NATURAL MICROFLORA OF RAW COW MILK AND THEIR ENZYMATIC SPOILAGE POTENTIAL

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Abstract: The aim of this work was to identify the main microbiota in raw cow milk from dairy farm of Slovakia and to describe the selected microorganisms responsible for thermostable protease and lipase production which can affected the quality of dairy products. The main bacterial classes identifying by MALDI-TOF MS were *Gammaproteobacteria* (62 %), *Actinobacteria* (19 %) and *Bacilli* (12 %). The dominant microbial genus of raw cow milk was *Pseudomonas*. From milk bacteria, the strain *Lactococcus lactis* and from the family *Enterobacteriaceae*, namely *Enterococcus faecalis*, *Hafnia alvei*, *Citrobacter braakii* and *Raoultella ornithinolytica* were observed in raw milk. The spoilage of milk products is caused by thermostable enzymes with lipolytic and proteolytic activity. Qualitative proteolytic and lipolytic activities were performed on skin milk agar and olive oil, respectively. From 16 identified microorganisms, only 8 strains (*P. fragii, P. gessardii, P. lundesis, H. alvei, C. braakii, R. ornithinolytica, Kocuria rhizophila* and *Candida inconspicua*) showed protease activity. Quantitative protease and lipase activities were determined by casein and olive oil, respectively. The highest both activities were measured for the genus *Pseudomonas.* While lipases produced by all isolated microbial species lose enzymatic activity at 77 °C for 30 – 40 min, almost proteases showed comparable activities during whole pasteurization experiment at selected experimental conditions (70 °C, 40 min).

Key words: milk, microflora, enzymes, protease, lipase, spoilage

1. Introduction

The milk is an important raw material for food industry. Its quality can be affected by various biochemical and microbial factors such as age and health of the production animals, their feed, season and some others (VANHAECKE *et al*., 1990). In the term of quality milk, the microbial population is very important. The microorganisms may come from the cowshed, bedding material, the seasonal feed, the teat purity and the dairy equipment (COUSIN, 1982; VACHEYROU *et al*., 2011). The main part of milk microflora includes bacteria such as *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Bacillus*, *Microbacterium*, *Micrococcus*, *Staphylococcus*, *Pseudomonas*, *Aeromonas* and *Acinetobacter* but some fungal species such as *Candida*, *Kluyveromyces* and *Pichia* can be present (DELAVENNE *et al*., 2011; SMARŽIJA *et al*., 2012; QUIGLEY *et al*., 2011; 2013).

The present of microorganisms in the cow milk is problematic for three main aspects, namely production of microbial toxins, biofilm production and production

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of undesirable enzymes. Production of microbial toxins can cause some health problems, but the present of these microorganisms is monitored and contaminated milk is not used in the next processing cycle. Production of biofilms allows some problematic microorganisms to survive during thermal technological operation such as pasteurization. Biofilm-producing microorganisms can cause faster spoilage of milk products. The last negative aspect of present of microorganism in the milk is production of enzymes which are stable at pasteurization temperature. These enzymes can cause physical and chemical instability of milk products.

The main problematic enzymes are proteolytic and lipolytic enzymes. Proteolytic enzymes may lead to the defects in the final dairy products, because the protein hydrolysis causes some negative properties such as bitter flavor in the milk and gelatinization of UHT milk during prolonged storage. Even small amounts of proteolytic enzymes can effectively hydrolyze peptide bonds in proteins and can change the certain properties of dairy products such as physico-chemical, functional and/or sensory properties (GUINOT-THOMAS *et al*., 1995; CELESTINO *et al*., 1997; CHEN *et al*., 2003; MARTINS *et al*., 2006; TEH *et al*., 2012). Lipolytic enzymes play the important role in increasing of oxidation lability of milk lipids by hydrolysis of triacylglycerols and production of more free fatty acids which are easily oxidized (TEH *et al*., 2011).

The aim of this work was to describe the key microorganisms responsible for enzymatic spoilage of milk products by production of thermostable enzymes with lipolytic and proteolytic activities which can affect the quality of milk and milk products.

2. Materials and Methods

2.1. Isolation and identification of bacterial strains

The raw cow milk was taken from Slovak dairy farm (Senica, Slovakia). The milk used in experiments was aseptically collected from storage tank. The collected samples were diluted and applied onto both nutrition agars, namely MPA and MRS agar. The microflora was incubated for determination of CFU for 48 hours at 6, 25 and 50 °C of psychrophiles, mesophiles and thermophiles, respectively. On the base of micro- and macroscopic properties of colonies, microbial isolates were collected and re-streaking on new solid media which were marked as isolates with the serial number.

2.2. Preparation of samples for MALDI-TOF MS analysis

Freshly biomass of each isolate was prepared by cultivation on MPA agar for 24 hours at 30 °C, before identification of microbial isolates by MALDI-TOF MS. After then, one colony of microbial isolate was taken off from agar plate and solvated in 300 µL of distilled water and after intensively mixing, 900 µL of pure ethanol was added. The mixture was two-times centrifuged at 12,000 x g for 2 min. The pellet

of washed microbial biomass was dried at laboratory temperature and after then, 10 µL of formic acid was added. After mixing, 10 µL of acetonitrile was added and mixture was centrifuged at 12,000 x g for 2 min. The supernatant was prepared for MALDI-TOF MS analysis.

2.3. Identification by MALDI-TOF MS

1 µL of supernatant, prepared on the base of method described in the Chapter 2.2., was applied on the MALDI board and after drying, 1 µL of saturated solution of α-cyano-4-hydroxycinnamic acid in 0.025 % (v/v) of trifluoracetic acid in 50 % (v/v) water solution of acetonitrile was added. Follows, sample was analyzed by MALDI-TOF MS (Microflex LT/SH, Bruker Daltonics, Germany) with Flex software and the results were obtained by Realtime classification software (Bruker Daltonics, Germany).

2.4. Enzyme determination

2.4.1. Agar diffusion method

The basic screening of ability of microbial isolates to produce of proteases and lipases was realized by agar diffusion method based on effect of enzyme on selected compounds, namely proteins and lipids forming turbidity of solid nutrition media. Skim milk powder was used as substrate for proteases and olive oil as substrate for lipases. These additives were added to nutrient agar in amount of 10 % (w/v). The microbial isolates were inoculated on the cooled solid nutrient agar and incubated at 30 °C for 24 and 48 hours. The enzyme production was visualized by clarification of zone around producer colony after incubation.

2.4.2. Protease production by selected microbial isolates

For determination of protease production ability, microbial isolates were cultivated in 10 % (w/v) water solution of dry milk at 30 °C for 48 hours. After this time, cultivation media were centrifuged at 4,000 x g for 10 min and supernatants were used for enzyme activity determination. Determination of protease activity was performed by the study of CHARNEY and TOMARELLI (1947) using azocasein as substrate. The enzyme reaction mixture contained 900 µL of 1 % (w/v) solution of azocasein in 5 mmol/L phosphate buffter (pH 7.4) and 100 µL of enzyme solution prepared in previous experiments. The enzyme reaction mixture was incubated at 30 °C for 15 min on the orbital shaker (150 rpm). After this time, 800 µL of 5 % (w/v) of water solution of trichloroacetic acid was added and mixture was intensively mixed on the vortex. Formed precipitate was eliminated by centrifugation at 10,000 x g for 5 min. 100 µL of the clear reaction solution was pipetted into the wells of microtitration plates contained 50 µL of 0.5 mol/L sodium hydroxide. The resulted absorbance was measured at 405 nm. The protease activity was calculated on the base of proteolytic activity of standard protease isolated from *Bacillus subtilis* (Sigma Aldrich, Germany).

2.4.3. Lipase production by selected microbial isolates

For determination of lipase production ability, microbial isolates were cultivated in 10 % (w/v) water solution of dry milk at 30 °C for 48 hours. After this time, cultivation media were centrifuged at 4,000 x g for 10 min and supernatant was used for enzyme activity determination. Determination of lipase activity was performed according to (BENZONANA and DESNUELLE, 1965). 100 µL of enzyme solution prepared in previous experiments was added into solution contained 5 g of olive oil and 12.5 mL of isopropanol. The enzyme reaction mixture was incubated at 30 °C for 30 min. After this time, 60 µL of standard solution of phenolphthalein was added and mixture was titrated by 0.25 mol/L potassium hydroxide to the pink color of solution stabile minimal 15 sec. The lipase activity was calculated on the base of proteolytic activity of standard lipase isolated from *Pseudomonas cepacia* (Sigma Aldrich, Germany).

2.5. Thermal inactivation of enzymes

The resistance of the enzymes to pasteurization was evaluated at 63 °C, 70 °C and 77 °C according to JANNESS and KOOPS (1962). The cultivation medium (100 µL) prepared by the method described in Chapter 2.1. was heated in the test microtubes using a thermomixer (Eppendorf, Germany) at selected temperature (63 °C, 70 °C and 77 °C, respectively) during 40 min. During this time, the samples were collected and frozen immediately in liquid N_2. After the measurement of the residual enzyme activity, samples were stored at -20 °C. The residual proteolytic and lipolytic activities were measured in all samples using the standard assays described in Chapter 2.4.2 and 2.4.3, respectively.

2.5.1. Determination of the enzyme half-life

The enzyme half-life was calculated according to BAUR *et al*. (2015). The first step was calculation of inactivation rate constant (K_d) by a first-order equation:

$$\frac{E_t}{E_0} = e^{-K_d t} \tag{1}$$

so that:

$$\ln\left[\frac{E_t}{E_0}\right] = -K_d t \tag{2}$$

where, K_d is equal to the slope of the regression line from a plot of $ln[E_t/E_0]$ versus t at a particular temperature. The resulting regression lines had high correlation coefficients ($r^2 > 0.95$), suggesting first order inactivation kinetics in the temperature range investigated. The half-life $t_{1/2}$ was calculated based on equation (2), using equation (3):

$$t_{1/2} = \frac{\ln 2}{K_d} \tag{3}$$

where E_t is the enzyme activity (U/mL) after a particular heating time, E_0 is the initial enzyme activity (U/mL), t is the heating time (s), and K_d is the inactivation rate constant (s^{-1}).

2.6. Statistical analysis

All experiments were realized in triplicate. The obtained data were evaluated by Excel (Microsoft, 2010).

3. Results and Discussion

The microbiota of raw milk is various due the possibility of contamination from cowshed, bedding material, the seasonal feed, the teat purity and the dairy equipment (GLÜCK *et al*., 2016). First step was determined the microbial population including psychrophilic, mesophilic and thermophilic microorganisms in raw cow milk by plate counting on selected media (MRS and MPA) (data not shown). Isolated colonies showing differences in micro- and macro-scopic properties were re-cultivated as one-colony culture. From the whole microbial population (10^7 CFU/mL), the psychrophilic (4.2×10^6 CFU/mL) and mesophilic (5.1×10^6 CFU/mL) bacteria represented the major part of microorganisms of raw cow milk. The thermophilic bacteria represent only 1.3×10^2 CFU/mL. Similarly, ERCOLINI *et al*. (2009) found that the milk samples contain mainly mesophilic bacteria (5.0×10^3 – 6.0×10^5 CFU/mL) and psychrophilic bacteria (1.2×10^3 – 1.6×10^6 CFU/mL). After an initial screening, 30 variable microbial isolates differencing in micro/macroscopic properties were characterized and identified by MALDI-TOF MS.

3.1. MALDI-TOF MS identification of microbial isolates

Prepared microbial isolates were identified by MALDI-TOF MS. Analysis was provided on the base of MALDI-TOF MS fingerprint of protein fraction microbial isolates. The obtained fingerprints were compared to the reference spectra of the BioTyper database and their similarity was expressed as BioTyper Log (score). MALDI-TOF MS fingerprint of the isolate from raw milk was compared to reference MALDI-TOF MS profiles of the BioTyper database. The dark grey, grey and colorless sticks in the top half colored panels indicating the peak matching (intensity and m/z value) between experimental and database reference MALDI-TOF MS profiles is excellent, medium and low match, respectively (Table 1). The Log(score) takes into account the number of matching peaks, the total number of peaks, the peak weight representing species specificity and a correlation factor related to the matching peak intensity (CHERKAOUI *et al.*, 2010).

The 30 isolates (6 psychrophilic, 11 mesophilic and 4 thermophilic) from raw cow milk isolated on MRS and MPA agar plates were identified by MALDI-TOF MS (7 isolates (27 %) with Log(score) $\geq$ 2.3; 11 isolates (33 %) with Log(score) 2.3 $\geq$

and ≤ 2.0; 8 isolates (27 %) with Log(score) 2.0 ≥ and ≤ 1.7. Only 4 isolates were not identified for low Log(score) (≤ 1.7). The 9 isolates differing in micro- and macroscopic properties after cultivation on MRS and MPA plates were identified as the same species. 7 Gram-positive and 9 gram-negative bacteria were identified among the isolates from milk samples. One of isolates was identified as the yeast *Candida inconspicua* (Table 1). ERCOLINI *et al.* (2009) found that some milk samples do not contain gram-positive bacteria. In general, the number of gram-negative bacteria was higher than the number of gram-positive bacteria (ERCOLINI *et al*., 2009).

Table 1. MALDI-TOF MS analysis of isolates from raw milk and peak matching between experimental and database reference MALDI-TOF MS profiles.

Number of isolate	Organism (best match)	Score value	Organism (second best match)	Score value
1	*Enterococcus faecalis*	2.405	*Enterococcus faecalis*	2.389
2	*Corynebacterium casei*	2.016	**not reliable identification**	1.503
3	*Candida inconspicua*	2.072	*Candida inconspicua*	2.020
4	*Aeromonas media*	1.909	*Aeromonas veronii*	1.881
5	*Citrobacter braakii*	1.716	**not reliable identification**	1.520
6	*Raoultella ornithinolytica*	2.376	*Raoultella ornithinolytica*	2.276
7	*Raoultella ornithinolytica*	2.425	*Raoultella ornithinolytica*	2.327
8	*Raoultella ornithinolytica*	2.390	*Raoultella ornithinolytica*	2.314
9	*Kytococcus sedentarius*	1.921	*Kytococcus sedentarius*	1.703
10	*Pseudomonas fragi*	1.749	**not reliable identification**	1.693
11	**not reliable identification**	1.684	**not reliable identification**	1.481
12	*Acinetobacter johnsonii*	2.035	*Acinetobacter johnsonii*	1.842
13	*Raoultella ornithinolytica*	2.392	*Raoultella ornithinolytica*	2.373
14	*Raoultella ornithinolytica*	2.353	*Raoultella ornithinolytica*	2.258
15	*Lactococcus lactis*	2.156	*Lactococcus lactis*	2.111
16	**not reliable identification**	1.576	**not reliable identification**	1.573
17	*Sphingobacterium multivorum*	1.987	**not reliable identification**	1.270
18	*Kocuria rhizophila*	1.950	*Kocuria varians*	1.837
19	*Pseudomonas gessardii*	1.930	*Pseudomonas proteolytica*	1.857
20	**not reliable identification**	1.323	**not reliable identification**	1.291
21	*Kocuria kristinae*	2.103	*Kocuria kristinae*	2.076
22	*Pseudomonas lundensis*	2.029	**not reliable identification**	1.634
23	**not reliable identification**	1.317	**not reliable identification**	1.297
24	*Pseudomonas fragi*	1.820	*Pseudomonas taetrolens*	1.773
25	*Pseudomonas lundensis*	2.068	*Pseudomonas taetrolens*	1.993
26	*Pseudomonas fragi*	2.159	*Pseudomonas taetrolens*	1.741
27	*Lactococcus lactis*	2.000	*Lactococcus lactis*	1.953
28	*Hafnia alvei*	2.092	*Hafnia alvei*	2.083
29	*Micrococcus luteus*	2.306	*Micrococcus luteus*	2.277
30	*Hafnia alvei*	2.080	*Hafnia alvei*	1.981

Legend: The dark grey (excellent match), grey (medium match) and colorless (low match) sticks in the colored panels indicated the peak matching between experimental and database reference MALDI-TOF MS profiles.

The Fig 1 shows the classification of identified microorganisms into groups of psychrophilic and mesophilic organisms presented in raw cow milk. The dominant psychrophilic microorganism in the milk sample was the bacterial genus *Pseudomonas* (77 %) (Fig. 1-A). Similarly, in the culture of mesophilic organisms, the most

abundant bacterial microorganism was the microbial genus *Pseudomonas* (20 %). It was observed the higher microbial diversity in the group of mesophilic microorganisms (Fig. 1-B). VITHANAGE *et al*. (2016) evaluated qualitative composition of microbial culture in raw cow milk by MALDI-TOF analysis. They found that the main bacterial classes are *Gammaproteobacteria, Bacilli* and *Actinobacteria*. The major isolates (approximately 42 %) belongs to *Gammaproteobacteria* (including *Pseudomonas*), followed by *Bacilli* (32 %) and *Actinobacteria* (15 %). In our study, the main bacterial classes were *Gammaproteobacteria* (62 %), *Actinobacteria* (19 %) and *Bacilli* (12 %). Similarly to this study, the genus *Pseudomonas* was the most abundant microbial genus in study of other authors (NEUBECK *et al.*, 2015; VITHANAGE *et al*., 2016) Milk bacteria belong to dominant group of mesophilic microorganisms of raw cow milk. Our isolate of milk bacteria was determined as *Lactococcus lactis*. In addition to this species, MARROK (2011) identified other species of milk bacteria such as *Lactococcus raffinolactis*, *Leuconostoc mesanteroides*, but the most numerous is *Lactococcus lactis*. XIN *et al.* (2017) found that bacterial composition of the raw milk is various and it affects different geographical areas.

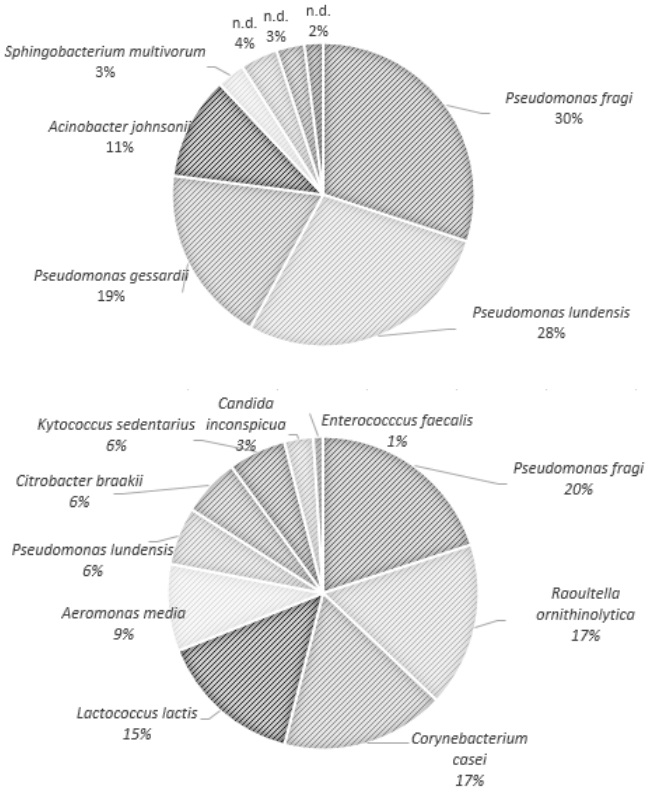

Fig. 1. Relative abundance of different microorganisms isolated from raw cow milk and identified by MALDI-TOF MS analysis. A – psychrophilic and B – mesophilic microorganisms; n.d. – not determined by MALDI-TOF MS analysis.

From the group of undesired microorganisms, many gram-positive bacteria were determined such as *Streptococcus*, *Enterococcus* and *Staphylococcus* (VITHANAGE *et al*., 2016). In our samples, *Enterococcus faecalis*, *Hafnia alvei*, *Citrobacter braakii* and *Raoultella ornithinolytica* were determined. KAGKLI *et al.* (2007) suggested that the possible source of *H. alvei* can be the water. Other undesired microorganism present in raw cow milk were *Clostridium* (ANDERSSON *et al*., 1995), *Moraxella*, *Listeria*, *Corynebacterium*, *Salmonella* and *Escherichia* (COUSIN, 1982; D´AOUST, 1991; STARK, 2000). Except to bacteria, the raw cow milk can contain also yeast and fungi. In our samples, we determined the presence of the yeast *Candida inconspicua*. Similarly, QUIGLEY *et al.* (2013) identified some yeasts such as *Candida*, *Kluyveromyces* and *Pichia* by next generation sequenation. DELAVENNE *et al.* (2011) describe that in the cow milk can be present also *Rhodotorula*, *Debaryomyces*, *Geotrichum*, *Trichosporon* and *Cyptococcus*.

3.2. Identification of proteolytic and lipolytic isolates

The ability of enzyme production by microbiota from milk was determined previously by several authors (HANTSIS-ZACHAROV *et al.*, 2007; TEH *et al*., 2011; BAUR *et al*., 2015; GLÜCK *et al*., 2016). In total, 17 isolates were obtained from raw caw milk. The first screening of the ability of microbial isolates to produce enzymes, namely proteases and lipases, it was evaluated agar diffusion method. This method is based on the elimination of turbidity of solid media with proteins from skim milk powder or lipids from olive oil. The analysis of cultivation media was realized after cultivation of each microbial isolate at 6, 20 and 30 °C for 48 hours. The results are shown in Table 2.

From 17 identified species from raw cow milk, some species such as *C. inconspicua*, *H. alvei*, *K. rhizophila*, *R. ornithinolytica* (mesophiles) and *C. braakii*, *P. fragii*, *P. gessardii* and *P. lundensis* (psychrophiles) showed proteolytic activity (Table 2). Similarly, GLÜCK *et al.* (2015) found that some microorganisms isolated from cow milk produced peptidase activity such as *Pseudomonas*, *Candida*, *Microbacterium* and *Chryseobacterium*. Similarly, ERCOLINI *et al*. (2007) observed that the genera *Pseudomonas*, *Hafnia* and *Citrobacter* were able to produce proteolytic activity on skim milk agar. Lipolytic activity was not observed by this method (Table 2). Although, BAUR *et al.* (2016) observed that some species of *Pseudomonas*, *Acinetobacter*, *Chryseobacterium*, *Candida*, *Lactococcus* and *Staphylococcus* produced lipases. The growth of microbial isolates and their production of proteolytic enzymes depend on cultivation temperature. Understandable, the temperature of 6 °C was suitable for growth of psychrophiles, but it was not suitable for growth of mesophiles (Table 2). Although, proteolytic activity was detected at 3 species from 6 identified psychrophilic bacteria from raw cow milk at 6 °C. All producers of proteolytic enzymes belong to the genus *Pseudomonas*. The temperatures of 20 °C and 30 °C were suitable for growth of all isolated microorganism, but proteolytic activity was detected only at 8 species from 17 identified microorganisms from raw cow milk. After an initial determination of enzyme production, these 8 species were used for precise determination of their proteolytic and lipolytic activity.

Table 2. The proteolytic and lipolytic ability of identified psychrophiles and mesophiles from raw cow milk.

	Microbial species	6 °C			20 °C			30 °C		
		Growth	PA	LA	Growth	PA	LA	Growth	PA	LA
Psychrophiles	*Acinetobacter johnsonii*	+	-	-	+	-	-	+	-	-
	Citrobacter braakii	+	-	-	+	+	-	+	+	-
	Pseudomonas fragi	+	+	-	+	+	-	+	+	-
	Pseudomonas gessardii	+	+	-	+	+	-	+	+	-
	Pseudomonas lundensis	+	+	-	+	+	-	+	-	-
	Sphingobacterium multivorum	+	-	-	+	-	-	+	-	-
Mesophiles	*Aeromonas media*	-	-	-	+	-	-	+	-	-
	Candida inconspicua	-	-	-	+	+	-	+	-	-
	Corynebacterium casei	-	-	-	+	-	-	+	-	-
	Enterococcus faecalis	-	-	-	+	-	-	+	-	-
	Hafnia alvei	-	-	-	+	+	-	+	-	-
	Kocuria kristinae	-	-	-	+	-	-	+	-	-
	Kocuria rhizophila	-	-	-	+	+	-	+	+	-
	Kytococcus sedentarius	-	-	-	+	-	-	+	-	-
	Lactococcus lactis	-	-	-	+	-	-	+	-	-
	Micrococcus luteus	-	-	-	+	-	-	+	-	-
	Raoultella ornithinolytica	-	-	-	+	+	-	+	-	-

Legend: PA – proteolytic activity; LA – lipolytic activity.

3.3. Determination of proteolytic and lipolytic activity

Although psychrotrophic bacteria are not resistant to heat treatment of milk, their enzymes can survive the heat treatment used in dairy industry and to reduce milk quality (XIN *et al*., 2017). They cause the coagulation and instability of milk (CALDERA *et al*., 2016). The proteolytic and lipolytic activities of microbial isolates from raw cow milk were determined on the base of their enzyme activity on their natural substrates such as azocasein and lipids (olive oil), respectively. The proteolytic activity was determined at pH 7.6 because it is known that thermophile peptidase produced by milk microbiota mainly belong to the class of metallopeptidase (EC 3.4.24.) and have an optimal pH between 6.5 and 8.0 (KOHLMANN *et al*., 1991; SCHOKKER and VAN BOEKEL, 1997; KOKA and WEIMER, 2000; RAJMOHAN *et al*., 2002; DUFOUR *et al*., 2008). The results are showed in the Table 3.

Table 3. Proteolytic and lipolytic activity of selected microbial isolates from raw cow milk after 48 hours cultivation at 30 °C.

Microbial species	Proteolytic activity [U/mL]	Lipolytic activity [U/mL]
Candida inconspicua	30.5±2.1	0.67±0.1
Kocuria rhizophila	24.9±0.2	1.50±0.2
Raoultella ornithinolytica	30.6±0.8	1.33±0.2
Acinetobacter johnsonii	19.9±2.1	1.17±0.4
Citrobacter braakii	20.5±1.1	1.00±0.1
Pseudomonas fragi	29.3±0.6	0.83±0.1
Pseudomonas gessardii	70.5±4.3	0.83±0.2
Pseudomonas lundensis	28.9±2.1	1.72±0.2

All of tested isolated species have proteolytic and lipolytic activity. From the literature, it is known that *Pseudomonas*, *Candida* and *Acinetobacter* produce the highest both enzymatic activities (BAUR *et al*., 2015; XIN *et al*., 2017). The genus *Pseudomonas* can simultaneously produce proteases and lipases because the gen *aprA* encoding metallopeptidase and the gen *lipA* encoding an extracellular lipase lie next to each other (WOODS *et al*., 2001; MCCARTHY *et al*., 2004; MARCHAND *et al*., 2009; HASAN *et al*., 2010; LOPER *et al*., 2012). The highest proteolytic activity was measured for the bacteria *P. gessardii* (70.5±4.3 U/mL) and the high lipolytic activity was observed for the bacteria *P. lundesis* (1.72±0.2 U/mL).

3.4. Determination of spoilage enzyme thermostability

The thermal inactivation of enzymes produced by microbial culture of milk is essential for obtaining of suitable dairy products (XIN *et al*., 2017). Some enzymes including lipases and proteases can withstand the thermal treatment during milk processing and then these enzymes can cause the spoilage of milk and dairy products.

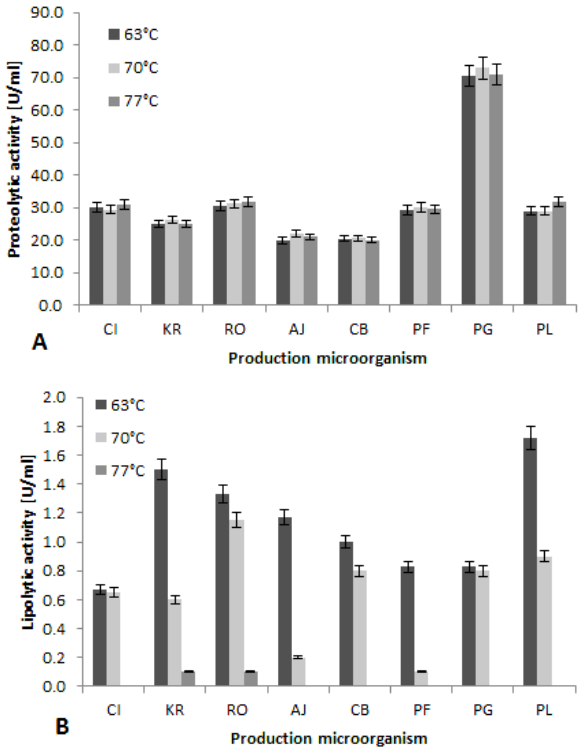

Fig. 2. Proteolytic (A) and lipolytic (B) activity of microbial isolates from raw cow milk after 40 min of pasteurization at selected temperatures (63 °C, 70 °C and 77 °C); CI – *Candida inconspicua*; KR – *Kocuria rhizophila*; RO – *Raoultella ornithinolytica*; AJ – *Acinetobacter johnsonii*; CB – *Citrobacter braakii*; PF – *Pseudomonas fragi*; PG – *Pseudomonas gessardii*; PL – *Pseudomonas lundensis*.

The thermal stability of enzymes presents in milk media fermented by our microbial isolates was determined. The analysis of enzyme thermostability during common pasteurization of cow milk was realized in the range 63 – 77 °C. The results are shown in Fig. 2 which shows residual activity of proteases and lipases in milk medium after pasteurized at described temperatures (63 °C, 70 °C and 70 °C) for 40 min.

The selected temperature conditions were appropriated for elimination of all studied microorganism (data not shown) but some enzymes were present and active also after pasteurization at the highest tested temperature (77 °C) for 40 min. Generally, proteases were more resistant to thermal treatment as lipases. Whereas lipases produced by all microbial isolates lose enzyme activity at 77 °C for 30 – 40 min, almost proteases showed the comparable activity during whole pasteurization experiment.

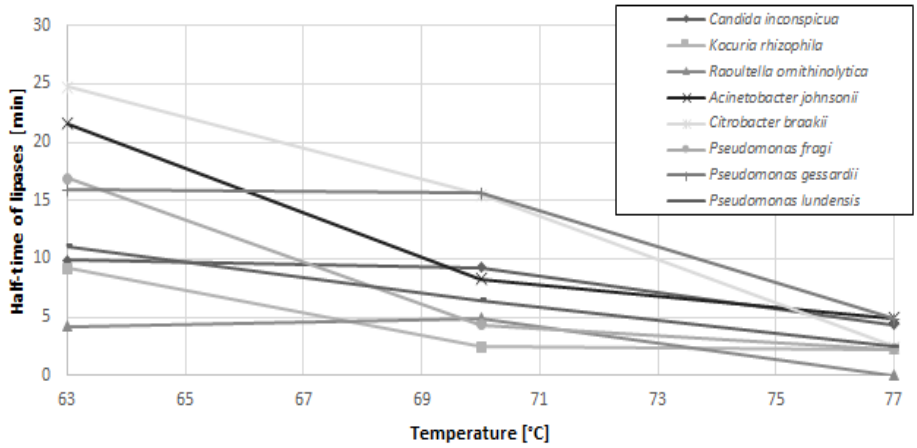

Fig. 3. The half-life of lipases produced by selected microflora of raw cow milk at described temperatures (63 – 77 °C) of pasteurization for 40 min.

These data are confirmed by other authors who described that peptidases from *Pseudomonas* spp. can withstand the heat treatments even in the UHT region (110 – 160 °C) (ADAMS *et al*., 1975; BARACH and ADAMS, 1977; MU *et al*., 2009). The thermolability of lipases is best documented by half-life of these enzymes (Fig. 3).

4. Conclusions

The dominant microbial genus of raw cow milk was *Pseudomonas*. Besides this, microbial genera *Aeromonas*, *Candida*, *Corynebacterium*, *Enterococcus*, *Hafnia*, *Kocuria*, *Kytococcus*, *Lactococcus*, *Micrococcus*, *Raoultella*, *Acinetobacter*, *Citrobacter* and *Sphingobacterium* were presented and identified in raw milk. The main producers of spoilage enzymes were *Candida inconspicua*, *Kocuria rhizophila*, *Raoultella ornithinolytica*, *Acinetobacter johnsonii*, *Citrobacter braakii*,

Pseudomonas fragi, *P. gessardii*, and *P. lundensis*. During pasteurization (30 – 40 min, 77 °C), lipolytic enzymes were denatured. Although, most proteases remained stable during pasteurization cycle (40 min, 77 °C).

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