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The evaluation of microbiological colonization of cows' mammary glands before and after herbal treatment of mastitis*

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The present paper is a report of multiple mammary gland colonization in dairy cows caused by bacteria before and 6 days after the end of an 8-day effective mastitis therapy with the use of a purely herbal medicine, which is in line with the European Green Deal (EGD) and the farm to fork (F2F) strategy program to reduce antibiotic use in farming. The microorganisms were isolated from milk samples from 45 cows of the native Polish Black and White (ZB) breed kept in a farm in Southern Poland. For *Staphylococcus aureus* isolates additional biochemical tests and genetic

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analyses were performed. All isolated bacteria, often the same for all cows, were Gram-positive cocci, rods and coryneforms, while tests showed also the presence of yeasts; the results indicate that the microorganisms adapted to the bovine mammary gland can even spread from one animal to another in a farm. Counts of some bacteria and yeast species was observed to decrease after the end of a successful herbal ointment therapy of mastitis; nevertheless, some of the same species of bacteria and yeasts were present both before and after therapy. There were no obligate pathogens evidenced, but many commensal and environmental species of bacteria and yeasts were reported. There is important discrimination of commensal microorganisms from obligate pathogens in such investigations. This paper shows a positive effect of herbal ointment application, broadens our knowledge on primary colonization and may prove important for epidemiological investigations in cases of secondary mammary gland infections in cows.

KEY WORDS: bacterial colonization / herbal therapy / cow mastitis / *Staphylococcus*

Staphylococci represent a genus of commensal bacteria colonizing animal or human organisms, although in many reported cases they are responsible for infections of various tissues/organs, manifestations and/or courses of the disease. As constituents of physiological microbiota the staphylococci colonize skin and mucosal membranes of humans and animals. Furthermore, under specific conditions known as facilitating infectious factors they become pathogens responsible for a broad range of clinical diseases [Lowy 1998, Międzobrodzki *et al*. 2002, O'Gara 2017]. There is a long list of factors associated with the clinical course of staphylococcal infections, both accute and chronic, including virulence factors such as toxins and enzymes, antimicrobial resistance, biofilm production, as well as particular susceptibility of the host [Oliver *et al*. 2009]. Additionally, surface staphylococcal proteins responsible for bacteria binding to fibrinogen, fibronectin, collagens, lactoferrin, laminin or other MSCRAMM (microbial surface components recognizing adhesive matrix molecules) play an important role in colonization and invasion of new tissues in the host organism [Międzobrodzki *et al*. 1989, Naidu *et al*. 1991, Foster 2019]. Thus, infection of the anterior nares in a large part of the human population is a major risk factor for staphylococcal infections, and in a minority cases also for other bacteria or yeasts such as *Candida* sp. [Dudko *et al*. 2010].

The staphylococci are divided into two main groups: coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS), although coagulase-variable ones are also reported [Becker *et al*. 2014]. Undoubtedly a much higher number of pathogenicity determinants are produced by CoPS with the leading pathogen *Staphylococcus aureus*, followed by the less prominent *S. intermedius* with *S. pseudintermedius*. Additionally, also CoNS are reported as pathogens, particularly for newborns or for immunocompromised patients. In veterinary microbiology there is an increasing number of data showing that CoNS have become leading causes of intramammary infections in cows, goats, and ewes, as reported recently [Jagielski *et al.* 2014, Lange *et al*. 2015, Puacz *et al*. 2015]. Research on the ecology and epidemiology of this group of staphylococci is urgently needed [Malinowski *et al.* 2003, Thompson-Crispi *et al*. 2014]. Recent knowledge on the distribution of the bacteria is poor, routine diagnostic procedures are not satisfactory and need extensive

projects in view of the dramatically increasing clinical significance of staphylococcal infections [Król *et al*. 2016]. However, advanced identification procedures are under development and within targeted chemotherapy they may bring expected results. Nevertheless, bacterial colonization of animals after the end of effective therapy remains an open question. This phenomenon is particularly relevant in relation to mammary gland infections in cows, where the udders are constantly and consequently liable to suffer an infection.

A group of CoNS with additional *Corynebacterium bovis* are still classified as minor pathogens, which usually are not responsible for the most severe forms of mastitis and are rarely associated with marked milk leukocytosis and clinical manifestations [Rainard and Poutrel 1988, Hacker *et al*. 2016]. CoNS colonize every niche in all ecosystems; however, the sites of their multiplication are localized in humans or in higher animals. Their distribution on the udder skin is under consideration: is it a physiological phenomenon with some protection against naturally occurring infections by major pathogens or is it the first period of developing infection? In the last decade a reclassification of CoNS was introduced, with over 50 species or subspecies distinguished when molecular methods were subjected to re-examination by analytical procedures. Also, an increasing number of methicillin-resistant CoNS isolated from bovine mastitis samples has been reported in the last few years [Gelasakis *et al*. 2015, Fernandes dos Santos *et al*. 2016]. The present project focused on isolation of gram-positive cocci bacteria (with particular reference to staphylococci) from milk samples collected from cows before and after the end of an effective therapy of mastitis. During effective therapy all the organisms present in milk were either completely eliminated or the number of bacteria was significantly decreased. The ecological microbial sterility keeps mammary glands free of pathogens until the end of an effective antibiotic concentration application. When the antibiotic concentration decreases in the tissue, invasion and colonization of the tissues by various microorganisms is observed. The anatomic niche is an absence of organisms, it means lack of antagonistic relations that opens a door for invasive organisms. This leads to an interesting question: what microorganisms are pioneers in such first colonization and then take part in the formation of new microbiota?

The bacterial colonization of bovine udders following an effective herbal therapy of previous mammary glands infections was investigated in this study. An effective therapy of mammary glands using antibiotics or other medicines results in killing of all bacteria present in particular anatomic niches that become free of bacteria, or results in a significant decrease in the levels of bacterial cells in milk samples (the number of bacterial cells in milk samples after the treatment were statistically non-significant). Those niches are progressively colonized by microorganisms if the medicine concentration gradually decreases. A filling of the ecological niche depends on the reconstruction of physiological microbiota or colonization with severe pathogens or commensal pathogens. The latter group can cause infection, in the short or long term, provided there are no additional factors which could facilitate infection. However,

little is known about the colonization of niches after the use of herbal medicines and which of the mentioned pathways will take place.

The aim of the study was to identify microorganisms belonging to new microbiota in the udder of healthy cows that have recovered from mastitis.

Material and methods

Collection of samples

Milk samples were collected before and after the end of mastitis therapy from 45 mastitic cows of the native, dual purpose Polish Black and White (ZB) breed, housed in a deep litter barn in a medium-sized farm in Southern Poland.

In this study a herbal ointment of the following composition was used: sage (*Salvia officinalis*), yarrow (*Achillea millefolium*), arnica (*Arnica montana*), marigold (*Calendula officinalis*), peppermint oil (*Mentha piperita*), natural camphor oil, and Creagel™. The ointment was used directly on the teats as liniment to the surface tissue after every milking.

After preliminary discrimination of 22 cows in lactations I-III, the degrees of infections in particular udder quarters were evaluated based on the California Mastitis Test (CMT) performed with a Mastirapid fluid tester. Milk samples were evaluated in the scale from 0 to 5 points: 0 – healthy milk, up to 2 x 10^5 somatic cell count (SCC), and 5 – mastitic milk, over 5 x 10^6 SCC.

The herbal ointment was used for 8 days and then milk samples were analyzed once again similarly as on the first day. Milk quality monitoring using CMT and microbiological analyses was continued for 14 days after the end of ointment administration. The number of somatic cells was analyzed in the control group of animals which were treated neither with the ointment nor other anti-mastitis agents during the entire period of the experiment. The control (23 cows) was constantly monitored in terms of their milking performance, with the SCC level evaluated using the AT4 method [ICAR, 2021].

Analytical procedures

The analyses were conducted on 130 milk samples. In most experiments two samples from each cow were collected (before and after therapy), while in one experiment the samples from 45 cows were collected in three phases of every milking (before milking, during milking, and from the final milk stream), as well as before and after treatment. Udder quarter milk samples were collected aseptically for bacteriological examination. Particular samples were inoculated on Mueller-Hinton blood agar (BioMerieux, Craponne, France) and incubated under standard conditions. The obtained colonies were identified based on their morphology followed by microscopic examination. Bacteria were identified following the cultures on blood agar TSA (CMR, Kraków) and then on SAID (BioMerieux, Craponne, France), a selective medium for staphylococci, on CPSE (BioMerieux, Craponne, France), a

selective medium for Gram-negative rods, and Sabouraud (BioMerieux, Craponne, France), a medium for yeast. After 3-day incubation of bacteria at 37°C and 7-day incubation of yeasts at 24°C all the colonies were examined, discriminated and provided for microscopic evaluation as well as further identification to genus and species using biochemical and molecular methods [Kasprowicz *et al.* 2018]. For *S. aureus* isolates additional biochemical tests for the haemolytic pattern, proteolytic, deoxyribonucleic activities and antibiogram were performed. Furthermore, Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) analyses, PCR-RFLP for the *gap* to confirm species identification, while MLVA analyses were performed to recognize phylogenetic relationships between the isolates. To construct the phylogenetic dendrogram presenting groups of bacteria distinguished on the basis of molecular similarity of *S. aureus* isolates the GelCompar II program (Applied Maths, Sint-Martens-Latem, Belgium) was used [Kosecka-Strojek *et al*. 2016].

Results and discussion

Staphylococcal colonization

Analysis of mammary gland colonization with staphylococci, which accounted for different staphylococci species a day before and 14 days after therapy, is shown in Figure 1. A wide variety of staphylococci species was identified, including 22 CoNS and 1 CoPS species *S. aureus*. Nineteen species were present before treatment and 15 species after treatment. In all the herds, the number of isolates was markedly lower after therapy than before treatment, as the total number of isolates for all the cows was 84 before and 57 after treatment. Before therapy the most frequently isolated staphylococci were *S. sciuri* (17 isolates) and *S. haemolyticus* with *S. equorum* (12 isolates each). Other species were represented by 8 isolates of *S. arlettae,* and 7 isolates each of *S. aureus* and *S. xylosus*. The other species were represented by single isolates. After treatment 8 CoNS species were completely eliminated, counts of 4 species were considerably reduced, whereas 4 species became more numerous and the other species retained their numbers. In two cases counts of *S. haemolyticus* and *S. aureus* species were unchanged, and the differences in colony counts ranged from 0 to 1-2.

Colonization by Gram-positive cocci

Analysis of mammary gland colonization by Gram-positive cocci other than *Staphylococcus* revealed the presence of 12 different species identified as *Aerococcus viridans, Enterococcus aquimarinus, E. faecalis, E. faecium, Lactococcus lactis, Micrococcus flavus, M.* spp.*, Streptococcus agalactiae, Str. dysgalactiae, Str. parasanguinis, Str. uberis,* and *Str. parauberis*. The most common species were *A. viridans* (9 isolates before and 13 isolates after treatment) and *E. faecalis* (5 and 6 isolates, respectively). The other species were represented by single isolates. The therapy did not cause any qualitative or quantitative changes in the analysed bacterial flora, which remained at a similar level.

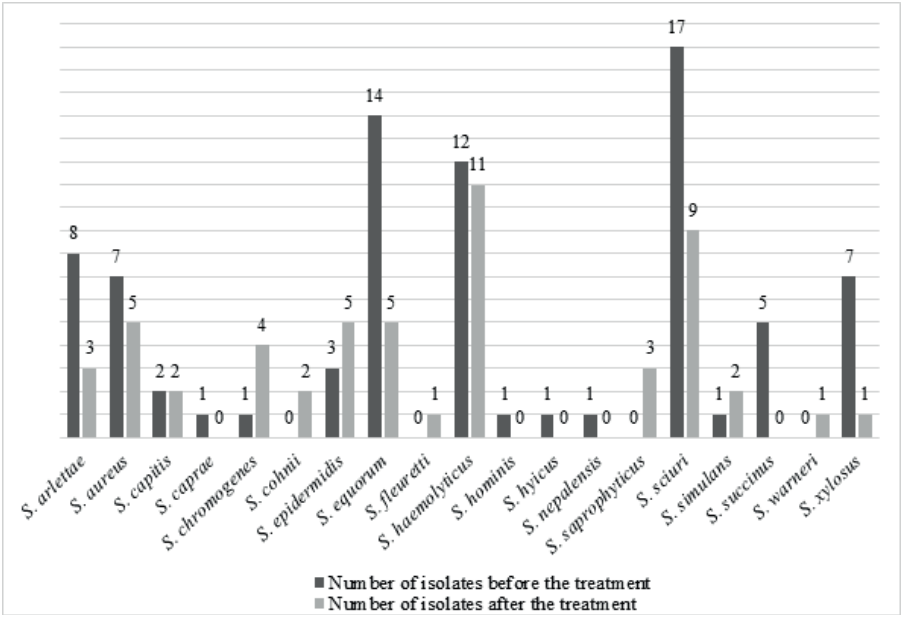

Fig. 1. Analysis of bacterial colonization. Comparison of *Staphylococcus* strains isolated from bovine mastitis samples before and after treatment.

Colonization by Gram-negative rods

The evaluation of that group of bacteria isolated from the mammary glands of mastitic cows before and after therapy showed a broad range of isolates identified as *Acinetobacter baumanii, A. lwoffii, A. towneri, A. ursingii*, *A.* sp., *Chryseobacterium indologenes, Comanomonas testosteroni, Enterobacter asburiae, Pantoea agglomerans*, *Providencia rettgeri, Pseudomonas aeruginosa, Ps. agarici, Ps. fulva, Ps. putida, Ps.* sp., *Raoultella ornithinolytica, Serratia grimesii, Stenotrophomonas maltophilia* and *St.* sp., which were represented by 1 or 2 isolates. The exceptions were *R. ornithinolytica* species, which were identified only 4 times before treatment and were not detected after the end of treatment, as well as specifically unidentified genus of *Acinetobacter* sp., which was represented as 1 isolate before and 3 isolates after the therapy.

Colonization by coryneform bacteria

The isolates belonging to coryneforms were identified in a broad range of species before and after treatment, as shown in Figure 2. The identification procedure revealed the following species: *Brevibacterium iodinum, B. linens, Corynebacterium ammoniagenes, C. aurimucosum, C. bovis, C. casei, C. comporealensis, C. glutamicum, C. mastitidis, C.* spp.*, C. xerosis, C. striatum, Dietzia maris, Escherichia*

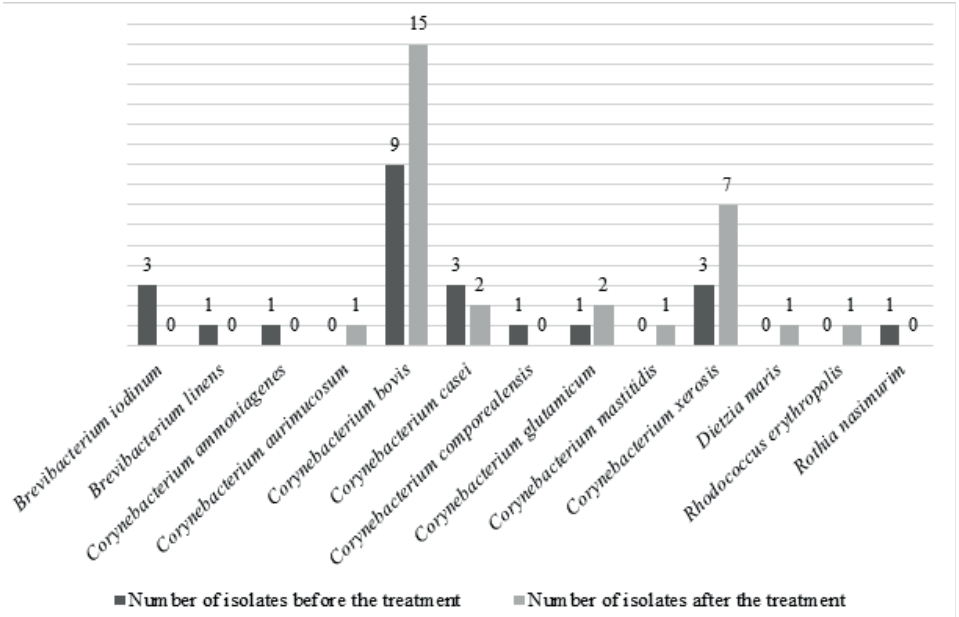

Fig. 2. Analysis of bacterial colonization. Comparison of coryneform bacterial strains isolated from bovine mastitis samples before and after treatment.

coli, Rhodococcus erythropolis and *Rothia nasimurim*. The majority of the reported species were represented by 1, 2 or 3 isolates in the entire group of cows, whereas only 2 species, *C. bovis* and *C.* sp., were represented by 9 and 27 isolates before treatment and 16 and 41 isolates after therapy, respectively.

Colonization by bacteria other than the groups presented above

Some other bacterial genera than those presented in previous sections were also identified, such as *Bacillus licheniformis, B.* sp., *Brachybacterium faecium, Flavobacterium flevense, Helococcus ovis* and *Kocuria carniphila.* All the bacteria were represented by single isolates from the milk samples collected either before or after treatment, with one exception of *Bacillus* sp., which was represented by 9 isolates before therapy and by 7 isolates after the end of therapy.

Fungal colonization

Tests for yeasts in milk samples before and after treatment revealed the presence of these microorganisms only in 9 cows, in which 9 isolates belonging to *Candida* spp., 2 isolates to *Trichosporon ovoidea,* and 1 isolate to *Trichoderma* sp. were identified. The therapy effectively eliminated the two latter isolates and reduced the presence of yeast-like fungi *Candida* spp. from 9 to 4 cases (Fig. 3).

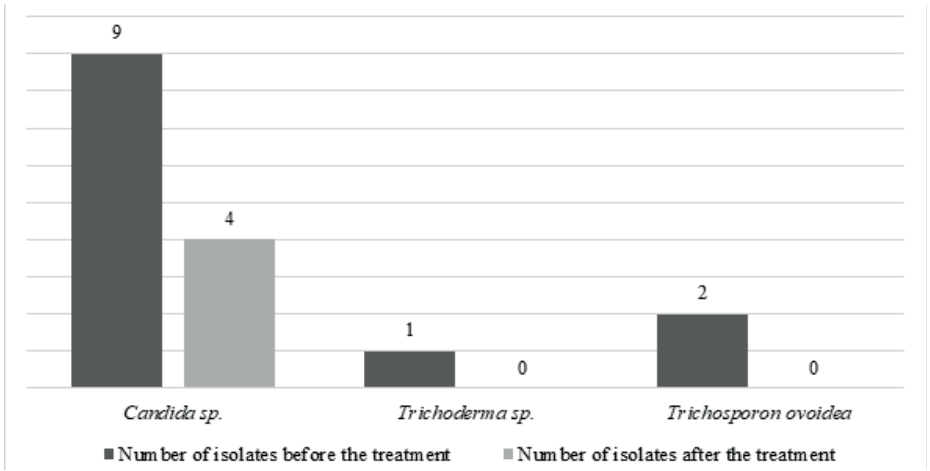

Fig. 3. Analysis of yeast colonization. Comparison of yeast strains isolated from bovine mastitis samples before and after treatment.

A relationship was observed for cows with the presence of aetiological fungal agent, in which colonization by cocci, including those of the *Staphylococcus* genus, was limited to just 1 or 2 species.

Evaluation of antibiotic profiles

The analyses of antibiotic resistance or susceptibility were performed for all 14 isolates of *S. aureus* originating from all the cows in the study. As shown in Table 1, the following twelve antibiotics were used in the analyses: amikacin, chloramphenicol, cefoxitin, ciprofloxacin, clindamycin, cotrimoxazole, doxycycline, erythromycin, gentamycin, levofloxacin, penicillin and tobramycin. Seven isolates of *S. aureus* were susceptible to all the antibiotics used, another 6 isolates presented resistance to 1 antibiotic (penicillin), while 1 isolate was resistant to 4 antibiotics (ciprofloxacin, clindamycin, levofloxacin and penicillin). Furthermore, there was no MRSA isolate reported among the tested bacteria.

Evaluation of enzymatic or toxic activities

The analyses of enzymatic or toxic profiles of the *S. aureus* isolates were performed using standard culture/biochemical methods on solid media enriched with specific substrates for particular enzymes or toxins (Tab. 2). The results obtained by these methods are general and preliminary values; nevertheless, they exhibit virulence profiles of the strains. High level proteolytic activity was presented by 8 isolates, moderate activity by 3 isolates and 1 isolate exhibited low activity. Also, only one isolate showed no proteolytic activity. Moderate DNAse activity was shown by 8 isolates, low activity was represented by 6 isolates, while no isolate exhibited high or no activity. The haemolysins were produced by eleven *S. aureus* isolates. Beta-

Table 1. Antibiotic susceptibility testing results of *Staphylococcus aureus* strains. S – susceptible, R – resistant

Strain	Penicillin	Cefoxitin	Cotrimoxazole	Doxycycline	Erythromycin	Clindamycin	Chloramphenicol	Gentamycin	Tobramycin	Ciprofloxacin	Amikacin	Levofloxacin
Mastitis16/44/Sau	R	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/45/Sau	S	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/46/Sau	S	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/47/Sau	S	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/48/Sau	R	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/49/Sau	R	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/50/Sau	R	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/51/Sau	R	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/52/Sau	R	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/53/Sau	S	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/54/Sau	S	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/55/Sau	S	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/110/Sau	S	S	S	S	S	S	S	S	S	S	S	S
Mastitis16/162/Sau	R	S	S	S	S	R	S	S	S	R	S	R

Table 2. Enzymatic activity of tested *Staphylococcus aureus* strains

Strain	Proteolysis	DNAse activity	Haemolysis
Mastitis/16/44/Sau	+++	++	α
Mastitis/16/45/Sau	+++	+	β +
Mastitis/16/46/Sau	+++	+	γ
Mastitis/16/47/Sau	+++	++	Double β ++
Mastitis/16/48/Sau	++	++	γ
Mastitis/16/49/Sau	+++	++	α
Mastitis/16/50/Sau	-	++	α
Mastitis/16/51/Sau	++	++	α
Mastitis/16/52/Sau	++	++	γ
Mastitis/16/53/Sau	+++	+	Double β ++
Mastitis/16/54/Sau	+++	+	β +
Mastitis/16/55/Sau	+++	+	β +++
Mastitis/16/110/Sau	+	++	β +
Mastitis/16/162/Sau	+	+	β +

α haemolysis – incomplete haemolysis with greenish zone; β haemolysis – complete haemolysis with transparent zone;
ɣ haemolysis – lack of haemolysis.
+ – low enzymatic activity; ++ – moderate enzymatic activity; ++ – high enzymatic activity.

haemolysin was presented by 7 isolates, of which 1 isolate showed high toxic activity, 2 isolates showed moderate beta and furthermore double haemolytic activities, whereas 4 other isolates showed low beta-haemolytic activity. Among the other isolates 4 isolates showed alpha-haemolytic activity and 3 isolates did not present any haemolytic activity (gamma haemolysis).

Analyses of phylogenetic relatedness between *Staphylococcus aureus* strains

A phylogenetic analysis was performed using the GelCompar II program (Applied Maths) and the results are presented as a dendrogram in Figure 4. The dendrogram is based on the percentage of DNA similarity obtained from the MLVA profiles of *S. aureus* isolates and shows 15 isolates – 14 originated from mastitic cows and additional 1 reference strain ATCC 25923 as a control. The dendrogram revealed two distinct clusters, which show 30% similarity. The first cluster includes 7 identical isolates, and at 50% similarity it combines the next two, which are 70% similar: 1 wild isolate and 1 reference strain ATCC 25923. The second identical group is formed by 5 isolates and there is another isolate with 50% similarity. Four MLVA clusters were identified at 100% similarity.

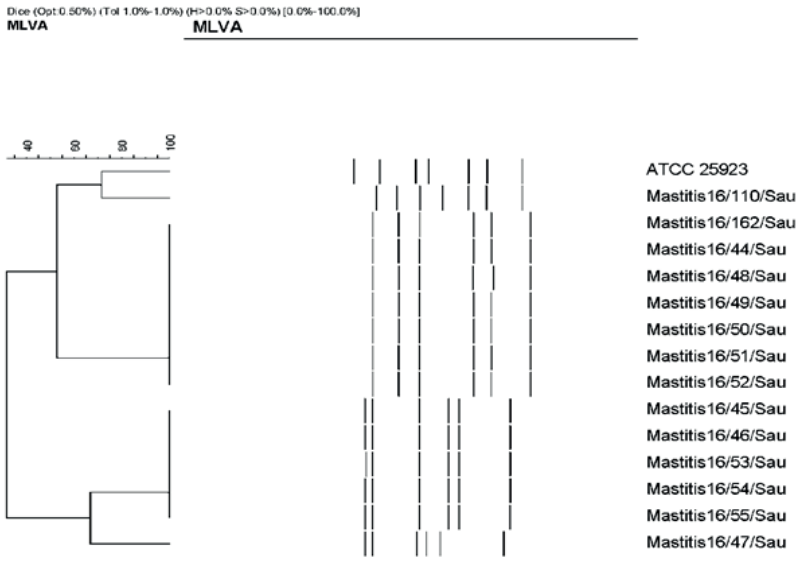

Fig. 4. Dendrogram showing evolutionary distances between analysed *Staphylococcus aureus* strains based on DNA similarity.

All ecosystems are known as complex environments enriched by various microorganisms, which do not respect any barriers and migrate in the biosphere. Some bacteria and yeast species show active mobility and can be transmitted in the air or during direct physical contact that frequently takes place between animals or between animals and humans, animals and equipment, e.g. milking systems [Kosecka-Strojek *et al*. 2018].

From a microbiological and ecological perspective, a livestock farm is a special environment, in which microorganisms come from various sources: from the physiological microbiota of farm staff or from other animals such as visiting birds and intruding small rodents which carry various bacteria, including staphylococci

[Hauschild *et al*. 2010]. Therefore, it is important to examine the bacterial flora from mastitic cows for several reasons. Pathogenic bacteria found in the mammary glands of cows cause a severe disease and their presence in food, milk and/or meat generates a major health hazard for consumers (food poisonings and infections) and leads to additional economic problems [Kuźma *et al*. 2005, Loncarevic *et al*. 2005].

For the reasons stated above, it is important to study the colonization of the mammary gland by microorganisms after the end of mastitis therapy. It needs to cover not only pathogenic, but also commensal microorganisms. Therefore the area of interest was identification of the course of normal colonization, the types of microorganisms involved in the process and potential risk of secondary infection. This is of great significance in the pathogenesis process and in the subsequent developmental stages of mastitis mechanisms, with negative effects on consumer health, the quality of milk and meat obtained from sick animals, as well as economic performance [Nawrotek *et al*. 2005].

The described analysis of milk from mastitic cows showed the presence of *Staphylococcus aureus* species in 14 samples, *Streptococcus agalactiae* in 3 samples, as well as many other species of bacteria and/or yeast-like fungi, which are commensal opportunistic pathogens, but not obligate pathogens. A high share of the commensals was composed by the bacteria forming the normal microbiota of cow udder skin, such as numerous species of coagulase-negative staphylococci and the *Micrococcus* genus. A particularly large proportion were bacteria typical for the natural environment. Moreover, no obligate pathogens or multi-drug resistant *S. aureus* strain (MRSA) was found in the analysed collection. The identification of the strains of this genus was confirmed by genetic methods and biochemical analysis revealed high enzymatic and toxic activities for all the 14 strains. As shown by the milk culture results obtained following treatment, during treatment certain species (mainly CoNS) were eliminated and the number of isolates was significantly decreased. *Corynebacterium* bacteria, Gram-negative streptococci and Gram-negative rods were present in the milk samples from some cows both before and after therapy. This proves that the udders and teat canals are permanently colonized. Although the cows' health was clinically confirmed, it may be assumed that the herbal ointment therapy was effective, but not in 100%. An alternative to the therapy applied in the study was the application of antibiotics, with all the negative consequences for the treatment of both single animals and for the global phenomenon such as increasing antibiotic resistance of pathogenic microorganisms [Sampimon *et al*. 2011, Taponen *et al*. 2016]. In the described experiment it was decided to treat sick cows with herbal medicines, considering all the positive and negative effects, the efficiency of which was verified by the colonization tests and presented in the paper. Although the main aim of the study was to investigate natural microbial colonization of cows after the end of the therapy process, with the application of herb medicines instead of antibiotics being an important aspect in line with the European Green Deal (EGD). An EGD program recommends gradual reduction of antibiotic use to 50% in farming by the end of 2030.

All the natural environments are characterized by the presence of microorganisms. In cattle breeding farms the udder skin is continuously colonized by bacteria and yeasts as a result of contact with bedding and other animals; that is why the study revealed a high representation of *Enterococcus, Bacillus, Corynebacterium, Acinetobacter, Pseudomonas* and other bacteria, which originate from the intestinal tracts, as well as typical environmental microorganisms. Thus, the exogenous or endogenous nature of the infections still needs to be clarified. An interesting observation was made for some analyzed milk samples, which were found to contain a yeast-like aetiological agent of mastitis with a qualitatively and quantitatively much lower percentage of bacteria, or even the complete absence of bacterial species. This result shows the phenomenon of antagonism and its mechanisms at cellular and molecular levels require thorough testing.

Identification of the sources of colonizing microorganisms as well as their spreading pathways is only possible through epidemiological studies using molecular research tools that recognize the genetic types of isolates and clonal complexes, which is the case in patient infection studies [Ilczyszyn *et al*. 2016]. Only then will it be possible to answer the question if colonization by a particular microorganism species will end with permanent colonization of the ecological niche or if colonization will be the first stage of infection leading to disease, elimination of sick cows from milking and financial losses.

The skin and mucous membranes in farm animals are frequently colonized by opportunistic microorganisms. In the animal body the occurrence of an additional factor predisposing to infection is a necessary condition for progression from colonization to endogenous infection. It is also essential to recognize the presence of any latent or subclinical form of mastitis without typical manifestation in a particular cow, which is important for studying the colonization of bovine udders.

The only signs of presence in unlike or subclinical stages of the disease are increased numbers of somatic cells in milk and the presence of pathogens, without other symptoms typical for mastitis. Such a phenomenon is known as asymptomatic infection and often leads to the chronic course of infections. Additionally, that stage of the disease hinders analysis of udder colonization after completed therapy. Therefore, the knowledge on physiological microbiota present in cows and careful investigation of microbiological colonization after mastitis therapy are of particular importance.

The results presented above contribute to science and broaden our knowledge on bacteria-host relationships during mastitis and after the end of therapy, facilitate recognition of aetiological factors and their sources, as well as improve diagnostics and early detection of the subclinical stage of mastitis that affects the final therapeutic success [Lisowska-Łysiak *et al*. 2018]. For the above reasons advanced diagnostic microbiological tests using genetic engineering methods have to be accompanied by the highest hygiene levels with regard to both single cows and sanitary conditions in farms.

This paper broadens our knowledge on microbial species accompanying mastitis in cows. The reported colonization confirmed the ecological and epidemiological processes in secondary infections of mammary glands in cows following previous mastitis successfully treated with herbal medicines instead of antibiotics. Isolated microorganisms were bacteria belonging to Gram-positive cocci, coryneforms, Gram-negative and Gram-positive rods, as well as yeasts. The presence of a wide range of bacterial species indicates that the microorganisms adapted to the bovine mammary gland can spread from one animal to another in the farm. A decrease in the counts of some bacteria and yeast species was observed after the mastitis therapy, with some of the same species of bacteria an yeasts being present both before and after therapy. There were no obligate pathogens evidenced, but many commensal and/or environmental species of bacteria and yeasts were reported. However, pathogenic and opportunistic bacteria found in the mammary glands in cows cause a severe disease and their presence in foods, milk and meat generates a major health hazard for consumers and leads to economic problems for farmers. For these reasons it is important to study the colonization of mammary glands by various microorganisms, not only obligate pathogens, after the end of mastitis therapy. A thorough microbiological analysis, including advanced methods of molecular diagnostics, makes it possible to recognize the course of colonization and reduce or eliminate the risk of a secondary infection.

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