Biofilm forming bacterial contaminants in small and medium-sized ewes' milk and meat processing enterprises in Slovakia

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Summary

Microbial contamination of production surfaces in small and medium-sized ewes' milk and meat processing enterprises in Slovakia was studied. Staphylococci and enterococci dominated in ewes' milk processing small and medium-sized enterprises (SME) on stainless steel at 10^3–10^4 CFU·cm^{-2} as well as on plastic surfaces at 10^1–10^3 CFU·cm^{-2}, and in meat processing SME at 10^1–10^2 CFU·cm^{-2} on both types of surfaces. In both types of SME, a reduction only by 0–1 orders of magnitude was observed in these contaminants after sanitation of stainless steel and plastic surfaces. Coliforms were not significantly resistant to sanitation. Eleven *Staphylococcus* isolates from ewes' milk processing SME and 8 isolates from meat processing SME were found to form biofilm. A few biofilm forming strains were isolated also among enterococci and coliforms. Pseudomonads were isolated only from one ewes' milk processing SME, but these were found to form biofilm and to resist sanitation. The levels of contamination of production surfaces in ewes' milk and meat processing SME in Slovakia were found to be considerably high and the effectiveness of sanitation was assessed to be considerably low.

Keywords

hygiene; sanitation; biofilm; surface; *Staphylococcus*

With the creation of the common European market, requirements for the control of hygiene in the production of food of animal origin have been adapted in order to ensure public health. Not only that food safety has become an issue increasingly dominating the public and political debates, but also the development of new analytical and microbiological methods has provided new insights in the topic [1, 2]. Issues of the hygiene of food production and distribution have been recently updated in the European food legislation [3–6]. Despite of the attention paid to these issues, problems persist in all technologically developed countries. A greater deal of food safety problems are connected with small and medium-sized enterprices (SME) processing food of animal origin. This represents an important topic in particular in countries where the majority of food production is processed by SME [7, 8].

Microbiological contamination belongs to the most important types of food contamination acquired during processing, as long as microbial contaminants may multiply during inappropriate distribution and storage of food products. The main routes of contamination are surfaces of the processing equipment, air, water, personel and pests. The most abundant microbial contaminants in milk processing, cheese producing and meat processing factories are *Escherichia coli* and other *Enterobacteriaceae*, *Pseudomonas* spp., *Micrococcus* sp., *Staphylococcus* sp., *Enterococcus* sp., *Bacillus* sp. and other bacteria, yeasts and fungi. Special attention is paid to contamination by pathogenic bacteria, such as *Salmonella enterica*, *Campylobacter* sp., *Staphylococcus aureus* or *Listeria monocytogenes*. In milk processing and cheese producing factories, the environment may be contaminated also by lactobacilli. Milking machines, surfaces of the production equipment, water and air were found to be microbially contaminated, and personel and pests have been identified as further routes of contamination [9–15].

Certain microbial contaminants are capable of forming biofilm on solid surfaces. These microbes are of particular interest because they are capable of persisting in the technology, are often more resistant to cleaning and disinfection, and may be important sources of secondary contamination of

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food products [16, 17]. The high resistance of bacterial biofilms to even intensive cleaning and disinfection has been previously proven by the isolation of bacterial contaminants in milk processing [18–22] and meat processing factories [17, 23, 24].

In Slovakia, there is a tradition of sheep farming and production of cheese from unpasteurized ewes' milk. A majority of such traditional ewes' cheese production is carried out by SME [25]. In the meat industry of Slovakia, SME are active in particular in the sector of regional specialities. Recent socio-economic changes in the country and the implementation of European legislation in food processing have lead to improvement in food technologies. In this concern, a need for relevant data on hygiene in these SME emerged. In this study, the current status of production hygiene in four ewes' milk processing and four meat processing SME in Slovakia was ascertained by tracing selected groups of bacteria in the plant environment. Isolated strains were identified to the species level and characterized for biofilm formation ability.

MATERIALS AND METHODS

Isolation of bacteria

Bacterial strains from the genera *Staphylococcus*, *Enterococcus* and *Pseudomonas*, species *Bacillus cereus* and coliforms were isolated from surfaces of the production equipment and from food products by the swab method. A surface of 100 cm^2 was swabbed with a cotton swab, which was subsequently vortexed in 9 ml of peptone water (PW) containing 0.1% bacteriological peptone (HiMedia, Mumbai, India) and 0.9% NaCl, 2–4 decimal dilutions in PW were prepared and 0.5 ml of individual dilutions was spread on Baird-Parker agar (HiMedia) for isolation of *Staphylococcus* sp. [26], on Slanetz-Bartley agar (HiMedia) for isolation of *Enterococcus* sp. [27], on *Pseudomonas* isolation agar (HiMedia) for isolation of *Pseudomonas* sp. [28], on mannitol-egg yolk polymyxin agar (Merck, Darmstadt, Germany) for isolation of *Bacillus cereus* [29] and on Chromocult coliform agar (Merck) for isolation of coliforms. Plates containing 15–300 typical colonies were counted and surface density of bacterial contamination was calculated in CFU·cm^{-2}.

Biochemical identification of isolates

Enterococcus isolates were subcultured on Brain-heart infusion agar (HiMedia) and other isolates were subcultured on Plate count agar (HiMedia) for 24–48 h at 30–37 °C. Then they were characterized on the basis of fermentation patterns using the kits Staphytest 16, Nefermtest 24 and Enterotest 24 (all from Pliva-Lachema Diagnostika, Brno, Czech Republic).

Identification using polymerase chain reaction

Pseudomonas isolates were typed using randomly-amplified microsatellite polymorphism (RAMP) [30]. *Staphylococcus* isolates were identified using $(GTG)_5$-polymerase chain reaction (PCR) in Czech Collection of Microorganisms, Brno, Czech Republic [31].

Characterization of the biofilm formation ability of the isolates

Individual strains were allowed to form a biofilm in tryptic soya broth (Merck) in polystyrene microtitre plates in static conditions and, after appropriate washing, the biofilm was quantified using crystal violet staining [32].

RESULTS AND DISCUSSION

Surfaces of various material of the production equipment in four ewes' milk processing and four meat processing SME in Slovakia were examined (Tab. 1). In ewes' milk processing SME, staphylococci and enterococci dominated before sanitation on stainless steel at 10^3–10^4 CFU·cm^{-2} as well as on plastic surfaces at 10^1–10^3 CFU·cm^{-2}. Coliforms were present in same densities on plastic surfaces, but in densities by an order of magnitude lower on stainless steel surfaces. Lower densities of coliforms were determined on wooden surfaces. In meat processing SME, staphylococci and enterococci dominated as well, but their counts were by

Tab. 1. Examined production equipment surfaces of ewes' milk processing and meat processing SME.

Material	Ewes' milk processing SME				Meat processing SME			
	E I	E II	E III	E IV	M I	M II	M III	M IV
stainless steel	5	–	5	–	2	5	4	3
plastics[a]	3	2	–	2	1	–	1	1
wood	–	4	–	1	1	–	–	–

a – polyethylene 500, polypropylene or silicone.

Tab. 2. Average bacterial counts on production equipment surfaces before and after sanitation.

	Material	Bacteria	Counts before sanitation [CFU·cm^{-2}]	Counts after sanitation [CFU·cm^{-2}]
Ewes' milk processing SME	stainless steel	*Staphylococcus* sp.	10^3–10^4	10^2–10^3
		Enterococcus sp.	10^3–10^4	10^2–10^3
		Coliforms	10^2–10^3	10^0–10^1
	plastics	*Staphylococcus* sp.	10^1–10^3	10^0–10^2
		Enterococcus sp.	10^1–10^3	10^0–10^2
		Coliforms	10^1–10^3	10^0–10^1
	wood (n = 5)	*Staphylococcus* sp.	10^1	10^1
		Enterococcus sp.	10^1	10^1
		Coliforms	10^1	10^1
Meat processing SME	stainless steel	*Staphylococcus* sp.	10^1–10^2	10^2–10^3
		Enterococcus sp.	10^1–10^2	10^2–10^3
		Coliforms	10^0–10^2	10^0–10^1
	plastics	*Staphylococcus* sp.	10^1–10^2	10^0–10^2
		Enterococcus sp.	10^1–10^2	10^0–10^2
		Coliforms	10^0–10^2	10^0–10^1
	wood (n = 1)	*Staphylococcus* sp.	10^1	10^0
		Enterococcus sp.	10^0	10^0
		Coliforms	10^1	10^0

1–2 orders of magnitude lower than those in ewes' milk processing SME. In both types of SME, a reduction by 1–2 orders of magnitude was observed after sanitation of stainless steel and plastic surfaces in coliforms, while numbers of staphylococci and enterococci were reduced to a lesser extent, only 0–1 orders of magnitude (Tab. 2).

Our results demonstrate a considerably high contamination of the production equipment surfaces in both types of SME and are similar or slightly higher than previously published data on microbiological contamination of surfaces in milk processing [14, 22] and in meat processing plants [33, 34]. They also demonstrate a considerably low efficiency of the routine sanitation processes. A practice that might have contributed to the low efficiency of sanitation in certain cases was drying the surfaces by towels after sanitation.

Since a possible reason for resistance of bacterial contaminants to sanitation may be their ability to form a biofilm [16], we examined the isolates for this feature. The greatest number of strains able to form a biofilm were from the genus *Staphylococcus*. These accounted for 11 isolates from ewes' milk processing SME and 8 isolates from meat processing SME (Tab. 3). Based on bio-

Tab. 3. Biofilm formation ability of isolates.

Plant/surface		*Staphylococcus* sp. Bf+/total	*Enterococcus* sp. Bf+/total	*B. cereus* Bf+/total	*Pseudomonas* sp. Bf+/total	Coliforms Bf+/total
Ewes' milk processing	stainless steel	1/9	1/37	–	4/4	3/14
	plastics	8/17	2/6	0/3	–	3/4
	wood	0/14	0/8	–	–	0/1
	products	1/11	0/12	0/14	17/17	9/36
	water, air	1/4	0/2	0/9	–	2/5
Meat processing	stainless steel	4/9	1/13	–	–	4/20
	plastics	1/5	0/3	0/1	–	2/10
	wood	2/2	–	–	–	0/2
	products	1/4	0/3	–	–	2/5
	water, air	–	–	–	–	–

Bf+/total – number of biofilm forming strains / number of all strains

Tab. 4. Identified *Staphylococcus* spp. with a biofilm forming ability.

Strain	Plant	Location	Species identity
7/1"/ Bč	E 1	plastic tubing	*St. haemolyticus* (B)
7/2"/ Bsd2	E 1	plastic tubing	*St. saprophyticus* (B, P)
7/3"/ Bs	E 1	stainless steel vessel	*St. hominis* (B)
4/3/ Bsd	E 2	plastic vessel	*St. xylosus* (B)
4/3/ Bč+ž	E 2	plastic vessel	*St. aureus* (B, P)
3/1/Bč2	M 1	meat product	*St. saprophyticus* (B)
3/3/Bs6ž	M 1	plastics	*Staphylococcus* sp. (B, P)
8/1/B3sda	M 1	wood	*St. succinus* (B, P)
8/1/B3sdb	M 1	wood	*St. succinus* (B, P)
10/1/Bčz	M 2	stainless steel	*St. saprophyticus* (B, P)
10/1/Bs2	M 2	stainless steel	*St. saprophyticus* (B, P)
11/6/Bsč	M 3	stainless steel	*St. saprophyticus* (B, P)
12/17/Bčs	M 4	stainless steel	*St. saprophyticus* (B, P)

E – ewes' milk processing plant, M – meat processing plant; B – identified by biochemical tests, P – identified by polymerase chain reaction.

chemical characterization, the strains were identified to belong to the species *St. saprophyticus*, *St. succinus*, *St. aureus*, *St. xylosus*, *St. hominis* and *St. haemolyticus* (Tab. 4). Strains *St. saprophyticus* 10/2 and 10/1 were found to be identical as long as they were indistinguishable by $(GTG)_5$-PCR, and identity was observed also with strains *St. succinus* 8/1a and 8/1b. With other *Staphylococcus* strains, $(GTG)_5$-PCR produced genuine profiles (data not shown) and provided useful taxonomical information complementary to biochemical characterization.

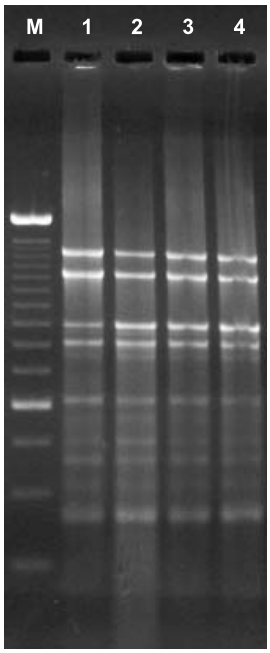

Fig. 1. RAMP profiles of 4 *Pseudomonas* sp. isolates from equipment surfaces from ewes' milk processing SME.
M – DNA molecular weight standard n.250 bp (a stronger band at 1000 bp).

A few biofilm forming strains were isolated also among enterococci and coliforms. Pseudomonads were isolated only from one ewes' milk processing SME, but these were contaminants which resisted sanitation and all 4 strains were found to form biofilm (Tab. 3). The high biofilm forming ability of pseudomonads is a well established feature of this species [16, 17]. The 4 strains were typed using RAMP and they were found to be indistinguishable (Fig. 1). They were also indistinguishable by RAMP from other 17 isolates from cheeses produced in this factory (data not shown). It can be assumed that all these strains belonged to one genotype which contaminated the entire production plant.

Regarding the effectiveness of sanitation, it was the lowest with staphylococci and enterococci on stainsless steel surfaces in both milk and meat processing plants, and it was also considerably low on plastic surfaces in both milk and meat processing plants. Staphylococci isolated from these surfaces contained several strains forming biofilm, which might have contributed to the low effectiveness of sanitation.

CONCLUSIONS

Working surfaces in small and medium-sized ewes' milk processing enterprises in Slovakia were found to be contaminated with staphylococci, enterococci, coliforms and pseudomonads. In meat processing enterprises, they were contaminated with staphylococci, enterococci and coliforms. The levels of contamination were considerably high and the effectiveness of sanitation was assessed to be considerably low. Staphylococci isolated from stainless steel and plastic surfaces contained several strains able to form biofilm.

Acknowledgements

This research was done in frames of the project "Development of progressive methods and procedures to support quality and safety of food protection and control" of the Department of Agriculture of the Slovak Republic. Authors wish to thank to G. Berta for RAMP analysis.

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Received 8 April 2009; accepted 4 May 2009.