Chapter

In vitro Evaluation of the Phagocytosis Activity of Neutrophils and Characterization of *Staphylococcus aureus* Mastitis in Dairy Cows of Small Family Farms

Valente Velazquez-Ordoñez, Nancy Montoya-Garcia, Ana María García-Gama, Gerardo Mancera Cuadros, Esvieta Tenorio-Borroto, Benjamín Valladares-Carranza, Carlos Bedolla-Cedeño, Patricia Cervantes-Acosta, Antonio Hernandez-Beltrán, Jorge Acosta-Dibarrat and Beatriz Silvana Carro-Techera

Abstract

A total of 269 cows in small family herds in the central region of México from different municipalities of México State were studied. Composed milk samples were obtained to detect subclinical mastitis and *S. aureus* infection and for characterization of phenotypes as follows: biotypes, capsular exopolysaccharide 5 and 8, ORSA/MRSA and MRSA strains; and *in vitro* phagocytosis neutrophil activity and apoptosis by *S. aureus* serotype 5. Results were evaluated by estimating proportions and chi-square test ($p < 0.05$). The microbial isolation rate was 46%; *S. aureus* isolation rate was 23.4–21.0% among cow herds; 39% of microbial isolates were in 1500–2500 cells/mL, with Wisconsin test. The phenotypes of *S. aureus* were: biotypes A and C are identified frequently that produce alpha and beta-hemolysin toxins, and a smaller proportion other hemolysins types. *S. aureus* isolates capsular serotypes 5 and 8 show differences in the *in vitro* neutrophil phagocytosis activity and apoptosis. The ORSA/MRSA isolates show that MRSA strains' mec A gene was confirmed by PCR. The *S. aureus* infection level in the dairy cow herds shows a wide municipal distribution, identifying different *S. aureus* pathotypes enclosed to virulence factors and MRSA to establish a potential health risk in small dairy cow herds in México.

Keywords: bovine mastitis, *Staphylococcus aureus*, neutrophil phagocytosis, milk quality, small dairy farms

1. Introduction

Bovine mastitis is a limiting disease of the production in dairy herd, commonly caused by *Staphylococcus aureus*. The inflammation of the glandular tissue constitutes a mechanism of natural resistance of the mammary gland infection in dairy cattle [1]. The appearance of inflammation in the mammary gland to increase the number of somatic cells (CCS), with an increased proportion of the leukocytes in milk is related to the agents present in the infection and the severity of the inflammatory response in the mammary gland [2, 3]. In addition, the inflammation provokes physical and chemical changes in the milk affecting the quality and its nutritional composition [4]. The significant increase in CCS in milk may be influenced by *S. aureus* herd infection level which stands out as one of the main contagious pathogens that are capable of producing a persistent infection and chronic inflammation in the mammary gland [5, 6]. The pathogenicity of *S. aureus* strains are related to the different virulence factors of the agent, considered primary in the development of mammary gland infection. The production of the alpha toxin shows a cytotoxic and cytolytic activity responsible for cases of gangrenous mastitis due to *S. aureus* [7]; the capsular exopolysaccharide is responsible for interfering with phagocytosis and complement activation [8]. In addition, colonization of udder skin in cows before parturition increases the risk of developing an intramammary postpartum infection, when the *S. aureus* infection level in the herd is high. The environment and body sites of animals are a frequent source of infection from cows with persistent *S. aureus* infection [9]. The potential risk for an epidemic colonization by *S. aureus* in the dairy herd occurs from the skin contamination of the nipple, and the udder lesions present favor the development of intraglandular infection. The first phase of lactation in cows is considered the most risky to new infections by *S. aureus* in cattle herds [10, 11]. Other environmental pathogens such as *Escherichia coli* and *Streptococcus agalactiae* increase the infection risk in fresh cows during postparturition period. The design of the milking parlors has a determining influence on the infection rate in the dairy herd and the elevated milk pipe lines producing large vacuum fluctuations that increase intramammary infection risk in dairy cows [12]. In comparison, the low pipe lines in the milking parlors reduce the proportion of new infections in the mammary glands. The risk of a cross infection increases at the milking time, when milking techniques are inappropriate or the milking equipment malfunctions. These factors increase the bacterial contamination of the nipple affecting the resistance mechanisms of the mammary gland. The reduction of the risk of bacterial colonization in the skin is achieved with the application of the nipple disinfectants, decreasing the skin contamination by *S. aureus* and other pathogens, without appreciably affecting the physical characteristics of the nipple skin. Keratin in the nipple duct is a natural barrier that reduces bacterial colonization and limits the development of the mammary gland infection [13, 14]. In the presence of mastitis, the chemical composition of keratin changes, and there is an increase in the proportion of polyunsaturated fatty acids and a decrease in the content of short-chain fatty acids [15, 16]. On the other hand, when the keratin is removed experimentally from the teat canal, the rate of infection with *Str. agalactiae* increases; consequently, there is a high excretion of bacteria in milk and an elevated somatic cell count in milk. This shows the importance of keratin as a natural barrier that contributes to reduce mammary glandular infection [17]. On the other hand, in infection of *S. aureus* in the mammary gland, significant changes of glandular tissue structure occur in which hyperplasia, stratification, and keratinization of the milk cistern and reduction of the glandular lumen are observed [18]. These changes are caused by the inflammatory

reaction and local leukocyte infiltration, with the formation of plaques of cellular debris and keratin present in the amorphous material. *S. aureus* experimental infection in dairy cows is affected by the lactation stage and the number of milk somatic cells [6]. The *S. aureus* strain type and the infective dose influence the development and the infection persistence in the mammary gland. In dairy cows with less than 4 months of lactation, when the number of somatic cells is less than 500,000 cells/mL, resistance to infection occurs. In turn, other studies indicate that the pathogenicity of *S. aureus* strains contributes in a decisive way to the development of mammary gland infection and its potential spread in the herd [19]. The persistence of infection by *S. aureus* in the udder of the cows increases the levels of antibodies present in the milk; this correlates positively with the number of lactations and the number of somatic cells in the cows [20]. The diagnosis and timely treatment decrease the evolution of clinical signs and the severity of damage to the mammary gland caused by *S. aureus, Str. agalactiae,* and *Str. uberi*s, resulting in a reduction in the incidence of bovine mastitis in the dairy herd [21]. The diagnostic tests allow early identification of physical-chemical milk changes caused by mastitis. The detection of agents, antigens, and antibodies favors the identification of an intramammary infection and its clinical evolution in the herd [22]. The diagnosis of the situation of subclinical mastitis in the herd, under a causal model based on clinical diagnosis, establishes an important association between the environmental factors and the causal conditions in the transmission of infectious agents in the population. In this case, the *S. aureus* infection is able to affect seriously the udder health and milk production. To evaluate herd infection level and to characterize *Staphylococcus aureus* phenotypes, dairy cows from small family farms in the Central Mexico region were studied.

2. Materials and methods

The diagnosis of the situation of subclinical mastitis in small dairy herds' family production was made under the causal model based on the clinical diagnosis of bovine mastitis and its association with the main infectious agents present in the herds and the *S. aureus* level infection and virulence factors in the different regions of the State of Mexico located in the Mexico central region. The determination of the *S. aureus* infection was associated to subclinical mastitis in dairy herds during the period of 2015–2017.

2.1 Determination of *Staphylococcus aureus* and frequency of subclinical mastitis

Milk samples obtained from 2749 cows of 182 dairy herds of family production in different municipal regions of the State of Mexico (Almoloya de Juarez, Zinacantepec, Chapultepec, Temoaya, Toluca, Tenango del Valle, and Lerma y Atlacomulco) were studied, to detect the mastitis frequency, the *S. aureus* herd level infection distribution, and dairy cow density. The isolation and identification of *S. aureus* in the milk samples was carried out using the protocol established by the National Mastitis Council [23]. The municipal regions of the entity were grouped taking into account the livestock inventory and the territorial extension of the municipalities studied to determine the population density expressed as the average number of cows per km^2. The population density of the region was classified as: low (BA), mean (ME), and high (AA). The results were evaluated using the proportion estimation test corresponding to the design ($p < 0.05$).

The level of association between the reaction of the Wisconsin Test and the isolation frequency of *S. aureus* in dairy herds was determined. A total of 243 milk pooled samples obtained from the four glandular quarters of the cows from different ages and lactation stages were obtained at random from small dairy herds of the Toluca Valley, Mexico. The inclusion criterion of cows in the study was a positive reaction to the mastitis Wisconsin Test [23]. The milk samples, were inoculated 0.01 mL, on blood agar plates, MacConkey and Vogel Jhonson agar plates (0.001 g/L potassium tellurite), blood agar plates CAMP-esculine, the plates were incubated at 37°C for 18–24 hours [24]. The identification of the isolates was carried out by routine bacteriological procedures Gram staining, coagulase test tube, catalase, Voges-Proskauer, and the standardized commercial systems API Staph and API 20E. The estimated number of somatic cells in milk was determined from tubes of the reaction level of the mastitis Wisconsin Test. The results were evaluated using the proportions estimation test corresponding to the design ($p < 0.05$).

2.2 *Staphylococcus aureus* isolation and phenotypic characterization

The isolation of *S. aureus* and its phenotypic characterization was carried out in a transversal longitudinal study during the fall-spring period of 2016, by randomly sampling 87 dairy family herds in the dairy system with an average herd size of 14.3 cows from different municipalities in the Valley of Toluca, State of Mexico. We obtained 1256 composed milk samples from the four glandular quarters to carry out the bacteriological study. The small-scale production system was characterized in the type of small dairy herd family production of rustic rural types. The racial phenotypes in dairy cow herds were Holstein, Creole and hybrid Holstein, European Swiss and *Bos indicus* crosses. A traditional productive management, hand dairy milking predominantly, feeding with diurnal grazing in native pastures and use of agricultural corn husks (*Zea mays*), a minimum supplementation diet with feedstuffs. Isolation of *S. aureus* was carried out by routine microbiological protocols [24]. About 0.01 mL was inoculated on Vogel Jhonson agar plates (0.001 g potassium tellurite/L). The agar plates were incubated at 37°C for 24 hours; colony forming units were identified by Gram stain, catalase, coagulase test tubes and aerobic fermentation of maltose, trehalose, and anaerobic mannitol tests. The final identification of the *S. aureus* was confirmed by API Staph system (Biomerieux Vitek, Durham, NC, USA).

The biotypes of *S. aureus* were identified from 90n isolates, those were grown on crystal violet media (brain heart infusion agar plates, added with 1:10000 violet crystal). The agar plates were incubated at 37°C for 24 hours, biotypes were identify when biotypes were identify when observing the colony forming units, associated with the biotypes A, B, C and D. The positive crystal violet reaction was considered with violet coloration and a slightly yellow halo formation. Biotype A was characterized as positive violet crystal, biotype B showed a whitish coloration, biotype C showed a yellowish color, and the absence of growth on the medium was related to biotype D.

The identification of the different types of hemolysins α, β, γ, and δ of *S. aureus* was made from the observation of hemolysis in blood agar plates supplemented with 7% erythrocytes obtained from: bovine, rabbit, equine, and human type O^+ [25]. The agar plates were incubated at 37°C for 24 hours under aerobic conditions and under a reduced atmosphere of CO_2, the type of hemolysis was observed in the different blood agar plates; they were compared with the control strains and the type was determined.

The *S. aureus* antibiotypes were characterized by *in vitro* sensitivity test performed by the agar diffusion method of National Committee for Clinical Laboratory Standards [26]. On the Mueller Hinton agar plates inoculated with the isolates of *S. aureus*, antibiotic discs were placed on the agar: penicillin 10 IU, 10 μg ampicillin, 1 μg dicloxacillin, 10 μg streptomycin, 30 μg cefotaxime, 30 μg cephalosporin, 2 μg lincomycin, 15 μg erythromycin, 30 μg novobiocin, and 100 μg spiramycin. Agar plates were incubated at 37°C for 18–24 hours. The bacterial growth inhibition halos on the agar plates were expressed in mm, compared to the bacterial growth inhibition halos of the control strain of *S. aureus* ATCC25923. The results were evaluated using the proportion estimation test corresponding to the design ($p < 0.05$).

2.2.1 Staphylococcus aureus *capsular polysaccharides characterization*

The capsular exopolysaccharide phenotypes were characterized from 90n *S. aureus* isolates obtained previously from small dairy family herds. They were studied for the expression of the capsule that was performed on Columbia agar added with 2% NaCl incubated at 37°C for 24 hours; the capsular expression was confirmed by capsule staining. The capsular type was observed in 4% whey soft agar tubes (brain and heart agar added with 4% v/v milk whey). The capsular serotype was determined with a rabbit polyclonal antiserum against the capsular serotypes 5 and 8 of *S. aureus*. The results obtained were evaluated by the Chi-square test ($p < 0.05$), based on the observed frequencies of bacterial isolation and the level of infection. The comparison between the growth inhibition halos was carried out by means of the hypothesis test of two proportions of the same group with mutually exclusive characteristics.

2.2.2 Staphylococcus aureus *capsular genes*

The identification of the cap5 and cap8 genes related to *S. aureus* capsular types was performed from isolates of *S. aureus*, using the polymerase chain reaction (PCR) test, were performed using the oligonucleotides Cap 5 k1 (5-GTCAAAGATT ATGTGATGCTACTGAG-3) and Cap 5 k2 (5-ACTTCGAATATAAACTTG AATCAATGTTATACAG-3) for the detection of the capsular typ. 5 and for the capsular typ. 8 the following primers 8 k1 (5GCCTTATGTTAGGTGATAAACC-3) 8 k2 (5-GGAAAAACACTATCATAGCAGG-3) were used, obtaining the PCR products amplified of 361 bp for cap 5 and 173 bp cap 8 [27].

2.3 *In vitro* induction of apoptosis in bovine neutrophils

The effect of the capsular serotyp. 5 of *S. aureus* on the induction of *in vitro* apoptosis of neutrophils from dairy cattle was evaluated by means of light field microscopy and smear staining method, May-Grünwald-Giemsa stain. The *in vitro* induction of apoptosis was used as a phagocytosis substrate of heat-inactivated *S. aureus* (120°C for 20 minutes) suspension (2×10^8 CFU/mL) at 4°C. The *in vitro* assay was performed in 1:10 neutrophil: bacteria ratio incubated during 1 hour, and then smears using May-Grünwald-Giemsa stain were prepared [28]. The microscopic observation of neutrophil apoptosis was confirmed under the epifluorescence microscope preparing ethidium bromide solution (100 μg/mL) and acridine orange (100 μg/mL). A number of apoptotic neutrophils were determined from the reddish coloration of the chromatin and nuclear condensation or fragmentation against viable neutrophils that showed a green coloration of the chromatin [29].

2.4 *Staphylococcus aureus* methicillin-resistant strains identification

With the identified resistant oxacillin and methicillin antibiotypes (ORSA/MRSA), the *in vitro* susceptibility test on antimicrobials with 90n *S. aureus* isolates was carried out by the agar diffusion method of National Committee for Clinical Laboratory Standards [30], *S. aureus* isolates were incubated in Mueller Hilton broth for 4 hours at 37°C, compared to McFarland standard 0.5; Mueller Hinton agar plates were inoculated by applying on the plates the antibiotic unidisks (BBL, Lawrence, KS, USA): 10 U penicillin, 30 μg ampicillin, 1 μg oxacillin, and 10 μg cephalothin. The *S. aureus* strains of ATCC25923 and ATCC 29213 and the *S. epidermidis* strain ATCC 12228 were used as controls. The strain of *S. aureus* ATCC 43300 was used as a control of methicillin resistance. To confirm the MRSA strains, Mueller Hinton agar plates containing 4% NaCl were inoculated and incubated at 35 and 42°C for 24 hours. The bacterial inhibition halos of the bacterial growth on the agar plates were expressed in mm and compared to the established values for the test with a difference >4 mm in diameter between the halos of inhibition containing unidisks with 1, 2, 4, and 6 μg of oxacicline [31]. The *in vitro* production of β-lactamase was determined by the modified iodometric method [32].

2.4.1 *Identification of the mec A gene in* Staphylococcus aureus *by polymerase chain reaction (PCR) test*

Isolation of chromosomal DNA was obtained from *S. aureus* 90n isolates, from the study groups of work strains 1, 56, 305, and 123, with the control strains of *S. epidermidis* ATCC 12228 and *S. aureus* ATCC 25923 as negative controls, and *S. aureus* ATCC 43300 as a positive control. The washed bacterial pellet was suspended in a buffered phosphate solution of pH 7.2 to proceed to bacterial lysis. The bacterial DNA solution was standardized at 0.5 ng per 1 μL and stored at −20° C. The PCR reactions were carried out with a commercial PCR kit (BioTecnologías Universitarias, México), using a thermal cycler (Genius Techne, Duckford, UK). The PCR for the identity of the *S. aureus* isolates was carried out by the amplification of nuc gene, using the oligonucleotides (Gibco BRL, Rockville, MD), Sa1 (5′GCGATTGATGGTGATACGGTT-3′), and Sa2 (5′AGCCAAGCCTTGACGAA CTAAAGC-3′) [33]; the amplification product of PCR obtained was 270 bp related to the nuc gene. The amplification of the mec A gene was performed to identify the MRSA strains of *S. aureus*, using the primers (Gibco BRL, Rockville, MD), mec1 (5′-TGGCTATCGTGTCACAATCG3′), and mec2 (5′-CTGGAACTTGTTGAGCA-GAG-3′) to obtain an amplification product of 310 bp; initial denaturation was performed at 92°C for 3 minutes and 30 cycles under the amplification conditions: 92°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, and a final extension of 2 minutes at 72°C. The amplified product was kept at 4°C, before being visualized on an agarose gel. The amplification conditions of the gene were similar with an alignment temperature of 56°C. The nuclear DNA amplification products were separated by electrophoresis in a horizontal chamber (Horizon 5B, Gibco BRL, Goithersburg, MD, USA), with a power supply of 70 Volts for 90 minutes applied to 2% agarose gel (Gibco BRL, Rockville, MD. USA), in TBE in a run with a 100 bp molecular weight marker (Gene Ruler, Fermentas, Burlington, Ontario, CA). The strains of *S. aureus* ATCC 29213, ATCC 43300, and *S. epidermidis* ATCC 12228 were used as a control. The gels were stained with ethidium bromide. The gels were photographed in a UV transluminator (UVP, Upland, CA. USA), visualized with a DC 120 digital camera (Kodak Eastman, Rochester, NY, USA) and image analysis program (ID image analysis software Windows; and 3.0, Kodak digital science, Rochester, NY, USA). The analysis of results was made from the frequency of

isolation and the expression of virulence factors of *S. aureus* related to the biotype, by estimating the absolute and relative frequency; The statistical evaluation was performed with the Pearson's Chi-square test ($\chi 2$), with an α level of 0.05 and a 95% confidence interval using Epi-Info 6.0 software (CDS, Atlanta, GA, USA). The *in vitro* sensitivity was estimated from the mean ± standard deviation of the inhibition halos (in mm) of the antibiotics.

3. Results

The *S aureus* subclinical mastitis frequency determination in dairy cow population density expressed as cows number/km^2 shows the municipalities that were identified as the regions: Low (1.12) Toluca and Metepec; and Median (2.7): Atlacomulco, Chapultepec, Lerma, Tenango del Valle, Temoaya, and Zinacantepec. The high municipal livestock density (5.6) was observed only at the municipality of Almoloya de Juárez (**Table 1**).The overall *S. aureus* rate of the isolates was 21%. The *S. aureus* infection level in the herds was higher in the municipal region of Almoloya de Juárez. The *S. aureus* infection rate showed a higher tendency when the density/km^2 of cows increased.

The bacterial isolation rate of reaction level in the Wisconsin Test obtained from 243 milk samples was 46%, the main agent isolated was *S. aureus* with an overall rate of 22.4% in the studied milk samples, and the coagulase-negative Staphylococcus frequency was 12.9% (**Table 2**). Low frequencies of other environmental pathogens and minor pathogens were identified in the bacterial isolates.

When evaluating the level of somatic cells in milk by the Wisconsin Test, a significant proportion of 39% of isolates were observed in the range 1700–2500 × 103 cells/mL of the Wisconsin Test reaction distribution and the proportion of the bacterial isolates in the population sample studied (**Table 3**).

The isolation frequency of *S. aureus* in the dairy cow herds studied was 22.8%, compared to 12.29% of coagulase negative *Staphylococcus* (SCN), observed in the study ($p < 0.001$). The identification of the types of hemolysin and their relationship with the biotypes is observed (**Table 4**). The expression of hemolysins in isolates of *S. aureus* alpha-toxin was higher than other types of the identified hemolysins.

The relationship among the *S. aureus* biotypes and hemolysin type was observed; the predominant hemolysin type was α-toxin mostly related to the biotypes C and A, and β hemolysin was observed with biotype A mainly and to a less proportion

Density (cows/km^2)	**Municipalities**	**Number of cows**	**Isolations *Staphylococcus aureus***	
				%
High [5.6]	Almoloya de Juárez	1021	268	46.2
Median [2.7]	Atlacomulco, Chapultepec, Lerma, Tenango del Valle, Temoaya, and y Zinacantepec	1237	211	36.3
Low [1.12]	Toluca, Metepec	491	101	17.4
Total		2749	580	21.0

$p < 0.01$.

Table 1.
Dairy cows density and frequency of staphylococcus aureus *isolates in municipalities of the State of Mexico.*

Agents	Isolation number	%
Staphylococcus aureus	82[a]	22.4
Staphylococcus coagulase negative	45[a]	12.29
Streptococcus agalactiae	14	3.82
Escherichia coli	6	1.63
Bacillus spp.	3	0.81
Micrococcus spp.	5	1.36
Klebsiella spp.	4	1.09
Enterobacter spp.	1	0.2
Streptococcus dysgalactiae	4	1.09
Streptococcus uberis	2	0.54
Negative isolations	197	53.82
Total	366	100

[a]*Significant differences ($p < 0.05$).*

Table 2.
Bacterial isolation frequency in dairy cows with subclinical mastitis.

Wisconsin test estimated somatic cells $\times 10^3$ mL	Bacterial isolates (%)
<100	10.0
100–500	8.0
500–1000	20.0
1000–1700	14.0
1700–2500	39.0
>2500	8.5
Total	100

[a]*Significant differences ($p < 0.05$).*

Table 3.
Distribution of mastitis Wisconsin Test reactions and bacterial isolates.

with C. The biotype C often expressed all hemolysin types: α-toxin, β, γ, and δ. The simultaneous expression among other hemolysin types such as α β and α β δ was observed in the biotype C.

The *S. aureus* antibiotypes resistant was frequent in β-lactam antibiotics with the highest observed proportion of antibiotics to the β-lactamases resistant was observed in the dicloxacillin (**Table 5**).

The distribution of *in vitro* sensitivity to antibiotics of the *S. aureus* isolates in the resistance pattern observed was: 65.7% and 90.3% for penicillin and ampicillin, 8.2% for dicloxacillin, 5.5% for cefotaxime, 6.0% for erythromycin, and 25.4% for lincomycin ($p < 0.05$). The evidence of the antibiotic resistance suggests a potential risk to health by antimicrobial resistance mainly to antibiotics β-lactam antibiotics and the possibility of identifying resistant strains ORSA/MRSA *S. aureus* methicillin and oxacillin resistant in the cow in the small family dairy herds studied [34].

The *in vitro* sensitivity to β-lactam antibiotics and the β-lactamase production was observed in a high percentage of the isolates evaluated (**Table 6**).

		Hemolysin types								
Biotype	**Total**	**α**	**β**	**γ**	**δ**	**α β**	**α δ**	**γ**	**α**	**α β δ**
A	48	9	11	0	2	19	2	0	3	1
C	36	23	4	1	1	5	3	1	2	0
B	6	0	0	3	0	0	0	0	0	0
Total	90	32	15	4	3	24	5	1	5	1
%	100	35.5	17.0	4.4	3.3	26.6	5.5	1.1	5.5	1.1

$p < 0.01$; total isolates of S. aureus *evaluated (90 n)*

Table 4.
Hemolysin types associated with biotypes of Staphylococcus aureus *in dairy herds of family production in Toluca Valley.*

Antimicrobial	**Isolations of *Staphylococcus aureus***		
	Sensitive (%)	**Intermediate (%)**	**Resistant (%)**
Penicillin	8.2	26.1	65.7
Ampicillin	8.2	1.5	90.3
Novobiocin	69.4 91.8	5.2 0.0	25.4 8.2
Dicloxacillin	90.3	1.5	8.2
Cephalosporin	8.2	40.3	5.5
Cefotaxime	91.8	2.2	6.0
Erythromycin	94.8	1.5	3.7
Spiramycin	69.4	5.2	25.4
Lincomycin	65.0	8.9	26.1

$p < 0.05$.

Table 5.
In vitro *sensitivity to* Staphylococcus aureus *antimicrobials in dairy herds of the Toluca Valley.*

The β-lactamase production was observed in relationship with β-lactam antibiotic in *S. aureus* resistant isolates mostly observed with penicillin and ampicillin. The oxacillin resistant isolates produce β-lactamase in a proportion of 20%.

The *S. aureus* capsular characterization results showed that total isolates of *S. aureus* expressed capsular exopolysaccharide phenotypes, expressed diffuse capsule, with the absence of the compact type in the milk serum soft agar in tube 63.33% (57/90) of the capsular strains were positive for serotyp. 5, 22.22% (20/90) for serotyp. 8 and 14.44% (13/90) were nontypable (NT) ($p < 0.05$). The municipal distribution of capsular serotypes 5 and 8 was similar ($p > 0.05$). In the Almoloya de Juárez region, a higher prevalence of capsular serotypes 5 and 8, 31.11 and 4.4%, respectively, was observed. In the municipality of Toluca, 1.75% *S. aureus* of the capsular serotyp. 5 was observed, indicating the absence of serotyp. 8; the results of the isolates were confirmed in the polymerase chain reaction (PCR) test (**Figure 1**).

The *S. aureus* capsular genes cap 5 and cap 8 were PCR confirmed, corresponding to the genes amplicons observed in the isolates obtained from dairy cows in small dairy family farms corresponding to the serotypes 5 and 8. In other hand, the serotypes non typiables NT, was not corresponded with the PCR evaluated amplicons.

Antimicrobial	Halo inhibition (mm) Halo growth inhibition				Strains resistant (%)	Production strains, β-lactamase (%)
	Maximum	Minimum	Average	SD±		
Penicillin	30	8	18.4	6.7	93.0	93.0
Ampicillin	29	10	17.3	5.7	93.0	93.0
Cefotaxime	30	0	19.1	10.2	7.0	59.0
Oxacicline	16	0	9.7	6.9	21.0	20.0

Table 6.
β-lactam In vitro *sensitivity of* Staphylococcus aureus *of cows with subclinical mastitis in small dairy family herds.*

The *in vitro* apoptosis of bovine neutrophils was evaluated by light field optical microscopy having a positive and negative control. The apoptosis values were 95.47 ± 3.07, compared with control groups. It was appreciated that *S. aureus* of capsular typ. 5 induced a greater proportion of neutrophils with apoptosis *in vitro*. The neutrophils apoptosis was confirmed in the May-Grunwald-Giemsa stained smears showing neutrophils with chromatin condensation and fragmentation.

On the other hand, the *in vitro* induction of apoptosis by *S. aureus* in bovine neutrophils was evaluated using light field and epifluorescence microscopy (**Figure 2**). The mean and standard deviation of the treatments are observed by the techniques of light field microscopy (CM) and fluorescence microscopy (MF); CC (+) positive control of neutrophils incubated with cyclophosphamide (400 μG/100 μL); negative control CC (−) only neutrophils; CC compact *S. aureus* strain; and CP capsular *S. aureus* strain serotyp. 5. The results showed differences between treatments ($p < 0.05$). The increased apoptosis induced by CP due to capsular serotyp. 5 was compared with the control groups. CC strain showed less *in vitro* neutrophil apoptosis induction.

The *S. aureus* mec A and nuc genes identified by PCR, results obtained from the characterization of the *S. aureus* phenotype isolates evaluated detecting the nuc gene (**Figure 3**), when confirming the MRSA strains mec A are shown with the PCR reaction products (**Figure 4**), that confirm presence of the amplicons in the *S.*

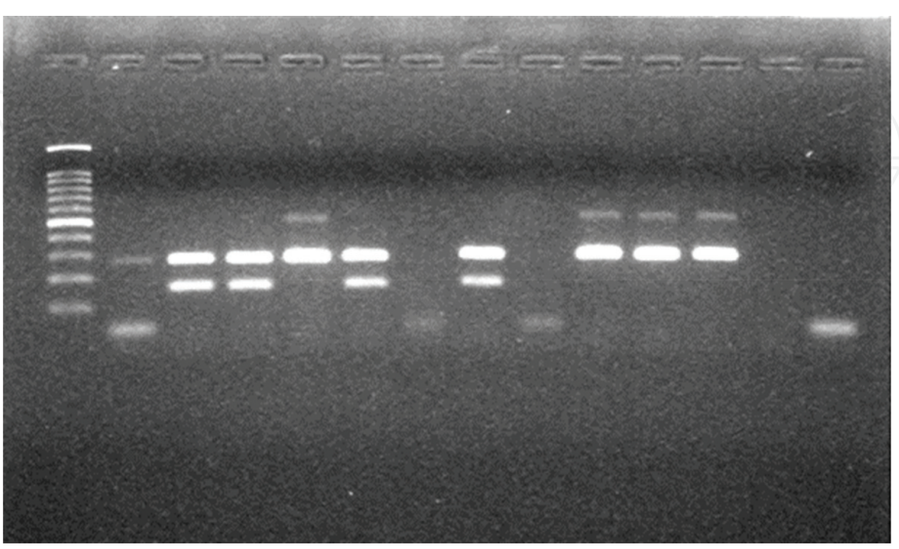

Figure 1.
Agarose gel showing the 173 bp amplicons obtained by PCR related to the cap 8 gene. Lanes 1: Molecular weight marker, 2: positive control Staphylococcus aureus *gene Nuc, 3 and 4: positive controls* Staphylococcus aureus *Cap8, 5: Positive control* Staphylococcus aureus *Cap5, 6 and 8: Positive samples of* Staphylococcus aureus *Cap8, 10–12: Positive samples for* Staphylococcus aureus *Cap5, 7, 9, 13, 14: Negative samples to* Staphylococcus aureus.

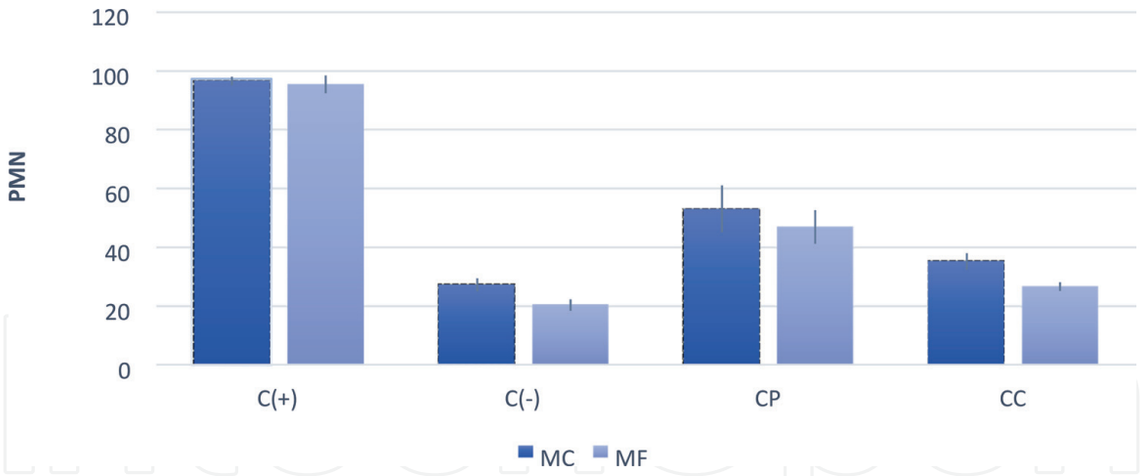

Figure 2.
Evaluation of the induction of apoptosis in vitro *in bovine neutrophils under light microscopy and epifluorescence.*

aureus isolates strains evaluated identified as *S. aureus* phenotypically as ORSA/MRSA, the nuc and mec A genes appreciated in the strains were identified is a such as 305, 123, 18, 25, 38, 44 and A53 previously identified as MRSA.

The MRSA strains were confirmed from ORSA/MRSA phenotypes detected previously; in the polymerase chain reaction (PCR) reactions, the *S. aureus* isolates were characterized by nuc gene. The MRSA isolates were identified as *S. aureus* methicillin resistant strains MRSA, identifying the mec A gene by PCR. In the ORSA/MRSA, isolates were considered to show phenotypical resistance in the different concentrations of oxacillin related to the production of β-lactamase. Those that showed resistant to 4 and 6 μg of oxacicline concentrations were confirmed by showing *in vitro* differences in the bacterial inhibition halos at 37 and 42°C, and they were considered presumptive MRSA strains. The results confirm that 90 *S. aureus* isolates were previously evaluated to detect β-lactam antibiotic-resistant phenotypes in the small dairy family farms studied, 93% the isolates produce β-lactamases and 20% of the isolates were considered ORSA/MRSA antibiotypes in which mec A gen of the MRSA strains confirmed by PCR were found.

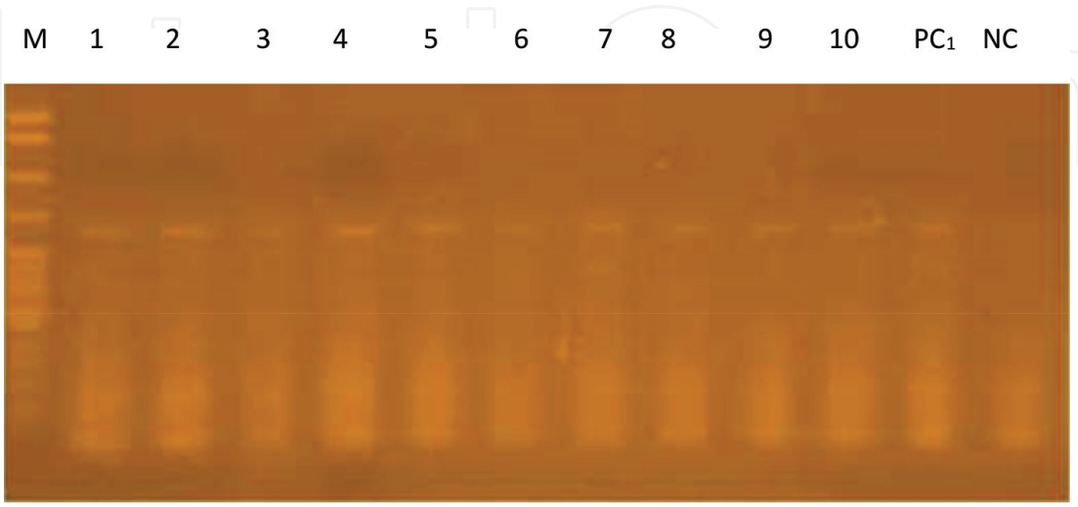

Figure 3.
PCR agarose gel electrophoresis, amplification products of the nuc gene of S. aureus *chromosomal DNA control strains ATCC 25293, ATCC 29213* S. aureus *strains as positive controls and ATCC 12228.* S. epidermidis *strain as negative control. Lanes: M, DNA manufacturer of molecular mass (100-bp ladder). Field strains; 1-8, 9-10, 305 and 113. PC1 (*S. aureus *ATCC 25293), NC (ATCC 12228* S. epidermidis*), PC2 (*S. aureus *ATCC 29213). All have an identical 270-Pb band pattern, corresponding to the* S. aureus *nuc gene.*

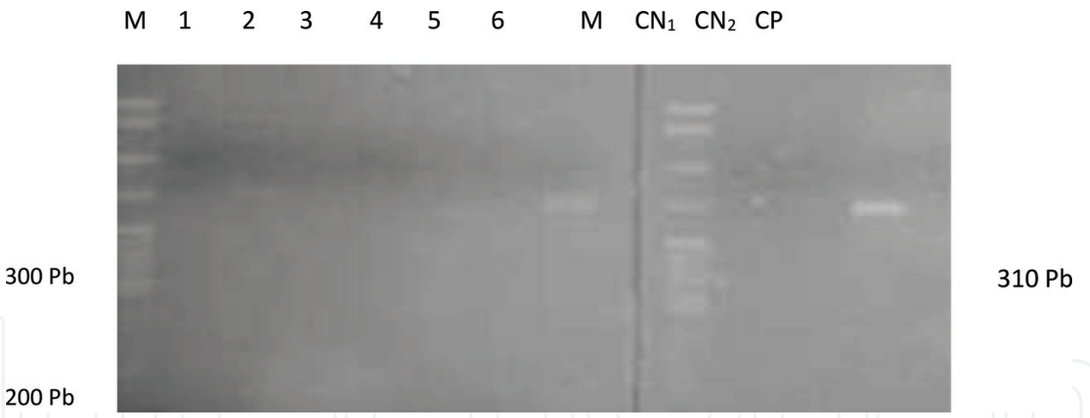

Figure 4.
Agarose gel electrophoresis shows the amplification products of the chromosomal DNA of the working strains of S. aureus*, the positive and negative controls line: M, molecular weight marker, field strains lines 1–6, the last lines correspond to SA 305, CN1 ATCC 12228* S. epidermidis *CN2 ATCC 25923 are negative controls, CP ATCC 43300* S. aureus*. SA 305 and ATCC 43300 showed a 310-bp amplicon corresponding to the mec A gene of* S. aureus*.*

4. Discussion

The wide municipal distribution of *S. aureus* mastitis and the dairy herd infection level by *S. aureus* in the studied cows were considered high as 22.4%, and *S. aureus* was identified as the main agent in the subclinical mastitis dairy cows family herds of Toluca Valley in the municipalities of central region of Mexico [35]. The herd infection level represents an important risk to the health herd and public health due to the contamination of the milk and fresh unpasteurized dairy products in the small dairy family farms in the municipal regions studied. The findings indicate the importance of *S. aureus* in the development and persistence of intramammary infection in subclinical mastitis in dairy cows [36, 37]. In it considered that dairy cows herds in family production in Mexico are widely distributed throughout the national territory, with an important contribution to regional socioeconomic development [38, 39]. In Toluca Valley, small-scale family-type production units are predominant with traditional production model, employment of family labor [40]. The danger of *S. aureus* mammary gland infection in the dairy herds occurred by poor hygiene at milking time, by increasing the bacterial contamination of the nipple and the challenge mammary gland resistance mechanisms [6, 41, 42]. In other cases, the possibility that the season of the year and the stress conditions that the cow undergoes are also indirectly affected mammary gland resistance mechanisms [43, 44]. Animal hygiene and udder health contributes to milk quality and safety food for the consumers, in opposite to subclinical mastitis, in which inflammatory reaction affects quality and milk safety [45].

Actually the importance of support of the sustainability of small cattle herds has an effect that can moderate the methane production and the adverse effects in the phenomenon of global climate change [46] because it will have a greater impact in agricultural production in geographic regions with less socioeconomic development affecting quality of life by increasing demand for food, deterioration of natural resources, water sources, and biodiversity [47]. One of the main expected effects of climate change is associated with changes in temperature and extreme weather disturbances that seriously affect the ecosystem, biodiversity, and agro-food production [48, 49]; there is a direct ecological and socioeconomic impact on human activities, the health of the human and animal population [50], and animal acclimation response in their adaptation processes [51]. Adaptive process in dairy cattle develops metabolic and behavioral physiological compensatory mechanisms to

reduce the adverse effects of climate related to the region's racial genotype [52, 53]. The risk of suffering thermal stress is increased in the animal population in certain regions with negative effects on livestock production and animal welfare [54, 55]. In extreme weather, events with a high ambient temperature, solar radiation, relative humidity and air velocity increase. Under these climatic conditions, cattle are susceptible to developing heat stress [56–59]. Thermotolerant animals expressed certain genes related to cellular stress induced by a high environmental temperature, increasing the secretion of growth hormone (b-GH), milk proteins β-casein (CSN3), and lactalbumin (LAA) [60–64].

Neutrophils phagocytosis activity in bovine mammary gland is the first line of cellular defense; its phagocytosis activity is reduced affecting its microbicidal capacity, by the presence of fat, casein [65, 66]. The bovine neutrophils are different in their capacity of phagocytosis on *S. aureus* in the mammary gland, the nipple canal and its permeability [67]. Leukocytic infiltration of the teat canal and glandular tissue occurs in response to *S. aureus*, infection at the time of mastitis [68]. Phagocytosis of leukocytes in the mammary gland shows differences in phagocytosis *in vitro*; bactericidal activity shown by neutrophils in the presence of milk whey stimulated is higher. The opsonization and intracellular killing of the *S. aureus* is affected by alpha toxin. It is possible that this effect increases intracellular survival causing failures in antibiotic therapy and mammary gland persistence infection, and the severity of the infection and the evolution of mastitis seriously affect the activity of phagocytosis with increased apoptosis and necrosis of neutrophils.

Other physiological factors of the cow modify the activity of phagocytosis in the mammary gland; during the first week of the dry period, the activity of phagocytosis increases, decreasing at the end of the dry period [69]. In other studies, one reveals a difference in the phagocytosis activity of neutrophils obtained from the glandular secretion of nulliparous and multiparous cows, when evaluating chemilumincence and peroxidase activity [70]. In lactating cows and heifers, peroxidase activation was associated with fat globules, casein, similar to that shown by zymosan phagosomes in the control group explained by the low activity of leukocyte xanthine oxidase. Other studies show differences in alkaline phosphatase of neutrophils from cows with mastitis and healthy cows. In the same way, another condition that can influence the resistance of the mammary gland is the ontogeny of the myeloid cells and their differentiation, by identifying the absence of the transferrin receptor and the expression of the antigens [43, 67, 68], BOCD11 A and BOWC5 [71]. The different surface receptors in the cell membrane of neutrophils are involved in chemotaxis, phagocytosis, and the activation of the respiratory explosion in neutrophils, evidencing the polymorphism of the functional sites of phagocytes and their modulation in phagocytosis [72]. Low neutrophils functional activity is shown at parturition, assuming an increase in susceptibility to infection at the beginning of lactation [73]. When evaluating the parameters of phagocytosis activity and milk production, a negative correlation was obtained. However, at present, there is a tendency to genetic selection of dairy cows to look for natural resistance to bovine mastitis, when choosing the progeny for the estimated data of the somatic cell count and the heritability index, evaluating the neutrophils phagocytosis activity and capacity [74].

During the *S. aureus* mammary gland infection, the somatic cells in milk are increased; these are composed of a cellular proportion of the mammary gland epithelium and another cellular portion of the leukocytes [75]. The leukocytes proportion present represent the severity inflammatory reaction affected by bovine mastitis, which causes physical-chemical and cellular alterations in milk that compromise the quality and safety of milk to increase the somatic cell count [76]. The changes that occur in milk are detected in the field and laboratory by diagnostic

techniques for detection of subclinical mastitis [77]. Other diagnostic methods for mastitis include determination of ions, leukocyte enzymes, proteins of the acute phase of inflammation, serum amyloid, and haptogloblin whose set of tests may determine the clinical course of the disease [78].

The mammary gland infection and their relationship between the average somatic cell in some cases to reflect observing persistent somatic cell counts in milk >1000,000 cells an infection with minor pathogens. The risk of intramammary infection increases when the somatic cell count of milk is >1500,000 cells/mL. When evaluating the dairy herds with high somatic cell counts in milk, the generated information is a useful collection by evolution to prevent the herd infection level [79]. Infection with *Escherichia coli* produces a pronounced increase in the somatic cell count in cows suffering from acute mastitis, in order to rapidly decline the somatic cell count after infection occurs [80, 81]. Unlike infection caused by contagious pathogens, cows have high somatic cell counts with a significantly high proportion of neutrophils [82]. The individual somatic cell count of milk and in the milk collection tank is a basic indicator of the level of mastitis in the dairy herd and health of the mammary gland of the cow [83].

The phagocytosis activity intervenes in cellular resistance and the modulation of glandular inflammation limiting the development of intraglandular mammary infection in the different stages of production of dairy cows [84]. *S. aureus* mammary gland infection in dairy cattle is important in the development of mastitis and epidemiological health risk to the population in animal and human. The phenotypic variation and genetic variability of strains of *S. aureu*s allows the expression of a greater pathogenicity potential of bacteria for the host, depending on the conditions of resistance and immunity of the gland mammary [85]. The infection by *S. aureus* is determined by the conditions of herd management and hygiene, due to the absence of measures of prevention and control of glandular disease. Intramammary infection by *S. aureus* causes a drastic reduction in production and deterioration of milk quality [86].

The infection by *S. aureus* in dairy herds develops from the contamination of the udder skin and the subsequent bacterial colonization of the nipple, which are determinants in the development of intramammary infection when proliferating the *S. aureus* in the glandular alveolus [87]. The colonization of the udder skin in cows before calving increases the risk of postpartum intramammary infection, when the level of infection in the herd by *S. aureus* is high. The production environment and the body sites of the animals are a source of infection of the agent from chronically infected cows.

The occurrence of *S. aureus* mastitis in cows increases the possibility of infection to the human population [88] through milk and unpasteurized dairy products. The pathogenicity of strains of *S. aureus* related to virulence factors are considered primary in the development of mammary infection. The production of the toxins shows a cytotoxic activity responsible for the cases of *S. aureus* mastitis. The biotypes A and C in the strains evaluated of *S. aureus* indicate their possible human and bovine origin, in which it suggests the possibility of cross infection of cow-man, extending the range of interspecies infection [89]. In isolates of *S. aureus*, the association of α and β toxins increases the cytotoxic and leukocidal capacity on neutrophils of the mammary gland, favoring the development and persistence of glandular infection [90]. The association of α hemolysin in the biotypes A and C manifests a potential risk to human health due to exotic strains of *S. aureus* of bovine origin. The capsular exopolysaccharide of *S. aureus* is responsible for interfering with phagocytosis and complement activation [67]. The capsular types of *S. aureus* predominant in dairy herds were of capsular serotypes 5 and 8. They show substantial differences in surface proteins and their ability to bind lactoferrin,

fibrinogen, fibronectin, and IgG in isolates of *S. aureus*. Different studies confirm the importance of serotypes 5 and 8 of *S. aureus* in the epidemiology of mastitis in dairy cattle [91]. The increasing occurrence of *S. aureus* strains carrying the R^+ factor to antibiotics modifies the response to antibiotic therapy. Antibiotic resistance and multidrug resistance frequently occur in isolates of *S. aureus* in livestock farms when antibiotics are used indiscriminately in drug therapy. Isolates of *S. aureus* showed resistance to β-lactam antibiotics associated with the production of β-lactamase and, to a lesser extent, related to ORSA/MRSA antibiotypes [92]. Since there is intraglandular infection due to *S. aureus*, dissemination may occur in cows and human. The mec A gene in the ORSA/MRSA phenotypes confirms a low proportion of MRSA strains in dairy cattle in family production herds. The results are similar with the low frequency reported in studies conducted in dairy herds from other countries. It is possible that human infection can occur through the consumption of dairy products contaminated with strains of animal origin and by the management of animals carrying ORSA/MRSA strains. The phenotypic expression of the virulence factors of *S. aureus* establishes additional risk conditions in the population for MRSA strains of an epidemic nature, related to their geographical distribution and genetic variability.

The MRSA strains identified in the study may be of the epidemic type (EMRSA), when related to the production of the α-toxin, which is considered a predictive marker of the virulence of *S. aureus* in EMRSA strains. The PCR amplification products demonstrated the nuc gene of *S. aureus*-specific thermonuclease in the strains evaluated. The detection of the genetic determinant of the mec A gene in the ORSA/MRSA phenotype allowed the identification of MRSA strains. The study confirms that the ORSA/MRSA phenotype carries the mec A gene that characterizes MRSA strains. The ORSA/MRSA phenotype also included the ORSA strains, which are considered sensitive methicillin (MSSA), which may include strains of β-lactamase producers called Border line that suggest a heterogeneous resistance. The use of the PCR allowed distinguishing the ORSA/MRSA isolates, the presence of MRSA strains. The procedure can be useful for the diagnosing and monitoring MRSA infection in dairy cattle [35, 93].

The case of bovine mastitis is a multifactorial disease in which several predisposing factors are identified, such as the stage of lactation, the number of births, the time of year, milking hygiene, the size, and technological level of the herd [94]. In the production environment, the presentation of subclinical mastitis is accentuated in the larger dairy herds compared with those of smaller size [95–97]. The monitory of the somatic cells in milk is an indicator of the inflammatory response of the mammary gland, and under stress, it suggests a condition of immunosuppression in cows [98, 99]. In the presence of mastitis, milk production and quality are affected by the disease presenting physicochemical and cellular alterations. When the somatic cells of total bacteria in milk increases and at the same time as it deteriorates the sanitary quality of the milk [100, 101], the poor milk quality contributes to the deterioration of the dairy products in the industrialization and increases the risk to the health of the consumers [102]. The physical-chemical alterations of milk are associated with the inflammatory reaction of the mammary gland, due to the increase in the number of leukocytes and the presence of enzymes and bacterial inhibitors that are incorporated into milk, as well as some components of blood plasma [103, 104]. The components of blood plasma contain proteases and lipases, which accelerate the decomposition of milk fat and proteins [105–107]. The increase in the number of somatic cells is related to the increase of proteins and nitrogenous substances in milk [108, 109]. The concentration of α-lactalbumin and β-lactoglobulin in milk serum decreases substantially [110]. The concentration of lactose decreases to maintain the ionic balance and the osmotic pressure of the milk,

thereby producing a variation of the mineral profile of the milk [111]. When this changes occurred, the thermotolerance of milk is reduced [112]. The glandular inflammation decreases the synthesis of casein, consequently decreasing the content of Zn, Ca, and P bound to the casein; the presence of blood serum in milk provokes an increase of the Cu, Fe, and Mn being united to serum albumin and ceruloplasmin, lactoferrin and transferrin [113, 114].

The milk pH increases from 6.6 to 7.0, due to the presence of bicarbonate, without affecting the titratable acidity and its buffer capacity and electrical conductivity, and increasing the freezing point of milk [110]. When the riboflavin and ascorbic acid concentration decreases in milk, it affects the fermentation and acidification capacity in dairy production [115]. There are many intrinsic and extrinsic factors that affect the quality of milk in the presence of mastitis, which is why a health problem is currently considered.

5. Conclusion

The *S. aureus* infection is prevalent in dairies of family production and associated to the expression of virulence factors that characterize the predominant *S. aureus* pathotypes in dairy cows in the Toluca Valley in México central region. Phenotypes and genotypes of *S. aureus* associated with the production of toxins, capsular exopolysaccharide, and MRSA strains, to establish a potential risk to health, are highlighted. The *S. aureus* infection level in the cow herds can be compromised with food safety and establishing animal and human health risk.

Acknowledgements

To the members collaborating in the project, “Red de Cuerpos Académicos en Salud Animal e Inocuidad Alimentaria: México-Uruguay”.

Author details

Valente Velazquez-Ordoñez[1*], Nancy Montoya-Garcia[1,2], Ana María García-Gama[1,2], Gerardo Mancera Cuadros[1,2], Esvieta Tenorio-Borroto[1], Benjamín Valladares-Carranza[1], Carlos Bedolla-Cedeño[3], Patricia Cervantes-Acosta[4], Antonio Hernandez-Beltrán[4], Jorge Acosta-Dibarrat[1] and Beatriz Silvana Carro-Techera[5]

1 Centro de Investigación y Estudios avanzados en Salud Animal FMVZ-UAEM, Toluca, Mexico

2 Programa de Ciencias Agropecuarias y Recursos Naturales UAEM-CONACYT, Toluca, Mexico

3 Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, Mexico

4 Facultad de Medicina Veterinaria y Zootecnia, Universidad Veracruzana, Mexico

5 Facultad de Veterinaria, Universidad de la Republica, Uruguay

*Address all correspondence to: vvo@uaemex.mx

References

[1] Gogoi-Tiwari J et al. Mammary gland pathology subsequent to acute infection with strong versus weak biofilm forming *Staphylococcus aureus* bovine mastitis isolates: A pilot study using non-invasive mouse mastitis model. PLoS One. 2017;**12**(1):e0170668

[2] Kehrli ME, Shuster DE. Factors affecting milk somatic cells and their role in health of the bovine mammary gland. Journal of Dairy Science. 1994; **77**(2):619-627

[3] Merle R, Schröder A, Hamann J. Cell function in the bovine mammary gland: A preliminary study on interdependence of healthy and infected udder quarters. The Journal of Dairy Research. 2007;**74**(2):174-179

[4] Paixão MG et al. Milk composition and health status from mammary gland quarters adjacent to glands affected with naturally occurring clinical mastitis. Journal of Dairy Science. 2017;**100**(9): 7522-7533

[5] Denis M et al. Bactericidal activity of macrophages against *Streptococcus uberis* is different in mammary gland secretions of lactating and drying off cows. Veterinary Immunology and Immunopathology. 2006;**114**(1–2): 111-120

[6] Cremonesi P et al. Response of the goat mammary gland to infection with *Staphylococcus aureus* revealed by gene expression profiling in milk somatic and white blood cells. BMC Genomics. 2012; **13**:540

[7] Valgaeren BR et al. Veal calves produce less antibodies against *C. perfringens* alpha toxin compared to beef calves. Toxins (Basel). 2015;**7**(7): 2586-2597

[8] Giovannini AEJ et al. Experimentally induced subclinical mastitis: Are lipopolysaccharide and lipoteichoic acid eliciting similar pain responses? Acta Veterinaria Scandinavica. 2017;**59**(1):40

[9] Stumpf PG, Flores M, Murillo J. Serious postpartum infection due to MRSA in an asymptomatic carrier: Case report and review. American Journal of Perinatology. 2008;**25**(7):413-415

[10] Wu J, Hu S, Cao L. Therapeutic effect of nisin Z on subclinical mastitis in lactating cows. Antimicrobial Agents and Chemotherapy. 2007;**51**(9): 3131-3135

[11] Hertl JA et al. Effects of pathogen-specific clinical mastitis on probability of conception in Holstein dairy cows. Journal of Dairy Science. 2014;**97**(11): 6942-6954

[12] Maye S et al. Bovine intra-mammary challenge with *Streptococcus dysgalactiae* spp. dysgalactiae to explore the effect on the response of complement activity. The Journal of Dairy Research. 2017; **84**(3):293-299

[13] Love SM, Barsky SH. Anatomy of the nipple and breast ducts revisited. Cancer. 2004;**101**(9):1947-1957

[14] Zucca-Matthes G, Urban C, Vallejo A. Anatomy of the nipple and breast ducts. Gland Surgery. 2016;**5**(1):32-36

[15] Strandberg Y et al. Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. Cytokine. 2005;**31**(1):72-86

[16] Pieper J, Hoedemaker M, Krömker V. Significance of the dry period for the development and prevention of new infections of the bovine mammary gland. Tierärztliche Praxis. Ausgabe G, Grosstiere/Nutztiere. 2013;**41**(5): 315-324; quiz 325

[17] Wellnitz O, Bruckmaier RM. The innate immune response of the bovine mammary gland to bacterial infection. Veterinary Journal. 2012;**192**(2):148-152

[18] Isobe N, Hosoda K, Yoshimura Y. Immunolocalization of lingual antimicrobial peptide (LAP) in the bovine mammary gland. Animal Science Journal. 2009;**80**(4):446-450

[19] Li T et al. Molecular characteristics of *Staphylococcus aureus* causing bovine mastitis between 2014 and 2015. Frontiers in Cellular and Infection Microbiology. 2017;**7**:127

[20] Boerhout EM et al. Reisolation of *Staphylococcus aureus* from bovine milk following experimental inoculation is influenced by fat percentage and specific immunoglobulin G1 titer in milk. Journal of Dairy Science. 2016; **99**(6):4259-4269

[21] Rainard P et al. Host factors determine the evolution of infection with *Staphylococcus aureus* to gangrenous mastitis in goats. Veterinary Research. 2018;**49**(1):72

[22] Heller M et al. Antigen capture ELISA using a monoclonal antibody for the detection of *Mycoplasma bovis* in milk. Veterinary Microbiology. 1993;**37** (1–2):127-133

[23] Wanner JM et al. Clinical mastitis in primiparous Holsteins: Comparisons of bovine leukocyte adhesion deficiency carriers and noncarriers. Journal of Dairy Science. 1999;**82**(11):2517-2523

[24] Boerlin P et al. Methods for identification of *Staphylococcus aureus* isolates in cases of bovine mastitis. Journal of Clinical Microbiology. 2003; **41**(2):767-771

[25] Růzicková V. A rapid method for the differentiation of *Staphylococcus aureus* hemolysins. Folia Microbiologia (Praha). 1994;**39**(2):112-114

[26] Pfaller MA et al. Proposed quality control guidelines for National Committee for Clinical Laboratory Standards Susceptibility Tests using the veterinary antimicrobial agent tiamulin. Diagnostic Microbiology and Infectious Disease. 2001;**40**(1–2):67-70

[27] Verdier I et al. Identification of the capsular polysaccharides in *Staphylococcus aureus* clinical isolates by PCR and agglutination tests. Journal of Clinical Microbiology. 2007;**45**(3): 725-729

[28] Sladek Z, Ryznarova H, Rysanek D. Macrophages of the bovine heifer mammary gland: morphological features during initiation and resolution of the inflammatory response. Anatomia, Histologia, Embryologia. 2006;**35**(2): 116-124

[29] Sládek Z, Rysánek D. Neutrophil apoptosis during the resolution of bovine mammary gland injury. Research in Veterinary Science. 2001;**70**(1):41-46

[30] d'Azevedo PA et al. Laboratory tests in the detection of extended spectrum beta-lactamase production: National Committee for Clinical Laboratory Standards (NCCLS) screening test, the E-test, the double disk confirmatory test, and cefoxitin susceptibility testing. The Brazilian Journal of Infectious Diseases. 2004;**8**(5):372-377

[31] Monsen T et al. Mueller-Hinton agar is superior to PDM blood agar for detection of methicillin-resistant *Staphylococcus aureus*. Clinical Microbiology and Infection. 2003;**9**(1): 61-64

[32] Rosenblatt JE, Neumann AM. A rapid slide test for penicillinase. American Journal of Clinical Pathology. 1978;**69**(3):351-354

[33] Ferreira DeC et al. Methicillin-resistant *Staphylococcus aureus* in HIV patients: Risk factors associated with

colonization and/or infection and methods for characterization of isolates - a systematic review. Clinics (São Paulo, Brazil). 2014;**69**(11):770-776

[34] Boyce JM et al. Induction of beta-lactamase and methicillin resistance in unusual strains of methicillin-resistant *Staphylococcus aureus*. The Journal of Antimicrobial Chemotherapy. 1990; **25**(1):73-81

[35] Hamdan-Partida A, Sainz-Espuñes T, Bustos-Martínez J. Isolation of community-acquired methicillin-resistant *Staphylococcus aureus* in healthy carriers in a Mexican community. International Journal of Infectious Diseases. 2014;**18**:22-26

[36] Fijałkowski K et al. Comparative analysis of superantigen genes in *Staphylococcus xylosus* and *Staphylococcus aureus* isolates collected from a single mammary quarter of cows with mastitis. Journal of Microbiology. 2014;**52**(5): 366-372

[37] Oliveira L et al. Enterotoxin production, enterotoxin gene distribution, and genetic diversity of *Staphylococcus aureus* recovered from milk of cows with subclinical mastitis. American Journal of Veterinary Research. 2011;**72**(10):1361-1368

[38] Sainz-Sánchez PA et al. Effect of stocking rate and supplementation on performance of dairy cows grazing native grassland in small-scale systems in the highlands of central Mexico. Tropical Animal Health and Production. 2017;**49**(1):179-186

[39] Plata-Reyes DA et al. Milk production and fatty acid profile of dairy cows grazing four grass species pastures during the rainy season in small-scale dairy systems in the highlands of Mexico. Tropical Animal Health and Production. 2018;**50**(8): 1797-1805

[40] Medina-Estrada I, López-Meza JE, Ochoa-Zarzosa A. Anti-inflammatory and antimicrobial effects of estradiol in bovine mammary epithelial cells during *Staphylococcus aureus* internalization. Mediators of Inflammation. 2016;**2016**: 6120509

[41] Wu AM et al. Role of STAT5 and epigenetics in lactation-associated upregulation of multidrug transporter ABCG2 in the mammary gland. American Journal of Physiology. Endocrinology and Metabolism. 2014; **307**(7):E596-E610

[42] Mao YJ et al. Methylation analysis of CXCR1 in mammary gland tissue of cows with mastitis induced by *Staphylococcus aureus*. Genetics and Molecular Research. 2015;**14**(4): 12606-12615

[43] Sol J et al. Factors associated with bacteriological cure during lactation after therapy for subclinical mastitis caused by *Staphylococcus aureus*. Journal of Dairy Science. 1997;**80**(11): 2803-2808

[44] Green MJ et al. Somatic cell count distributions during lactation predict clinical mastitis. Journal of Dairy Science. 2004;**87**(5):1256-1264

[45] El-Deeb W et al. Methicillin resistant *Staphylococcus aureus* among goat farms in Eastern province, Saudi Arabia: Prevalence and risk factors. Preventive Veterinary Medicine. 2018; **156**:84-90

[46] Qaderi MM, Reid DM. Methane emissions from six crop species exposed to three components of global climate change: temperature, ultraviolet-B radiation and water stress. Physiologia Plantarum. 2009;**137**(2):139-147

[47] Tyczewska A et al. Towards food security: Current state and future prospects of agrobiotechnology. Trends in Biotechnology. 2018;**12**:1219-1229

[48] Nolan C et al. Past and future global transformation of terrestrial ecosystems under climate change. Science. 2018; **361**(6405):920-923

[49] Li D et al. Vulnerability of the global terrestrial ecosystems to climate change. Global Change Biology. 2018

[50] Iqbal MS, Islam MMM, Hofstra N. The impact of socio-economic development and climate change on E. coli loads and concentrations in Kabul River, Pakistan. Science of the Total Environment. 2018;**650**(Pt 2): 1935-1943

[51] Chevin LM, Lande R, Mace GM. Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. PLoS Biology. 2010; **8**(4):e1000357

[52] Vicedo-Cabrera AM et al. A multi-country analysis on potential adaptive mechanisms to cold and heat in a changing climate. Environment International. 2018;**111**:239-246

[53] Hanna M et al. Gaps in the capacity of modern forage crops to adapt to the changing climate in northern Europe. Mitigation and Adaptation Strategies for Global ChangeMitigation and Adaptation Strategies for Global Change. 2018;**23**(1):81-100

[54] Corbett J et al. Inter-individual variation in the adaptive response to heat acclimation. Journal of Thermal Biology. 2018;**74**:29-36

[55] Bröde P et al. Estimated work ability in warm outdoor environments depends on the chosen heat stress assessment metric. International Journal of Biometeorology. 2018;**62**(3):331-345

[56] Leon LR. Common mechanisms for the adaptive responses to exercise and heat stress. Journal of Applied Physiology (Bethesda, MD: 1985). 2016; **120**(6):662-663

[57] Esperk T et al. Plastic and evolutionary responses to heat stress in a temperate dung fly: negative correlation between basal and induced heat tolerance? Journal of Evolutionary Biology. 2016;**29**(5):900-915

[58] Turk R et al. The effect of seasonal thermal stress on lipid mobilisation, antioxidant status and reproductive performance in dairy cows. Reproduction in Domestic Animals. 2015;**50**(4):595-603

[59] Battini M et al. Feasibility and validity of animal-based indicators for on-farm welfare assessment of thermal stress in dairy goats. International Journal of Biometeorology. 2016;**60**(2): 289-296

[60] Schüller LK, Burfeind O, Heuwieser W. Impact of heat stress on conception rate of dairy cows in the moderate climate considering different temperature-humidity index thresholds, periods relative to breeding, and heat load indices. Theriogenology. 2014; **81**(8):1050-1057

[61] Renaudeau D et al. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animal. 2012;**6**(5):707-728

[62] Macías-Cruz U et al. Thermoregulation of nutrient-restricted hair ewes subjected to heat stress during late pregnancy. Journal of Thermal Biology. 2013;**38**(1):1-9

[63] Liu J et al. Feeding glycerol-enriched yeast culture improves performance, energy status, and heat shock protein gene expression of lactating Holstein cows under heat stress. Journal of Animal Science. 2014; **92**(6):2494-2502

[64] Hansen PJ. Genetic variation in resistance of the preimplantation bovine embryo to heat shock. Reproduction,

Fertility, and Development. 2014;**27**(1): 22-30

[65] Della Libera AM et al. Effects of bovine leukemia virus infection on milk neutrophil function and the milk lymphocyte profile. Veterinary Research. 2015;**46**:2

[66] Grinberg N et al. Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic *Escherichia coli*. Infection and Immunity. 2008;**76**(6): 2802-2807

[67] Barrio MB, Rainard P, Poutrel B. Milk complement and the opsonophagocytosis and killing of *Staphylococcus aureus* mastitis isolates by bovine neutrophils. Microbial Pathogenesis. 2003;**34**(1):1-9

[68] Bassel LL, Caswell JL. Bovine neutrophils in health and disease. Cell and Tissue Research. 2018;**371**(3): 617-637

[69] Mukherjee R. Selenium and vitamin E increases polymorphonuclear cell phagocytosis and antioxidant levels during acute mastitis in riverine buffaloes. Veterinary Research Communications. 2008;**32**(4):305-313

[70] Li W, Jin W. Measurement of peroxidase activity in single neutrophils by combining catalyzed-enzyme reaction and epi-fluorescence microscopy. Talanta. 2006;**70**(2):251-256

[71] Li HN et al. Effects of neutrophils alkaline phosphatase on functions of neutrophils in vitro. Zhonghua Xue Ye Xue Za Zhi. 2016;**37**(5):405-411

[72] Li H et al. Critical role of neutrophil alkaline phosphatase in the antimicrobial function of neutrophils. Life Sciences. 2016;**157**:152-157

[73] Swain DK et al. Formation of NET, phagocytic activity, surface architecture, apoptosis and expression of toll like receptors 2 and 4 (TLR2 and TLR4) in neutrophils of mastitic cows. Veterinary Research Communications. 2014;**38**(3):209-219

[74] Blagitz MG et al. The neutrophil function and lymphocyte profile of milk from bovine mammary glands infected with Streptococcus dysgalactiae. The Journal of Dairy Research. 2015;**82**(4): 460-469

[75] de Haas Y, Barkema HW, Veerkamp RF. The effect of pathogen-specific clinical mastitis on the lactation curve for somatic cell count. Journal of Dairy Science. 2002;**85**(5):1314-1323

[76] Olatoye O et al. Bulk tank somatic cell count and associated microbial quality of milk from selected dairy cattle herds in Oyo State, Nigeria. Italian Journal of Food Safety. 2018; **7**(2):7130

[77] Hiitiö H et al. Elimination of experimentally induced bovine intramammary infection assessed by multiplex real-time PCR and bacterial culture. Journal of Dairy Science. 2018; **101**(6):5267-5276

[78] Gosselin VB et al. Use of MALDI-TOF to characterize staphylococcal intramammary infections in dairy goats. Journal of Dairy Science. 2018;**101**(7): 6262-6270

[79] Nyman AK et al. Sensitivity and specificity of PCR analysis and bacteriological culture of milk samples for identification of intramammary infections in dairy cows using latent class analysis. Preventive Veterinary Medicine. 2016;**135**:123-131

[80] de Haas Y et al. Associations between pathogen-specific cases of clinical mastitis and somatic cell count patterns. Journal of Dairy Science. 2004; **87**(1):95-105

[81] de Haas Y et al. Associations between somatic cell count patterns and the incidence of clinical mastitis. Preventive Veterinary Medicine. 2005; **67**(1):55-68

[82] Gonçalves JL et al. Using milk leukocyte differentials for diagnosis of subclinical bovine mastitis. The Journal of Dairy Research. 2017;**84**(3):309-317

[83] De Vliegher S et al. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention, and control. Journal of Dairy Science. 2012;**95**(3):1025-1040

[84] Curone G et al. What we have lost: Mastitis resistance in Holstein Friesians and in a local cattle breed. Research in Veterinary Science. 2018;**116**:88-98

[85] Banos G et al. The genomic architecture of mastitis resistance in dairy sheep. BMC Genomics. 2017; **18**(1):624

[86] Sun J et al. MicroRNA expression profiles of bovine milk exosomes in response to *Staphylococcus aureus* infection. BMC Genomics. 2015;**16**:806

[87] Penadés JR et al. Role of an intramammary device in protection against experimentally induced staphylococcal mastitis in ewes. American Journal of Veterinary Research. 1993;**54**(5):732-737

[88] Liu CLS, Hall AC. Optimizing the composition of irrigation fluid to reduce the potency of *Staphylococcus aureus* α-toxin: Potential role in the treatment of septic arthritis. Cartilage. September 6, 2018. DOI: 10.1177/1947603518798888

[89] Espinosa-Gongora C et al. Phenotypes and genotypes of old and contemporary porcine strains indicate a temporal change in the *S. aureus* population structure in pigs. PLoS One. 2014;**9**(7):e101988

[90] Rodríguez-Calleja JM et al. Molecular and phenotypic typing of *Staphylococcus aureus* isolates from rabbit meat. Research in Microbiology. 2006;**157**(5):496-502

[91] Vlkova H et al. Epidemiology of intramammary infections with *Staphylococcus aureus* and mastitis streptococci in a dairy cattle herd with a history of recurrent clinical mastitis. Polish Journal of Veterinary Sciences. 2017;**20**(1):133-139

[92] Nobrega DB et al. Prevalence and genetic basis of antimicrobial resistance in non-aureus staphylococci isolated from Canadian dairy herds. Frontiers in Microbiology. 2018;**9**:256

[93] Jeremias G et al. Synthesizing the role of epigenetics in the response and adaptation of species to climate change in freshwater ecosystems. Molecular Ecology. 2018;**27**(13):2790-2806

[94] McDougall S et al. A review of prevention and control of heifer mastitis via non-antibiotic strategies. Veterinary Microbiology. 2009;**134** (1-2):177-185

[95] Vanderhaeghen W et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Veterinary Microbiology. 2010;**144** (1-2):166-171

[96] Özkan Gülzari Ş, Vosough Ahmadi B, Stott AW. Impact of subclinical mastitis on greenhouse gas emissions intensity and profitability of dairy cows in Norway. Preventive Veterinary Medicine. 2018;**150**:19-29

[97] Dobson H et al. Is stress really all that important? Theriogenology. 2001; **55**(1):65-73

[98] Eitam H et al. Caloric stress alters fat characteristics and Hsp70 expression

in milk somatic cells of lactating beef cows. Cell Stress & Chaperones. 2009; **14**(2):173-182

[99] Lecchi C et al. In vitro assessment of the effects of temperature on phagocytosis, reactive oxygen species production and apoptosis in bovine polymorphonuclear cells. Veterinary Immunology and Immunopathology. 2016;**182**:89-94

[100] Sert D et al. Effects of milk somatic cell counts on some physicochemical and functional characteristics of skim and whole milk powders. Journal of Dairy Science. 2016;**99**(7):5254-5264

[101] Martí-De Olives A et al. Composition, proteolysis indices and coagulating properties of ewe milk as affected by bulk tank somatic cell count. The Journal of Dairy Research. 2015; **82**(3):344-349

[102] Delgadillo-Puga C et al. Fatty acid content, health and risk indices, physicochemical composition, and somatic cell counts of milk from organic and conventional farming systems in tropical south-eastern Mexico. Tropical Animal Health and Production. 2014; **46**(5):883-888

[103] Paape M et al. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. Journal of Mammary Gland Biology and Neoplasia. 2002;**7**(2): 109-121

[104] Burvenich C et al. Role of neutrophil polymorphonuclear leukocytes during bovine coliform mastitis: physiology or pathology? Verhandelingen - Koninklijke Academie voor Geneeskunde van België. 2004; **66**(2):97-150 discussion 150-3

[105] Bucky AR, Hayes PR, Robinson DS. Enhanced inactivation of bacterial lipases and proteinases in whole milk by a modified ultra high temperature treatment. The Journal of Dairy Research. 1988;**55**(3):373-380

[106] Hinz K et al. Proteolytic and proteomic changes in milk at quarter level following infusion with *Escherichia coli* lipopolysaccharide. Journal of Dairy Science. 2012;**95**(4):1655-1666

[107] Albenzio M et al. Activities of indigenous proteolytic enzymes in caprine milk of different somatic cell counts. Journal of Dairy Science. 2015; **98**(11):7587-7594

[108] Andrews AT. Proteinases in normal bovine milk and their action on caseins. The Journal of Dairy Research. 1983;**50**(1):45-55

[109] Le Roux Y et al. Proteolysis in samples of quarter milk with varying somatic cell counts. 2. Component PP3 and beta-casein-1P (f29-105 and f29-107) of the proteose-peptone fraction. Journal of Dairy Science. 1995; **78**(6):1298-1305

[110] Ogola H, Shitandi A, Nanua J. Effect of mastitis on raw milk compositional quality. Journal of Veterinary Science. 2007;**8**(3):237-242

[111] Ghedini CP et al. Replacing ground corn with incremental amounts of liquid molasses does not change milk enterolactone but decreases production in dairy cows fed flaxseed meal. Journal of Dairy Science. 2018;**101**(3): 2096-2109

[112] Basiricò L et al. Cellular thermotolerance is associated with heat shock protein 70.1 genetic polymorphisms in Holstein lactating cows. Cell Stress & Chaperones. 2011; **16**(4):441-448

[113] Warken AC et al. Mineral supplementation stimulates the immune system and antioxidant responses of dairy cows and reduces somatic cell

counts in milk. Anais da Academia Brasileira de Ciências. 2018;**90**(2): 1649-1658

[114] Zwierzchowski G, Ametaj BN. Minerals and heavy metals in the whole raw milk of dairy cows from different management systems and countries of origin: A meta-analytical study. Journal of Agricultural and Food Chemistry. 2018;**66**(26):6877-6888

[115] Svanborg S et al. The composition and functional properties of whey protein concentrates produced from buttermilk are comparable with those of whey protein concentrates produced from skimmed milk. Journal of Dairy Science. 2015;**98**(9):5829-5840