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Changes in Biochemical Properties during Ripening Process of Swiss-Type Cheeses Produced with Different *Lactobacillus helveticus* Strains

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ABSTRACT

The presence of various biotypes of *Lactobacillus helveticus* seems to be related to the specificity of the ecosystem, which is one of the main determinants of the unique features of cheese produced in particular regions. So far, it has been proved that even biotypes isolated from the same niche differ significantly from each other and many features exhibited by these bacteria are strain-dependent. Therefore, the new strains of *L. helveticus* T104 and T105 (isolated from traditionally fermented Polish dairy product) were applied in the production of ripened cheeses due to their potential health-promoting properties. We determined changes in fat, fatty acids, protein, amino acids, and some physicochemical characteristics, e.g. antioxidant properties of the produced cheeses (in three stages of ripening). Tricine-SDS-PAGE and MALDI-TOF MS analysis showed some differences in protein and peptide profiles. Final products obtained using *L. helveticus* T105 exhibited the greatest amount of free amino acids, which are important precursor of cheese aroma and flavor. The research indicated that the tested strains could be applied in the manufacture of cheeses. Moreover, the cheese produced using the said strain exhibited the highest free radical scavenging capacity (88.89% after pre-ripening and 92.74% in the final products) even in comparison to the control cheese variant produced using the industrial *L. helveticus* strain. Obtained findings indicate that the tested strains exhibit technological and functional potential that provide a reference for further study and might contribute to the development of functional food products with novel, valuable characteristics.

Keywords: Antioxidant activities, Cheese aroma and flavor, Fatty acids, Functional food.

INTRODUCTION

According to the European Commission research report (European Commission, 2015), the milk supply in the European Union is predicted to rise by 13 million tons in the coming years (0.8% a year). Simultaneously, the consumption of cheese, butter, and fresh cream per capita in the EU is steadily increasing and cheese consumption is predicted to rise to 16 kg per capita in the European Union countries by 2025. The report data indicate that cheese production is to increase by 1.15 million tons in the next decade and 11.2 million tons by 2025.

Microflora plays a decisive role in cheese production and maturation. The ripening process is the most significant stage determining the specific characteristics and quality of final products. A number of

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complex and dynamic biochemical reactions occurring in the cheese matrix determine the properties of cheese. These reactions depend on the composition of bacterial starter cultures, duration and conditions of the ripening process (Tunick, 2000; McSweeney, 2004). Microorganisms contribute to the development of cheese aroma, flavor, and texture by their involvement in the biochemical conversions of fat, protein, and lactose. Moreover, the metabolic activity of microorganisms leads to the formation of some bioactive health-promoting compounds (such as bioactive peptides) during cheese maturation (Griffiths and Tellez, 2013).

The ability of microflora to adapt to changing environmental conditions in natural niches can contribute to the improvement of starter cultures used in the dairy industry and to the introduction of new technologically useful properties (Gatti *et al.*, 2014). *L. helveticus* has been widely applied in the production of various cheese types; however, individual strains from this bacterial genus exhibit great diversity in their technological properties and health-promoting characteristics (Fortina *et al.*, 1998; Hannon *et al.*, 2007; Sheenan *et al.*, 2007).

It has been suggested that the impact of *L. helveticus* on aroma formation in cheeses seems to be a strain-dependent feature (Petersen *et al.*, 2010). Therefore, the aim of this study was to determine the potential of application of new Polish strains of *L. helveticus* T104 and T105 exhibiting some health-promoting characteristics in cheese manufacture (Skrzypczak *et al*., 2015; Skrzypczak *et al.*, 2017a and 2017b). Besides, we aimed to investigate the biochemical changes occurring during cheese ripening, with particular emphasis on determination of the content of free fatty acids and amino acids.

Moreover, an attempt was made to analyze the antioxidant activities of products obtained using the novel *L. helveticus* strains. The potential applicability of the strains in the dairy industry was evaluated as well. The experimental cheeses were compared with cheeses made using a commercial starter culture.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Native strains of *Lactobacillus helveticus* T104 and T105 were isolated from traditional Polish products of fermented milk and kindly provided by Prof. Łucja Łaniewska - Trokenheim (University of Warmia and Mazury in Olsztyn, Poland). The microorganisms were deposited in the internal collection of strains at Faculty of Food Science and Biotechnology (University of Life Sciences in Lublin). The taxonomic affiliation of the tested strains to *L. helveticus* and some of their potential probiotic properties have been already confirmed (Skrzypczak *et al.*, 2015; Skrzypczak *et al.*, 2017a, 2017b). The strains have not been industrially applied yet.

The following components of inoculums were used for cheese production: Commercial frozen concentrated (F-DVS) cultures for direct inoculation (Chr. Hansen. Denmark), mesophilic aromatic cultures type DL producing flavor and CO_2 (CHN - 19) containing *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*; thermophilic culture (ST - B05) containing a single *Streptococcus thermophilus* strain and secondary culture (PS- 4) containing the *Propionibacterium freudenreichii* subsp. *shermanii* strain. The F-DVS LH-32 culture (Chr. Hansen. Denmark) was used as the reference strain of *L. helveticus* to produce a control cheese variant.

All *L. helveticus* strains were cultured in MRS broth (BTL, Poland) according to Waśko *et al.* (2014), while the other F-DVS cultures were stored in accordance with the

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manufacturer's recommendations until direct use.

Cheese Production and Sampling Procedure

Three types of cheeses (A, B and C) were produced in triplicate and analyzed. Each type of cheese was produced from 10 L of standardized, microfiltered, and pasteurized (74˚C for 30 seconds) cow milk (fat 3.2%, protein 3.2%, carbohydrates 4.7%). For improved milk curdling and concise curd structure, 2 mL of $CaCl_2$ (40% solution) was added into the milk.

The cheese was made according to a technical brochure of Emmental cheese production provided by the manufacturer of the starter cultures (Chr. Hansen, 2002). Briefly, 10 L of milk (33°C) were inoculated with a combination of commercial starter cultures: 1% of mesophilic starter culture CHN–19; 1% of PS–4; 2.5% of ST-B05, and 0.5% of the *L. helveticus* strain (T104, T105 or LH–32).

Suitable bacterial cell suspensions were prepared to ensure an equal contribution of each *L. helveticus* strain to the composition of each inoculum variant. The single *L. helveticus* strains were cultivated in MRS broth (at 37˚C) and harvested in the exponential phase of growth (OD_{600}= 0.8) by centrifugation at 8,000×g for 10 minutes. at 4˚C. Cell pellets were washed in 0.9% NaCl and resuspended in saline to obtain proper cell suspensions (OD_{600}= 0.7).

Each cell suspension of the *L. helveticus* strains was incorporated into the combination of commercial starter cultures to prepare three different cheese inoculum variants. LH–32 was applied to produce the control cheese (variant A), while T104 and T105 were used in the B and C cheese variants, respectively. Table 1 presents the combinations of the starter cultures composition used for the production of three cheese variants.

After milk inoculation and incubation in a cheese vat (33˚C for 45 minutes), rennet (CHYMOGEN Premium Plus®, Chr. Hansen, Denmark) was added according to the manufacturer's instructions. Coagulation was held at 33 ˚C for 35 min. The curd was cut into 3-5 mm cubes, left to rest for 10 min., and then gently stirred for 15 min. Subsequently, 40% of whey was removed and replaced by the same amount of warm water (40˚C). Subsequently, the temperature was gradually increased up to 50˚C (with continuous stirring of the cheese grains).

After prior scalding of the cheese grains (50˚C for 45 minutes) and final stirring, whey was removed to the level of the cheese mass in the vat. The curd was divided into equal portions (400 g), placed in a perforated form, and subjected to pressing.

Table 1. Combinations of the composition of the starter cultures used for the production of three cheese variants.

Cheese variant	*Lactobacillus helveticus* strain	Mesophilic, aromatic cultures (Type DL)	Thermophilic culture	Secondary culture
A (Control)	LH–32 (Chr. Hansen. Denmark)	CHN–19 (Chr. Hansen. Denmark)	ST-B05 (Chr. Hansen. Denmark)	PS–4 (Chr. Hansen. Denmark)
B	T104	CHN–19 (Chr. Hansen. Denmark)	ST-B05 (Chr. Hansen. Denmark)	PS–4 (Chr. Hansen. Denmark)
C	T105	CHN–19 (Chr. Hansen. Denmark)	ST-B05 (Chr. Hansen. Denmark)	PS–4 (Chr. Hansen. Denmark)

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The initial pressure of 2 kg on the mold (13×7 cm) was increased progressively during the pressing with a rate equal to 2 kg/1.5 h. To drain the whey effectively, the fresh chesses were rotated every 45 minutes, molded, pressed (18˚C/4.5 h), and salted in saturated brine (18%; pH= 4.8; Temp.= 11˚C/2 h) with cheese rotation every 30 minutes. Subsequently, the cheeses were dried thoroughly, transferred into a cool ripening room (13˚C/15 days, RH= 85%), and next moved to a warm ripening room (23˚C, RH= 85%) for the next two months.

In order to protect against excessive drying out, cheeses were coated with commercially available, inedible cheese coating (SEROWAR s.c. Edyta Kardaszewicz, Jakub Krężel, Poland). A thin layer of the paste (the colorless, water-based polyvinyl acetate emulsion containing 0.025% natamycin) was applied with a brush to the surface of the cheeses according to manufacturer`s instruction. During ripening process, the cheese circles were turned from one side to another every day

Cheese samples (40 g) were collected for analysis after 15 days of pre-ripening in a cold room, and after one and two months of ripening in the warm room. Before the tests, the paste layer was removed from the samples.

Physicochemical Analysis

The physicochemical analysis of all cheeses (at tested ripening intervals) included determination of salt content (NaCl) with the method of flame Atomic Absorption Spectrometry (AAS) according to EN 15505:2008 and pH was measured in triplicate using the pH meter (HI 221, Hanna Instruments, Poland). The analyses of fat, fatty acid, protein, peptides, and amino acids are described below.

Analysis of Fat and Fatty Acids

Samples of cheeses were frozen (-20°C/24 h) and then lyophilized (72 h/Maintaining the vacuum at 0.8 mbar; condenser temperature setting at –60°C) in the Alpha 1–2 LD plus freeze-dryer (Martin Christ Osterode am Harz, Germany). Before future analyses, the obtained lyophilized materials were ground (Społem WŻ-1 mill, Warsaw, Poland). Fat extraction from lyophilized cheese samples was based on the Soxhlet method (AOAC, 1995) using Soxtec Avanti® (Tecator). Hexane was used as the organic solvent. The sample extraction dishes were weighed, dried (103±2˚C), and reweighed after the extraction. The fat content was calculated on the basis of the weight differences.

To determine the profiles of fatty acids, methyl esters were prepared and analyzed by gas chromatography in accordance with ISO 12966-4: 2015.

The methyl esters were separated in a gas chromatograph Varian 450-GC (Software Galaxie ™ Chromatography Data System) with a flame-ionization detector using a capillary column Select ™ Biodiesel for FAME (30 m×0.32 mm×0.25 µm). Helium was used as the carrier gas with a flow rate of 2.5 mL min^{-1}. Fatty acids were identified by comparing the retention times with the standards.

Analysis of Protein, Peptides, and Amino Acids

The total protein content in the cheese samples was determined with the Kjeldahl method (AOAC International, 2000; method 920.123) using an automatic analyzer Kjeltec 2300 (FOSS). Protein was determined on the basis of the total nitrogen content using the conversion factor 6.38.

Water-soluble peptides were extracted from all cheese samples with the method described by Pritchard *et al.* (2010).

Antioxidant activities of all extracts were determined by measurement of their capacity of free radical scavenging using 60 µM 1,1-DiPhenyl-2-PicrylHydrazyl (DPPH) as described by Apostolidis *et al.* (2007)

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with the modification developed by Pritchard *et al.* (2010).

Electrophoresis was conducted on 10% Tricine-SDS-PAGE gel as described by Schägger and Von Jagow (1987) in order to compare the profiles of low molecular weight proteins and peptides in the extracts of cheeses after pre-ripening (after 15 days in the cold room) and after 2 months of ripening in the warm room.

Physiological amino acids were detected in the cheese samples after the pre-ripening and samples of final products by ion-exchange chromatography using an amino acid analyzer AAA 400 INGOS (Czech Republic, Prague). Samples were deproteinized by 1% picric acid and the precipitated proteins were absorbed on Dowex ion exchange resin. The clear filtrate (filtered through 0.45 μm syringe filters) was dosed on a column (0.37×45 cm) filled with ion exchanger Li (Ostion LG FA). The elution was carried out at 74˚C.

Cheese samples were prepared for mass spectrometry analysis (MALDI-TOF-MS) according to Adaszek *et al.* (2014) applying an HCCA (α-Cyano-4HydroxyCinnamic Acid) matrix solution (suspended in a standard solution recommended by the manufacturer, Bruker Daltonics) to each dose.

Three analyses with an UltrafleXtreme mass spectrometer (Bruker) were performed for each sample within the molecular mass range from 700 to 4,000 Da. The spectrometric analysis was conducted using the flex Control 3.3 (build 108) program. The spectra were analyzed with the flex Analysis 3.3 (build 80) program.

Data Analysis

Statistical analysis was carried out using the STATISTICA 13.1 program (StatSoft, Inc., USA). Analysis Of Variance (ANOVA) followed by Tukey's HSD post hoc test was performed to determine significant differences ($P< 0.05$) among the average values of the measured parameters.

RESULTS AND DISCUSSION

Analysis of Free Fatty Acids

The process of cheese maturation involves many complex biochemical reactions. Most of the specific properties of the final products like texture, flavors, and aromas are mainly developed during cheese matrix ripening due to changes in proteins and fat. Therefore, the concentration of these compounds was analyzed during the maturation period.

With the extended ripening time, the protein, fat, and salt concentration increased in all tested cheeses (Table 2). The results can be explained by the reduction of moisture that took place during cheese ripening (Aminifar *et al.*, 2014).

Analyses of changes in the pH value revealed lower values of this parameter in all the cheese variants at the end of the second ripening stage (compared to the pre-ripening stage), which increased in the next stage of maturation (Table 2). Moreover, the pH values of cheeses from variant C after the entire ripening period were significantly lower than pH determined in the final control samples. In terms of pH, the final products were similar to Swiss cheeses obtained after 28 days of maturation in a warm room and produced using *Lactobacillus helveticus* (L1 and L2) and *Propionibacterium freudenreichi* ssp. *shermanii* (P1 and P2) (White *et al.*, 2003). Moreover, the pH values of final products of the obtained Swiss-type cheeses (variants A, B and C) were similar to the pH values (5.35 -5.4) characteristic for some Italian cheeses (Hill, 2007).

The formation of Free Fatty Acids (FFAs) during the cheese maturation is a result of many complex transformations related to catabolism of amino acids, lipolysis, and lactose fermentation (Ganesan *et al.*, 2007).

Enzymatically produced short- and medium-chain fatty acids are important precursors of many flavor and aroma components such as alcohols, ketones, methyl lactones, esters, and secondary alcohols contributing directly to the characteristic

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properties of cheeses (Curioni and Bosset, 2002; McSweeney, 2004; Wilkinson 2007). Moreover, the autolysis of *L. helveticus* cells has been shown to contribute to the increase in the concentration of FFAs in cheese during ripening (Hannon *et al.*, 2007). Therefore, the content of fatty acids in the cheese produced with the different *L. helveticus* strain was also analyzed during the ripening (Table 3).

Significant differences in the fatty acid profile were observed especially between the first and the last stage of the maturation period. The content of individual FFAs increased in the cheeses along the ripening time, but the concentration of individual FFAs was similar in all cheese variants with respect to the maturation time (Table 3). All products were characterized by the highest share of palmitic (C16:0), oleic (C18:1n9c), and elaidic (C18:1n9t) acids as well as myristic acid (C14: 0). These results correspond to the findings presented by Mangia *et al.* (2011), who analyzed the fatty acid composition in Pecorino Romano cheese. They recorded the highest concentrations of all the above-mentioned fatty acids as well as butyric acid (C4:0).

Furthermore, an analysis of the FFA composition in Emmental (Maturation conditions: 12 days at 12˚C/85% RH, 28 days at 21˚C/80% RH, 8-15 days at 4˚C) showed the dominance of C14:0, C16:0, and C18:0 constituting 80-86% of all fatty acids, regardless of the maturation phase (Lopez *et al.*, 2006). The amounts of C8:0, C10:0, C12:0, C18:0, C18:1, and C18:2 determined in the analyzed cheese variants (A, B, and C) correspond to the results obtained by Lopez *et al.* (2006).

As already suggested, proteolysis increases the level of volatile fatty acids (C4:0; C6:0, C8:0 and C10:0), which are formed from amino acids (Domagała *et al.*, 2013). In all the cheese variants, only caprylic (C8:0) and decanoic (C10:0) acids were detected as volatile fatty acids. The share of these acids was inconsiderable in comparison to the other analyzed FFAs.

The quantity of medium-chain FFAs in all the cheese variants increased throughout ripening and achieved the highest level in the final stage of maturation. It was noted that palmitic acid (C16:0) was the most abundant, followed by oleic (C18:1) and myristic (C14:0) acids. These results are consistent with the reports by Aminifar *et al.*

Table 2. Comparison of the analyzed parameters in the tested cheese varieties during ripening

Cheese variant	Stage of cheese maturation			Analyzed parameter
	15 Days/13˚C	1 Month/23˚C	2 Months/23˚C	
A	5.46^{Aa}±0.01	5.38^{Ba}±0.02	5.44^{Aa}±0.01	pH
B	5.22^{Bc}±0.01	5.14^{Cb}±0.02	5.39^{Aab}±0.04	
C	5.41^{Ab}±0.03	5.10^{Bc}±0.01	5.37^{Ab}±0.03	
A	1.01^{Ba}±0.12	1.40^{ABa}±0.17	1.67^{Aa} ±0.2	Content of NaCl [%]
B	1.22^{Aa}±0.15	1.23^{Aa}±0.15	1.53^{Aa}±0.18	
C	1.08^{Ba}±0.13	1.42^{ABa}±0.17	1.70^{Aa}±0.2	
A	27.08^{Ca}±0.11	28.30^{Bb}±0.05	28.74^{Aa}±0.01	Total protein content [%]
B	26.00^{Bb}±0.14	28.48^{Aa}±0.04	28.58^{Aa}±0.27	
C	25.44^{Bc}±0.16	26.91^{Ac}±0.02	27.17^{Ab}±0.24	
A	28.33^{Aa}±0.01	27.15^{Bb}±0.16	28.38^{Aa}±0.56	Total fat content [%]
B	27.06^{Bc}±0.26	29.49^{Aa}±0.3	29.14^{Aa}±0.5	
C	25.60^{Bb}±0.07	26.74^{Ab}±0.23	27.05^{Ab}±0.44	

(A-C) Different upper case letter express significant differences (P <0.05) among maturation stages for the same cheese variant. **(a-c)** Different lower case letter express significant differences (P <0.05) among cheese samples for the measured parameter in the same maturation stages. Explanation notes: The results are given as mean values±Standard Deviation (SD).

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Table 3. Changes in the fatty acid content during cheese ripening.

Fatty acid	Cheese variant[a]	Ripening stage					
		After 15 days/13˚C		After 1 month/23˚C		After 2 months/23˚C	
		[%][b]	[g 100 g^{-1}][c]	[%]	[g 100 g^{-1}]	[%]	[g 100 g^{-1}]
C8:0	A	0.94^{Aa}±0.01	0.27	0.93^{Aa}±0.01	0.25	0.94^{Aab}±0.02	0.27
	B	0.93^{Aa}±0.02	0.25	0.92^{Aa}±0.02	0.27	0.92^{Ab}±0.01	0.27
	C	0.97^{Aa}±0.02	0.25	0.96^{Aa}±0.01	0.25	0.96^{Aa}±0.01	0.25
C10:0	A	3.18^{Aa}±0.02	0.91	2.88^{Ba}±0.01	0.78	2.90^{Ba}±0.05	0.83
	B	3.05^{Aa}±0.12	0.85	2.84^{Ba}±0.06	0.84	2.90^{ABa}±0.02	0.84
	C	3.10^{Aa}±0.03	0.79	2.84^{Ba}±0.02	0.75	2.88^{Ba}±0.01	0.76
C12:0	A	3.75^{Ba}±0.01	1.07	3.81^{Aa}±0.01	1.04	3.82^{Aa}±0.04	1.09
	B	3.75^{Aa}±0.07	1.01	3.77^{Aa}±0.04	1.12	3.83^{Aa}±0.02	1.11
	C	3.58^{Bb}±0.03	0.91	3.67^{Ab}±0.01	1.00	3.70^{Ab}±0.01	0.99
C14:0	A	12.42^{Ba}±0.01	3.54	12.56^{Aa}±0.01	3.42	12.61^{Aa}±0.06	3.58
	B	12.50^{Aa}±0.17	3.36	12.47^{Aa}±0.06	3.68	12.63^{Aa}±0.03	3.66
	C	12.12^{Bb}±0.05	3.10	12.31^{Ab}±0.03	3.27	12.35^{Ab}±0.02	3.34
C14:1	A	1.56^{Ba}±0.01	0.45	1.58^{ABa}±0.01	0.43	1.59^{Aa}±0.02	0.45
	B	1.56^{Ba}±0.02	0.42	1.58^{ABa}±0.01	0.47	1.60^{Aa}±0.01	0.46
	C	1.50^{Cb}±0.01	0.38	1.53^{Bb}±0.01	0.41	1.56^{Ab}±0.01	0.42
C15:0	A	1.41^{ABa}±0.01	0.40	1.40^{Ba}±0.01	0.38	1.42^{Aa}±0.01	0.40
	B	1.42^{Aa}±0.02	0.38	1.40^{Aa}±0.01	0.41	1.42^{Aa}±0.01	0.41
	C	1.37^{Cb}±0.01	0.35	1.36^{Bb}±0.01	0.36	1.37^{Ab}±0.01	0.37
C16:0	A	34.93^{Cb}±0.04	9.95	35.06^{Ba}±0.06	9.54	35.21^{Aa}±0.04	10.00
	B	35.29^{Aa}±0.20	9.53	34.89^{Bb}±0.04	10.29	35.19^{Aa}±0.01	10.19
	C	34.14^{Cc}±0.03	8.74	34.18^{ABc}±0.02	9.09	34.20^{Ab}±0.02	9.26
C16:1	A	2.28^{Ba}±0.01	0.65	2.29^{Bb}±0.01	0.623	2.42^{Aa}±0.02	0.69
	B	2.28^{Ba}±0.02	0.61	2.30^{Ba}±0.00	0.68	2.43^{Aa}±0.01	0.70
	C	2.20^{Bb}±0.00	0.56	2.21^{Bc}±0.00	0.59	2.36^{Ab}±0.01	0.64
C18:0	A	9.76^{Ab}±0.02	2.78	9.71^{Aa}±0.03	2.64	9.78^{Ab}±0.04	2.77
	B	9.88^{Ab}±0.07	2.69	9.72^{Bb}±0.05	2.86	9.79^{ABb}±0.03	2.83
	C	10.47^{Aa}±0.04	2.68	10.42^{ABa}±0.02	2.77	10.41^{Ca}±0.02	2.82
C18:1n9c and C18:1n9t	A	23.72^{Bb}±0.03	6.76	23.69^{Bc}±0.02	6.45	23.90^{Ab}±0.09	6.78
	B	23.78^{Ab}±0.15	6.46	23.99^{Ab}±0.12	7.06	23.86^{Ab}±0.05	6.91
	C	24.47^{Ba}±0.08	6.26	24.23^{Ca}±0.03	6.45	24.70^{Aa}±0.05	6.69
C18:2n6c and C18:2n6t	A	2.16^{Ba}±0.01	0.62	2.26^{Ab}±0.03	0.61	2.26^{Aa}±0.01	0.64
	B	2.12^{Cb}±0.02	0.58	2.31^{Aa}±0.01	0.68	2.26^{Ba}±0.01	0.65
	C	2.08^{Bc}±0.01	0.53	2.23^{Ab}±0.01	0.59	2.22^{Ab}±0.01	0.60

[a] A: control variant (containing commercial strain *L. helveticus* LH-32 in the composition of the cheese starter); B: Cheese variant (containing strain T104 in the composition of the cheese starter); C: Cheese variant (containing strain T105 in the composition of the cheese starter.

[b] The results show the percentage share of individual fatty acids in the fat extracted from a cheese variant (analyzed at the end of a given maturation period)±Standard Deviation (±SD); n= 3. The same letter designations (a–c) express no statistically significant differences (P< 0.05) between the values in a given column for the content of a given acid; the same letters (A–C) express no statistically significant differences (P< 0.05) between the average values in a row referring to a specific fatty acid. [c]Values expressing the content of individual fatty acids in grams per 100 g of the tested product (cheese variant)

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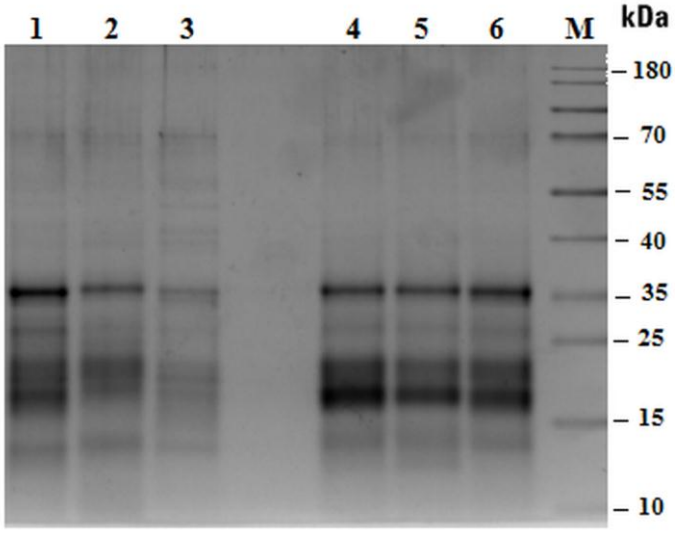

Figure 1. Comparison of protein profiles in final products. Explanation notes: Lanes= (1) Variant A; (2) Variant B; (3) Variant C and in pre-ripened cheese (after 15 days of maturation at 13˚C) Lanes= (4) Variant A; (5) Variant B, and (6) Variant C, Lane M= Molecular mass marker.

(2014). Interestingly, similar findings were obtained for Turkish raw ewe's cheese-Orgu (Türkoglu, 2011).

The content of individual fatty acids in cheese produced using *Lactobacillus helveticus* T105 and T104 corresponded to the composition of FFAs in the control variant obtained by application of industrial strain LH-32.

Analysis of Peptides and Amino Acids

The Tricine- SDS PAGE of the water-soluble extracts of cheeses (A, B and C variants) was conducted to visualize the differences (proteolytic changes) between the products in the pre-maturation stage and in the final products (Figure 1). Peptides in the range from 35 to > 10 kDa were apparent in all samples. Moreover, protein with a molecular mass of ca. 35 kDa was the main product present in the analyzed samples in all the ripening stages. It was suggested that bands with a molecular mass higher than 30 kDa were characteristic for whey proteins (bovine serum albumin, lactoferrin) and bacterial low molecular weight intracellular proteins (Gagnaire *et al.*, 2004). In turn, the main native casein fractions α_{s1}, α_{s2}, β, and κ were characterized by molecular weights of 22-23.7, 25, 24, and 19 kDa, respectively.

The main differences between the profiles of cheeses from the pre-ripening stage and the final products were found for bands ranging from 25 to 15 kDa. In samples of cheeses from the early ripening stage, the bands of the fractions were more intensive and visible, whereas products derived from the later ripening stage were visible on the electropherogram in the form of smudges and slightly differed between the cheese variants. Obtained products of proteolysis characterized by the same, or very similar to each other, molecular weighs formed a kind of smear, visible on the separating gel as a very smudged regions of the separation path. They are visible in the final products, where the time of hydrolysis was longer than the pre-ripening stage (Figure 1). The considerable changes between these two stages of maturation were clearly visible in the variant C and B samples, compared to the control cheese (variant A). The stronger 35 kDa band after ripening in the control cheese was probably related to the fact that in this variant of the cheeses the process of proteolysis occurred less intensive compared to the other cheeses variants obtained using the *L. helveticus* T105 or strain T104. Also, observed differences might be associated

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with various activities of the proteolytic system of *L. helveticus* strains.

These results are consistent with the findings presented by Aminifar *et al.* (2014), who analyzed electrophoretic changes in the protein profile in cheese during ripening. Their results indicated that products of β- and α_S- casein degradation were apparent as fainter bands than the corresponding primary bands.

To compare the peptide profiles, all cheese variants were analyzed using the MALDI-TOF MS technique. The spectra of the peaks obtained for the analyzed samples (in accordance with the charge, molecular weight, and time of flight of ions) confirmed the differences in the protein-peptide profiles between the cheeses. Moreover, the analysis exhibited differences in peptide profile patterns that were dependent on the stage of the ripening process (Figure 2).

The cheese samples analyzed after the completed maturation process (Figure 2- Sections A3, B3, and C3) were characterized by a greater share of peptides in a mass range of 1,500-2,000 Da (I), which can be related to the intensity and degree of proteolysis during the ripening process. Interestingly, the peptide with a molecular mass of approximately 2,763 Da (Figure 2– Area II) was a characteristic element occurring in all spectra. In addition, the presence of a 826 Da peptide (Figure 2- Black circles) was confirmed in all extracts of the final products (Figure 2– Sections A3, B3, C3) and in variant B of pre-ripened cheeses (Figure 2– Section B1). However, the cheese samples from the final ripening stage were distinguished by the presence of products in a mass range from 1,363 to 1,377 Da. The differences observed in the spectral images may be a result of the varied specificity of bacterial proteolytic enzymes, as in the case of *L. helveticus*, which exhibits the strongest proteolytic system among all lactic acid bacteria (Christiansen *et al.*, 2008; Nielsen *et al.*, 2009). Moreover, it has also been demonstrated that the distribution as well as the activity and specificity of *L. helveticus* Cell Envelope

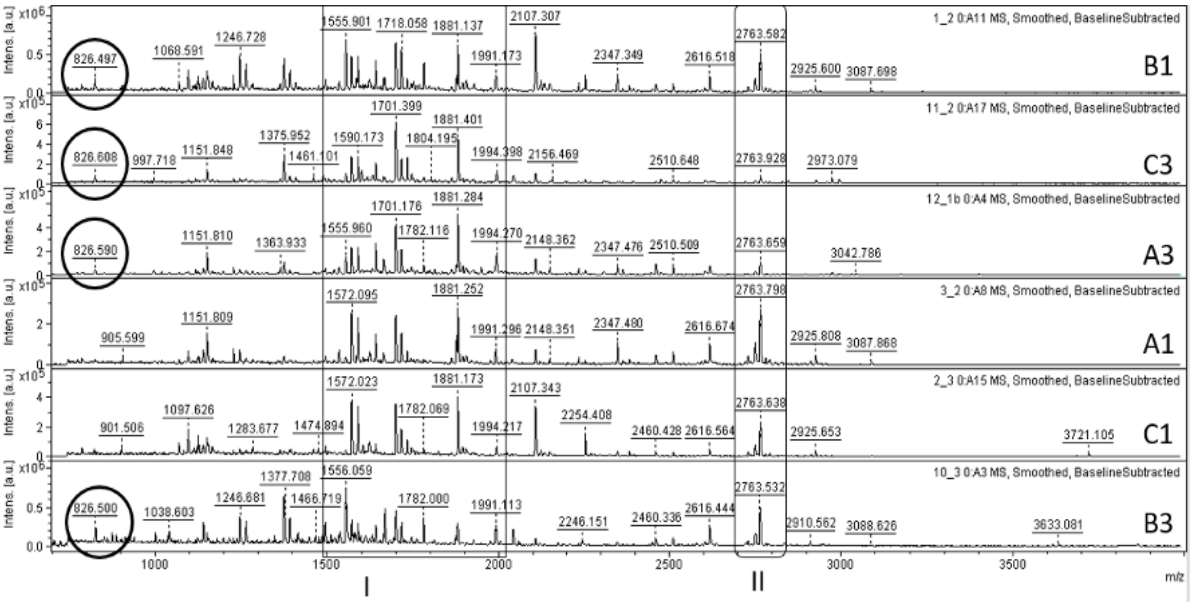

Figure 2. Analysis of the MALDI-ToF-MS spectra of peptides from cheeses samples derived from two stages of ripening process. Explanatory notes: A1, A3, B1, B3, C1, and C3 are samples of cheeses variants, (A) Control variant containing the commercial strain of L. helveticus LH-32 in the composition of starter culture; (B) Variant containing the L. helveticus T104 in the composition of the starter culture; (C) Variant containing strain L. helveticus T105 in the composition of the starter culture) analyzed after stages of maturation: (1) After ripening in cool room (15 days at 13°C, RH= 85%); (3) After ripening in cool room (13˚C/15 days; RH= 85%) and 60 days of ripening in warm room (23˚C, RH= 85%)

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Proteinases (CEPs) to individual casein fractions may vary and is a strain-dependent property, which may explain the differences in the proteolysis products (Hébert *et al*., 2008; Sadat -Mekmene *et al*., 2011; Griffiths and Tellez, 2013; Gatti *et al.*, 2014).

The results obtained by Fenelon *et al.* (2002) proved that *L. helveticus* is responsible for important biochemical changes in cheese during ripening. Their investigation has revealed that *L. helveticus* strain DPC4571 used as the only adjunct culture in cheese production improved the cheese flavor and increased the content of low-molecular-mass and peptide free amino acids. Moreover, the results obtained by Celik and Tarakci (2017) on analyses of the α_{s1}-casein degradation in cheeses, suggest that their applied supplemental bacteria including *Streptococcus thermophilus*, *Lac. lactis* subsp. *lactis* biovar. *diacetylactis*, *Lb. bulgaricus* and also *Lb. helveticus* (which exhibited different protein degradation capacities) were the main microorganisms influencing process of proteolysis.

Proteolysis contributes to the formation of free amino acids that are substrates for further catabolic changes (McSweeney, 2004). Furthermore, intracellular enzymes of *Lactobacillus helveticus* significantly support the production of aroma compounds by catabolism of amino acids that play a crucial role in cheese flavor development (Klein *et al.*, 2001; Widyastuti *et al.*, 2014). Therefore, the contents of Glutamic acid (Glu), Alanine (Ala), Lysine (Lys), Serine (Ser), Threonine (Thr), Asparagine (Asp), Histidine (His), Phenylalanine (Phe), Isoleucine (Ile), Methionine (Met), Valine (Val), Glycine (Gly), Proline (Pro), Leucine (Leu), and Tyrosine (Tyr) in the cheese samples were analyzed in the first stage and at the end of maturation (Table 4).

Only three amino acids were detected in the first ripening stage. During the ripening period, the content of amino acids in the cheeses changed, leading to variation in the concentration of the amino acids (Table 4).

The highest quantity and concentration of

Table 4. Content of free amino acids identified in cheese after the pre-ripening stage and in final products (mg g^{-1} of cheese).

Amino acid	Cheese variant[a]					
	A		B		C	
	Ripening stage					
	15 Days/13˚C	2 Months/23˚C	15 Days/13˚C	2 Months/23˚C	15 Days/13˚C	2 Months/23˚C
Glu	0.0013	0.119	0.0509	0.1	ND	0.261
Ala	0.0304	0.249	0.0339	0.167	0.0526	0.319
Lys	0.0039	0.613	0.0043	0.471	0.004	0.687
Ser	ND	0.081	ND	0.108	ND	0.096
Thr	ND	0.15	ND	0.121	ND	0.136
Asp	ND	0.007	ND	ND	ND	0.102
His	ND	0.078	ND	0.065	ND	0.091
Phe	ND	0.228	ND	0.197	ND	0.305
Ile	ND	0.204	ND	0.187	ND	0.174
Met	ND	0.098	ND	0.074	ND	0.078
Val	ND	0.356	ND	0.249	ND	0.352
Gly	ND	0.087	ND	0.07	ND	0.069
Pro	ND	0.324	ND	0.213	ND	0.402
Leu	ND	0.556	ND	0.429	ND	0.505
Tyr	ND	0.152	ND	0.128	ND	0.284

[a] A: Control variant (containing the commercial strain of *L. helveticus* LH-32 in the composition of the cheese starter); B: Cheese variant (containing strain T104 in the composition of the cheese starter); C: Cheese variant (containing strain T105 in the composition of the cheese starter); ND: Not Detected.

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the individual amino acids in the pre-ripening stage were detected in cheese B with *L. helveticus* T104 contribution. In turn, after two months of ripening, the greatest diversity in terms of the presence of flavor compound precursors was noted for cheese C, where *L. helveticus* T105 was applied. Glutamic acid and aspartic acid belong to salty-umami taste groups of amino acids. In the presence of α-ketoglutarate in the medium, *L. helveticus* is able to produce approx. 80% of acids by degradation of leucine (Helinck *et al.*, 2004).

After summing the concentration values of all analyzed Free Amino Acids (FAAs) for each cheeses variant (Table 4), the highest number of FAAs was recorded in cheeses from variant C. Interestingly, final products belonging to this variant were characterized by the lowest content of fat and total protein (Table 2), wherein exhibited the highest antioxidant activity (Figure 3) in comparison to final products from other variants (control and B). This may indicate that, during ripening, the process of proteolysis and other biochemical changes occurred more intensively in cheeses belonging to C variant (produced with using strain T105) than in final products of the control and B variant

The differences in the amino acid profiles may affect their further catabolic reaction, which influences the formation of flavor and aroma compounds. It was suggested that the capacity of lactic acid bacteria of amino acid degradation into flavor compounds is strictly strain dependent (Yvon and Rijnen, 2001).

Our study also revealed that the antioxidant activity (Figure 3) was higher in extracts derived from the final products (after the whole ripening period). This can be explained by the findings reported by Gupta *et al.* (2009), who concluded that the proteolytic activity of bacterial enzymes increased the number of peptides that contribute to the antioxidant activity of products. Furthermore, antioxidant activity depends on the rate of formation of soluble peptides (Perna *et al.*, 2015). Also, obtained in our study the results of electrophoretic separations, determination the ability to scavenging free radicals and analysis the spectra of peptides from cheeses samples (received using MALDI-ToF-MS) suggest that formation of peptides may influence the antioxidant activity of tested material.

Investigations of antioxidant peptide formation during milk fermentation induced by lactic acid bacteria indicated that the radical scavenging activity was a strain-dependent property, while radical scavengers were associated with proteolysis (Hernandez *et al.*, 2005; Virtanen *et al.*, 2007). In our research, the highest ability to scavenge free radicals in the first stage and at the end of maturation was demonstrated by cheeses from variant C, which were produced with the contribution of

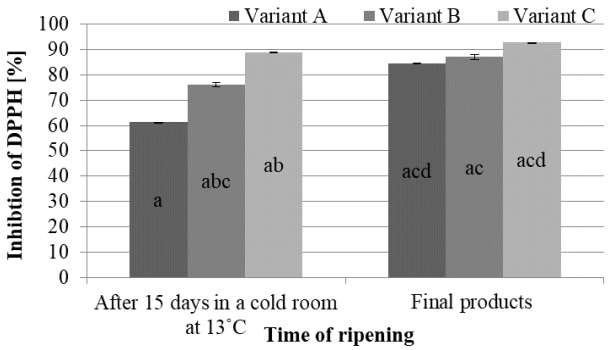

Figure 3. Comparison of the antioxidant activity of cheeses at the beginning and at the end of the ripening process. Data show mean values±standard deviation (n= 5) of the percentage of DPPH inhibition by cheese peptide extracts (ratio: 0.5 mL DPPH:1 mL extract). The same letter designations express there is no statistically significant differences (P< 0.05) between the means values.

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Lactobacillus helveticus T105 (88.89% in cheeses after ripening and 92.74% in the final product). Our results might indicate that the highest values of antioxidant activity (as well as small difference in values of this parameter between samples analyzed after the first maturation stage and the final products) for cheeses samples from variant C may be related to the high proteolytic activity of the strain T105. Our previous studies of proteolytic activity (and analysis of genetic determinants affecting these enzymatic properties) of the tested *L. helveticus* strains have revealed differences in the activity and enzymatic specificity of the proteolytic system among analyzed strains (Skrzypczak *et al.,* 2017b; Skrzypczak *et al.,* 2018). Moreover, strain T105 distinguished from other tested Polish strains of *L. helveticus* showed the highest values of proteolytic activity (Skrzypczak *et al.,* 2018).

It has been shown that the sequence of peptides exhibiting an ability to inhibit free radicals contains amino acids such as histidine and tyrosine as well as tryptophan, methionine, lysine, phenylalanine, and arginine in the form of free amino acids, which have antioxidant properties (Saiga *et al.,* 2003; Pihlanto and Mäkinen, 2013).

The present results have practical relevance and indicate that *Lactobacillus helveticus* T105 and T104 have great potential to be used in the dairy industry. Based on the results of the profiles of FFAs and amino acids, i.e. precursors of flavor compounds, it can be concluded that the analyzed strains can be promising flavor adjunct cultures incorporated into industrial cultures for production of ripened cheese.

The final products obtained using *L. helveticus* T105 exhibited the greatest amount of free amino acids and free radical scavenging capacity, even higher than the control cheeses containing the commercial strain.

Strains isolated from their native environment such as traditional fermented dairy products can be a good reservoir of new microorganisms exhibiting high technological potential in the development of some products with new characteristics, e.g. functional food.

Our results show possibilities of application of the new *L. helveticus* strains in ripened cheese production. Given the promising findings presented in this paper, further investigations should be conducted to verify the rheological and textural characteristics as well as other technological parameters.

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تغییرات خواص پنیر نوع سوئیسی تهیه شده با سویه های مختلف *Lactobacillus helveticus* درطی فرایند رسیدن

ک. اسکرزیپزاک، و. گوستاو، ا. واسکو، و ت. باناچ

چکیده

به نظر می رسد که وجود بیوتیپ های مختلف *Lactobacillus helveticus* با ویژگی های زیست بوم در ارتباط باشد که این ویژگی ها از عوامل تعیین کننده خصوصیات اصلی پنیر در مناطق خاص است. تا کنون، چنین ثابت شده که حتی بیو تیپ های جداسازی شده از یک مکان و جایگاه به طور معناداری با هم اختلاف دارند و بسیاری از ویژگی هایی که این باکتری ها نشان میدهند وابسته به سویه می باشد. در این پژوهش، سویه های جدید *helveticus* T104 و *helveticus* ۱۰۵ جداسازی شده از تخمیر سنتی محصولات لبنی لهستان) به لحاظ خواص آن ها درارتقای سلامت، در تولید پنیر رسیده (*ripened cheeses*) به کار برده شد. در طی این کار، تغییرات در چربی، اسیدهای چرب، پروتئین، و برخی ویژگی های فیزیکوشیمیایی مانند خواص آنتی اکسیدانی پنیر تهیه شده(در سه مرحله از فرایند رسیدن) تعیین شد. تحلیل های *Tricine-SDS-PAGE* و *MALDI-TOF MS*چند تفاوت در پروفیل های پروتیئن و پپتید نشان داد. محصول نهایی به دست آمده از *helveticus L.*۱۰۵ بیشترین مقداراسید های آمینه آزاد را نشان داد که مواد اولیه مهمی در عطر و مزه پنیر هستند. این پژوهش اشارت داشت که سویه های آزمون شده را می توان در تهیه و تولید پنیر به کار بست. افزون بر این، پنیر تولیدشده با استفاده از این سویه حتی در مقایسه با پنیر گونه شاهد که با استفاده از سویه *L. helveticus* مخصوص تولید صنعتی تهیه شده بود، بیشترین ظرفیت اصلاح رادیکال آزاد (free radical scavenging capacity) را نشان داد (برابر با ۸۸/۸۹٪ بعد از رسیدن و ۹۲/۷۴٪ در محصول نهایی). نتایج به دست آمده حاکی از آن است که سویه های آزمون شده پتانسیل فناوری و غنی سازی از خود نشان میدهد که می تواند مرجعی برای مطالعات آینده باشد و احتمالا می تواند به تهیه و تولید محصولات غذایی غنی شده و مناسب برای سلامتی (functional food) با ویژگی های نو و ارزشمند کمک کند.

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