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Original Scientific Paper

SENSITIVITY OF BACTERIAL STRAINS ISOLATED IN CASES OF COW MASTITIS TO ANTIBIOTICS AND ESSENTIAL OILS

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Summary

Mastitis is an inflammatory reaction of the udder caused by microorganisms, chemical or mechanical injuries. Bacterial mastitis, caused by several types of bacteria, is divided into two main groups: contagious mastitis caused by *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Mycoplasma bovis* and mastitis caused by the ubiquitous bacteria *Escherichia coli*, *Pseudomonas aeruginosa* or *Proteus spp*. Currently, instead of using antibiotics, new strategies are being sought to reduce this clinical health problem. The aim of this study is to determine the sensitivity of bacterial strains causing mastitis to antibiotics and essential oils of oregano (*Origanum compactum*, *Origanum majorana*) and thyme (*Thymus serpyllum*). Mammary gland secretion samples, obtained by mechanical and manual methods, from dairy cows from the Kalesija and Gradačac areas were used. A total of 200 samples from dairy cows in different periods of lactation and age were examined. Mastitis control included determining somatic cells count in milk with the California mastitis test, or counting somatic cells on the Fossomatik apparatus, after which all samples with an increased number of somatic cells were microbiologically tested. Based on the obtained results, it can be concluded that there are justified reasons why the use of essential oils should be considered as an alternative to the use of antibiotics in the therapy of animals suffering from mastitis.

Key words: mastitis, pathogens, antibiotics, essential oils.

INTRODUCTION

Mastitis is an inflammatory reaction of the udder tissue, mostly of microbial etiology. Infection occurs when an infectious factor penetrates the mammary gland

through the teat canal, begins to multiply and damage the udder tissue with its harmful metabolic products. The most common bacterial causes of mastitis are: *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*Str. agalactiae*), *Streptococcus uberis*, *Echerichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Proteus species* (Bannerman, 2004). As a result of the infection, the pathogens and their toxins are excreted into the milk, which is than a harmful to the health of the consumer. In the fight against the causative factor of mastitis, various strategies are applied, including regular udder health controls, prophylactic therapy during the dry period, vaccination, as well as the exclusion of diseased animals from production. When mastitis occurs during lactation, antibiotic therapy is often used. Long-term use of one antimicrobial substance destroys sensitive strains of pathogenic bacteria and selection that favors resistant strains of microorganisms occurs, which over time lead to the resistance. Bacteria have increasing antimicrobial resistance, and that represent one of the biggest public health problems that attracts the modern scientific public (Bačić, 2009). In the antimicrobial therapy of mastitis, various alternative substances with an antibacterial effect can be used, leading to the prevention of growth and reproduction or the destruction of the causative factor. For udder infections, essential oils and/or their active components can be used for preventive and therapeutic purposes. Essential oils that can be used in mastitis therapy come from plants such as: coriander (*Coriandrum sativum L.*), oregano (*Origanum vulgare L.*), thyme (*Thymus vulgaris L.*), rosemary (*Rosmarinus officinalis L.*), cinnamon (*Cinnamomum zeylanicum Breyn*.), sage (*Salvia officinalis L.*), thyme (*Thymus serpyllum*) and cloves (*Eugenia caryophyllata Thunb*). Among others, carvacrol, eugenol, cinnamaldehyde and thymol were isolated as active components of these essential oils. The combined therapy of conventional antibiotics and essential oils is currently very interesting and represents a potential area for future research.

The aim of the study is to determine the sensitivity of the bacterial strains causing mastitis to some antibiotics and essential oils of oregano (*Origanum compactum, Origanum majorana*) and thyme (*Thymus serpyllum*). In order to achieve the goal of the study, the following tasks were set:

- isolation and identification of the causative factor of subclinical mastitis of cows using conventional bacteriological techniques,
- determining somatic cells count,
- determining the sensitivity of isolated strains of the causative factor of mastitis to antibiotics,
- examination of the antibacterial effect of *Origanum compactum, Origanum*

majorana and *Thymus serpyllum* essential oils on isolated strains causing mastitis,

- determination of minimum inhibitory and minimum bactericidal concentrations for essential oils and their synergistic effect.

MATERIALS AND METHODS

Mammary gland secretion samples of dairy cows from the Kalesija and Gradačac areas, obtained by machine milking, were used in this study. Milk samples taken from 200 dairy cows (100 samples from each area) were analyzed.

Isolation and identification of bacterial strains causing mastitis were carried out in the bacteriological laboratory of the PI Veterinary Institute of Tuzla Canton, while testing of the bacterial isolates sensitivity to antibiotics and essential oils was obtained in the microbiological laboratory of the Faculty of Science and Mathematics, University of Tuzla.

Milk samples for microbiological tests were taken aseptically, before milking. First, the udder was washed with warm water and soap, especially the papillae, and wiped with a clean cloth. Disinfection of the papillae tips was carried out with a cotton swab soaked in 70% ethanol, using the "to yourself" principle. First the further, and then closer quarters were disinfected, in order to avoid contamination of the already disinfected papillae. Three jets of milk were squeezed out of each quarter of the udder before sampling. After disinfection, each quarter was milked separately into sterile plastic bottles with a volume of 50 ml (Figure 1). After sampling, the milk samples were placed in a hand-held refrigerator (temperature 4°C) and transported to the laboratory for bacteriological examination.

Figure 1 Samples of udder secretions from diseased quarters for bacteriological analysis

Mastitis control included determining the somatic cells count in milk by the California mastitis test (CMT) (White et al., 2005), then counting somatic cells on the Fossomatik apparatus, after which all samples with an increased number of somatic cells were microbiologically tested.

The somatic cells count (SSC) is an indicator of the hygienic quality of milk and is a general indicator of the health of the udder. SSC directly indicates the mammary gland health. Somatic cells in milk originate from the udder and blood, and are most often leukocytes. An upper limit of 400 000 somatic cells (SS)/ml has been established for cow's milk, above which subclinical mastitis occurs. In the milk of healthy quarters, the number of somatic cells is less than 200 000 per ml, and they consist of epithelial cells and leukocytes (polymorphonuclear neutrophils, lymphocytes, macrophages). The Regulation on the method of sampling, classification and calculation of the price of milk (Regulation, 1994) defines that those who process milk can only buy milk that meets the criteria prescribed by the Regulation on raw milk (Regulation, 2011) and the calculation of the geometric mean.

Samples with a geometric mean of 400 000 SCC/ml are excluded from the classification. Blood agar is used to isolate *S. aureus* from cow mammary gland secretion samples, as it enables the growth of the most common bacterial pathogens under aerobic incubation conditions. For routine inoculation of udder secretions on blood agar, disposable plastic swabs with a volume of 0.01 ml are used. For larger volumes, swab sticks or pipettes can be used. The seeded substrates are incubated for 24 to 48 hours at 37ºC in an inverted position. After incubation for 24 hours on blood agar, round, smooth, shiny colonies, with straight edges, slightly convex, pigmented, white or cream-colored, sometimes yellow and even orange, 1-2 mm in diameter grow. Also, thay may show hemolysis. If there are no grown colonies, the incubation of the seeded samples is extended for another 24 hours. Gram-positive cocci with a characteristic arrangement in the form of clusters are visible on the Gram-stained microscopic preparation from the culture grown on blood agar. Characteristic beta hemolytic colonies from blood agar are inoculated on a selective Baird-Parker agar (BPA) medium on which staphylococci form colonies that are black, convex and shiny, 1-1.5 mm in diameter. Bacteria from the genus *Staphylococcus* produce the enzyme catalase, i.e. they are catalase positive. Confirmation of the production of this enzyme is done with 3-5% H_2O_2. Species of the genus *Staphylococcus* are differentiated according to their ability to coagulate plasma using the coagulase enzyme. *S. aureus* is coagulase positive, while other species of the genus *Staphylococcus* are coagulase negative. Bound coagulase is determined by a plate test, and free coagulase by a test tube. Certified reference strains were used as positive and negative controls (positive control *Staphylococcus*

aureus WDCM 00034, negative control *Staphylococcus epidermidis* WDCM 00036). A positive result is visible formation of clumps within 10 seconds, and a negative result means no visible formation of clumps. Isolation and identification of *Streptococcus* and *Streptococcus agalactiae* was done by incubating a sample of udder secretions on a blood medium for 24 hours at 37°C, and after incubation, the growth of characteristic colonies was visible on the plates. Colonies are small, grayish or airy, convex and compact (complete, rounded). Some strains require the presence of carbon dioxide to grow. Streptococci show hemolysis on blood agar. Colonies of *Enterococcus spp*. can have a zone of α hemolysis or be without hemolysis, they are grayish, shiny and not compact. Colonies that morphologically correspond to streptococci are inoculated onto the selective esculin azide agar medium and incubated for 24 hours at 37°C. Gram-positive cocci, round or ovoid, with a diameter of 0.6-1μm, arranged in pairs and chains of different lengths, were found on a Gram-stained microscopic preparation from a culture grown on blood agar. To confirm *Str. agalactiae*, the CAMP test is used. This test detects the presence of CAMP factor (Christie, Atkins and Munch Peterson) – an extracellular protein that enhances beta hemolysis of *S. aureus*. It is positive at *Str. agalactiae*. The test is performed by dragging the staphylococcal culture along the blood agar with 10% sheep's blood, and then the tested strain of streptococcus is sown perpendicular to it in the form of a line starting from the end of the plate to near the staphylococcal line. A strain of *S. aureus* with a double zone of hemolysis is required to perform this test. After incubation at 35-37°C for 24 hours, it is observed whether there is a spear-shaped increase in hemolysis of streptococci near the staphylococcal line (Maksimović and Rifatbegović, 2015). Further identification was based on the analysis of a confirmatory test for the presence of intestinal enterococci by the breakdown of esculin. One typical colony was subcultured on bile esculin agar and incubated at 44°C for 48 hours. The identification of isolates to the species level was based on Gram staining of preparations, a negative catalase test and a biochemical test for the breakdown of sugars (sorbitol and arabinose). One colony was transferred with a sterile swab to the test tubes containing the mentioned sugars, and then the test tubes were incubated overnight at a temperature of 37°C. A positive reaction was read in the form of a change in the color of the sugar from pink to red, with noticeable turbidity, which is the main identification factor by which it is possible to distinguish between the two species, *Enterococcus faecalis* and *Enterococcus faecium*. *Enterococcus faecalis* has the ability to degrade sorbitol, but does not degrade arabinose, while *Enterococcus faecium* degrades arabinose, but does not degrade sorbitol. Isolation and identification of *E. coli* from udder secretion samples was also carried out by inoculating the sample and incubating it on a blood

medium for 24 hours at 37°C, and after incubation, large, smooth, shiny and round colonies appeared on the plates. Characteristic colonies are spread on MacConkey agar. On MacConkey agar, colonies change color to pink due to lactose fermentation. Identification with a Gram-stained microscopic preparation reveals Gram-negative bacteria in the shape of sticks, arranged individually or in pairs. For further identification, the API 20 E test (BioMérieux, 2002) was used, which is a standardized identification system for *Enterobacteriaceae* and other Gram-negative bacteria. API 20 E strip consists of 20 micro tubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension that reconstitutes the medium. During incubation, there is a change in color that is detected spontaneously or by the addition of reagents. Reactions are read in accordance with the attached table, and identification is done by referring to the Analytical Profile Index or using identification software. The oxidase test must be performed according to the manufacturer's instructions. The result should be kept on the result sheet because it is an integral part of the final profile (Schuster, 1997). The sensitivity test to antibiotics and essential oils was performed on three bacterial isolates, and the Disc diffusion method was used according to the CLSI M02-A11 standard (Clinical and Laboratory Standards Institute) (CLSI, 2012) with the use of antibiogram discs (Conda pronadis, Micro & Molecular Biology , Spain): Amoxicillin/clavulanic acid 30µg, Amoxicillin 30µg, Cephalexin 30µg, Doxycycline 30µg, Enrofloxacin 10µg, Gentamicin 30µg, Lincomycin 30µg, Oxytetracycline 30µg, Penicillin 10µg, Trimethoprim/sulphamethoxazole 25µg. Interpretation of categories was done according to the same standard (CLSI, 2014). Due to its simplicity, the disc diffusion method has been routinely used for many years to determine the sensitivity of bacteria to antibiotics. The inoculum is prepared by diluting an 18-24 hours old pure bacterial culture. Four to five colonies are picked and transferred to 5 ml of physiological solution. The suspension is vortexed, and then the density of the suspension (turbidity) is adjusted by comparison with the 0.5 McFarland standard. The inoculum is sown evenly on the surface of the substrate with a swab, gauze or glass rod (Bauer, 1966). After sowing the inoculum, it is preferable to leave the substrate for 10-15 minutes at room temperature to dry the surface. Disc placement is performed with a dispenser or sterile tweezers. The distance between the discs is at least 3 cm, and the distance from the edge of the container is at least 1 cm. A maximum of 6 discs are applied to a plate with a diameter of 10 cm. The seeded substrates with antibiotic discs are incubated at the appropriate temperature in aerobic, microaerophilic or anaerobic conditions, depending on the type of bacteria being tested, and the plates are turned with the lid up. Since the thickness and composition of the nutrient medium can affect the results, the procedure is carried

out in accordance with the principles that standardize the methodology. After incubation at 37°C for 24 hours, the diameter of the growth inhibition zone of the tested bacterial strain for individual antibiotics is measured with a meter with a millimeter division. According to the CLSI M02-A11 standard, based on the measured values of the diameter of the growth inhibition zone, the degree of sensitivity is assessed and classified into one of three categories: S – sensitive - infection caused by a sensitive strain is treated with usual (optimal) therapeutic doses.; I- intermediate or moderately sensitive - infection caused by a moderately sensitive strain is treated with higher therapeutic doses of antibiotics; R- resistant - an infection caused by a resistant strain cannot be treated with the tested antibiotics. To test the antimicrobial sensitivity of bacteria to three essential oils *Thymus serpyllum*, *Origanum majorana* and *Origanum compactum* (Fadli, 2012), 10 isolates of coagulase-negative staphylococci (CNS), *Enterococcus faecalis* and *E. coli* were used. Bacteria were cultured on Baird-Parker agar, Slanetz Bartley agar, and MacConkey agar overnight to obtain single colonies. Colonies were then suspended in 0.9% sterile saline to achieve a turbidity equal to 0.5 McFarland standard (108 CFU/ml). After that Mueller Hinton agar was inoculated with bacterial suspension. 50 µl of each essential oil was applied to each plate in wells with a diameter of 6 mm. The plates were incubated for 24 hours at 35°C. Interpretation of the results was carried out by measuring the diameter of inhibition zones in millimeters. The criteria for evaluating the antimicrobial sensitivity of essential oils predict that an inhibition zone of less than 10 mm indicates the insensitivity and resistance of bacteria to the essential oil, 10 to 15 mm indicates a weak antimicrobial effect of the oil, 16 to 20 mm to a moderate antimicrobial effect and a zone of 20 mm to pronounced antimicrobial activity. The broth microdilution test (MIC) and the determination of the minimum bactericidal concentration of antimicrobial substances (MBC) were performed according to the standard (Lambert, 2001). MBC was performed after determining the MIC value, by spreading bacteria from the wells of the microtiter plate onto Mueller Hinton agar, both from the wells of the MIC value and from the wells of the four next higher concentrations. The diffusion test was also used to determine the antimicrobial effect of oregano and thymus essential oils on bacterial isolates (Choi et al., 2012). The minimum inhibitory concentration (MIC) was determined using the microdilution method, which represents the lowest tested concentration of essential oil at which there is no visible growth. This method is performed on microtiter plates containing 96 wells using MH broth, bacterial suspension and different concentrations of essential oil. The determination of the minimum inhibitory concentration was done by the use of the indicator substance 2,3.5-triphenyltetrazolium chloride (TTC solution), which, due

to a change in color caused by a change in the pH value during culture growth, facilitates the determination of the obtained results.

The synergistic effect between essential oils on bacterial isolates causing cow mastitis was determined by the checkerboard method. This method determines the interaction and strength of two tested substances when they are used simultaneously, that is, the potential power of two essential oils as well as essential oils and antibiotics compared to their individual effect. Thus, this method determines the interaction and strength of two test substances (A and B) when used simultaneously, which is expressed as an FIC value (fractional inhibitory concentration). In this case, the antibiotic was designated as substance A, while the essential oil represented substance B. Based on previously determined MIC values, series of standard dilutions of substances A and B were made in order to test their combined interaction (initial concentrations for the preparation of dilutions are the MIC values of the tested substances and they decreased horizontally in the microtiter plate for the antibiotic, and vertically for the essential oil). After the preparation of serial dilutions of antimicrobial substances in microtiter plates, bacterial suspensions of the tested strains were prepared and added in the same way as in the tests for testing the antimicrobial activity of individual substances. In a microtiter plate with 96 wells, 90 μL of inoculated MH broth and 10 μL of a serial dilution of the combination of substances A and B were added in a certain order. The final volume in each well was 100 μL, the final density of bacterial cells was 106 CFU/ml. The microtiter plates were incubated overnight at 35ºC, and the results were read as described above with the addition of TTC. The lowest concentration of substance in the combination required to prevent the appearance of a red color was considered the MIC value. The obtained MIC values of the tested combinations were used to determine the FIC index and interpret the type of interaction between the antimicrobial components. We also analyzed the total number of somatic cells in the milk samples using the instrumental method of counting with the Fossomatic device (Fossomatic counting).

RESULTS

According to the CMT in the Kalesija area, 41 out of 100 processed samples were identified as unusable (25 were in trace, 23 were weak positive, 17 were distinct positive, and 1 was strongly positive). There were no cases of clinical mastitis in the Kalesija area. Given that CMT is an orientation procedure that indirectly provides insight into the number of somatic cells, we were interested in comparing the results obtained by the CMT procedure and Fossomatic counting.

Table 1 shows a comparison of the results of the CMT test and Fossomatic counting for the Kalesija area.

Table 1 Comparison of SSC obtained by CMT and the instrumental method - Kalesija area

Evaluation of the results of the instrumental method	Number of samples	Mean SSC	Assesment of reaction CMT	Number of samples	Mean SCC
negative (-)	47	119 124	negative (-)	34	241 778
weak positive (+)	17	386 273	trace (+/-)	25	337 561
pozitive (++)	19	1 259 682	weak positive (+)	23	906 385
strong positive (+++)	17	2 832 250	distinct positive (++)	17	1 851 941
			strong positive (+++)	1	5 971 000
			clinical mastitis	0	0

The geometric mean of negative samples was 119 124 for 47 samples according to the instrumental method in the Kalesija area, while it was 241 778 for 34 samples according to CMT.
Table 2 shows a comparison of the results of the CMT test and Fossomatic counting for the Gradačac area.

Table 2 Comparison of SSC obtained by CMT test and instrumental method - Gradačac area

Evaluation of the results of the instrumental method	Number of samples	Mean SCC	Assesment of reaction CMT	Number of samples	Mean SCC
negative (-)	49	205 846	negative (-)	35	125 090
weak positive (+)	7	373 333	trace (+/-)	19	267 231
positive (++)	21	938 500	weak positive (+)	16	841 263
strong positive (+++)	23	8 422 000	distinct positive (++)	27	1 447 867
			strong positive (+++)	2	6 474 000
			clinical mastitis	1	6 290 000

The geometric mean (mean value) of the somatic cells count in the Gradačac area, for negative samples, was 125 090 for 35 samples according to CMT, while it was 205 846 for 49 samples for the instrumental method. In accordance with the Regulation on raw milk (Regulation, 2011), samples were processed by the instrumental method, and if a geometric mean was 400 000 SCC/ml and more, samples were put out of classification and such milk was withdrawn from purchase. CNS as the cause of mastitis was identified in 8.5% of samples (17/200) (Table 3).

Table 3 CNS isolated from mammary gland secretions of cows

The causative factor	% of isolates from the Kalesija area	% of isolates from the Gradačac area
CNS	8	9

Enterococcus faecalis as the causative factor of mastitis was identified in 5% of samples (10/200) (Table 4).

Table 4 *Enterococcus faecalis* isolated from mammary gland secretions of cows

The causative factor	% of isolates from the Kalesija area		% of isolates from the Gradačac area
Enterococcus faecalis	6		4

E. coli as the cause of mastitis was identified in 26% of samples (52/200) (Table 5).

Table 5 *E. coli* isolated from mammary gland secretions of cows

The causative factor	% of isolates from the Kalesija area		% of isolates from the Gradačac area
E. coli	22		30

Results of somatic cells count using the instrumental method by Fossomatic apparatus and isolation of the causative factor (Table 6) indicate that the samples with increased SCC were also unusable according to the bacteriological findings, and that bacteria are the reason for their increase.

Table 6 Comparasion of somatic cells count using the instrumental method and isolated causative factors of mastitis

Gradačac area		Kalesija area	
% of unusable samples determined by the instrumental method of counting somatic cells	Total number of isolated causative factors	% of unusable samples determined by the instrumental method of counting somatic cells	Total number of isolated causative factors
44	43	36	36

In order to test the sensitivity profile of isolates of coagulase-negative staphylococci, *Enterococcus faecalis* and *E. coli* to antibiotics, 10 isolates of each species were analyzed. The results are presented in Tables 7, 8 and 9. Interpretation of the results was carried out by measuring the diameter of inhibition zones in millimeters.

Table 7 Susceptibility of CNS isolates to antibiotics

KNS	AMC	AX	CEF	DO	ENR	GM	L	T	P	TMP
1.	S	R	R	R	S	R	R	R	S	S
2.	S	R	R	R	S	R	R	R	S	R
3.	S	R	R	R	S	R	R	R	R	R
4.	S	R	R	R	S	R	R	R	R	R
5.	S	R	R	R	S	R	R	R	R	S
6.	S	R	R	R	S	R	R	R	S	R
7.	S	R	R	R	S	R	R	R	R	R
8.	S	R	R	R	S	R	R	R	S	R
9.	S	R	R	R	S	R	R	R	R	R
10.	S	R	R	R	S	R	R	R	R	S

R – antibiotic resistance; S – sensitivity; AMC - amoxicillin/clavulanic acid; AX – amoxicillin; CEF – cephalexin; DO – doxycycline; ENR – enrofloxacin; GM – gentamicin; L – lincomycin; T – oxytetracycline; P– penicillin; TMP - trimethoprim/sulphamethoxazol

All ten CNS isolates showed sensitivity to AMC and ENR, four CNS isolates were sensitive to penicillin 10μg, while the other six isolates were resistant. Three isolates were sensitive to TMP, while seven other isolates were resistant to this antibiotic. CNS showed 100% resistance to AX, CEF, DO, GM, L and T. Resistance to penicillin was established in 6 isolates (60%), and to TMP in 7 isolates (70%).

The highest resistance (100%) of *Enterococcus faecalis* was found to antibiotics AMC, AX, CEF, ENR, TMP, then to lincomycin (80%) and penicillin (60%). The highest sensitivity (100%) of *Enterococcus faecalis* was found to the antibiotics doxycycline and gentamicin, which is shown in Table 8.

Table 8 Susceptibility of *Enterococcus faecalis* isolates to antibiotics

Enterococcus faecalis	AMC	AX	CEF	DO	ENR	GM	L	T	P	TMP
1.	R	R	R	S	R	S	S	S	R	R
2.	R	R	R	S	R	S	R	R	S	R
3.	R	R	R	S	R	S	R	R	R	R
4.	R	R	R	S	R	S	R	R	R	R
5.	R	R	R	S	R	S	S	S	S	R
6.	R	R	R	S	R	S	R	R	S	R
7.	R	R	R	S	R	S	R	R	R	R
8.	R	R	R	S	R	S	R	S	S	R
9.	R	R	R	S	R	S	R	S	R	R
10.	R	R	R	S	R	S	R	R	R	R

R – antibiotic resistance; S – sensitivity; AMC - amoxicillin/clavulanic acid; AX – amoxicillin; CEF – cephalexin; DO – doxycycline; ENR – enrofloxacin; GM – gentamicin; L – lincomycin; T – oxytetracycline; P – penicillin; TMP - trimethoprim/sulphamethoxazol

Testing the sensitivity of *E. coli* isolates to antibiotics, 100% sensitivity to AMC, AX and penicillin, and 60% to ENR was established. The highest resistance of *E. coli* to antibiotics was found to CEF, DO, GM, L, T, TMP, which is shown in Table 9.

Table 9 Susceptibility of *E. coli* isolates to antibiotics

E. coli	AMC	AX	CEF	DO	ENR	GM	L	T	P	TMP
1.	S	S	R	R	S	R	R	R	S	R
2.	S	S	R	R	R	R	R	R	S	R
3.	S	S	R	R	R	R	R	R	S	R
4.	S	S	R	R	R	R	R	R	S	R
5.	S	S	R	R	S	R	R	R	S	R
6.	S	S	R	R	S	R	R	R	S	R
7.	S	S	R	R	R	R	R	R	S	R
8.	S	S	R	R	S	R	R	R	S	R
9.	S	S	R	R	S	R	R	R	S	R
10.	S	S	R	R	S	R	R	R	S	R

R – antibiotic resistance; S – sensitivity; AMC - amoxicillin/clavulanic acid; AX – amoxicillin; CEF – cephalexin; DO – doxycycline; ENR – enrofloxacin; GM – gentamicin; L – lincomycin; T – oxytetracycline; P – penicillin; TMP - trimethoprim/sulphamethoxazol

Sensitivity profiles of CNS isolates to essential oils were determined by the disk-diffusion method as shown in Table 10. The essential oils of *Origanum compactum* and *Thymus serpyllum* have the greatest antimicrobial potential against CNS isolates. All ten isolates showed sensitivity to these essential oils. Four isolates were sensitive and six isolates intermediate sensitive to Origanum majorana essential oil.

Table 10 Sensitivity of CNS isolates to essential oils

KNS	*O. compactum*	*O. majorana*	*T. serpyllum*
1.	S	S	S
2.	S	I	S
3.	S	I	S
4.	S	I	S
5.	S	I	S
6.	S	S	S
7.	S	I	S
8.	S	I	S
9.	S	S	S
10.	S	S	S

R – resistance; S – sensitive; I – intermediate

The sensitivity profile of *Enterococcus faecalis* to essential oils using the disc diffusion method is shown in Table 11.

Table 11 Susceptibility of *Enterococcus faecalis* isolates to essential oils

Enterococcus faecalis	*O. compactum*	*O. majorana*	*T. serpyllum*
1.	S	R	S
2.	R	R	I
3.	S	R	S
4.	S	R	S
5.	S	R	I
6.	S	R	S
7.	S	R	S
8.	S	R	S
9.	S	R	S
10.	S	R	S

R – resistance; S – sensitive; I – intermediate

In the case of *Enterococcus faecalis* isolates, the second sample showed resistance to *Origanum compactum* and *Origanum majorana* essential oils, while all other isolates showed resistance only to the *Origanum majorana* essential oil. Eight isolates of *Enterococcus faecalis* were sensitive to the *Thymus serpyllum* essential oil, while two isolates showed intermediate sensitivity. *Origanum compactum* had better antimicrobial activity against *Enterococcus faecalis* isolates than *Thymus serpyllum* essential oil.

The sensitivity profile of *E. coli* to essential oils using the disc-diffusion method is shown in Table 12. The essential oils of *Origanum compactum* and *Thymus serpyllum* had the greatest antimicrobial potential against *E. coli* isolates, while the essential oil of *Origanum majorana* had intermediate effect on 6, and resistant to 4 isolates (Table 12).

Table 12 Sensitivity of *E. coli* isolates to essential oils

E. coli	*O. compactum*	*O. majorana*	*T. serpyllum*
1.	S	R	S
2.	S	I	S
3.	S	R	S
4.	S	R	S
5.	S	I	S
6.	S	R	S
7.	S	I	S
8.	S	I	S
9.	S	I	S
10.	S	I	S

R – resistance; S – sensitive; I – intermediate

In this study, minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined for three types of essential oils *O. compactum*, *O. majorana* and *T. serpyllum* tested on three isolates, namely *E. coli* isolate no. 10, CNS isolate 5 and *Enterococcus faecalis* isolate 10. All 10 *E. coli* and CNS isolates showed sensitivity to all three types of essential oils *O. compactum*, *O. majorana* and *T. serpyllum*. However, all 10 isolates of *Enterococcus faecalis* were resistant to the essential oil of *O. majorana*, and therefore the minimum inhibitory and bactericidal concentration could not be determined. Table 13 shows the MIC and MBC of the tested essential oils.

Table 13 MIC and MBC of essential oils tested on 3 isolates of *E. coli*, CNS and *Enterococcus faecalis*

Essential oil	*O. compactum*		*O. majorana*		*T. serpyllum*	
Izolates	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
E.coli izolate 10	12	21	28.6	43	26	39
CNS izolate 5	14	21	17.2	43	19.5	39
Enterococcus faecalis izolate 10	10.5	16.8	R	R	17.2	43

Based on obtained results, it can be observed that the essential oil of *O. compactum*

has the lowest minimum inhibitory and minimum bactericidal concentrations, and was identified as the oil with the strongest antimicrobial activity on all three isolates tested. MIC values for *O. compactum* range from 10.5 mg/ml to 14 mg/ml, in contrast to *O. majorana* which has the lowest MIC value of 17.2 mg/ml and *T. serpyllum* which has the lowest MIC value also of 17.2 mg/ml.

Minimum inhibitory and minimum bactericidal concentrations for the antibiotic amoxicillin/clavulanic acid showed the highest antimicrobial activity against the tested isolates. All 10 *E. coli* isolates and all 10 CNS isolates were sensitive to the antibiotic amoxicillin/clavulanic acid, and therefore this antibiotic was chosen for further testing. However, all 10 isolates of *Enterococcus faecalis* showed resistance to this antibiotic, and the MIC and MBC values of this antibiotic were not determined on this isolate. MIC and MBC values of amoxicillin/clavulanic acid were tested on two isolates, namely *E. coli* isolate 10 and CNS isolate 5. From the obtained results, it can be observed that the antibiotic amoxicillin/clavulanic acid shows significantly lower MIC and MBC values compared to ethereal oils. Such a result was expected considering that this antibiotic is known as an extremely strong antibacterial substance. However, the antibacterial activity of essential oils should not be ignored, and they should be taken into consideration as possible alternative antibacterial substances, because it has been proven that they have strong effect on the isolates tested in this study. A total of 3 isolates (*E. coli* isolate 10, CNS isolate 5 and *Enterococcus faecalis* isolate 10) were analyzed to determine the synergistic effect of essential oils and antibiotics (FIC). Interactions between combined antimicrobial components were determined after determining the MIC value by calculating the fractional inhibitory concentration index (FICI) for each combination of antimicrobial components (*O. compactum* + *O. majorana*, *O. compactum* + *T. serpyllum*, *O. majorana* + *T. serpyllum*) according to the following formula:

FIC of antimicrobial component A = MIC of component A in combination/MIC of component A alone

FIC of antimicrobial component B = MIC of component B in combination / MIC of component B alone

FICI (FIC indeks) = FIC of component A + FIC of component B

The obtained FIC index values were interpreted as follows:

- synergistic effect of components FICI ≤ 0.5
- additive effect of components $0.5 < \text{FICI} \leq 1.0$
- indifferent effect of components $1.0 < \text{FICI} \leq 4.0$
- antagonistic effect of components FICI > 4.0 (62).

All experiments were performed in two independent replicates. The obtained results are presented in Table 14 and Figure 2.

Table 14 Synergistic effect between essential oils

Izolates	*O. compactum* + *O. majorana*		*O. compactum* + *T. serpyllum*		*O. majorana* + *T. serpyllum*	
E. coli izolate 10	FIC	Effect	FIC	Effect	FIC	Effect
	0.31	SYNERGISM	0.13	SYNERGISM	0.18	SYNERGISM
*KNS i*zolate 5	FIC	Effect	FIC	Effect	FIC	Effect
	0.18	SYNERGISM	0.21	SYNERGISM	0.20	SYNERGISM
Enterococcus faecalis izolate 10	FIC	Effect	FIC	Effect	FIC	Effect
	1	INDIFFERENT	0.16	SYNERGISM	1	INDIFFERENT

FIC – Fractional inhibitory zone

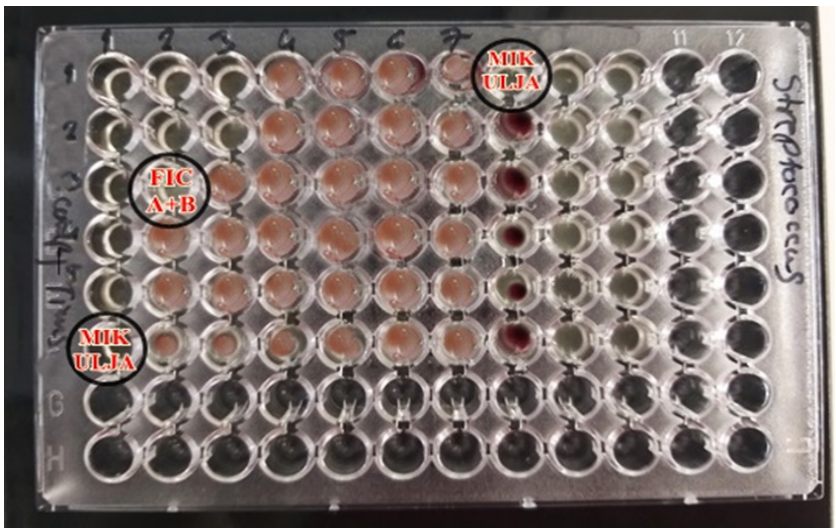

Figure 2 Synergistic interaction between essential oils of *O. compactum* and *T. serpyllum* identified on *Enterococcus faecalis* isolate no. 10. FIC index 0.16

All three essential oils (*O. compactum*, *O. majorana* and *T. serpyllum*) tested on *E. coli* isolate 10, KNS isolate 5, *Enterococcus faecalis* isolate 10 showed mutual

interaction marked as synergism. The combinations of all three essential oils did not exceed FIC values higher than 0.5, whereby the interactions of these essential oils are marked as synergistic, which means that individual components of one essential oil significantly enhance the effect of another essential oil, and give a combined positive effect. An exception is the isolate *Enterococcus faecalis* 10, in which the combination of the essential oil of *O. compactum* with *O. majorana*, and the combination of *O. majorana* with *T. serpyllum* were identified as indifferent. As previously stated, this strain was resistant to the essential oil of *O. majorana*, and the mixture of this oil with the other two (*O. compactum* or *T. serpyllum*) did not give a positive interaction between the oils. The effect of this combination is indifferent, which means that *O. majorana*, regardless of the fact that it is combined with some other oil, does not show an antimicrobial effect on this isolate.

DISCUSSION

Mastitis is the most common disease of dairy cows and has recognized negative effects on animal welfare and the profitability of dairy farms. A more complete understanding of mastitis was not possible until the microscope was developed, since it provided detection of the microorganisms that are the primary etiological factors. Initial concern about mastitis was based on public health and focused on reducing the number of bacteria in raw milk (Choi et al., 2012). The development of reliable tests for the detection of mastitis is a priority for researchers to ensure public safety, produce high-quality milk products and have practical tools of managing cows with mastitis. Detection methods include direct microscopic examination, milk somatic cells counting and isolation of the causative factor (Adkins, 2017). Those were the methods we used in our study.

The somatic cells count is a good indicator of the infection. If the SCC is higher, the probability that the mammary gland or individual quarters are infected is also higher. The Regulation on the method of sampling, classification and calculation of the price of milk (Regulation, 1994) defines that milk processors in Bosnia and Herzegovina can only buy milk that meets the criteria prescribed by the Regulation on raw milk (Regulation, 2011). The classification of raw milk in Bosnia and Herzegovina is based on the calculated geometric mean for somatic cells for the last three months, and for microorganisms for the last two months. Samples with a geometric mean of 400 000 SCC/ml are excluded from the classification. Such milk is withdrawn from purchase, and cattle are treated with antibiotics after analysis of mammary gland secretions. After the therapy and withholding period, the milk is analyzed again for SCC and the presence of antibiotics. If the value after repeated

analysis is less than 400 000 SCC/ml of milk, it is classified and purchased. A total of 44 out of 100 processed samples from the Gradačac area in our study using the instrumental method of somatic cell counting, and with a geometric mean value of 400 000 SCC/ml and more, were put out of classification and such milk was withdrawn from purchase. From the Kalesija area, 36 out of 100 samples processed by the instrumental method of counting somatic cells, with a geometric mean value of 400 000 SCC/ml and more, were put out of classification and such milk was withdrawn from purchase.

In the study obtained by Murphy (1947) it was noted that more than 20 species of bacteria can cause mastitis in cows, and at least 99% of these infections are caused by *Str. agalactiae*, other streptococci, staphylococci and bacilli (including coliform bacteria, *Pseudomonas spp*., etc.). Eberhart (1977) focused his study on coliform bacteria as a cause of mastitis. The publication provoked awareness of the growing importance of mastitis caused by environmental opportunistic organisms. In the study conducted in 2017 in the Zenica region (Bosnia and Herzegovina), the most common causes of mastitis were coagulase-negative staphylococci in 13.04% samples, *Klebisella pneumoniae* in 13.04% samples, *Enterococcus spp*. in 8.70% samples, *E. coli* in 8.70% samples, *Enterobacter*, *Serratia spp.* and *Yersinia enterocolitoca* in 4.35% samples (Burović, 2020).

The results of our study showed a high percentage of bacterial causes of mastitis. The causative factors of mastitis were found in 43% samples from the Gradačac area and 36% samples from the Kalesija area. The results of isolate identification showed that 8.5% of the isolates had characteristic of CNS, 5% had characteristics of *Enterococcus faecalis*, and 26% had characteristics of *E. coli*. These results indicate that the causative factors of mastitis are opportunistic bacteria from the environment. From the results of counting somatic cells using Fossomatic apparatus and isolation of the causative factors, it is clear that the samples with increased SCC were also unusable according to microbiological indicators, and that bacteria are the reason for their increase.

Bacterial isolates of clinical mastitis are mostly sensitive to antibiotics used in therapy, but due to increasing selective pressure due to the uncontrolled use of antibiotics in veterinary medicine, the percentage of resistant strains of the causative factor of mastitis is constantly increasing. Additional attention should be focused on herd management, with special emphasis on hygiene. Bacterial isolation and antimicrobial susceptibility testing are of great importance for the choice of treatment and the monitoring of antimicrobial resistance. Coagulase-negative staphylococci were isolated in 8.5% cases. They are the most common isolate of bovine mastitis in many countries and have been described as new mastitis

pathogens. Based on current knowledge, it is difficult to determine if coagulase-negative staphylococci are infectious or environmental pathogens (Petrović, 2006). Pitkälä et al. (2004) showed that *Enterococcus spp*. is sensitive to penicillin and ampicillin, and the study by Lambert et al. (2001) showed sensitivity to ampicillin and resistance to tetracycline, while all our *Enterococcus faecalis* isolates showed sensitivity to doxycycline and gentamicin, and complete resistance to amoxicillin/clavulanic acid.

More recently, numerous authors have studied effective alternatives to antibiotics in the treatment of mastitis in cows. In this sense, essential oils are a safe alternative, because they show good antimicrobial characteristics after prolonged exposure (Lambert et al., 2001). Previous study has shown that oregano essential oil has good antimicrobial properties to all *Staphylococcus species* that were resistant to antibiotics, as well as to *E. coli* that was resistant to several drugs in the treatment of mastitis in cows. The antibacterial mechanism of oregano essential oil indicates an increase in the permeability of the bacterial membrane, which further affects pH, homeostasis and the balance of inorganic ions. Phenolic compounds in oregano essential oil inhibit bacterial growth by altering the surface of bacterial cells, which can inhibit bacterial adhesion to mammary gland epithelial cells. There are many studies in the world to evaluate the antimicrobial effect of essential oils on the causative factors of mastitis, and for many essential oils and their components, minimum inhibitory and minimum bactericidal concentrations have been determined.

According to study done by Choi et al. (2012), oregano essential oil injected into the inflamed quarters of the mammary gland twice a day for 3 days in a single and double dose led to an improvement of the udder condition. SCC decreased significantly compared to those before treatment, and *S. aureus* and *E. coli* were not detected in milk on the day four after treatment. In the literature, there is a small amount of data on the effect of essential oils in the treatment of bovine mastitis in Bosnia and Herzegovina. The results of our study showed that the essential oils of *Origanum compactum* and *Thymus serpyllum* have the greatest antimicrobial potential against CNS isolates, the causative factor of mastitis, while these isolates showed moderate sensitivity to the essential oil of *Origanum majorana*. *Origanum compactum* essential oil also showed a high antimicrobial effect on *Enterococcus faecalis* and *E. coli* isolates, and *Origanum majorana* essential oil had a intermediate sensitive effect. In studies comparing the antibacterial activity of essential oils with antibiotics, the MIC values of the active components of essential oils significantly exceed the MIC of antibiotics. Also results in our study indicated that the antibiotic amoxicillin/clavulanic acid shows significantly lower MIC and

MBC values compared to essential oils. Such a result was expected considering that this antibiotic is known as an extremely strong antibacterial factor. However, the antibacterial activity of essential oils should not be ignored, and they should be taken into consideration as possible alternative antibacterial factors, because it has been confirmed that they have an extremely high antimicrobial potential against the isolates tested in this study.

Many essential oils have significant antibacterial, antifungal and antiinflammatory effects. Some of their main components, such as terpinen-4-ol, act by inhibiting proinflammatory cytokine expression, stimulating the production of antiinflammatory cytokines. Such observations can be used to stimulate biotherapy against mastitis. The use of synthetic antibiotics is discouraged, as their presence in milk can have potential consequences for population health and the agri-food supply chain (Souza et al., 2013).

CONCLUSION

Based on the obtained results, the following conclusions were made:

1. From the results of somatic cells counting using the instrumental method Fossomatic apparatus (40% of unusable samples) and isolation of the causative factor (79 identified causative factors), it is clear that the samples with increased SCC were also unusable according to microbiological indicators, and that bacteria are the reason for their increase. Based on our results, and in accordance with the Regulation on raw milk, a total of 40% of the milk samples were out of classification and were withdrawn from purchase, and then the affected milk cows were adequately treated with antibiotics.
2. By examining the sensitivity of isolates to antibiotics, the highest resistance in CNS was observed to the antibiotic amoxicillin 30μg, cephalexin 30μg, doxycycline 30μg, gentamicin 30μg, lincomycin 30μg and oxytetracycline 30μg, while the highest sensitivity was observed to amoxicillin/clavulanic acid 30μg and enrofloxacin 10μg. The highest antibiotic resistance of *Enterococcus faecalis* isolates was observed to amoxicillin/clavulanic acid 30μg, amoxicillin 30μg, cephalexin 30μg, enrofloxacin 10μg, trimethoprim/sulphamethoxazole 25μg. The sensitivity of all isolates was observed to the antibiotics doxycycline 30μg and gentamicin 30μg. Testing the sensitivity of *E. coli* isolates, sensitivity to following antibiotics was observed: amoxicillin/clavulanic acid 30μg, amoxicillin 30μg, penicillin 10μg. Resistance in all isolates was observed to cephalexin 30μg, doxycycline

30µg, gentamicin 30µg, lincomycin 30µg, oxytetracycline 30µg and trimethoprim/sulphamethoxazole 25µg.

3. The greatest antimicrobial potential was shown by the essential oils of *Origanum compactum* and *Thimus serpyllum* on CNS isolates (100% sensitivity), while 40% of the isolates were sensitive to the essential oil of *Origanum majorana*, and the other isolates (60%) were intermediate sensitive to this essential oil. Isolates of *Enterococcus faecalis* (90%) were sensitive to essential oil of *Origanum compactum*, 80% of isolates to *Thymus serpyllum*, and 100% showed resistance to essential oil of *Origanum majorana*. The essential oils of *Origanum compactum* and *Thymus serpyllum* exhibited the greatest antimicrobial potential against *E. coli* isolates. *Origanum majorana* essential oil had a intermediate sensitive effect on 60% of the isolates, and 40% of the isolates were resistant to this essential oil.
4. By testing the MIC and MBC of essential oils on three isolates, *E. coli*, CNS and *Enterococcus faecalis*, it was observed that the essential oil of *O. compactum* has the strongest antimicrobial activity on all three tested isolates.
5. All three essential oils (*O. compactum*, *O. majorana* and *T. serpyllum*) tested on *E. coli* isolate 10, CNS isolate 5, *Enterococcus faecalis* isolate 10 showed mutual interaction, which means that individual components of one essential oil significantly enhance the effect of another essential oil, and give a common positive effect. An exception is one isolate of *Enterococcus faecalis*. The combination of the essential oil of *O. compactum* with *O. majorana* and the combination of *O. majorana* with *T. serpyllum* on the *Enterococcus faecalis* isolate had an indifferent effect, which means that *O. majorana*, regardless of whether it is combined with another oil, does not show antimicrobial activity to this isolate.
6. The antibacterial activity of essential oils should be taken into consideration, as it can represent an interesting choice and alternative for reducing the use of antibiotics in the treatment of animals suffering from mastitis.

Conflict of interest statement: The authors declare that there is no conflict of interest.

REFERENCES

Adkins P. R. F., Middleton J. R., Fox L. K., Pighetti G., Petersson-Wolfe C. (2017): Laboratory handbook on bovine mastitis. National Mastitis Council. 2017.

Bačić G. (2009): Diagnosis and treatment of mastitis in cows. Veterinary Faculty University of Zagreb.

Bannerman D. D. (2004): *Escherichia coli* and *Staphylococcus aureus* Elicit Differential Innate Immune Responses following Intramammary Infection. *Clinical and Diagnostic Laboratory Immunology*, 11(3):463-72.

Bauer A. W., Kirby W. M., Sherris J. C., Turck M. (1966): Antibiotic susceptibility testing by a standardized single disk method. *Tech Bull Regist Med Technol*., 36(3):49-52.

BioMérieux (2002): API 20E - Identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods. Biomerieux.

Burović J. (2020): Isolation of bovine clinical mastitis bacterial pathogens and their antimicrobial suspectibility in the Zenica region in 2017. *Veterinarska stanica*, 51:47-52.

Choi J.-Y., Damte D., Lee S.-J., Kim J.-C., Park S.-C. (2012): Antimicrobial Activity of Lemongrass and Oregano essential oil against standard antibiotic resistant and field isolates from chronic mastitis cow. *International Journal of Phytomedicine*, 4(1):134-139.

CLSI (2014): M100-S24 Performance standards for antimicrobial susceptibility testing. Clinical and Laboratory Standards Institute.

CLSI (2012): M02-A11 Performance standards for antimicrobial disk susceptibility tests. Clinical and laboratory standards institute.

Eberhart R. J. (1977): Coliform mastitis. *J. Am. Vet. Med. Assoc*., 170:1160-1163.

Fadli M., Saad A., Sayadi S., Chevalier J., Mezrioui N.-E., Pagès J.-M., Hassani L. (2012): Antibacterial activity of Thymus maroccanus and Thymus broussoneti essential oil against nosocomial infection bacteria. *Phytomedicine*, 19(5):464-471.

Lambert R. J. W., Skandamis P. N., Coote P. J., Nychas G. J. E. (2001): A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol*., 91:453-462.

Maksimović Z., Rifatbegović M. (2015): Basic principle of clinical bacteriology. Veterinary Faculty Sarajevo.

Murphy J. M. (1947): The genesis of bovine udder infection and mastitis; the occurrence of streptococcal infection in a cow population during a seven-year period and its relationship to age. *Am. J. Vet. Res*., 8;29-42.

Petrović M. D., Petrović M. M., Nenadović, G., Kurćubić V., Marinkov G. (2006): Chemical-microbial quality parameters of raw cow milk. *Biotechnology in Animal Husbandry*, 22(5-6):109-119.

Pitkälä A., Haveri M., Pyörälä S., Myllys V., Honkanen-Buzalski T. (2004): Bovine mastitis in Finland 2001-prevalence, distribution of bacteria, and antimicrobial resistance. *J. Dairy Sci.*, 87(8):2433-2441.

Regulation (1994): Regulation on the method of sampling, classification and calculation of the price of milk. Official Journal of Bosnia and Herzegovina, 1/94.

Regulation (2011): Regulation on Raw Milk. Oficial Gazzete of Bosnia and Herzegovina, 21/11.

Schuster D. E., Kchirli M. E., Rainard P., Paape M. (1997): Complement fragment C5a and inflammatory cytokines in neutrofilic recruitment during intramammary infection with E. coli. *Infect. Immun*., 65:3286-92.

Souza E. L., Oliveira C. E. V., Stamford T. L. M., Conceição M. L., Gomes Neto N. J. (2013): Influence of carvacrol and thymolon the physiological attributes, enterotoxin production and surface characteristics of *Staphylococcus aureus* strains isolated from foods. *Braz J Microbiol*., 44:29-35.

White D., Walmsley M., Liew A., Claycomb R., Mein G. (2005): Chemical and rheological aspects of gel formation in the California Mastitis Test. *Journal of Dairy Research*, 72(1):115-121.

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