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Short Paper

Fatty acid profile of ewe's milk infected with *Staphylococcus* spp.

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Abstract

Background: Mastitis in sheep caused by *Staphylococcus* spp. is a serious concern for dairy farming. **Aims:** The objective of this study was to evaluate the impact of the intramammary infection (IMI) caused by *Staphylococcus* spp. on the long-chain fatty acid profile and composition of ewe's milk. **Methods:** The experiment was conducted in a herd of Zoslachtena Valaska sheep. Half-udder milk samples were collected from 20 weight-matched sheep at the peak of their first or second lactation. The basic physicochemical composition of milk, somatic cell count (SCC), *Staphylococcus* spp. infection, and total bacterial contamination (TBC) were determined. The fatty acid profile of the milk fat was determined using gas chromatography. **Results:** The SCC in milk infected with *Staphylococcus* spp. was 3.25 times higher ($P<0.01$) than that in the uninfected milk samples. The content of lauric acid (C12:0) was higher ($P<0.05$) in the milk fat of infected ewes. A significant increase ($P<0.05$) in the share of linoleic acid (C18:2n6t), arachidonic acid (C20:4n6), and a decrease ($P<0.01$) in the vaccenic acid (C18:1n7t) were observed in the milk collected from ewes infected with *Staphylococcus* spp.. *Staphylococcus* spp. infection increased the ratio of n-6 to n-3 polyunsaturated fatty acids. **Conclusion:** Changes in the fatty acid profile of milk caused by *Staphylococcus* spp. infection decrease the value of ewes' milk as a health-promoting product.

Key words: Ewe, Fatty acid, Milk, Staphylococci

Introduction

Over 400 individual fatty acids have been identified in milk fat. Some fatty acids are found in very small amounts, but they contribute to the unique and desirable flavor of milk fat and butter. For example, the C14:0 and C16:0 β-hydroxy fatty acids form lactones upon heating which enhances the flavor of butter (Guetouache *et al*., 2014). In sheep's milk, 5 fatty acids (C10:0, C14:0, C16:0, C18:0, and C18:1) account for >75% of the total fatty acids (Park *et al*., 2007; Ptáček *et al*., 2019). Moreover, sheep's milk is the richest source of conjugated linoleic acid and α-linoleic acid when compared with cow's and goat's milk, which makes this product more desirable as a functional food (Markiewicz-Keszyńska *et al*., 2013; Cividini and Simčič, 2015).

Mastitis negatively influences milk's usefulness in the cheese industry and the quality of the products (Raynal-Ljutovac *et al*., 2007). Mastitis may be associated with changes in the fatty acid composition of milk, which may further affect the organoleptic and the health-promoting properties of raw milk and cheese products. However, some mammary gland pathogens are thought to produce significant changes in the composition of milk while others are believed to barely affect milk's properties (LeMarechal *et al*., 2011). This may be associated with the virulence of the strategy of bacteria. For example, most of the staphylococci were found to produce a fatty acid modifying enzyme (FAME) as opposed to *Escherichia coli* and *Streptococcus uberis* (Lu *et al*., 2012). Fatty acid modifying enzyme might be one of the factors affecting milk's fatty acid composition in mastitis caused by *Staphylococcus* spp. (Mortensen *et al*., 1992).

Staphylococci are considered the main etiological agents in intramammary infections (IMIs) of small ruminants, with *Staphylococcus aureus* being isolated more often in clinical cases and coagulase-negative staphylococci (CNS) species in subclinical IMI. Coagulase-negative staphylococci, followed by *S. aureus*, were the most frequently isolated bacteria in sick dairy sheep and goats regardless of the form of mastitis (Takano *et al*., 2018). It is also established that enterotoxin gene positivity of milk-derived staphylococci constitutes a potential risk for consumers' health (Nazari

et al., 2014).

A bacterial IMI is one of the main reasons for lowering the milk yield and quality in sheep. The fatty acid composition of milk, to a large extent, determines its nutritional quality for both consumers and newborn animals. *Staphylococcus* spp. express FAME, which may change the fatty acid composition in milk. Since staphylococci are the main cause of mastitis in sheep, the aim of our study was to determine the influence of IMI caused by *Staphylococcus* spp. on the long-chain fatty acid profile and composition of milk in these animals.

Materials and Methods

Animals

Experiments were carried out on 20 clinically healthy sheep belonging to the Zoslachtena Valaska (Zošlachtená Valaška) breed in northern Slovakia. The animals in their first or second lactation (25-30 days in milk) were kept in typical buildings that complied with the European Union Directive (Directive 2010/63/EU of the European Parliament). The daily diet of the sheep was comprised of hay (*ad libitum*), wheat (250 g/head), and silage (3 kg/head).

Milk sampling was conducted in the morning before feeding on one day. Before sampling, lambs were separated from ewes for 4 h. Samples of milk from each half of the udder (50 ml) were collected in sterile containers and transported to the laboratory at a temperature of 4°C. Forty milk samples for analysis were obtained.

Microbiological studies

Samples infected with *Staphylococcus* spp. were detected by the procedure described by Vasil *et al*. (2016). Briefly, all isolates were characterized by primo-cultivation on 5% blood agar and consistent cultivation on specific cultivation media. Based on the morphology of the colonies, the *Staphylococcus* spp. were tested for coagulase activity (Staphylo PK, ImunaPharm, Slovakia). The species of *Staphylococcus* bacteria was determined based on the biochemical enzymatic properties of the bacteria with the help of a STAPHY test 24 with the identification program TNW 7.0 (Erba-Lachema, Brno, Czech Republic). The accuracy of detection is over 90.0%. Moreover, analysis of the spectrum of proteins by a Maldi-Biotyper (Bruker, USA; values in the range [2.300-3.000]) was used to provide highly probable species identification.

Analysis of milk's physical and chemical properties

An Infrared Milk Analyzer 150 (Bentley Instruments Inc., Chaska, MN, USA) was used to determine the content of fat, total protein, lactose, and dry matter (DM). The somatic cell count (SCC) in the milk samples was determined using a Somacount 150 (Bentley Instruments Inc., Chaska, MN, USA). A Bactocount 70 analyzer (Bentley Instruments Inc., Chaska, MN, USA) was also used to determine the total bacterial contamination (TBC).

Fatty acid analysis

Milk fat from milk samples was extracted by homogenization in the chloroform-methanol mixture (2:1) and spontaneous separation according to Folch's method (Bligh and Dyer, 1959). The ISO 15884:2002 [IDF 182:2002] method was used to obtain methyl esters of the fatty acids. The amount of fatty acids in the samples was determined using an Agilent Technologies 7890A gas chromatograph with a Flame Ionization detector (FID) (Agilent Technologies, USA) and an external standard (Supelco 37 Component FAME Mix, Sigma-Aldrich, USA). The resulting peaks of the fatty acids were identified by comparing them with the content of the methyl esters of fatty acids (Sigma, Aldrich, USA).

Statistical analysis

Milk samples from 40 udder halves (25 uninfected and 15 infected) obtained from 20 ewes were statistically analyzed using a one-factor analysis of variance (ANOVA) in Statistica 10.0 (StatSoft Poland, Krakow, Poland). $P<0.05$ and $P<0.01$ were considered significant, and the differences between means with $0.05<P<0.01$ were regarded as tendencies.

Results

Four pathogenic *Staphylococcus* species were identified in the milk of ewes. *Staphylococcus epidermidis* and *S. aureus* were predominant (Fig. 1). The ratio of CNS to coagulase-positive staphylococci (CPS) was 60:40.

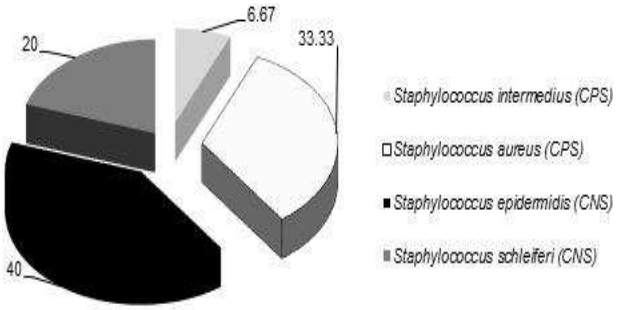

Fig. 1: Percentages of particular pathogens in the *Staphylococcus* spp. group in the ewe's milk (%). CPS: Coagulase-positive *Staphylococcus*, and CNS: Coagulase-negative *Staphylococcus*

The SCC in the affected milk samples was 3.25 times higher ($P<0.01$) compared to the uninfected milk samples (Table 1).

The fatty acid composition of the milk fat from udder halves, infected and uninfected by *Staphylococcus* spp., is presented in Table 2. The C12:0 concentration in the milk fat was significantly higher in milk with *Staphylococcus* spp. ($P<0.05$). The total number of saturated fatty acids in the milk from infected udder halves tended to be higher.

Table 1: Physical and chemical properties of ewe's milk

Parameter	Uninfected (n=25)	*Staphylococcus* spp. (n=15)	SEM	P-value
SCC·1000 m/L	220.32***	715.93***	81.93	0.003
Fat (%)	11.61	12.52	0.43	0.358
Protein (%)	8.07	8.74	0.24	0.214
DM (%)	24.50	26.24	0.65	0.250
Non-fat DM (%)	12.90*	13.73*	0.30	0.094
Lactose (%)	3.64	3.74	0.09	0.250
TBC 10000 m/L	7091.28	6875.80	801.54	0.905

* Trend, and *** P<0.01. SEM: Standard error of the mean, SCC: Somatic cell count, DM: Dry matter, and TBC: Total bacterial count

Table 2: Fatty acid composition in the fat of ewe's milk

Parameter	Uninfected (n=25)	*Staphylococcus* spp. (n=15)	SEM	P-value
C4:0[1]	0.24	0.31	0.03	0.275
C6:0[1]	0.56	0.57	0.04	0.817
C8:0[1]	0.85	0.93	0.05	0.479
C10:0[1]	3.33	3.82	0.17	0.154
C11:0[1]	0.18	0.16	0.01	0.413
C12:0[1]	2.53**	2.87**	0.094	0.046
C13:0[1]	0.07	0.08	0.00	0.133
C14:0[1]	8.35	8.83	0.19	0.108
C15:0[1]	1.01	1.00	0.01	0.538
C16:0[1]	18.54	18.42	0.17	0.843
C17:0[1]	0.65	0.65	0.02	0.930
C18:0[1]	10.94	11.22	0.24	0.776
ΣSFA	47.14*	48.72*	0.47	0.083
C14:1[1]	0.82	0.79	0.02	0.378
C15:1[1]	0.26	0.27	0.01	0.638
C16:1[1]	5.03	4.97	0.05	0.648
C17:1[1]	0.27	0.27	0.01	0.267
C18:1n9c[1]	24.44	23.99	0.31	0.892
C18:1n9t[1]	1.58	1.62	0.06	0.321
C18:1n7t[1]	3.42**	2.98**	0.14	0.034
C18:2n6c[1]	1.96	1.82	0.04	0.721
C18:2n6t	3.15***	3.27***	0.08	0.001
CLA[1]	0.27	0.26	0.01	0.447
C18:3n6[1]	1.53	1.44	0.04	0.202
C18:3n3[1]	2.34	2.18	0.07	0.583
C20:4n6[1]	0.10**	0.11**	0.00	0.018
DHA	0.19	0.19	0.01	0.734
ΣUFA	45.48	44.15	0.46	0.194

* Trend, ** P<0.05, and *** P<0.01. SEM: Standard error of the mean. [1] g/100 g of the total fat concentration. ΣSFA: Sum saturated fatty acids, CLA: Conjugated linoleic acid, DHA: Docosahexaenoic acid, and ΣUFA: Sum unsaturated fatty acids

A significant decrease (P<0.05) in the proportion of vaccenic acid (C18:1n7t), and arachidonic acid (C20:4n6), and an increase (P<0.01) in the level of linoleic acid (C18:2n6t) were observed in the milk obtained from udder halves infected with *Staphylococcus* spp. compared to the levels observed in the milk from healthy udders.

Discussion

According to Fragkou *et al*. (2014), a SCC below 500·1000 m/L indicates a healthy udder in sheep while SCC values above 1000·1000 m/L are indicative of clinical or subclinical mastitis, and the range between these two values remains a gray zone. We obtained SCC values within the gray zone. Therefore, microbiological culture may be a reliable tool to improve mastitis detection in sheep. On the other hand, SCC values above 700·1000·m/L might be considered as confirming the disease, especially as *Staphylococcus* spp. pathogens are recognized as the most frequent causative factors in mastitis in sheep (Dore *et al*., 2016; Vasileiou *et al*., 2018). Consistent with our findings, *S. epidermidis* is recognized as the most frequent pathogen in milk, usually causing subclinical mastitis of a milder course (Abbondio *et al*., 2019).

In our study, *S. aureus* is the second most frequent bacteria among the staphylococci. This is in agreement with the findings of Merz *et al*. (2016), who state that *S. aureus* shows a pronounced adaptation to small ruminants. However, *S. aureus* is believed to be a predominant pathogen isolated from cases of clinical mastitis, rather than subclinical infections (Gelasakis *et al*., 2015; Takano *et al.*, 2018). A probable reason for the

discrepancy between our results and other studies may be a different susceptibility to the pathogen in the studied breed (Fragkou *et al*., 2007).

The unaffected physicochemical composition and thus the overall quality of the ewes' milk in our study were similar to the results reported for milk from cows infected with *Staphylococcus xylosus* and *Staphylococcus warneri* (Vasil *et al*., 2016). However, the quality of ewe's milk, as a substrate for cheese production, is evaluated in terms of its technological and coagulation properties, which also depend on the SCC (Nudda *et al*., 2014). Therefore, the unchanged physicochemical composition does not exclude the lowering of the usefulness of raw milk in cheese production.

The alteration in the fatty acid composition of sheep milk in our study may be regarded as a result of subclinical mastitis *per se*. Similarly, subclinical mastitis in dairy cows was associated with changes in the long-chain fatty acid profile in milk (Chang *et al*., 2011). On the other hand, particular changes in fatty acid composition in milk may be induced by *Staphylococcus* spp. specifically. In dairy cows, *S. xylosus* and *S. warneri* infections caused an increase in C8:0, C10:0, C18:3n3, and C17:1, and a decrease in C20:1 and eicosapentaenoic acid (EPA) concentrations in milk fat (Vasil *et al*., 2016), whereas an infection of the mammary gland with *S. uberis* resulted in an elevated content of CLA, C18:1n7t, and C18:0 in the milk (Pecka-Kiełb *et al*., 2016). Moreover, in human milk few correlations between fatty acids and milk bacteria were identified (Moossavi *et al*., 2019). These associations are still unclear; however, they may be governed by the utilization of fatty acids by the bacteria as an energy source, structural elements or regulating factors (Fujita *et al*., 2007). Some fatty acids seem to act as prebiotics (Endo *et al*., 2006). Natural trans fatty acids in milk, like vaccenic acid whose concentration decreased in the milk from infected udders in our study, were shown to stimulate the growth of *Lactobacillus gasseri* known for its probiotic properties and bacteriocin synthesis (Itoh *et al*., 1995; Endo *et al*., 2006). Finally, some fatty acids might be produced by the microbiota or have a direct antibacterial effect (Kelsey *et al*., 2006). It is not excluded that FAME produced by *Staphylococcus* spp. is one of the factors affecting fatty acid composition in milk (Lu *et al*., 2012).

An increase in lauric acid (C12:0) concentration may be associated with the cholesterol-raising effect in humans (Mensink *et al*., 1994). Moreover, a higher concentration of C18:2n6t and C20:4n6 n-6 PUFAs and concomitant elevation in the ratio of n-6 to n-3 may limit the value of sheep's milk because a diet with an increased n-6:n-3 polyunsaturated fatty acid (PUFA) ratio coincides with chronic inflammatory diseases (Patterson *et al*., 2012). This indicates that the subclinical mastitis caused by *Staphylococcus* spp. in sheep reduces the quality of their milk as a health-promoting product and alternative to cow's milk.

The changes in the fatty acid profile of milk caused by *Staphylococcus* spp. infection decreased the value of ewe's milk as a health-promoting product.

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