PRODUCTION OF BENZOIC ACID IN FERMENTED GOAT'S AND SHEEP'S MILK*

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The content of hippuric acid in raw goat's and sheep's milk and the content of benzoic acid in model fermented milk drinks prepared using different cultures of bacteria of milk fermentation were determined. All fermented milk drinks contained benzoic acid in the range 5.0–78 mg kg^{-1}. Higher quantity of benzoic acid in fermented sheep's milk drinks corresponded also to significantly higher quantity of hippuric acid in raw sheep's milk. No significant differences among used cultures of bacteria of dairy fermentation and the quantity of benzoic acid in fermented milk drinks were recorded. In addition, the quantity of benzoic acid in various commercial goat's and sheep's cheeses produced directly in farms was analyzed. The contents of benzoic acid in cheeses ranged from 5.1 to 90 mg kg^{-1}. No significant difference in the quantity of benzoic acid from goat's and sheep's cheeses was found, whereas significant differences were registered in the quantity of benzoic acid in cheeses produced in individual farms.

hippuric acid; benzoic acid, fermented milk drinks, raw milk, HPLC

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INTRODUCTION

The production of goat's and sheep's milk in the Czech Republic has had a growing tendency in recent years, primarily on private farms. Here most of the milk is processed into fermented dairy products. These products show high digestive, nutritive, and organoleptic properties (J e n n e s , 1980; S l a č a n a c et al., 2010).

Minor components of milk are also organic acids, which can be obtained from hydrolysis of milk fat. The organic acid content of milk depends on a variety of factors such as animal breed diet and duration of the lactation period. Some organic acids including hippuric and benzoic acids can reflect the health condition of the animals yielding the milk and they are present in varying quantities (B u i a r e l l i et al., 2003). Although cow's milk contains only a few mg per kg of benzoic acid, fermented dairy products such as yoghurt and cheese can contain up to 50 mg kg^{-1} benzoic acid with most mean values around 20 mg kg^{-1} (S i e b e r et al., 1995). According to N i s h i m o t o et al. (1968, 1969) lactic acid bacteria convert hippuric acid present in milk to benzoic acid (Fig. 1) so that the latter could also be considered as a natural component of milk and dairy products.

The level of benzoic acid depends primarily on the level of hippuric acid in raw milks and may also depend on the process of dairy product manufacture, especially on the used microorganisms (H e j t m á n k o v á et al., 2000a). In addition, *Lactococcus lactis*, *Lactobacilus casei*, *Streptococcus thermophiles, Lactobacillus helvetius*, *Escherichia coli* and *Pseudomonas fluorescens* can synthesize benzoic acid in milk (S a j k o et al., 1984; G a r m i e n e et al., 2010). According to S i e b e r et al. (1995) another possibility of benzoic acid forming is auto-oxidation of benzaldehyde and phenylalanine degradation. This substance is often identified in fermented dairy products and it is produced by bacteria of milk fermentation. Another way to enhance the content

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of benzoic acid in milk products is their bleaching. Benzoyl peroxide, a whey bleaching agent, degrades to benzoic acid and may elevate its concentration in dried whey products (L i s t i y a n i et al., 2011).

Benzoic acid is generally recognized as safe, however adverse effects such as asthma, urticaria, metabolic acidosis, and convulsions were observed in sensitive persons even if it was present at low doses. Some weak clastogenic activity was noted in *in vitro* assays (P i n g et al., 2009).

Recently new analytical methods for determination of benzoic acid in raw milk, milk powder, and milk products have been developed (W a n g et al., 2013; M a et al., 2014).

In the year 1996, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) assessed the acceptable daily intake (ADI) of 0–5 mg benzoic acid per kg body weight. Although since the year 2004 there has not been established hygienic limit for the content of benzoic acid in fermented dairy products in the Czech Republic, due to the inhibitory effect of benzoic acid on some enzymes and the possible irritant effect on sensitive persons, the lowest content of benzoic acid in fermented dairy products is desirable.

The objective of this study is to determine the content of hippuric acid in raw goat's and sheep's milk and the content of benzoic acid in fermented goat's and sheep's milk drinks prepared using different starter cultures of lactic acid bacteria during the whole lactation period and the content of benzoic acid in goat's and sheep's fermented dairy products produced on private farms.

MATERIAL AND METHODS

Production of fermented milk drinks

Bulk milk samples from two farms (farm A – goat's milk, Czech White Shorthaired breed; farm B – sheep's milk, East Friesian breed) were collected monthly during the whole lactation period from April to October. In the laboratory, the milk was treated by heating in a water bath at 95°C for 5 min. Model fermented drinks were prepared from raw milks using different types of dairy cultures. The milk was inoculated with 1% (wt/wt) of working starter culture.

Dairy cultures

Yoghurt starter cultures (multiple-strain cultures) were *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (CCDM 176 – origin Germany, CCDM 528 – origin Canada). Cultivation was carried out at 42°C for 3 h.

Butter starter cultures (multiple-strain cultures) were *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetilactis* (CCDM 1 – basic dairy culture, origin Danmark; CCDM 12 – recommended for cheese and cottage cheese making, origin Danmark; CCDM 973 – basic dairy culture, origin Slovakia). Cultivation was carried out at 37°C for 16 h.

Probiotic strain was *Enterococcus faecium* (CCDM 922). Cultivation was carried out at 37°C for 16 h.

A maintainer and distributor of all the used cultures is the company MILCOM a.s., Czech Republic.

Sampling of dairy products

Various types of fermented dairy products (cheeses, cottage cheeses, and yoghurts) were additionally purchased at 5 different private farms (Farms 1–5) and subsequently the content of benzoic acid was determined in the laboratory. Dairy products were purchased regularly once a month from April to September, however the products were not available in the whole range for the whole period. Each farm was keeping different breeds of small ruminants. The products were prepared from milk of Czech White Shorthaired (WSH), *Brown Shorthaired (BSH), and* Anglo Nubian (AN) goat breeds and from milk of East Friesian (EF) and Romanov (R) sheep breeds.

Compositional analysis

Determination of the hippuric and benzoic acids was carried out using an HPLC system UltiMate 3000 (Dionex Softron GmbH, Germering, Germany) on reverse phase with diode array detector after removal of fat and proteins by precipitation from a slightly alkaline aqueous solution of the sample using *clearing agent* solutions Carrez I (potassium ferrocyanide 10.6%) and Carrez II (zinc sulfate 21.6%) and dilution of both acids in methanol. Preparation of the sample was conducted according to I S O 9 2 3 1 : 2 0 0 8 (I D F

Fig. 1: Conversion of hippuric acid to benzoic acid

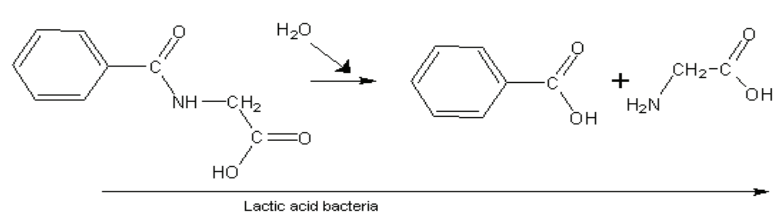

Table 1. Content of hippuric acid in raw milk (mg/kg)

Milk type	Goat's milk (farm A)	Sheep's milk (farm B)
April	20.66^{Aa}	38.73^{Ba}
May	5.78^{Ab}	55.58^{Bb}
June	8.06^{Ac}	44.21^{Bc}
July	21.20^{Aa}	38.57^{Ba}
August	7.56^{Ac}	51.78^{Bd}
September	10.35^{Ad}	20.29^{Be}
October	25.11^{Ae}	54.15^{Bb}
Average	15.48	43.33
Standard deviation	8.31	12.34

$^{a–eAB}$means within a column not followed by the same superscript differ ($P < 0.001$)

$^{a–e}$related to differences of hippuric acid in milk sampled in a particular month

^{AB}related to differences of hippuric acid in a different milk type

Table 2. Average content of benzoic acid (mg/kg) in fermented goat's or sheep's milk, time effect

Month of sampling	Goat's milk (farm A)			Sheep's milk (farm B)		
	mean	median	SD[1]	mean	median	SD[1]
April	42.35^{c}	46.53	12.14	53.32^{b}	55.27	17.88
May	12.84^{ab}	13.15	8.64	14.89^{a}	16.90	7.35
June	9.73^{a}	10.12	2.87	35.09^{ab}	34.54	6.98
July	18.81^{ab}	16.96	11.39	26.97^{a}	28.66	7.18
August	13.34^{ab}	10.70	10.70	17.68^{a}	17.76	7.92
September	15.01^{ab}	14.89	8.13	22.97^{a}	20.68	13.00
October	30.19^{bc}	30.79	7.54	35.45^{ab}	36.00	13.70

SD = standard deviation

$^{a–c}$means within a column not followed by the same superscript differ ($P < 0.05$)

139: 2008) Standard. For separation of both acids a chromatographic column Grom-Sil 120 ODS-5 ST 5 μm Column 150 × 4 mm (*Grace* Davison Discovery Sciences, Deerfield, USA) was used. Mobile phase consisted of methanol (A) and 0.02M acetate buffer (acetic acid/sodium acetate, 57 : 43, v/v, pH = 4.5)(B).

Gradient elution was realized at 35°C for 21 min at a flow rate of 0.8 ml min^{-1}. In brief, for the first 4 min the initial elution was isocratic 15% A, 85% B, followed by 1 min linear gradient elution until 20% A, 80% B, and then isocratic elution continued for 12 min under the same conditions. Then the system returned to the initial terms 15% A, 85% B in 2 min and was set for initial conditions during the last 2 min. The runs were monitored at 280 nm. Hippuric and benzoic acids were used as standards (Sigma-Aldrich, St. Louis, USA), eluting at 7.3 and 10.6 min, respectively. All samples were analyzed in triplicate.

The detection limit of the benzoic acid was 0.89 μg ml^{-1} of analyzed sample solution, i.e. 4.7 μg g^{-1} of raw milks. The linear working range was in the interval of 0–30 μg ml^{-1}. The detection limit of the hippuric acid was 0.36 μg ml^{-1} of the analyzed sample solution, i.e. 1.9 μg g^{-1} of raw milks. The linear working range was in the interval 0–20 μg ml^{-1}. The benzoic as well as hippuric acid recovery was identically 100%.

Statistical analysis

The measured values were processed by the analysis of variance (ANOVA) at the significance level α = 0.05 using the STATISTICA software (Version 8.0, 2007, the Tukey's HSD test was performed for more detailed evaluation. In the case of the milk products, similar products (cheese, yoghurt) with the same number of values in the measured set were gathered.

RESULTS

The contents of hippuric acid in raw milks as well as the contents of benzoic acid in fermented milk drinks were rather variable during the lactation period. A significantly ($P < 0.001$) lower quantity of hippuric acid (15.5 ± 8.3 mg.kg^{-1}) was established in goat's milk than in sheep's milk (43.3 ± 12.3 mg kg^{-1}). The

Table 3. Content of benzoic acid (mg/kg) in fermented goat's or sheep's milk, culture effect

Culture	Goat's milk (farm A)			Sheep's milk (farm B)		
	mean	median	SD	mean	median	SD
Butter starter culture[1] (CCDM 1)	19.66^{a}	11.95	15.62	27.92^{a}	27.09	17.02
Butter starter culture (CCDM 12)	17.36^{a}	16.34	10.31	23.72^{a}	25.23	14.35
Butter starter culture (CCDM 973)	17.82^{a}	15.71	16.92	35.50^{a}	30.34	20.70
Yoghurt starter culture[2] (CCDM 176)	20.46^{a}	11.03	16.08	30.20^{a}	32.97	16.22
Yoghurt starter culture[2] (CCDM 528)	21.84^{a}	18.51	13.34	31.59^{a}	24.61	18.86
Enterococcus faecium (CCDM 922)	24.79^{a}	25.28	14.53	27.95^{a}	22.26	12.08

SD = standard deviation

[a]means within a column not followed by the same superscript differ ($P < 0.05$)

[1]multiple-strain cultures consist of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetilactis*

[2]multiple-strain cultures consist of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (for details see the section of *Dairy cultures*)

Table 4. Average content of benzoic acid (mg/kg) in fermented dairy products produced on farms

Type of product	Milk of breed	Farm No.	Sample No.	Mean	SD
Cream cheese	WSH–G	1	6	28.75^{a}	18.48
D'Ampier cheese	BSH–G	2	6	7.46^{b}	6.78
Rohan cheese	BSH–G	2	6	5.92^{b}	2.23
Bio cream cheese	EF–S	4	6	25.95^{a}	6.03
Cream cheese	R–S	5	6	16.01^{c}	9.30
Bio yoghurt	EF–S	4	6	69.62^{A}	26.09
Yoghurt	AN–G	3	6	7.87^{B}	2.21
Curd cheese	AN–G	3	2	30.67	9.51
Cheese	AN–G	3	1	12.86	–
Cottage cheese	WSH–G	1	1	89.97	–
Curd cheese "Brynza"	R–S	5	3	15.6	8.51
Cheese "Oštěpek"	R–S	5	4	12.97	3.15
Hard cheese	R–S	5	3	16.40	4.59

WSH–G = Czech White Shorthaired goat, BSH–G = *Brown Shorthaired* goat, AN–G = Anglo Nubian goat, EF–S = East Friesian sheep, R–S = Romanov sheep, SD = standard deviation

[a–cAB]means within a column not followed by the same superscript differ ($P < 0.0015$)

[a–c]related to differences of benzoic acid in cheese

[AB]related to differences of benzoic acid in yoghur

contents of hippuric acid in raw goat's milk ranged from 5.8 to 25.1 mg kg^{-1}, and in raw sheep's milk from 20.3 to 55.6 mg kg^{-1} (Table 1).

A significantly ($P < 0.05$) lower quantity of benzoic acid (20.3 ± 13.9 $mg.kg^{-1}$) was established in fermented products prepared from goat's milk than from sheep's milk (29.5 ± 16.1 $mg.kg^{-1}$) (Table 2). The contents of benzoic acid ranged from 5.0 to 52.8 $mg.kg^{-1}$ in fermented goat's milk drinks, and from 4.9 to 77.8 $mg.kg^{-1}$ in fermented sheep's milk drinks. There was no correlation between the time of sample collection at different stages of lactation and the content of benzoic acid in fermented milk drinks made from goat's and sheep's milk as in the case of hippuric acid content in raw goat's and sheep's milk.

The difference in the effect of various bacterial cultures on milk fermentation was no significant (Table 3). In addition, the contents of benzoic acid in various commercial goat's and sheep's fermented dairy products made directly on farms were determined (Table 4). The contents of benzoic acid in cheeses ranged from 5.1 to 90 $mg.kg^{-1}$. No significant difference in the quantity of benzoic acid in goat's and sheep's cheeses was found, whereas differences in the quantity of benzoic acid in cheeses produced on individual farms were observed. Both the lowest and the highest contents of benzoic acid were determined in goat's cheeses.

The contents of benzoic acid in yoghurts produced directly on farms ranged from 5.2 to 10.5 $mg.kg^{-1}$ in yoghurt made from goat's milk and from 27.8 to

106.2 mg.kg^{-1} in yoghurt made from sheep's milk, i.e. significant differences in the content of benzoic acid in yoghurts made at two different farms were found out.

Correlation dependence between hippuric and benzoic acid in fermented dairy products from goat's milk is close to very close (correlation coefficient is 0.776).

DISCUSSION

Very similar results of the average content of hippuric acid in whole cow's milk (15.4 ± 0.9 mg.kg^{-1}) were determined by Marsili et al. (1981), whereas Hatanaka, Kaneda (1986) recorded 196 mg of hippuric acid in 1 kg of skimmed cow's milk. Although according to Svensen (1974) and Carpio et al. (2010) hippuric acid content in milk depends on the season, there was no correlation between the time of sample collection at different stages of lactation and hippuric acid content in goat's or sheep's milk either Svensen (1974) stated a lower hippuric acid content in milk in the winter than in the summer period. The content of this acid in cow's milk ranged 13.0–45.9 mg.kg^{-1} throughout the year.

However, in this study, a relatively high variability of the hippuric acid content was found out in sheep's milk (28.5%) and in goat's milk (53.7%). According to Carpio et al. (2010) the content of hippuric acid in milk depends on the origin of the milk (organic vs conventional milk). The result led to a presumption, that by using hippuric acid as a marker, it could be distinguished between milk from organically and conventionally fed goats. Average content of hippuric acid in organic and conventional goat's milk was determined at 132 ± 7 and 59 ± 7 mg. l^{-1}, respectively. Therefore it depends significantly on the type of farming and animal feed ration composition, which may change during the year. Caprio et al. (2013) claimed that the highest hippuric acid content was found in samples from grazing animals corresponding to the organic group and that a high content of hippuric acid in milk is associated with nutrition based mainly or exclusively on green grass (grazing), independently on the production system. In this case we can expect a higher level of benzoic acid in fermented milk products.

In China, benzoic acid (0.51–13 mg kg^{-1}) was also detected in pasteurized and UHT milk and in milk powder and infant formula it was 11–110 mg.kg^{-1} (Ping et al., 2009). The objective of study by Rukkwamsuk et al. (2012) was to evaluate the concentrations of benzoic acid in milk within 6 h after collection. At 0 and 6 h after collection, average concentrations were 0.09 ± 0.06 and 0.13 ± 0.03 mg.kg^{-1}, respectively. These results confirmed that benzoic acid is normally present in fresh milk and its concentrations increase with time.

In this study, no significant differences in milk fermentation depending on the bacterial cultures used were found (Table 3). The variability of benzoic acid content in fermented milk drinks depending on the culture was sufficiently low (2.7% in goat's milk and 4.0% in sheep's milk). The production of benzoic acid by yoghurt starter cultures (mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*), butter starter cultures (mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetilactis*), and *Enterococcus faecium* was very similar. Garmine at al. (2010) arrived to different conclusion in their study aimed at determining the influence of some lactic acid bacteria species and strains on production of benzoic acid in cultured milk samples. According to them all strains of *Lactobacillus acidophilus, Lactobacillus casei*, *Streptococcus thermophilus,* and *Lactobacillus helveticus* used for milk fermentation produced similar levels of benzoic acid in cultured milk samples. However, selected strains of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *lactis*, *Lactobacillus lactis* subsp. *cremoris*, *Lactobacillus lactis* subsp. *lactis* biovar. *diacetylactis* produced different levels of benzoic acid. In addition, strains used for manufacturing yoghurt based on *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* and strains used for manufacturing quark, sour cream, cheese, and cultured milk products based on *Lactobacillus lactis* subsp. *lactis*, *Lactobacillus lactis* subsp. *cremoris,* and *Lactobacillus lactis* subsp. *lactis* biovar. *diacetylactis* produced low levels of benzoic acid.

In Korea, benzoic acid in fermented milk was detected after the fermentation stage by addition of starter culture at the level of 2.28–10.48 mg.kg^{-1}, and when the culture was added at < 0.18–16.5 mg.kg^{-1} in the commercial fermented milk products, no benzoic acid was detected (Lim et al., 2013).

A wide range of benzoic acid content (from traces to 341 mg.kg^{-1}) in various types of cheese was also reported by Sieber et al., 1995. On the other hand, in cheeses made from cow's milk and purchased in the market network in the Czech Republic the extent of specified levels of benzoic acid was narrower and its contents were significantly lower, from 1.2 to 22.5 mg.kg^{-1} (Hejtmánková et al., 2000b). Similar results of benzoic acid content were found in Korea (Lim et al., 2013). In the case of cheese products, the benzoic acid level was influenced by the curd formation (Camembert cheese) and the quality of natural cheese (processed cheese), by the way, the benzoic acid level of commercial natural cheese was < 0.18–4.2 mg.kg^{-1}, in processed cheese it was < 0.18–20.8 mg.kg^{-1}. Based on this result, it may be possible to utilize for the systematic control the level of natural benzoic acids in raw material, processing and final products of animal origin. A slightly higher content of benzoic acid in dairy products from cow's milk purchased in supermarkets was detected in Turkey in the period 2009–2010 (Yildiz et al., 2012). The levels of benzoic acid in cheese, yoghurt, and ayran samples were in the range of 3.17–56.77 $mg.kg^{-1}$, 8.94–28.30 $mg.kg^{-1}$, and 1.54–16.57 $mg.l^{-1}$, respectively.

Contents of benzoic acid in goat's yoghurts **analyzed in this study** were lower than in the model-prepared yoghurt from cow's milk using different yoghurt cultures (13.1–24.1 $mg.kg^{-1}$), but they were **moreover (even)** significantly lower than in the model-prepared yoghurt from goat's milk (34.2 $mg.kg^{-1}$) (Hejtmánková et al., 2000a). In contrast, the content of benzoic acid in sheep's yoghurt was significantly higher. Higher contents of benzoic acid (9–56 $mg.kg^{-1}$) in various types of yoghurts made from cow's milk were detected by Sieber et al. (1995).

CONCLUSION

The contents of hippuric acid in raw milk and the contents of benzoic acid in fermented milk drinks are very variable and the contents of both acids in milk significantly change during the lactation period. However, the contents of benzoic acid in fermented milk drinks are not significantly influenced by the starter culture used. The results indicated that the contents of hippuric acid in raw milks and after that the level of benzoic acid in fermented goat's and sheep's dairy products were not influenced by the differences between goat's and sheep's milk, but mainly by specific breeding conditions on individual farms.

Due to the higher values of benzoic acid exceeding the *legislative* requirement repeatedly determined in dairy products from some of the farms, and the possible adverse effects of benzoic acid on human organism, occasional monitoring of the content of benzoic acid in dairy products manufactured on private farms would be desirable. In addition, the determination of benzoic and hippuric acid contents in milk can serve for checking the health status of the animals yielding the milk.

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