *Lactobacillus delbrueckii* ssp. *bulgaricus* J24. We prepared 3 samples with different media. The media of the first sample was pasteurized cow milk and 2.9% SCN. The media of the second sample was pasteurized cow milk, 2.7% SCN and 1% MPC 52. The media of the third sample was pasteurized cow milk, 2.7% SCN and 1% MPC 48. The fourth batch was prepared using strains *Lactobacillus helveticus* A1, *Lactobacillus delbrueckii* ssp. *bulgaricus* J24 and *Streptococcus thermophilus* TN1. The media was prepared by pasteurized cow milk, 5% MPC 48 and 1% SMP. The fifth batch was prepared with the same strains as the fourth, but we prepared two samples. The media of first sample was pasteurized cow milk and 2.7% MPC 52. The media of second sample was pasteurized cow milk and 2.7 % WPC 80. The batches 3 to 5 were prepared as mentioned above for batch 1 and 2. The yoghurt-making process is presented in Figure 1.

Evaluation of ACE-inhibiting peptides Isoleucine-Proline-Proline (IPP) and Valine-Proline-Proline (VPP)

Evaluation of tripeptides IPP and VPP was performed by Ultra high-pressure liquid chromatography with tandem mass spectrometry (UPLC-MS^2). Selected Reaction Monitoring method was applied using certified peptide standards. The UPLC separation was carried out on reverse phase column TG-RP 2.1 x 150 mm (120 A, 1.9 µm) using gradient of acetonitrile in 0.1% formic acid from 20% to 60% at flow rate of 0.2 ml/min. The temperatures of the vaporizer and capillary tubes were 250°C and 350°C, respectively. The ionization potential was 4.5 kV. The software Xcalibur (Thermo Scientific) was used for the quantitative assessment by the method of the internal standard.

Physicochemical and sensory analyses

The pH of yoghurt samples was measured at 1, 7, 14 and 21 days of storage at 5°C by using a Mettler Toledo-Seven Compact pH/Ion meter S220. Yoghurt samples, contained in white plastic covered cups and coded randomly, were scored in terms of: aroma; color; the presence of whey; consistency after breaking the gel with a spoon; the presence of lumps after stirring; taste and overall preference.

Bacterial cells enumeration

The spread plate technique was used to determine viable cell counts (cfu.mL^{-1}). Ten-fold serial dilutions were prepared and plated on MRS and M17 agar to enumerate *Lactobacillus helveticus, Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* cells, respectively. Plates were incubated for 48 – 72 h at 37°C using anaerobic jars with an anaerobic kit for lactobacilli. The colony enumeration was carried out by Stuart Scientific colony counter. Plates showing 25 – 250 colonies were counted and expressed as colony-forming units per mL (cfu.mL^{-1}) of the sample. All the plating was performed in duplicate.

http://www.jbb.uni-plovdiv.bg
DOI: 10.69085/jbb20181031

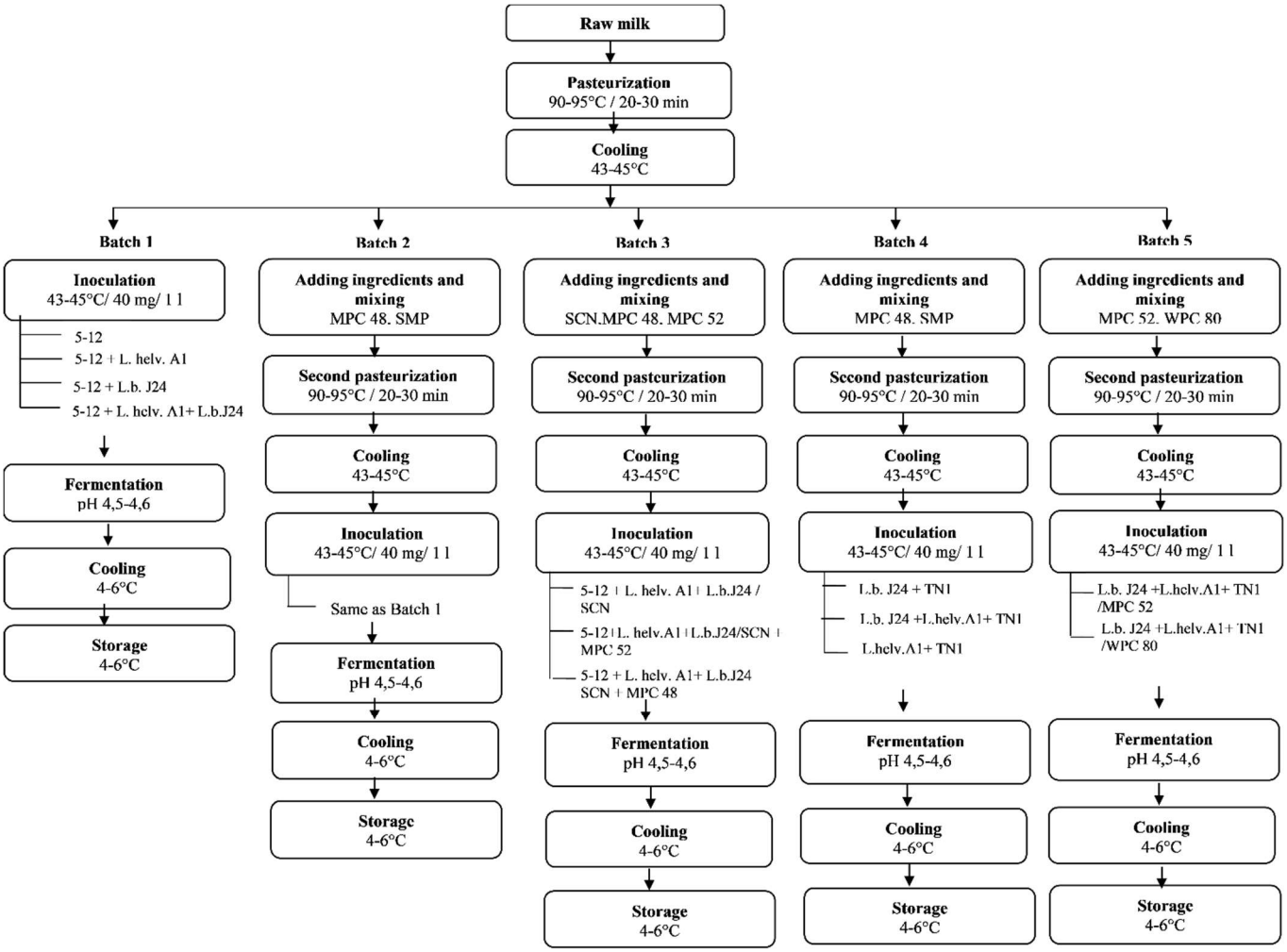

Figure 1. *Flowchart of yoghurt-making process used in the study.*

Results

Detection and quantification of peptides with ACE-inhibitory activity

The concentration of VPP and IPP in the different samples was between 2.8 and 36.6 ppm. The addition of milk proteins and especially WPC 80 led to increasing of the production of VPP and IPP several (up to thirteen) times. VPP and IPP are released from β-casein (f84-86 and f74-76, respectively). But the inclusion in the composition of the samples of whey protein, which does not contain VPP and IPP sequences, increased the VPP and IPP content much higher than the addition of sodium caseinate, which contains VPP and IPP sequences. This means that the WPC 80 is an inducer of the proteolytic activity of the selected starter strains and leads to increased release of low molecular weight peptides such as VPP and IPP.

On Figure 2 full scan of VPP after collision-induced dissociation (CID) is shown. The selected precursor ion 312.3 m/z was subjected to CID which led to three product ions: 169.1, 197.09, and 213.09 m/z. The product ion 197.09 m/z was selected for SRM quantification assay because of its highest intensity in comparison of the other two ions.

On Figure 3 full scan of IPP after collision-induced dissociation is shown. The selected precursor ion 326.4 m/z was subjected to CID which led to three product ions: 183.05, 211.09, and 213.09 m/z. The product ion 211.09 m/z was selected for SRM quantification assay because of its highest intensity in comparison of the other two ions. Another reason to select product ions 197.09 m/z for SRM of VPP and 211.09 m/z for SRM of IPP is that the ion 213.09 is common for the two peptides.

In Table 1 the results of the total concentration of VPP and IPP are shown using different formulations of the composition. Everywhere the previously selected as ACE-inhibitor strain *Lactobacillus helveticus* A1 was used the concentration of VPP and IPP was remarkably increased. Additionally, the inclusion of WPC 80 led to the higher release rate of the two tripeptides.

http://www.jbb.uni-plovdiv.bg
DOI: 10.69085/jbb20181031

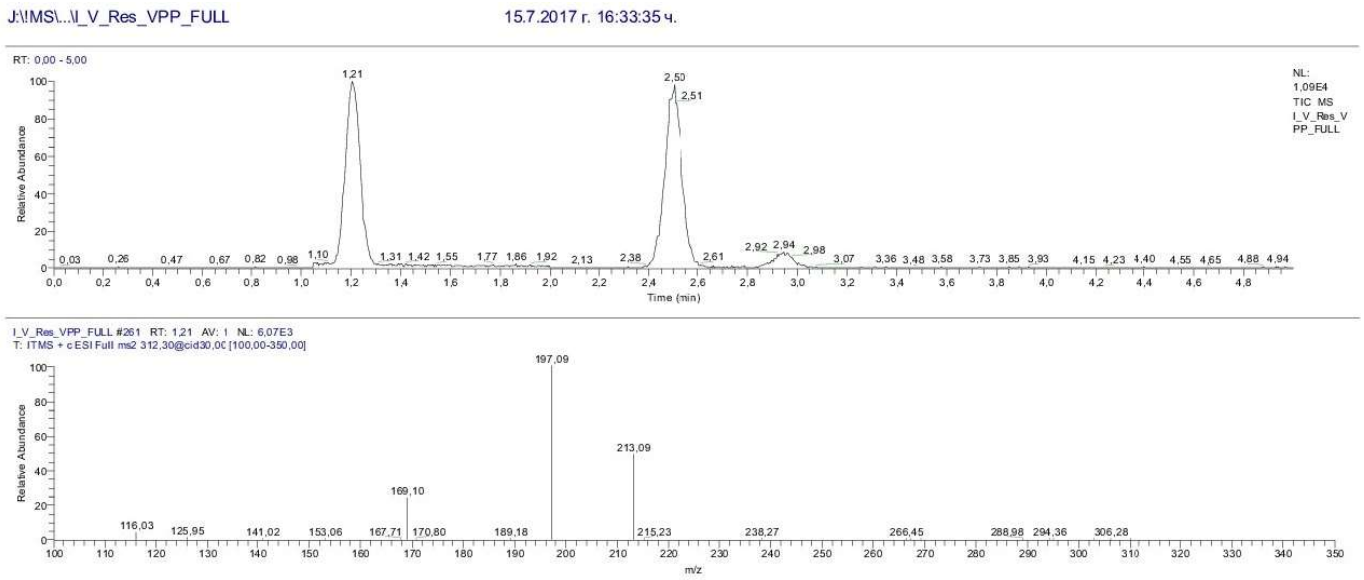

Figure 2. *Full scan of VPP after collision-induced dissociation.*

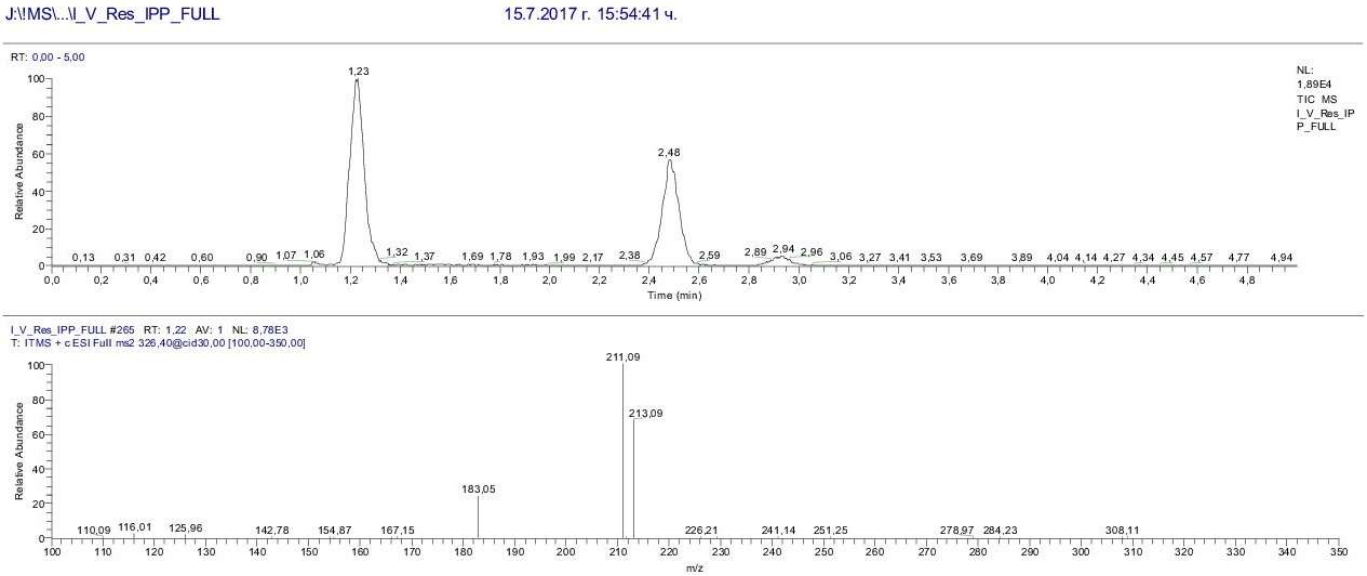

Figure 3. *Full scan of IPP after collision-induced dissociation.*

Physicochemical analyse

The results of the acidification process during storage of yoghurt samples with high concentration of ACE-inhibiting peptides are presented in Figure 4. The pH at 1 day of the samples varied in the range of 4.30 – 4.62. Sample with pH 4.62 was from Batch 4 which was produced with MPC 48 and SMP. As a starter culture was used combination of *Lactobacillus helveticus* A1, *Lactobacillus bulgaricus* J24 and *Streptococcus thermophilus* TN1. Sample with pH 4.30 was from Batch 5 which was produced with WPC 80. As a starter culture was used as mentioned above combination. At 21 day of storage, the pH varied 4.08 – 4.17 for the sample from Batch 1 and sample from Batch 4, respectively. Presented results show that no significant difference in pH was observed during storage in all samples. The addition of protein-based ingredients did not significantly affect the acidification process. Samples were organoleptically tested at 1, 7, 14 and 21 days during storage. There were no significant differences in taste, flavor and overall appearance between samples prepared with and without proteins. (The results are not presented).

Bacterial cells enumeration

The results of the viability of LAB during storage of yoghurt samples with high ACE-activity are presented at Figure 5. The bacterial count of lactobacilli were 3.7 – 5.5.10^8 cfu.mL^{-1} for the sample from Batch 1 which was produced without protein-based ingredients and for the sample from

http://www.jbb.uni-plovdiv.bg
DOI: 10.69085/jbb20181031

Batch 5 which was produced with WPC 80, respectively. The results of the viability of *Streptococcus thermophilus* cells were similar as mentioned above 2.4 – 3.2.10^9 cfu.mL^{-1}. The survival trend was retained until the 21st day of storage. The viability of samples shown above was 1.3 – 3.6.10^8 cfu.mL^{-1} for lactobacilli and 1.1 – 2.1.10^9 cfu.mL^{-1} and for *Streptococcus thermophilus* cells.

Discussion

In this study, the influence of different kind of milk protein-based ingredients was evaluated on the release of ACE-inhibiting peptides by LAB. Analysis of the variety of combinations showed that there is a correlation between bacterial growth, the different type of media and the strain specificity to obtain samples with a higher concentration of ACE-inhibiting peptides. Combination of *L. helveticus* A1, *L. bulgaricus* J24 and *S. thermophilus* TN1 with WPC 80 showed the highest total concentration of VPP and IPP - 36.60 ppm. It should be noted that the bacterial growth in this sample was 5.5.10^8 cfu.mL^{-1} for lactobacilli and 3.2.10^9 cfu.mL^{-1} for streptococci at the beginning of storage. Good concentration of VPP and IPP was observed in the second sample of the fourth batch – 14.92 ppm. The same strains of microorganisms were involved, but the ingredients were different. The milk-protein ingredients were MPC 48 and SMP. The number of cells was also preserved during storage. The number of lactobacilli was 4.9.10^8 cfu.mL^{-1} and 2.9.10^9 cfu.mL^{-1} for streptococci on the first day of storage. These results indicate that sample with WPC 80 has a higher concentration of VPP and IPP compared with the sample with MPC 48 and SMP.

Table 1. *Total concentration of VPP and IPP using different formulations.*

	Yogurt samples	
Batch	**Starter culture**	**Result ppm**
	Samples without ingredients	
Batch 1	5-12	2.8
	5-12 + L.helv. A1	5.32
	5-12 + L.b. J24	4.70
	5-12 + L.helv. A1 + L.b. J24	5.94
	Samples with MPC 48 and SMP	
Batch 2	5-12	2.85
	5-12 + L.helv. A1	12.82
	5-12 + L.b. J24	8.12
	5-12 + L.helv. A1 + L.b. J24	13.80
	Samples with SCN, MPC 48 and MPC 52	
Batch 3	5-12 + L.helv. A1+ L.b. J24 / SCN	7.84
	5-12 + L.helv. A1+ L.b. J24 / SCN + MPC 52	10.06
	5-12 + L.helv. A1+ L.b. J24 / SCN + MPC 48	11.20
	Samples with MPC 48 and SMP	
Batch 4	L.b.J24 + TN1	8.84
	L.helv.A1 + L.b.J24 + TN1	14.92
	L.helv.A1 + TN1	9.95
	Samples with MPC 52 and WPC 80	
Batch 5	L.helv.A1 + L.b.J24 + TN1/ MPC 52	12.90
	L.helv.A1 + L.b.J24 + TN1/ WPC-80	36.60

Similar results were observed in sample 4 from Batch 2 which was produced with the same ingredients (MPC 48 and SMP) but with different strains. The amount of VPP and IPP was 13.80 ppm. Beside to the samples presented above, commercial DVS starter culture LBB BY 5-12, *L. helveticus*

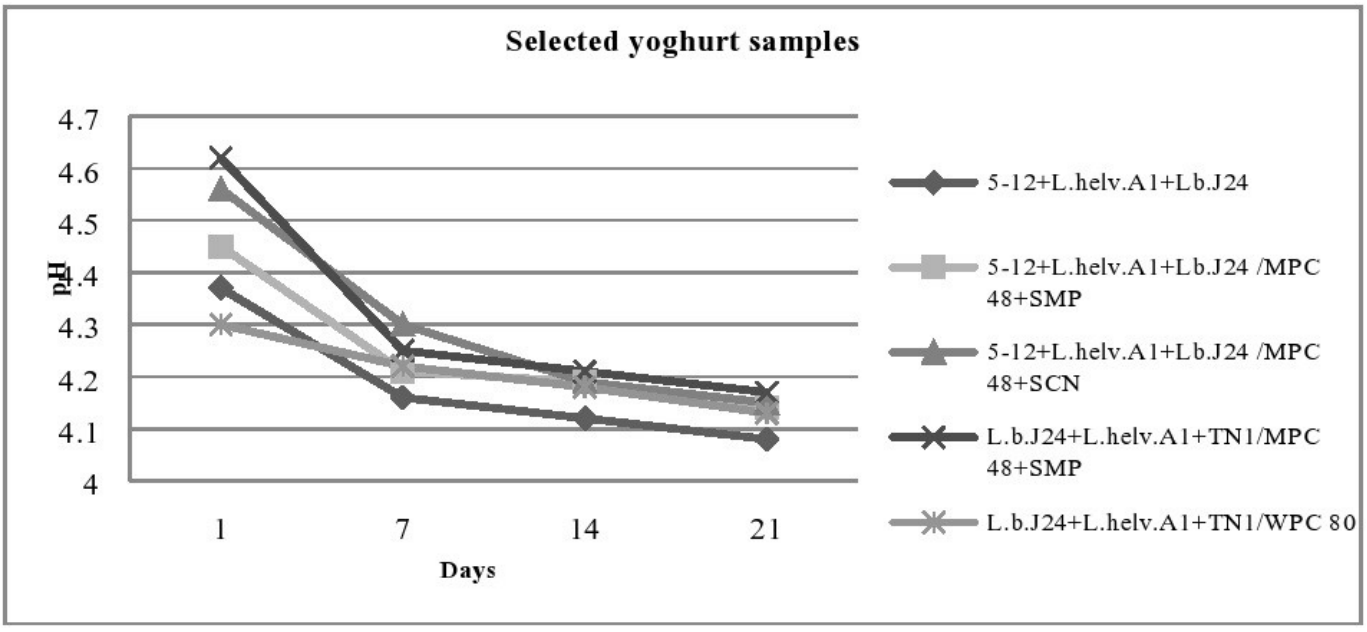

Figure 4. *Acidification process of selected yoghurt samples (with high ACE-activity) during storage.*

http://www.jbb.uni-plovdiv.bg
DOI: 10.69085/jbb20181031

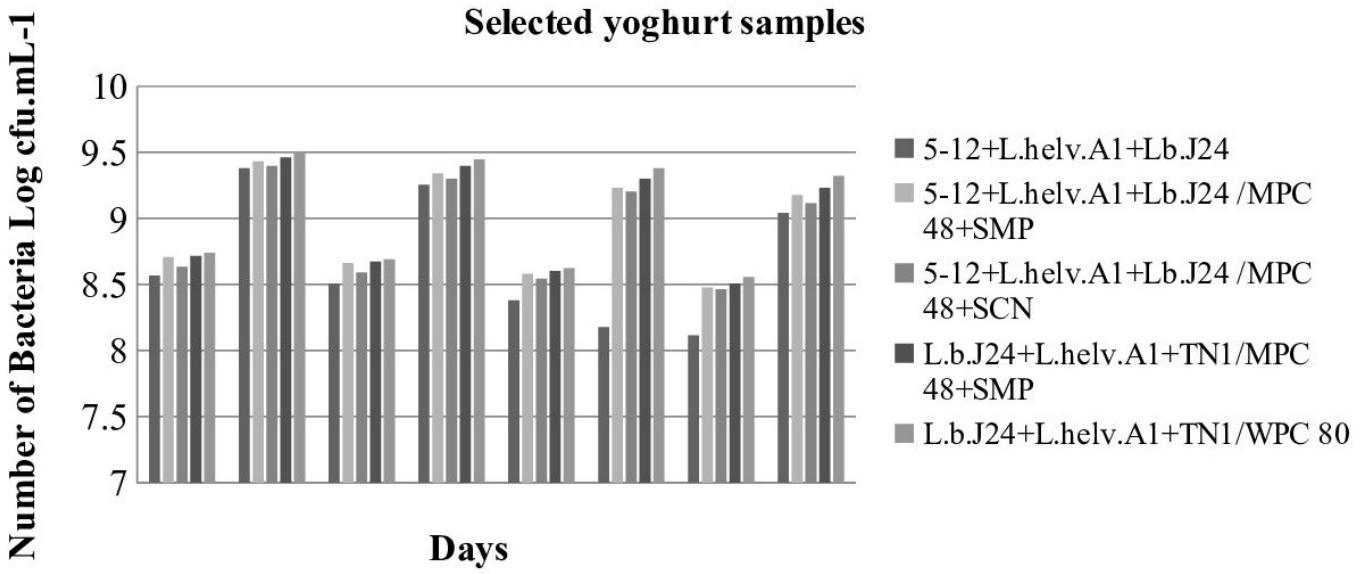

Figure 5. *Distribution of LAB of selected yoghurt samples (with high ACE-activity) during storage.*

A1 and *L. bulgaricus* J24 were used here. The number of lactobacilli was $4.7.10^8$ cfu.mL^{-1} and $2.7.10^9$ cfu.mL^{-1} for streptococci on the first day of storage. The presented results show that replacement of *S. thermophilus* TN1 with the combination LBB BY 5-12 did not significantly affect the release of bioactive peptides. The same combination of starter cultures was used with MPC 48 and SCN. Table 1 shows that concentration of VPP and IPP was 11.20 ppm. This indicates that, when replacing the SMP with SCN, it leads to a decrease in the release of bioactive peptides. The number of cells is also lower than the above-mentioned samples (Figure 5). The combination with LBB BY 5-12, *L. helveticus* A1 and *L. bulgaricus* J24 was used without milk-protein ingredients. The concentration of VPP and IPP was 5.94 ppm and the viability of lactobacilli and streptococci on the first day of storage was $3.7.10^8$ cfu.mL^{-1} and $2.4.10^9$ cfu.mL^{-1}, respectively. It's obvious that the concentration of the released bioactive peptides was minimal when no protein additives were used. Also, the lowest number of microorganisms was observed. These results show that the use of appropriate protein additives, as well as the proper selection of the strains, is of great importance in obtaining a high content of bioactive peptides. Therefore, in order to obtain a functional dairy product containing ACE-inhibiting peptides at increased concentration, numerous tests were carried out and the best protein additive and the suitable strains were selected. Our study showed that samples with *L. helveticus* A1 produced considerable ACE inhibitory activity after fermentation. *L. helveticus* is known to possess a cell-wall associated protease, capable of forming antihypertensive peptides from casein (Vermeirssen et al., 2003). This variant of fermented milk with selected protein additive and proper strains can be considered to have a hypotensive potential. ACE-inhibiting peptides are food derived natural preventives used to control hypertension and could lead to a decrease in the requirement of medicines which exert some side effects.

Conclusion

Our study has shown that milk fermented with selected LAB is an excellent source of bioactive peptides with ACE-inhibitory activity. The highest concentration of ACE-inhibiting peptides (36.60 ppm) was achieved with the combination of *Lactobacillus helveticus* A1, *Lactobacillus bulgaricus* J24 and *Streptococcus thermophilus* TN1. It should be noted that the release of ACE-inhibiting peptides VPP and IPP is a strain-dependent and the inclusion of *L. helveticus* A1 strain in the starter led to a higher release of the two tripeptides. It is known that whey proteins possess high nutritional value and versatile functional properties in food products (De Wit, 1998). The addition of WPC 80 led to a significant increase of the two tripeptides. Normal acidification process during storage was observed. After 21 days of storage, substantial numbers of the viable LAB were presented in fermented milks ($10^8 – 10^9$ cfu.mL^{-1}). This high survival of LAB during storage contributes to high nutritive and biological value of fermented milks. After 21 days of storage, yoghurt samples had good technological parameters and organoleptic assessment.

References

Alkalin AS, Unal G, Dinkci N, Hayaloglu AA. 2012. Microstructural, textural, and sensory characteristics of probiotic yoghurts fortified with sodium caseinate or whey protein concentrate. J. Dairy Sci., 95 (7): 3617-3628.

http://www.jbb.uni-plovdiv.bg
DOI: 10.69085/jbb20181031

Chobert JM, El-Zahar K, Sitohy M, Dalgalarrondo M, Metro F, Choiset Y, Haertlé T. 2005. Angiotensin I-converting-enzyme (ACE)-inhibitory activity of tryptic peptides of ovine β-lactoglobulin and of milk yoghurts obtained by using different starters. Dairy Sci. Technol., Issue Lait, 85 (3): 141-152.

De Wit JN. 1998. Nutritional and functional characteristics of whey proteins in food products. J. Dairy Sci., 81 (3): 597-608.

Dimitrov Zh, Chorbadjiyska E, Gotova I, Pashova K, Ilieva S. 2015. Selected adjunct cultures remarkably increase the content of bioactive peptides in Bulgarian white brined cheese. Biotechnol. Biotec. Eq., 29, (1): 78-83.

FitzGerald RJ, Meisel H. 2000. Milk-protein derived peptide inhibitors of angiotensin-I-converting enzyme. Brit. J. Nutr., 84 (S1): 33-37.

FitzGerald RJ, Murray BA, Walsh DJ. 2004. Hypotensive peptides from milk proteins. The Journal of Nutrition, 134 (4): 980S-988S.

Lollo PCB, Morato PN, Moura CS, Almada CN, Felicio TL, Esmerino EA, Barros ME, Amaya-Farfan J, Sant'Ana AS, Raices RRS, Silva MC, Cruz AG. 2015. Hypertension parameters are attenuated by the continuous consumption of probiotic Minas cheese. Food Res. Int., 76 (Pt 3): 611-617.

Mohanty DP, Mohapatra S, Misra S, Sahu PS. 2016. Milk derived bioactive peptides and their impact on human health. Saudi Journal of Biological Sciences, 23 (5): 577-583.

Vermeirssen V, Van Camp J, Decroos K, Van Wijmelbeke L, Verstraete W. 2003. The impact of fermentation and in vitro digestion on the formation of angiotensin-I-converting enzyme inhibitory activity from pea and whey protein. J. Dairy Sci., 86(2): 429-438.

http://www.jbb.uni-plovdiv.bg
DOI: 10.69085/jbb20181031