Acta Scientiarum
http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-8664
Doi: 10.4025/actascitechnol.v42i1.45231

Enterotoxins production, biofilm formation and antimicrobial resistance of *Staphylococcus aureus* strains isolated from refrigerated raw cow milk

Camila Lampugnani[1], Maike Taís Maziero Montanhini[1], Maria Emilene Martino Campos-Galvão[2], Luís Augusto Nero[2] and Luciano dos Santos Bersot[1*]

[1]Programa de Pós-graduação em Ciência Animal, Universidade Federal do Paraná, Rua Pioneiro, 2153, 85950-000, Palotina, Paraná, Brazil. [2]Programa de Pós-Graduação em Medicina Veterinária, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. *Author for correspondence. E-mail: lucianobersot@ufpr.br

ABSTRACT. This study aimed to isolate *Staphylococcus aureus* in refrigerated raw cow milk, and identify the presence of enterotoxin-expression genes, enterotoxin production and adherence ability, and antimicrobial resistance potential of the isolated strains. Fifty raw milk samples obtained in different dairy farms were analyzed for *S. aureus* and evaluated in the isolates the presence of genes associated with the production of major staphylococcal enterotoxins and biofilm formation. *In vitro* assays were also performed to evaluate the production of enterotoxins and adherence ability, and the antimicrobial resistance. One half (25/50) of raw milk samples presented coagulase-positive staphylococci and 95.2% of the isolates were confirmed to be *S. aureus*. Among them, 42.4% were carrying genes for enterotoxins production; however, only one isolate was able to produce enterotoxins. All *S. aureus* isolates were carrying at least two genes associated with biofilm formation and 95.2% isolates was able to adhere upon the *in vitro* assay. All isolates demonstrated antimicrobial resistance potential to one or more of the tested antibiotics.

Keywords: biofilm; enterotoxins; food poisoning; raw milk; staphylococci.

Received on November 5, 2018
Accepted on July 19, 2019

Introduction

Staphylococcus aureus is a Gram-positive coccal bacterium from the Staphylococcaceae family that occurs as a commensal colonizer of the mucocutaneous membranes of warm-blooded animals (Podkowik, Park, Seo, Bystroń, & Bania, 2013). This foodborne pathogen is considered one of the world's leading causes of disease outbreaks related to food consumption, being responsible for a variety of diseases (Hennekinne, 2018). Staphylococcal food poisoning (SFP) results from ingestion of staphylococcal enterotoxins in food produced by enterotoxigenic strains of staphylococci, mainly coagulase-positive staphylococci (CPS), with *S. aureus* being the most prominent (Hennekinne, 2018). This variety of disease is attributed to a number of virulence factors, including pathogenic antigens localized in the bacterial cells, enzymes, and toxins, such as staphylococcal enterotoxins (SEs) (Hu, Wang, Fang, Okamura, & Ono, 2018). This microorganism can produce 24 different SEs or SELs (staphylococcal enterotoxins like), including the five major antigenic variants: *sea*, *seb*, *sec*, *sed*, and *see* (Johler, Sihto, Macori, & Stephan, 2016). These toxins are globular single-chain proteins with molecular weights ranging from 22 to 29 kDa (Hennekinne & Dragacci, 2014).

S. aureus is also one of the most common causes of clinical mastitis and is the pathogen most frequently isolated in cases of subclinical mastitis worldwide (Beuron et al., 2014; Artursson, Söderlund, Liu, Monecke, & Schelin, 2016). Although milk-producing animals are the most important sources of *S. aureus* contamination, there are other factors that may affect the risks of contamination, such as human handling, water quality, milking equipment, and the environment (Thiran et al., 2018). Because *S. aureus* shows a strong ability and genetic potential to produce biofilms in the milking environment, this contamination can persist in the system (Kim, Chang, Rimal, Yang, & Schaefer, 2018); the cells within the biofilm are very resistant to sanitation procedures and antimicrobial agents (Lee et al., 2014). Antimicrobial agents are administered to animals either to inhibit bacterial infections or as a growth promoter in terms of performance. Excessive application of these agents has led to the emergence of resistant strains as a

growing problem in developed countries (Fair & Tor, 2014). High resistance levels of *S. aureus* have been reported against some antimicrobials, indicating the potential of this microorganism for developing resistance to antimicrobial agents (Jamali, Paydar, Radmehr, Ismail, & Dadrasnia, 2015)

In the present study, we aim to isolate *S. aureus* strains in refrigerated raw cow milk, as well as identify the genotypic (presence of genes responsible for expressing enterotoxins and adhesion ability) and phenotypic (enterotoxins production and antimicrobial resistance potential) characteristics of the isolated strains.

Material and methods

Isolation and enumeration

Fifty dairy farms located in the western region of Paraná state in southern Brazil were selected for raw milk sampling. For detection and quantification of CPS, according to Bennett and Lancette (2001), the samples were streaked onto Baird-Parker agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 5% egg yolk and tellurite. Colonies with a typical black appearance and surrounded by a clear zone were enumerated and submitted to Gram coloration and biochemical tests (coagulase production, catalase activity, anaerobic use of mannitol, thermostable nuclease production, Voges-Proskauer reaction and hemolysis).

DNA extraction and PCR amplification

The DNA amplification was conducted based on the primers described in Table 1. The thermonuclease (*nuc*) genes were amplified to identify *S. aureus* strains, the *sea*, *seb*, *sec*, *sed*, and *see* genes for the expression of enterotoxins production, and the *bap*, *icaA*, *icaD*, *sarA*, and *sasG* genes for the expression of biofilm production. New primers were designed for the detection of two genes for biofilm formation of 16S rRNA: *sarA* and *sasG*. The primer sequences were checked for specificity against available *S. aureus* genomes with the BLAST utility (Basic Local Alignment Search Tool, National Center for Biotechnology Information, Bethesda, MD, USA, available through www.ncbi.nlm.nih.gov). For these genes, the PCR conditions were as follows: denaturation at 94ºC for 1 minute, followed by 35 cycles at 94ºC for 1 minute, annealing at 55ºC for 1 minute, and extension at 72ºC for 2 minutes. For the other genes, the procedures were performed as described in Table 1. The size of the PCR products was analyzed by electrophoresis on 1.5% (w/v) agarose gel with 3X GelRed™ (Biotium Inc., Hayward, CA, USA). The positive controls for the reaction were *S. aureus* ATCC® 13565 for *sea*, ATCC® 19095 for *sec*, ATCC® 14458 for *seb*, and ATCC® 27664 for *see* and ATCC® 23235 for *sed* and *nuc* (American Type Culture Collection, Manassas, VA, USA).

Table 1. List of primers used for DNA amplification.

Target gene	Primer	Prime sequence (5'-3')	Expected size (pb)	Reference
nuc	Nuc AUR F Nuc AUR R	TCGCTTGCTATGATTGTGG GCCAATGTTCTACCATAGC	359	Sasaki et al. (2010)
sea	ESA-1 ESA-2	GAATGATATTAATTCGCATC TGCATGTTTTCAGAGTTAATC	544	Rosec and Gigaud (2002)
seb	ESB-1 ESB-2	GAATGATATTAATTCGCATC TCTTTGTCGTAAGATAAACTTC	416	Rosec and Gigaud (2002)
sec	ESC-1 ESC-2	GACATAAAAGCTAGGAATTT TCTTTGTCGTAAGATAAACTTC	416	Rosec and Gigaud (2002)
sed	SED-1 SED-2	CTAGTTTGGTAATATCTCCT TAATGCTATATCTTATAGGG	317	Johnson et al. (1991)
see	SEE-1 SEE-2	TAGATAAAGTTAAAACAAGC TAACTTACCGTGGACCCTTC	170	Johnson et al. (1991)
bap	Sasp-6m Sasp-7c	CCTATATCGAAGGTGTAGATTGCAC GCTGTTGAATTAATACTGTACCTGC	971	Cucarella et al. (2004)
icaA	icaAF icaAR	ATGGTCAAGCCAGACAGAG CGTGTTTTCAACATTTAATGCAA	188	Arciola, Collamati, Donati, and Montanaro (2001)
icaD	icaDF icaDR	ATGGTCAAGCCCAGACAGAG CGTGTTTTCAACATTTAATGCAA	198	Arciola et al. (2001)
sarA	sarAF sarAR	CGTTGTTTGCTTCAGTGATTCG GCTGTATTGACATACATCAGCGA	250	Designed for this study
sasG	sasGF sasGR	CTGCACCACAGGGTGTAGAA CCGCCTGAATTTACCAGACCT	571	Designed for this study

The isolated strains were reactivated in TSB for 16 - 18 hours at 37ºC, and their DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The DNA concentration was estimated using NanoDrop™ spectrophotometry (Thermo Scientific, Wilmington, DE, USA).

In vitro enterotoxin production

The isolates of *S. aureus* were tested for staphylococcal enterotoxin production using the kit Ridascreen® SET Total (R-Biopharm, Darmstadt, GER) according to the manufacturer's instructions. Ridascreen® SET Total is a sandwich enzyme immunoassay for the combined detection of *Staphylococcus* enterotoxins *sea*, *seb*, *sec*, *sed*, and *see* in fluid and solid foods, as well as in bacterial cultures.

Adherence capability

Adherence capability was measured according Stepanović, Vuković, Dakić, Savić, and Švabić-Vlahović (2000). All confirmed *S. aureus* isolates were inoculated into TSB and incubated for 24 hours at 35ºC. The bacterial cultures were adjusted to a 0.5 McFarland scale and aliquots (200 µL) of this suspension were inoculated into sterile 96-well polystyrene microplates for 24 hours at 35ºC. The material was fixed with 200 µL of methanol, and after 15 minutes, microplates were emptied and left to dry. Then, plates were stained with crystal violet 1% for 5 minutes. After the plates were air-dried, the dye bound to the adherent cells was solubilized with 160 µL of 33% (v/v) glacial acetic acid per microplate. The optical density (OD) of each well was measured at 492 nm using a Polaris® spectrophotometer (Celer SA, Belo Horizonte, Brazil). The quantification of adherence capability was based on the difference between the OD measurements of each microplate of isolates and that obtained for the negative control (OD_c). Following the classification proposed by Stepanović et al. (2000) based upon the ODs of bacterial films, the *S. aureus* isolates that presented the potential for adherence were considered to be: non-adherent ($OD \leqslant OD_c$), weakly adherent ($OD_c < OD \leqslant 2xOD_c$), moderately adherent ($2xOD_c < OD \leqslant 4xOD_c$), or strongly adherent ($OD > 4xOD_c$).

Antibiotic resistance or susceptibility potential

The assay to evaluate the antibiotic *in vitro* resistance or susceptibility potential of isolated strains, was performed by the brakepoint method (Höffler & Pulverer, 1984). An aliquot of 20 µL of stationary-phase bacterial cell cultures grown in TSB were added using a 96-pin replicator (Boekel Scientific, Feasterville, PA, USA) to a Mueller-Hinton agar (BD Difco™; Becton Dickinson and Company, Franklin Lakes, NJ, USA) containing each evaluated antibiotic (Sigma Chemical, St. Louis, MO, USA): streptomycin (STR; 10 µg/mL), gentamicin (GEN; 10 µg mL^{-1}), kanamycin (KAN; 30 µg mL^{-1}), ampicillin (AMP; 10 µg mL^{-1}), ciprofloxacin (CIP; 5 µg mL^{-1}), chloramphenicol (CHL; 30 µg mL^{-1}), sulfamethoxazole (SUL; 300 µg mL^{-1}), rifampin (RIF; 5 µg mL^{-1}), and vancomycin (VAN; 30 µg mL^{-1}). A standard culture of *S. aureus* (ATCC® 25923; American Type Culture Collection, Manassas, VA, USA) was used as the reference strain for quality-control purposes.

Statistical analysis

Proportions of positive:negative results for the adherence capability and antimicrobial resistance potential of *S. aureus* were compared by testing the differences among groups by the Fisher's exact Chi-Squared test. Statistical analyses were performed using the software Statistica 12.0 (Statsoft, Inc., Tulsa, OK, USA), with 95% reliability (Hill & Lewicki, 2007).

Results

One half (25/50) of raw milk samples presented CPS counts with levels ranging from 1 to 4.9 log CFU mL^{-1}. A total of 62 isolates were recovered from these samples, and 95.2% (59/62) of them were confirmed by PCR to be carrying the *nuc* gene, confirmed to be *S. aureus*. Thus, only those 59 *nuc*-gene-carrying strains were used for the following evaluations.

Twenty-five *S. aureus* strains (42.4%) were carrying some gene for the expression of enterotoxins production, *see* being the most frequently carried gene present in 22% (13/59) of the isolates, followed by *sec* or *sea* representing 15.3% (9/59) of the isolates, but no isolate carrying the *seb* gene. Five strains showed more than one enterotoxin gene for the expression of enterotoxins production (Figure 1). However, only one isolate, which was carrying alongside the *sec*, *sed*, and *see* genes, was able to produce enterotoxins within the *in vitro* assay.

Fifty-six *S. aureus* isolates (95.2%) showed adherence capability. Among all isolates, 13.5% (8/59) were classified as strongly adherent, 10.2% (6/59) moderately adherent, 69.5% (41/59) weakly adherent, and 5.1%

(3/59) non-adherent or non-biofilm producers (Table 2). All *S. aureus* isolates were carrying *icaD* and *sarA* genes for the expression of biofilm formation, whereas 88.1% (52/59) and 83.1% (49/59) of isolates were carrying *bap* and *sasG* genes, respectively (Figure 2). All strains were negative for *icaA* gen.

Table 2. Relationship between genes for biofilm formation (*bap*, *icaD*, *sasG*, and *sarA*) and the classification of the adherence capability† of *Staphylococcus aureus* strains isolated from cow milk samples (N = 59).

Carrying genes	Non-adherent	Weakly adherent	Moderately adherent	Strongly adherent
icaD-sarA	0 (0%)	0 (0%)	0 (0%)	2 (100%)^{a}
icaD-sarA-sasG	0 (0%)^{B}	3 (37.5%)^{AB}	0 (0%)^{B}	5 (62.5%)^{Aa}
bap-icaD-sarA	0 (0%)^{B}	5 (100%)^{A}	0 (0%)^{B}	0 (0%)^{Bb}
bap-icaD-sarA-sasG	3 (6.8%)^{B}	33 (75%)^{A}	7 (15.9%)^{B}	1 (2.3%)^{Bb}
Total	3 (5.1%)^{B}	41 (69.5%)^{A}	7 (11.9%)^{B}	8 (13.6%)^{B}

†According to the classification proposed by Stepanović et al. (2000). ^{Aa} Different small letters in the same column or different capital letters in the same row indicates significant differences among proportions by Chi-Squared test.

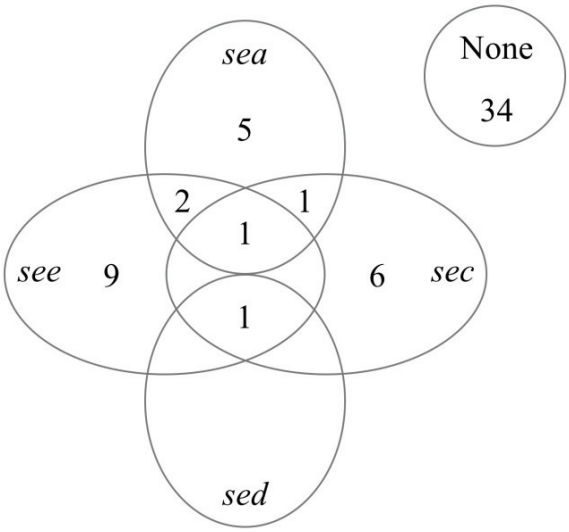

Figure 1. Grouping plot of *Staphylococcus aureus* strains isolated from cow milk samples (N=59) carrying the genes for enterotoxin production (*sea*, *sec*, *sed*, and *see*).

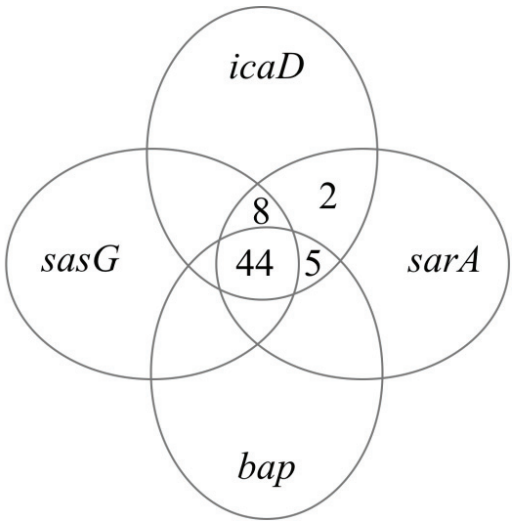

Figure 2. Grouping plot of *Staphylococcus aureus* strains isolated from cow milk samples (N=59) carrying the genes for biofilm formation (*bap*, *icaD*, *sasG*, and *sarA*).

All *S. aureus* isolates demonstrated potential resistance to one or more antibiotics at their respective dosages in this study. The highest ($p < 0.05$) resistance potential was seen against sulfamethoxazole in 94.9% (56/59) of the isolates, followed by streptomycin in 81.3% (48/59), ciprofloxacin in 49.1% (29/59), ampicillin in 35.6% (21/59), gentamicin in 32.2% (19/59), kanamycin in 18.6% (11/59), and rifampin in 8.5% (5/59). On the other hand, all isolates showed susceptibility potential to chloramphenicol and vancomycin

(Table 3). Besides, most *S. aureus* isolates were resistant to more than one tested antibiotic, with 27.1% (16/59) resistant to two different antibiotics, 20.3% (12/59) resistant to three, 18.6% (11/59) resistant to four, 13.5% (8/59) resistant to five, and 8.5% (5/59) resistant to six antibiotics.

Table 3. Antibiotic resistance potential of *Staphylococcus aureus* strains isolated from cow milk samples (N = 59), arranged by those antibiotics with more potential.

Antibiotic (dosage)	Resistant strains	Susceptible strains	Resistance potential
Sulfamethoxazole (300 μg mL^{-1})	56	3	94.9%[a]
Streptomycin (10 μg mL^{-1})	48	11	81.4%[b]
Ciprofloxacin (5 μg mL^{-1})	29	30	49.2%[c]
Ampicillin (10 μg mL^{-1})	21	38	35.6%[cd]
Gentamicin (10 μg mL^{-1})	19	40	32.2%[cd]
Kanamycin (30 μg mL^{-1})	11	48	18.6%[de]
Rifampin (5 μg mL^{-1})	5	54	8.5%[ef]
Chloramphenicol (30 μg mL^{-1})	None	59	0.0%[f]
Vancomycin (30 μg mL^{-1})	None	59	0.0%[f]

[a] Different letters in the same column indicates significant differences among proportions by Chi-Squared test.

Discussion

According to Artursson et al. (2016), *S. aureus* is one of the most common bacteria causing bovine mastitis and is frequently isolated from milk produced by cows with mastitis. Several studies reported a high prevalence of *S. aureus* in raw milk (Mehli, Hoel, Thomassen, Jakobsen, & Karlsen, 2017; Obaidat, Roess, Mahasneh, & Al-Hakimi, 2018). In this study, we have found a significant prevalence (50%) of this microorganism in raw milk samples. Still, we can't state that the average contamination level of positive samples (up to 5 log CFU mL^{-1}) is considered to be non-compliant with local regulation, because the Brazilian legislation has no specifications for *S. aureus* in raw milk. The European Union countries local legislation establishes that the maximum allowed count for *S. aureus* in raw milk intended for direct human consumption is 2.7 log CFU mL^{-1} or, if intended for further processing, 3.3 log CFU mL^{-1} (EU, 1992). Therefore, if enterotoxinogenic staphylococci are able to grow in raw milk to high numbers (more than 5 log CFU mL^{-1}) before they are killed by thermic processing, there is still a risk to consumers of dairy products (Kadariya, Smith, & Thapaliya, 2014).

Staphylococcal enterotoxin-associated outbreak has always been an irrefutable threat to farm dairies, and frequent reports of raw milk-product contamination with different serotypes of *S. aureus* clearly demonstrate the impact of this pathogen on dairy production (Beuron et al., 2014; Jamali et al., 2015). We analyzed five major *S. aureus* enterotoxins (SEs); a significant number of isolates in this study contained one or more of these enterotoxin genes, particularly the *see* gene. The majority of studies working on SEs isolated from across several sources of food, including raw milk or dairy products, have reported different frequencies for each gene (Khoramrooz et al., 2016). Mehli et al. (2017) identified sixteen different toxin gene profiles in 87.5% of isolates, with *sec* and *tst* being the most frequent (52.5%), followed by *seg* and *seh*. Carfora et al. (2015) found 57.4% of 54 samples tested positive for enterotoxin genes; the genes most frequently detected were *sed*, present in 40% of isolates, followed by *sec* (34.3%), *sea* and *selj* (both present in 31.4% of isolates) and *ser* (28.6%); none of the isolates had *see* or *selp* genes. Obaidat et al. (2018) found 19.5% (33/169) of *S. aureus* isolates with enterotoxin genes; specifically, *sec* was the most frequently carried gene (25/169; 14.8%), followed by *sei* (9/169, 5.3%) and *seg* (5/169, 3%). Two isolates and one isolate carried the *sea* and *sed* genes, respectively; none of the isolates carried *seb*, *see,* or *seh*. In a classical epidemiological study in the UK, the analysis of 120 *S. aureus* strains involved in foodborne intoxication showed that 113 (94.2%) of outbreaks resulted from *sea-see* enterotoxins; therefore, about 5% did not. Among those 113, 88 (77.9%) of the enterotoxinogenic strains produced *sea* and 48 (42.5%) *sed*, either alone or together with another toxin.

Enterotoxigenic *S. aureus* often differs in the number of mobile genetic elements and SEs/SELs genes therein, as well as in the enterotoxins they produce. The *sea* gene, either alone or together with other SEs/SELs, is the enterotoxin most commonly reported in foods, and is also considered the main cause of SFP (Argudín, Mendoza, & Rodicio, 2010). In the present study only 1.7% *S. aureus* isolates were a toxin producer *in vitro*. Morandi, Brasca, Lodi, Cremonesi, and Castiglioni (2007) detected 67% *S. aureus* strains with some enterotoxin genes, but only 52% produced a detectable number of enterotoxins. Non-

correspondence was observed between the presence of the enterotoxin genes and their expression. This finding is in disagreement with the results of investigators who reported a positive correlation between PCR assays and immunoassay methods (Cremonesi et al., 2005). Therefore, the presence of enterotoxin genes does not imply their expression in any condition. Detection of SEs genes without determining the status of their expression should be considered with caution. According to Valihrach, Alibayov, Zdenkova, and Demnerova (2014), the milk environment dramatically changes the expression profiles of several enterotoxin genes, although staphylococcal growth is not affected. The mechanism of the reduction may be explained by inhibitory effect over the regulatory genes. To assess real safety hazards, the phenotype ought to play a crucial role, rendering genotypic studies a screening tool (Podkowik et al., 2013). The data obtained in this study probably underestimated the enterotoxigenic properties of the evaluated strains, since the possible presence of newly described SEs was not considered.

S. aureus is a strong biofilm producer. Its ability in biofilm formation is a virulence factor and facilitates the adhesion and colonization of microorganisms on mammary epithelium cells, evasion from host immunological response, and resistance against antibiotics. There is a possibility of correlation between enterotoxin genes with biofilm formation (Khoramrooz et al., 2016). Khoramrooz et al. (2016) found significant associations between the presence of *ica*D, *ica*A, *fnb*A, *clf*A, and *can* genes and biofilm formation. On the other hand, Marques et al. (2017) did not observe a relation between the intensity of biofilm formation and presence of *icaA* and *icaD* genes. In a study with 215 *S. aureus* strains collected from human and dairy cow infections, Khoramian, Jabalameli, Niasari-Naslaji, Taherikalani, and Emaneini (2015) found that approximately 70% of the isolates were able to produce biofilm and most (59.3%) were producers of weakly adherent biofilms. The most prevalent gene was *icaD* and *icaA*, found in 88.4% and 87.9% of the isolates, respectively; however, the authors did not detect *bap* genes in any of the isolates. Castelani, Pilon, Martins, Pozzi, and Arcaro (2015) detected the *icaA* and *icaD* genes in 98% and 100% of 110 *S. aureus* isolates, respectively; however, author reported that only 55.5% of all isolates produced a biofilm on Congo red agar. Several environmental factors, including glucose, oleic acid, osmolality, temperature, and anaerobiosis, have been reported to affect biofilm formation in *S. aureus* (Xue, Chen, & Shang, 2014).

There are contradictory results between the proportion of isolates with the ability to form biofilm detected by phenotypic tests and the presence of specific genes associated with adhesion and biofilm formation detected in different studies. This suggests that there is no direct correlation between the different methods or among the currently established classification criteria. Factors including incubation time, growth surface, and nutrients are thought to influence biofilm formation in staphylococci measured by static or dynamic model assays. On the other hand, the detection of a limited number of genes as a criterion to characterize an isolate as a biofilm producer should be revised, since their presence does not always correlate with the biofilm-producing phenotype presented by the same isolates (Thiran et al., 2018).

It has been widely noted that *S. aureus* is an extraordinarily adaptable pathogen with a proven ability to develop resistance to antimicrobials (Chambers & DeLeo, 2009; Jamali et al., 2015). Actually, it can exemplify better than any other pathogen the adaptive evolution of bacteria in the antibiotic era (Pantosti, Sanchini, & Monaco, 2007). In our study, isolates showed high resistance potential to sulfamethoxazole, streptomycin, ciprofloxacin, ampicillin, gentamicin and, in less significant levels, to kanamycin and rifampin. The high number of isolates with a multidrug-resistance phenotype is another notable finding of this study. Other reports also indicate a high percentage of multidrug-resistant *S. aureus* isolates from animals (Castelani et al., 2015; Marques et al., 2017). According to Chambers and DeLeo (2009), this microorganism is naturally susceptible to virtually every antibiotic that has ever been developed; however, *S. aureus* has demonstrated a unique ability to quickly respond to each new antibiotic with various resistance mechanisms, starting with penicillin and methicillin and up to the most recently developed antibiotics. Resistance mechanisms include enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes), alteration of the target with decreased affinity for the antibiotic (e.g. penicillin-binding protein of methicillin-resistant strains and peptidoglycan precursors of vancomycin-resistant strains), trapping of the antibiotic (for vancomycin and possibly daptomycin), and efflux pumps (fluoroquinolones and tetracycline). Complex genetic arrays have been acquired by *S. aureus* through horizontal gene transfers, while resistance to other antibiotics, including some of the most recent ones (e.g. fluoroquinolones, linezolid and daptomycin), have developed through spontaneous chromosomal mutations and positive selection via bacterial competition (Pantosti et al., 2007).

The presence of antibiotic-resistant *S. aureus* strains in milk and dairy products and its transmission via contaminated food is a public health concern (Jamali et al., 2015). The difficult therapeutic problem of antibiotic-resistant *S. aureus* is an important example of the diminishing efficacy of antimicrobial agents for the treatment of bacterial infections. This trend is particularly alarming because of the severity and diversity of disease caused by this uniquely versatile pathogen. Our understanding of the forces that direct the evolution of virulent and antibiotic-resistant *S. aureus* is imperfect, but overuse and misuse of antibiotics in veterinary practice is clearly the major contributing factor. Discovery and development of new antimicrobials, while necessary, is unlikely to solve the problem of drug resistance for very long. New technologies leading to improved and more rapid and precise diagnostics, a better understanding of pathogenesis of staphylococcal mastitis in dairy cows, and alternative approaches to the prevention and treatment of infection will also be needed to overcoming the non-antibiotic-dependent practice (Chambers & DeLeo, 2009).

Conclusion

Our study suggests that the contamination of raw cow milk by *S. aureus* is a critical issue for the dairy industry, and emphasizes the need to consider not only the control of this microorganism by the use of antibiotics, which has been shown to be less and less effective as long as these bacteria develop news means of resistance against the principal antibiotics used in veterinary practice, but also the interactions between this bacteria and the dairy environment - such as their adherence ability in milking equipment - and their capacity to release enterotoxins in the milk, which can easily reach the final consumers through industrial procedures incapable of effectively inactivating these toxins.

Acknowledgements

This study was funded by Brazilian Coordination for the Improvement of Higher Education Personnel (CAPES; grants 88881.068400/2014-01 and 88887.175768/2018-00).

References

Arciola, C. R., Collamati, S., Donati, E., & Montanaro, L. (2001). A rapid PCR method for the detection of slime-producing strains of *Staphylococcus* epidermidis and *S. aureus* in periprosthesis infections. *Diagnostic Molecular Pathology*, *10*(2), 130-137. DOI: 10.1097/00019606-200106000-00010

Argudín, M. Á., Mendoza, M. C., & Rodicio, M. R. (2010). Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins*, *2*(7), 1751-1773. DOI: 10.3390/toxins2071751

Artursson, K., Söderlund, R., Liu, L., Monecke, S., & Schelin, J. (2016). Genotyping of *Staphylococcus aureus* in bovine mastitis and correlation to phenotypic characteristics. *Veterinary Microbiology*, *193*(1), 156-161. DOI: 10.1016/j.vetmic.2016.08.012

Bennett, R. W., & Lancette, G. A. (2001). *Staphylococcus aureus*. In L. A. Tomlinson, & D. H. Hughley (Eds.), *Bacteriological analytical manual* (p. 1-5). Silver Spring, US: U.S. Food and Drug Administration.

Beuron, D. C., Cortinhas, C. S., Botaro, B. G., Macedo, S. N., Gonçalves, J. L., Brito, M. A. V. P., & Santos, M. V. (2014). Risk factors associated with the antimicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis. *Pesquisa Veterinária Brasileira*, *34*(10), 947-952. DOI: 10.1590/S0100-736X2014001000004

Carfora, V., Caprioli, A., Marri, N., Sagrafoli, D., Boselli, C., Giacinti, G., ... Amatiste, S. (2015). Enterotoxin genes, enterotoxin production, and methicillin resistance in *Staphylococcus aureus* isolated from milk and dairy products in Central Italy. *International Dairy Journal*, *42*(1), 12-15. DOI: 10.1016/j.idairyj.2014.10.009

Castelani, L., Pilon, L. E., Martins, T., Pozzi, C. R., & Arcaro, J. R. P. (2015). Investigation of biofilm production and *icaA* and *icaD* genes in *Staphylococcus aureus* isolated from heifers and cows with mastitis. *Animal Science Journal*, *86*(3), 340-344. DOI: 10.1111/asj.12284

Chambers, H. F., & DeLeo, F. R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews Microbiology*, *7*(9), 629-641. DOI: 10.1038/nrmicro2200

Cremonesi, P., Luzzana, M., Brasca, M., Morandi, S., Lodi, R., Vimercati, C., ... Castiglioni, B. (2005). Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. *Molecular and Cellular Probes*, *19*(5), 299-305. DOI: 10.1016/j.mcp.2005.03.002

Cucarella, C., Tormo, M. Á., Úbeda, C., Trotonda, M. P., Monzón, M., Peris, C., ... Penadés, J. R. (2004). Role of biofilm-associated protein bap in the pathogenesis of bovine *Staphylococcus aureus*. *Infection and Immunity*, *72*(4), 2177-2185. DOI: 10.1128/IAI.72.4.2177-2185.2004

EU (1992). *Health rules for the production and placing on the market of raw milk, heat-treated milk and milk-based products*, Council Directive 92/46/EEC. Paris, FR: Council of the European Communities.

Fair, R. J., & Tor, Y. (2014). Antibiotics and bacterial resistance in the 21st century. *Perspectives in Medicinal Chemistry*, *6*(1), 25-64. DOI: 10.4137/PMC.S14459

Hennekinne, J. A. (2018). *Staphylococcus aureus* as a leading cause of foodborne outbreaks worldwide. In A. Fetsch (Ed.), *Staphylococcus aureus* (p. 129-146). London, UK: Academic Press.

Hennekinne, J. A., & Dragacci, S. (2014). Staphylococcal enterotoxins. In D. Liu (Ed.), *Manual of security sensitive microbes and toxins* (p. 533-544). Boca Raton, US: Taylor & Francis Group.

Hill, T., & Lewicki, P. (2007). *Statistics: Methods and applications.* Tulsa, US: StatSoft Inc.

Höffler, U., & Pulverer, G. (1984). Antibiotic sensitivity testing of anaerobic bacteria by the Breakpoint Method. *Chemotherapy*, *30*(6), 392-397. DOI: 10.1159/000238299

Hu, D. L., Wang, L., Fang, R., Okamura, M., & Ono, H. K. (2018). *Staphylococcus aureus* enterotoxins. In A. Fetsch (Ed.), *Staphylococcus aureus* (p. 39-55). London, UK: Academic Press.

Jamali, H., Paydar, M., Radmehr, B., Ismail, S., & Dadrasnia, A. (2015). Prevalence and antimicrobial resistance of *Staphylococcus aureus* isolated from raw milk and dairy products. *Food Control*, *54*(1), 383-388. DOI: 10.1016/j.foodcont.2015.02.013

Johler, S., Sihto, H. M., Macori, G., & Stephan, R. (2016). Sequence variability in staphylococcal enterotoxin SEB, SEC, and SEB. *Toxins*, *8*(6), 169. DOI: 10.3390/toxins8060169

Johnson, W. M., Tyler, S. D., Ewan, E. P., Ashton, F. E., Pollard, D. R., & Rozee, K. R. (1991). Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. *Journal of Clinical Microbiology*, *29*(3), 426-430. DOI: 0095-1137/91/030426-05$02.00/0

Kadariya, J., Smith, T. C., & Thapaliya, D. (2014). *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. *BioMed Research International*, 2014, 827965. DOI: 10.1155/2014/827965

Khoramian, B., Jabalameli, F., Niasari-Naslaji, A., Taherikalani, M., & Emaneini, M. (2015). Comparison of virulence factors and biofilm formation among *Staphylococcus aureus* strains isolated from human and bovine infections. *Microbial Pathogenesis*, *88*(1), 73-77. DOI: 10.1016/j.micpath.2015.08.007

Khoramrooz, S. S., Mansouri, F., Marashifard, M., Malek Hosseini, S. A. A., Akbarian Chenarestane-Olia, F., Ganavehei, B., ... Darban-Sarokhalil, D. (2016). Detection of biofilm related genes, classical enterotoxin genes and agr typing among *Staphylococcus aureus* isolated from bovine with subclinical mastitis in southwest of Iran. *Microbial Pathogenesis*, *97*(1), 45-51. DOI: 10.1016/j.micpath.2016.05.022

Kim, S. J., Chang, J., Rimal, B., Yang, H., & Schaefer, J. (2018). Surface proteins and the formation of biofilms by *Staphylococcus aureus*. *Biochimica et Biophysica Acta. Biomembranes*, *1860*(3), 749-756. DOI: 10.1016/j.bbamem.2017.12.003

Lee, S. H. I., Mangolin, B. L. C., Gonçalves, J. L., Neeff, D. V., Silva, M. P., Cruz, A. G., & Oliveira, C. A. F. (2014). Biofilm-producing ability of *Staphylococcus aureus* isolates from Brazilian dairy farms. *Journal of Dairy Science*, *97*(3), 1812-1816. DOI: 10.3168/jds.2013-7387

Marques, V. F., Motta, C. C., Soares, B. S., Melo, D. A., Coelho, S. M. O., Coelho, I. S., ... Souza, M. M. S. (2017). Biofilm production and beta-lactamic resistance in Brazilian *Staphylococcus aureus* isolates from bovine mastitis. *Brazilian Journal of Microbiology*, *48*(1), 118-124. DOI: 10.1016/j.bjm.2016.10.001

Mehli, L., Hoel, S., Thomassen, G. M. B., Jakobsen, A. N., & Karlsen, H. (2017). The prevalence, genetic diversity and antibiotic resistance of *Staphylococcus aureus* in milk, whey, and cheese from artisan farm dairies. *International Dairy Journal*, *65*(1), 20-27. DOI: 10.1016/j.idairyj.2016.10.006

Morandi, S., Brasca, M., Lodi, R., Cremonesi, P., & Castiglioni, B. (2007). Detection of classical enterotoxins and identification of enterotoxin genes in *Staphylococcus aureus* from milk and dairy products. *Veterinary Microbiology*, *124*(1), 66-72. DOI: 10.1016/j.vetmic.2007.03.014

Obaidat, M. M., Roess, A. A., Mahasneh, A. A., & Al-Hakimi, R. A. (2018). Antibiotic-resistance, enterotoxin gene profiles and farm-level prevalence of *Staphylococcus aureus* in cow, sheep and goat bulk tank milk in Jordan. *International Dairy Journal*, *81*(1), 28-34. DOI: 10.1016/j.idairyj.2018.02.001

Pantosti, A., Sanchini, A., & Monaco, M. (2007). Mechanisms of antibiotic resistance in *Staphylococcus aureus*. *Future Microbiology*, *2*(3), 323-334. DOI: 10.2217/17460913.2.3.323

Podkowik, M., Park, J. Y., Seo, K. S., Bystroń, J., & Bania, J. (2013). Enterotoxigenic potential of coagulase-negative staphylococci. *International Journal of Food Microbiology*, *163*(1), 34-40. DOI: 10.1016/j.ijfoodmicro.2013.02.005

Rosec, J. P., & Gigaud, O. (2002). Staphylococcal enterotoxin genes of classical and new types detected by PCR in France. *International Journal of Food Microbiology*, *77*(1), 61-70. DOI: 10.1016/S0168-1605(02)00044-2

Sasaki, T., Tsubakishita, S., Tanaka, Y., Sakusabe, A., Ohtsuka, M., Hirotaki, S., ... Hiramatsu, K. (2010). Multiplex-PCR method for species identification of coagulase-positive staphylococci. *Journal of Clinical Microbiology*, *48*(3), 765-769. DOI: 10.1128/JCM.01232-09

Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, *40*(2), 175-179. DOI: 10.1016/S0167-7012(00)00122-6

Thiran, E., Di Ciccio, P. A., Graber, H. U., Zanardi, E., Ianieri, A., & Hummerjohann, J. (2018). Biofilm formation of *Staphylococcus aureus* dairy isolates representing different genotypes. *Journal of Dairy Science*, *101*(2), 1000-1012. DOI: 10.3168/jds.2017-13696

Valihrach, L., Alibayov, B., Zdenkova, K., & Demnerova, K. (2014). Expression and production of staphylococcal enterotoxin C is substantially reduced in milk. *Food Microbiology*, *44(1)*, 54-59. DOI: 10.1016/j.fm.2014.05.020

Xue, T., Chen, X., & Shang, F. (2014). Effects of lactose and milk on the expression of biofilm-associated genes in *Staphylococcus aureus* strains isolated from a dairy cow with mastitis. *Journal of Dairy Science*, *97*(10), 6129-6134. DOI: 10.3168/jds.2014-8344