

# Virulence Factor Profile of *Staphylococcus aureus* Isolated from Bovine Milk from Brazil

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## Abstract

This study investigates the biofilm formation, presence and distribution of virulence genes and the capacity to induce an inflammatory response in strains of *Staphylococcus aureus* isolated from milk samples in Bahia, Brazil. A total of 132 samples of raw milk were collected from four dairy farms (designated A to D) located in southwestern Bahia, in the municipality of Vitória da Conquista, from October/2009 to September/2010. After processing of the samples, 94 (71.2%) *S. aureus* isolates were obtained. These strains were subjected to the antibiogram method MIC (Minimum Inhibitory Concentration). As for the pathogenicity, tests were performed *in vitro* biofilm formation induced by glucose. Moreover, we performed PCR for their virulence genes: *sea* (enterotoxin A), *seb* (B), *sec* (C), *pvl* (Panton-Valentine Leukocidin), *clfA* (Clumping Factor A) and *spa* (protein A) and analysis of cytokine induction in the inflammatory response of J774 macrophages by exocellular lipoteichoic acid. No isolates were resistant to oxacillin and vancomycin. In biofilm production, 5.31% (5/94) isolates did not produce biofilm, 5.31% (5/94) of the samples were poor producers, 15.96% (15/94) strains were moderate producers, 18.09% (17/94) were producers and 55.32% (55/94) of isolates were strong biofilm producers. One (1.06%) isolate expressed the *seb* gene, one (1.06%) *sec*, 18 (19.2%) *clfA* and 44 (46.8%) had *spa*. There was no expression of *sea* and *pvl* between isolates analyzed. The analysis of cytokine induction in the inflammatory response did not show statistical difference in the levels of IL-6, TNF- $\alpha$  and IL-10 induction. However, there was statistical difference in IL-1 induction between isolates from different farms. Thus, it

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appears that the results obtained in this study show significant effects for the region studied, since it is an important dairy region, hence the need for further studies, with the intent of attracting funding that contributes to improving prevention and control in both dairy farms and dairy industries, since milk contamination poses a serious potential health risk to consumers.

## Keywords

Milk, *Staphylococcus aureus*, Resistance, Virulence Factors

## 1. Background

Milk is considered an outstanding food source, as it is rich in proteins, fats, carbohydrates, minerals and vitamins. Yet the quality of the milk produced is often a major barrier to its marketing [1] [2]. The quality of the milk produced is often a major barrier in their marketing. Milk considered to be of good quality must have satisfactory organoleptic, nutritional, sensory, microbiological and physical-chemical characteristics.

Due to its constitution, milk is an excellent culture medium for the growth of microorganisms, and may be responsible for transmission of harmful zoonoses. Therefore, its quality is an important public health concern [3]. *Staphylococcus aureus* is a pathogen that can be transmitted by milk and dairy products, which is of major concern in the epidemiology of foodborne illnesses due to its high prevalence and potential risk during food production [4].

*S. aureus* is the bacterial agent most commonly isolated from bovine mastitis, being identified by the International Dairy Federation (IDF) as the main pathogen of this pathology [5]. Mastitis is considered the main disease of dairy animals, because of its high frequency, economic aspects (both due to decreased production as well as reduced profits for the dairy industry) and aspects related to public health, since this milk can be a vehicle for pathogens, toxins and antimicrobial residues [5].

The genus *Staphylococcus* is among various pathogens undergoing significant changes in antimicrobial susceptibility over the years [6]. In addition to resistance, the pathogenicity of these organisms is an extremely important feature to be understood. The ability of *S. aureus* to cause various infections and intoxication, results from the production of different extracellular and surface virulence factors with adhesive properties targeting a range of molecules (MSCRAMMs). The extracellular products include especially toxins with superantigenic properties, namely enterotoxins A-E, G-K, M-O and Q, exfoliative toxins A and B, toxic shock syndrome toxin-1 as well as, for example, Panton-Valentine leukocidin [7]. Another mechanism of resistance and pathogenicity is biofilm production. This mechanism occurs through the production of extracellular polysaccharide substances, causing bacterial cells to form clusters in multilayer biofilm, thus preventing the action of antibiotics and the immune system [8].

Contaminated milk, upon being ingested raw and unprocessed becomes a potential source of food intoxication due to the presence of staphylococcal toxins. Furthermore, detection of potential strains of *Staphylococcus* biofilm formations may represent a toxic risk factor for consumers [9]. Based on these data, the aim of this study was to investigate the biofilm formation, presence and distribution of virulence genes and the capacity to induce an inflammatory response in strains of *Staphylococcus aureus* isolated from milk samples in southwestern Bahia, Brazil.

## 2. Methods

### 2.1. Samples

A total of 132 samples of raw mastitic milk from four dairy farms (designated A to D) located in the southwestern region of Bahia, in the municipality of Vitória da Conquista, from October/2009 to September/2010. These farms were selected because they produce and supply milk to the dairy industry in the region. The animals were milked twice a day by a milking machine (Properties A and C) or manually (Properties B and D). After sanitizing, 10ml of sample was collected in sterile vials. For samples from farm B (30/30) and farm D (7/31), the collection procedure was the same, however, the samples came from bulk tanks. The samples were transported at 4°C and processed for 12 hours. The milk samples were plated onto mannitol salt agar. Cultures were incubated at 37°C for 48 hours. Suspect colonies, which revealed acidification of mannitol, were subjected to identification procedures. Colonies

with coagulase-positive, Gram-positive cocci and catalase positive were selected as possible *S. aureus* and identified by PCR.

## 2.2. Susceptibility Testing

Antimicrobial susceptibility testing was performed by the broth microdilution method, following recommendations of the Clinical and Laboratory Standards Institute [10]. Oxacillin and vancomycin were obtained from the respective manufacturers, and the plates were prepared and used on the same day as testing. The strain was sub-cultured on mannitol salt agar at 37°C overnight. On the day of experiment, bacterial suspension was prepared by sodium chloride 0.9% solution and the inoculum was adjusted by spectrophotometer. Susceptibility results were interpreted according to CLSI document. The tests were read 24 h after incubation at 35°C. Quality control was performed by testing *S. aureus* ATCC 29213 and ATCC 43300. All experiments were performed in triplicate with three independent repetitions.

## 2.3. Genotypic Characterization to Pathogenic Genes

Staphylococci cultures in 2 mL of TSB medium were used for DNA extraction according to the method described by Fan *et al.* [11]. The isolates were submitted to PCR for detection of virulence factors genes: *sea* (Staphylococcal enterotoxins type A), *seb* (Staphylococcal enterotoxins type B), *sec* (Staphylococcal enterotoxins type C), *pvl* (Panton-Valentine Leucocidin), *clfA* (Clumping factor) and *spa* (IgG-binding region and the X-region of protein A) using primers described by Proietti *et al.* [12]. The primer sequences of the *sea*, *seb*, *sec*, PVL, *spa* and *CflA* genes are described in Table 1. Amplified products were separated by agarose gel electrophoresis (1% agarose containing 0.5 mg ethidium bromide in 0.5× Tris-EDTA electrophoresis buffer) at 100 V and photographed under UV illumination.

## 2.4. Biofilm Assay

Biofilm assays were performed in 96-well polystyrene microplates, using trypticase soy broth (TSB/Difco) with 1% (w/v) glucose (TSB-1% Glc) [13]. Briefly, cultures of staphylococci in 5 mL were incubated in a shaker with 250 rpm at 37°C for 18 h. Cultures were diluted 1:100 in TSB-1% Glc and 200 µL were inoculated into each well. The microplate was incubated at 37°C for 20 h. Supernatants were removed from each well and biofilms were gently washed twice with PBS, then dried and fixed at 65°C for 1 h. Finally, the plates were stained with crystal violet 1% used in Gram-stain and gently washed twice with PBS. The absorbance at 492 nm was calculated in a spectrophotometer. The samples were compared with cultures of *Streptococcus pyogenes* ATCC75194. The *S. aureus* isolates were classified as non-biofilm producers, weak producers, moderate producers, producers, and strong producers. Because the production of biofilm depends on phase variation, tests were repeated four times. At least two independent experiments were carried out for each test. The cutoff point for the production was taken into account, the absorbance obtained by *S. pyogenes* (O.D.492 0.07). The mean value was used for the statistical calculation.

**Table 1.** Sequence of primers used for detection of *S. aureus* from raw milk samples collected from dairy farms located in the municipality of Vitória da Conquista, Bahia, Brazil.

Gene	Primers	Sequence
<i>Sea</i>	Primer 1 (forward)	AAAGTCCCGATCAATTTATGGCTA
	Primer 2 (reverse)	GTAATTAACCGAAGTTCTGTAGA
<i>Seb</i>	Primer 1 (forward)	TCGCATCAAACCTGACAAACG
	Primer 2 (reverse)	GCAGGTACTCTATAAGTGCC
<i>Sec</i>	Primer 1 (forward)	GACATAAAAGCTAGGAATTT
	Primer 2 (reverse)	AAATCGGATTAACATTATCC
<i>PVL</i>	Primer 1 (forward)	ATCATTAGGTAATAATGTCTGGACATGATCC
	Primer 2 (reverse)	GCATCAASTGTATTGGATAGCAAAGC
<i>CflA</i>	Primer 1 (forward)	GGCTTCAGTGCTTGTAGG
	Primer 2 (reverse)	TTTCAGGGTCAATATAAGC
<i>Spa</i>	Primer 1 (forward)	CAAGCACAAAAGAGAA
	Primer 2 (reverse)	CACCAGGTTTAAACGCAT

In addition, to confirm the differences between biofilm phenotypes, as determined by BU values, confocal laser scanning microscopy (CLSM) was used to obtain the structural images of the biofilms [14]. Here, the biofilm assays were performed at the same way, but after being fixed, the bacterial cells were stained with 25nM SYTO9 and propidium iodide (Live/Dead Bacteria-Invitrogen) for 15 min in the dark. The stain was gently removed and biofilms were observed with a Confocal Laser Scanning Microscope-CLSM (Carl Zeiss LSM 510, Germany, equipped with Argon laser, 488 nm, and 2 helium/neon 543 nm wavelengths) to visualize the luminescence of fluorochromes.

## 2.5. Cytokines Induction in Murine Macrophages Assay

Staphylococcal cells were homogenized in 0.9% sodium chloride solution and the suspensions were adjusted to  $0.5 \times 10^8$  CFU/mL by spectrophotometer. Then an aliquot of 100 mL was mixed with 2 ml of Minimum Essential Medium-MEM with 2 mM of L-glutamine and Earle's balanced salts, supplemented with 10% fetal calf serum (Cult Lab, São Paulo, Brazil), and incubated in a shaker at 250 rpm at 35°C for 24 hours. Subsequently, the cultures were filtrated through 0.22-micrometer pores. The filtrates were inoculated into J774 murine macrophages. The sets of inoculated cells were incubated at 37°C in 5% CO<sub>2</sub> atmosphere for 24 hours. The supernatants were removed and the cytokines TNF- $\alpha$ , IL-1, IL-6 and IL-10 were measured using ELISA, according to manufacturer instructions (eBioscience, San Diego, CA).

## 2.6. Statistical Analysis

The differences between samples were analyzed using One-way ANOVA followed by Kruskal-Wallis test and Dunn's post-test. Analyses were performed using GraphPad Prism<sup>®</sup> software (version 5.0, GraphPad Software, San Diego, CA, USA). Statistical differences were considered significant at p values < 0.05 in a confidence interval of 95%.

## 3. Results

### 3.1. Isolation and Susceptibility

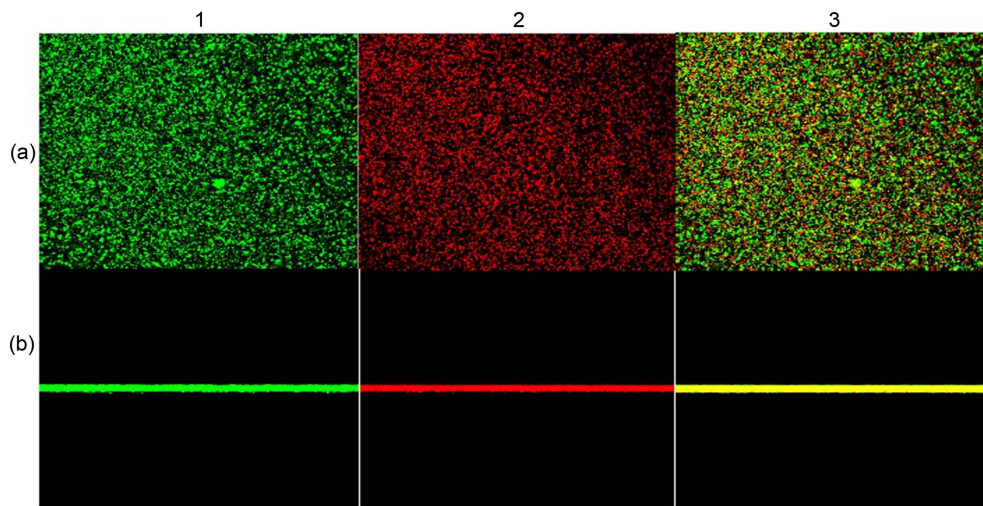
After processing the samples, 94 (71.2%) *S. aureus* isolates were obtained (Table 2). As shown in Table 2, farms A and B had the highest isolation frequency: 100% (30/30) and 90% (27/30), respectively. Farms C and D had 80.6% (25/41) and 29.3% (12/31) isolates, respectively. Among these isolates, 39.4% (37/94) were collected from bulk tanks. All samples from bulk tanks were positive for *S. aureus*. There was a statistical difference between isolates obtained from samples collected from udders and from bulk tanks (p < 0.05). No isolates were resistant to oxacillin and vancomycin.

### 3.2. Biofilm Assay

Evaluation of biofilm production was performed with all isolates (Table 3). In this respect, 5.31% (5/94) of isolates did not produce biofilm, 5.31% (5/94) of the samples were poor producers of biofilm; 15.96% (15/94) strains were moderate producers biofilm; 18.09% (17/94) were producers, and 55.32% (55/94) isolates were strong biofilm producers. Figure 1 shows the biofilm having a thickness of approximately 23  $\mu$ m. There was no

**Table 2.** Isolation of *S. aureus* from raw milk samples collected from dairy farms located in the municipality of Vitória da Conquista, Bahia, Brazil.

	Samples	<i>S. aureus</i> isolation (%)
Farm A	30	27 (90%)
Farm B	30	30 (100%)
Farm C	41	12 (29.3%)
Farm D	31	25 (80.6%)
<b>TOTAL</b>	<b>132</b>	<b>94 (71.2%)</b>



**Figure 1.** Confocal microscopy showing biofilm formation of *Staphylococcus aureus* samples isolated, and (a) shows the top view of the biofilm and (b) the side view of the biofilm. The microorganisms were marked with SYTO9 (green, 1) and unviable with propidium iodide (red, 2). Image 3 is an overlay of images 1 and 2. Magnification 2 $\times$ .

**Table 3.** Biofilm production of *S. aureus* isolated from raw milk samples collected from dairy farms located in the municipality of Vitória da Conquista, Bahia, Brazil.

Biofilm	Farm A		Farm B		Farm C		Farm D		Total	
	n	%	n	%	n	%	n	%	n	%
Non-producers	0	0	1	3.33	0	0	4	16	5	5.3
Weak producers	1	3.7	0	0	0	0	4	16	5	5.3
Moderate producers	10	37.03	1	3.33	3	25	1	4	15	15.96
Producers	5	18.52	2	6.66	4	33.3	6	24	17	18.09
Strong producers	11	40.74	26	86.7	5	41.7	10	40	52	55.32

statistical difference in biofilm formation among isolates obtained from udders and from bulk tanks ( $p > 0.05$ ). However, there was statistical difference in biofilm formation among isolates from different farms ( $p < 0.05$ ) (Figure 2).

### 3.3. Genotypic Characterization to Pathogenic Genes

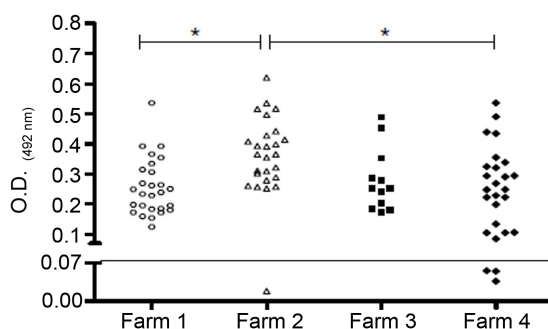
The isolates were genotyped by PCR for detection of genes *sea* (enterotoxin A), *seb* (B), *sec* (C), *pvl* (Panton-Valentine Leukocidin), *clfA* (Clumping Factor A) and *spa* (protein A). One (1.06%) isolate expressed the *seb* gene, one (1.06%) *sec*, 18 (19.2%) *clfA* and 44 (46.8%) had *spa*. There was no detection of *sea* and *pvl* between isolates analyzed (Table 4).

### 3.4. Cytokine Induction in Murine Macrophage Assays

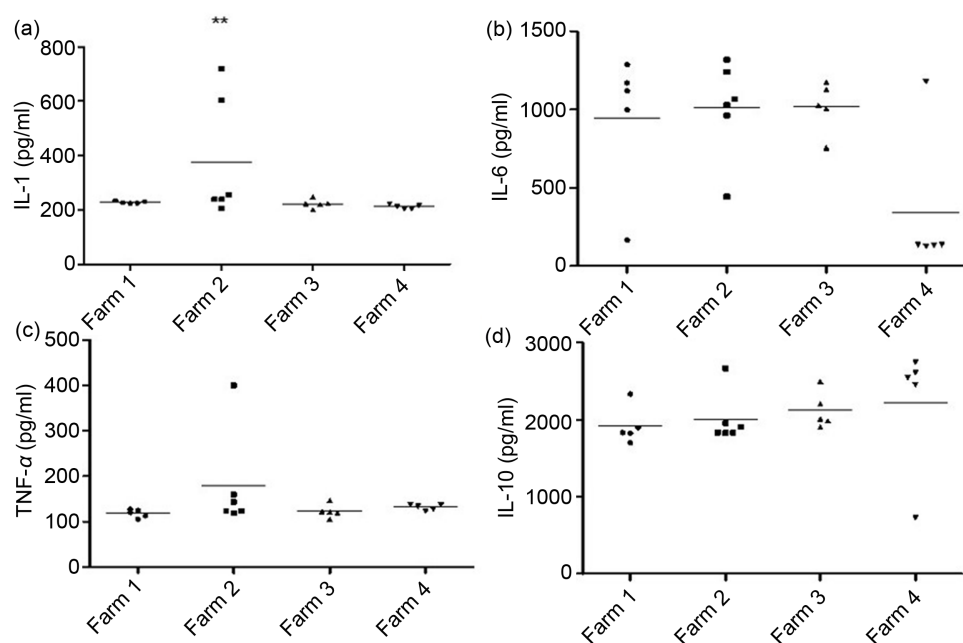
The analysis of cytokine induction in the inflammatory response of J774 macrophages by different farms isolates showed no statistical difference in the levels of IL-6 (Figure 3(b)), TNF- $\alpha$  (3C) and IL-10 (3D) induction ( $p > 0.05$ ). However, there was statistical difference in IL-1 (3A) induction among isolates from different farms ( $p < 0.05$ ).

## 4. Discussion

Research of *S. aureus* in raw milk is important because of its historical importance and its clinical epidemiology



**Figure 2.** Dispersion analysis of the samples in relation to production of biofilm of *Staphylococcus aureus* isolated from different farms. As the cutoff point for the production was taken into account, the absorbance obtained by *S. pyogenes* (O.D.<sub>492</sub> 0.07). There was statistical difference in biofilm formation among isolates from different farms ( $p < 0.05$ , One-way ANOVA test, Graph Pad Prism®).



**Figure 3.** Production of cytokines involved in the inflammatory response by *Staphylococcus aureus* isolated from different farms. There was no statistical difference in the levels of IL-6 (**Figure 3(b)**), TNF- $\alpha$ (3C) and IL-10 (3D) induction ( $p > 0.05$ ). However, there was statistical difference in IL-1 (3A) induction among isolates from different farms ( $p < 0.05$ , One-way ANOVA test, GraphPad Prism®).

**Table 4.** Determination of the *sea*, *seb*, *sec*, *pvl*, *spa* and *cflA* genes in *S. aureus* isolated from raw milk samples collected from dairy farms located in the municipality of Vitória da Conquista, Bahia, Brazil.

	Isolates	<i>Sea</i>		<i>Seb</i>		<i>Sec</i>		<i>Pvl</i>		<i>CflA</i>		<i>Spa</i>	
		n	%	n	%	n	%	n	%	n	%	n	%
<b>Farm A</b>	27	0	0	0	0	1	3.7	0	0	1	3.7	11	40.74
<b>Farm B</b>	30	0	0	1	3.33	0	0	0	0	15	50	14	46.6
<b>Farm C</b>	12	0	0	0	0	0	0	0	0	0	0	5	41.6
<b>Farm D</b>	25	0	0	0	0	0	0	0	0	2	8	14	0
<b>TOTAL</b>	<b>94</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>1.06</b>	<b>1</b>	<b>1.06</b>	<b>0</b>	<b>0</b>	<b>18</b>	<b>19.2</b>	<b>44</b>	<b>46.8</b>

of foodborne diseases. Moreover, because this microorganism normally inhabits human skin, it is an important subject of study for preventing cross-infection in nosocomial environments [15]. The isolation rates of *S. aureus* observed in the present study are consistent with findings in other studies. Zafalon *et al.* [16] identified a total of 245 strains of *S. aureus*, among which 61.2% were derived from milk. In another study, Borges *et al.* [17] observed *S. aureus* in 67% (16/24) of isolates from raw milk. Worldwide, several studies suggest that the *S. aureus* isolation rates in milk can vary from 13.5% to 64.7% [12] [18] [19]. Furthermore, in the present study, a high frequency of isolation of *S. aureus* was observed in samples collected directly from bulk tanks.

One factor that could explain the high rates of *S. aureus* isolation in dairy herds is bovine mastitis. Some authors suggest that mastitis caused by these microorganisms are generally in a subclinical form, which unlike clinical mastitis, is milder and more difficult to detect, because the cows appear sound, and the udder inflammation and milk appear normal despite detection of microorganisms and somatic cells in high numbers [20]-[22]. This factor may be associated with cross-contamination, poor hygienic conditions during milking, contaminated water, infected milkers, and other conditions involved in the development of this agent in milk products [23]-[26].

In the present study, 94.7% (89/94) of *S. aureus* isolates obtained from milk samples produced biofilm, while 5.3% (5/94) did not. Similar results were reported by Fox *et al.* [27], Vasudevan *et al.* [28], Melo *et al.* [29] reported that 41%, 68.6% and 95.7%, respectively, of samples between biofilm-producing *S. aureus* isolated from milk. The results of this study indicate, therefore, that the high frequency of isolation of *S. aureus* in milk, indicating the presence of bovine mastitis, tends to have higher biofilm production, suggesting a possible relationship between the occurrence of bovine mastitis and this factor virulence. Some authors suggest that *S. aureus* ability to form biofilms increased ability to initiate and trigger persistent intramammary infections [30]-[32].

The ability of microbial adhesion and biofilm formation may occur as a result of deposition of microorganisms on a surface of contact, where they attach and begin growing [33]. The main problem is recontamination of milk and thus there is a high microbial load in the product. This can put health of consumers at risk, besides causing financial damage to the industry due to decreased shelf life of food products.

In the present study, some virulence genes were analyzed in isolates. One isolate expressed the *seb* gene, one *sec* gene, 18 *CflA* and 44 had *spa*. There was no expression of *sea* and *pvl* between isolates analyzed. The virulence genes of *S. aureus* described in the literature show variations [34] [35]. Spano *et al.* [36] observed that strains carrying one or more genes for the production of enterotoxins and other virulence factors and some of the virulence factors investigated could be considered important determinants for the host-pathogen relationship providing information that allows for tracing the most probable source of the contamination. Ote *et al.* [37] demonstrated a large variation in the presence of virulence genes in *S. aureus* isolates and the considerable diversity of strain populations capable of causing mastitis in cows. Moreover, the presence of isolates carrying genes coding for toxins involved in important human infections renders the milk of cows with mastitis a potential reservoir for these toxins, and therefore a potential danger in human health, which underscores the importance of carefully scrutinizing raw milk for consumption and its processing. Zeccone *et al.* [4] reported that the presence of a subclinical mastitis showed the role of *spa* and *sej* gene as risk factors.

The analysis of cytokine induction by different farm isolates showed no statistical difference in the levels of IL-6, TNF- $\alpha$  and IL-10 induction. However, there was statistical difference in IL-1 induction among isolates from different farms. In fact, these compounds are induced mainly by the exocellular lipoteichoic acid of *S. aureus* [38]. Jones *et al.* [39] demonstrated that staphylococcal exocellular lipoteichoic acid is a potent activator of pro-inflammatory cytokines (TNF- $\alpha$ , IL-6 and IL-1) and nitric oxide in a murine macrophage cell line. The exocellular lipoteichoic acid is significantly more active than that of lipoteichoic acid, peptidoglycan or wall teichoic acid, especially for TNF- $\alpha$  and nitric oxide production. Lee *et al.* [40] propose that the compromised upregulation of inflammatory cytokines in *S. aureus* infected glands may, at least partially, contribute to the chronic course of infection caused by this pathogen. Further research identifying factors responsible for the differentially expressed cytokine profiles may be fundamental to developing strategies that mitigate the outcome of bovine mastitis.

Other virulence factors could be associated with the intensive inflammatory response, such as enterotoxins [41]. Fijalkowski *et al.* [42] [43] observed that exogenic virulence factors secreted by *S. aureus* isolates significantly influenced the digestion efficiency and phagocytosis carried out by bovine polymorphonuclear neutrophils *in vitro* and cytokine gene expression and cytokine secretion. In the present study, the relationship between the presence of these genes and increased production of cytokines was not observed.

To reduce the risk of the presence of *S. aureus* and other microorganisms in raw milk, it is necessary to im-

plement measures to reduce the prevalence of intramammary infections as well as increase the development of guidelines and support for dairy producers to improve production techniques that enhance the quality of milk in terms of microbiological, physical-chemical, organoleptic and nutritional aspects [15] [44]. Consequently, in the current milk production chain, it is important to educate producers about good hygiene practices, as well as the harmful effects of low quality milk, such as the transmission of diseases, the associated economic and human costs, as well as decreased quality of dairy products, among others.

## 5. Conclusion

Thus, it appears that the results obtained in this study have important implications for the region studied, since it is characterized as an important dairy region, demonstrating the need for further studies of this nature, with the purpose of bringing subsidies that contribute to the improvement of prevention and control in both dairy farms and dairy industries, since milk contamination poses a potential risk to the health of consumers.

## Competing Interests

The authors declare that they have no competing interests.

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