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Functional and Technological Potential of Whey Protein Isolate in Production of Milk Beverages Fermented by New Strains of *Lactobacillus helveticus*

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Abstract: With their desired functional and technological properties, whey protein preparations are used in the food industry. In turn, lactic acid fermentation may contribute to release of a wide range of biologically active peptides (BAPs) (known also as bioactive peptides or biopeptides) from whey and milk proteins, which are perceived as potential therapeutic tools and important constituents of personalized food suitable for prevention of many civilization and diet-related diseases. Therefore, the objective of this study was to determine the suitability of new *Lb. helveticus* strains for production of fermented milk beverages (drinking type) supplemented with whey protein isolate (WPI). Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) was employed to assess if WPI (water solution) might be a suitable precursor for BAPs produced by selected strains of *Lb. helveticus.* In order to identify the bioactivities of the peptides generated in WPI hydrolysates, the procedures indicated in databases were used. The fermented products differed from each other in some texture parameters, the content of protein, total nitrogen, and non-protein nitrogen, and the proteolysis index, which was dependent on the strain. Strain B734 was found to exhibit technological potential for development of new health-oriented fermented milk beverages with characteristics of functional food. Additionally, it proved to be able to release a wide range of BAPs from WPI with antioxidative, antibacterial, and immuno- and cyto-modulatory effects, as well as ACE (angiotensin-converting enzyme) inhibitory and antihypertensive activities.

Keywords: *Lactobacillus helveticus*; biopeptides; fermented milk beverages

1. Introduction

Whey and whey-derived preparations are no longer seen as a problematic by-product of cheese manufacture. Many current scientific reports and the latest research results indicating pro-health activity of whey proteins on the human body and a wide range of beneficial health effects contribute to the noticeably growing interest in whey and whey protein preparations in recent years [1]. Moreover,

whey protein preparations also exhibit desired technological properties, including improvement of some textural properties, and modify some characteristics of final food products, e.g., they increase water binding, emulsifying, or foaming properties and contribute to lowering the costs of production by reduction of expenditure of raw materials (for instance, they allow decreasing the addition of milk powder) [2,3]. Furthermore, milk-based ingredients, such as whey protein concentrate, sodium caseinate, and others, can be used in the manufacture of novel foodstuffs, contributing to development of products with health-promoting properties [1,4,5]. Similarly, some protein preparations can be used in the food industry as substitutes of ingredients in formulations of many foodstuffs, such as the dynamically developing novel products with reduced contents of fat and/or sugar [6,7]. In addition, the application of different dry dairy ingredients (derived from whey) allows obtaining products with diversified protein composition and influences the textural and rheological properties of fermented milk beverages [1,8].

It is worth emphasizing that, in addition to their high nutritional value and functional properties, whey protein preparations do not negatively influence the taste of products [1] and can be incorporated in protein supplements for physically active people, sportsmen, convalescents, or individuals with specific nutritional requirements. Moreover, milk and whey proteins are a source of precursors of biologically active peptides (BAPs, also known as bioactive peptides or biopeptides) that exhibit a wide range of beneficial health effects. However, when enclosed in native protein structures, they are not active. Sequences of biopeptides can be released from the native structure of proteins through hydrolysis performed by digestive enzymes and by the proteolytic enzyme system of lactic acid bacteria (some dairy starter cultures) during the fermentation process [9,10].

Lactic acid fermentation contributes to generation of BAPs, which are perceived as one of the potential therapeutic tools and can be constituents of personalized food in prevention of many civilization and diet-related diseases. Additionally, whey protein preparations exhibit various functional and desired technological properties. Therefore, the objective of this study was to determine the suitability of new *Lb. helveticus* strains (isolated in Poland) to produce fermented milk beverages (drinking type) supplemented with whey protein isolate (WPI). The study was also focused on analysis of some biochemical properties of the fermented products obtained. An additional aim of the study was to detect and identify BAPs in the hydrolysates of WPI (water solution) obtained by application of the selected strains.

2. Materials and Methods

2.1. Bacterial Strains, Culture Conditions, and Preparation of Inoculums

In the study, *Lactobacillus helveticus* T80, T105, and B734 were used, derived from traditionally fermented Polish milk products and kindly provided by Prof. Łucja Łaniewska-Trokenheim (University of Warmia and Mazury in Olsztyn, Poland). The strains were previously analyzed to determine their taxonomic affiliation, genetic and microbiological characteristics, and some biochemical properties, including enzymatic activity [11–16]. *Lb. helveticus* DSMZ 20075 (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was used as the reference microorganism.

Cultures of all the tested strains were stored at −80 °C in a stock solution containing 15% glycerol [17,18]. Before analysis, the microorganisms were systematically transferred (2% *v*/*v*) into fresh and sterile Man-Rogosa-Sharpe broth (BTL, Poland) and incubated in anaerobic conditions at 42 °C for 18 h [18].

The inoculums of each strain were separately prepared in accordance with the description provided by Beganović et al. [19] (with some modifications) and applied in the study to hydrolyze a 1% water solution of whey protein isolate (WPI) with 91.87% protein content (DAVISCO Foods International, Le Seur, MN, USA) and to carry out the fermentation of milk samples 13% regenerated skim milk (RSM) purchased from OSM Krasnystaw, Poland) containing a 1% addition of the tested protein preparations.

Briefly, samples containing 100 mL of sterile De Man, Rogosa and Sharpe (MRS)broth were inoculated with the individual *Lb. helveticus* strain cultures (obtained after 18 h anaerobic incubation in MRS broth at 42 °C) to the optical density (OD) of the bacterial cell solutions at the level of OD_{550} = 0.5, which were subsequently incubated (at 42 °C) to reach the value of OD_{550} = 0.8. Thereafter, biomass was collected by centrifugation (8000× *g*, 4 °C at 15 min). The pellets were washed three times in a sterile saline solution and re-suspended (also in saline) to obtain equal suspensions of each strain exhibiting OD_{550} = 0.7.

2.2. Texture Profile Analysis of Fermented Milk Beverages

Samples of 13% RSM containing 1% (*w*/*v*) addition of WPI after the process of pasteurization (in a water bath, 80 °C/30 min) and cooling down to 35 °C were inoculated (1% *v*/*v*) with one of the previously prepared strain inoculums. The inoculated milk variants were transferred (in equal 40 mL portions) into sterile closable unit packs. The process of fermentation was carried on using the thermostatic method (42 °C/12 h). Then, received final fermented products were cooled and stored, refrigerated at 4 °C for 12 h before further analysis.

The texture profile analysis (TPA) of the fermented beverages included determination of the values of hardness, cohesiveness, springiness, gumminess, chewiness, and resilience. It was performed with the use of TA-XT2i (Stable Micro Systems, Godalming, UK) according to the procedure described by Gustaw et al. [20]. The analysis was performed in triple repetition.

2.3. Determination of the Protein and Nitrogen Content and the Proteolysis Index of Fermented Milk Beverages

The protein content in the fermented beverages, RSM (pasteurized, non-fermented 13% regenerated skim milk without any additives), and control variants (pasteurized and non-fermented RSM containing 1% addition of WPI) was determined in accordance with EN ISO 8968-1:2014 with the Kjeldahl method [21]. The determination of the content of non-protein-nitrogen was performed according to ISO 8968-4:2001 [22], whereas the proteolysis index was calculated from the following equation [23]:

$$\text{PI (proteolysis index)} = 100 \times \text{non-protein nitrogen/total nitrogen} \quad (1)$$

The analyses were carried out in triplicate.

2.4. Hydrolysis of the Whey Protein Preparation

The reference strain and also one of the tested *Lb. helveticus* strains (T105, T80, or B734) that exhibited the most desirable technological properties ensuring desired texture properties (suitable for fermented milk beverages intended for consumption in a drinking form) were chosen for further studies determining the ability of the microorganisms to generate BAPs from WPI. The selection of the strain was based on the results of TPA of the analyzed fermented milk beverages containing WPI (the lowest value of the hardness parameter). Moreover, another criterion considered in the choice of the strain was the highest values of the proteolysis index (which is associated with the proteolytic activity of the microorganism and the ability to release peptide sequences from protein matrix).

The samples of water solutions (1% *w*/*v*) of WPI were prepared with a method described previously [16]. The samples containing the tested protein preparation were pasteurized in a water bath (80 °C/30 min), cooled down to 37 °C, and inoculated by introducing one of the *Lb. helveticus* strains into each sample (1% *v*/*v*) (all strain inoculums were prepared previously, as specified above). Then, the inoculated samples were anaerobically incubated (42 °C/24 h). After incubation, the samples were heated in a water bath (100 °C/5 min) to deactivate enzymes and inhibit the hydrolysis process. After cooling down, the material was collected, filtered (sterile syringe filter Ø = 0.45 μm), and used in further analyses.

2.5. Detection of Sequences of Biopeptides in the Hydrolysates

In order to detect the sequences of BAPs derived from tested WPI hydrolysates, liquid chromatography-high-resolution mass spectrometry (LC-HRMS) was performed following a procedure described previously [13]. The analysis was carried out using the Agilent nano-HPLC chromatograph series 1200 coupled to an Agilent LC/MS QTOF 6538 equipped with a chip-cube ion source. The Agilent HPLC-Chip G4240–62001 consisting of a 40 nL enrichment column and a 75 µm × 943 mm separation column packed with Zorbax 300 SB-C18 5 µm material was used for separation of peptides. Positive ions were generated and registered at the m/z range of 100–1700. The full scan MS mode using Agilent Mass Hunter acquisition B.05.01 software was applied for acquiring the data, whereas Agilent Mass Hunter qualitative analysis B.07 with integrated Bioconfirm add-on software was applied for data analysis and peptide mapping.

Products with molecular mass in the range of 300–3000 Da were selected from the abundant range of peptides obtained from the analyzed hydrolysates for further investigations.

The biological activities of the analyzed peptides were identified in accordance with the procedure provided in the Milk Bioactive Peptide Database [24], BIOPEP [25–28], and BioPepDB [29–31] databases.

2.6. Statistical Analysis

Statistical analysis was performed using the STATISTICA 13.1 program (StatSoft, Inc., Tulsa, OK, USA).where Tukey's HSD (honest significant difference) test was applied in analysis of variance (ANOVA) to estimate the significance of the differences between the mean values (at $p < 0.05$).

3. Results

3.1. Analysis of Fermented Milk Beverages

3.1.1. Texture Profile Analysis

The milk products exhibited similar properties of cohesiveness, springiness, gumminess, chewiness, and resilience (Table 1). The differences in the mean values of the textural parameters between the analyzed fermented milk beverages were not statistically significant ($p > 0.05$). In turn, variability in the hardness of the tested products was noted. The beverages obtained with the use of *Lb. helveticus* T105 were characterized by the highest value of this texture parameter; moreover, they had the strongest acidic milk gel with intensive syneresis. The application of strain B734 yielded a product with the most liquid consistency (homogeneous, smooth), in comparison to all the fermented beverages. This variant also had the lowest value of hardness but the highest springiness (Table 1), which are the most desired features of a product intended for the consumption in the drinking form.

Table 1. Comparison of the texture parameters of the fermented milk beverages.

Fermented Product *	Texture Parameter					
	Hardness (N)	Cohesiveness	Springiness	Gumminess (g)	Chewiness (g)	Resilience
WPI_DSMZ	0.336 ± 0.035^{ab}	0.153 ± 0.012^{a}	0.514 ± 0.029^{a}	0.051 ± 0.006^{a}	0.026 ± 0.002^{a}	0.082 ± 0.007^{a}
WPI_B734	0.310 ± 0.045^{a}	0.154 ± 0.066^{a}	0.543 ± 0.328^{a}	0.046 ± 0.015^{a}	0.028 ± 0.020^{a}	0.084 ± 0.038^{a}
WPI_T80	0.352 ± 0.023^{ab}	0.161 ± 0.051^{a}	0.445 ± 0.183^{a}	0.056 ± 0.015^{a}	0.027 ± 0.017^{a}	0.088 ± 0.030^{a}
WPI_T105	0.410 ± 0.035^{b}	0.096 ± 0.001^{a}	0.213 ± 0.048^{a}	0.039 ± 0.004^{a}	0.008 ± 0.002^{a}	0.050 ± 0.010^{a}

Explanatory notes: * the name of analyzed fermented products variants are presented as follows: the name of the additive introduced in the milk (WPI—whey protein isolate) followed by the *Lb. helveticus* strain designation that was used in the fermentation process (T80, B734, T105, or DSMZ denoting the reference strain DSMZ 20075). The results are given as mean values ± standard deviation (x ± SD). The lowercase letters (^{a}, ^{b}) in the same column with the texture parameter indicate statistically significant differences ($p < 0.05$).

The results do not show statistically significant differences between the hardness of the product obtained with the use of the reference strain (DSMZ 20075) and the beverage (WPI_T80) fermented with

Lb. helveticus T80 (Table 1). The consistency of these products was semi-liquid, but these beverages were much denser than WPI_B734 obtained using strain B734 (and had consistency with looser structure than beverages produced by applying T105).

3.1.2. Determination of the Protein and Nitrogen Content and Calculation of the Proteolysis Index

The samples of non-fermented 13% pasteurized milk without any addition of protein preparations (RSM) and the control variant (RSM with 1% addition of WPI) had the lowest content of total nitrogen among all the tested products (Table 2); however, the differences in the mean values of this parameter (between these two types of products) were not statistically significant ($p > 0.05$). In turn, the differences in the non-protein nitrogen content between the examined products were much more pronounced.

Table 2. Comparison of the content of protein, total nitrogen, and non-protein nitrogen, and the proteolysis index in the analyzed products.

Product *	Total Nitrogen (g/100g)	Non-Protein Nitrogen (g/100g)	Protein (g/100g)	Proteolysis Index (%)
RSM	0.812 ± 0.08 ^{a}	0.137 ± 0.01 ^{a}	5.18 ± 0.52 ^{a}	17.04 ± 1.13 ^{a}
WPI_DSMZ	0.851 ± 0.01 ^{a}	0.192 ± 0.01 ^{c}	5.43 ± 0.07 ^{a}	22.50 ± 0.96 ^{c}
WPI_B734	0.881 ± 0.03 ^{a}	0.190 ± 0.01 ^{c}	5.62 ± 0.22 ^{a}	21.61 ± 0.87 ^{c}
WPI_T80	0.857 ± 0.01 ^{a}	0.180 ± 0.01 ^{bc}	5.47 ± 0.01 ^{a}	20.96 ± 0.36 ^{bc}
WPI_T105	0.873 ± 0.04 ^{a}	0.170 ± 0.01 ^{b}	5.57 ± 0.29 ^{a}	19.35 ± 1.38 ^{b}
Control	0.832 ± 0.03 ^{a}	0.134 ± 0.01 ^{a}	5.31 ± 0.18 ^{a}	16.11 ± 0.57 ^{a}

Explanatory notes: * the name of analyzed fermented products variants are presented as follows: the name of the additive introduced in the milk (WPI—whey protein isolate) followed by the *Lb. helveticus* strain designation that was used in the fermentation process (T80, B734, T105, or DSMZ denoting the reference strain DSMZ 20075), RSM—regenerated skim milk (non-fermented 13% pasteurized milk without any addition of the protein preparation), Control—non-fermented 13% pasteurized milk (RSM) with 1% addition of WPI. The results are given as mean values ± standard deviation (x ± SD). The lowercase letters (^{a}–^{c}) in the same column with the analyzed parameter indicate significant differences ($p < 0.05$).

The lowest content of non-protein nitrogen was noted in the non-fermented products (RSM and the control variant), while the highest value of this component was found in products fermented by the reference strain (WPI_DSMZ) and in beverages obtained with the use of *Lb. helveticus* B734 (WPI_B734). Moreover, the products obtained with the use of the reference strain and the fermented milk beverages (Table 2) produced by strain B734 were characterized by the highest proteolysis index (22.50% ± 0.96% and 21.61% ± 0.87%, respectively) and differences between the values were not statistically significant ($p > 0.05$).

The results (Table 2) showed that the protein content in all products supplemented with WPI (including fermented ones) was higher than in RSM, but the differences between the mean values were not statistically significant ($p > 0.05$).

3.2. Analysis of the Sequences of Biopeptides Detected in the Hydrolysates

For the analyses, the reference strain was selected and also one of the tested strains that allowed to obtain the fermented milk beverages containing WPI (drinking type) that were characterized by the lowest value of hardness (determined through TPA analysis) and exhibited the highest values of the proteolysis index (because it is linked to the efficiency and ability to generate BAPs.)

The findings revealed that the selection criteria were best met by *Lb. helveticus* strain B734. Therefore, it was used as an inoculum for hydrolysis of WPI (1% water solution). The bioactive peptide sequences obtained in this process are presented in Table 3.

Table 3. Sequences of biopeptides detected in the hydrolysates of the tested whey protein preparation.

Biopeptide Sequence	Mass (Da)	Designation Allocated to the Sequence in the Database (ID [1, 2] or References [3])	Biological Activity Reported in the Database	Tested Strain of *Lb. helveticus* Generating the Biopeptide
RPKHPIKHQGLPQ	1534.88	biopep01213 [1]	antihypertensive	B734, DSMZ
TQSLVYP	806.42	biopep01306	antihypertensive	DSMZ
		3333/BIOPEP-UWM[2]	ACE inhibitory	
SRY [4]	424.21	biopep01260	antihypertensive	B734
GKEKV	559.33	biopep00360	antihypertensive	B734, DSMZ
ENLHLP	721.37	biopep00179	antihypertensive	B734, DSMZ
		[32]	ACE inhibitory	
AQTQSL	646.33	biopep00067	antihypertensive	B734
FAQTQS	680.31	biopep00212	antihypertensive	B734
YPFP	522.25	biopep04755, 2868/BIOPEP-UWM, [33]	opioid	B734
		[34]	anticancer	
GLPQE	542.27	8163/BIOPEP-UWM	antibacterial	B734
		9375/BIOPEP-UWM	ACE inhibitory	
VQVTSTAV	803.44	biopep01445	antihypertensive	B734, DSMZ
		8264/BIOPEP-UWM	antibacterial	
		[35]	antimicrobial	
VPSERYL	862.45	biopep01442	antihypertensive	B734
		9250/BIOPEP-UWM, [36]	ACE inhibitory	
INQF	520.26	biopep00551	antihypertensive	B734
TVY [4]	381.19	biopep01319	antihypertensive	B734
		[37]	ACE-inhibitory	
FPPQSVL	786.43	biopep00270	antihypertensive	B734
LQPEVMGVSK	1086.57	biopep00867	antihypertensive	DSMZ
AYPS	436.20	8472/BIOPEP-UWM	antioxidative	B734, DSMZ
		8380/BIOPEP-UWM	ACE inhibitory	
VRSP	457.26	biopep01460	antihypertensive	B734, DSMZ
		8309/BIOPEP-UWM	ACE inhibitory	
LVYPFPGPI	1001.56	biopep00927	antihypertensive	B734, DSMZ
		[38]	ACE-inhibitory	
NENLLRFFVAPFPE	1691.87	biopep00991	antihypertensive	B734
LQPEVMG	772.38	biopep00866	antihypertensive	DSMZ
FVAPFPEVFGKEKVNE	1835.94	biopep00305	antihypertensive	B734
LEQL	501.28	biopep00755	antihypertensive	B734, DSMZ
ENLLRF	790.43	biopep00182	antihypertensive	B734
		[39]	ACE inhibitory	
FPPQS	574.27	9378/BIOPEP-UWM	ACE inhibitory	DSMZ
FVAPFPEVFGK	1236.65	biopep00304	antihypertensive	B734, DSMZ

Table 3. *Cont.*

Biopeptide Sequence	Mass (Da)	Designation Allocated to the Sequence in the Database (ID [1, 2] or References [3])	Biological Activity Reported in the Database	Tested Strain of *Lb. helveticus* Generating the Biopeptide
RELEELNVPGEIVESLSSSEESITR	2801.39	biopep04772, 3055/BIOPEP-UWM	mineral-binding	B734, DSMZ
		[40]	caseinophosphopeptide	
		[41]	immunomodulatory	
RPKHPIKHQGLPQEVLNEN	2233.21	biopep01215	antihypertensive	B734
ERF [4]	450.22	biopep00189	antihypertensive	B734, DSMZ
VVPP	410.25	biopep01483	antihypertensive	B734
		8308/BIOPEP-UWM, [42]	ACE inhibitory	
LLR [4]	400.28	biopep00827	antihypertensive	B734, DSMZ
		8484/BIOPEP-UWM	antioxidative	
FAL [4]	349.20	biopep00207	antihypertensive	DSMZ
		7823/BIOPEP-UWM	ACE inhibitory	
IKH [4]	396.25	biopep00532	antihypertensive	B734
RHPHP	642.33	8469/BIOPEP-UWM	antioxidant	B734
		8373/BIOPEP-UWM	ACE inhibitory	
LAY [4]	365.19	biopep00734	antihypertensive	DSMZ
		3558/BIOPEP-UWM	ACE inhibitory	
HIQKEDVPSER	1336.67	9559/BIOPEP-UWM	antioxidative	B734, DSMZ
FLLY	554.31	biopep00734	antihypertensive	B734, DSMZ
QEPVLGPVRGPFPIIV	1716.99	[43]	ACE-inhibitory	DSMZ
SQSKVLPVPQ	1081.61	[44]	ACE-inhibitory	DSMZ
VYPFPGPIPN	1099.57	[45]	antioxidant, ACE-inhibitory	B734
LLY [4]	407.24	biopep04791	immuno- and cytomodulatory peptide	B734
		3065/BIOPEP-UWM, [46]	immunostimulatory	

Explanatory notes: database: [1] Bioactive Peptide Database BioPepDB [29], [2] BIOPEP-UWM database [25], [3] Milk Bioactive Peptide Database [24]. [4] Based on accurate mass matching < 5 ppm. DSMZ denotes *Lb. helveticus* DSMZ 20075 (reference strain).

The most widespread bioactivity among all of the detected biopeptides sequences with molecular mass in the range of 300–3000 Da (Tables 3 and 4) was antihypertensive.

It was also observed that some of BAPs sequences were generated only by one strain (DSMZ or B734). Moreover, thirty different biopeptides were released in the process of WPI hydrolysis by B734, while twenty BAPs were obtained by application of the reference strain (Table 4). In addition, the use of *Lb. helveticus* B734 to hydrolyze WPI yielded sequences of immuno- and cyto-modulatory (LLY) and opioid (YPFP) peptides, which were absent in hydrolysates obtained with application of strain DSMZ 20075.

Table 4. Comparison of the number of biologically active peptides (BAPs) sequences generated by the tested strains of *Lb. helveticus*.

Biological Activity of Identified BAPs	Number of the BAPs Generated by the Tested *Lb. helveticus* Strain	
	B734	DSMZ 20075
immunostimulatory/immuno- and cyto-modulatory peptide	2	1
caseinophosphopeptide	1	1
mineral-binding	1	1
opioid	1	-
antibacterial/antimicrobial	2	1
anticancer	1	-
antioxidant	5	3
antihypertensive	24	16
ACE inhibitory	11	9
Total number of biopeptide sequences	48	32

4. Discussion

The novel methods facilitate effective separation and purification of whey proteins, which are nowadays widely used in cosmetic, pharmaceutical, or food industries.

It has been suggested that whey proteins ensure a smooth and creamy texture of fermented milk beverages with enhanced nutritional values and might be applied for production of food supplements or functional foods [47–50]. The possibility of using the selected whey protein preparation (WPI) to obtain fermented milk beverages (drinking type) was investigated in our research. This type of product may meet the expectations of consumers preferring drinking yogurts or may be dedicated for individuals favoring products that are alternative to conventional yogurts or traditional fermented milk variants. Moreover, with their high potential of functional properties, the beverages may meet the requirements of consumers that particularly consider the health-promoting character of food products.

The fermented products had similar values of cohesiveness, springiness, gumminess, chewiness, and resilience, whereas differences were found in the hardness of the products. Despite the same WPI addition (1%), the products differed in some textural characteristics depending on the *Lb. helveticus* strain used. This indicates that the texture profile of the beverages was influenced by the microorganism involved in the fermentation process. Furthermore, the strongest milk gels were noted in the variants supplemented with applying *Lb. helveticus* T105, whereas strain B734 yielded a drinking type of fermented milk beverages with a delicate creamy texture (and the most liquid structure). In turn, Gursel et al. [51] reported that the addition (2% wt/vol) of WPI into goat milk in yogurt production produced the hardest structure in all fermented product samples in comparison to yogurts obtained by fortification with skim goat milk powder, sodium caseinate, whey protein concentrate, or a yogurt texture improver. However, only one type of commercial DVS (Direct Vat Set) freeze-dried yogurt culture (YO-MIX 572, Danisco-DuPont Group, Copenhagen, Denmark) was applied in the manufacture of all variants of yogurts (produced from goat milk); therefore, no considerable differences were recorded by the authors between the samples of WPI-containing products.

The differences in the values of some parameters of the TPA profiles in the fermented beverages observed in our study may be explained by the fact that introduction of whey protein preparations (or other dry dairy ingredients substituting skim milk powder) to milk affects the proteolytic activity of lactic acid bacteria due to the different contents and fractions of proteins available in milk [4,52,53]. Moreover, Beganović et al. [19], Broadbent et al. [54], and Sadat-Mekmene et al. [55] proved that the proteolytic activity of *Lb. helveticus* is a strain-dependent feature, which is strongly determined genetically and refers to various variants of genes encoding cell envelope proteinases (genetic biodiversity of CEP paralogs). This also corresponds to the findings of our previous study [14] and the present results of the proteolytic index assay, which displayed a high variability of the measured parameter depending on the strain used. Moreover, the differences in the texture properties observed between the variants of the fermented milk beverages containing WPI might also be related to the

different levels of protein hydrolysis carried out by the tested strains (different enzymatic specificity of bacterial proteases as well as different effectiveness and extent of the proteolysis process). Similar observations were reported by Tzvetkova et al. [56], who also noted that proteolytic activity toward whey proteins was dependent on the strain of bacteria.

The lower values of the hardness parameter noted in the variants of beverages containing *Lb. helveticus* B734 is probably related to the specificity and activity of the proteolytic system of the strain. This is in agreement with findings described by Albenzio et al. [57], who analyzed different bacterial cultures used in cheese production and revealed that the hardness values of fermented products (mainly related to the primary proteolysis, breakdown of both α- and β fractions of casein) were substantially lower in fermented products obtained by addition of probiotic bacteria. Moreover, Amani et al. [58] noted that samples of yoghurt obtained with the application of strains with low proteolytic activity exhibited more hardness than samples of the products received by using bacteria with strong proteolytic activity. This may suggest that *Lb. helveticus* B734 exhibited higher activity towards the fermented matrix (RSM supplemented with WPI) referring to lower hardness value than the other tested strains (also for this reason, strain B734 was subjected to further analysis in the investigations). In addition, the obtained findings may also be associated with the fact that different peptides (with different amino acid sequences) were released from milk and whey proteins during fermentation via the enzymatic activity of different *Lb. helveticus* strains, which probably influenced the structure of the gel network, i.e., a very dynamic system dependent on diverse interactions between the individual components (including milk proteins, denatured whey proteins, and calcium phosphate crosslinks) in the complex composition of fermented matrix [59].

It is also worth emphasizing that proteolysis processes (occurring also during lactic acid fermentation) not only lead to changes in the physical structure of the product but also contribute to the production of biopeptides [60–62]. Many BAPs derived from milk and whey proteins exhibit multiple bioactivities, bringing desired health-promoting effects, which contributes to the constantly increasing importance of the biopeptides and potential applications in pharmacy and medicine [34,60,63–65]. The desired therapeutic effects of BAPs extracted from milk and whey precursors have been confirmed, especially in terms of their ability to reduce blood pressure [63–67].

Our findings have revealed that a wide range of various BAPs can be obtained from 1% water solutions of WPI using strains of *Lb. helveticus* (B734 and DSMZ 20075). The antihypertensive and ACE inhibitory effects were the predominant activities of the identified BAPs sequences. One of the thoroughly tested peptides with ACE inhibitory and antihypertensive activities (which was also detected in the WPI hydrolysates obtained with the use of *Lb. helveticus* B734) is VPSERYL. It was revealed that this peptide at a concentration of 249.5 μM inhibited 50% of the original ACE activity [36], whereas the value of 50% inhibitory concentration (IC_{50}) for SQSKVLPVPQ (sequence released from WPI by the reference strain DSMZ 20075) was at the level of 92.00 μM [44]. This peptide was also detected by Ali et al. [61] in samples of WPI fermented with *Lb. helveticus* LH-2 CFSM.

Due to their influence on blood pressure and desirable cardiovascular effects, opioid peptides are other important sequences of BAPs generated from whey and milk proteins (mainly from α-lactalbumin and β-lactoglobulins) [66]. The present study revealed that strain B734 was able to generate opioid peptide sequences (YPFP) from WPI. This tetrapeptide (known as morphiceptin, present in the enzymatic digest of bovine casein) is a selective ligand of the μ-opioid receptor. Furthermore, it has been indicated that the biopeptide exhibits considerable opioid potency and is a selective ligand of the μ-opioid receptor (MOR), inducing strong supraspinal antinociception through application directly to the central nervous system [33]. Moreover, the application of morphiceptin analogues in the control of tumor growth and cell proliferation is investigated, which is particularly important for the diagnosis and treatment of various cancers [67].

The present results correspond to findings described by Ali et al. [61], who noted that some BAPs derived from WPI hydrolyzed by a strain of *Lb. helveticus* (LH-2 CFSM) have multifunctional properties that may influence two or more physiological processes. An example of such a

sequence is RELEELNVPGEIVESLSSSEESITR, i.e., a caseinophosphopeptide with mineral-binding, immunomodulatory [40,41], and anti-caries activities [68,69]. The mineral-binding bioactivity of this peptide has been confirmed in in vivo studies in an animal model (rats) and in humans [68,70]. This BAP was identified in the WPI hydrolysates obtained with the use of strain B734 and in samples supplemented with the reference strain (DSMZ 20075).

Interestingly, the VQVTSTAV sequence with antihypertensive and antimicrobial activities (present in WPI hydrolysates obtained using the reference strain and in samples with *Lb. helveticus* B734) was also detected by Ali et al. [61] in unfermented (control) samples of WPI, which indicates the activity of endogenous milk proteases (plasmin, elastase, and cathepsin D, B, and G) and the ability to form some BAPs [71]. Moreover, efficient antimicrobial effects of this peptide (at a concentration of 5 mM) have been confirmed against *Staphylococcus carnosus* CECT 231, *Serratia marcescens* CECT 854, and *Escherichia coli* ATCC 25922, whereas the strongest antibacterial activity was noted toward *Listeria innocua* CECT 910T [35]. This antibacterial peptide was also detected in water-soluble extracts of the Pecorino Romano and Canestrato Pugliese cheeses [72].

It is worth mentioning that *Lb. helveticus* is considered the most proteolytic species in the genus *Lactobacillus* [73,74]. These lactic acid bacteria are involved in the manufacture of long-ripening cheeses, and the proteolytic system of these microorganisms influences the processes of protein hydrolysis contributing to development of characteristics of cheeses (including texture, taste, and flavor) and affect generation of BAPs.

The present results indicate that certain sequences were released by only one of the tested *Lb. helveticus* strains (DSMZ 20057 or B734), suggesting that the ability to generate BAPs (in terms of the amount of different sequences) from the selected protein matrix is a strain-dependent feature. This may imply that, depending on the *Lb. helveticus* strain used, it is possible to obtain hydrolysates of the protein preparation with different BAP profiles and various functional properties. This is supported by results described by Ahn et al. [75], who revealed that whey fermented by *Lactobacillus brevis* exhibited higher ACE inhibitory activity than other samples, which were fermented by other bacteria (*Lb. acidophilus*, *Lb. plantarum*, *Lb. casei*, *Lb. helveticus*, *Lb. lactis*, *Lb. paracasei*, *Lb. reuteri*, and *Lb. bifermentans*). Similar observations were reported by Ali et al. [61], i.e., depending on the microorganisms, WPI hydrolysates differed in the number and amino acid sequences of generated BAPs. Moreover, 19 sequences associated with several categories of bioactivity (ACE inhibitory, opioid, antimicrobial, and antioxidant activities) were detected in hydrolysates obtained by *Lb. helveticus* LH-2 CFSM, whereas *L. acidophilus* La-5 CFSM was able to generate only 5 BAPs (with ACE-inhibitory, anti-caries, antimicrobial, and antioxidant activities).

Our findings revealed that strain B734 had higher potential to release a larger amount of BAP sequences from WPI than the reference strain. The strain exhibits potential and suitability for development of new health-oriented fermented milk-derived food products.

5. Conclusions

The investigations addressed a significant issue of the technological and functional aspects associated with the addition of WPI to milk subjected to lactic acid fermentation. The results indicate the potential of three industrially unused strains of *Lactobacillus helveticus* to produce fermented milk beverages supplemented with the tested protein preparation. Moreover, the findings suggest that the application of *L. heveticus* B734 and WPI might contribute to the development of new health-oriented fermented milk beverages with potential functional food characteristics. The strain is able to release a wide range of BAPs from WPI with antioxidative, antibacterial, and immuno- and cyto-modulatory effects as well as ACE inhibitory and antihypertensive activities, which can be used in production of nutraceuticals or in medical and pharmaceutical applications. These promising results encourage further analysis, including determination of the concentration of individual BAPs generated by bacteria as well as the in vitro and in vivo effects of the bioactivity.

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