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Proteolysis in Raw Milk in Relation to Microbiological Indicators

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Abstract

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Proteolysis in raw milk is a crucial parameter indicating both cow's mastitis and the technological problems or spoilage risk of final products. However, a suitable analytical method for its early detection in practice is still missing. Thus, we proposed a spectrophotometric determination of milk proteolysis equivalent (MPE). We tested this method on 104 bovine raw milk samples in relation to their somatic cell count (SCC) as an indicator of native proteolysis, and the total count of mesophilic bacteria (TCMB) and the total count of psychrotrophic bacteria (TCPB) as indicators of microbial proteolysis. Correlation coefficients between log TCMB and MPE and log TCPB and MPE were 0.3651 and 0.4152, respectively (both $P < 0.001$). SCC was not correlated with MPE ($P > 0.05$). We estimated the MPE limit indicating an incipient risk of proteolysis in the range from 0.9366 to 1.02 mmol/l. The determination of MPE seems to be a promising method applicable in the control of raw milk.

Keywords: primary amino groups; proteolytic enzymes; psychrotrophic microorganisms; quality of bovine raw milk; somatic cell count; total count of microorganisms

Proteolysis in raw milk is an important factor influencing the quality of final dairy products and technological certainty, especially deterioration of heat-stability and fouling in heat-exchangers (Gaucher *et al.* 2008), gelation, off-flavours, or bitterness of pasteurised milk (McKellar 1981) and UHT milk (Valero *et al.* 2001), as well as the functionality and sensory changes of milk powders (Chen *et al.* 2003). Moreover, the concentration of proteolytic enzymes can be an indicator of cow's health as well as the conditions of milking, milk storage, and treatment (Topcu *et al.* 2006).

Proteolysis can be caused by both native and bacterial enzymes present in raw milk. Native enzymes are especially plasmin and enzymes originating from somatic cells, e.g. cathepsins (Upadhyay *et al.* 2004). Proteolytic bacteria belong to various genera, e.g. *Pseudomonas, Bacillus, Clostridium, Proteus, Escherichia, Micrococcus, Microbacterium, Flavobacterium, Chryseobacterium*, and others. Moreover, proteolytic yeasts also occur in raw milk (Baur *et al.* 2015). However, psychrotrophic bacteria seem to be the most important (Champagne *et al.* 1994) and *Pseudomonas* spp. predominate among them (von Neubeck *et al.* 2015). Generally, proteolytic changes in milk occur during the storage of raw milk (Button *et al.* 2011).

Such variability in proteolytic enzymes, their low concentration as well as the natural variability and complexity of proteins in raw milk lead to problems with the diagnostics. For researchers and service laboratories, instrumental methods already exist, e.g. an HPLC method described by Datta and Deeth (2003) or Le *et al.* (2006). On the other hand, various simple analytical methods available to dairy-plant laboratories usually have too low sensitivity to provide practically useful information. For this reason, a classical microbiological analysis was recommended

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(Němečková *et al.* 2009). However, the microbiological analysis is too time-consuming.

Hence in this work, we focus on another promising simple method potentially applicable in practice – the determination of milk proteolysis equivalent (MPE) – and its correlation with microbiological indicators.

MATERIAL AND METHODS

Milk samples. Samples of bulk raw milk were collected periodically twice a month from January to March 2015 in the Olomouc region (altitude 260–360 m) in the Czech Republic. A total of 13 commercial herds of Holstein cattle were included. Each herd involved from 30 to 500 cows with average milk yield from 6700 kg to 11 050 kg. The herds differed in milking parlours and in the ratio of silages and concentrates in their feed rations.

After morning milking, the bulk samples were collected separately or in the pairs of subsamples and transported to a laboratory at a temperature below 4°C.

In terms of analyses and evaluation, the samples were divided into sets I, II, III, IV, and T. Set I (n = 10) contained the samples collected separately and analysed immediately after their delivery; set III (n = 47) contained the first subsamples which were analysed after their delivery. All samples analysed after delivery (I + III) constituted set II (n = 57). Set IV (n = 47) contained the second subsamples which were incubated at 10°C for 24 h to induce spontaneous proteolysis (Chramostová *et al*. 2014). The total set T (n = 104) originated from sets II + IV. Set II represented the common procedure of milk quality control. Set IV simulated worsened care of samples or potential failures in the system of milk quality control during an alternate day collection and was used to emphasise prospective dependences between parameters in set T. Furthermore, set T represented the total possible effect of hygiene during milk handling on its proteolysis.

Milk proteolysis equivalent. MPE was expressed as the concentration of primary amino groups in mmol/l determined spectrophotometrically using an OPA reagent. The reagent contained 0.04 g *o*-phthaldialdehyde and 0.05 g *N*-acetyl-L-cysteine (both Merck, Darmstadt, Germany) dissolved in 1 ml of methanol, 40 ml of $Na_2B_4O_7 \cdot 10\ H_2O$ (0.1 mol/l, pH 9.55), and 2.5 ml of sodium dodecyl sulphate (20% w/w) (all Lach-ner, Neratovice, Czech Republic) filled to 50 ml with demineralised water. It was left to stand 1 h in darkness. Afterwards, 1 ml of the reagent was mixed with 1 ml of milk sample diluted with demineralised water at a ratio of 1 : 200. After a 12 min reaction in darkness, absorbance at a wavelength of 335 nm was measured using SPEKOL 11 (Carl Zeiss AG, Jena, Germany). Data were evaluated using a calibration curve of glutamic acid 0–0.16 mmol/l (Čurda & Dryáková 2003).

Somatic cell count. SCC (in 10^3/ml) was determined by flow cytometry (ISO 13366-2:2006) using Somacount 300 (Bentley Instruments, Chaska, USA).

Basic milk composition. The contents of fat, crude protein, lactose monohydrate, and solids-non-fat (SNF) were measured using a MilkoScan 133 B infrared analyser with filter technology (Foss Electric, Hillerød, Denmark).

Cultivation microbiological analyses. The total count of mesophilic bacteria (TCMB) and the total count of psychrotrophic bacteria (TCPB) were determined according to ISO 4833:2013 and ISO 8552:2004, respectively.

Statistical analysis. The arithmetic mean (x), standard deviation (SD), coefficient of variation (v), geometric mean (xg), median (m), minimum (min), and maximum (max) were determined. For microbiological data, the logarithmic transformation ($\log_{10}$) was used due to the absence of normal frequency distribution (Hanuš *et al.* 2011). For the statistical significance of zero hypothesis the t-criterion was used. The relationships between milk indicators were evaluated by linear regression and correlation coefficients (r) using MS Excel (Microsoft, Redmond, USA).

RESULTS AND DISCUSSION

For set T, the statistical data on milk parameters can be seen in Table 1. The arithmetic mean of milk proteolysis equivalent (MPE) was 0.9404 ± 0.068 mmol/l and the coefficient of variation was 7.3%. The geometric mean of the total count of mesophilic bacteria and the total count of psychrotrophic bacteria was 85.083 and 71.936 CFU/ml, respectively. These relatively high values were due to the incubation of some samples at 10°C for 24 h, which was performed to induce proteolysis.

The statistical data of set II are summarised in Table 1. The arithmetic mean of MPE was 0.9165 ± 0.063 mmol/l with the coefficient of variation 6.9%.

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Table 1. Statistical data on the milk parameters in the total set T and set II

	MPE (mmol/l)	SCC (log 10^3/ml)	TCMB (log CFU/ml)	TCPB (log CFU/ml)	SNF (% w/w)	Fat (% w/w)	Lactose (% w/w)	Protein (% w/w)
Total set T (n = 104)								
x	0.9404	2.3490	4.9248	4.8569	8.95	3.78	4.95	3.36
xg	–	223	85.083	71.936	–	–	–	–
SD	0.068	0.2090	1.0420	1.1960	0.18	0.29	0.06	0.16
vx	7.3	–	–	–	2.00	7.50	1.20	4.60
min	0.754	1.8388	3.1139	2.7782	8.49	3.26	4.74	2.98
max	1.162	2.7566	7.2041	7.3010	9.26	4.95	5.05	3.66
m	0.944	2.3910	4.6580	4.4983	8.96	3.77	4.97	3.35
Set II (n = 57)								
x	0.9165	2.3582	4.1220	3.9808	8.96	3.79	4.96	3.36
xg	–	228	13.245	9.5670	–	–	–	–
SD	0.063	0.2070	0.4110	0.5640	0.18	0.31	0.06	0.16
vx	6.9	–	–	–	2.00	8.10	1.20	4.70
min	0.754	1.9345	3.1139	2.7782	8.50	3.28	4.76	3.00
max	1.075	2.7566	5.1461	5.1461	9.26	4.95	5.05	3.66
m	0.932	2.4065	4.1072	3.9542	8.96	3.75	4.97	3.36

x – arithmetic mean; xg – geometric mean (in original indicator unit); SD – standard deviation; vx – coefficient of variation (in %); m – median; MPE – milk proteolysis equivalent; SCC – somatic cell count; TCMB – total count of mesophilic bacteria; TCPB – total count of psychrotrophic bacteria; SNF – solids-non-fat

The geometric means of TCMB (13 245 CFU/ml) and TCPB (9 567 CFU/ml) were in accordance with the standard requirements for raw milk quality (Regulation /EC/ No. 853/2004). It corresponded to the arithmetic mean of log TCMB and log TCPB (4.1220 and 3.9808) with the respective variability. The respective medians showed similar values as the geometric means. SCC and chemical parameters were in accordance with the characteristics of Holstein breed in the Czech Republic (Janů *et al.* 2007).

The parameters MPE, TCMB, TCPB, and protein were important for the evaluation of results for sets III and IV (Table 2). As expected, TCMB and TCPB significantly increased during sample incubation. The geometric mean of TCMB and TCPB increased (P < 0.001) from 13 713 and 10 728 to 811.927 and 830 819 CFU/ml, respectively. The respective increases in median values were from 4.1139 and 4.0000 (13 000 and 10 000 CFU/ml) to 6.0792 and 6.2041 (1 200 000 and 1 600 000 CFU/ml). Such an increase in the microbial contamination was accompanied by a significant (P < 0.001) increase of the MPE mean value from 0.9188 to 0.9694 mmol/l, which was about 5.5%. An increase in primary amino groups in the majority of samples was observed also by Chramostová *et al.* (2014) in a model experiment with artificial microbial contamination after two-day storage. The content of fat and protein did not change (P > 0.05) and lactose content decreased by 0.02% (P < 0.001).

Data sets for the regression analysis were selected according to the experimental design, physiology of lactation, and technology of milking process. Moreover, the closeness of evaluated correlations was taken into account. For the selected set combinations, the relations between milk parameters can be seen in Table 3 and Figures 1–3.

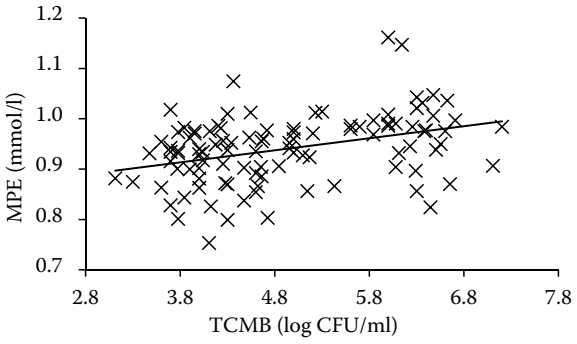

Figure 1. Linear regression between log total count of mesophilic bacteria (TCMB) and milk proteolysis equivalent (MPE) in set T (n = 104; r = 0.3651; P < 0.001)

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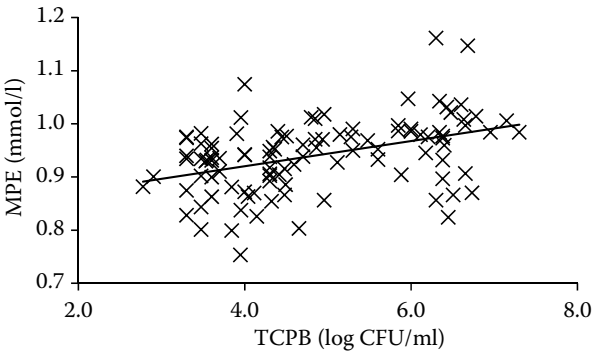

Figure 2. Linear regression between log total count of psychrotrophic bacteria (TCPB) and milk proteolysis equivalent (MPE) in set T (n = 104; r = 0.4152; P < 0.001)

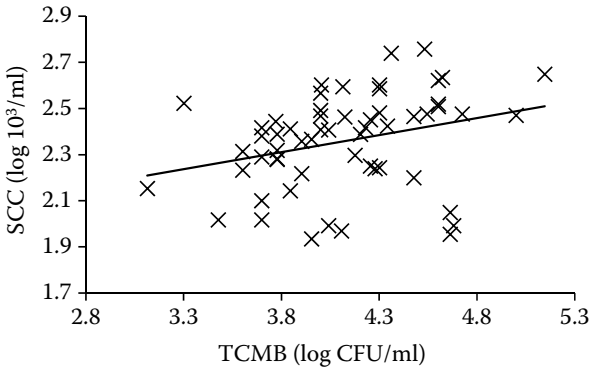

Figure 3. Linear regression between log total count of mesophilic bacteria (TCMB) and log somatic cell count (SCC) in set II (n = 57; r = 0.2941; P < 0.05)

In set III, no significant relation between MPE and log SCC was found (Table 3; P > 0.05) although mastitis can probably increase milk proteolysis (Le Roux *et al.* 1995). It can be explained by the fact that the SCC geometric mean (Table 3; set III) was 224 × 10^3/ml and the health of the monitored herds was good with regard to mastitis. Proteolysis by native enzymes can occur in milk with SCC as low as 250 × 10^3/ml (Le Roux *et al.* 1995). Variations in SCC could explain about a half of variations in plasmin activity in milk (Le Bars & Gripon 1993; Le Roux *et al.* 1995; McSweeney & Fox 1995; Barbano 2000).

Table 2. Statistical data on the milk parameters in reference set III (n = 47) and experimental set IV (n = 47)

	MPE (mmol/l)	SCC (log 10^3/ml)	TCMB (log CFU/ml)	TCPB (log CFU/ml)	SNF (% w/w)	Fat (% w/w)	Lactose (% w/w)	Protein (% w/w)
Reference set IV (n = 47)								
x	0.9188	2.3511	4.1371	4.0305	8.95	3.78	4.97	3.35
xg	–	224	13.731	10.728	–	–	–	–
SD	0.058	0.2060	0.4180	0.5870	0.18	0.26	0.06	0.15
vx	6.3	–	–	–	2.0	7.0	1.1	4.6
min	0.754	1.9345	3.1139	2.7782	8.5	3.28	4.76	3.00
max	1.018	2.7566	5.1461	5.1461	9.21	4.44	5.05	3.62
m	0.935	2.3892	4.1139	4.0000	8.95	3.78	4.98	3.34
Experimental set IV (n = 47)								
x	0.9694	2.3377	5.9095	5.9195	8.94	3.78	4.95	3.35
xg	–	218	811.927	830.819	–	–	–	–
SD	0.064	0.2140	0.6610	0.8430	0.18	0.26	0.06	0.15
vx	6.6	–	–	–	2.1	6.9	1.2	4.5
min	0.824	1.8388	4.6532	3.9542	8.49	3.26	4.74	2.98
max	1.162	2.7497	7.2041	7.3010	9.22	4.42	5.04	3.62
m	0.976	2.3820	6.0792	6.2041	8.96	3.77	4.96	3.35
Difference significance (IV–III)								
t-criterion	6.89	2.69	18.87	20.97	2.05	0.16	5.79	1.42
DFS	***	*	***	***	*	ns	***	ns

x – arithmetic mean; xg – geometric mean (in original indicator unit); SD – standard deviation; vx – coefficient of variation (in %); m – median; DFS – significance of differences (ns – insignificant P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001); MPE – milk proteolysis equivalent; SCC – somatic cell count; TCMB – total count of mesophilic bacteria; TCPB – total count of psychrotrophic bacteria; SNF – solids-non-fat

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Table 3. Linear regression analysis of the relations between the milk parameters

Data file	Indicators		*n*	Equation	R^2	*r*	DFS
I, III, and IV (T)	log TCPB	MPE	104	$y = 0.02370x + 0.8252$	0.1724	0.4152	***
	log TCMB	MPE	104	$y = 0.02390x + 0.8224$	0.1333	0.3651	**
I and III (II)	SCC	lactose	57	$y = -0.00009x + 4.9811$	0.0265	– 0.1628	ns
	SCC	SNF	57	$y = 0.00008x + 8.9379$	0.0023	0.0480	ns
	TCMB	SCC	57	$y = 0.00148x + 222.5103$	0.0977	0.3126	*
	log TCMB	log SCC	57	$y = 0.14790x + 1.7486$	0.0865	0.2941	*
	TCMB	TCPB	57	$y = 0.78823x + 4680.1849$	0.4295	0.6554	***
	log TCMB	log TCPB	57	$y = 0.88874x + 0.3174$	0.4204	0.6484	***
III	log SCC	MPE	47	$y = 0.02170x + 0.8677$	0.0059	0.0768	ns
	TCPB	MPE	47	$y = 0.0000006119x + 0.9042$	0.1041	0.3226	*
	log TCPB	MPE	47	$y = 0.02310x + 0.8255$	0.0545	0.2335	ns
	log TCMB	MPE	47	$y = 0.00300x + 0.9062$	0.0005	0.0224	ns

R^2 – coefficient of determination; *r* – correlation coefficient; DFS – significance of difference (ns – insignificant $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$); TCPB – total count of psychrotrophic bacteria (CFU/ml); TCMB – total count of mesophilic bacteria (CFU/ml); SCC – somatic cell count (10^3/ml); MPE – milk proteolysis equivalent (mmol/l); lactose (% w/w); SNF – solids-non-fat (% w/w); lactose (% w/w)

The elevation of plasmin or other proteases derived from somatic cells leads to the breakdown of casein and the influx of blood proteins (immunoglobulins, IgG, and bovine serum albumin) into milk due to increased permeability of the mammary epithelium, which results in an increased non-casein nitrogen content (Le Roux *et al*. 1995; Coulon *et al*. 2002).

In contrast to SSC, the microbial contamination was significantly related to MPE (Table 3). In set T, the significant ($P < 0.01$) positive correlation coefficient 0.3651 (Table 3 and Figure 1) was found out between MPE and log TCMB. Contrary to set III, this relation was insignificant (Table 3; $P > 0.05$). Despite this fact, it can be stated that a unit increase in log TCMB (Figure 1) results in an increased concentration of primary amino groups by more than 0.02 mmol/l. It is evident that only the analysis of samples without incubation enabled the discovery and quantification of this relation.

Another significant ($P < 0.001$) positive correlation coefficient 0.4152 was found between MPE and log TCPB (Table 3 and Figure 2). It means that 17.2% of the variability in MPE was explainable by the variations in log TCPB. Moreover in this case, positive correlations between the same parameters (Table 3; set III) were found in the samples analysed after delivery (0.2335 and 0.3226; $P > 0.05$ and $P < 0.05$). It is evident that TCPB is more closely related to MPE than TCMB. Furthermore, proteolysis in milk seems to be affected more strongly by the microbial contamination than by SCC and mastitis. The predominant contribution of psychrotrophic bacteria to proteolysis in raw milk was reported also by Özer (2000) and Topcu *et al*. (2006).

To detect the incipient risk of increased proteolysis in bovine milk, the MPE limit was estimated in the interval from 0.9366 to 1.02 mmol/l. The first value was calculated from the linear regression (Figures 1 and 2) for the hygienic limits of TCMB (100 000 CFU/ml) and TCPB (50 000 CFU/ml) (Regulation /EC/ No. 853/2004) at 0.9419 and 0.9366 mmol/l. The second value was estimated at convention for a 95% confidence interval as the MPE mean + SD × 1.64 (Table 1, set II) at 1.02 mmol/l.

Besides those mentioned above, other correlations were detected (Table 3). For example, the correlation coefficient between SCC and lactose was insignificant (–0.1628, $P > 0.05$). The relation between TCMB and SCC or log TCMB and log SCC showed significant ($P < 0.05$) positive correlation coefficients 0.3126 and 0.2941, respectively (Figure 3). In mastitis milk, higher TCMB probably includes a higher quantity of pathogens. Some earlier results (Vyletělová *et al*. 1999) were confirmed by the significant positive ($P < 0.001$) relation between log TCMB and log TCPB (Table 3; $r = 0.6484$). It means that 43% of the variability in TCPB values was explainable by the variations in TCMB.

Furthermore as shown in Figure 4, no significant relationship was found out between milk protein con-

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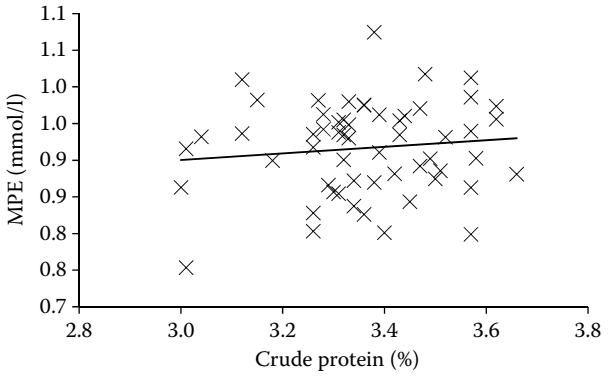

Figure 4. Linear regression between milk protein content (%) and milk proteolysis equivalent (MPE) in set II (n = 57; $y = 0.045x + 0.7652$; $R^2 = 0.0129$; $r = 0.1136$; $P > 0.05$)

tent and MPE in non-cultivated milk samples (set II: $n = 57$, $r = 0.1136$, $P > 0.05$). This was confirmed by the outcome of set III ($n = 47$, $r = 0.2207$; $P > 0.05$). Therefore, samples with high protein content cannot be automatically considered as samples with high proteolysis. To confirm this interpretation, also the total correlation of the respective relationship (uncultivated and cultivated samples) was insignificant (set T: $n = 104$, $y = 0.0531x + 0.762$, $r = 0.1204$; $P > 0.05$). From this point of view, future research can be focused on the determination of MPE in a supernatant after previous protein precipitation or on the calculation of free amino acids in the average milk protein and the subsequent correction of MPE according to the particular milk protein content in a sample.

CONCLUSION

Proteolysis is an important indicator of bovine milk quality. Our results indicate that it can be monitored by the simple determination of MPE. This parameter significantly depends on microbial contamination expressed as TCMB or TCPB. In terms of milk quality control, we propose the MPE limit indicating the incipient risk of increased proteolysis in the interval of 0.9366–1.02 mmol/l. This parameter could be implemented into the control system of raw milk quality.

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